UNIVERSITY OF CRETE
MEDICAL SCHOOL
MOTHER CHILD SECTION
HUMAN REPRODUCTION LAB

‘STUDY OF THE EXPRESSION, THE REGULATION AND THE BIOLOGICAL ROLE OF THE CORTICOTROPIN RELEASING HORMONE (CRH) IN ENDOMETRIOSIS’

‘ΜΕΛΕΤΗ ΤΗΣ ΕΚΦΡΑΣΗΣ, ΤΗΣ ΡΥΘΜΙΣΗΣ ΚΑΙ ΤΟΥ ΒΙΟΛΟΓΙΚΟΥ ΡΟΛΟΥ ΤΟΥ ΕΚΛΥΤΙΚΟΥ ΠΑΡΑΓΟΝΤΑ ΤΗΣ ΚΟΡΤΙΚΟΤΡΟΠΙΝΗΣ (CRH, corticotropin releasing hormone) ΣΤΗΝ ΕΝΔΟΜΗΤΡΙΩΣΗ’

PhD Thesis

Aikaterini Vergetaki
Biologist

HERAKLION 2013

HUMAN REPRODUCTION LAB
MOTHER CHILD SECTION
MEDICAL SCHOOL
UNIVERSITY OF CRETE

PhD thesis

'STUDY OF THE EXPRESSION, THE REGULATION AND THE BIOLOGICAL ROLE OF THE CORTICOTROPIN RELEASING HORMONE (CRH) IN ENDOMETRIOSIS'

Aikaterini Vergetaki
Biologist

PhD supervisor 1: Prof. Antonios Makrigiannakis
Medical School, University of Crete

Co-Supervisor 2: Prof. Achilleas Gravanis
Medical School, University of Crete

Co- Supervisor 3: Assosiate Prof. Christos Tsatsanis
Medical School, University of Crete

Consulting Committee

- **Prof. Antonios Makrigiannakis, Obstetrics & Gynecology Medical School, University of Crete (PhD supervisor 1)**

- **Prof. Achilleas Gravanis, Pharmacology, Medical School, University of Crete (Co-Supervisor 2)**

- **Associate Prof. Christos Tsatsanis, Clinical Chemistry, Medical School, University of Crete (Co- Supervisor 3)**

- **Assistant Prof. Evangelia Papakonstanti, Biochemistry, Medical School, University of Crete**

- **Associate Prof.Konstantinos Relakis, Obstetrics & Gynecology, Medical School, University of Crete**

- **Prof. Efstathios Stathopoulos, Pathology of anatomy, Medical School, University of Crete**

- **Assosiate Prof. Eleni Dimitriou, hematology and Child Oncology, Medical School, University of Crete**

Στη μητέρα μου και την αδερφή μου

Αφιερωμένο ιδιαιτέρως στον μπαμπά μου Νικήτα
που μου λείπει πολύ και θα ήθελα να είναι εδώ……..

Acknowledgments

This PhD thesis was performed at Human Reproduction lab, Medical School, University of Crete, under the supervision of Professor Antonis Makrigiannakis, Professor of the Obstetrics and Gynecology, Director of In vitro fertilization Unit of PAGNI Hospital, at Heraklion, Crete.

Firstly, I would very much like to express my gratitude and I would like to thank very much my Supervisor Prof. Antonis Makrigiannakis who gave me the opportunity to be a member of the Human Reproduction Lab and have my PhD thesis at a very interesting area concerning reproduction. Moreover, I am grateful to Prof. Antonis Makrigiannakis for the guidance, the scientific consulting, experimental design and support in the experimental procedures, the study of the scientific background and scientific writing in order this PhD thesis to begin, to be successfully organized and productively finished.

Furthermore, I would very much like to thank my co-Supervisors Prof. Achilleas Gravanis and Associate Prof. Christos Tsatsanis for their valuable scientific support and guidance during my PhD. In addition, I would like to thank Prof Udo Jesckhe, University of Munich, Germany, for the cell lines he provided me with and his valuable help in order to perform my experiments.

I am truly grateful and I would very much like to thank the Assistant Prof. Evangelia Papakonstanti for the valuable and priceless scientific guidance in experimental design and scientific writing and for her support in the conduction of the experiments for my PhD thesis in her lab. Furthermore, I am really thankful for the inestimable productive conversations concerning science which helped me to address lots of questions in my PhD thesis and conversations about lots of other aspects which have been an inspiration and motivation for me as a person and scientist. Furthermore, I am really grateful to Assos.Prof. Georgios Koumoundouros, Biology Department, University of Crete, for his scientific support during the final years of my PhD and the opportunity to be part of his lab.

I would very much like to express my warmest feelings and gratitude to Dr Eirini Taliouri, valuable member of the Human Reproduction lab, who was a friend and a lab partner during my PhD. Firstly, I am really grateful to Eirini as she had learnt to me most of the techniques I needed for my PhD thesis and gave me generously all the knowledge I needed to begin and keep on my PhD. Moreover, I would like to thank her for her time, willingness and true interest to stand by me during these years and up to present. I will never forget the priceless support of her and I am really thankful for having a new friend.

Also, I would like to thank all the Human Reproduction lab and Biochemistry lab members. I would like especially to thank Dr Eirini Neofytou and MSc Niki Tzenaki who were really generous and helpful to me in learning new techniques and the know-how of lots of experimental procedures in a very explicable and detailed way, for their support during my PhD and in having a pleasant environment in and outside the labs. I would like to thank Dr Georgios Petsas and Dr Thomas Vrekoussis for their scientific guidance as well. Many thanks as well to the PhD students of the Pharmacology lab (Sifis Pediaditakis and Pascalis Efstathopoulos) and of the Marine Biology lab (Anastasia Georgiou), University of Crete for their assistance in experimental procedures.

Moreover, I would like to thank Assist. Prof. Emmanouil Zoumakis, Medical School of Athens, for his scientific guidance and support concerning antalarmin manipulation.

I would like to thank all of my friends in Crete, Thessaloniki, Athens, Cyprus, United Kingdom and Ireland who are next to me in every aspect of my life, as in for these years having my PhD, with patience and tireless mood for support, lots of conversations and true entertainment and quality of life. I would like to thank my special 'friend' as well, as he helped me very effectively with his companionship and priceless support during these years and was a reason to be positive and full of life. All of my friends help was crucial and of high importance to me in order to be optimistic and fulfilled as a person, trying to keep up and do my best every day despite the life challenges.

Last but not least, I am more than grateful to my father, mother and sister who gave me the opportunity to do my PhD and to do my best for my studies during all these years. There are not enough words to thank them for being my partners in life, my motivation, my role models and a priceless continuous help spiritually and economically in my everyday life. I thank them very much as they have taught me that everything is possible *a posse ad esse*.

Aikaterini Vergetaki

Curriculum Vitae

Name: Aikaterini Vergetaki, **Father's Name**: Nikitas Vergetakis
Adress: Kosta Varnali 2, Heraklion, Crete, postal code: 71305
E-mail: katerina.verg@googlemail.com
Nationality: Greek
Date of Birth: 05/01/1984

EDUCATION:

❖ **(April 2008 – April 2013): PhD thesis**
Medical Scholl, University of Crete
Human Reproduction Lab
Supervisor: Prof. Antonis Makrigiannakis
PhD thesis title: Study of the expression, regulation and biological role of corticotropin releasing hormone (CRH) in endometriosis

Parallel Experimental activity (February 2010- up to present): Biochemistry Lab Supervisor: Assist.Prof. Evangelia Papakonstanti

❖ **(September 2006- August 2007)**: **Master of Research**
MRes in Medical and Molecular Biosciences
Newcastle upon Tyne University, United Kingdom
Modules included: Genetic Basis of complex diseases, Cancer Studies, Applied Immunobiology, Statistical Techniques in Medical Research, Bioethics and Safety, Library and Information Technology Skills, Twenty week research project.
Research Project (February 2007-present): Institute of Human Genetics, Newcastle University, Centre for Life, NE1 3BZ.
MRes Project Title: Non-canonical Wnt signalling in cardiac development
Supervisors: Dr Deborah Henderson and Dr Bill Chaudhry.

Overall Result: Merit (67%)

❖ **(September 2001– July 2006): Bachelor Degree**
Department of Biology
University of Crete, Greece
Bachelor Graduation score: 6.7 / 10.
Research Project Dissertation: Molecular Toxicology Laboratory, Medicine School, University of Crete, Greece.
Dissertation title: ''Study of the distribution of Q192R polymorphism in PON1 gene, in Cretan population and correlation of this polymorphism with potential pathological conditions''

January- March 2005: Practical research exercise

Analytical Toxicology Laboratory, University of Crete, Greece.

Research practical exercise title: 'Study of the influence of passive smoking in children according to cotinine and nicotine levels.'

❖ **(September 1998 – June 2001)**: Greek Senior High School
Aristotelian College of Thessaloniki
Graduation Score: 18.5 /20.

LANGUAGE SKILLS AND QUALIFICATIONS

- Fluent speaker of English:

1. 2005, University of Cambridge, Proficiency, Certificate of Proficiency in English
2. International English language testing system IELTS- overall score: 7

- Good knowledge of French: DELF A1,A2,A3,A4.(Evidence available on request)
- Familiarity with computer applications Windows XP and using the internet.

RESEARCH EXPERIENCE:

- ❖ PhD project placement: Cell and tissue RNA extraction, cDNA synthesis, RT-PCR, agarose gel electrophoresis, cell culture, immunofluorescence, immunohistochemisrty, Western blotting, protein extraction, mice macrophage extraction, microscopy.
- ❖ MRes project placement:Non-canonical Wnt signalling in cardiac development. My project involved examining the formation of branchial arches in zebrafish with mutations in this pathway. I managed several zebrafish lines and collected and processed my own embryos. Key techniques were histological examination after paraffin sectioning and wholemount *in-situ* hybridisation. I have gained experience of molecular techniques such as use of competent cells and plasmids to make riboprobes. Morpholino injections were included too.
- ❖ Bachelor dissertation placement: DNA extraction from human blood samples, Polymerase Chain Reaction (PCR), Restriction Fragment Length Polymorphisms (RFLPs) and agarose gel electrophoresis.
- ❖ Practical Research project exercise: Study of the influence of passive smoking in children according to cotinine and nicotine levels: Solid Phase Extraction(SPE),Gas Chromatography (GC), Liquid Chromatography(LC): Thin Layer Chromatography (TLC), Column Chromatography(CC), Partition Column Chromatography (PCC), Ion Exchange Column Chromatography (IXCC), Thin Layer Chromatography(TLC) and hair homogenization.

CONFERENCES ATTENDED:

- 4th Greek Conference of Forensic Medicine and Toxicology, Athens, September 2005
- Onassis Foundation Lectures Series ''Programmed Cell Death and Cell Signalling in the Development and Disease'', organised by FORTH (Foundation of Research and Technology Hellas), Heraklion, July, 2005.
- 5th Annual MSRM meeting-"RECENT ADVANCES IN REPRODUCTION-THE CUTTING EDGE" , Heraklion,27-29/04/2006
- MSRM Workshop- "New Trends on Controlled Ovarian Stimulation and Ovulation Induction" , Mediterranean Conference for Reproductive Medicine, Heraklion,3-4 / 5/ 2008
- 3^0 Hellenic Endometriosis Conferense and 3^0 Symposium Introspection, Heraklion, 17-19/ 10/ 2008
- MSRM Workshop- "The Many Faces of Polycystic Ovarian Syndrome", Mediterranean Conference for Reproductive Medicine, Heraklion,26-27/ 9/ 2009
- International Conferense of Reproduction, Chania, Crete, 25-26/09/2010
- 45th Annual scientific meeting of ESCI, Heraklion, Crete 13-16/4/2011
- MSRM-International meeting: ENDOMETRIOSIS: "Current management and future trends" 21-23 October 2011, Gran Melia, Vathi, Agios Nikolaos, Crete,Greece.
- 62^o Hellenic Conferense of Hellenic Society for Biochemistry and Molecular Biology, Athens, Eugenides Foundation, 9-11 December, 2011.
- MSRM Conference in Controversies in Human Reproduction, 28-30 September 2012, Daios Cove, Agios Nicolaos, Crete
- 63^o Hellenic Conferense of Hellenic Society for Biochemistry and Molecular Biology, FORTH(ITE), Heraklion, Crete, 9-11 December 2012

CONFERENCES ORAL PRESENTATIONS AND POSTER:

1. **Poster presentation:**
   Differential expression of CRH, UCN, CRHR1 AND CRHR2 in eutopic and ectopic endometrium of women with endometriosis.
   Vergetaki A., Jeschke U., Vrekoussis T., Sabatini L., Taliouri E., Christoforaki V., Papakonstanti E.A., Makrigiannakis A.
   63^o Hellenic Conferense of Hellenic Society for Biochemistry and Molecular Biology, FORTH (ITE), Heraklion, Crete, 9-11 December 2012

2. **Oral Presentation:**
   Galectin-1 is overexpressed in endometriotic tissue and upregulated by CRH, UCN and CSF-1.
   Vergetaki A., Jeschke U., Taliouri E., Christoforaki V., Papakonstanti E.A., Makrigiannakis A.
   MSRM Conference in Controversies in Human Reproduction, 28-30 September 2012, Daios Cove, Agios Nicolaos, Crete

3. **Oral Presentation:**
   CSF-1 receptors are located at the nuclear envelope and activated via the p110_ PI3K-dependent translocation of extracellular CSF-1 to the nucleus
   Zwaenepoel O., Tzenaki N., **Vergetaki A.,** Makrigiannakis A., Vanhaesebroeck B., Papakonstanti E.A.
   62^{o} Hellenic Conferense of Hellenic Society for Biochemistry and Molecular Biology, Athens, Eugenides Foundation, 9-11 December, 2011

4. **Oral Presentation:**
   The effect of CRH on Galectin-1 pattern in endometriosis and Galectin-1 expression in ectopic endometrium of women with endometriosis.
   Vergetaki A, Jeschke U, Taliouri E, Petsas G, Kardari I , Friese K, Makrigiannakis A
   MSRM-Internation meeting:ENDOMETRIOSIS: "Current management and future trends" 21-23 October 2011, Gran Melia, Vathi, Agios Nikolaos, Crete,Greece.

5. **Oral Presentation:**
   Overexpression of CRH, UCN, CRHR1 and CRHR2 in ectopic endometrium of women with endometriosis.
   Vergetaki A, Taliouri E, Neofytou E, Petsas G, Kardari I , Jeschke U, Friese K, Makrigiannakis A
   MSRM-Internation meeting: ENDOMETRIOSIS: "Current management and future trends" 21-23 October 2011, Gran Melia, Vathi, Agios Nikolaos, Crete,Greece.

CONFERENCES ANNOUNCEMENTS:

1. Poster Presentation:
   CRH is expressed in the Human cervical carcinoma cells(Hela), cervical adenocarcinomas and intraepithelial neoplasias and up-regulates the expression of FasL in vitro. Taliouri E, Vrekoussis T, ***Vergetaki A***, Tsentelierou E, Stamataki A, Stathopoulos E, Makrigiannakis A. 63 ^{o} Hellenic Conferense of Hellenic Society for Biochemistry and Molecular Biology, FORTH (ITE), Heraklion, Crete, 9-11 December 2012
2. Oral Presentation:
   *Neuropeptides expression in HPV infected cells?*Taliouri E, Vrekoussis T, Tsentelierou E, ***Vergetaki A***, Stathopoulos E, Agorastos T, Makrigiannakis A. MSRM Conference in Controversies in Human Reproduction, 28-30 September 2012, Daios Cove, Agios Nicolaos, Crete
3. Oral Presentation:
   Possible correlation between endometriosis and cervical cancer immune – mediated mechanisms?
   Taliouri E, ***Vergetaki A***, Vrekoussis T, Petsas G, Neofytou E,D Miliaras, AgorastosT,MakrigiannakisA. MSRM-Internation meeting:ENDOMETRIOSIS: "Current management and future trends" 21-23 October 2011, Gran Melia, Vathi, Agios Nikolaos, Crete,Greece.

4. Poster Presentation:
   p110δ PI3K inhibitors induce the activity of the tumor suppressor PTEN in cancer cells
   Tzenaki N., Andreou M., Stratigi K., ***Vergetaki A***., Makrigiannakis A., Vanhaesebroeck B. and Papakonstanti E.A.
   62^{o} Hellenic Conferense of Hellenic Society for Biochemistry and Molecular Biology, Athens, Eugenides Foundation, 9-11 December, 2011

PUBLICATIONS

1. **Aikaterini Vergetaki**, Udo Jeschke, Thomas Vrekoussis, Eirini Taliouri, Luca Sabatini, Evangelia A. Papakonstanti, Antonis Makrigiannakis. **Differential Expression of CRH, UCN, CRHR1 and CRHR2 in Eutopic and Ectopic Endometrium of Women with Endometriosis.** PLOS ONE Journal (2013) 8(4): e62313

2. Taliouri E, Vrekoussis T, **Vergetaki A**, Agorastos T, Makrigiannakis A. **Corticotropin-releasing hormone (CRH) is expressed in the human cervical carcinoma cells (HeLa) and upregulates the expression of Fas ligand.**Tumour Biol. (2013) 34(1):125-30.

3. Zwaenepoel O, Tzenaki N, **Vergetaki A**, Makrigiannakis A, Vanhaesebroeck B, Papakonstanti EA. FASEB J. **Functional CSF-1 receptors are located at the nuclear envelope and activated via the p110δ isoform of PI 3-kinase**. *FASEB* J. (2012) 26(2):691-706.

4. Tzenaki, N., Andreou, M., Stratigi, K., **Vergetaki, A.,** , Makrigiannakis, A., Vanhaesebroeck, B., Papakonstanti, E.A. **High level of p110δPI3K expression in solid tumour cancer cells suppresses PTEN activity, generating cellular sensitivity to p110δ inhibitors through PTEN activation**. *FASEB* J. (2012) 26(6):2498-508.

TABLE OF CONTENTS

Abbreviations

- **ACTH (adrenocorticotropin hormone)**
- **AICD (activation-induced cell death)**
- **APO1(apoptosis antigen 1)**
- **AVP (arginine- vasopressin)**
- **Bcl2 (B-cell lymphoma 2)**
- **c/EBPs (enhancer binding proteins)**
- **cAMP (cyclic adenosine monophosphate)**
- **CEACAM1(Carcinoembryonic antigen-related cell adhesion molecule 1)**
- **CNS (central nervous system)**
- **COX2 (cyclooxygenase-2)**
- **CRDs (carbohydrate recognition domains)**
- **CRH(corticotrophin releasing hormone)**
- **CRHBP (corticotrophin releasing hormone binding protein)**
- **CRHR1/CRHR2(corticotrophin releasing hormone receptor1/2)**
- **CRHRs(corticotrophin releasing hormone receptors)**
- **CSF1 (colony stimulating factor 1)**
- **E2 (estradiol)**
- **ECM (extracellular matrix)**
- **EGF(epidermal growth factor)**
- **ER (estrogen)**
- **ERK-2 (Extracellular regulated kinase)**
- **ESCs (endometrial stromal cells)**
- **EVTs (Extravillous trophoblast cells)**
- **FasL (Fas ligand)**
- **FasR (Fas receptor)**
- **Foxo1 (Forkhead box protein O1)**
- **FSH (follicle stimulating hormone)**
- **Gal-1 (galectin-1)**
- **GnRH (Gonadotrophin- releasing hormone)**
- **hCG(human chorionic gonadotropin)**
- **HESC(human endometrial stromal cells)**
- **HPA (hypothalamic-pituitary adrenal)**

- **HRT (Hormone Replacement Therapy)**
- **ICAM (Intercellular Adhesion Molecule)**
- **IFN(interferon)**
- **IL(interleukin)**
- **IL-1ra(interleukin 1 receptor a)**
- **IVF (in vitro fertilization)**
- **LH(luteinizing hormone)**
- **LIF(leukemia inhibitory factor)**
- **LUFs (luteinized unruptured follicle syndrome)**
- **MCP-1(monocyte chemoattractant protein)**
- **MCSF1R (monocyte colony stimulating factor 1 receptor)**
- **MMPs (matrix metalloproteinase)**
- **MRI (magnetic resonance imaging)**
- **NK cells (natural killer cells)**
- **P (progesterone)**
- **PAR2 (protease activated receptors)**
- **PDGF (platelet‐derived growth factor)**
- **PGE2(prostaglandin E2)**
- **PGI2 (prostacyclin)**
- **PGs (progestins)**
- **PR (progesterone receptors)**
- **PVN (paraventricular nucleus)**
- **RLX (relaxin)**
- **ROS (reactive oxygen species)**
- **SF1(steroidogenic factor 1)**
- **SicAM-1(soluble intercellular adhesion molecule-1)**
- **SRC (sarcoma)**
- **STAT5(Signal Transducer and Activator of Transcription 5)**
- **TAMs(tumor- associated macrophages)**
- **TGF(transforming frowth factor)**
- **TIMPs (tissue inhibitors of metalloproteinase)**
- **TMEM(tumour microenvironment of metastasis)**
- **TNFα (tumour necrosis factor α)**
- **TNFRSF6(tumour necrosis factor receptor superfamily member 6)**
- **UCN (urocortin)**
- **VEGF(vascular endothelial growth factor)**

SUMMARY

Endometriosis is one of the most important benign chronic diseases affecting the 6-10% of women of reproductive age, being mainly associated with pelvic pain, adhesion formation and infertility. Endometriosis is characterised by the ectopic presence of endometrial stroma and epithelium**.** Endometriosis has been proven to be both an estrogen-dependent and a chronic inflammatory disease. In that context, endocrine/paracrine influences and immunological aspects have been investigated. Thus, several growth factors, cytokines, immune cells and hormones in eutopic and ectopic endometrium, are considered to be involved in the pathophysiology of endometriosis-related infertility**.**

CRH (Corticotropin Releasing Hormone) is a 41-amino acid neuropeptide, synthesised in the hypothalamus, regulating the hypothalamus-pituitary-adrenal axis and its expression and biological functions are mediated by its membrane receptors, CRH-R1 (α, β, γ, c-h) and CRH-R2 (α, β, γ). CRH receptors are also activated by other endogenous agonists, such as urocortin (UCN), which is a 40-amino acid peptide belonging to the corticotropin-releasing hormone family and is structurally related to CRH. Apart from the central nervous system, CRH and its receptors are expressed in several sites of the female reproductive system, including the endometrial glands, decidualized stroma, trophoblast, syncytiotrophoblast and placental decidua. CRH and UCN are secreted at inflammatory sites, acting as proinflammatory factors. Reproductive CRH has been shown to serve as an autocrine and paracrine modulator and to participate in decidualization, embryo implantation and maintenance of human pregnancy. In addition, CRH and UCN mRNA have been found to be expressed by endometriotic cells, while endometriotic lesions show a strongly positive staining reaction for CRH and UCN. The expression and function of CRH and UCN have also been found to be impaired in eutopic endometrium of women with endometriosis. These data suggest a crucial role of CRH and UCN in pathogenesis of endometriosis. However, the relative expression of CRH, UCN and their receptors in eutopic and ectopic endometrium of endometriotic women and in eutopic endometrium of healthy women and women with endometriosis have never been investigated. Additionally, CRH receptors and in particular their subtypes have never been identified in endometriotic lesions. In this current study, the relative expression levels of these molecules in eutopic and ectopic endometrium of endometriotic and healthy women have been investigated, indicating a potential role of CRH receptors in infertility profile of endometriotic women. As a result it is shown, in this current study, that CRHR1 and CRHR2 are expressed in endometriotic tissue and their expression levels are higher in eutopic endometrium of women with endometriosis compared to their expression levels in eutopic endometrium of healthy women. Moreover, CRH, UCN CRHR1 and CRHR2 have been found to be more abundantly expressed in ectopic rather than eutopic endometrium of the same endometriotic women.

As endometriosis is characterized by the release of several growth factors and expression of integrins, cadherins and lectins that regulate cell migration, invasion, angiogenesis, immune functions and apoptosis, galectins, which are characterized as members of a large family of animal lectins which bind b-

galactoside should play an important role in this inflammatory disease. Galectins have strongly been implicated in inflammation and may be useful targets for anti-inflammatory therapy. As far as the immune system concerned, galectins are found in activated macrophages and B cells. They play a vital role in T cell homeostasis and survival. Galectin-1 plays a major role in initiation, activation and resolution of inflammatory responses and can act as a pro-inflammatory or an anti-inflammatory cytokine. Galectin-1 induces inhibition of cell growth and cell-cycle arrest and promotes the apoptosis of activated immune cells. Galectin-1 has been found to be expressed in human endometrium and decidua apart from other tissues but it has not been investigated in endometriotic tissue and in this current study it was a motivation in order to examine the galectin-1 expression in eutopic endometrium and in endometriotic tissue and compare it to that in eutopic endometrium of endometriotic women and healthy women. So, as a result, in this study, it is shown that galectin-1 shows much higher expression levels at the late secretory phase of eutopic endometrium and its expression levels are higher in ectopic endometrium compared to eutopic endometrium of endometriotic and healthy women. Moreover, the evaluation of its regulation by CRH, UCN and FasL was performed in Ishikawa cell line and peripheral macrophages as to possibly set new light concerning galectin-1 inflammatory role in endometriosis and infertility. So, as a result, it is shown that CRH, UCN and CSF-1 upregulate galectin-1 expression in both the cells types and the upregulative effect of the former one is mediated by CRHR1.

Colony stimulating factor- 1 is a secreted cytokine, characterized as a haematopoietic growth factor which influences hematopoietic stem cells to differentiate into macrophages or other related cell types**.** Macrophages and other cells of the female reproductive tract are also regulated by locally produced CSF-1. CSF-1 plays an important role in placental growth and differentiation. As endometriosis is an inflammatory disease, there are several cytokines and growth factors that are implicated in this immune-disequilibrium status. As secretory cells, monocytes and macrophages are vital to the regulation of immune responses and the development of inflammation. These cytokines are produced by peritoneal macrophages, lymphocytes, mesothelial cells and endometriosis implants. Furthermore, women with endometriosis are characterised by increased number of peritoneal macrophages and by increased expression of CSF-1 compared to healthy ones. So, it was of quite interest, to examine the possible co-localization of galectin-1 and macrophage expression in endometriotic tissue, the CSF-1 expression in eutopic and ectopic endometrium of endometriotic and healthy women and how CSF-1 is regulated by CRH, UCN and CSF-1 in Ishikawa cell line and peripheral macrophages. In this current study, it is shown that there is a co-localization of macrophages and galectin-1 in ectopic sites. CSF-1 has been found to be much more highly expressed in ectopic endometrium compared to eutopic endometrium of endometriotic and healthy women. CRH, UCN and exogenously added CSF-1 have been shown to upregulate CSF-1 expression in Ishikawa cell line and macrophages and the upregulative effect of the former one is mediated by CRHR1.

Fas ligand is a type-II transmembrane protein that belongs to the tumor necrosis factor (TNF) family. Its binding with its receptor induces apoptosis. Fas ligand/receptor interactions play an important role in the regulation of the immune system. FasL is expressed on the surface of fetal cytotrophoblasts as well as

maternal decidual cells of the placenta and maternal endometrium. Abnormalities of maternal immune tolerance to the fetal semi-allograft and vise versa have been implicated in several pregnancy malfunctions such as recurrent early miscarriage, pre-eclampsia and eclampsia. CRH produced locally by decidual cells and extravillous trophoblasts acts in an autocrine/paracrine fashion, through CRH-R1, to stimulate FasL expression and to potentiate the ability of these cells to cause apoptosis of activated maternal T lymphocytes in order proper decidualization to occur. As FasL is implicated in inflammatory processes, it has been found that Fas is expressed randomly in both eutopic and ectopic endometrial tissues. Higher expression of FasL by endometriotic tissues contributes to their survival and the development of endometriosis. It has been suggested that the levels of soluble/active FasL are higher in serum and peritoneal fluid in women with moderate to severe endometriosis than in women with early‐stage disease or in healthy women. Consequently, ectopic endometrial cells escaping from immune surveillance in the peritoneal cavity of women with endometriosis may contribute to the maintenance of the disease, so it was of high importance to study how FasL is regulated by stress neuropeptides such as CRH and UCN and by an important macrophage factor such as CSF-1, in Ishikawa cell line and peripheral macrophages. As a result, in this study it is shown that CRH, UCN and CSF-1 upregulate FasL expression in both the cell types and the upregulative effect of the former one is mediated by CRHR1.

In conclusion, the findings of this current study point to a new inflammatory- modulatory pathway in which CRH, UCN, CRHR1 and CRHR2 are involved acting by an autocrine/paracrine pathway in eutopic and ectopic endometrium potentially affecting the pathogenesis of endometriosis and the infertility profile of endometriotic women. These results suggest that a new therapeutic intervention could potentially based on blockage of CRH, UCN and their receptors leading to the improvement of the quality of endometriotic women's life. Furthermore, it is proposed that galectin-1 could play a vital role in the pathogenesis of the disease. In addition, CRH, UCN and CSF-1 have been found to upregulate galectin-1, CSF-1 and FasL expression in Ishikawa cell line and macrophages. As all the peptides causing these upregulative effects and the molecules that are regulated by them are implicated in inflammation procedures such as endometriosis and in reproductive functions such as decidualization and implantation, these results could possibly set new light to the immune disequilibrium of endometriosis and the infertility profile of endometriotic women. Finally, this study results suggest that the potential use of antalarmin could be further considered in accessing the immune disequilibrium noticed in eutopic and ectopic endometrium of women with endometriosis.

ΠΕΡΙΛΗΨΗ

Η ενδομητρίωση είναι μια από τις πιο σημαντικές χρόνιες ασθένειες που εμφανίζεται στο 10% περίπου των γυναικών της αναπαραγωγικής ηλικίας. Τα βασικότερα συμπτώματα αυτής της ασθένειας είναι ο πόνος στην πυελική περιοχή, ο σχηματισμός συμφύσεων και η υπογονιμότητα. Η ενδομητρίωση χαρακτηρίζεται από την εμφάνιση ενδομητριοτικού ιστού (στρώμα και επιθήλιο) σε έκτοπες –μη φυσιολογικές θέσεις. Η ενδομητρίωση είναι ορμονοεξαρτώμενη και φλεγμονώδης νόσος. Πληθώρα αυξητικών παραγόντων, κυτοκινών, κυττάρων που σχετίζονται με την ανοσοβιολογική απόκριση αλλά και ορμονών συνδέονται με την παθοφυσιολογία της νόσου και την υπογονιμότητα που χαρακτηρίζει την συγκεκριμένη ασθένεια.

Η εκκριτική ορμόνη της κορτικοτροπίνης, CRH(corticotropin releasing hormone) είναι ένα νευροπεπτίδιο 41 αμινοξέων, αρμόδιο για τις ενδοκρινείς, ανοσολογικές, και συμπεριφορικές αποκρίσεις των θηλαστικών σε συναισθηματικούς παράγοντες (stress). Η ορμόνη αυτή συντίθεται κυρίως στον υποθάλαμο, και ο πιο σημαντικός ρόλος της είναι η ρύθμιση του υποθάλαμο-υποφυσιακού επινεφριδιακού άξονα HPA(hypothalamic-pituitary-adrenal axis). Η CRH εμπλέκεται στην ρύθμιση του καρδιοαγγειακού, γανστρενετρικού, ανοσολογικού και αναπαραγωγικού συστήματος. Η δράση και έκφραση της CRH επιτελείται μέσω της σύνδεσης της με ειδικούς μεμβρανικούς υποδοχείς που συνδέονται με G πρωτεΐνες και την αδενυλική κυκλάση. Τρεις τύποι υποδοχέων έχουν βρεθεί μέχρι σήμερα και είναι οι εξής: CRHR1, CRHR2, CRHR3. Εκτός από την CRH , το πεπτίδιο της UCN(urocortin), που έχει πρόσφατα απομονωθεί και ανήκει στην οικογένεια των CRH νευροπεπτιδίων, προσδένεται πολύ ισχυρά στους CRH υποδοχείς. Εκτός από την έκφραση και τον λειτουργικό ρόλο της CRH στο κεντρικό νευρικό σύστημα και στους περιφερικούς ιστούς , είναι σημαντικό να αναφερθούμε και στον ρόλο της CRH στο ανοσοποιητικό σύστημα. Η CRH έχει αντι-φλεγμονώδεις ιδιότητες οι οποίες προκύπτουν μέσα από αλληλεπιδράσεις του ανοσοποιητικού συστήματος και του άξονα HPA. Η CRH παράγεται επίσης σε περιφερικές θέσεις φλεγμονής στον άνθρωπο και τον επίμυ. Η CRH, σε αντίθεση με την συστηματική της έμμεση ανοσοκατασταλτική δράση, πιστεύεται ότι δρα ως αυτοκρινής ή παρακρινής προ-φλεγμονώδης ρυθμιστής . Η τοπικά παραγόμενη CRH έχει δράσεις προ-φλεγμονώδους κυτοκίνης, ενεργοποιεί δηλαδή τις φλεγμονώδεις δράσεις των κυτοκινών και άλλων ρυθμιστών, ενώ η ανοσοεξουδετέρωσή της οδηγεί σε καταστολή της φλεγμονής. Η CRH παράγεται σχεδόν από όλους τους ιστούς που συνδέονται με τη γυναικεία αναπαραγωγή, συμπεριλαμβανομένων των ωοθηκών, της μήτρας, της βλαστοκύστης και του πλακούντα. Η CRH που σχετίζεται με την αναπαραγωγή είναι μία μορφή CRH ιστών (CRH που βρίσκεται στους περιφερειακούς ιστούς) ανάλογη με την ανοσοαντιδρώσα μορφή της CRH που ανιχνεύεται σε περιφερειακές φλεγμονώδεις εστίες. Η ανοσοαντιδρώσα CRH που σχετίζεται με το αναπαραγωγικό σύστημα συμμετέχει σε διάφορες αναπαραγωγικές λειτουργίες όπως η ωορρηξία, η φθαρτοποίηση, η εμφύτευση του εμβρύου και η εγκυμοσύνη. Η ανοσοαντιδρώσα CRH έχει προ-φλεγμονώδεις ιδιότητες στις

ανοσολογικές αποκρίσεις και σχετίζεται με την παραγωγή και λειτουργία κυτοκινών και αυξητικών παραγόντων σε διάφορους ιστούς του αναπαραγωγικού συστήματος. Επιπρόσθετα, η CRH και η UCN εκφράζονται σε κύτταρα ενδομητρίωσης και ενδομητριοτικές εστίες. Η έκφραση και η λειτουργία της CRH και της UCN έχει βρεθεί να είναι μειωμένη στο εύτοπο ενδομήτριο γυναικών με ενδομητρίωση και αυτό υποδεικνύει έναν πολύ σημαντικό ρόλο της CRH και της UCN στην παθογένεση της ενδομητρίωσης. Στην παρούσα διδακτορική διατριβή μελετήθηκε η έκφραση των υποδοχέων της CRH σε ιστούς ενδομητρίωσης-έκτοπο ενδομήτριο, καθώς και έγινε σύγκριση της έκφρασης όλων αυτών των μορίων(CRH, UCN και των υποδοχέων τους) σε εύτοπο-φυσιολογικό ενδομήτριο υγιών γυναικών αλλά και σε εύτοπο και έκτοπο ενδομήτριο γυναικών με ενδομητρίωση. Έτσι, σε αυτή την μελέτη της διδακτορικής διατριβής βρέθηκε για πρώτη φορά ότι οι υποδοχείς CRHR1β και CRHR2α εκφράζονται σε ιστούς ενδομητρίωσης και η έκφραση τους είναι υψηλότερη στο εύτοπο ενδομήτριο των γυναικών με ενδομητρίωση σε σχέση με το εύτοπο ενδομήτριο υγιών γυναικών. Επίσης η έκφραση των CRH, UCN, CRHR1 και CRHR2 βρέθηκε να είναι μεγαλύτερη στο έκτοπο ενδομήτριο σε σχέση με αυτή στο εύτοπο ενδομήτριο γυναικών που πάσχουν από ενδομητρίωση.

Καθώς η ενδομητρίωση είναι μία φλεγμονώδης νόσος και χαρακτηρίζεται από την έκφραση πολλών αυξητικών παραγόντων, ιντεγκρινών, καντχερινών και λεκτινών που ρυθμίζουν την μετακίνηση των κυττάρων, την εισβολή κυττάρων, την αγγειογένεση, ανοσολογικές λειτουργίες και την απόπτωση, οι galectins, που είναι μέλη μιας μεγάλης οικογένειας λεκτινών, θα μπορούσαμε να υποθέσουμε ότι παίζουν πολύ σημαντικό ρόλο στην φλεγμονώδη αυτή νόσο, την ενδομητρίωση. Οι galectins είναι άρρηκτα συνδεδεμένες με διάφορες φλεγμονώδεις καταστάσεις και θα μπορούσαν να χρησιμοποιηθούν ως αντι-φλεγμονώδη θεραπεία. Σε ότι αφορά στο ανοσοβιολογικό σύστημα, οι galectins εκφράζονται από ενεργοποιημένα μακροφάγα και Β λεμφοκύτταρα. Παίζουν σημαντικό ρόλο στην ομοιόσταση και επιβίωση των Τ λεμφοκυττάρων. Πιο συγκεκριμένα η galectin-1 , η οποία μελετήθηκε στην συγκεκριμένη διδακτορική διατριβή, παίζει πολύ σημαντικό ρόλο στην έναρξη, την ενεργοποίηση και το τέλος των φλεγμονών αντιδράσεων και λειτουργεί ως προ- ή αντί- φλεγμονώδης κυτοκίνη. Η galectin-1 μπλοκάρει την ανάπτυξη των κυττάρων και επάγει την απόπτωση την ενεργοποιημένων κυττάρων του ανοσοποιητικού. Επίσης έχει βρεθεί ότι εκφράζεται στο ανθρώπινο ενδομήτριο και φθαρτό αλλά δεν έχει μελετηθεί ποτέ ως τώρα η έκφραση της στην ενδομητρίωση και αυτό αποτέλεσε ένα κίνητρο για την παρούσα διδακτορική διατριβή. Έτσι λοιπόν στην συγκεκριμένη διδακτορική διατριβή, αφού επιβεβαιώθηκε η έκφραση της στην εκκριτική φάση του φυσιολογικού ενδομητρίου και πιο συγκεκριμένα ότι είναι αρκετά αυξημένη η έκφραση της στην ‘late secretory phase’, βρέθηκε ότι η galectin-1 εκφράζεται σε εστίες ενδομητρίωσης και η έκφραση της είναι υψηλότερη στο έκτοπο ενδομήτριο από ότι στο φυσιολογικο- εύτοπο ενδομήτριο των γυναικών με ενδομητρίωση καθώς και ακόμα ότι είναι υψηλότερη η έκφρασή της στο ευτοπο ενδομήτριο γυναικών με ενδομητρίωση σε σχέση με το εύτοπο ενδομήτριο των υγιών γυναικών. Επίσης, στην συγκεκριμένη διδακτορική διατριβή παρουσιάζεται και η ρύθμιση της galectin-1 από τα εξής μόρια: CRH, UCN και CSF-1 στην κυτταρική σειρά Ishikawa cell line (επιθηλιακό αδενοκαρκίνωμα ενδομητρίου) καθώς και σε περιφερειακά μακροφάγα. Τα προαναφερθέντα μόρια λοιπόν αυξάνουν την έκφραση της galectin-1

σε σχέση με τον χρόνο διέγερσης της από αυτά και η επαγωγή της έκφρασης της galectin-1 από την CRH επιτελείται μέσω του υποδοχέα της, CRHR1. Αυτά λοιπόν τα αποτελέσματα, θα μπορούσαμε να υποθέσουμε ότι έχουν μεγάλη σημασία για τον ανοσοβιολογικό ρόλο της galectin-1 στην ενδομητρίωση.

Ο CSF-1(colony stimulating factor), που χαρακτηρίζεται ως παράγοντας διέγερσης αποικιών μακροφάγων, παίζει σημαντικό ρόλο στην διαφοροποίηση των αιμοποιητικών βλαστοκυττάρων σε μακροφάγα ή άλλους τύπους κυττάρων. Τα μακροφάγα και άλλοι τύποι κυττάρων του γυναικείου αναπαραγωγικού συστήματος ρυθμίζονται από τον τοπικά παραγόμενο CSF-1. Ο CSF-1 πάιζει σημαντικό ρόλο στην ανάπτυξη και διαφοροποίηση του πλακούντα. Η ενδομητρίωση είναι μία φλεγμονώδης νόσος, τα μακροφάγα έχουν πολύ σημαντικό ρόλο στην ανοσοβιολογική απόκριση και πολλές από τις κυτοκίνες που σχετίζονται με την ενδομητρίωση παράγονται από τα μακροφάγα του περιτοναίου και εστίες ενδομητρίωσης. Οι γυναίκες που πάσχουν από ενδομητρίωση παρουσιάζουν αυξημένο αριθμό περιτοναϊκών μακροφάγων και αυξημένη έκφραση του CSF-1 σε σύγκριση με τις υγιείς γυναίκες. Στην συγκεκριμένη διδακτορική διατριβή παρουσιάζεται ο συνεντοπισμός της έκφρασης των μακροφάγων και της galectin-1 σε εστίες ενδομητρίωσης. Επίσης επιβεβαιώνεται η έκφραση του CSF-1 στην ενδομητρίωση και παρατηρήθηκε ότι η έκφραση του CSF-1 είναι μεγαλύτερη στις εστίες ενδομητρίωσης- έκτοπο ενδομήτριο σε σχέση με την έκφραση του στο αντίστοιχο εύτοπο- φυσιολογικό ενδομήτριο. Επίσης, παρατηρήθηκε ότι η έκφραση του CSF-1 είναι μεγαλύτερη στο ευτοπο-φυσιολογικό ενδομήτριο των γυναικών με ενδομητρίωση απο ότι στο ευτοπο ενδομήτριο των υγιών γυναικών. Ακόμα παρουσιάζεται η επαγωγή της έκφρασης του CSF-1 από τα εξής μόρια: CRH, UCN και CSF-1 στην κυτταρική σειρά Ishikawa cell line (επιθηλιακό αδενοκαρκίνωμα ενδομητρίου) καθώς και σε περιφερειακά μακροφάγα. Τα προαναφερθέντα μόρια λοιπόν αυξάνουν την έκφραση του CSF-1 σε σχέση με τον χρόνο διέγερσης της από αυτά και η επαγωγή της έκφρασης του CSF-1 από την CRH επιτελείται μέσω του υποδοχέα της, CRHR1. Αυτά λοιπόν τα αποτελέσματα θα μπορούσαν να συνεισφέρουν περεταίρω στην κατανόηση του ρόλου του CSF-1 και των μακροφάγων στην ενδομητρίωση.

Ο Fas είναι μία τύπου-ΙΙ διαμεμβρανική πρωτεΐνη που ανήκει στην οικογένεια παραγόντων νέκρωσης όγκου (TNF- tumour necrosis factor family). Η σύνδεσή του με τον υποδοχέα του επάγει την απόπτωση. Η σύνδεση του Fas/FasL(συνδέτη / υποδοχέα) παίζει σημαντικό ρόλο στη ρύθμιση του ανοσοποιητικού συστήματος. Ο FasL εκφράζεται στην επιφάνεια των εμβρυϊκών και μητρικών τροφοβλαστικών κυττάρων καθώς και σε κύτταρα του φθαρτού και του ενδομητρίου. Διάφορες μη φυσιολογικές καταστάσεις σε ό,τι αφορά στην ανοσοανοχή της μητέρας καθώς και του εμβρυικού ημιμοσχεύματος έχουν συσχετιστεί με διάφορες δυσλειτουργίες στην εγκυμοσύνη όπως η αποβολη του εμβρύου, η προεκλαμψία και η εκλαμψία. Η CRH που παράγεται τοπικά από τα κύτταρα του φθαρτού και την τροφοβλάστη λειτουργεί με αυτοκρινή ή παρακρινή τρόπο μέσω του CRHR1 και διεγείρει την έκφραση του FasL, δίνοντας την δυνατότητα σε αυτά τα κύτταρα να προκαλέσουν απόπτωση των μητρικών Τ λεμφοκυττάρων ώστε να επιτευχθεί επιτυχής φθαρτοποίηση. Ο FasL , καθώς συμμετέχει στις φλεγμονώδεις αντιδράσεις, έχει βρεθεί ότι εκφράζεται από το εύτοπο και το έκτοπο ενδομήτριο. Η υψηλή έκφραση του FasL στις εστίες ενδομητρίωσης συνεισφέρει ώστε να επιβιώνει και να αναπτύσσεται ο ιστός της ενδομητρίωσης- έκτοπο ενδομήτριο. Επίσης έχει βρεθεί ότι η έκφραση του FasL είναι υψηλότερη σε

γυναίκες που πάσχουν από ενδομητρίωση προχωρημένου σταδίου σε σχέση με χαμηλότερου σταδίου ή τις υγιείς γυναίκες. Με δεδομένα όλα τα παραπάνω , στην παρούσα διδακτορική διατριβή μελετήθηκε η ρύθμιση του FasL στην κυτταρική σειρά Ishikawa και σε περιφερειακά μακροφάγα και βρέθηκε ότι τα εξής μόρια: CRH, UCN και CSF-1 αυξάνουν την έκφραση του σε σχέση με τον χρόνο και η επαγωγή της έκφρασης του από την CRH επιτελείται μέσω του υποδοχέα της, CRHR1.

Συμπερασματικά, τα ευρήματα της παρούσας διδακτορικής διατριβής, στοχεύουν σε ένα νέο ανοσορυθμιστικό μονοπάτι, στο οποίο τα μόρια CRH, UCN, CRHR1 και CRHR2 λειτουργούν με αυτοκρινή ή παρακρινή τρόπο στο εύτοπο και έκτοπο ενδομήτριο με πιθανή την εμπλοκή τους στην παθογένεια της ενδομητρίωσης και το προφίλ υπογονιμότητας των γυναικών με ενδομητρίωση. Μέσα από αυτά τα αποτελέσματα διαφαίνεται η πιθανή χρήση θεραπευτικών μεθόδων για την ενδομητρίωση που θα μπορούσαν να εμπεριέχουν το μπλοκάρισμα των μορίων CRH, UCN, CRHR1 και CRHR2 στοχεύοντας στην βελτίωση της ποιότητας ζωής των γυναικών με ενδομητρίωση. Επιπροσθέτως, το ότι η galectin-1 εκφράζεται στην ενδομητρίωση, καταδεικνύει την πιθανή εμπλοκή της στην παθογένεση της ενδομητρίωσης. Έπειτα, βρέθηκε σε αυτή την μελέτη ότι τα εξής μόρια: CRH, UCN και CSF-1 αυξάνουν την έκφραση των galectin-1, CSF-1 και FasL. Καθώς αυτά τα μόρια που προκαλούν την επαγομένη αυτή έκφραση και τα μόρια που ρυθμίζονται από αυτά συμμετέχουν σε φλεγμονώδεις καταστάσεις, όπως η ενδομητρίωση αλλά και σε αναπαραγωγικές διαδικασίες όπως η φθαρτοποίηση και η εμφύτευση του εμβρύου, αυτά τα αποτελέσματα θα μπορούσαν να διαφωτίσουν περεταίρω την έλλειψη ισσοροπημένης ανοσοβιολογίας της ενδομητρίωσης και του προφίλ υπογονιμότητας των γυναικών που πάσχουν από ενδομητρίωση. Τέλος, μέσω αυτής της μελέτης προτείνεται η πιθανή χρήση της antalarmin, που αποτελεί αναστολέα έκφρασης του υποδοχέα CRHR1, στην προσπάθεια βελτίωσης της ανοσοβιολογικής λειτουργίας του εύτοπου και του έκτοπου ενδομητρίου.

PhD thesis

INTRODUCTION

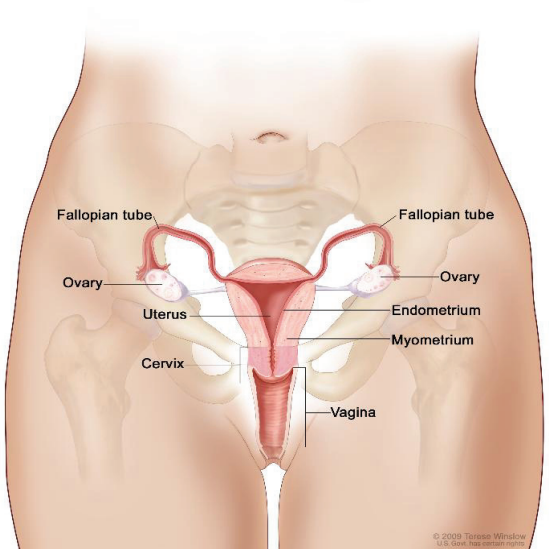
CHAPTER 1

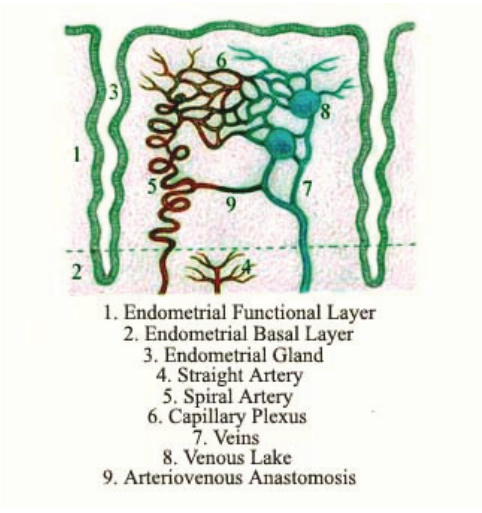
ENDOMETRIUM

1.1 GENERAL

The normal-eutopic endometrium is the inner glandular layer of the mammalian uterus, preventing adhesions between the opposed walls of the myometrium. The endometrium consists of epithelium, stroma and connective tissue. Endometrium thickness varries according to hormonal influences. Tubular uterine glands reach from the endometrial surface through to the base of the stroma, which also carries a rich blood supply of spiral arteries. In a woman of reproductive age, two layers of endometrium can be distinguished. The endometrium is divided in two layers:

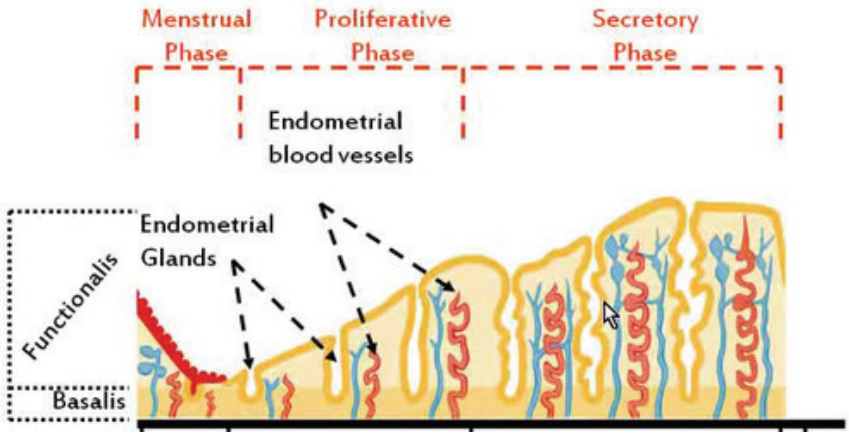
- The **functional layer** which is adjacent to the uterine cavity. This layer is created after the end of menstruation during the first part of the previous menstrual cycle. Estrogen induce proliferation of endometrium (follicular phase of menstrual cycle), and then progestrone of the corpus luteum (luteal phase) stimulate further changes creating the proper environment for implantation, decidualization and embryo growth. This layer is shed during menstruation.
- The **basal layer** which is adjacent to the myometrium and below the functional layer, is not shed during the menstrual cycle, and from it the functional layer arises.





(http://www.yalemedicalgroup.org/stw/Page.asp?PageID=STW022188)

(http://www.nelsonginecologia.med.br/localmechmenstr_engl.htm)

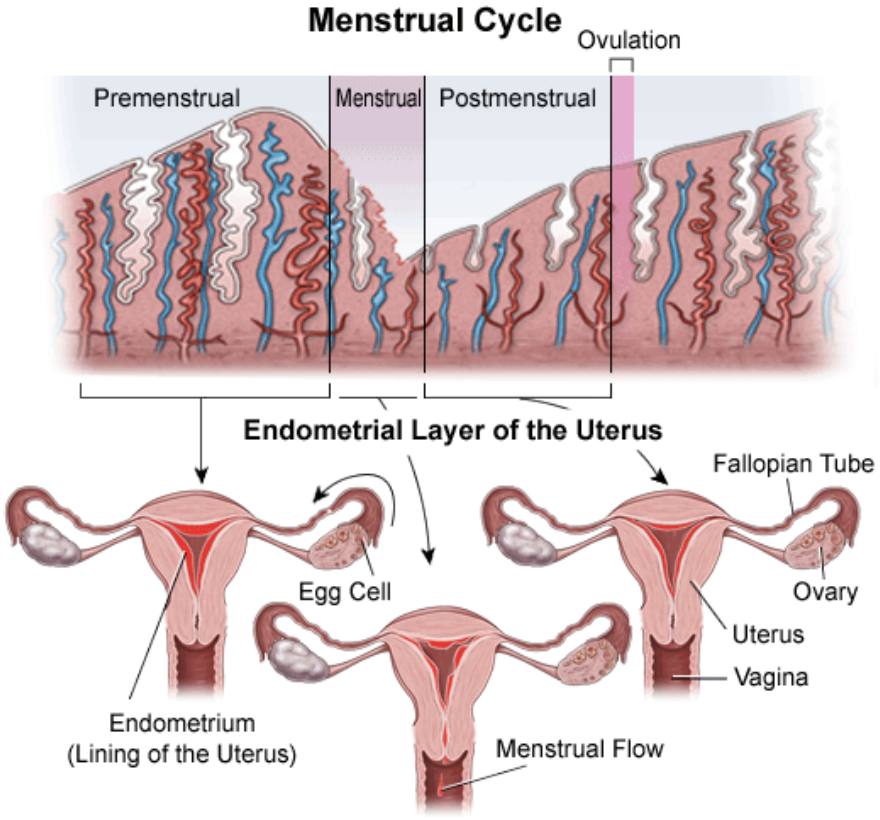


http://quizlet.com/6841533/familiarize/embedv2?&m

In the absence of progesterone, the arteries supplying blood to the functional layer compress, so that cells in that layer become ischaemic and die, leading to menstruation. The phase of the menstrual cycle can be identified by the histological differences noticed in endometrium. During the menstrual cycle, the endometrium grows to a thick, blood vessel-rich, glandular tissue layer. This represents an optimal environment for the implantation of a blastocyst. The endometrium is central, echogenic (detectable using ultrasound scanners), and has an average thickness of 6.7 mm. It is shed each month during the menstrual cycle. During pregnancy, the glands and blood vessels in the endometrium further increase in size and number. Vascular spaces fuse and become interconnected, forming the placenta, which supplies oxygen and nutrition to the embryo and foetus. The menstrual cycle occur in fertile women. The human menstrual cycle can vary around an average of ~28 days per cycle. It is under the control of the endocrine system. There are three phases of the menstrual cycle: the follicular phase, ovulation, and the luteal phase. Menstrual cycle counts from the first day of menstrual bleeding**[1,2].**

1.1.1. Follicular phase – Proliferative phase

This phase is called proliferative as the lining of the uterus grows, or proliferate. Through the follicle stimulating hormone (FSH) during the first days of the cycle, ovarian follicles are stimulated. The follicles secrete increasing amounts of estradiol, which belong to estrogens. The estrogens stimulate the formation of the proliferative endometrium and the lining of the uterus thickens. When FSH causes follicles to grow and develop, these follicles produce estradiol, activin and inhibin, which, in turn, provide a feedback mechanism to control the hypothalamus-pituitary-ovarian axis. While the follicles are growing in size, the cells within the follicle are changing.Within the follicle, follicular cells express luteinizing hormone (LH) receptors, which prepare the follicle for ovulation **[1,2]**.



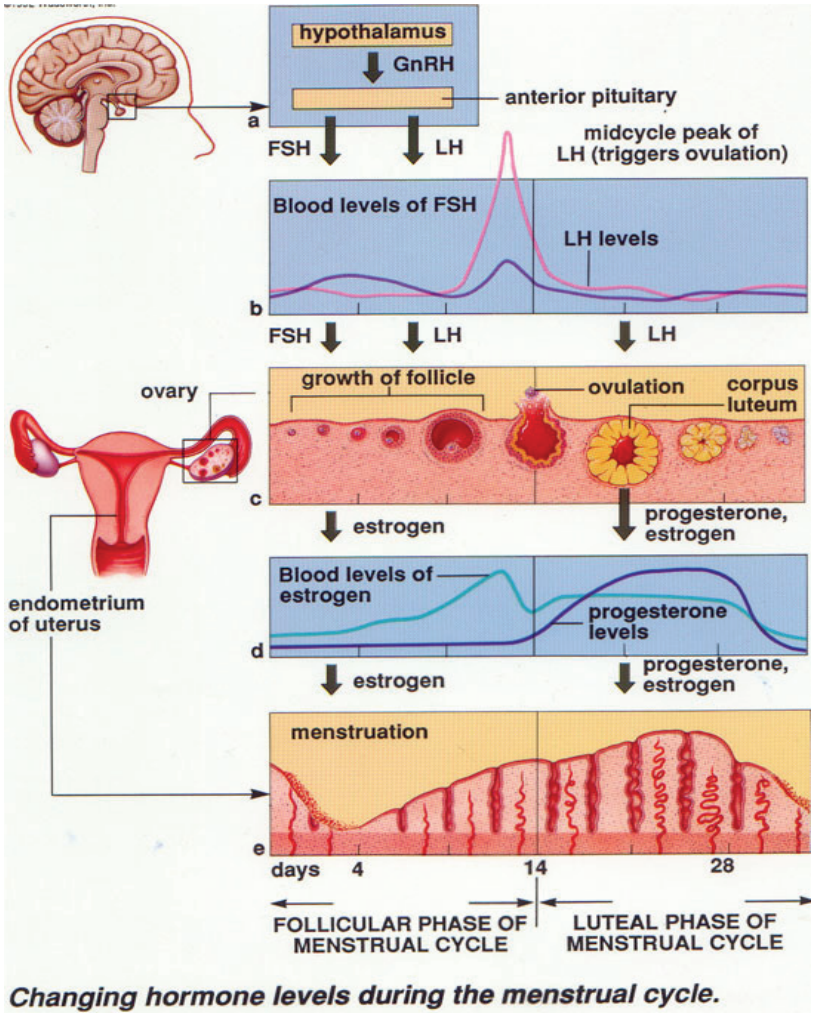
(http://www.yalemedicalgroup.org/stw/Page.asp?PageID=STW029029)

1.1.2. Ovulation

During the follicular phase, estradiol suppresses the production of luteinizing hormone (LH) from the anterior pituitary gland. Levels of estradiol reach a threshold when the oocyte is matured and this effect is reversed and estrogen stimulates the production of a large amount of LH. This process is known as the LH surge and accounts around day 12 of the average cycle and may last 48 hours. The release of LH leads to the oocyte maturation and weakening of the wall of the follicle in the ovary, causing the fully developed follicle to release its secondary oocyte. The secondary oocyte promptly matures into an ootid and then becomes a mature ovum. The mature ovum has a diameter of about 0.2 mm. Which of the two ovaries—left or right—ovulates appears essentially random. Occasionally, both ovaries will release an ovum; if both oocytes are fertilized, the result is fraternal twins. After being released from the ovary, the ovum is swept into the fallopian tube by the fimbria, which is a fringe of tissue at the end of each fallopian tube. After about a day, an unfertilized egg will disintegrate or dissolve in the fallopian tube. Fertilization by a spermatozoon, when it occurs, usually takes place in the ampulla, the widest section of the fallopian tubes. A fertilized oocyte immediately begins the process of embryogenesis, or development. The developing embryo takes about three days to reach the uterus and another three days to implant into the endometrium. It has usually reached the blastocyst stage at the time of implantation**[1,2]**.

1.1.3 Luteal phase – secretory phase

The luteal phase is also called the secretory phase. An important role is played by the corpus luteum, the solid body formed in an ovary after the ovum has been released from the ovary into the fallopian tube. This body continues to grow for some time after ovulation and produces significant amounts of hormones, particularly progesterone. Progesterone plays a vital role in endometrium receptivity leading to implantation of the blastocyst and effective early pregnancy, by increasing blood flow and uterine secretions and reducing the contractility of the smooth muscle in the uterus. After ovulation, the pituitary hormones FSH and LH leads to the remaining parts of the dominant follicle transformation into the corpus luteum, with progesterone to be produced. The increased progesterone in the adrenals starts to induce estrogen production. The hormones produced by the corpus luteum also suppress production of the FSH and LH that the corpus luteum needs to maintain itself. Consequently, the level of FSH and LH fall quickly over time, and the corpus luteum subsequently atrophies. Falling levels of progesterone trigger menstruation and the beginning of the next cycle. From the time of ovulation until progesterone withdrawal has caused menstruation to begin, the process typically takes about two weeks, with 14 days considered normal. For an individual woman, the follicular phase often varies in length from cycle to cycle; by contrast, the length of her luteal phase will be fairly consistent from cycle to cycle. The loss of the corpus luteum can be prevented by fertilization of the oocyte; the resulting embryo produces human chorionic gonadotropin (hCG), which is very similar to LH and which can preserve the corpus luteum. Because the hormone is unique to the embryo, most pregnancy tests look for the presence of hCG **[3,4].**



Changing hormone levels during the menstrual cycle.

(http://dentistryandmedicine.blogspot.gr/2011/07/menstrual-cycle-gynecology-lecture.html)

1.2. ENDOMETRIAL FUNCTION IN REPRODUCTION

1.2.1 Decidualization

Decidualization is characterized as the postovulatory process of endometrial remodelling in preparation for pregnancy. Changes that occur during the secretory phase, induced by progesterone, in stroma are known as decidualization, which is an inflammatory process characterized by morphological transformation of stromal cells and secretion of prolactin, IL-15, insulin-like growth factor- binding protein 1 and widening of the wall of the blood vessels, muscle layer of arteries and increased secretion of inflammatory mediators and cytokines by the endometrial gland cells **[5]**. Decidualization is a characteristic of the endometrium of the pregnant uterus. It is a response of maternal cells to progesterone. Decidualization may be used to describe any change due to progesterone. These changes include the eosinophilic proliferation around arterioles after ovulation or progesterone action on endometrium which increases glandular epithelial secretion, stimulates glycogen accumulation in stromal cell cytoplasm, and promotes stromal vascularity (spiral arterioles) and edema. The process is decidualization, the endometrium is now

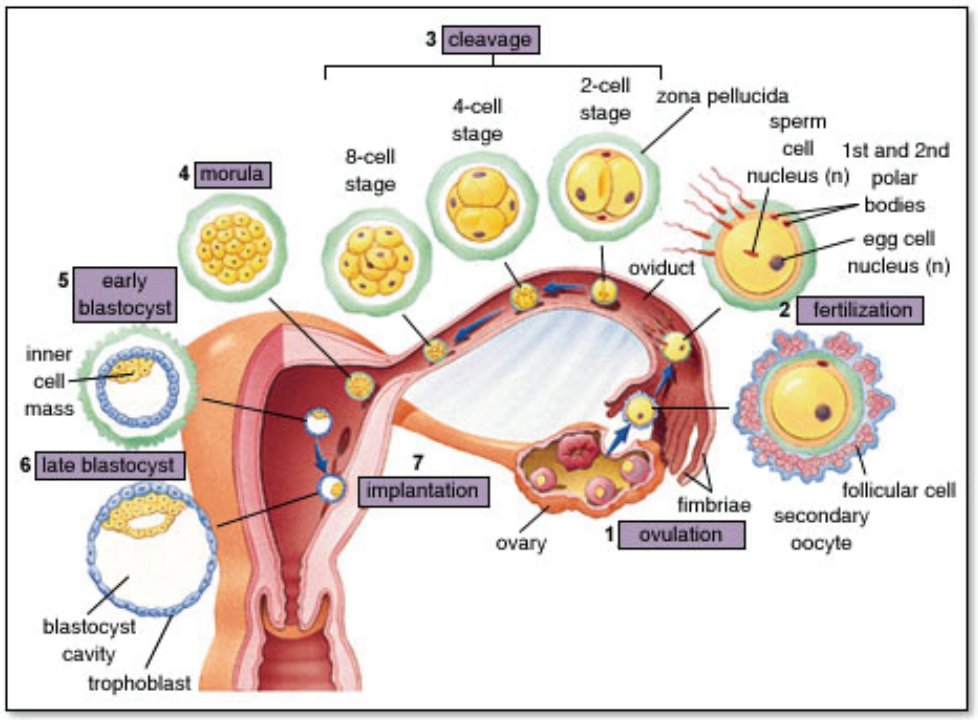
called decidua and it is ready for the implantation of the embryo. After ovulation, in mammals, the endometrial lining becomes transformed into a secretory lining in preparation of accepting the embryo. Without implantation, the secretory lining will be absorbed (estrous cycle) or shed (menstrual cycle). With implantation the lining now termed decidua evolves further during the pregnancy. The decidua is shed during the third phase of the parturition process.

Decidualization includes secretory changes of the uterine glands, differentiation of stromal cells, accumulation of specialized uterine natural killer cells and vascular remodelling. It is the progesterone-induced differentiation of fibroplast – like endometrial stromal cells (ESCs), located in the proliferative estrogen primed endometrium, into decidual cells. There are histological changes as well, so there are larger and rounder cells surrounding the spiral arteries and spread through most of the endometrium. Decidualizing endometrial stromal cells acquire the capacity of regulate trophoblast invasion, resist inflammatory and oxidative insults and diminish local maternal immune response. This is mediated by several growth factors, cytokines, neuropeptides, free radical scavengers and extracellular matrix components. As a result, decidualization is important for successful embryo implantation and maintenance of pregnancy, as otherwise infertility, recurrent miscarriages, uteroplacental dysfunctions, endometriosis and endometrial cancer may occur[**6]**.

The inflammatory reaction taking place in deciduas differs from the one in response to injury. In deciduas, the recruitment of leykocyte is dominated by natural killer (NK) cells. The transformation of the uterine stromal cells into decidual cells in mediated by prolactin, IL-15 and activation of the arachidonic acid pathway.The regulation of endometrial changes during decidualization is under ovarian hormone control. So, progesterone initiates decidualization and exerts its actions via the progesterone receptor (PR). It has been shown that activated PR, has an important role for both the direct and indirect recruitment of other activated transcription factors in response to cyclic adenosine monophosphate (cAMP) signalling such as signal transducer and activator of transcription (STAT5), CCAAT enhancer binding proteins(C/EBPs) and forkhead box O (FOXO1). In that way, activated PR has control of diverse gene families involved in decidualization **[6]**. Moreover, after ovulation, the endometrium is highly exposed to a variety of local and endocrine factors including prostaglandin (PGE2), relaxin (RLX), corticotrophin releasing hormone (CRH), LH, and FSH that are capable of stimulating cAMP production in hESCs. The activation of cAMP pathway is an essential event that starts the decidual process **[7]**. Furthermore, the tyrosine kinase activity of SRC(sarcoma) is increased in decidualization, as it is a non-receptor tyrosine kinase that associates with integrins and many surface receptors including those for growth factors, cytokines and G-couples protein receptors **[8,9]**. Also, PGE2 can directly affect the PI3K-related signalling pathway by increasing phosphorylation of PKB/AKT in endometrial cells [**10]**.

Endometrial changes during decidualization process are not only under hormone regulation, but mediators that induce an inflammatory process in endometrium such as IL-1, tumour necrosis factor α (TNF-α) and progestins (PGs) are produced. IL-1 induces PGs, whose synthesis is regulated by COX (cyclooxygenase -2) enzymes. The mechanism implicated in decidualization is the action of PGE2 and PGI2 through production of cytokines and growth factors. Moreover, endometrium requires PGs during decidualization especially for vascular changes. Furthermore, decidualization requires the recruitment of NK cells, whose differentiation can be induced by PGE2. In addition, endometrium during decidualization is characterized by the production of growth factors such as EGF (epidermal growth factor) and vascular endothelial growth factor (VEGF). Endometrial angiogenesis and vessel remodeling are driven by a network of signaling molecules that include the VEGF family **[5].**

1.2.2. Embryo implantation- blastocyst- endometrium crosstalk



http://aagklithopedion.wordpress.com/normal-pregnancy/

Firstly, concerning the pre-implantatory phase, the embryo begins when the oocyte is fertilized by a spermatozoid; the first stage of the embryo is named zygote. Mitotic divisions of the zygote give rise to two, four and eight cells until the morula. After that, a cell polarization process in morula follows, where outermost cells form the trophoectoderm and result in blastocele. Pluripotent cells, named as the inner cell

mass, are established in a pole of the embryo between the trophoectoderm and the blastocele. So, changes give rise to blastocyst, the final step of early embryo development **[5]**.

Embryo implantation can be defined as the process by which the embryo attaches to the endometrium, invades the decidualised stroma and reaches the maternal microvasculature. Blastocyst implantation is a very complicated developmental process which involves a series of events leading to an effective invasive trophoblast cell- maternal endometrium crosstalk. These events are affected by lots of endocrine, paracrine and autocrine factors. The success of implantation relies on achieving the embryo development to the blastocyst stage and its invasion to the decidualized endometrial stroma. Moreover, there are lots of hormones, cytokines and growth factors participating in implantation as the table indicates **[11]**.

Table 1. Cytokines, prostaglandins, and growth factors implicated in embryo-endometrium interaction.

Compounds	Biological actions	Reference
LIF	Blastocyst implantation	Stewart et al., 1992 (40)
	Early embryogenesis as an embryotrophin	Lavranos et al., 1995 (16)
	Decidualization	Chen et al., 2000 (41)
LIFR	Decidualization and placentation	Ni et al., 2002 (31)
IL-1	Successful implantation	Sheth et al., 1991 (9)
	Induces LIF production	Arici et al., 1995 (43)
	Induces COX-2	Huang et al., 1998 (26)
	Endometrial changes	McMaster et al., 1992 (23)
	GM-CSF and CSF-1 production	Garcia-Lloret et al., 1994 (57)
IL-1 ra	Failure of implantation	Simon et al., 1994 (49)
IL-1R tI	Embryo development	Kruessel et al., 1997 (10)
EGF	Blastocyst differentiation	Kaye, 1997 (1)
	Angiogenic factor	Moller et al., 2001 (37)
	Trophoblastic differentiation	Kliman et al., 1990 (55)
	Induces LIF production	Arici et al., 1995 (43)
HB-EGF	Blastocyst growth, zona-hatching and trophoblast outgrowth	Das et al., 1994 (39)
		Leach et al., 1999 (36)
	Implantation reaction	Wang et al., 1994 (46)
IL-15	Decidual marker	Okada et al., 2000 (19)
IGFBP-1	Decidual marker	Dimitriadis et al., 2002 (20)
TNF-α	Endometrial changes	von Wolff et al., 1999 (24)
	GM-CSF and CSF-1 production	Garcia-Lloret et al., 1994 (57)
PGE_2	Endometrial changes	Jabbour et al., 2003 (33)
	Multiple female reproductive failures	Lim et al., 1997 (28)
		Reese et al., 2001 (29)
	Differentiation of NK cells	Linnemeyer et al., 1993 (35)
VEGF	Angiogenic factor	Moller et al., 2001 (37)
GM-CSF	Trophoblastic differentiation	Garcia-Lloret et al., 1994 (57)
CSF-1	Trophoblastic differentiation	Garcia-Lloret et al., 1994 (57)

LIF = leukemia inhibitory factor; LIFR = LIF receptor; IL-1 = interleukin-1; IL-1 ra = IL-1 receptor antagonist; IL-1R tI = IL-1 receptor type I; EGF = epidermal growth factor; HB-EGF = heparin binding-EGF; IL-15 = interleukin-15; IGFBP-1 = insulin-like growth factor-binding protein 1; TNF-α = tumor necrosis factor alpha; PGE_2 = prostaglandin E_2; VEGF = vascular endothelial growth factor; GM-CSF = granulocyte-macrophage colony-stimulating factor; CSF-1 = colony stimulating factor-1.

(Castro-Rendon WA, Castro-Alvarez JF, Guzman-Martinez C, Bueno-Sanchez JC (2006) Blastocyst-endometrium interaction: intertwining a cytokine network. Braz J Med Biol Res 39: 1373-1385.)

The endometrium is modified in structure by progesterone and estrogens. These changes facilitate the phases of implantation which are: the apposition, the attachment and the invasion. Those three phases are called 'implantation window' **[12]**. The 'window of implantation' is defined as the limited period of time during which the uterine environment is conducive to blastocyst implantation. The simultaneous development of the embryo to the blastocyst stage and the differentiation of the uterus to the receptive state are very important at the implantation process **[13]**. The endometrial differentiation in order the blastocyst to be successfully implanted is stimulated by progesterone (P) and estrogen. Endometrial sensitivity to

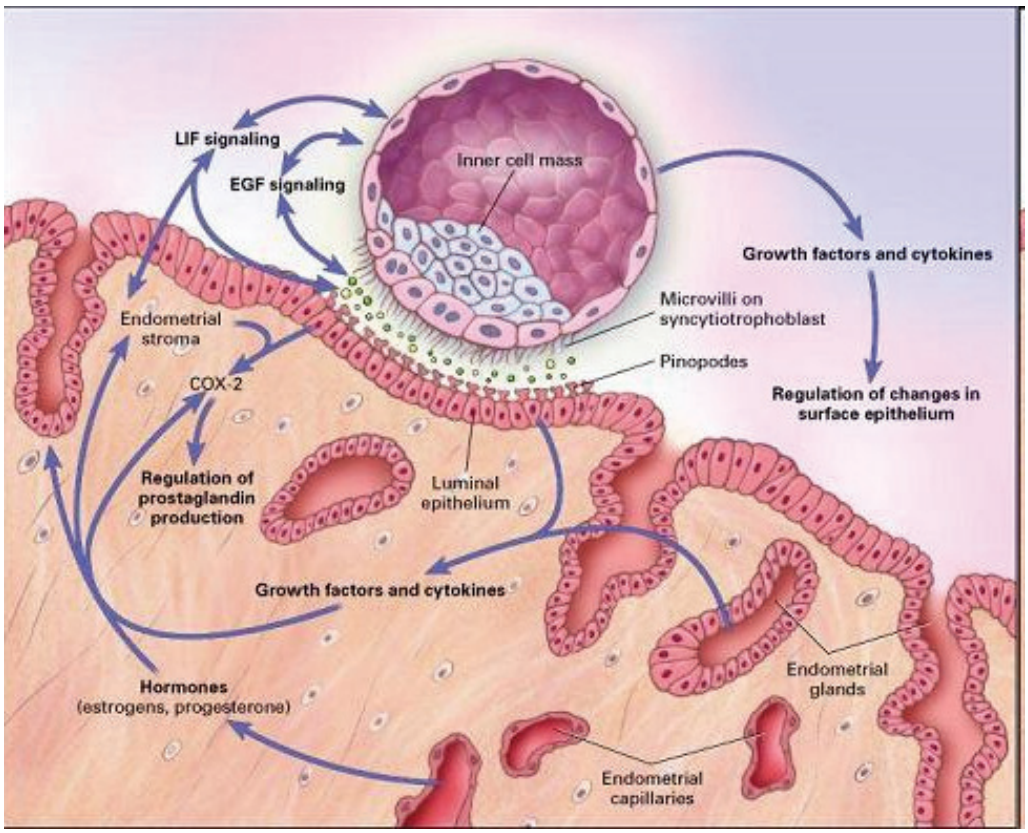
implantation due to ovarian steroids is programmed into three phases: prereceptive, receptive and nonreceptive. Blastocysts implant in the receptive phase, where all the morphological changes occur to endometrium.

During this phase, the plasma membrane of the luminal epithelial cells has no microvilli and develops large ectoplasmic projections, called pinopodes. Pinopodes are markers of endometrial receptivity and their appearance corresponds to the 'window of implantation' **[11,14]**. In humans, E2 (estradiol) induces ER (estrogen) and PR peptide synthesis in endometrial glands and stroma during the follicular phase. Estrogen exerts its proliferative effect on the endometrium, priming it for secretory transformation, which will follow under P stimulation**[15]**.

Moreover, it has been shown that chorionic gonadotropin (CG) implicates in implantation by direct effects on the endometrium and that the blastocyst modulates the uterine environment before implantation through CG signalling (reviewed by [**11]**). Furthermore, an important factor having a vital role in implantation is CRH (corticotrophin releasing hormone). The endometrial glands are characterised by high levels of CRH during the secretory phase of the menstrual cycle. Estrogens and glucocorticoids inhibit the promoter of the human CRH gene in transfected human endometrial cells **[16]**. P induces the production of CRH in primary cultures of human endometrial stromal cells. In turn, CRH induces stromal decidualization and potentiates the decidualizing effect of P **[17]**. Additionally, CRH regulates local modulators of the decidualization process; for example, it inhibits the enhancer PGE2, induces the inhibitor interleukin (IL) 1 and stimulates the inducer IL-6. These modulators exert a positive effect on the synthesis of endometrial CRH, completing this endometrial paracrine network, which could act as a local fine-tuner of decidualization **[18,19]**. Furthermore, it has been proposed that CRH has a role in establishing the 'immune privilege' of the maternal–fetal interface, thus accommodating blastocyst implantation. Indeed, CRH induces the synthesis of proapoptotic Fas ligand (FasL) in human invasive extravillous trophoblasts (EVTs) and maternal decidual cells. Therefore, it potentiates the ability of these cells to induce apoptosis of surrounding activated maternal T cells bearing the Fas receptor on their surface. This effect was reversed by antalarmin, a specific type 1 CRH receptor (CRHR1) antagonist. Administration of antalarmin in female rats decreased the number of implantation sites **[20]**. Finally, a recent study suggested a role for CRH in trophoblast invasion. CRH inhibited in vitro the invasion of human EVTs. This effect was mediated by CRHR1 and involved downregulation of the synthesis of the carcinoembryonic antigen-related cell adhesion molecule 1 by EVTs **[21]**.

Concerning the implantation window phase, despite the important role of ovarian steroids in implantation there are lots of P- and E2- regulated factors involved in implantation such as cytokines, growth factor, matrix metalloproteinase (MMPs), adhesion molecules, extracellular matrix components and homeobox element- containing genes **[22,23]**. One of the factors that plays important role in implantation is EGF. EGF promotes PG production and during pregnancy, increased EGF production leads to trophoblast differentiation **[24]**. In addition, transforming growth factor β(TGF-β) have been found to be expressed in

endometrial epithelium and stroma, to modulate maternal immune tolerance during implantation and to regulate several implantation related molecules such as VEGF, MMP-9, IGFBP-1 and LIF (leukemia inhibitory factor), a molecule that appears to be necessary for murine blastocyst implantation **[25,26**]. Furthermore, IL-11 is another cytokine involved in implantation, as it promotes P-induced decidualization of human stromal cells in vitro **[27]**. In primary cultures of human endometrial and decidual epithelial cells, estrogen induced, whereas P reduced, IL-11 secretion and IL-11 signalling was found to participate in the regulation of trophoblast invasion **[28**]. A recent study on women with recurrent miscarriage reported decreased synthesis of IL-11 in the endometrial epithelium **[29].** Also, CSF-1(colony stimulating factor) which is responsible for macrophage proliferation and differentiation has been found to be expressed in tissues in the maternal-fetal interface during implantation and early pregnancy and lack of CSF-1 has shown to provoke infertility problems in mice (reviewed by **[11]**).



Signalling and implantation system.

(Norwitz ER, Schust DJ, Fisher SJ (2001) Implantation and the survival of early pregnancy. N Engl J Med 345: 1400-1408)

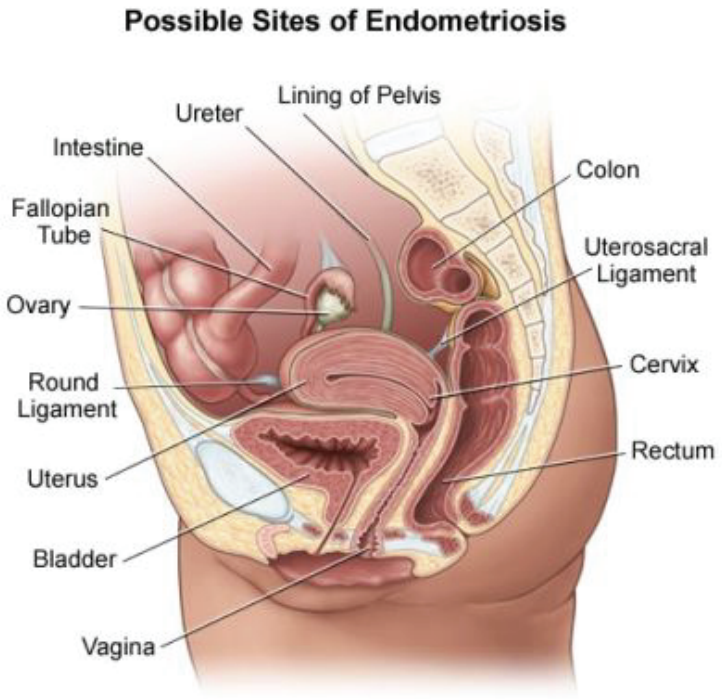
CHAPTER 2

ENDOMETRIOSIS

2.1. Definition and sites of endometriosis

Endometriosis is defined as the growth of endometrial glands and stroma at extra-uterine sites that responds to hormonal stimuli. Endometriosis is an inflammatory estrogen-dependent disease affecting 5 to 10% of women of reproductive age. It is characterized by the presence of endometrium-like tissue in sites outside the uterine cavity. The most popular sites of endometriosis are **[30]** :

- Ovaries (the most common site)
- Fallopian tubes
- The back of the uterus and the posterior cul-de-sac
- The front of the uterus and the anterior cul-de-sac
- Uterine ligaments such as the broad or round ligament of the uterus
- Pelvic and back wall
- Intestines, most commonly the rectosigmoid
- Urinary bladder and ureters

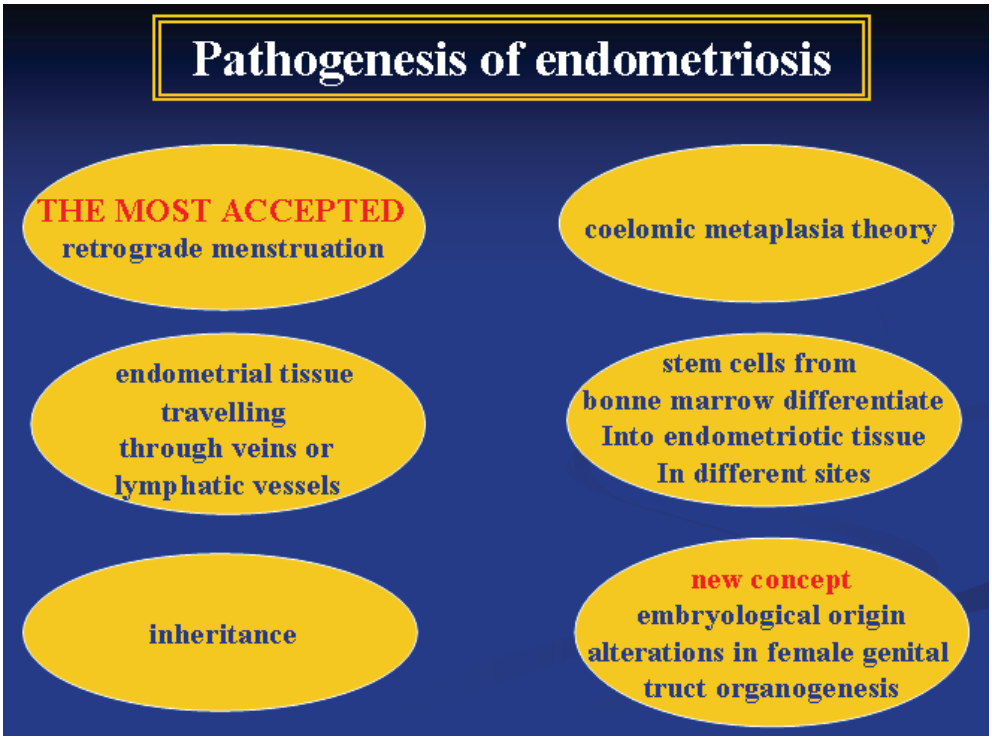


(http://www.endo-resolved.com/symptoms.html)

As previously mentioned, the normal-eutopic endometrium is shed each month during the menstrual cycle. In endometriosis, the endometrium grows outside the uterus and is unable to exit the body. The ectopic endometrium – endometriotic sites still detach and bleed such as eutopic- normal endometrium and internal bleeding, degenerated blood, tissue shedding, pain, formation of new tissue and inflammation of the surrounding areas usually occur**[30].**

2.2. Pathogenesis of endometriosis

In 1690, was the first time that endometriosis has been described by the German physician Daniel Shroen. There are many theories concerning its pathogenesis as the image displays:



Retrograde menstruation: The most common theory for the pathogenesis of endometriosis**[31]**. So, it has been proposed that the adhesion and growth of endometrial fragments into the peritoneal cavity happens as the menstrual endometrium pass backward through the fallopian tubes and implants in the peritoneal cavity. Although, this theory shows lots of controversies as it fails to explain the formation of endometriosis in very distant sites such as lungs, skin, lymph nodes, breasts, in early puberty and newborns, in male and in women affected by the syndrome of Mayer- Rokitansky – Kuster – Hauser who are characterised by congenital aplasia of the uterus **[30]**.

***Coelomic metaplasia*:** this theory claims that the formation of endometriotic lesions is a result of differentiation of mesothelial cells into endometrium-like tissue – metaplasia of the coelomic epithelium **[32]**.

Endometrial tissue travelling through veins and lymphatic vessels **[31]**

Bonne marrow stem cells differentiation to endometriotic tissue*:* a theory which claims that circulating stem cells originating from bonne marrow or from the basal layer of the endometrium could differentiate into endometriotic tissue at various sites **[33]**.

Vasculogenesis*:* Up to 37% of the microvascular endothelium of ectopic endometrial tissue originates from endothelial progenitor cells, which result in *de novo* formation of microvessels by the process vasculogenesis rather than the conventional process of angiogenesis [**34]**.

Embryological origin of endometriosis: suggesting alterations in the fine tunning of female genital structure organogenesis [**35]**.

2.3. Epidemiology and Risk factors

Endometriosis is a disease affecting mostly women of the reproductive age but two to four percent of women with endometriosis are postmenopausal exposed to the use of Hormone Replacement Therapy (HRT). It has been found that mostly Asian women suffer from endometriosis comparing to Caucasian women and lowest in African women. However, these studies are not always including factors like socioeconomic status and the availability of healthcare facilities which makes the results uncertain. There is a 7 times increased risk of endometriosis when a first degree relative has this disease, indicating that genetic factors are important. Obstruction of menstrual outflow (e.g., mullerian anomalies), exposure to diethylstilbestrol in utero, prolonged exposure to endogenous estrogen (e.g., because of early menarche, late menopause, or obesity), short menstrual cycles, low birth weight and exposure to endocrine-disrupting chemicals are thought to be the most common risk factors for endometriosis. Twin and family studies suggest a genetic component. Consumption of red meat and trans fats is associated with an increased risk of laparoscopically confirmed endometriosis, and eating fruits, green vegetables, and n−3 long-chain fatty acids is associated with a decreased risk. Prolonged lactation and multiple pregnancies are protective factors for endometriosis. Endometriosis is associated with increased risks of autoimmune diseases and ovarian endometrioid and clear-cell cancers, as well as other cancers, including non-Hodgkin's lymphoma and melanoma **[36-38].** Concerning the environmental toxins implication in endometriosis, the prevalence of endometriosis has been linked by several studies with exposure to dioxins but the mechanisms are poorly

understood **[39]**. Dioxin-exposed monkeys showed immune abnormalities similar to those observed in women with endometriosis [**40]**

2.4. Genetics

Inheritance and genetic predisposition has been linked to endometriosis [**41]**. Familiar studies have shown that daughters or sisters of patients with endometriosis are at higher risk of developing endometriosis; low progesterone levels may be genetic, and may contribute to a hormone imbalance. It has been suggested that there is a 6-fold increased incidence in women with an affected first-degree relative. Endometriosis can occur due to a series of multiple hits within target genes, a mechanism similar to that of cancer development and the mutation can be either somatic or heritable. Individual genomic changes associated with endometriosis are those referring to changes in chromosome 10 at region 10q26 and changes in the 7p15.2 region. There are many findings of altered gene expression and epigenetics, but these can be a result of environmental factors or altered metabolism. Moreover, there are lots of positive correlation between polymorphisms and endometriosis. Polymorphisms that mainly refer to cytokines and inflammation, steroid-synthesizing enzymes and detoxifying enzymes and receptors, estradiol metabolism, other enzymes and metabolic systems, adhesion molecules and matrix enzymes, hormone receptors, growth factor systems, and especially human leukocyte antigen system components showed strong correlation. Although, polymorphisms related to apoptosis, cell-cycle regulation and oncogenes have been found to be negatively correlated with endometriosis **[42]**.

2.5. Signs and Symptoms

The main clinical symptoms of women with endometriosis are chronic pelvic pain, adhesion formation and infertility. Apart from them there are lots of others symptoms as the table indicates [**38]**.

Pain symptoms	Bleeding symptoms	Bowel & Bladder symptoms	Other symptoms
Painful periods	Heavy periods with or without clots	Painful bowel movements	Tiredness/Lack of energy
Pain on ovulation	Prolonged bleeding	Bleeding from the bowel	Depression
Pain during an internal examination	'Spotting' or bleeding between periods	Symptoms of irritable bowel (diarrhoea, constipation, bloating – particularly during your period)	Back pain
Pain during or after sex	Irregular periods	Pain when passing urine	Leg pain
Pelvic pain	Loss of 'old' or 'dark' blood before a period	Pain before or after passing urine or opening bowel	

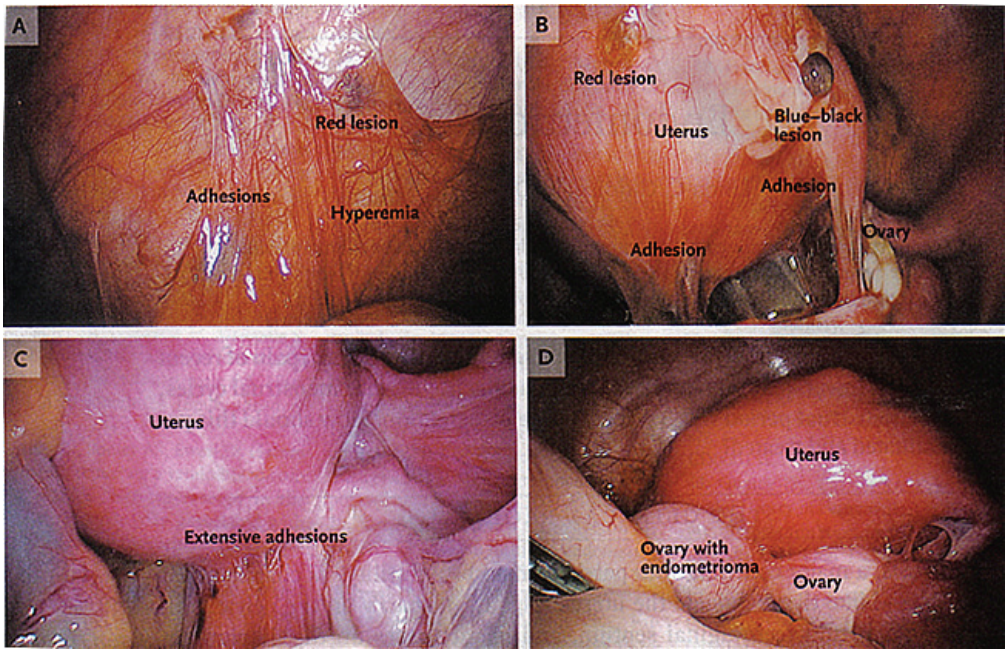
The pain can range from mild to severe that occurs on both sides of the pelvis, in the lower back and rectal area, and even down the legs. Pain is not correlated to the extent or stage (1 through 4) of endometriosis. Symptoms of endometriosis-related pain may include **[30]** :

- dysmenorrhea – painful, sometimes disabling cramps during menses; pain may get worse over time (progressive pain), also lower back pains linked to the pelvis
- chronic pelvic pain – typically accompanied by lower back pain or abdominal pain
- dyspareunia – painful sex
- dysuria – urinary urgency, frequency, and sometimes painful voiding

As endometriotic lesions are related to hormonal stimulation, they may "bleed" at the same time of menstruation by causing swelling, and inflammatory responses and at these circumstances pain may be caused. Pain can also occur from adhesions binding internal organs to each other, causing organ dislocation. Fallopian tubes, ovaries, the uterus, the bowels, and the bladder can be bound together in ways that are painful, not just during menstrual periods. Also, as endometriotic lesions develop their own nerve system, this may be another cause of severe pain **[43]**.

2.6. Diagnosis and staging

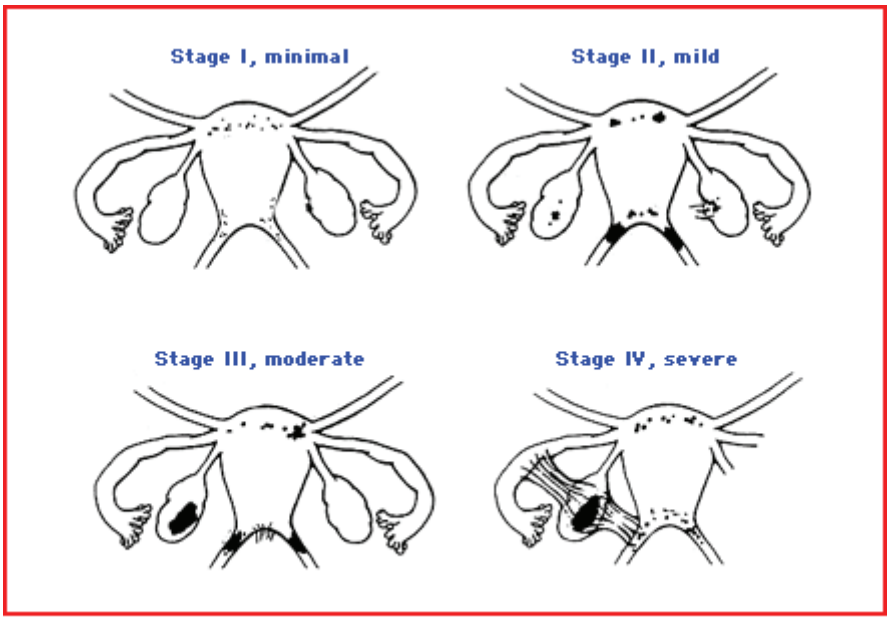
The definite method for diagnosis and staging of endometriosis is surgery as the figure shows



Peritoneal Lesions and an Ovarian Endometrioma Due to Endometriosis*[38]** *'Panel A shows an endometriotic implant (red lesion), adhesions, and hyperemia in the peritoneum. Panel B shows peritoneal implants, including red and blue–black lesions and adhesions. Panel C shows extensive adhesions distorting the normal pelvic anatomy. Panel D shows an endometrioma adherent to the posterior uterus and distending the ovarian capsule. (Images courtesy of Dr. Christopher Herndon, University of California, San Francisco.)'* ***[38]

Endometriosis can be staged I–IV (Revised Classification of the American Society of Reproductive Medicine) through surgery **[44]**. This point system assesses lesions and adhesions in the pelvic organs, but not the level of pain or infertility.

Stage I (Minimal): Findings restricted to only superficial lesions and possibly a few filmy adhesions
Stage II (Mild): In addition, some deep lesions are present in the cul-de-sac
Stage III (Moderate): As above, plus presence of endometriomas on the ovary and more adhesions.
Stage IV (Severe): As above, plus large endometriomas, extensive adhesions.



Examples of the classification of endometriosis Modified from the American Society for Reproductive Medicine.

http://www.rockymountainfertility.com/diagnosis/endometriosis/#

Endometrioma is sometimes misdiagnosed as ovarian cysts. The cyst fluid in endometriomas is thick and dark brown because it contains hemosiderin (hence, the name "chocolate cysts"), this color is not specific to endometriomas. Apart from surgery there are other nonsurgical diagnostic approaches such as transvaginal ultrasonography and magnetic resonance imaging (MRI) but the detection of peritoneal and ovarian implants and adhesions are poorly detected. Both imaging methods can successfully detect ovarian endometriomas. Transvaginal ultrasonography is preferred over MRI in the diagnosis of endometriomas due to lower cost. Doppler ultrasonography may help in establishing the diagnosis; it shows characteristically scant blood flow to an endometrioma, normal flow to normal ovarian tissue, and enhanced flow to an ovarian tumor. Moreover, levels of CA-125 may be elevated in endometriosis, but it is not recommended for diagnostics as it is characterised by poor sensitivity and specificity **[38]**.

2.7. Treatment of endometriosis

Treatment of endometriosis is divided in two categories depending on the pain management and the infertility management.

2.7.1. Pain management

Pain can be managed by a variety of medical therapies and surgery therapy. Medical treatment refers to pain killers and/or NSAID (nonsteroidal anti-inflammatory drugs). As far as the hormonal treatment concerned, it is used in order to minimize the estrogen stimulation of the endometriotic lesions including endometrial atrophy and amenorrhea eliminating the pain. Hormonal treatment is often used in combination with surgical treatment and/or to prevent recurrence of the disease after surgery. The types of hormonal treatment for endometriosis that have been used are since 1960's are displayed on the table above. Apart from medical treatment of pain, there is surgical therapy as well. The most common surgical procedure performed is laparoscopy. Surgical can relieve pain and improve fertility, by diminishing inflammatory response in the pelvic region and improving quality of patient's life. The removal of adhesions, fibrotic tissues and endometriotic cysts can reduce pain and improve fertility as well. In severe cases total hysterectomy and/or bilateral oophorectomy is performed to induce amenorrhea and thereby reduce inflammation and pain**[38]**.

Medical and Surgical Therapies for Endometriosis-Related Pelvic Pain **[38]**

Treatment	Indication	Type of Therapy	Side Effects and Complications	Comments
Medical therapy				
NSAIDs	Dysmenorrhea	First-line	Nausea, vomiting, gastrointestinal irritation, drowsiness, headache	Initiate treatment at beginning of or just before menses; somewhat decreased menstrual flow
Combined oral contraceptives				
Cyclic	Dysmenorrhea	First-line	Nausea, weight gain, fluid retention, depression, breakthrough bleeding, breast tenderness, headache, decreased menstrual flow	
Continuous	Dysmenorrhea, noncyclic chronic pelvic pain	Second-line	Nausea, weight gain, fluid retention, depression, breakthrough bleeding, breast tenderness, headache, amenorrhea	
Progestins				
Medroxyprogesterone acetate	Dysmenorrhea, noncyclic chronic pelvic pain	Second-line	Nausea, weight gain, fluid retention, breakthrough bleeding, depression, amenorrhea, delayed return of ovulation	
Levonorgestrel intrauterine system	Dysmenorrhea, dyspareunia	Second- or third-line	Bloating, weight gain, headache, breast tenderness	Especially beneficial for symptomatic rectovaginal endometriosis; hypomenorrhea or amenorrhea for 6–12 mo; can be used for up to 5 yr; not FDA-approved for endometriosis

GnRH agonists	Dysmenorrhea, dyspareunia	Second- or third-line	Hypoestrogenism (vasomotor symptoms, vaginal dryness, decreased libido, irritability, loss of bone mineral density)	FDA-approved for endometriosis pain; estrogen-progestin add-back therapy used to mitigate loss of bone mineral density
Aromatase inhibitors	Dysmenorrhea, noncyclic chronic pelvic pain	Third-line	Hypoestrogenism, induction of ovulation	Combined with progestagens, combined oral contraceptives, and GnRH agonists because ovulation may be induced; not FDA-approved for endometriosis pain
Danazol	Dysmenorrhea, noncyclic chronic pelvic pain	Second- or third-line	Hyperandrogenic side effects (acne, edema, decreased breast size)	Side effects limit widespread use

Treatment	Indication	Type of Therapy	Side Effects and Complications	Comments
Surgical therapy				
Laparoscopy				
Fulguration, ablation, and excision	Dysmenorrhea, noncyclic chronic pelvic pain, dyspareunia	First-or second-line	Risk associated with anesthesia and risk of infection, damage to internal organs, new adhesions, hemorrhage	First-line therapy for pelvic mass; commonly second-line therapy for pelvic pain resistant to medical therapy
Excision or drainage and ablation	Endometrioma >3 cm in diameter, chronic pelvic pain	First-line	Risk associated with anesthesia and risk of infection, damage to internal organs, new adhesions, hemorrhage	Potential for decreased ovarian reserve; excision is preferable to drainage and ablation
LPSN, nerve-pathway interruption (with conservative surgery)	Dysmenorrhea, dyspareunia, deep central pain	Third-line	Bleeding in the adjacent venous plexus, urinary urgency, constipation, painless first-stage labor	Technically challenging surgery; should be performed by surgeons with experience in LPSN
Hysterectomy, bilateral oo-phorectomy (abdominal, laparoscopic, total, or supracervical)	Noncyclic chronic pelvic pain	Fourth-line	Persistent or recurrent pain in 10% of patients, residual ovarian tissue	Reoperation may be necessary; measure FSH level to check ovarian remnant; add progestin to postoperative estrogen-replacement therapy for vasomotor symptoms
Adjunctive medical therapy after conservative surgery				
GnRH agonist	Dysmenorrhea, noncyclic chronic pelvic pain	Third-line	Hypoestrogenism	Used primarily in stage III or IV disease
Medroxyprogesterone acetate, danazol, combined oral contraceptives	Dysmenorrhea	Third-line	Medroxyprogesterone acetate: nausea, weight gain, fluid retention, breakthrough bleeding, depression; danazol: hyperandrogenic side effects (acne, edema, decreased breast size); combined oral contraceptives: nausea, weight gain, fluid retention, depression, breakthrough bleeding, breast tenderness, headache	Not commonly used

*FDA denotes Food and Drug Administration, FSH follicle-stimulating hormone, GnRH gonadotropin-releasing hormone, LPSN laparoscopic presacral neurectomy, and NSAID nonsteroidal antiinflammatory drug.

2.7.2. Fertility treatment

One of the main characteristics and symptoms of endometriosis is infertility, although women with endometriosis have reduced fecundity rate, they are not sterile. Several causal factors have been proposed; adhesions, ovulatory dysfunction, reduced implantation and decidualization capacity, embryotoxicity and fagocytosis of the sperm cells. Surgical intervention has been found to improve fertility rates of women with endometriosis **[30,38,45].** Gonadotropin therapy and intrauterine insemination, as well as in vitro fertilization (IVF), are efficacious treatments in women with infertility and endometriosis. Ablation of endometriotic lesions with lysis of adhesions is recommended for the treatment of infertility related to stage I or II endometriosis **[46,47]**.

2.8. Immunology in endometriosis

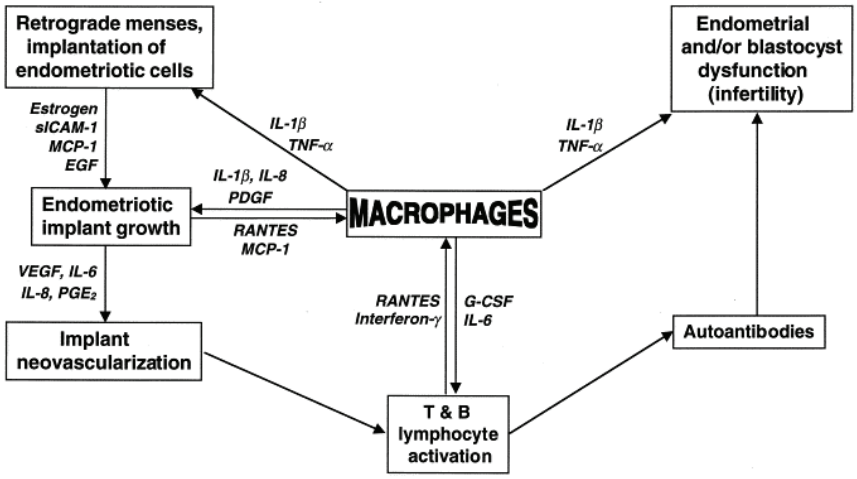
Endometriosis and altered eutopic endometrium

There are lots of studies suggesting that alterations in the immune response (cell-mediated immunity and humoral immunity) in endometriotic lesions may contribute to the development and progression of the disease and contribute to pain and infertility symptoms.

Endometriosis affects women of all ethnicics and approximately 5-10 % of women of reproductive age. It has been shown that 25-50% of infertile women have endometriosis and that 30-50% of women with endometriosis are infertile (reviewed by [**48]**). To better understand the association between infertility and endometriosis, an insight of the altered immune response characterising endometriosis, the 'autoimmunity' profile of endometriosis and the alteration of endometrium of women with ednometriosis would be of quite interest to be discussed.

Endometriosis is an inflammatory disease characterised by increased concentrations of cytokines, growth factors and macrophages. Macrophages are implicated in innate immune system and are responsible for secreting growth factors, cytokines and complement components, prostagladins and hydrolytic enzymes. Peripheral blood monocytes from women with endometriosis promote proliferation of cocultured autologous endometrial cells in contrast with monocytes from healthy women which suppress endometrial cell proliferation. Apart from the growth stimulatory effect of macrophages to endometriotic implants, macrophage products are connected with pathophysiology of endometriosis, referring to pain and infertility **[49,50]**. The concentration of peritoneal macrophages are highly increased in women with endometriosis compared to healthy ones and this can be linked to increased levels of CSF-1, monocyte chemoattractant protein (MCP-1), deriving from endometrial/endometriotic cells or peritoneal macrophages respectively **[51-54]**. It has been found that peritoneal environment controls the differentiation of macrophage precursors, leading them to an alternatively activated status. The alternatively activated macrophages are quite important for ectopic tissue to vascularize and grow **[55]**. Peripheral monocytes and peritoneal macrophages are altered

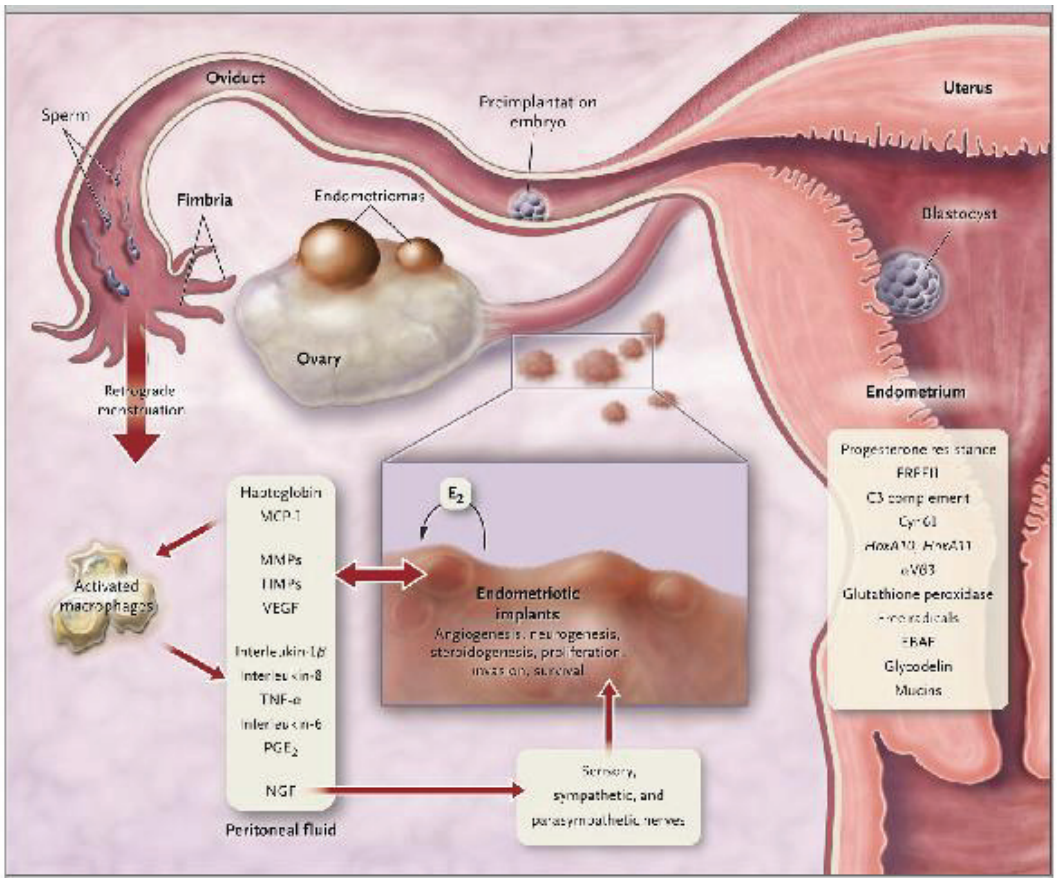
in women with endometriosis, more specifically, the peripheral monocytes of women with endometriosis do not differ in number with those of healthy women but they differ in their activation mode, as their activation status is increased in women with endometriosis. Peritoneal macrophages are increased in number, concentration and activation mode (reviewed by [**56]**). Increased macrophages are also related to the stage of endometriosis **[57]**. Macrophages play also a very important role in growth and development of endometriotic lesions and in generation of pain through interaction with nerve fibres as well. There is an accumulation of nerve fibres in peritoneal endometriotic lesions, which are stimulated by several factors such as ILs, VEGF, TNF- α, EGF and many others which are secreted by macrophages. So macrophages and their products had been found to play important role in stimulation, growth and repair of nerve fiber, inducing pain. Moreover, as VEGF is increased in women with endometriosis and had been found to act as a neurotrophic factor, stimulating the growth of nerve factors, might also play a vital role in nerve fiber density in endometriotic sites **[58]**. Furthermore, peritoneal fluid of women with endometriosis favorably induces monocyte differentiation in macrophages rather than dendritic cells (DCs) and this is mediated by IL-6. This can play a vital role in pathogenesis of endometriosis as DCs are quite important for the induction of immunity and tolerance **[59].**



Macrophages play a central role in the immunobiology of endometriosis[60]

Macrophages secrete cytokines and growth factors and concerning endometriosis, these macrophage products can promote stimulation of endometrial cell proliferation, implantation of endometrial cells or tissue, increased tissue remodeling through regulation of matrix metalloproteinase and increased angiogenesis of the ectopic endometrial tissue **[56,61]**. Increased number of macrophages in endometriotic sites equals increased number of cytokine production as well. Cytokines can act in an autocrine or paracrine way and have a role in initiation, propagation and regulation of immune and inflammatory responses and can have proliferative, cytostatic, chemoattractant or differentiative effects. Cytokines such as IL-1, IL-4, IL-6, IL-8, IL-10, mitochondrial protein (B-cell lymphoma 2, Bcl2), reactive oxygen species (ROS), soluble intercellular adhesion molecule (sic AM-1), TGF, VEGF, RANTES, and antioxidants are increased in the

peritoneal fluid of women with endometriosis. On the other hand, cytokines such as IL-1, IL-5, IL-13 and IFN are decreased in peritoneal fluid of endometriotic women. In the follicular fluid, IL-1, IL-6, IL-8, TNFα, MCP-1, endothelin-1 and immune cells including NK cells, B lymphocytes and monocytes increase but VEGF decreases [**48]**. All these cytokines are not only produced by macrophages but from endometriotic lesions as well. One example of this fact is that IL-6 has been found to be expressed by endometriotic stromal cells **[62]**. Some of the cytokines such as IL-1, IL-6, IL-8, IL-10 and TNF-α when they are found in elevated levels in peritoneal fluid of women with endometriosis they have been linked with embryotoxicity and infertility **[63]**. Another very important factor of immune response, mast cells, have been found to be elevated in the stroma of peritoneal endometriotic lesions compared to eutopic endometrium **[64]** and as mast cells in deep infiltrating endometriosis were found near the nerve fibers, this lead to the suggestion that mast cells might play a role in the endometriosis related pain **[65]**.



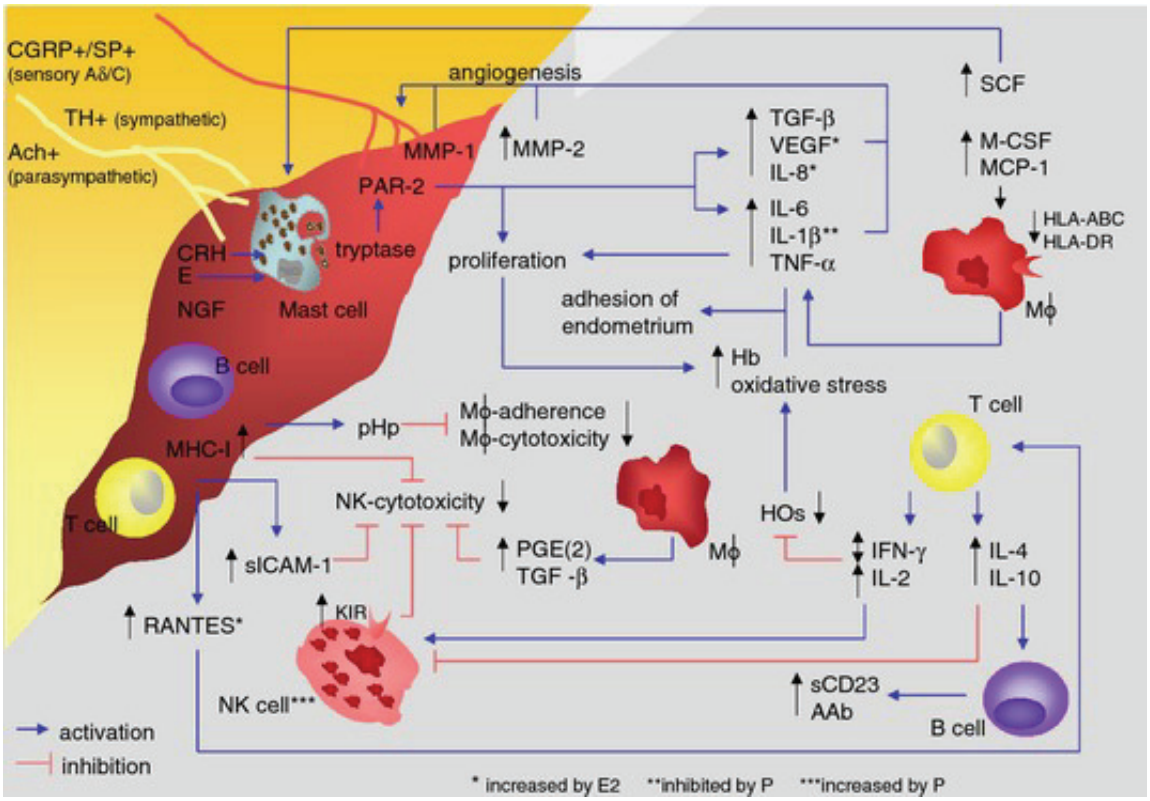
*Giudice LC (2010) Clinical practice. Endometriosis. N Engl J Med 362: 2389-2398.**[38]***

Natural killer cells (NK) cells are also altered in endometriotic women, as peripheral and peritoneal NK cells from women with endometriosis present decreased cytotoxicity to autologous and heterologous endometrium compared with those in healthy women. This reduced cytoxicity of NK cells has been correlated with the establishment of ectopic endometrium in the peritoneal cavity and this has also been linked to the severity of the disease **[56]**. Another cytokine promoting the adhesion creation for endometriotic implants is IL-8, as it stimulates the ability of endometrial stromal cells to adhere to an

extracellular matrix protein fibronectin **[61]**. There are several adhesion molecules implicated in endometriosis favoring the persistence of endometriotic tissue such as increased levels of intercellular adhesion molecule (ICAM). Factors promoting vascularization, angiogenesis and tissue remodeling in endometriosis are the elevated levels of VEGF and MMPS [**45]**.

Stress neuropeptides such as CRH and UCN, related to inflammation and pain are also present in endometriotic sites. CRH can activate mast cells to release tryptase activating PAR-2 (protease activated receptors) which leads to increased levels of VEGF, IL-8, IL-6 and proliferation of ectopic tissue (reviewed by **[45,66]**).

In addition to alterations in cell-mediated immunity in women with endometriosis, there are several humoral immunological changes including autoimmune phenomena such as autoantibodies against endometrial antigens, anti-laminin-1 autoantibodies and other autoimmune antibodies and polyclonal B cell autoimmune activation **[48]** .



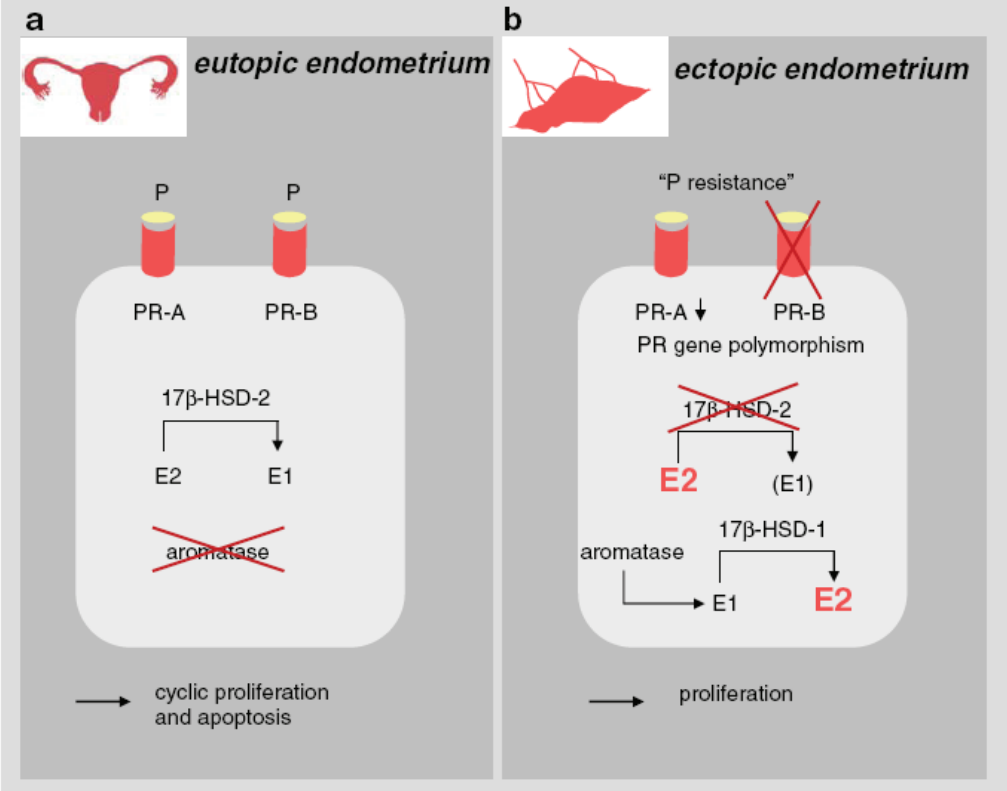
AAb autoantibodies, *Ach* achetylcholine, *CGRP* calcitonin-gene-related peptide, *CRH* corticotropin-releasing hormone, *E* estrogens, *E2* estradiol, *Hb* hemoglobin, *HO* heme oxygenases, *IL* interleukin, *HLA* human leukocyte antigen, *IFN-γ* interferon-γ, *KIR* killer cell inhibitory receptor, *M-CSF* macrophage colony stimulating factor, *MCP-1* monocyte chemotactic protein-1, *MHC-I* major histocompatibility complex class-I, *MMP* matrix metalloproteinase, *NGF* nerve growth factor, *NK* natural killer, *P* progesterone, *PAR-2* protease-activated receptor-2, *PGE(2)* prostaglandin E(2), *pHp* peritoneal haptoglobin, *RANTES* regulated upon activation normal T cell expressed and secreted, *sCD23* soluble CD23, *SCF* stem cell factor, *sICAM-1* soluble intercellular adhesion molecule-1, *SP* substance P, *TGF-β* transforming growth factor-β, *TH* tyrosine hydroxylase, *TNF-α* tumor necrosis factor-α, *VEGF* vascular endothelial growth factor. [**45]**

2.9. Steroid hormones in endometriosis and molecular mechanisms in eutopic endometrium (of endometriotic women and healthy women)

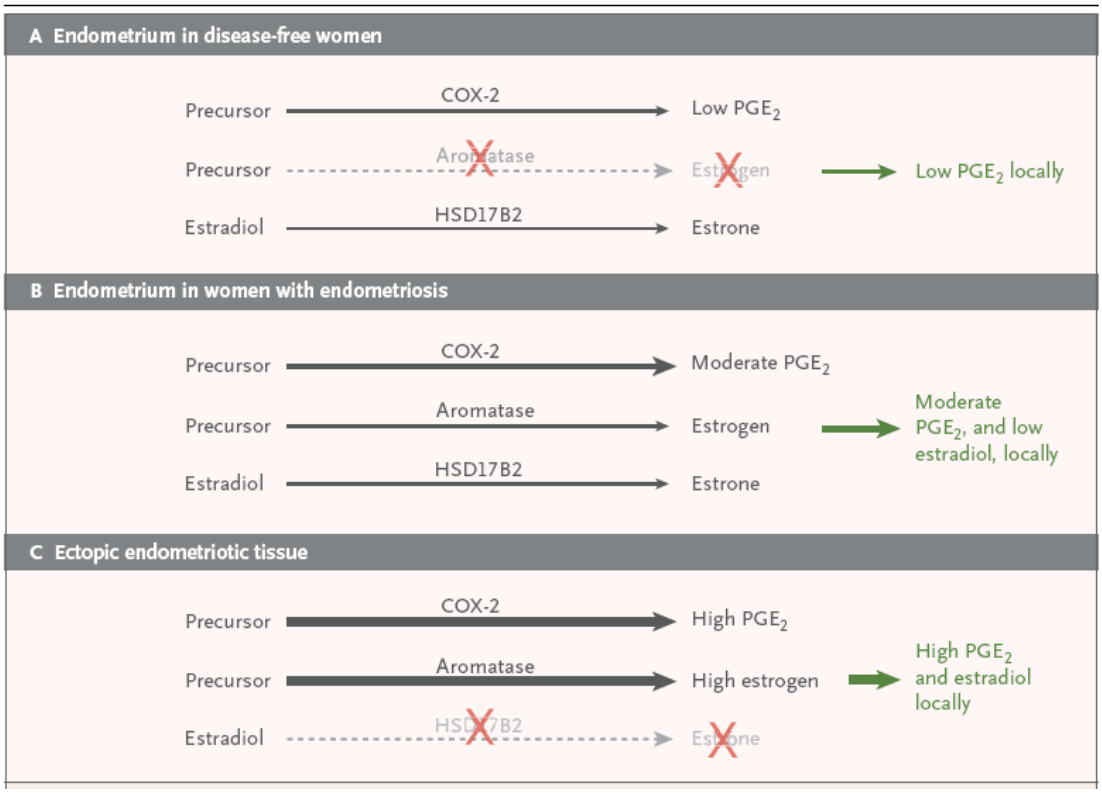
There are several differences between the eutopic and ectopic endometrium in endometriotic patients such as the overproduction of estrogen, prostagladins and cytokines in endometriotic tissue. Apart from that, eutopic endometrium of endometriotic women has different gene expression concerning implantation failure, infertility and progesterone resistance when compared to eutopic endometrium of healthy women **[30]**. As previously mentioned, inflammation, immune response, angiogenesis and apoptosis are altered in endometriosis in order to establish the survival of endometriotic tissue. These processes are regulated by estrogen or progesterone. It is of high importance to study the overproduction of estrogen and prostagladin and the progesterone resistance in endometriosis as there is a therapeutic target of aromatase in the estrogen biosynthesis procedure, COX-2 in the prostagladin pathway and the progesterone receptor which reduces pelvic pain or laparoscopically visible endometriosis. These targets have been associated with epigenetic markers (hypomethylation) that cause overexpression of nuclear receptors steroidogenic factor 1 (SF1) and estrogen receptor β **[67,68]**.

Steroid hormones play a very important role in the establishment and maintenance of endometriosis. Apart from the fact that endometriosis is an estrogen-dependent disease, recent studies suggest that progesterone fails to regulate the expression of genes during endometrial differentiation and this is critical for the disease process. Progesterone inhibits estradiol (E2)-dependent proliferation in the uterine epithelium. In humans and other vertebrates, the biological activities of progesterone are mediated by interaction with specific progesterone receptors (PRs) that are members of nuclear receptor superfamily of transcription factors. In contrast to healthy endometrium, hormone receptors remain at constant levels during the menstrual cycle in endometriosis **[69]**, which points at a disruption of the normal cyclic responsiveness to hormonal changes with regular apoptosis of the epithelium in endometriotic lesions. So, there is an important role of progesterone in endometriosis. Firstly, there is a PR gene polymorphism associated with endometriosis **[70]**. Secondly, there are low levels of PR isoform A (PR-A) and PR-B is not detected in extraovarian endometriosis **[71]**. Thirdly, progesterone-dependent regulation of target genes was found to be disrupted in endometriosis. In endometriotic tissue E2 accumulates and probably induces proliferation of endometrial tissue. Moreover, the enzyme aromatase is exclusively found in endometriotic stroma **[72]** and provides E1, which is further converted to E2 by 17β-HSD type 1 (17β-HSD-1), contributing to the accumulation of E2. The importance of aromatase for the endometriotic growth has been demonstrated by its genetic or enzymatic disruption **[73]**. Besides, E2 might also be involved in endometriosis related inflammation, as it stimulates several mediators of inflammation such as IL-8 and RANTES **[74]**. Levels of MMP-2 in peritoneal fluid positively correlate with E2, but inversely with progesterone, and estrogens can also co-stimulate peritoneal mast cell activation in rats **[75]**. Furthermore, both E2 and progesterone increase the release of VEGF from peritoneal macrophages **[76]** and may promote angiogenesis in endometriotic

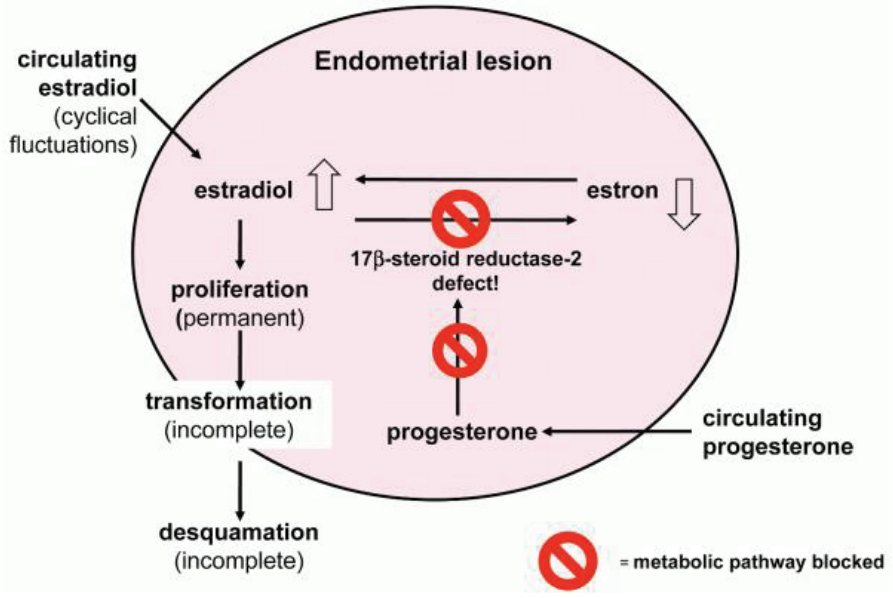
lesions. Given the high levels of E2 within the lesion, E2 might be more relevant in this context. In contrast to estrogens, progesterone or synthetically produced progestogens called progestins exert potent immunosuppressive properties, and mechanisms of dampening inflammation have been demonstrated for the progestin dydrogesterone **[77-79]**. Using other progestins available to date, a reduction in transplant growth, a degeneration of transplants, an increase in NK cells in peritoneal fluid, and a decrease in IL-1β secretion from peritoneal macrophages could be demonstrated **[80-82]**. In conclusion, E2 promotes endometriosis and progesterone/progestins dampen it, which is the rationale why progestins are widely used to treat endometriosis. In general, they act as agonists on the PR and have a functional anti-proliferative effect on endometrial tissue and endometriotic lesions. In addition, progestin treatment is generally better tolerated than other endometriosis therapies, which are associated with rather severe side effects **[83]**. Importantly, progestins are known to be effective in the control of pain symptoms in general or related to endometriosis **[79,83,84]**. However, not all patients respond to this regimen **[84]**. This clinical observation may be explicable by the partial resistance of endometriotic tissue to progesterone action due to the down-regulation of its receptors**[45]**.



Progesterone resistance and estradiol accumulation in endometriosis **[45]**

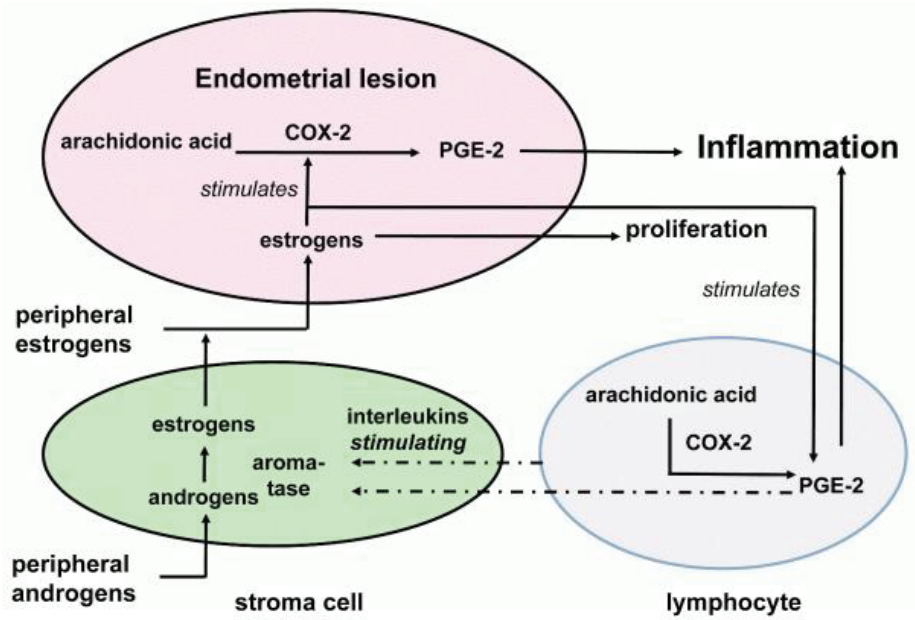


Normal endometriosis and endometriosis PGE2 production[30]



Schweppe KW et al. Journal für Reproduktionsmedizin und Endokrinologie - Journal of Reproductive Medicine and Endocrinology 2013; 10 (Sonderheft 1): 102-119 ©

Estrogen metabolism in endometrial lesions
*Estrogen metabolism in endometrial lesions: Defective 17-beta-steroid-dehydrogenase type 2. The bioactive estradiol cannot be converted into the less active estron. In the normal endometrium, progesterone activates this 17-beta-HSD Type 2 and has an antiproliferative effect; this mechanism is disrupted in endometrial lesions (so-called progesterone block).**[85]***



Schweppe KW et al. Journal für Reproduktionsmedizin und Endokrinologie - Journal of Reproductive Medicine and Endocrinology 2013; 10 (Sonderheft 1): 102-119 ©

Vicious circle in the endometrial focus
*Vicious circle in the endometrial focus maintains the proliferation and inflammatory reaction: (1) local estrogen is produced by aromatase activation; (2) estrogens stimulate prostaglandin synthesis via the activation of the COX-2 enzyme; (3) Prostaglandin E-2 again stimulates aromatase. Whether the aromatase is activated in the endometrial lesion itself or in the surrounding tissue (fat, peritoneum) is controversial.**[85]***

2.10. Altered endometrium in women with endometriosis

Despite the fact that eutopic end ectopic endometrium of women with endometriosis are histologically similar, these tissues have some biochemical differences. Apart from that, there are several differences between eutopic endometrium of healthy women and endometriotic women including differences in structure, proliferation, immune components, adhesion molecules, proteolytic enzymes and inhibitors, steroid and cytokine production and responsiveness, gene expression and protein production **[86].**

Apart from the differences presented in tables, there are several genes with differential expression in endometriotic lesions, responsible for the loss of cellular homeostasis in endometriotic lesions **[87]**.

Qualitative and quantitative differences between endometriotic lesions and eutopic endometrium[88]

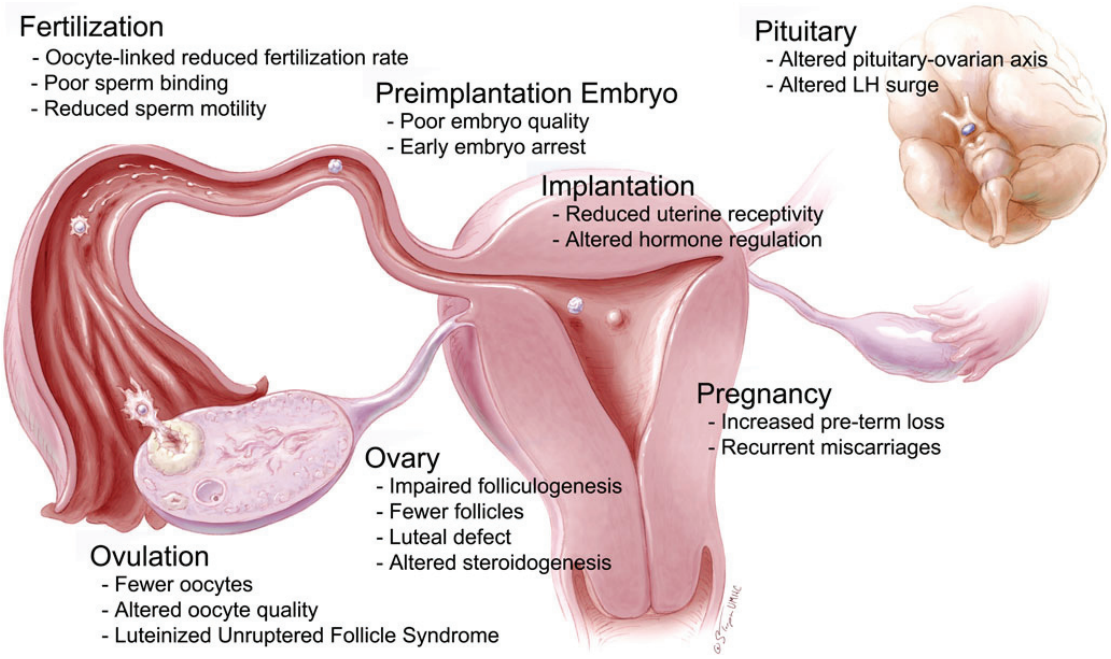
Anomaly	Description in endometriotic lesions
Steroid hormone responsiveness & receptor content	• Less hormonally regulated than eutopic endometrium and heterogeneous content and altered cyclic pattern of expression of steroid receptors
Growth factor responsiveness and receptor content	• Diminished response to EGF • Fibroblast growth factor similar to eutopic endometrium • Increased expression of GM-CSF in the secretory stage • Increased INF g mRNA and receptors • Deficiency of IL-1 receptor antagonist
Protein production	• Synthesize and secrete more CA 125 • Lack of estrogen regulation of complement component 3 • Significant expression of ENDO-I = endometriotic haptoglobin
Expression of enzymes and their inhibitors	• Higher levels of cathepsin D • Continuous expression of several MMPs • Overexpression of TIMPs *in vitro* • High levels of uPA

Endometrial anomalies in eutopic endometrium from women with endometriosis[88]

Anomaly	Description
Structure	• Increased heterogeneity in surface epithelium, reduced glandular and stromal mitoses, basal vacuolated cells • Reduced endometrial thickness
Proliferation	• Increased numbers of proliferating endometrial epithelial, stromal, and endothelial cells • No differences in endometrial cell proliferative activity
Apoptosis	• Impaired spontaneous apoptosis. • No significant difference in apoptosis or Bcl-2
Immune components	• Increased secretion of complement component C3 • No difference in presence of C3 or C4 • Presence of endometrial antigens of MW 60 and 66 kDa of the IgG class • Decreased mitogenicity for autologous lymphocytes • Trend for fewer T-suppressor/cytotoxic ($CD8^+$) cells and endometrial granulated lymphocytes but more T-helper/inducer ($CD4^+$) cells, $CD68^+$cells and $CD16^+$ cells • No difference in defined stromal leukocyte subpopulations • Increased numbers of $CD45^+$, $CD43^+$, and $CD3^+$ intraepithelial leukocytes • Increased resistance to the cytotoxic effect of heterologous lymphocytes • Increased expression of heat shock protein 27
Cell adhesion molecules	• Lack of αv3 expression on endometrial epithelium • No difference in endometrial epithelial αv3 expression • Increased expression of β-1 integrins and E-cadherin on endometrial glandular epithelium
Proteases and their inhibitors	• Aberrant production of matrix metalloproteinases (MMPs) and their inhibitors, the tissue inhibitor of metalloproteinases (TIMPs)
Steroid and cytokine production and responsiveness	• Expression of aromatase P450 • No difference in estrogen nor progesterone receptor expression • Increased secretion of hepatic growth factor • Elevated production of IL-6 • Elevated responsiveness to IL-1 and IL-6 • Increased production of MCP-1

2.11. Endometriosis and infertility

Infertility caused by endometriosis refers to almost every part of the female reproductive system.



Factors associated with reduced fecundity in women with endometriosis ***[89]***

2.11.1. Infertility – endometriosis – Pituitary

In the normal cycle of fertile women, the pituitary secretes FSH and LH to stimulate growing ovarian follicles. These follicles provide positive and negative feedback to the pituitary culminating in an LH surge to signal ovulation. However, in women with endometriosis, a pituitary-ovarian axis dysfunction preventing normal cyclic changes in the ovary as the length of the follicular phase is extended in endometriosis **[90,91]** and women with endometriosis have delayed LH secretion and lower levels of LH leading to abnormal urinary hormone profiles **[90,92-94]**. All these can impair follicular growth, ovulation and corpus luteum development in the ovary specifically with respect to the timing of ovarian events**[89].**

2.11.2. Infertility – endometriosis – ovary

Follicular phase

Folliculogenesis is impaired in women with endometriosis. The number of preovulatory follicles, follicular growth, dominant follicle size and follicular estradiol concentrations are reduced in ovaries of endometriosis patients. The follicular fluid of patients with endometriosis has been reported to have altered hormone profiles including reduced estrogen, androgen and progesterone and increased activin. Further, the follicular fluid from patients has been shown to contain factors such as cytokines and growth factors that might

promote the maintenance of endometriotic lesions and lead to a suboptimum follicular environment (reviewed by **[89]**).

Ovulation phase

In women with endometriosis, mechanisms of ovulation are impaired. The LH surge might be altered and there is a deficiency in follicular LH receptors **[95]**. In addition, lower levels of estrogen and progesterone have been found in the serum and urine of women with endometriosis. Changes in proteolytic enzymes, cytokines, inflammatory molecules and the vasculature, all of which are required for normal ovulation, can also be found in the follicles of women with endometriosis. Taking everything into consideration, these altered events could cause ovulatory dysfunction in endometriosis such as the fact that oocytes become trapped in a luteinizing corpus hemorrhagicum, defined as luteinized unruptured follicle syndrome (LUFs), has been associated with endometriosis and infertility in women. Peritoneal concentrations of steroid hormones, including progesterone and estradiol, are reported to decrease in women with LUFs (reviewed by **[89]**).

Luteal phase

Disrupted luteal function has been found in endometriosis patients and affecting both large and small luteal cells. Women with endometriosis with luteal defects secrete less progesterone than those from healthy patients and they are more likely to experience infertility **[96,97]**.

2.11.3. Infertility – endometriosis - Ooocyte quality

Women with endometriosis ovulate fewer oocytes than healthy women **[98,99]** and those oocytes ovulated by women with endometriosis are sometimes not of good quality **[100]**. There are several factors contributing to the failure of a spermatozoon to fertilize a potentially compromised oocyte in women with endometriosis. Increased peritoneal macrophages during endometriosis can lead to increased phagocytosis of healthy spermatozoa **[101]**. Interleukin-6 (IL-6) present in the peritoneal fluid of women with endometriosis **[102]** reduce sperm motility **[103]**.

2.11.4. Infertility – endometriosis - Embryo development

Endometriosis negatively impacts on embryo development including cytoplasmic fragmentation, darkened cytoplasm, reduced cell numbers and increased frequency of arrested embryos leading to significantly fewer transferable blastocysts. So women with endometriosis are characterised by reduced quality of embryos **[99]**. Inflammatory cytokines in the peritoneal fluid of women with endometriosis play also an important role in decreased embryo quality. Murine embryos cultured in the presence of peritoneal fluid from women with endometriosis have decreased rates of development after the two-cell stage, increased rates of DNA fragmentation and apoptosis compared with treatment by control peritoneal fluid **[104]**.

Further, embryos cultured in the presence of IL-6 stayed at the blastocyst stage or earlier **[105]**. Increased concentrations of inflammatory cytokines or reactive oxygen species (ROS) have been found to be embryo toxic causing apoptosis or programmed cell death **[106]**.

2.11.5. Infertility – endometriosis - Decidualization – uterine receptivity

Proper decidualization and uterine receptivity allows the developing embryo to implant in endometrium and this process includes regulation by hormones, cytokines, adhesion molecules and other factors **[107]** . Integrins which are cell surface receptors that mediate intracellular signals play a major role in this process specifically αVβ3 **[108]**, as about 50% of women with endometriosis have decreased or, in some cases, absent expression of endometrial αVβ3. Other uterine biomarkers of implantation such as glycodelin A, osteopontin, leukemia inhibitory factor and lysophosphatidic acid receptor 3 are reduced in women with endometriosis **[109]**. Moreover, concerning decidualization, steroid hormone pathways are altered in endometriosis as previously mentioned so, in women with endometriosis there is an upregulation of endometrial estrogen receptors, aromatase is also aberrantly expressed by the endometrium of women with endometriosis, increasing the amount of active estradiol **[110]**, altered estrogen during receptivity even further, 17β- hydroxysteroid dehydrogenase-2 is downregulated thereby inhibiting estradiol inactivation leading to a local increase in estrogen action **[109]**, a progesterone resistance in endometriosis **[111]** both in eutopic and ectopic endometrium **[112]**, differential expression of the isoforms of the progesterone receptor occurs in endometriosis: isoform A is present but B is not, most likely because of aberrant methylation of its promoter **[71,113]**. Reduced progesterone receptors and decreased levels of estrone lead to high levels of estradiol furthering the progesterone resistance. These data provide evidence for mechanisms involved in reduced uterine receptivity.

2.11.6. Infertility – endometriosis - Embryo implantation

Women with endometriosis are reported to experience implantation failure more often than controls **[99,114]**. Defects in embryo implantation might be associated with hormone level alterations, embryo anomalies and/or endometrial anomalies as described. For example, embryo anomalies can include slow growth and delayed blastocyst hatching, which are detrimental for implantation of the embryo in the uterine endometrium **[115]**. Concerning the hypothalamic pituitary adrenal axis and its main regulatory molecules as CRH and UCN are characterised, it has been found that eutopic endometrium of women with endometriosis is characterised by a decreased expression of CRH and UCN during menstrual cycle by a reduced response to CRH and UCN and an impaired expression of CRHR1 suggesting a disrupted local CRH/UCN/CRHR1 pathway in the disease and this may explain the reduced fertility characterizing patients with endometriosis as CRH is one of the main regulators of decidualization and implantation **[116]**. Moreover, peripheral CRH, increasing upon high psychological stress, might contribute to the peritoneal inflammation present in endometriosis associated with infertility problems involving unsuccessful implantation and decidualization.

2.11.7. Infertility – endometriosis – miscarriage

Women with endometriosis can have an increased risk of miscarriage and recurrent miscarriage **[117]** due to B cell immunodeficiency and autoantibodies **[118]**. Also, women with endometriosis have an increased risk of spontaneous abortion.

2.11.8. Infertility – endometriosis – altered peritoneal milieu

Endometriotic lesions secrete proteins including prostaglandins, haptoglobin, cytokines such as IL-1, IL-6, IL-8 and IL-10; growth factors, such as vascular endothelial growth factor, nerve growth factor, transforming growth factor-β1 and 2, insulin-like growth factor-2, cellular remodelling enzymes, such as the matrix metalloproteinases (MMPs) and their inhibitors (tissue inhibitors of metalloproteinase, TIMPs **[89]**.

CHAPTER 3

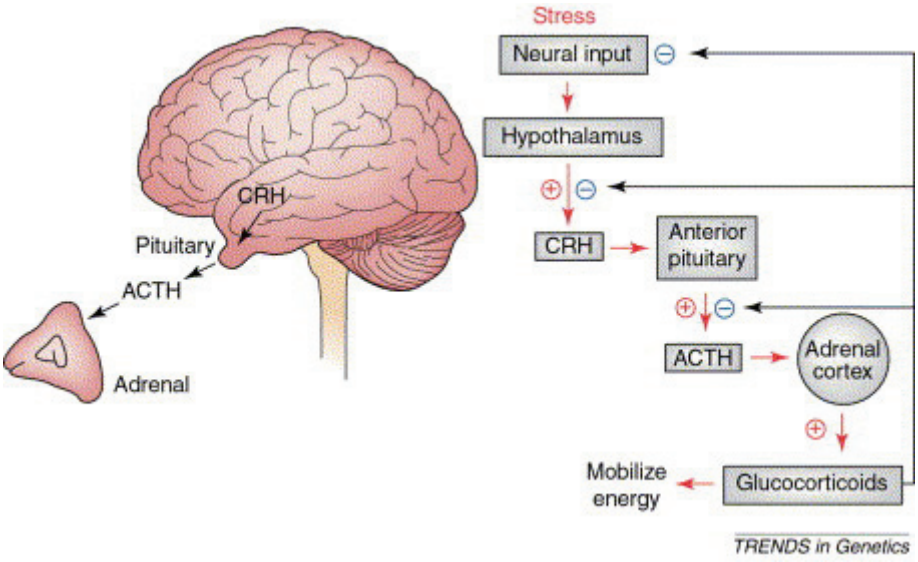
Corticotropin releasing hormone (CRH)

Corticotropin-releasing hormone (CRH) is a 41-amino acid peptide derived from a 196-amino acid prehormone. The human CRH gene maps in the long arm of chromosome 8 and the size of its transcript is 1.3kb. CRH is secreted by the paraventricular nucleus (PVN) of the hypothalamus in response to stress. CRH is the main regulator of the hypothalamic- pituitary adrenal axis (HPA) together with arginine- vasopressin (AVP), both produced by parvicellular neurons of the paraventricular nucleus of the hypothalamus into the hypophyseal portal system. HPA together with the arousal and autonomic nervous system constitute the stress system **[119]**.

3.1. CRH - HPA axis

The **hypothalamic-pituitary-adrenal axis (HPA or HTPA axis)** is a complex set of direct influences and feedback interactions among the hypothalamus, the pituitary gland (a pea-shaped structure located below the hypothalamus), and the adrenal (also called "suprarenal") glands (small, conical organs on top of the kidneys). HPA axis is a neuroendocrine system that controls reactions to stress and regulates many body processes, including digestion, the immune system, mood and emotions, sexuality and energy storage and expenditure. The paraventricular nucleus of the hypothalamus contains neuroendocrine neurons that synthesize and secrete vasopressin and corticotropin-releasing hormone (CRH). The anterior lobe of the pituitary gland where CRH and vasopressin stimulate the secretion of adrenocorticotropic hormone (ACTH), once known as corticotropin. ACTH in turn acts on the adrenal cortex, which produces glucocorticoid hormones (mainly cortisol in humans) in response to stimulation by ACTH. Glucocorticoids in turn act back on the hypothalamus and pituitary (to suppress CRH and ACTH production) in a negative feedback cycle.

CRH and vasopressin are released from neurosecretory nerve terminals at the median eminence. CRH is transported to the anterior pituitary through the portal blood vessel system of the hypophyseal stalk and vasopressin is transported by axonal transport to the posterior pituitary. There, CRH and vasopressin act synergistically to stimulate the secretion of stored ACTH from corticotrope cells. ACTH is transported by the blood to the adrenal cortex of the adrenal gland, where it rapidly stimulates biosynthesis of corticosteroids such as cortisol from cholesterol. Cortisol is a major stress hormone and has effects on many tissues in the body, including the brain. In the brain, cortisol acts on two types of receptor - mineralocorticoid receptors and glucocorticoid receptors, and these are expressed by many different types of neurons **[120]**.



The hypothalamic-pituitary-adrenal (HPA) axis. The initial changes in the axis in response to stressful stimuli are shown in red. Stress increases corticotropin-releasing hormone (CRH) release from the hypothalamus. CRH binds to CRH receptors on anterior pituitary cells resulting in increased adrenocorticotropic hormone (ACTH) release. ACTH, in turn, is carried via the blood to the adrenal cortex where it stimulates glucocorticoid production and release. Glucocorticoids cause metabolic changes that allow one to respond to the stressor. They also provide negative feedback (shown in blue) to decrease synthesis and release of CRH and ACTH to terminate the stress response and return the system to homeostasis. ***[121]***

3.2. CRH receptors and CRHBP

CRH is involved in the control of cardiovascular, gastrointestinal, immune and reproductive systems. CRH exerts its effects by binding to plasma membrane receptors. Corticotropin-releasing hormone receptors (CRHRs), also known as corticotropin-releasing factor receptors (CRFRs) are a G protein-coupled receptor family that binds corticotropin-releasing hormone (CRH). Three CRH receptor genes had been identified so far CRHR1, CRHR2, CRHR3 – identified only in catfish **[122-124]**. In mammals, only CRHR1 and CRHR2 have been identified. CRHR1 binds CRH with high affinity and eight slice variants have been identified that encode different isoforms R1α,β, c, d, e, f, g, h. CRHR2 is encoded by a distinct gene that has three slice mRNA variants encoding CRHR2 α,β,γ receptor subtypes with unique tissue distribution. The two CRH receptor families share 70% homology at the amino acid level. The wide distribution of CRH receptors (central and peripheral nervous system, adrenals, retina, spleen, heart and skeletal muscles, skin, ovary, testis and myometrium) suggests that CRH plays an important role in the physiology of these organs. CRHR1 has

been detected in human endometrial stromal cells in both the proliferative and the secretory phase and is also involved in decidualization of stroma **[125]**. CRH and CRHR1 have also been detected in human endometrial adenocarcinoma Ishikawa cell line **[126].** Ishikawa cell line expresses almost all steroid hormone receptors and represents an extensively used in vitro model for the study of steroid hormone effects on human endometrium **[127-129]**. CRHR2α and UCN (one the native ligand for CRHR2α) is expressed in human endometrium **[130]** and pregnant myometrium. CRHRs are present on resident macrophages and on endothelial cells of all pregnancy tissues from trophoblasts, myometrium, deciduas and fetal membranes **[131]**.

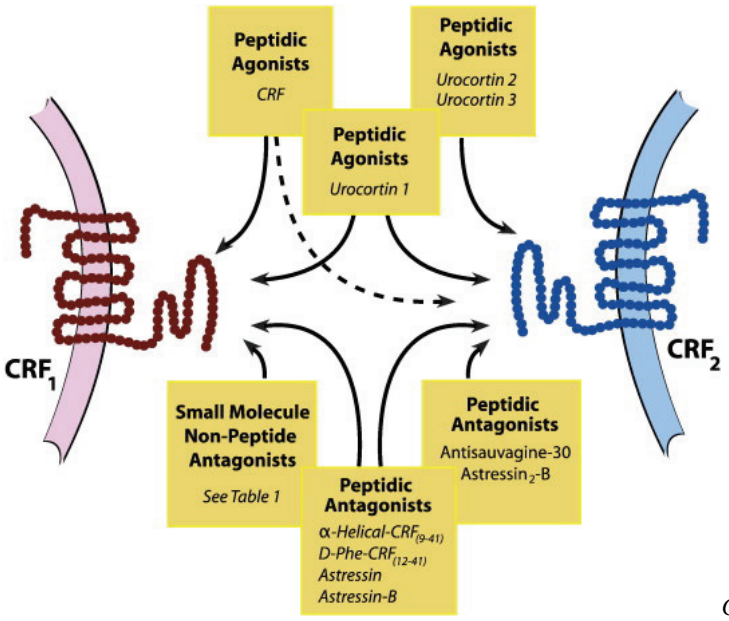
Moreover, CRH modulate its biological effects by a soluble high- affinity CRH-binding protein (CRH-BP). CRHBP is circulating in humans and its physiological role is to control the availability of the free ligand. Corticotropin-releasing factor-binding protein is a protein that in humans is encoded by the *CRHBP* gene. It belongs to corticotropin-releasing hormone binding protein family. In neuronal sites, CRH-BP regulation is under the control of CRH and protein kinase A, effectors such as cAMP, but also by known mediators of the CRH- system such as glucocorticoids and cytokines. CRH-BP binds with high affinity to CRH and consists of 322 amino acids. It is synthesised in liver and expressed by various sites such as brain and human placenta. In human, its gene is localized in the long arm of chromosome 5(5q11.2 –q13.3) **[132]**.

3.3. Urocortin

Urocortin belongs to the corticotrophin releasing hormone family. The CRH family include a variety of neuropeptides and consists of CRH, urocortin 1, urocortin 2 (stresscopin-related peptide), urocortin 3(stresscopin) **[133]**. Urocortin is a 40-amino acid peptide which share 45% sequence homology with CRH. Its affinity for CRHR1 is the same as CRH but it binds with higher affinity, even higher than CRH does with CRHR2, suggesting that this could be the endogenous ligand for this receptor subtype **[134,135]**. UCN is expressed in several sites in the nervous system controlling behaviour, stress response and food ingestion. UCN is synthesised in cell bodies of the Edinger Westphal nucleus, lateral superior olive and supraotic nucleus in the brain as well as in non-neuronal site such as the gastrointestinal tract, adipose tissue, heart, testis, kidneys, adrenals, pancreas, cardiac myocytes, skin. It is expressed in immune and cardiovascular system but reproductive system as well (ovaries, myometrium, endometrium, placenta, fetal membranes) **[21]**. Moreover, urocortin has been found to be correlated to maintenance of effective labor, onset of labor, spontaneous abortion and preeclampia **[133]**.

3.4. CRH receptor antagonists

CRH and UCN 1, 2, 3 act as endogenous peptide agonists with varying relative affinities for the CRHR1 and CRHR2. Peptidic antagonists are either non-selective for the two subtypes (a- helical- CRH(9-41), D-phe- CRH(12-41) , Astressin, Astressin-B) or are selective for the CRHR2(anti-sauvagin-30, Astressin2-B). Small molecule, non-peptidic antagonists show a high degree of selectivity for the CRHR1, such as antalarmin **[136]**. Antalarmin is a close chemical analog of CP-154, 526 and it is a non-peptidic CRH antagonist, selective for the CRHR1 receptor subtype **[137,138]**. Antalarmin is a non-peptide drug that blocks the CRHR1 and, as a consequence, reduces the release of ACTH in response to chronic stress **[139]**.This has been demonstrated in animals to reduce the behavioural responses to stressful situations **[140]** and it is proposed that antalarmin itself, or more likely newer CRH antagonist drugs still under development **[141]** could be useful for reducing the adverse health consequences of chronic stress in humans, as well as having possible uses in the treatment of conditions such as anxiety, depression, and drug addiction **[142].** Chronic antalarmin treatment also showed anti-inflammatory effects and has been suggested as having potential uses in the treatment of inflammatory conditions such as arthritis **[143]** as well as stress-induced gastrointestinal ulcers **[144]** and irritable bowel syndrome **[145,146]** Antalarmin has been used to elucidate the roles of CRH in stress, inflammation and reproduction, suggesting that this could be a regulatory key in reproductive functions with an inflammatory component, such as ovulation, luteolysis, implantation and parturition. Antalarmin has been found to decrease FasL expression and inhibited apoptosis of T-lymphocytes in implantation sites **[147,148]**.



CRH ligands and receptors. ***[136]***

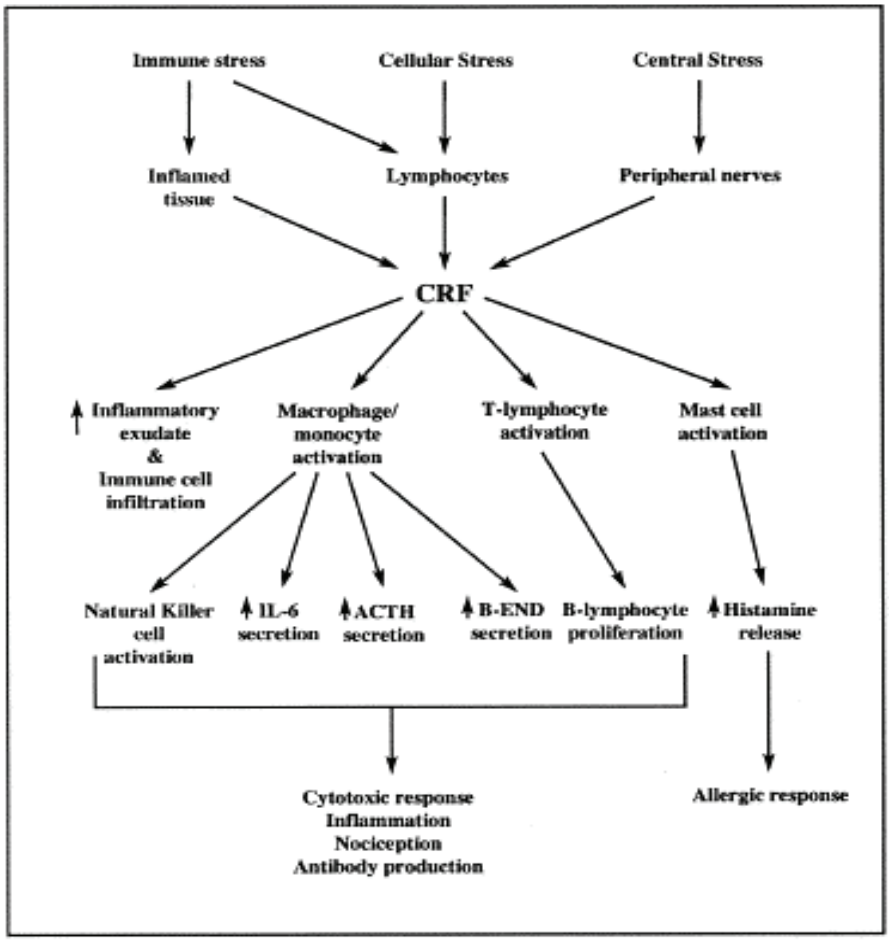
CRF_1 receptor antagonists. This table summarizes current publically-available information about compounds reported to be (or presumed to be, if data are not available) non-peptidic, potent, selective, orally active antagonists of the CRF_1 receptor. Numerous agents listed are not in development but are used as pharmacological probes in animal studies. Some agents have reached clinical testing in various disorders, as described by publications and/or company press releases, and some are reported to be discontinued. "CRF_1 Aff." refers to reported or estimated affinity in CRF_1 receptor binding assays (IC_{50} or K_i, in nM). (n.a. = not available).

Name	Company/Stakeholder	Chemical Name	CRF^1 Aff.	Status (estimated farthest advanced)	References
Antalarmin		N-butyl-N-ethyl-2,5,6-trimethyl-7-(2,4,6-trimethylphenyl) pyrrolo[3,2-e]pyrimidin-4-amine	<10	Preclinical	(Seymour et al., 2003)
CP-154,526	Pfizer	(2,5-dimethyl-3-(6-dimethyl-4-methylpyridin-3-yl)-7-dipropylamino-pyrazolo [1,5-a]pyrimidine)	<10	Preclinical	(Schulz et al., 1996; Seymour et al., 2003)
CP-316,311	Pfizer	3,6-dimethyl-4-(pentan-3-yloxy)-2-(2,4, 6-trimethylphenoxy)pyridine	<10	Phase II, depression; double blind, placebo controlled;no difference vs placebo	(Binneman et al., 2008); www.bindingdb.org
CRA1000	Taisho	2-[N-(2-methylthio-4-isopropylphenyl)-N-ethylamino]-4-[4-(3-fluorophenyl)-1,2,3,6-tetrahydropyridin-1-yl)-6-methylpyrimidine	20–40	Preclinical	(Chaki et al., 1999; Okuyama et al., 1999)
CRA1001	Taisho	2-[N-(2-bromo-4-isopropylphenyl)-N-ethylamino]-4-[4-(3-fluorophenyl)- 1,2,3, 6-tetrahydropyridin-1-yl)-6-methylpyrimidine	20–40	Preclinical	(Chaki et al., 1999; Okuyama et al., 1999)
CRA0450	Taisho	1-[8-(2,4-dichlorophenyl)-2-methylquinolin-4-yl]-1,2,3, 6-tetrahydropyridin e-4-carboxamide benzenesulfonate	40–60	Preclinical	(Dawe et al., 2001; Chaki et al., 2004)
DMP696	BMS	4-(1,3-Dimethoxyprop-2-ylamino)-2,7-dimethyl-8-(2, 4-dichlorophenyl)pyrazolo[1,5-a]-1,3,5-triazine	<10	Preclinical	(Li et al., 2005)
DMP904	BMS	[4-(3-pentylamino)-2,7-dimethyl-8-(2-methyl-4-methoxyphenyl)-pyrazolo-[1,5 -a]-pyrimidine]	n.a.	Preclinical	(Li et al., 2005)
GSK561679	GSK/ Neurocrine	n.a.	n.a.	Phase II, depression; double blind, placebo controlled; registered 08/08; completed but results not published	www.clinicaltrials.gov
				Phase I, social anxiety disorder; registered 11/07; double blind; completed but results not published	www.clinicaltrials.gov
				Phase II, PTSD, females;double blind, placebo controlled; registered 11/09	www.clinicaltrials.gov
				Experimental, human startle, females; registered	www.clinicaltrials.gov
				Experimental, emotional processing (fMRI); double blind; registered 8/07; completed but results not published	www.clinicaltrials.gov
GSK586529	GSK/Neurocrine	n.a.	n.a.	Phase I, depression	www.clinicaltrials.gov
GW876008	GSK/Neurocrine	n.a.	n.a.	Phase II, social anxiety disorder; double blind, placebo controlled; no difference vs placebo	www.clinicaltrials.gov
				Phase II, IBS; double blind, placebo controlled; completed; results not published	www.clinicaltrials.gov; www.neurocrine.com
				Phase I, emotional processing (fMRI); registered 1/07; completed but results not published	www.clinicaltrials.gov
MJL-1-109-2		pyrazolo[1,5-a]-1,3,5-triazin-4-amine,8-[4-(bromo)-2-chlorophenyl]-N, N-bis(2-methoxyethyl)-2,7-dimethyl-(9Cl)	<10	Preclinical	(Zhao et al., 2007)
MPZP	Salk Institute	N,N-bis(2-methoxyethyl)-3-(4-methoxy-2-methylphenyl)-2, 5-dimethyl-pyrazolo [1,5-a] pyrimidin-7-amine	<10	Preclinical	(Richardson et al., 2008)
MTIP	NIAAA/Eli Lilly	3-(4-Chloro-2-morpholin-4-yl-thiazol-5-yl)-8-(1-ethylpropyl)-2, 6-dimethyl- imidazo[1,2-b]pyridazine	<10	Preclinical	(Gehlert et al., 2007)
NBI3b1996	Neurocrine	(N-Cyclopropylmethyl-2,5-dimethyl-N-propyl-N'-(2,4, 6-trichloro-phenyl)-pyr imidine-4,6-diamine)	<10	Preclinical	(Gehlert et al., 2005)
NBI-34041	GSK/Neurocrine	2-(2,4-dichlorophenyl)-4-methyl-6-(1-propylbutyl)-7, 8-dihydro-6H-1,3,6,8a-tetraazaacenaphthylene	<10	Reduced stress-induced ACTH release and corticosterone in Trier Social Stress Test	(Ising et al., 2007)
ONO-2333Ms	Ono	n.a.	n.a.	Program discontinued due to lack of efficacy (07/08)	www.clinicaltrials.gov
Pexacerfont (BMS-562086)	BMS	8-(6-methoxy-2-methylpyridin-3-yl)-2,7-dimethyl-N-[(1R)-1-methylpropyl]pyrazolo[1,5-a]-1,3,5-triazin-4-amine	n.a.	Phase II, depression; listed as completed 10/07; results conveyed as personal communication from V. Coric, M.D.	www.clinicaltrials.gov;
				Phase II, GAD	(Coric et al., 2010)
				Phase II, IBS; listed as completed 01/08 but results not published	www.clinicaltrials.gov
PF-572778	Pfizer	n.a.	n.a.		
R121919 (NBI27914)	GSK/Neurocrine	(2,5-dimethyl-3-(6-dimethyl-4-methylpyridin-3-yl)-7-dipropylamino-pyrazolo [1,5-a]pyrimidine)	<10	Phase II (open-label; depression); active; discontinued due to abnormal liver function tests	(Chen et al., 1996; Saunders & Williams, 2001)
R27899 (CRA0450)	Taisho/Johnson and Johnson	1-[8-(2,4-dichlorophenyl)-2-methylquinolin-4-yl]-1,2,3, 6-tetrahydropyridin e-4-carboxamide benzenesulfonate	50–60	Preclinical	(Chaki et al., 2004)
R317573	Taisho/Johnson and Johnson		46	Phase IIa; double-blind, placebo controlled; regional cerebral glucose metabolism PET study	(Schmidt et al., 2010)
				Phase IIa; double-blind, randomized, placebo-controlled	(Dawson et al., 2009)
SSR125543	Sanofi-Aventis	4-(2-Chloro-4-methoxy-5-methylphenyl)-N-[(1S)-2-cyclopropyl-1- (3-fluoro-4-methylphenyl)ethyl]5-methyl-N-(2-propynyl)-1,3-thiazol-2-amine hydrochloride	<10	Preclinical	(Gully et al., 2002)

CRH receptor antagonists ***[136]***

3.5. CRH and the immune system

CRH and its receptors have been identified not only throughout the central nerve system (CNS) but also in various organ systems such as the immune system and the reproductive system. CRH has been identified in peripheral inflammatory sites and reproductive organs such as the ovaries, endometrial glands, decidualised endometrial stroma, placental trophoblast, syncytiotrophoblast, deciduas and the testes **[123,149,150].** UCN has been found in immune and reproductive systems as well **[151]**.



Sources of peripheral CRH and its proposed roles in enhancing the immune response. Abbreviation: B-END: β-endorphin. **[152]**

CRH is secreted at inflammatory sites (such as spleen, thymus and inflamed tissues) and exerts proinflammatory properties both in innate and acquired immune processes with potential pathogenic effects in autoimmune inflammatory disorders, in stress-induced allergic or vasokinetic conditions such as asthma, eczema and migraine headaches **[149]**. Despite the fact that CRH via glucocorticoids and catecholamines have anti-inflammatory role in CNS by inhibiting inflammation, locally secreted CRH promotes local inflammation and acts as an autocrine or paracrine inflammatory cytokine as for example in rheumatoid arthritis patients or ulcerative colitis. UCN has been found to be increased in inflammatory situations and

also to be expressed by spleen, thymus, macrophages and lymphocytes as well **[21,152]**. CRH is expressed in the cytosol of endothelial cells, macrophages and tissue fibroblasts. Immune CRH expressed in inflammatory sites has been found to be identical to hypothalamic CRH **[153]**. One major mechanism by which CRH and UCN exerts their local immune effects is the degranulation of mast cells. Mast cells are necessary for allergic reactions but they are interestingly implicated in acquired immunity and inflammatory diseases having stress as a factor for severity. Mast cells synthesize and secret CRH, acting in autocrine or paracrine way in allergic inflammatory disorders worsened by stress **[154]**. CRH increases vascular permeability and mast cell degranulation in a dose-dependent manner via CRHR1 **[155]**. CRH also stimulates IL-1 secretion from monocytes, IL-2 from lymphocytes and IL-6 from mononuclear cells. Moreover, CRH promotes lymphocytes proliferation and IL-2 receptor expression and chemotaxis by mononuclear leukocytes and production of oxygen radicals in macrophages **[149]**.

3.6. CRH and the female reproductive system

The female reproductive system is regulated by the HPA axis. The HPA axis, activated by stress, has an inhibitory effect on the female reproductive system. Gonadotrophin- releasing hormone (GnRH) is the principal regulator of the HPA axis. GnRH stimulates LH, FSH, estradiol and progesterone secretion. CRH and CRH-induced proopiomelanocortin peptides, such as b-endorphin inhibit GnRH secretion and glucocorticoids suppress female gonadal axis function at the hypothalamic, pituitary and uterine level. Glucocorticoids reduce LH response to intravenous GnRH, having an inhibitory effect in the pituitary gonadotrophin and inhibits estradiol and progesterone as well. Furthermore, the HPA axis is responsible for amenorrhea of stress in cases such as anxiety and depression, malnutrition, eating disorders and chronic excessive exercise and for the hypogonadism of the Cushing Syndrome **[156]**.

As previously mentioned CRH is expressed by lots of tissues of the reproductive system including the ovaries, the uterus and placenta) and participates as an inflammatory component in ovulation, luteolysis, implantation and parturition, as the table indicates but I will focus more in intrauterine (endometrial decidualization and blastocyst implantation) CRH and placental CRH **[149]**

Reproductive CRH [149]

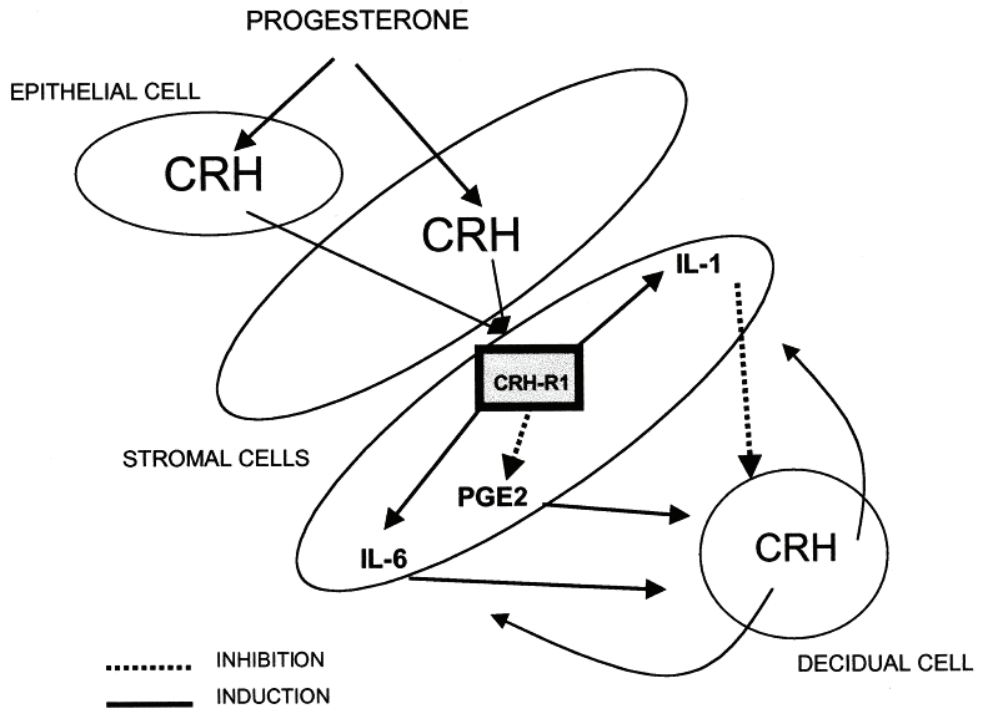
TABLE 1 Reproductive CRH: Sites of Production, Proposed Functions, and Potential Participation in Pathophysiologic Phenomena

Reproductive CRH	Potential roles in female physiology	Potential roles in female pathophysiology
Ovaries		
• Theca • Stroma • Ovum	• Follicular maturation • Steroid production • Inflammatory-like phenomena (ovulation, luteolysis) • Inhibition of female sex steroid production	• Premature ovarian failure • Anovulation • Corpus luteum dysfunction • Ovarian dysfunction
Uterus		
• Endometrial glands • Decidualized stroma	• Inflammatory-like phenomena (decidualization, blastocyst implantation) • Early maternal tolerance	• Infertility • Spontaneous abortion • Implantation failure
Blastocyst		
• Invasive trophoblast	• Blastocyst implantation • Early maternal tolerance • Trophoblast invasion	• Spontaneous abortion
Placenta		
• Cytotrophoblast • Syncytiotrophoblast • Amnion • Chorion	• Regulation of fetoplacental circulation • Fetal adrenal steroidogenesis • Induction of labor • Maternal hypercortisolism	• Premature labor • Delayed labor • Preeclampsia and eclampsia

3.6.1. Uterine CRH – endometrial decidualization

CRH is expressed in uterus **[154,157]**.The main source of endometrial CRH is epithelial cells while stromal cells express it only after decidualization occurs **[125,157-159]**. Moreover, CRHR1 and CRHR2 are also expressed by epithelial and stromal endometrial cells and myometrium **[125,160,161]**. Epithelial cells are the main source of UCN, CRHR1β and CRHR2α as well in human endometrium. Foskolin, 8-bromo-cAMP and epidermal growth factor (EGF) are the inducers of hypothalamic CRH which stimulate the activity of endometrial CRH as well. cAMP regulates endogenous hypothalamic and placental CRH by inducing its increase **[16]**. Estrogens decrease CRH promoter activity in endometrium but having an opposite effect in hypothalamic CRH. Glucocorticoids decrease CRH promoter activity in endometrium. The inhibitory effect of glucocorticoids on endometrial CRH is similar to that in hypothalamus and opposite to that found in human placenta, indicating that the regulation of the transcription of CRH gene is tissue specific. The cytokines IL-1 and IL-6 stimulate the CRH promoter activity, an effect possibly mediated by prostagladins both in hypothalamus and placenta **[162]**. Endometrial stroma decidualization occurs in the luteal phase of the menstrual cycle. In human endometrium, a phenomenon characterised as an aseptic inflammatory reaction takes place during the differentiation of endometrial stroma. During both the luteal

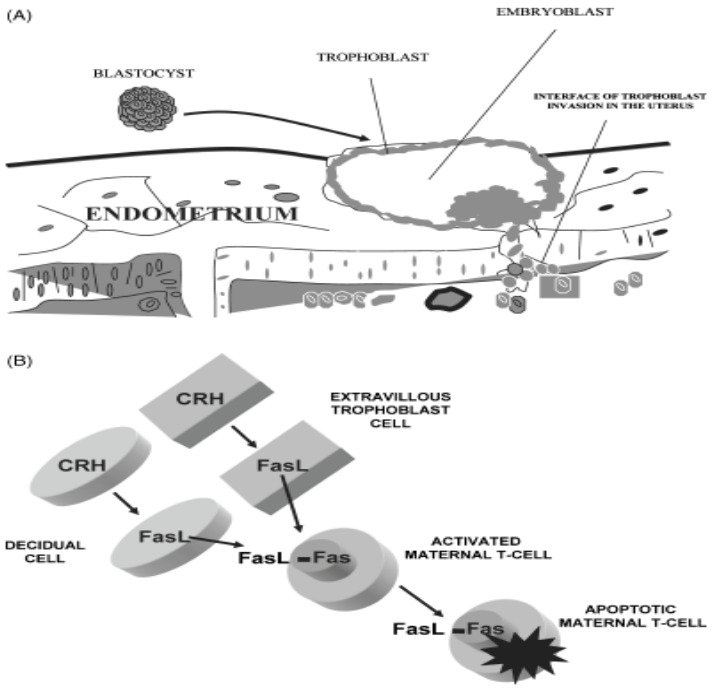
and the follicular phase of the cycle, endometrial glands show an excessive CRH expression, but CRH levels are higher in luteal phase as CRH induces the decidualization of endometrial stroma and potentiate the decidualizing effect of progesterone **[157-159]**. Endometrial CRH expression is stimulated by progestins in a cAMP manner **[17].** In stromal cells CRH mediate via CRHR1 the cAMP-dependent part of the decidualizing effect of progesterone, an effect blocked by cAMP inhibitors. Apart from progesterone, there are lots of locally produced proinflammatory immune factors such as prostagladins and interleukins exert a decidualizing effect. In humans, PGE2 enhances the decidualizing effect of progesterone while IL-1 inhibits it. CRH induces the decidualization of human endometrial stromal cells and during the decidualization process, CRH interacts with local factors. As for example, CRH inhibits PGE2 production by human endometrial cell. Endometrial CRH may also alter the decidualizing action of progesterone via locally produced PGE2. Moreover, CRH promotes the production of IL-1 and IL-6 in human endometrial stromal cells **[19]**. IL-1 is an important modulator of decidualization, as it blocks the differentiation of human endometrial stromal cells induced by ovarian steroids or cAMP **[163]**. The stimulatory effect of CRH on stromal IL-1 indicated that CRH may exert its decidualizing effect either directly or indirectly, as a modulator of progesterone, a classical decidualizing effector. To summarise, progesterone in addition to its decidualizing effect, stimulates endometrial CRH production, CRH participates in stromal decidualization and regulates local modulators of this process by inhibiting the enhancer PGE2, inducing the inhibitor IL-1 and stimulating the inducer IL-6 and endometrial PGE2, IL-1 and IL-6 exert positive control on the expression of endometrial CRH and complete the endometrial paracrine network **[164]**.



Endometrial CRH under the influence of progesterone [164]

3.6.2. Uterine CRH – blastocyst implantation

Implantation is a process that endocrine, paracrine, autocrine and autonomic factors are involved. Synchronised interactions between a blastocyst competent for implantation and an adhesive endometrium is required. This process, involves an effective cross-talk between invasive trophoblast cells and maternal endometrium. The success of implantation depends on achieving the embryo development to the blastocyst stage and the invasion of the latter into the decidualised endometrium **[11]**. During blastocyst implantation, the maternal endometrial response to the invading fetal semi-allograft has characteristics of an acute, aseptic inflammatory response **[20]**. Once implanted, the embryo suppresses this response and prevents rejection. At the same time, mother's immune system prevents a graft vs host reaction deriving from the fetal immune system **[20]**. During implantation, the invading blastocyst secretes several inflammatory mediators such as CRH, IL-1, IL-6, leukaemia inhibitory factor(LIF) and PGE2. Blastocyst derived IL-1 plays an essential role in implantation and blockage of its effect by IL-1ra antagonist in mice inhibits implantation. LIF has a similar importance as well **[123]**. CRH is expressed and released by decidual and trophoblast cells, which means that CRH is produced simultaneously by both maternal and fetal sides of implantation. Also, FasL – a proapoptotic molecule is expressed by embryonic trophoblast and maternal decidual cells. The major function of Fas-FasL system is that it induces apoptosis of activated cells carrying Fas in cells located at the interface between the fetal placenta and maternal endometrium **[20]**. CRH induces the expression of apoptotic FasL on invasive extravillous trophoblast cells (EVT) and maternal decidual cells at the fetal-maternal interface. Furthermore, CRH increases the apoptosis of activated T lymphocytes through FasL induction and participates in the processes of both implantation and early pregnancy tolerance. This effect of CRH is specifically mediated through CRH-R1 **[20]**. So, CRH participates on both implantation and early pregnancy immune tolerance. Blockage of CRH-R1 had an antinidation effect when it was administered at a very early stage of pregnancy. However, antalarmin did not completely abolish nidation; this suggested the presence of other redundant mechanisms. This was compatible with the observation that CRH- and CRH-R1-deficient mice are not entirely sterile **[13,165]**. In summary, CRH induces FasL expression on invasive EVT and maternal decidual cells at the fetal-maternal interface, antalarmin decreases the implantation rate and CRH increases apoptosis of activated T lymphocytes suggesting that CRH participates in implantation and early pregnancy tolerance along with a combination of proinflammatory and anti-rejection properties. Moreover, CRH inhibits in vitro the invasion of human EVTs **[21]** and this effect is mediated by CRHR1 and involves downregulation of carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1) synthesis by EVTs. So, CRH controls proper trophoblast invasion as well by regulating CEACAM1.



(A) The early phase of implantation. (B) The involvement of the CRH/Fas system in the early phase of implantation.[123]

3.6.3. Placental CRH

Large amounts of CRH are produced by placenta and fetal membranes **[166]**. CRH is present in high concentrations in the maternal and fetal blood and in amniotic fluid. In pregnant women, plasma concentrations of CRH, which are low during the first trimester, rise from mid-gestation to term to reach concentrations as much as 1000-fold greater during the last 6–8 weeks of pregnancy compared to those in the plasma of non-pregnant women. CRH-BP concentrations decrease during the last 6 weeks of pregnancy, leading to elevations of free CRH. Thus, placental CRH is responsible for the hypercortisolism observed during the latter half of pregnancy. This hypercortisolism is followed by a transient suppression of hypothalamic CRH secretion in the postpartum period, which may explain the blues/depression and autoimmune phenomena that may occur during this period. Placental CRH secretion is stimulated by glucocorticoids, inflammatory cytokines, and anoxic conditions, including the stress of preeclampsia or eclampsia, whereas it is suppressed by estrogens **[150].** CRH and its related peptide UCN may control human parturition not only by affecting myometrial contractility but also by increasing local matrix metalloproteinase-9 (MMP-9) activity in placenta and fetal membranes **[167]**, contributing to membrane rupture with the onset of labor. Increased plasma CRH and ACTH level may be associated with idiopathic preterm labor **[168]**. Moreover, CRH and UCN expression is upregulated in abortions, an effect mediated by CRHR1 as well **[169]**.

3.6.4. CRH – antalarmin- reproduction

Antalarmin-mediated CRH-R1 blockade may prevent implantation by reducing the inflammatory-like reaction of the endometrium to the invading blastocyst **[20]**. Antalarmin and other CRH-R1 antagonists represent a new class of nonsteroidal inhibitors of pregnancy at its very early stages and as CRH antagonists could be used in the therapy of depression and anxiety disorders **[156]**, there is a potential to cause hypofertility or early miscarriages. Nevertheless, in rats, administration of antalarmin after gestation day 5 and until the end of pregnancy did not affect the embryos; this suggests that mechanisms other than CRH-mediated FasL expression are present in mid- and late gestation that assists in nonrejection of the fetus. Therefore, it has been suggested that CRH antagonists could be used to protect the fetus from maternal stress and/or to prevent premature labor, another potential use of this class of compounds. Furthermore, CRH-R1 antagonists have been used experimentally to elucidate the role of CRH on blastocyst implantation and invasion, early fetal immunotolerance, and premature labor **[141]**.

3.6.5. CRH – endometriosis

CRH and UCN are neuropeptides expressed by human endometrium **[130,170]** with an mRNA expression highest in secretory phase endometrium **[125,158,159]**, most probably taking part in the process of human endometrial stromal cell (HESC) decidualization. Through a paracrine mechanism, CRH and UCN act by CRH receptors type 1 (CRH-R1) and type 2 (CRH-R2), synergistically with progesterone in activating decidualization **[158,171]** .Moreover, CRH and UCN also have an immunomodulatory activity **[161,172]** and their mRNA is expressed by ectopic endometriotic cells **[173,174]**. Endometrium of women with endometriosis is characterized by a deranged expression of CRH and UCN during menstrual cycle by a reduced response to CRH and UCN, and an impaired expression of CRH-R1 mRNA suggests a disrupted local CRH/Ucn/CRH-R1 pathway in the disease and may explain the reduced fertility characterizing patients with endometriosis **[116]**. Chronic pain and infertility are characterised as endometriosis symptoms which interfere with the patients quality of life and perceived as persistent stressor. High levels of stress have been proposed to contribute to endometriosis progression. It may be proposed that high levels of stress promote the dissemination of endometriosis via CRH- dependent pathways, as peripheral CRH is found increased in psychological stress **[45]**. Peripheral CRH, increasing upon high psychological stress, might contribute to the peritoneal inflammation present in endometriosis. The therapeutic application of progesterone derivatives, CRH blocking agents as well as improvement of stress may disrupt the vicious cycle between the chronic peritoneal inflammation and high perception of psychological stress in endometriosis. CRH is highly expressed in endometriotic lesions **[64]** and has been proposed to stimulate mast cells to secrete VEGF. This would facilitate angiogenesis in endometriotic lesions and perpetuate the dissemination of the disease. CRH – like immunoreactivity was demonstrated in splenic nerve fibres, in dorsal horn of the spinal cord and dorsa root ganglia as well as sympathetic nerve fibres pointing at neuronal source of immune CRH in descending

nerve fibres. Because sympathetic nerve fibres are present in endometriotic tissue, it appears possibly that CRH contributes to peritoneal inflammation in endometriosis **[45].** High numbers of activated mast cells are present in endometriotic sites and proteases secreted from mast cells play important role in fiibrogenesis. Mast cells are essential for the initiation of inflammatory reactions by releasing several mediators including histamine, proteases, cytokines, granulocyte macrophage colony stimulating factor (GM- CSF), TNF-α, TGF- β. Also mast cells synthesize and secrete CRH and UCN. CRH and UCN exert local effects in inflammatory disorders, worsened by stress through activation of mast cells. Mast cells in endometriotic sites are strongly positive for CRH and UCN. So, CRH and UCN may activate mast cells and contribute to the fibrosis and inflammation in endometriosis **[64]**.

CHAPTER 4

Fas – Fas ligand system

4.1. GENERAL

The FAS receptor (FasR), also known as apoptosis antigen 1 (APO-1 or APT), cluster of differentiation 95 (CD95) or tumor necrosis factor receptor superfamily member 6 (TNFRSF6) is a protein encoded by the *TNFRSF6* gene in humans. The Fas receptor is a death receptor on the surface of cells that leads to programmed cell death (apoptosis). It is one of two apoptosis pathways, the other one is the mitochondrial pathway. FasR is located on chromosome 10 in humans. FAS receptor is located on the long arm of chromosome 10 (10q24.1) in humans and on chromosome 19 in mice. The gene lies on the plus (Watson strand) and is 25,255 bases in length organised into 9 protein encoding exons. Fas ligand (FasL or CD95L) is a type-II transmembrane protein that belongs to the tumor necrosis factor (TNF) family. Its binding with its receptor induces apoptosis. Fas ligand/receptor interactions play an important role in the regulation of the immune system and the progression of cancer. Fas ligand or FasL is a homotrimeric type II transmembrane protein. It signals through trimerization of FasR, which spans the membrane of the "target" cell. This trimerization usually leads to apoptosis, or cell death. Soluble Fas ligand is generated by cleaving membrane-bound FasL at a conserved cleavage site by the external matrix metalloproteinase MMP-7. FasL expression by cells such as eye, testis and placenta may be involved in maintaining the immune privilege of these tissues by inducing apoptosis of Fas- positive immune effector cells **[175]**.

Fas-Fas ligand induced apoptosis plays an important in the regulation of the immune system concerning different aspects of it such as:

T-cell homeostasis: the activation of T-cells leads to their expression of the Fas ligand. T cells are initially resistant to Fas-mediated apoptosis during clonal expansion, but become progressively more sensitive the

longer they are activated, ultimately resulting in activation-induced cell death (AICD). This process is needed to prevent an excessive immune response and eliminate autoreactive T-cells. Humans and mice with deleterious mutations of Fas or Fas ligand develop an accumulation of aberrant T-cells, leading to lymphadenopathy, splenomegaly, and lupus erythematosus.

Cytotoxic T-cell activity: Fas-induced apoptosis is one of the main mechanisms by which cytotoxic T lymphocytes induce cell death in cells expressing foreign antigens **[176]**

Immune privilege: Cells in immune privileged areas such as the cornea or testes express Fas ligand and induce the apoptosis of infiltrating lymphocytes. It is one of many mechanisms the body employs in the establishment and maintenance of immune privilege.

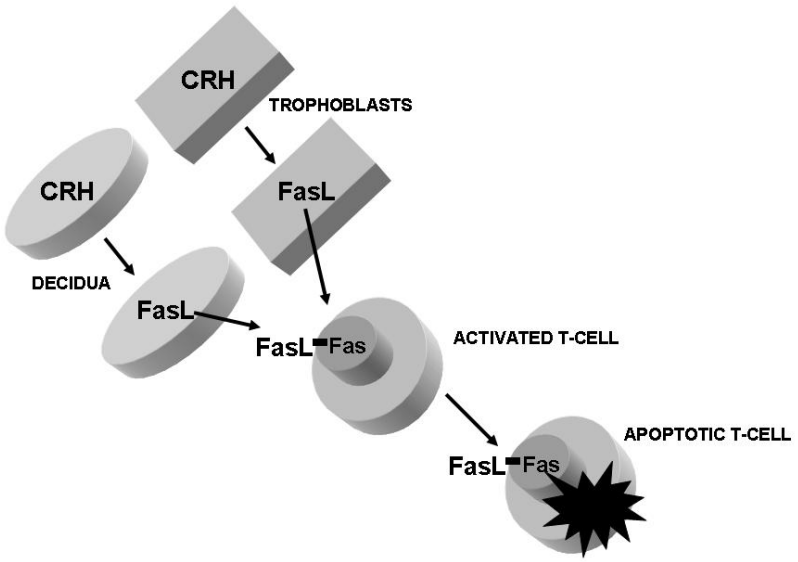
Tumors: Fas-FasL systems helps tumour cells to escape the immune surveillance. Tumors may over-express Fas ligand and induce the apoptosis of infiltrating lymphocytes, allowing the tumor to escape the effects of an immune response **[177]**. The up-regulation of Fas ligand often occurs following chemotherapy, from which the tumor cells have attained apoptosis resistance. In HeLa cells (cervical cancer cell line), CRH can significantly induce both FasL transcription and FasL translation. Taking into account previous studies already establishing a connection between FasL expression and tumor immunoescape in cervical cancer, it can be concluded that such immunoescape could be CRH dependent **[178]**.

4.2. Fas/FasL in normal endometrium - Maternal tolerance

Fas ligand plays an important role in leukocyte trafficking between the mother and the fetus. Fas and FasL are expressed in human endometrium throughout the menstrual cycle **[179-181]**. During the late proliferative phase, these proteins are unable to interact and induce apoptosis. In contrast, during the secretory phase, these proteins are extracted as parts of the cellular membranes, where Fas can bind FasL and turn on apoptotic signals **[180,182]**. Fas immunostaining on human endometrial glandular cells was determined to be stronger during the secretory phase than during the proliferative phase **[180,181,183]**, demonstrated that withdrawal of estrogen and/or progesterone from endometrial cells in culture induced apoptosis, causing a significant decrease in cell viability. This coincided with increased Fas and FasL expression. These data indicate that Fas‐mediated apoptosis is important for endometrial cycling. Selam *et al*. (2001) showed that estradiol and progesterone up‐regulated FasL protein expression in cultured endometrial glandular and stromal cells **[184]**. There are two forms of FasL: membrane‐bound and soluble. The membrane‐bound FasL is converted to the active soluble FasL by the action of some matrix metalloproteinases (MMP) **[185]**. In the proliferative phase, endometrial glandular cells scarcely undergo apoptosis. This may be due to the localization of FasL in the glandular cells in a membrane‐bound inactivated form**[182]** and/or its localization into the cell's Golgi apparatus. There are increased activities of

MMP, such as MMP‐1, ‐3 and ‐9, before and during the menstruating phase **[186]**. The membrane‐bound FasL localized on the apical membrane of glandular cells is cleaved by the action of MMP during the secretory phase, resulting in an increase in the amount of soluble FasL in the glandular lumen and therefore can bind with Fas on the cell surface of glandular cells, inducing their death **[182]**.

CRH participates in the nidation of the fertilized egg by inhibiting local maternal immune response to the implanted embryo. CRH stimulates the expression of the pro-apoptotic FasL protein in the decidual and the trophoblastic cell, potentiating their ability to induce apoptosis of the maternal T lymphocytes activated by the presence of the embryo. Expression of the FasL by the fetal extravillous trophoblast cells can induce apoptosis of activated T lymphocytes expressing increasing numbers of the Fas membrane protein. Another role that is attributed to maternal and fetal FasL is that it limits the migration of fetal cytotrophoblast cells into maternal tissue and vise versa. The intra-uterine presence of CRH, both in the maternal (decidua) and fetal (trophoblast) sites suggests that locally produced CRH regulates FasL production, thus affecting the invasion process through a local auto/paracrine regulatory loop of cytotrophoblast cells, regulating their own apoptosis. Inadequate CRH- mediated self induction of FasL in extravillous trophoblasts might be involved in the pathophysiology of infertility and recurrent fetal absorption or miscarriage **[187].** Moreover, aberrant expression of CRH in pre-eclampsia may activate the FasL-positive decidual macrophages, impair the physiological turnover of EVT and eventually disturb placentation **[188]**.



Uterine CRH affects the maternal immune tolerance[187]

Uterine CRH affects the maternal immune tolerance. CRH produced locally by decidual cells and extravillous trophoblasts acts in an autocrine/paracrine fashion, through CRH-R1, to stimulate FasL expression and to potentiate the ability of these cells to cause apoptosis of activated maternal T lymphocytes (Fas receptor positive) **[187]**.

4.3. Fas/FasL system in endometriosis:

Harada *et al*. (1996) found that Fas is expressed randomly in both eutopic and ectopic endometrial tissues. The authors suggested that the expression of Fas antigen may be less involved in apoptosis of eutopic and ectopic endometrial tissues as an apoptosis - regulator **[189]**. Watanabe *et al*. (1997) also observed Fas expression in glandular cells of both ectopic and eutopic endometrium. In contrast with the cyclic expression pattern of Bcl - 2, Fas expression was constant in both tissues throughout the menstrual cycle **[183]** . Differences in the expression of Fas were found between ovarian, cervical and endometrial carcinoma tissues compared with normal tissues. Tumour cells had significantly decreased levels of Fas **[190]**. In addition, there was a higher Fas expression in ovarian endometriotic cells compared with benign ovarian tumours but the difference did not reach significance **[191]**. Higher expression of FasL by endometriotic tissues contributes to their survival and the development of endometriosis. Garcia - Velasco *et al*. (2002) suggests that levels of soluble/active FasL are higher in serum and peritoneal fluid in women with moderate to severe endometriosis than in women with early - stage disease or in disease - free women. Higher levels of soluble FasL in the peritoneal fluid of women with endometriosis may contribute to increased apoptosis of Fas - bearing immune cells in the peritoneal cavity, leading to their decreased scavenger activity **[179]**. This may result in prolonged survival of endometrial cells in the peritoneal cavity. The sources of the elevated levels of soluble FasL in the peritoneal cavity were endometriotic lesions and peritoneal fluid leukocytes. Several authors have shown that endometrial glandular and stromal cells express FasL, at both the mRNA and protein levels. This membrane - bound FasL can be shed by matrilysin, producing an active, soluble form of the ligand **[192].** Peritoneal fluid leukocytes are another plausible source for high levels of soluble FasL in women with endometriosis, because human - activated peripheral blood mononuclear cells were shown to express FasL messenger RNA **[193].** Macrophage - derived growth factors, such as platelet - derived growth factor (PDGF) and transforming growth factor (TGF), are increased in the peritoneal fluid of women with endometriosis **[179,194]** showed that macrophage - conditioned media containing PDGF and TGF - β induced FasL expression by endometrial stromal cells, suggesting that peritoneal macrophages in endometriosis might stimulate a Fas - mediated apoptosis of immune cells. Expression of FasL by the endometriotic cells may protect them from attack by T - cells. Consequently, ectopic endometrial cells escaping from immune surveillance in the peritoneal cavity of women with endometriosis may contribute to the maintenance of the disease. It is therefore possible that many

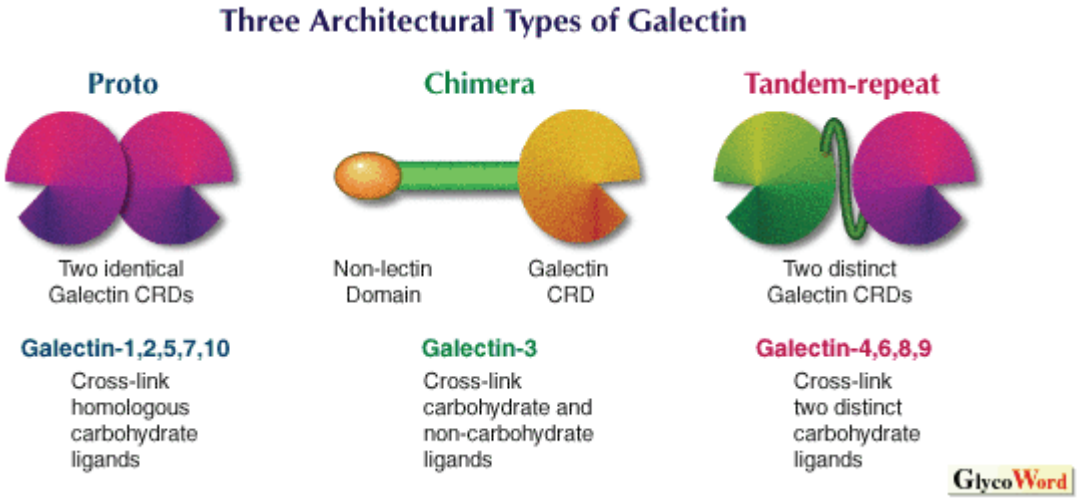
endometriotic cells not only become resistant to Fas‐mediated apoptosis, but also acquire the ability to utilize this pathway to their advantage by launching a 'Fas counter‐attack' against the host's immune system. MMP have been implicated in the conversion of TNF‐α and FasL to active soluble forms, suggesting that these molecules can activate or release factors involved in the apoptotic process **[195,196]**. MMP‐2, MMP‐9 and Mt1‐MMP mRNA expression levels are significantly higher in endometriotic lesions compared with normal eutopic endometrium **[197]**, implying that endometriotic tissue has a greater capacity for invasion. Up‐regulation of FasL expression by endometriotic cells could be induced after the adhesion of these cells to the extracellular matrix proteins laminin, fibronectin and collagen IV **[198]**. FasL expressed on endometriotic cells may induce apoptosis of the local immune cells including activated T lymphocytes, thereby reducing attacks by host immune surveillance and promoting the survival of endometrial stromal cells during the initial attachment of endometrial implants. Early lesions of endometriosis reportedly invade the extracellular matrix of the peritoneum. FasL expression that occurs when endometrial stromal cells attach to the extracellular matrix may be one of the critical events in the development of endometriosis. Under these observations, it was speculated that the expression levels of soluble/active FasL may be enhanced in shedding endometrial cells presenting in the peritoneal environment and protect endometrial cells from the immune effector cells of the peritoneal cavity. Interleukin‐8 (IL‐8), a chemokine for neutrophils and a potent angiogenic agent, is elevated in the peritoneal fluid of women with endometriosis. IL‐8 promotes proliferation of stromal cells derived from endometriotic tissues **[199,200]**, suggesting that it may facilitate growth of endometriotic implants. Selam *et al*. (2002b) examined whether IL‐8 may up‐regulate FasL expression in endometrial cells and may be relevant for the development of a relative local immunotolerance in endometriosis **[198]**. They demonstrated a concentration‐dependent increase in the protein expression of FasL by IL‐8 in endometrial stromal cells. The authors speculated that elevated peritoneal fluid IL‐8 levels, via stimulation of FasL‐induced apoptosis in activated T lymphocytes, contribute to an immune‐privileged environment around the endometriosis implants, supporting their survival. IL‐8 exerts a chemotactic activity primarily on neutrophils and inhibits their apoptosis even in the presence of Fas engagement **[201]**. Kwak *et al*. (2002) investigated the effects of plasma and peritoneal fluid (PF) from patients with advanced endometriosis on apoptosis of neutrophils. Adding plasma and PF in neutrophil culture reduced spontaneous apoptosis. Neutralizing IL‐8 antibody abrogated the delay of neutrophil apoptosis induced by PF, suggesting that IL‐8 is one of the neutrophil survival factors in the PF of endometriosis patients. The impaired clearance of cells responsible for innate immunity in the peritoneal fluid of patients with endometriosis may be associated with the development of the disease **[202]**.

CHAPTER 5

GALECTINS

5.1. GENERAL

Galectins are members of a large family of animal lectins which bind b- galactoside and are highly conserved throughout evolution. Twelve galectins are present in humans and the family consists of fourteen mammalian galectins that have been identified in several tissues and animal species **[203]** .



http://www.glycoforum.gr.jp/science/word/lectin/LEA01E.html

The members of this family share sequence similarities in the carbohydrate- recognition domain (CRD). The galectin carbohydrate recognition domain (CRD) is a beta-sandwich of about 135 amino acids. Based on their biochemical structure galectins have been classified by Hirabayashi and Kasai in:

Prototype galectins (gal-1, -2, -5, -7, -10, -11, -13 and -14): which exist as monomers or noncovalent homodimers of a CRD.

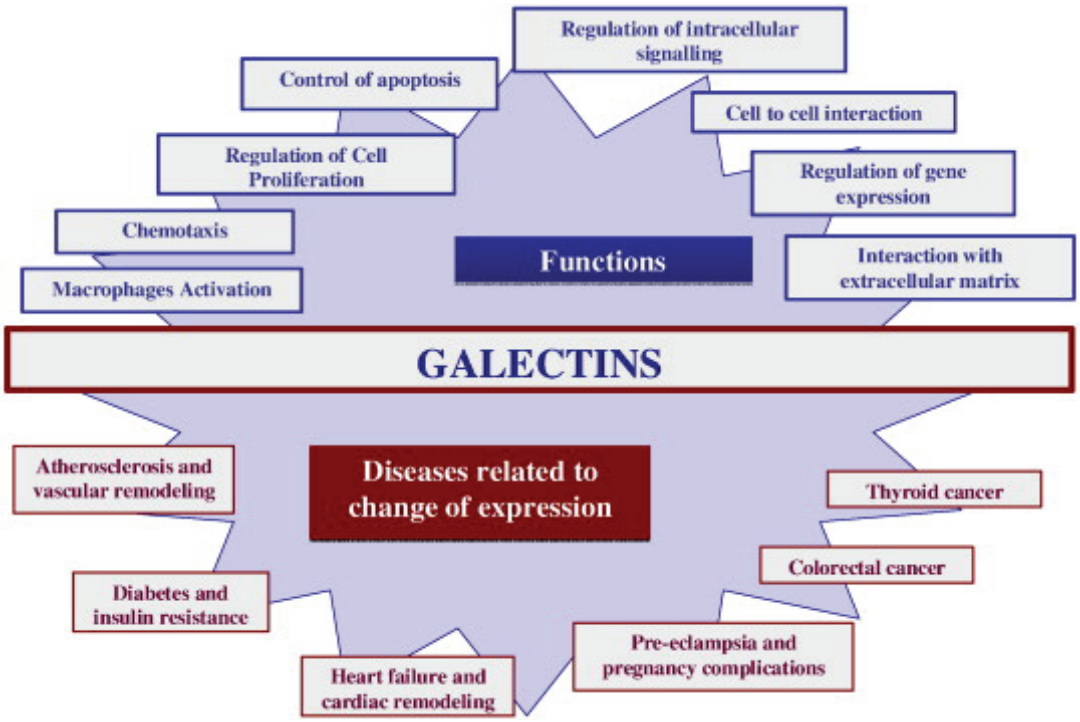
Chimera type (gal-3): composed of a non-lectin domain bind to a CRD.

Tandem repeat type (gal-4, -6, -8, -9 and -12): which consist of two different CRD in a single polypeptide chain.

Galectins are present intracellularly in the cell cytosol and in the nucleus as well. Galectins lack a secretion signal peptide required for export by classical vesicle- mediated exocytosis. Galectins are synthesised in the cytosol and released from the cell by a secretory mechanism that bypasses the endoplasmic reticulum and the Golgi. After galectins are released into the extracellular medium, they

crosslink β- galactoside- containing cell surface glycoconjugates, resulting in the modulation of cell signaling, adhesion and cell survival. Generally galectins are characterized as soluble proteins confined to cytosolic compartments or released to the extracellular milieu but recently galectin-3 has been found to contain a transmembrane – spanning domain **[204,205].** Extracellular galectins crosslink cell surface and extracellular glycoproteins and can modulate cell adhesion and induce intracellular signals. Moreover galectins may also bind intracellular non-carbonhydrate ligands and have intracellular regulatory roles in processes such as RNA splicing, apoptosis and the cell cycle **[206]**.

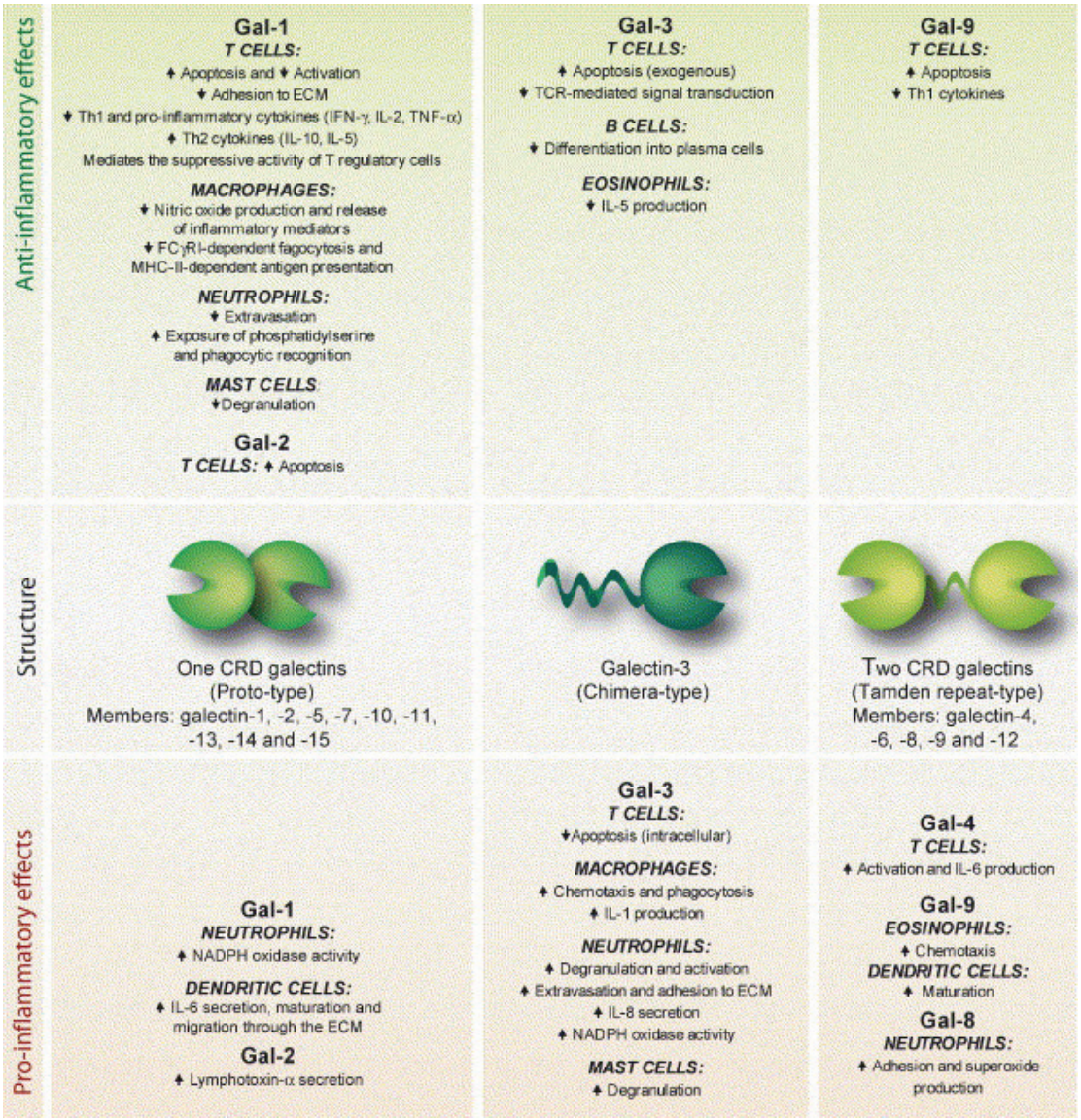
Galectins have strongly been implicated in inflammation, autoimmunity and cancer and may be useful targets for anti-inflammatory and anti-cancer therapy. Galectins are important modulators of neoplastic transformation, cancer cell survival, angiogenesis and tumour metastasis. As far as the immune system concerned, galectins are found in activated macrophages, activated B cells and activated B cells. They play a vital role in T cell homeostasis and survival. The expression of galectins is modulated during the activation and differentiation of immune cells and may be significantly altered under several pathological conditions **[204,205]**.



Functions of galectins and diseases related to changes of galectins expression.[207]

Galectins may play a role in initiation, activation and resolution of acute and chronic inflammatory processes. The same galectins may exert pro- or anti- inflammatory roles depending on their concentration in the inflammatory sites, the extracellular microenvironment and the cell types. In addition to the fact that

galectins participate in cell growth and survival acting as cytokines, chemokines or growth factors, they can potentiate or inhibit cell- cell and cell-matrix interactions **[205]**.



Toscano MA, Ilarregui JM, Bianco GA, Campagna L, Croci DO, et al. (2007) Dissecting the pathophysiologic role of endogenous lectins: glycan-binding proteins with cytokine-like activity? Cytokine Growth Factor Rev 18: 57-71.[208]

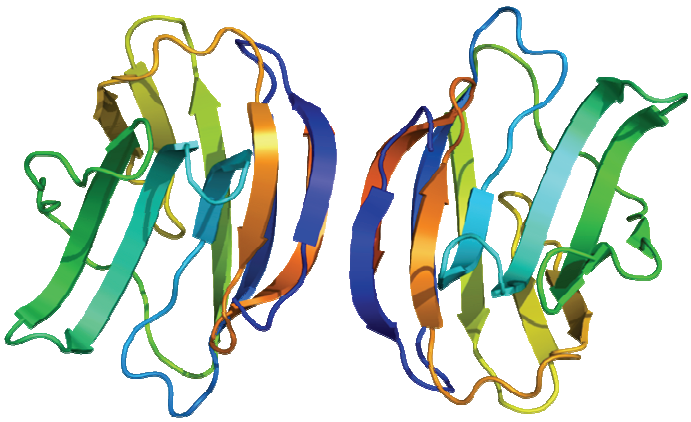
Galectins	Localization	Apoptosis	Cell adhesion	Inflammation	Cytokine production	Macrophage functions	Chemotaxis	Effects in autoimmune diseases
Galectin-1	Widely distributed in placenta, ovary, testis, liver, lymph node, thymus, spleen, macrophages, T cells, B cells	⬆ Immature thymocytes, activated peripheral T cells and infected macrophages	⬆⬇Opposite effects on different cell types ⬇Activated T cells	⬇PLA_2- induced edema, neutrophil extravasation and mast cell degranulation	⬇ TNF-α; IL-2 and IFN-γ from activated T cells ⬇ IL-12 from infected macrophages ⬆ IL-5 release	⬇ Arachidonic acid release and PGE_2 production	NA	⬇ Ameliorates EAE, CIA, EAMG, Con A-induced hepatitis. Decreased expression in synovial infiltrates
Galectin-3	Macrophages, epithelial cells, fibroblasts and tumors	⬇ Activated T cells and tumors	⬆Neutrophil adhesion to laminin and favors dendritic cell adhesion to naïve lymphocytes	⬆ Mast cell activation, NADPH activation and superoxide production by neutrophils	⬇ IL-5 production from eosinophils and allergen-specific T cells	⬆ Potentiates LPS-induced IL-1 production	⬆ Monocytes and macrophages	Increased expression in synovial infiltrates. Modulates rat nephrotoxic nephritis
Galectin-7	Keratinocytes	⬆ Keratino-cytes	NA	NA	NA	NA	NA	NA
Galectin-8	Liver, kidney, cardiac muscle, lung and brain	⬆ Lung carcinoma	Regulates tumor cell adhesion	NA	NA	NA	NA	NA
Galectin-9	Thymus, kidney, Hodgkin's lymphoma	⬆ Immature thymocytes	NA	NA	NA	NA	⬆ Eosinophils	Modulates rat nephrotoxic nephritis induced by anti-basement membrane antibodies
Galectin-12	Adipocytes	⬆ Adypocytes	NA	NA	NA	NA	NA	NA

Role of galectins in inflammatory and immunomodulatory processes. [205]

5.2. Galectins- reproduction

Apart from other tissues galectins have been identified in the reproductive tract as well. As endometrial function and implantation involve many inflammatory mediators, galectins may contribute to endometrial immune system regulation. As galectins play a vital role in cell adhesion, migration and chemotaxis, galectins might play a role in regulation of leukocyte regulation. Moreover, galectins are involved in defense against invading micro-organisms, so they might contribute to protection of endometrium against bacteria **[209]**. Gal-1, -3, -9, -15 have been found to be expressed in human endometrium and deciduas apart from other tissues. Galectin-1 has been found to be expressed by stromal cell and galectin-9 by glandular epithelial cells in human endometrium and has been characterized as an endometrial epithelial marker and in human uteroderme as well **[210,211]**. Galectin-3 is expressed by secretory- phase epithelial glandular cell as well **[212]**. Furthermore, gal-1 and gal-3 are overexpressed on EVTs in preecramptic and low platelet syndrome (HELLP) placentas **[213]**.

5.3. Galectin-1 – one of the most extensively studied galectin



Galectin-1 is the first galectin that have been discovered in the galectins family. It is a prototype galectin, consisting of two identical carbonhydrate recognition domains (CRDs) that may aggregate into homodimers. It is encoded by the LSGALS1 gene located on chromosome 22q12. Gal-1 occurs inside and outside the cells and has intracellular and extracellular functions. Apart from the fact that galectin-1, as all galectins, is characterized a lectin, it is involved in protein –protein interactions as well. Extracellularly, gal-1 acts as a lectin and intracellularly exerts its protein-protein interactions and that is why it has lots of binding partners such as integrins, actin, fibronectin, laminin, H-ras **[214]**.

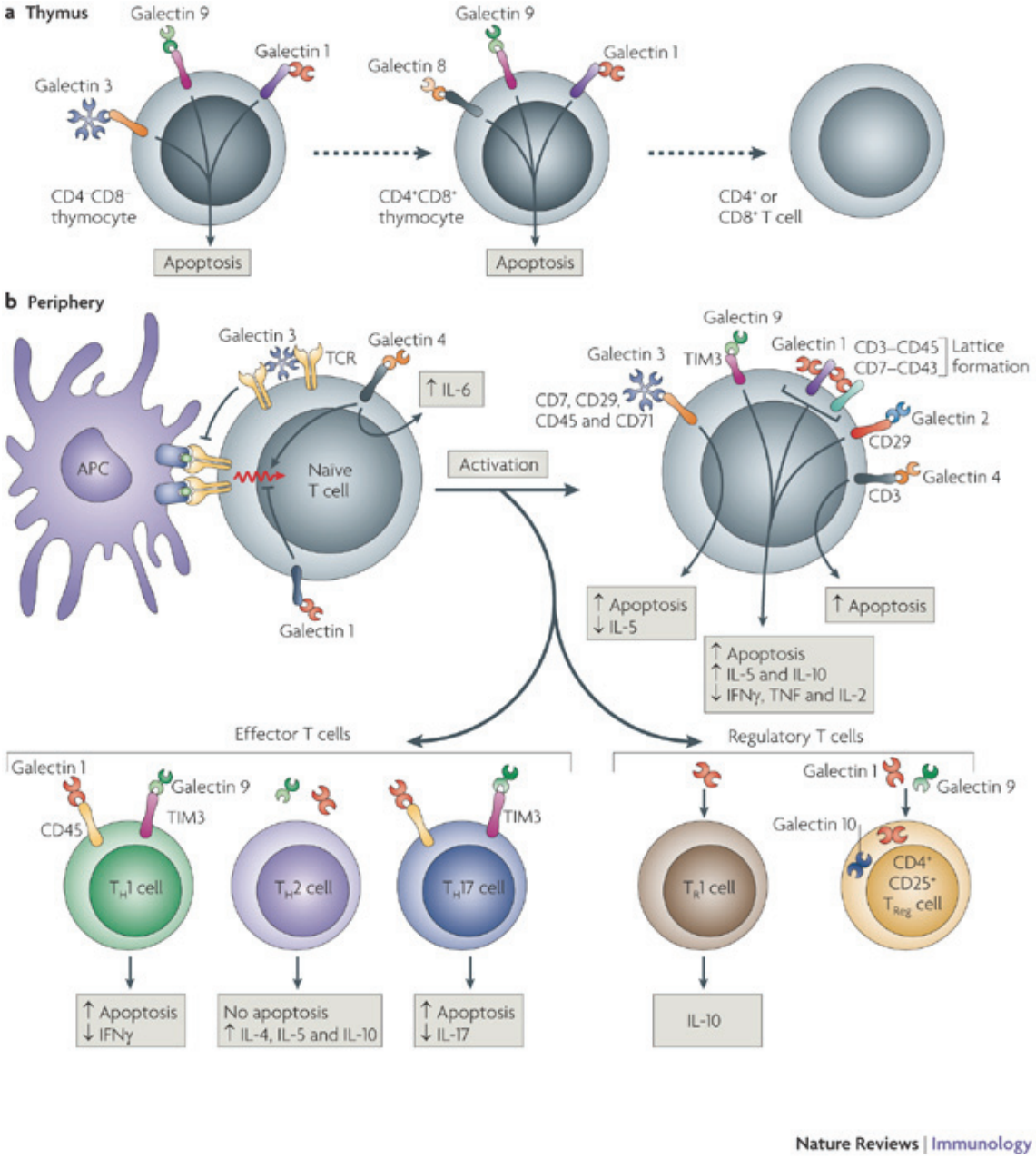
5.3.1. Galectin-1 - cell signalling

Galectin-1(gal-1) has various roles in cell signaling pathways. It regulates cell growth. Extracellular galectin-1 has no effect on growth of naïve T cells but is mitogenic for normal or pathological murine and human cells **[215]**. Gal-1 can also regulate cell cycle progression in human mammary tumor cells **[216]**. The positive or negative effects of gal-1 on cell growth depend on the cell type, cell activation status, monomeric or dimeric form and extracellular or intracellular localization. Furthermore, galectin-1 modifies cell adhesion, motility and invasion. It increases adhesion of various normal and cancer cells to the ECM (extracellular matrix) via crosslinking of integrins, laminin and fibronectin. Moreover, it increases motility causes reorganization of actin skeleton of glioma cells. The membrane expression of gal-1 is an indication of cell invasiveness **[217]**.

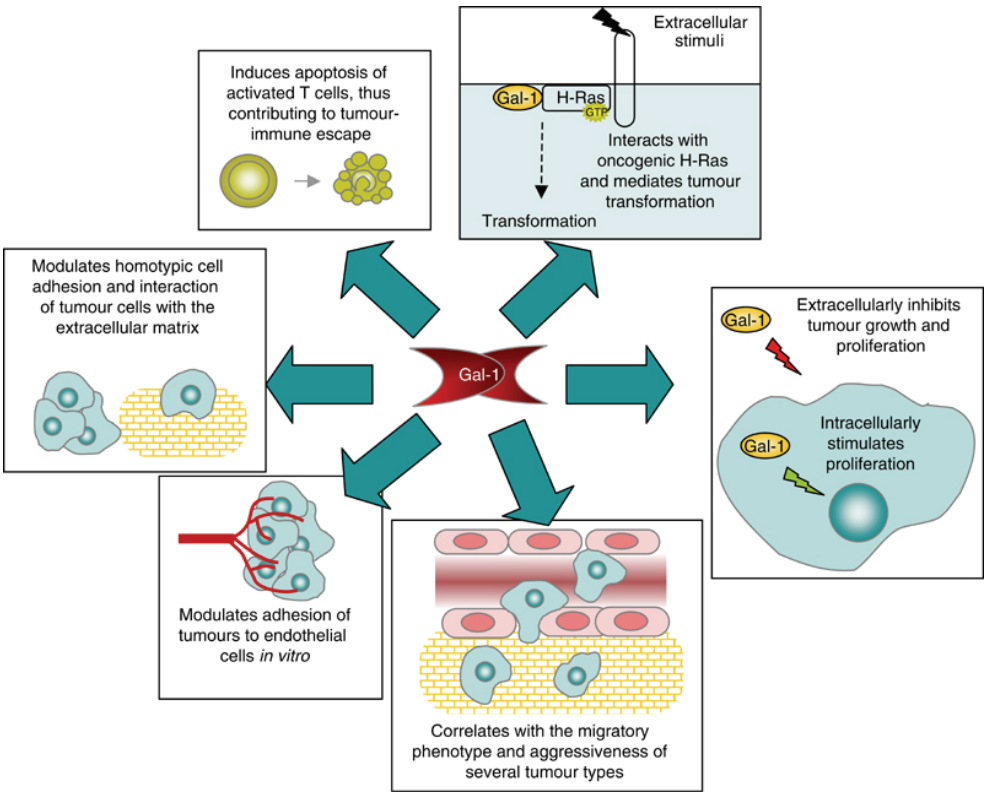
5.3.2. Galectin-1 – immune system – inflammation- cancer

Galectin-1 plays a major role in initiation, activation and resolution of inflammatory responses **[218]** and can act as a pro-inflammatory or a anti-inflammatory cytokine. Gal-1 induces inhibition of cell growth and cell-cycle arrest and promotes the apoptosis of activated immune cells. Galectin-1 is expressed by a variety of cells in central and peripheral immune compartments including thymic epithelial cells, antigen-

primed T cells, macrophages and activated B cells **[205]**. Galectin-1 modulates central monocyte /macrophage function. By inhibiting T-cell function via macrophage priming , galectin-1 links innate and adaptive immune system **[219]**.Galectin-1 inhibits rachinoid acid release, blocks nitric oxide synthesis and increases arginase activity, suggesting a role for this protein in triggering a state of alternative activation in cells of the monocyte macrophage lineage. So, galectin-1 has been characterized as a modulator of monocyte/macrophage functions. Furthermore, galectin-1 inhibits the proliferation of activated T cells and reduces the clonal expansion of antigen primed CD8+ T cells and human leukemia T cells in a saccharide-dependent manner **[220]**. Galectin-1 induces cell-cycle arrest and/or apoptosis of human and murine T cells during development in the thymus and after stimulation in the periphery. Galectin-1 also suppresses the secretion of the pro-inflammatory cytokine IL-2 and favors the secretion of the anti-inflammatory cytokine IL-10. The amount of gal-1 secreted in ECM is sufficient to kill T cells **[221]**.Galectin-1 binds to CD45, CD43and CD7 on the surface of the thymocytes and T-cell lines and as a result, the receptors are clustered into domains on the cell surface. CD-7 is an important receptor for galectin-1 and the loss of CD7 in several autoimmune diseases and T- cell lymphomas allow the survival of autoreactive and neoplastic T cells **[222]**. The anti-inflammatory properties of galectin-1 have been evaluated in several models of chronic inflammation and autoimmunity including autoimmune encephalomyelitis, arthritis, colitis, hepatitis, diabetes and chronic pancreatitis **[214]**. Moreover, galectin-1 has the ability to reduce the levels of interferon γ (IFNγ), TNFα, IL-2 and to increase the levels of IL-5. Moreover, the ability of galectin-1 to suppress the allogenic T cell response through apoptotic or non-apoptotic mechanisms suggests its potential use for immunosuppression in organ transplantation and graft versus host reaction **[223]**. Galectin-1 initiates a variety of signal transduction such as extracellular regulated kinase (ERK-2) phosphorylation, calcium influx, activation of specific transcription factors such as AP-1 and Bcl-2 downregulation to influence T- cell physiology and survival. Galectin-1 acts as T-cell receptor ligand. Despite the fact that gal-1 inhibits T cell proliferation and survival , it stimulates proliferation of vascular endothelial cells **[205]**. Galectin- 1 also participates in acute and allergic inflammation. Also, galectin-1 plays an important role in survival, differentiation and synaptic plasticity of neurons, preventing neuronal loss in case of injury in the central nervous system **[224]**. Moreover galectin-1 is involved in tumor progression and tumor-immune escape. Galectin-1 expression in cancer cells has been linked to aggressiveness of tumors **[214]**, the secretion of galectin-1 by tumor cells may be a mechanism by which immusuppresive microenvironment is created in tumor sites. So, galectin-1 contributes to immune privilege of tumors by negatively regulating the survival of effector T cells and taking part in the balance in Th2-predominant cytokine milieu. As a result, galectin-1 can be used as a target for therapeutic intervention in cancer **[208]**.



Galectins regulate a range of T cell processes including T cell signalling, activation, apoptosis, cytokine secretion and regulatory T (T_{Reg}) cell expansion. a | In the thymic microenvironment, galectin 1 induces apoptosis in double-negative ($CD4^-CD8^-$) or double-positive ($CD4^+CD8^+$) thymocytes, suggesting a possible role for these galectins in regulating central tolerance. b | Once in the periphery, galectin 1 blocks early T cell receptor (TCR)-mediated activation signals and prolongs the survival of naive T cells. Following T cell activation, galectin 1 binds to particular glycosylated receptors and T cell immunoglobulin domain and mucin domain protein 3 (TIM3) and triggers distinct intracellular events to induce T cell death. Galectins can also regulate the secretion of pro- or anti-inflammatory cytokines and promote the expansion of IL-10-producing regulatory T (T_R1) cells. In addition, galectin 1 contribute to the suppressive activity of $CD4^+CD25^+$ T_{Reg} cells. APC, antigen-presenting cell; IFNγ, interferon-γ; TNF, tumour necrosis factor. ***[225]***

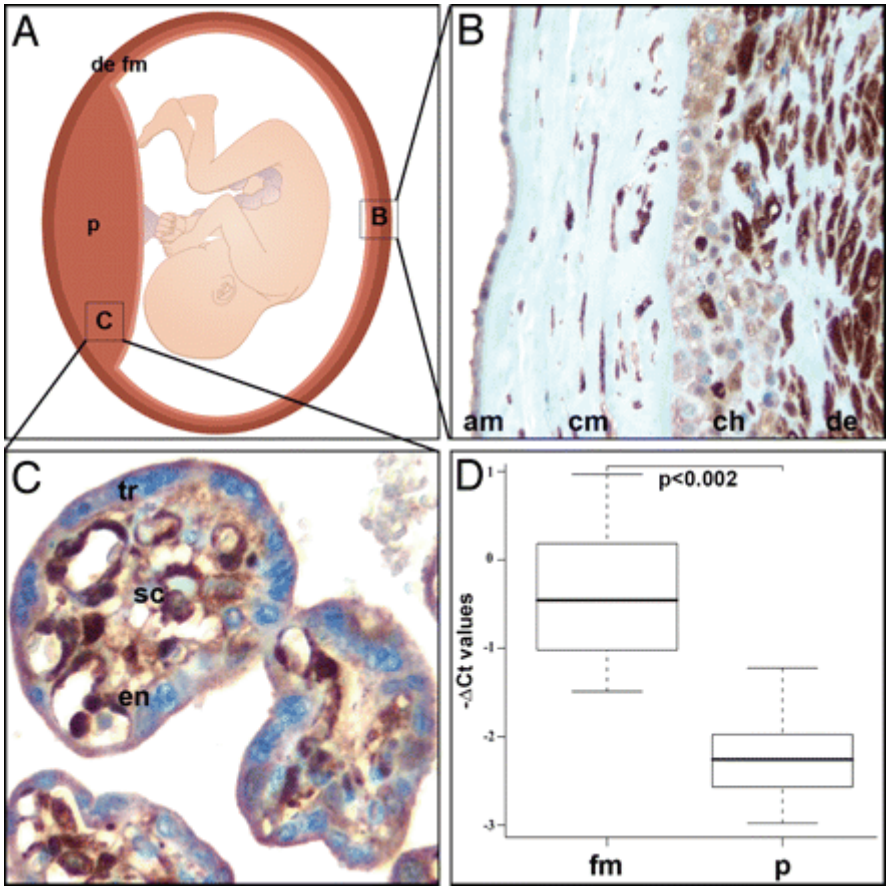


Contribution of galectin-1 to tumour progression.[226]

5.3.3. Galectin-1 – female reproduction

Galectin-1 has been identified in male and female reproductive systems as well. The abundance of proapoptotic galectin-1 in immune privileged sites such as placenta, brain and reproductive organs suggests that galectin-1 might trigger the death of T cells protecting these sites from tissue damage induced by T cell derived proinflammatory cytokines **[205].** Galectin-1 has a similar expression pattern in placentas and extraembryonic membranes. Gal-1 is abundantly expressed in human third trimester placentas and extraembryonic membranes and is upregulated in placentas in preeclampsia and in extraembryonic membranes in chorioamnionitis suggesting a role in a fetal contribution to anti-inflammatory response. Galectin-1 is upregulated in uterine natural killer cells and a key modulator of regulatory T cell functions. Galectin-1 induces generation of tolerogenic dendritic cells and regulatory T cells, so knock out mice of galectin-1 led to a higher rate of fetal loss in allogenic mating. Additionally, galectin-1 is involved in immune- endocrine cross-talk, maternal – decidual expression of gal-1 is regulated by progesterone and galectin-1 increases progesterone concentrations, suggesting its role in the maintenance of pregnancy **[227]**. Galectin-1 localizes in the placentomal/interplacentomal regions, smooth muscle cells in blood vessels and especially the reticular layer of the maternal stroma **[228]**. Concerning the female genital tract, exogenous added gal-1 has an inhibitory effect in steroidogenic activity on granulose cell in the ovaries **[229,230]**. It is expressed in endometrium and its expression levels vary according to the phase of the menstrual cycle and during the early phase of gestation. Galectiin-1 expression increases significantly at late secretory phase of

endometrium and deciduas and shows a specific pattern of expression in trophoblastic tissue. During embryogenesis, specifically on the first trimester, galectin-1 is expressed in connective tissue, smooth and striated muscles, in epithelial such as skin, gonads, thyroid gland and the kidneys **[212]**. This protein was the first isolated in human placenta, among placental trophoblast cells, gal-1 was immunolocalizes in the cytotrophoblast. Galectin-1 is synthesized prior to implantation in the trophoectoderm of blastocysts, suggesting a role in the attachment of the embryo in the uterine epithelium. Galectin-1 has been implicated in some of the pathological conditions involving trophoblast, such as early pregnancy loss, preeclampsia and trophoblastic malignant disease. Galectin-1 has also been recently qualified as a member of human trophoblast cell invasion machinery **[231]**. Galectin-1 is involved in the immune-mediated fetal tolerance during pregnancy promoting the generation of tolerogenic dendritic cells, inducing IL-10 expressing T regulatory cells and a Th2 cytokine shift by provoking apoptosis of susceptible Th1 cells. Moreover galectin-1 is under the control of ovarian steroids during blastocyst implantation **[232]** and is initially synthesized in the trophoectoderm. Galectin-1 plays an important role in the organization of extracellular matrix and the regulation of trophoblast differentiation and cell motility during placentation **[231,233]**. So, galectin-1 is very important in trophoblast differentiation and plays a major role in pregnancy maintenance. Circulating gal-1 levels could serve as a predictive factor fro pregnancy success in early human gestation as compared with normal pregnant women, circulating gal-1 levels were significantly decreased in patients who suffered a miscarriage **[234].**



Galectin-1 immunostaining and LGALS1 *expression in normal-term placenta and extraembryonic membranes. (*A*) Galectin-1 expression, shown with shades of brown, is strong in the placenta (p) and fetal membranes (fm), and the most intense in maternal decidua (de). (*B*) All layers of the fetal membranes [amnion (am); chorioamniotic mesenchyma (cm); chorion (ch)] stained for galectin-1; maternal decidua (de) is the richest source of galectin-1. (*C*) All placental cell types (trophoblasts [tr], stromal cells [sc], villous endothelium [en]) stained for galectin-1. Immunohistochemisrty, haematoxylin counter stain, magnification ×200 (*B*) and ×400 (*C*). (*D*)* LGALS1 *expression was 3.6-fold higher in the membranes including maternal decidua (*n = 6*) than in placentas (*n = 6*) of the same patients. Boxes represent medians and interquartile ranges, whereas whiskers represent the most extreme data points.* ***[227,235]***

5.3.4. Galectins- endometriosis

As endometriosis is characterized by the release of several proteins including growth factors, integrins, cadherins and lectins that regulate cell migration, invasion, angiogenesis, immune functions and apoptosis, galectins should play an important role in this inflammatory disease. So far, only galectin-3 has been found to be overexpressed in various forms of endometriosis and by having a higher expression compared to eutopic endometrium and by having a higher expression in eutopic endometrium of women with endometriosis than in eutopic endometrium of women without endometriosis **[236]**.

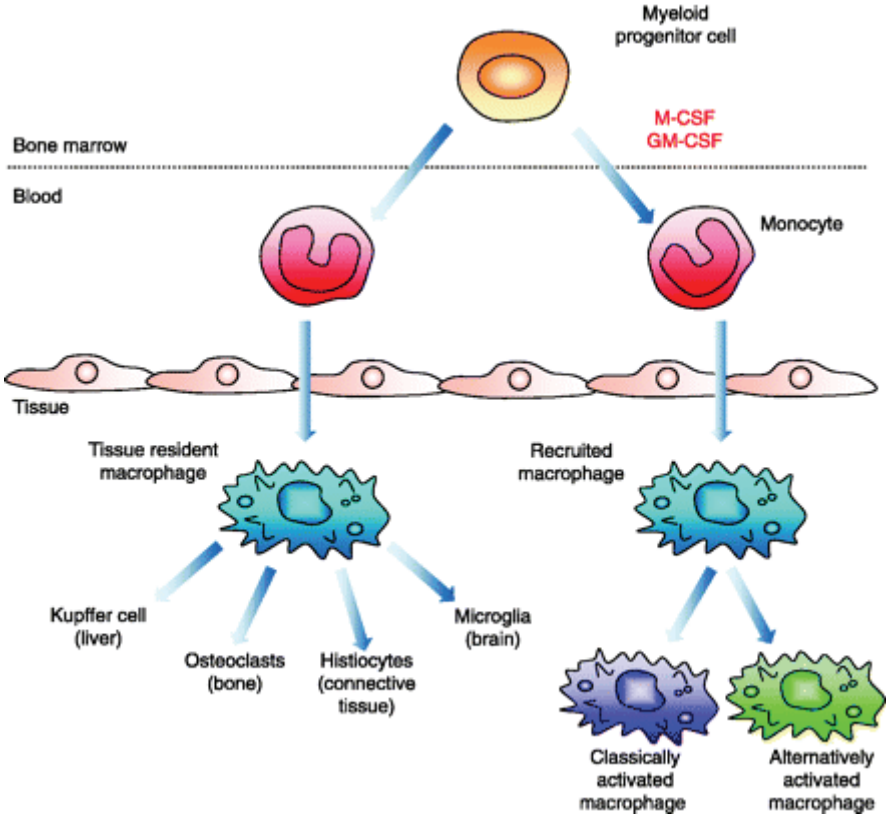
CHAPTER 6

Colony stimulating factor-1 (CSF-1)

6.1. GENERAL

Colony stimulating factor- 1, is also known as macrophage colony stimulating factor is a secreted cytokine, characterized as a hematopoetic growth factor which influences hematopoietic stem cells to differentiate into macrophages or other related cell types. Bone marrow-derived monocytes enter peripheral blood and circulate for several days in an inert state before entering tissues and differentiating into tissue resident macrophages. Tissue resident macrophages include histiocytes (connective tissue), microglial cells (nervous system), Kupffer cells (liver) and osteoclasts (bone). Macrophages are recruited to tissues to restore resident populations or respond to diverse inflammatory and immune stimuli. The acquired phenotype of recruited macrophages reflects signals from the microenvironment in which they reside. These macrophages are broadly classified as classically or alternatively activated macrophages Eukaryotic cells also produce M-CSF in order to combat intercellular viral infection. M-CSF binds to the Colony stimulating factor 1 receptor. CSF-1 regulates the survival, proliferation and differentiation of mononuclear phagocytes to macrophages **[237,238]**. M-CSF1 is produced by fibroblasts, endothelial cells, monocytes and macrophages. M-CSF is a cytokine. The active form of the protein is found extracellularly as a disulfide-linked homodimer, and is thought to be produced by proteolytic cleavage of membrane-bound precursors. Four

transcript variants encoding three different isoforms have been found for this gene. So, there are three forms of CSF-1: a secreted glycoprotein, acting by humoral route, a secreted proteoglycan, which is an extracellular matrix-anchored form acting locally and a cell-surface glycoprotein **[239]**.



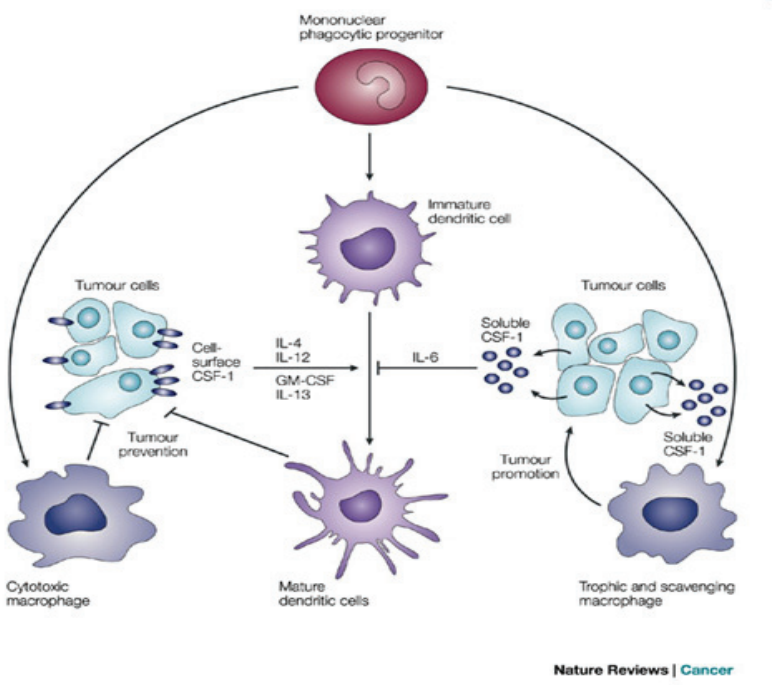
M-CSF, macrophage colony stimulating factor; GM-CSF, granulocyte-macrophage colony stimulating factor. [240]

CSF-1 exerts its functions and acts via a high affinity transmembrane tyrosine kinase receptor, M-CSF1R (macrophage colony stimulating factor receptor), which is the product of the c-fms oncogene, a cellular variant of the v-fms oncogene encoded by the feline sarcoma virus. In humans, CSF1R is expressed in monocytes, macrophages, trophoblast, syncytiotrophoblast, fetal Hofbauer cell, endometrium and in several tumours such as adenocarcinoma of the ovary, endometrium and breast.CSF-1 has immunomodulatory roles in tumours and inflammation. Studies with op/op mouse, having an inactive mutation in the CSF-1 gene, have shown that CSF1 regulates macrophage production **[241]**.

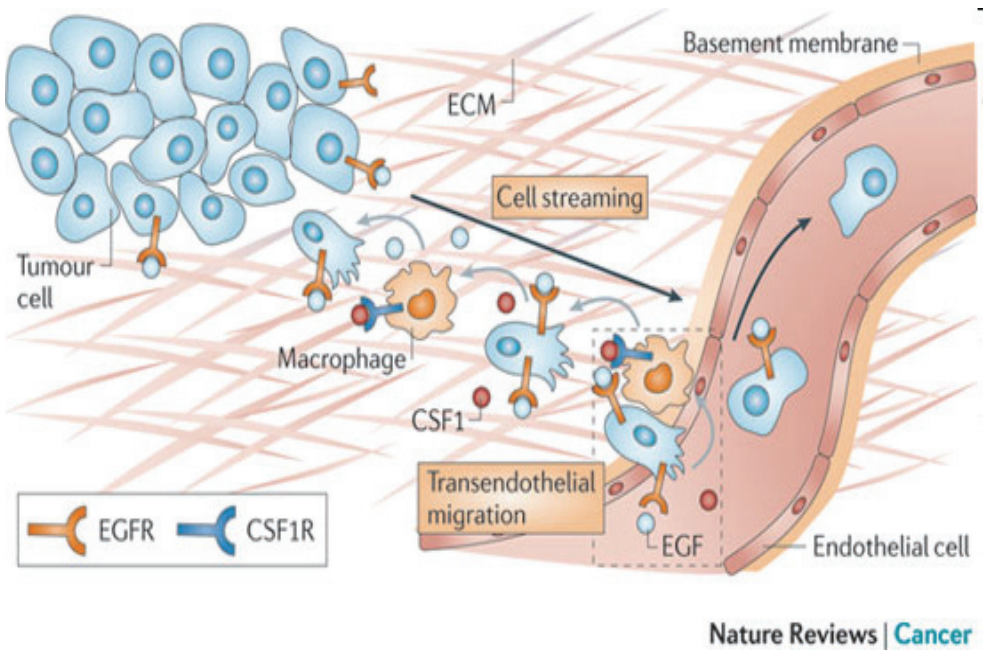
6.2. CSF-1 and cancer

One of the nonhematopoetic roles of CSF-1 and CSF-1R is that in cancer. It has been shown that several human carcinomas and carcinoma derived cell lines are characterised by abnormal expression of CSF-1R. Moreover, increased levels of CSF-1R in breast, ovarian, cervical and endometrial carcinomas have been linked with poor outcome and tumourigenesis. Furthermore, CSF-1R plays a vital role in the

accumulation of macrophages in tumor sites and abundant tumor- associated macrophages (TAMs) are indications of poor prognosis in breast and ovarian cancers. Tumours are populated by macrophages and dendritic cells that are derived from mononuclear phagocytic progenitor cells. In many tumours, a high concentration of soluble colony-stimulating factor-1 (CSF-1) educates macrophages to be trophic to tumours and, together with interleukin-6 (IL-6), inhibits the maturation of dendritic cells. This creates a microenvironment that potentiates progression to metastatic tumours. By contrast, CSF-1 presented in a transmembrane form on the tumour surface activates macrophages to kill tumour cells. This — together with high concentrations of IL-4, IL-12, IL-13 and GM-CSF — causes dendritic cells to mature, allowing the presentation of tumour antigens to cytotoxic T cells, with the consequent rejection of the tumour. Both streaming migration and intravasation require macrophages. In the metastatic tumour microenvironment shown, initiation of chemotaxis to epidermal growth factor (EGF) that is supplied by macrophages promotes colony-stimulating factor 1 (CSF1) production by tumour cells. Macrophages chemotax towards CSF1, resulting in relay chemotaxis between the two cell types. Relay chemotaxis results in paracrine-dependent carcinoma cell streaming and transendothelial migration. The close proximity of invasive tumour cells, macrophages and endothelial cells leads to the formation of the tumour microenvironment of metastasis (TMEM) which has also been found as an anatomical landmark in tumour tissues from patients with breast cancer. Black arrows indicate the direction of cell migration, grey arrows indicate the gradient direction of chemotactic factors: from the cell that secretes to the cell that responds to the factor. CSF1R, CSF1 receptor; ECM, extracellular matrix; EGFR, EGF receptor **[242]**.



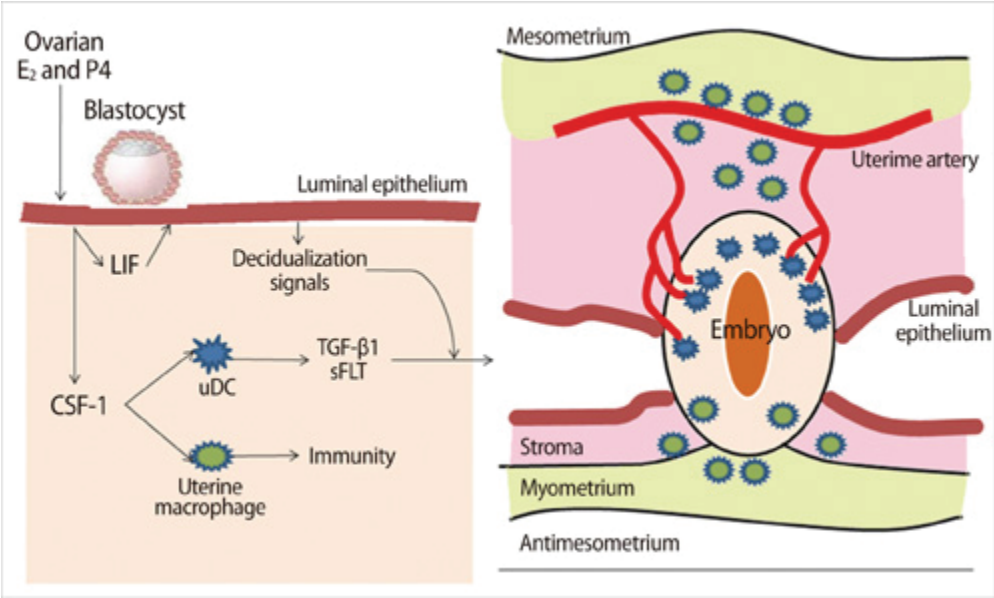
Tumour-educated macrophages promote tumour progression and metastasis [243]



Chemotaxis in cancer [242]

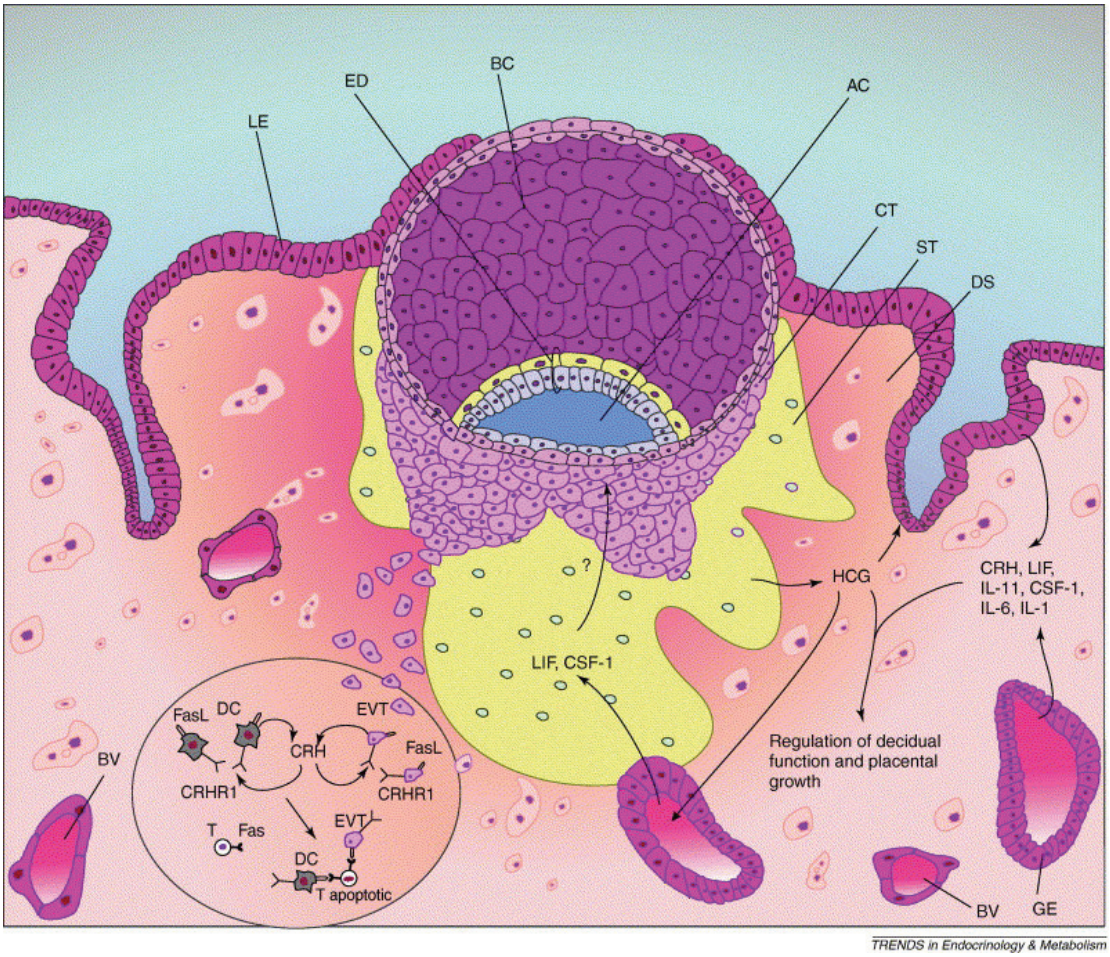
6.3. CSF-1 and reproduction

Macrophages and other cells of the female reproductive tract are also regulated by locally produced CSF-1. CSF-1 plays an important role in placental growth and differentiation. CSF1 has important roles in non-hematopoetic cells too, including endometrium. CSF1 and its receptor are expressed by endometrial stromal cells, endometrial epithelial cells and peritoneal mesothelial cells. It has also been detected in endometrial tissue, amniotic fluid and placenta. The concentration of CSF-1 in uterus increases during pregnancy. Progesterone and estradiol regulate CSF-1 synthesis by the luminal and glandular secretory epithelial cells of the uterus. Although, the regulation of locally produced CSF-1 during pregnancy is separate from the regulation of circulating CSF-1. Also, CSF-1 stimulates endometrial epithelial cells in an autocrine mechanism. Knock down of CSF-1 in endometrial epithelial cells results in decreased proliferation and transmethothelial invasion of endometrial epithelial cells **[244]**. Levels of colony-stimulating factor (CSF)-1 synthesized by the uterine epithelium increase at the time of implantation and continue to elevate dramatically throughout the process of placentation. This CSF-1 is the major regulator of the mononuclear phagocytic lineage and controls the proliferation, migration, viability, and function of DCs and macrophages and affects decidual cells and trophoblasts **[245]**.



Role of dendritic cells (DCs) and macrophages in implantation. Ovarian steroid hormones, E2, and progesterone (P4) stimulate synthesis of growth factors from endometrial epithelial cells, including colony-stimulating factor (CSF-1) and leukemia inhibitor factor (LIF). Uterine DCs are required for efficient decidualization of the endometrium. Macrophages play a largely immune role at this TGF, transforming growth factor; sFLT, soluble FMS-like tyrosine kinase. [246]

A role for CSF-1 in implantation is indicated by studies in osteopetrotic mice, which lack functional CSF-1. Homozygous females with a mutated CSF-1 gene (op/op) were consistently infertile when mated with males with the same genotype. Pregnancies were partially rescued in crosses between op/op females and op/+ males, probably as a result of fetal or seminal fluid- derived CSF-1 or CSF-1- induced factors, which compensated for the absence of maternal CSF-1 **[247]**. Moreover, it has been indicated that CSF-1 increase the rate of preimplantation mouse embryo development and the number of trophoectodermal cells in the blastocyst **[248]**. In humans, there is an increased local production of CSF-1 in tissues found at the maternal-fetal interface during the time of implantation and early pregnancy. As CSF-1R is expressed in these tissues, CSF-1 might have an important role in decidual function and placental growth **[249]**. Low CSF-1 serum levels have been correlated with unexplained recurrent spontaneous abortion in both the preconceptional and conceptional phases. Moreover, decidual T cells clones from women suffering from unexplained recurrent abortion display decreased production of CSF-1, as well as of other cytokines, including LIF **[250]** .



Hormonal and cytokine factors acting locally at the maternal–fetal interface during the early phase of blastocyst implantation. As shown in the bottom-left hand side of the diagram, CRH produced locally by EVTs and decidual cells acts through CRHR1 to stimulate FasL synthesis and to potentiate the ability of these cells to cause apoptosis of activated maternal T cells (Fas receptor positive). CRH, LIF, IL-11, ***CSF-1****, IL-6 and IL-1 produced by the endometrial epithelium participate in the regulation of decidual function and placental growth (right-hand side of the diagram). HCG produced by the syncytiotrophoblast acts at the stroma to promote decidualization, as well as at the glandular and luminal epithelium to induce glycodelin secretion and epithelial plaque reaction, respectively. The effect of endometrial-derived LIF and CSF-1 (middle of the diagram) in mammalian blastocysts is still a matter of debate. Abbreviations: AC, amniotic cavity; BC, blastocyst cavity; BV, blood vessel; CRH, corticotropin-releasing hormone; CRHR1, CRH receptor type 1; CT, cytotrophoblast; DC, decidual cell; DS, decidualized stroma; ED, embryonic disk; EVT, extravillous trophoblast; Fas, Fas receptor; FasL, Fas ligand; GE, glandular epithelium; HCG, human chorionic gonadotropin; IL, interleukin; LE, luminal epithelium; LIF, leukemia inhibitory factor; ST, syncytiotrophoblast; T, T lymphocyte. [11]*

6.4. CSF-1 and endometriosis

As endometriosis is an inflammatory disease, there are several cytokines and growth factors that are implicated in this immune-disequilibrium status. As secretory cells, monocytes and macrophages are vital to the regulation of immune responses and the development of inflammation. These cytokines are produced by peritoneal macrophages, lymphocytes, mesothelial cells and endometriosis implants **[251]**. Furthermore, women and female baboons with endometriosis are characterised by increased number of peritoneal macrophages **[252]**. Women with endometriosis are characterised by increased expression of CSF-1 compared to healthy ones **[53]**. Although, it is unclear whether increased levels of CSF-1 in patients with endometriosis originate from inflammation associated macrophages or it is produced by the endometriotic

lesions. Therefore, one study supports that CSF-1 and its receptor are involved in the genesis of early endometriotic lesions and that endometriotic lesions contribute to elevated CSF-1 levels in the peritoneal fluid of women with endometriosis and that elevated levels of CSF-1R in endometrial cells from women with endometriosis after co-culture with peritoneal mesothelial cell suggests that endometrial tissue is highly responsive to CSF-1 signalling **[253,254]**.Moreover, endometrial tissue from CSF-1 knockdown mice develop significantly fewer endometriotic lesions than control tissue in a syngeneic mouse model of endometrial tissue transplantation into the pelvic cavity **[255]**.

AIM OF THE STUDY

CHAPTER 7

AIM OF THIS STUDY

The aim of this study was:

i) To examine the expression of CRH, UCN, CRHR1, CRHR2 and their subtypes CRHR1β and CRHR2α at endometriotic sites.

ii) To compare the expression of CRHR1 and CRHR2 in eutopic endometrium of endometriotic and healthy women.

iii) To evaluate and compare the expression levels of CRH, UCN and their receptors in endometriotic – ectopic sites with those in eutopic endometrium of endometriotic women.

iv) To examine galectin-1 expression in eutopic and ectopic endometrium of women with endometriosis and in eutopic endometrium of healthy women.

v) To evaluate how CRH, UCN and CSF-1 regulate galectin-1 expression in Ishikawa cell line and macrophages.

vi) To examine CSF-1 expression in eutopic and ectopic endometrium of women with endometriosis and in eutopic endometrium of healthy women.

vii) To evaluate how CRH, UCN and CSF-1 regulate CSF-1 expression in Ishikawa cell line and macrophages.

viii) To evaluate how CRH, UCN and CSF-1 regulate FasL expression in Ishikawa cell line and macrophages.

MATERIALS AND METHODS

CHAPTER 8

MATERIALS AND METHODS

8.1. Tissue sample collection

Endometrial biopsy specimens (at secretory phase, as it was confirmed by the progesterone levels of the women) were taken from healthy women (10 patients) undergoing hysteroscopy for diagnostic reasons as their most recent menstrual cycles were characterised by spontaneous spotting haemorrhage. Placenta and myometrium tissue of healthy women had been used as well. The outcome of their hysteroscopy showed that they were all healthy apart from 3/10 having small polyps. Endometrial and Endometriotic tissue biopsies (stage III and IV) at secretory phase, as it was confirmed by the progesterone levels of the patients, were obtained from 16 patients diagnosed with endometriosis on different sites (peritoneal nodule, rectovaginal nodule, rectouterine nodule, right and left ovarian cyst endometriosis, left and right uterosacral legiment nodule), sharing all the same pathology, in the Department of Obstetrics and Gynaecology, St Bartholomew's Hospital of Queen Mary University, London, UK (Research Ethics Committee Reference Number: 05/Q0604/44). This research protocol was approved by the Ethics Committee of Queen Mary University, London, UK. It is important to notice that the most critical reproductive hormone levels of both healthy and endometriotic patients did not affect the outcome of our research protocols as they ranged among: FSH levels (day 3 of the menstrual cycle): healthy patients 6-8mlU/ml, endometriotic patients 7-9mlU/ml, E_2 levels (day 3 of the menstrual cycle): healthy patients 45±7pg/ml, endometriotic patients 50±11pg/ml and Progesterone levels (day 21 of the menstrual cycle): healthy patients 17±2ng/ml, endometriotic patients 15±3ng/ml.

8.2. Cell culture

8.2.1. Materials for cell culture:

- Trypan Blue (Seromed Biochrom, Germany)
- Cover slips 22 x 22 mm και 24 x 32 mm (Deckglaser, Knittel glaser)
- Haemocytometer (Neubaeur)
- DMEM Glutamax Dulbecco's Modified Eagle's Medium, (GIBCO-BRL Co)
- DMEM (GIBCO, REF no. 41966-029).
- RPMI medium, GIBCO-BRL Co)
- FBS (GIBCO-BRL Co)
- DMSO(Sigma)
- Thrypsin EDTA 0,25% (GIBCO-BRL Co, MD, USA)
- Peniccilin Strepromycin (GIBCO, Invitrogen, Carlsbad, USA)
- Sodium Pyruvate (GIBCO, Invitrogen, Carlsbad, USA)

- Anti – Mycotic (GIBCO, Invitrogen, Carlsbad, USA)
- PBS,phosphate buffured saline (pH=7,4) (Sigma-Aldrich, USA)
- B-mercaptoethanol (Sigma Aldrich, M7522)
- Non-essential amino acids (Gibco 11140-035)
- Lcells solution : prepared in lab
- 2ml cryovials(greiner bio-one GmbH)
- Pipettes (Gilson)
- 100x20mm Cell culture dish cell star(greiner bio-one GmbH)
- Cell culture flasks 175cm^2(greiner bio-one GmbH)
- Petril dish(Bector Dincinson)
- 1.5 ml eppendorfs(greiner bio-one GmbH)
- 5,10,25ml pipettes(greiner bio-one GmbH)
- 15, 50ml falcon (greiner bio-one GmbH)
- Centrifuge (5415 eppendorf)
- Versene (Gibco)
- Filters 0.22 pore(Corning incorporated life sciences)
- Reverse microscope (Olympus, Japan)

8.2.2. Cell lines and macrophages:

Ishikawa cell line: Human endometrial adenocarcinoma cell line. Ishikawa cells show mixed characteristics of glandular and luminal epithelium and express many of the same enzymes and structural proteins present in normal endometrium, along with functional steroid receptors, which makes them suitable for the study of endocrine signaling in endometrium. Ishikawa cells are widely considered a good model for studying endometrium function **[256].**

Jeg-3 cell line: Human placental choriocarcinoma cell line. The human placenta is a highly invasive tumor-like structure in which a subpopulation of placental trophoblast cells known as the "extravillous trophoblast" (EVT) invades the uterine decidua and its vasculature to establish adequate fetal-maternal exchange of molecules. In vitro-propagated short-lived EVT cell lines such as JEG-3 cells are used to study molecular mechanisms responsible for this invasiveness, which are identical to those of cancer cells; however, unlike cancer cells, their proliferation, migration, and invasiveness in situ are stringently controlled by decidua-derived transforming

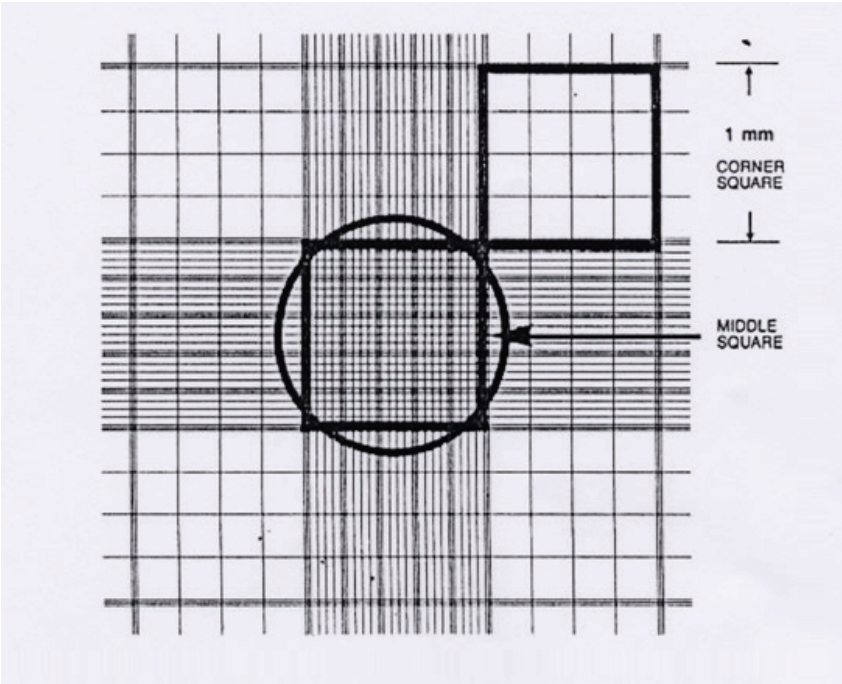
growth factor (TGF)-beta. These epithelial-trophoblast-like cells are therefore used in identifying genetic changes underlying epithelial tumor progression as well as gonadotrophin secretion studies.

Ishikawa and Jeg-3 cell lines were purchased from the European Collection of Cell Cultures (ECACC, Salisbury, UK) and was a generous gift from Prof Udo Jeschke. Both cell lines were cultured in DMEM Glutamax supplemented with 10% FBS (inactivated at 56^0C for 1hr), 1% Penicilline/Streptomysin, 1% Sodium Pyruvate and 1% Anti – Mycotic at a humidified incubator in a 5% CO2 atmosphere at 37 ^{0}C. The medium used to be changed every 48hrs. Cells were cultured in cell culture dishes and Flasks.

8.2.3. Cell counting

- Ensure the cover-slip and haemocytometer are clean (use alcohol to clean).
- Attach cover-slip to the haemocytometer.
- Mix equal volumes of 0.4% trypan blue stain and a well mixed cell suspension e.g. mix 10µl trypan blue stain with 10 µl cell suspension (cells suspended in medium).
- Pipette trypan blue/cell mix (approximately 10µl) at the edge of the cover-slip and allow running under the cover slip.
- Visualise the haemocytometer grid under the microscope
  Live cells appear colourless and bright (refractile) under phase contrast.
  Dead cells stain blue and are non-refractile.
- Count viable (live) in 4 large corner squares and record cell counts.
- To calculate cell concentration per ml:

(No of cells on corner square 1+ 2+ 3+ 4)/4 * 10 * 10^4) = cells/ml



8.2.4. Protocol for freezing cells for long-term cell line storage

In order to produce large amounts of cells, cells where cultured in dish/flask and when they were filled up to 80%, the follow procedure was performed:

- Remove medium from one dish / flask, wash with 1x sterile PBS and trypsinize for 15min at 37^0C
- Once cells are detached, add back 5-10 ml medium and transfer to centrifuge tube (50ml sterile centrifuge tube).
- Count the cells using trypan blue as described above.
- Centrifuge at 1200 rpm for 10 minutes and remove medium.
- Resuspend cells in enough freezing medium(FBS+10%DMSO) to create a cell suspension of $4x10^6$ cells per ml. Pipette up and down to ensure even mixture and aliquot about 2 ml into cryovials. This will provide $8x10^6$ cells per cryovial.
- Transfer cells immediately to by -80°C.

8.2.5. Defreeze and recovery of cells

- Remove cryovial from -80°C and suspend frozen cell in warmed 10ml medium
- Centrifuge at 1200rpm for 10min, throw supernatant
- Suspend palette with medium and place cell in disk/flask at 37°C.

8.2.6. Murine bone marrow- derived primary macrophages

In this protocol, 6 to 8 week old mice were used. Bone marrow cells are harvested and cultured in the medium containing macrophage colony–stimulating factor (M-CSF;[**257**]). After 7 days in culture, contaminating nonadherent cells are eliminated and adherent cells are harvested for assays. All of the cells in this population of macrophages have the ability to respond to exogenous stimuli, and, therefore, relatively uniform populations of macrophages can be generated and harvested.

8.2.6.1. Protocol:

- Euthanize mice.
- Using aseptic technique, peel the skin from the top of each hind leg and down over the foot. Cut off the foot along with the skin and discard. Cut off the hind legs at the hip joint with scissors, leaving the femur intact. Place femurs in plastic dish containing sterile PBS.
- Remove excess muscle from legs by holding end of bone with forceps and using scissors to push muscle downward away from forceps. Alternatively, excess muscle can be removed from the legs by holding the end of the bone and pushing muscle away from bones with fingers.
- Using sharp scissors or razor blade soaked in ethanol, carefully sever leg bones proximal to each joint.
- Attach 10-ml syringe to 25-G needle and fill with cold sterile wash medium:

8.2.6.2. Normal Growth Medium of primary mice macrophages

- RPMI
- 10% inactivated FBS
- 5ml pen/strep
- 5 ml sodium pyruvate
- 5 ml non-essential amino acids
- β-mercaptoethanol– make a diluted stock, 4 µl β-ME in 10 ml RPMI then add 2.5 ml of this to a 500 ml bottle of medium
- 10% L-cell conditioned medium (source of CSF-1, recipy and preparation of LC medium described later)

- Insert needle into bone marrow cavity of femur and tibia. Flush bone cavity with 2 to 5 ml of the wash medium, until bone cavity appears white. Allow wash medium to collect in a sterile 50-ml conical centrifuge tube on ice.(5ml medium for each pair of legs)
- Centrifuge cells 10 min at 1200rpm, room temperature.
- Discard supernatant. Resuspend cell pellet at 10^6 cells/ml in macrophage complete medium and pipetting up and down.
- Culture bone marrow cells at 10^6 cells/ml per Petri dishes (Falcon, 100 * 15 mm, 10 ml for these dishes). Leave 3 days. Incubator 5 or 10% CO_2 and 37°C. – these are BACTERIAL PETRI DISHES.
- Grow on bacterial Petri dishes (Falcon) NOT tissue culture treated. These cells are so adherent that they can not be detached.
- Collect the non adherent population, count and freeze down in cryovials in growth media containing 10% DMSO Cell Culture (~ 4.10^6 cells/vial) and/or seed them on Petri dishes for 4 more days.

- Day 4^{th} (7^{th} in total): You need to split and resuspend them in medium and plate as required for your experiment (You can now use tissue culture treated dishes)
- Lcell medium needs to be added every two days during cell culture.

8.2.6.3. Detachment of macrophages

Remove cells by washing twice with approx 2 ml versene (4 ml for 100 mm dishes) then incubate in 2 ml (for 60 mm dish) versene for 5-10 min. Wash cells off by pipetting over versene with a 1 ml Gilson. Add 8 ml starvation medium then mix and count.

8.2.6.4. Starvation medium for macrophages ~ 16hrs prior to experiment

No more than 16hrs before the experimental procedure, add all ingredients of normal growth medium for macrophages, as described above, no L-cell conditioned medium. Cells can be confluent for stimulation (unlike epithelial cells).

8.2.7. Preparation of LCells medium

The medium needed for LCells preparation is: D-MEM including: 4500 mg/L glucose, + L-glutamine,+ pyruvate. With heat inactivated serum at 10% and 5 mL penicillin/streptomycin (10000 iu/mL).

- Culture the cells in 75 square cm vented flasks. Cells are recovered from frozen into a flask in 20 mL medium **(** for 3-4 days (or until confluent). For continued culture, cells will require splitting 1 in 5, twice weekly using 4 mL trypsin-EDTA per flask.
- For harvesting: expand cells as required, usually we reseed the cells from 4 flasks of 75 cm^2 into 10 flaks of 150 cm^2. The cells from the 5^{th} flask are frozen down into 5 vials (1:5).
- Allow 24 hours for cells to adhere and then close the vented lids of the flasks. Cells will now begin conditioning the medium with CSF-1. Leave the medium to condition for about three weeks but it can be harvested after two weeks.
- To collect the medium: Check each flask under microscope to ensure cells are okay and still adhered to plastic (there may be some non-adherent cells at this stage, but there should still be a fully confluent adherent layer.
- Aspirate medium from cells and collect into 50mL falcon tubes. Spin tubes for 5 minutes at 1000 rpm. Pool the supernatants and filter under vacuum (filter pore size: 0.22um). Aliquot medium and store at 4C.

8.3. Tissue and cell homogenisation - RNA Extraction - cDNA synthesis

8.3.1. Materials

- Trizol (Trisol reagentTM, Sigma, USA)
- Chlorophorm (Sigma, USA) kept at -20°C
- 2- propanol, 75% Ethanol (Merk,Germany) kept at -20°C
- DEPC- H_20 (Diethylpyrocarbonate (DEPC)–H2O)
- RT-PCR cDNA Synthesis Kit, ThermoScript (Invitrogen)
- Centrifuge (5415 eppendorf)
- Eppendorfs (greiner bio-one GmbH)

- ❖ 15, 50ml falcons(greiner bio-one GmbH)
- ❖ Spectrophotometer (perkin-elmer lamda computer)
- ❖ Tissue homogenator(Quiagen)

RNA was isolated from Ishikawa cell line, JEG3 cell line, endometrium of healthy women (10 patients) and eutopic endometrium and endometriotic tissue samples from 16 patients with endometriosis were homogenised in Trizol (Invitrogen, Carlsbad, USA). RNA was measured in a spectrophotometer by measuring ultraviolet absorbance at 260 nm and used for the cDNA synthesis according to the cDNA synthesis Kit (Thermoscript, Invitrogen, Carlsbad, USA).

8.3.2. Homogenization - RNA extraction Protocol:

- Homogenise tissue samples (100mgr each) and pellet cells(5-10*10^6 cells) in 1ml of Trizol reagent and incubate the samples with trizol for 5min at RT.
- Add 0.2ml of chlorophorm, shake the tubes vigorously for 15sec up and down, leave at RT for 5min.
- Centrifuge at 12.000xg for 15min at 4°C.
- Transfer the upper phase, where RNA remains in a fresh tube, add 0.5ml of 2-propanol and incubate at RT for 10min.
- Centrifuge at 12.000xg for 10min at 4°C. The RNA precipitates and forms a gel like pellet on the side and bottom of the tube.
- Remove the supernatant, wash the RNA pellet with 75% Ethanol, vortex and centrifuge at 7500xg for 5min at 4°C
- Dry the RNA pellet and dissolve RNA with DEPC water(~0.010ml) and incubate for 10min at 55-60°C and store at -80°C till it will be used.
- RNA was measured in a spectrophotometer at 260nm.

8.3.3. First-Strand cDNA Synthesis Using ThermoScript. RT cDNA synthesis protocol:

Add the following components to a nuclease-free microcentrifuge tube:

❖ 1 µl random primers

❖ 2.5 µg total RNA

❖ 2 µl 10 mM dNTP Mix

❖ 12 µl Sterile, distilled water

- Incubate mixture at 65°C for 5 min. and
- Collect the contents of the tube by brief centrifugation and add:

❖ 5X cDNA Synthesis Buffer 4 µl

❖ 0.1 M DTT 1 µl

❖ RNaseOUT. (40 units/μl) 1 μl

❖ Sterile, distilled water 1 μl

❖ ThermoScript. RT (15 U/μl)** 1 μl

- incubate tube at 25°C for 10 min.
- Mix contents of the tube gently and incubate at 50°C for 60 min.
- Terminate the reaction by heating at 85°C for 5 min.
- To remove RNA complementary to the cDNA 1 μl (2 units) of *E. coli* RNase H and incubate at 37°C for 20 min
- Store at -20°C

8.4. RT-PCR (Reverse transcription - polymerase chain reaction) detection of CRH, UCN, CRHR1β and CRHR2α.

8.4.1. Materials

- ❖ dNTPs 10mM (Life Technologies, ΗΠΑ)
- ❖ 50mM $MgCl_2$ (Promega)
- ❖ Buffer (Promega)
- ❖ Taq Polymerase(Promega)
- ❖ Primers 10Mm stock (MWG- Biotech AG, VBG- GENOMICS, Biosciense Research GmbH)
- ❖ Water for injection
- ❖ Ladder(Fermentas 50bp DNA ladder), 100bp ladder promega G210A
- ❖ Agarose(Invitrogen)
- ❖ TBE (Tris, Boric Acid, EDTA, Biorad labs, BDH)
- ❖ Ethidium Bromide (Sigma)
- ❖ Alpha imager
- ❖ PCR machine (esco swift maxi)
- ❖ Orange dye (Fermentas)
- ❖ Electrophoresis machine (Power Pac Biorad Labs, USA)

Reverse transcription polymerase chain reaction (RT-PCR) is one of many variants of polymerase chain reaction (PCR). This technique is commonly used in molecular biology to detect RNA expression levels. RT-PCR is used to clone expressed genes by reverse transcribing the RNA of interest into its DNA complement through the use of reverse transcriptase. Subsequently, the newly synthesized cDNA is amplified using traditional PCR.

PCR is used to amplify a specific region of a DNA strand (the DNA target). Most PCR methods typically amplify DNA fragments of between 0.1 and 10 kilo base pairs (kb), although some techniques allow for amplification of fragments up to 40 kb in size. The amount of amplified product is determined by the available substrates in the reaction, which become limiting as the reaction progresses.

Each PCR reaction is prepared in a PCR hood, completely sterile environment and incubated in a thermal cycler. Each PCR reaction contains these components:

❖ DNA template that contains the DNA region (target) to be amplified.

❖ Buffer solution providing a suitable chemical environment for stability of the DNA polymerase.

❖ $MgCl_2$ 25Mm

❖ dNTPs, the building-blocks from which the DNA polymerase synthesizes a new DNA strand.

❖ Taq DNA Polymerase with a temperature optimum at around 70 °C.

❖ Primers complementary to the 3' (three prime) ends of each of the sense and anti-sense strand of the DNA target.

8.4.2. General PCR procedure

- Denaturation step: This step is the first regular cycling event and consists of heating the reaction to 94–98 °C for 20–30 seconds. It causes DNA melting of the DNA template by disrupting the hydrogen bonds between complementary bases, yielding single-stranded DNA molecules.

- Annealing step: The reaction temperature is lowered to 50–65 °C for 20–40 seconds allowing annealing of the primers to the single-stranded DNA template. Typically the annealing temperature is about 3-5 degrees Celsius below the Tm of the primers used. Stable DNA-DNA hydrogen bonds are only formed when the primer sequence very closely matches the template sequence. The polymerase binds to the primer-template hybrid and begins DNA formation.

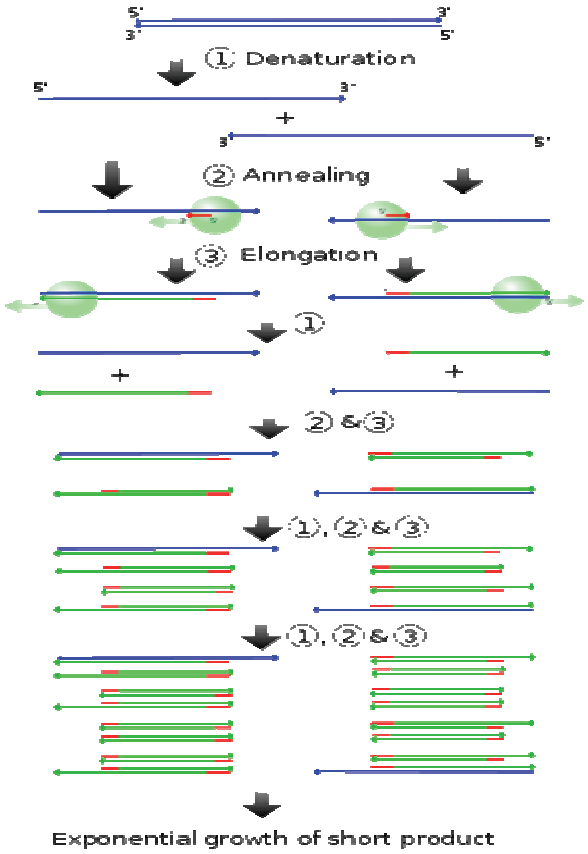
- Extension/elongation step: The temperature at this step depends on the DNA polymerase used; Taq polymerase has its optimum activity temperature at 75–80 °C, and commonly a temperature of 72 °C is used with this enzyme. At this step the DNA polymerase synthesizes a new DNA strand complementary to the DNA template strand by adding dNTPs that are complementary to the template in 5' to 3' direction, condensing the 5'-phosphate group of the dNTPs with the 3'-hydroxyl group at the end of the nascent (extending) DNA strand. The extension time depends both on the DNA polymerase used and on the length of the DNA fragment to be amplified. As a rule-of-thumb, at its optimum temperature, the DNA polymerase will polymerize a thousand bases per minute. Under optimum

conditions, i.e., if there are no limitations due to limiting substrates or reagents, at each extension step, the amount of DNA target is doubled, leading to exponential (geometric) amplification of the specific DNA fragment.

- Final elongation: This single step is occasionally performed at a temperature of 70–74 °C for 5–15 minutes after the last PCR cycle to ensure that any remaining single-stranded DNA is fully extended.

- Final hold: This step at 4–15 °C for an indefinite time may be employed for short-term storage of the reaction.

To check whether the PCR generated the anticipated DNA fragment (also sometimes referred to as the amplimer or amplicon), agarose gel electrophoresis is employed for size separation of the PCR products. The size(s) of PCR products is determined by comparison with a DNA ladder (a molecular weight marker), which contains DNA fragments of known size, run on the gel alongside the PCR products.



(1) Denaturing at 94–96 °C

(2) Annealing at ~65 °C

(3) Elongation at 72 °C.

Four cycles are shown here. The blue lines represent the DNA template to which primers (red arrows) anneal that are extended by the DNA polymerase (light green circles), to give shorter DNA products (green lines), which themselves are used as templates as PCR progresses.

To quantify mRNA expression of CRH, UCN, CRHR1β and CRHR2α in eutopic endometrium of healthy(10 women) and endometriotic women(16 women) and in endometriotic tissue from 16 patients, as well as in Ishikawa cell line and JEG3 cell line, reverse transcription PCR (RT-PCR) of CRH, UCN, CRHR1β and CRHR2α. was performed. Ten microliters of the amplification products (CRH: 413bp, UCN: 146bp, CRHR1β:554bps, CRHR2α:322bps) was separated on a 2% agarose gel and visualized by ethidium bromide staining. Human CRH primer sequences were as follows: Forward: 5'- CAC CCT CAG CCC TTG GAT TTC -3', Reverse: 5'- GCC CTG GCC ATT TCC AAG AC -3'. UCN primer sequences were as follows: Forward: 5'- CAG GCG AGC GGC CGC G-3', Reverse: 5'- CTT GCC CAC CGA GTC GAA T-3'. CRHR1β primer sequences were as follows: Forward: 5'- ATG GAC GCG GCA CTG CTC CA-3', Reverse: 5' – CAC GGC CTC TCC ACG AGG G-3'. CRHR2α primer sequences were as follows: Forward: 5'- GGC CAG GCT GCA CCC ATT G-3', Reverse: 5'- TCG CAG GCA CCG GAT GCT C-3'. All primers were provided by VBC Biotech, Vienna, Austria. Placenta and myometrium homogenated total RNA was used as positive controls and GAPDH (primers for GAPDH were: forward: 5′-GCCACATCGCTCAGACACCA-3′and reverse: 5′-GATGACCCTTTTGGCTCCCC-3′) as a house keeping gene. Band intensities of mRNA of interest were normalized with band intensities of GAPDH and expressed as arbitrary units (a.u.).

8.4.3. RT- PCR reaction recipies (Promega) – Total volume of 20 μl

PCR mix (per sample)	**CRH**	**UCN**	**CRHR1 - CRHR2**	**GAPDH**
D H_2O μl	**12.3**	**10.7**	**12.3**	**13.1**
Buffer μl	**4**	**4**	**4**	**4**
$MgCl_2$ μl	**1.2**	**1.2**	**1.2**	**1.2**
dNTPs μl	**0.5**	**0.5**	**0.5**	**0.5**
Primer forward μl	**0.4**	**1.2**	**0.4**	**0.5**
Primer Reverse μl	**0.4**	**1.2**	**0.4**	**0.5**
Taq μl	**0.2**	**0.2**	**0.2**	**0.2**
cDNA μl	**1**	**1**	**1**	**1**

8.4.4. 2% agarose gel recipy:

- 100ml 0.5x TBE (stock of 2lt of 5x TBE: 108gr Tris, 55gr Boric Acid, 7.44gr EDTA, pH8.3)
- 0.5μgr/ml EtBr

8.4.5. PCR Conditions

mRNA	CRH	CRHR1	CRHR2	GAPDH
PCR Conditions	98 °C for 5min 95°C for 1min 60°C for 1min 72°C for 11min 72°C for 71min 4°C forever X 40 cycles	98°C for 5min 95°C for 1min 62°C for 1min 72°C for 1 min 4°C forever x 40 cycles	98°C for 5min 95°C for 45 sec 62°C for 45 sec 72°C for 45sec 72°C for 1 min 4°C forever x 40 cycles	94°C for 30sec 58°C for 30sec 72°C for 60sec 4°C forever x 30 cycles

PCR Conditions

UCN	
1. 95 °C for 2min 2. 95°C for 30sec 3. 68°C for 30sec 4. 72°C for 30sec 5. Go to step1 for 1 time 6. 95°C for 30sec 7. 66°C for 30sec 8. 72°C for 30sec 9. Go to step 6 for 1 time 10. 95°C for 30sec 11. 64°C for 30sec 12. 72°C for 30sec	13. Go to step 10 for 1 time 14. 95°C for 30sec 15. 62°C for 30sec 16. 72°C for 30sec 17. Go to step 14 for 1 time 18. 95°C for 30sec 19. 60°C for 30sec 20. 72°C for 30sec 21. Go to step 18 for 1 time 22. 72°C for 5min 23. 4°C for ever

8.5. Immunohistochemical analysis

8.5.1. Materials

- Xylole (Merk, Germany)
- Ethanol (Merk, Germany)
- Tris(Merk, Germany)
- NaCl (Sigma)
- Citric acid (Merk, Germany)
- Sodium Citrate(Merk, Germany)
- H_2O_2 (Sigma)
- BioGenex Lig DAB substrate Pack (BioGenex Laboratories Inc, Fremont, CA, USA)
- Primary antibodies(Santa Cruz, Phoenix Pharmaceuticals, Abcam)
- BioGenex Lig DAB substrate Pack (BioGenex Laboratories Inc, Fremont, CA, USA)

- SS Multilink(Biogenex)
- Donkey Anti-goat secondary(Santa Cruz)
- Mayer's Haematoxylin((Dako, Carpinteria, CA, USA)
- Glycergel (Dako, Carpinteria, CA, USA)
- Cover slips 22 x 22 mm και 24 x 32 mm (Deckglaser, Knittel glaser)
- Slides (Superfrost plus, microscopic slides knittel glaser)
- Reverse Microscope (Zeiss IM-35)

8.5.2. Protocol:

Formalin-fixed, paraffin-embedded tissue sections (4μm thick) of eutopic and ectopic endometrium from 16 patients were at 37C for 1hr and overnight at 56^0C and the day of the experiment , sections are incubated at 56^0C for 10min, deparaffinized in xylene and rehydrated through graded concentrations of ethanol.

- 2X 100% xylole 5min each
- 3x 100% ethanol 5min each
- 3x 96% ethanol 5min each
- 1x 80% ethanol 5min each
- 1x 70% ethanol 5min each

After that, section had been washed with 1x TBS(made from stock solution of 10x: 50nmol/lt Tris, 150nmol/lt NaCl) and antigen retrieval (350W, 3 cycles, 5 min each in citrate buffer: 10% citric acid mix – 9ml citric acid and 41ml sodium citrate in 450ml ddH_20) followed. After inhibition of endogenous peroxidases with 3% H_2O_2 (5min), unspecific antibody binding was blocked with 10% power block (BioGenex Lig DAB substrate Pack, BioGenex Laboratories Inc, Fremont, CA, USA) for 10 min. Serial sections were then incubated with primary antibodies against human CRH (1:200, H-019-06, Rabbit Anti-Corticotropin Releasing Factor, Phoenix Pharmaceuticals, Belmont, USA), UCN (1:200, H-019-14, Rabbit Anti-Urocortin Serum, Phoenix Pharmaceuticals, Belmont, USA), galectin-1 (rabbit polyclonal anti-galectin-1 antibody, Abcam) overnight at 4^oC. Both blocking as well as detection and visualization of staining were performed by using the BioGenex Supersensitive link-label Detection System (BioGenex Laboratories Inc, Fremont, CA, USA) followed by the BioGenex Lig DAB substrate Pack (BioGenex Laboratories Inc, Fremont, CA, USA), according to the manufacturer's protocols. Finally the slides were counterstained with Mayer's heamatoxylin (Dako, Carpinteria, CA, USA) for 3 min, washed in tap water and covered using Glycergel (Dako, Carpinteria, CA, USA). Negative controls were performed by replacing the primary antibody with normal rabbit or goat IgG as isotype control and placental tissue was used as negative tissue control. The sections were examined by light microscopy. The intensity and distribution of the staining reaction were evaluated by two blinded, independent observers, including a gynaecological pathologist, using the semiquantitative immunoreactive score (IRS). The IRS was calculated by multiplication of optical

staining intensity including glandular and stromal staining (graded as 0=no reaction, 1=weak, 2=moderate and 3=strong staining) and the percentage of positive-stained cells (0=no positive, 1=<25% of the cells, 2=25–50% of the cells, 3=51–75% of the cells and 4=>75% of the cells). The IRS score derived from both the glandular and the stromal staining of the tissues.

Intensity of staining	Percentage of positive cells	IRS points-classification
0=no reaction	0=no positive	0-1= negative
1=weak staining	1=<25% of the cells	2-3= mild
2=moderate staining	2=25–50% of the cells	4-8= moderate
3=strong staining	3=51–75% of the cells	9-12=strongly positive
	4=>75% of the cells	

8.6. Immunofluorescense Analysis

8.6.1. Materials

- 6 well plate(greiner bio-one GmbH)
- Formaldehyde(BDH)
- PBS (Sigma)
- Tween20 (Sigma)
- Saponin(Sigma)
- BSA(Sigma)
- FBS (GIBCO-BRL Co)
- Dako Antigen Retreval Solution
- Primary Antibodies (Santa Cruz and Phoenix Pharmaceuticals, invitrogen, Abcam)
- Secondary antibodies (goat anti-rabbit IgG A11008 Alexa 488 lifetechnologies, and Cy3 anti-goat IgG, Jackson Immunology research laboratories)
- Coverslips Cover slips 22 x 22 mm και 24 x 32 mm (Deckglaser, Knittel glaser)
- Slides (Superfrost Plus)
- Glycergel (Dako)
- DAPI (molecular probes)
- SlowFade AntiFade kit(molecular probes)
- Nail polish
- Fluorescent microscope (Leica DMLC, Leica Microsystems, Wetzlar, Germany)
- Leica DC300F camera (Leica Microsystems, Wetzlar, Germany)

8.6.2. Cell immunofluorescense Protocol

1. Cells (Ishikawa cell line) are grown in a 6 well plate on a sterile coverslip, 300.000 – 500.000 cells/well using 2 ml medium/well.
2. 18 h before experiment change to starvation medium
3. Cells are washed once with cold 1x PBS
4. Cells are fixed with 3.7% formaldehyde in PBS for 20 min at RT (2ml/well)
5. Wash 3X 10 min with PBST (Tween 0.05%) (2ml/well) at RT, with gentle shaking
6. Cells are permeabilised with 0.2% Saponin in PBS for 10 min at RT (2ml/well)
7. Wash 3X 10 min with PBST (Tween 0.05%) (2ml/well) at RT, with gentle shaking
8. Block with 2% BSA in PBS for 40 min at RT (2ml/well)
9. Incubate with primary Antibody or ab-mix diluted in PBST (Tween 0.05%) overnight at 4C in a dark humid box.
10. Wash 3X 10 min with PBST (Tween 0.05%) (2ml/well) at RT
11. Incubate with second Antibody or ab-mix for 2-3 hours (in dark humid box). Sec Ab's are used at 1:200 (1ul in 100ul, because of the glycerol) in PBST (Tween 0.05%)
12. Wash 3X 10 min with PBST (Tween 0.05%) (2ml/well) at RT
13. Coverslips are put on the slides using a SlowFade AntiFade kit. Let it dry for 10 – 15 min
14. Borders of the coverslips are covered using nail polish. Make sure not to move the coverslips during this step. Start with fixing the corners and let them dry first, afterwards cover the sides.
15. Samples can be stored in the dark at 4C and examined at a Fluorescent microscope.

8.6.3. Paraffin embedded tissue immunofluorescense protocol

Paraffin embedded tissues sections are incubated at 37C for 1hr and overnight at 56^0C and the day of the experiment, sections are incubated at 56^0C for 10min, deparaffinized in xylene and rehydrated through graded concentrations of ethanol.

- 3X 100% xylole 5min each, shaking every 30sec in any wash.
- 2x 100% ethanol 5min each
- 1x 90% ethanol 5min each
- 1x 80% ethanol 5min each
- 1x 70% ethanol 5min each
- 2x dH_2O
- Slides are incubated with 200ml (20ml stock in180ml dH_2O) Dako target retrieval solution (preheated at 95C) in water bath at 95C for 30min, at RT for 20min and 5min at running tap water.
- Wash each slide with 200µl PBS

- Incubate slides with 200µl IFF/ per slide (PBS + 1% BSA) and 2% FBS in a dark humid box for 40min
- Incubate with primary Ab or Ab-mix for double immunofluorescence overnight at 4C in a dark humid box. Ab's are used at 1:50 in IFF
- 3x 5min wash with PBS
- Incubate with secondary antibody diluted in IFF for 1hr in dark humid box at RT
- 3x 5min wash with PBS
- Coverslips are put on the slides using Dako glycergel. Let it dry for 10 – 15 min
- Borders of the coverslips are covered using nail polish. Make sure not to move the coverslips during this step. Start with fixing the corners and let them dry first, afterwards cover the sides.
- Samples can be stored in the dark at 4C and examined at a fluorescent microscope.

Ishikawa cells, eutopic and ectopic endometrium had been used in immunofluorescent analysis. The primary antibodies that had been used were against CRH -at a dilution of 1:200- (H-019-06, Rabbit Anti-Corticotropin Releasing Factor, Phoenix Pharmaceuticals, Belmont, USA), UCN-at a dilution of 1:200- (H-019-14, Rabbit Anti-Urocortin Serum, Phoenix Pharmaceuticals, Belmont, USA), CRH RI -at a dilution of 1:200- (CRF-RI (V-14) goat polyclonal antibody, Santa Cruz Biotechnology, Bergheimer, Germany) and CRH RII -at a dilution of 1:200- (CRF-RII (C-15) goat polyclonal antibody, Santa Cruz Biotechnology, Bergheimer, Germany), galectin-1 (-at a dilution of 1:1000- rabbit polyclonal anti-galectin-1 antibody, Abcam) and CD-14 -at a dilution of 1:200- (human CD14 R-PE conjugated invitrogen MHCD1404) diluted in PBS-T. The secondary antibodies used were: goat anti-rabbit IgG A11008 Alexa 488 (1:100) lifetechnologies and Cy3 anti-goat (1:100) diluted in PBS-T. The results were viewed under a fluorescent microscope (Leica DMLC, Leica Microsystems, Wetzlar, Germany) and photographed using Leica DC300F (Leica Microsystems, Wetzlar, Germany) camera. For negative controls, the primary antibodies were omitted.

8.7. Western Blot Analysis

8.7.1. Materials

- Peptides (CRH, UCN: Tocris, Biosciense, Sigma, M-CSF-1 peptide: recombinant human peptide 216MC, R&D systems)
- Secondary antibodies: (chemicon international)
- Primary antibodies (santa cruz biotechnology, abcam, cell signalling)

- Antalarmin (Provided by Prof E. Zoumakis)
- PBS(Sigma)
- Tris (Merk, Germany)
- Biorad protein assay for Bradford(Biorad)
- Acrylamide (Serva, Germany)
- SDS(Biorad labs, USA)
- EDTA(BDH)
- Na(Sigma)
- F(Sigma)
- NaCl(Merk, Germany)
- Glycine (GIBCO)
- TEMED(Biorad labs)
- Ammonium Persulfate(Sigma)
- b- mercaptoethanol
- glycerol(Merk, Germany)
- bromophenol blue(Sigma)
- Nitrocelluse membrane(Schleicher & Schuell)
- Bis – acrylamide(Promega)
- TrisBase(Sigma)
- NaCl(Sigma)
- Tween20(Sigma)
- Methanol (Fluka)
- Skimmed milk powder(Regilait)
- ECL(GE healthcare/life sciences)
- Whatmann paper(Whatmann 3MM, Chr)
- Marker (Nippon genetics Europe GmbH)
- Electrophoresis machine: (Mini PROTEANsystem- Biorad Labs, USA, Power Pac Biorad Labs, USA)
- Film(Kodak X-0mat AR films)
- Developing films machine (Kodak)
- T-PER(Thermoscientific)
- Tissue homogenator(Quiagen)

8.7.2. Cells incubated with peptides

Cells had been incubated with the appropriate peptide according to each experimental protocol, before the protein lysate extraction protocol.

CRH peptide (Stock of 10^{-4}M): 10^{-7}M final concentration in cells – medium
Incubation of cells for 0,2,8,24hrs

UCN peptide (Stock of 10^{-4}M): 10^{-7}M concentration in cells – medium
Incubation of cells for 0,2,8,24hrs

CSF-1 peptide (Stock of 10μgr): 30 μgr/ml cells – medium
Incubation of cells for 0,2,8,24hrs

Antalarmin (Stock of 10^{-3}M): 10^{-6}M final concentration in cells – medium together with CRH peptide.
Incubation of cells for 8,24hrs

8.7.3. Cell Protein Lysates extraction

Protein lysate has been extracted from Ishikawa cell line and macrophages according to the following protocol:

- After cells had been treated with the appropriate peptides added in cell – medium for the time period needed according to each experiment as described above, remove cells- medium with peptide and wash the cells with 1x PBS.
- Add 0,8ml – for 100x100 cell culture plates - General Lysis Buffer (recipy described below) and scab cell from plates and place them in a tube, leave on ice for 20min by continuous vortexing.
- Centrifuge at 13000 rpm for 5 min.
- The supernatant at this step is the protein lysate. The whole quantity should be removed very carefully in a new tube and placed on ice till used.
- Determine the protein concentration using Bradford protein assay method and a spectrophotometer. Proteins are mixed with Biorad (1ml Biorad (1Biorad :5 water for injection H_2O) + 5 μl of protein extract) are measured at 495nm.
- 100 μg of protein is mixed with 4x sample buffer and boiled at a heat blocker at 95^0C for 5 min
- Samples can be stored at -20^0C, till to be used for western blotting analysis.

General Lysis buffer

	200 ml
50 mM Tris, pH 7.5	10 ml of stock Tris 1 M pH 7.5
1 mM EDTA	400 ul of stock EDTA 0.5 M
150 mM M NaCl	6 ml of stock NaCl 5M
1 % Igepal	2 ml Igepal
50 mM NaF	0.42g or 10 ml of stock NaF 1 M

On day of cell lysis add the following inhibitors:

10 μM leupeptin	1:1000 of stock leupeptin 10 mM
1mM PMSF	1:100 of stock 100 mM PMSF (in isopropanol).
10 ug/ml aprotinin	
1 mM Na4V03	1:100 of stock 100 mM Na4V03

Sample Buffer, Store at -20^{0}C: - 5ml Tris 1M
- 1.6ml b-mercaptoethanol
- 1.6 gr SDS
- 1ml glycerol
- 0.008gr BPB

8.7.4. Tissue Protein Extraction

- Make the T-PER mix by adding inhibitors (as described above)
- Add 1,5ml of T-PER mix per 0.100gr of tissue and homogenate tissue
- Centrifuge at 10.000 g for 10 min.
- The supernatant at this step is the protein lysate. The whole quantity should be removed very carefully in new tubes and placed on ice till used.
- Determine the protein concentration using Bradford protein assay method and a spectrophotometer. Proteins are mixed with Biorad (1ml Biorad {1Biorad :5 water for injection H_2O} + 5 μl of protein extract) are measured at 495nm.
- 100 μg of protein is mixed with 4x sample buffer and boiled at a heat blocker at 95^{0}C for 5 min
- Samples can be stored at -20^{0}C, till to be used for western blotting analysis.

100 μg of proteins were extracted from Ishikawa cell line, macrophages, healthy women's eutopic endometrium (10 patients) and eutopic endometrium and endometriotic tissue from endometriotic patients (16 patients), placenta and myometrium followed by SDS-PAGE analysis in 10% acrylamide gel, and electrotransfer onto a nitrocellulose membrane. The membrane was blocked in 5% skim milk powder in 0.1% Tris-buffered saline/Tween for 20 min. The membrane was then incubated with CRHRI (CRF-RI (V-14) goat polyclonal antibody, Santa Cruz Biotechnology, Bergheimer, Germany) or CRH RII (CRF-RII (C-15) goat polyclonal antibody, Santa Cruz Biotechnology, Bergheimer, Germany), galectin-1 (rabbit polyclonal anti-galectin-1 antibody, Abcam), M-CSF ((D-4), sc-12381, mouse monoclonal antibody, Santa Cruz Biotechnology), FasL (Q20 sc956 rabbit FasL antibody, Santa Cruz Biotechnology, Bergheimer, Germany), at a dilution of 1:1000, followed by incubation with the peroxidase-conjugated donkey antigoat IgG, goat anti-rabbit IgG AP132P and goat anti-mouse IgG AP194P secondary antibodies(Chemicon International, multipore). GAPDH (rabbit GAPDH antibody 14C10, Cell signalling) was used as a house

keeping gene. Protein extracts from placenta and myometrium used as positive controls. The specificity of CRHR1 and CRHR2 polyclonal antibodies was confirmed by absorption with blocking peptides sc-12381P and sc-20550P (Santa Cruz Biotechnology) respectively. The blocking peptides for CRHR1 and CRHR2 antibody were mixed with the CRHR1 and CRHR2 antibodies with a five-fold (by weight) excess of blocking peptides and incubated overnight at 4°C. We had the same samples loaded twice so that we could cut the nitrocelluse membrane in two lanes having identical samples and one lane was incubated with the polyclonal antibody and the other one with the blocked antibody followed by followed by incubation with the peroxidase-conjugated donkey antigoat IgG secondary antibody. The lane which had been incubated with the blocked peptide had its staining disappeared was specific to the antibody. Band intensities of protein of interest were normalized with band intensities of GAPDH and expressed as arbitrary units (a.u.)

8.7.5. Solutions for Electrophoresis

30% Acrylamide stock(100ml): - 2902gr acrylamide
- 0.8 gr Bis - acrylamide
- Filter solution through 0.45 pore filter and store at 4^0C

Running gel Solution(for 2 gels): - 6ml 30% acrylamide
- 3.75 ml separating buffer
- 4.95ml wfi H2O
- 150ul of 10%APS
- 8 ul TEMED

Stacking gel Solution (for 2 gels): - 1.275ml Acrylamide 30%
- 0.937ml stacking buffer
- 5.1ml H2O
- 0.075ml of 10% APS
- 8.5ul TEMED

Separation gel buffer 100 ml : - 18.16gr Tris 1.5 M pH 8.8
- 0.4 g SDS, 0.4%w/w

Store at 4^0C

Stacking gel buffer 100 ml: - 6.05grTris 0.5 M pH 6.8
- 0.4 g SDS

Store at 4^0C

10% Ammunium Persulfate(APS) 10ml : 1gr APS and 10ml wfi H2O, store at 4^0C

Electrophoresis buffer 10x (1l): - 30.3 g Tris
- 144.2 g glycine
- 10 g SDS

Store at RT

Transfer Buffer (1.5lt): - 300ml Methanol
- 150ml 10x electrophoresis
- 1050ml dH2O

Store at 4^0C

8.8. Evaluation and Statistical Analysis

Western blot and RT-PCR gel bands were analysed via image analysis software (Scion Corporation, Release Beta 4.0.2, Frederick, MD, USA). Statistical analysis was performed using the unpaired two-tailed Student's *t*-test. Any statistical difference at $p<0.05$ was considered significant.

RESULTS

CHAPTER 9

RESULTS

❖ 9.1. CRH, UCN, CRHR1 and CRHR2 expression in the endometriotic sites.

In order to verify the expression of the CRH, UCN, CRHR1 and CRHR2 in endometriotic lesions, the expression of these transcripts was tested by RT-PCR. Total RNA was extracted from endometriotic tissues obtained from sixteen patients with confirmed endometriosis. cDNA was synthesized and was screened for the presence of both the ligand transcripts (CRH and UCN) and their receptors' transcripts (CRHR1β and CRHR2α). Both CRH and UCN genes were found to be transcribed in endometriotic tissues (Figure 1A, 1B). Interestingly, both CRHR1β and CRHR2α were also present in endometriotic tissues (Figure 1C, 1D). The above finding was further verified by evaluating the protein expression levels of CRHR1 and CRHR2 receptors in endometriotic sites. Total protein extracts from endometriotic tissues (obtained from 16 patients) were used for CRHR1 and CRHR2 detection by western blot. As shown in Figure 1E and 1F, both CRHR1 and CRHR2 are expressed in endometriotic sites. Placental and myometrial tissue were used as positive controls and GAPDH (Figure1G) as a house keeping gene for Western blotting experiments.

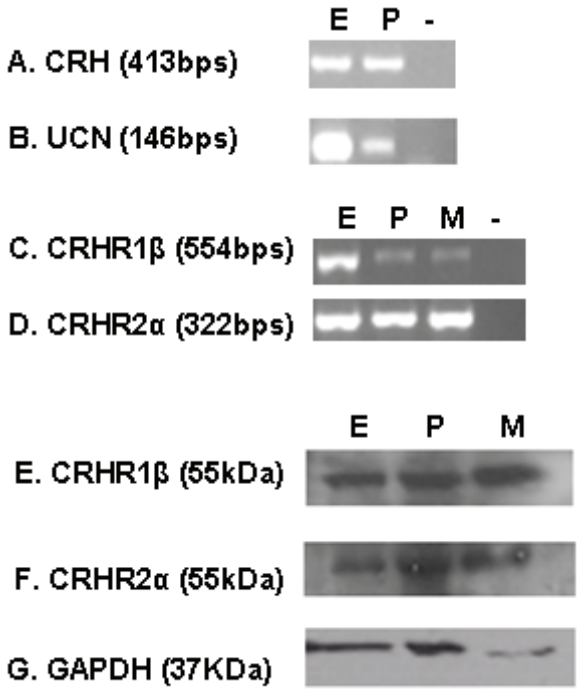


Figure 1. CRH, UCN, CRHR1 and CRHR2 expression in endometriotic sites.
A: CRH (413bps), B: UCN(146bps), C: CRHR1β (554bps) and D: CRHR2α (322bps) mRNA expression and CRHR1 (55kDa) and CRHR2 (55kDa) protein expression in endometriotic tissue(E) (1E, 1F respectively), having placental tissue (P) and myometrial tissue (M) as positive control, negative sample(-), GAPDH(G) house keeping gene. (representative data)

❖ 9.2. Expression of CRH, UCN, CRHR1 and CRHR2 in Ishikawa cell line

Apart from the results presented above in endometriotic tissue, the presence of CRH/UCN and their receptors were evaluated in Ishikawa cells, a well-accepted model of epithelial endometrial cell physiology. RT-PCR was performed in order to investigate CRH, UCN, CRHR1β and CRHR2α mRNA expression in Ishikawa cell line. Furthermore, concerning the protein expression of CRH, UCN, CRHR1 and CRHR2, immunodetection was performed by immunofluorescence. As shown in Figure 2, both, CRH and UCN as well as CRHR1 and CRHR2 were identified in Ishikawa cells at an mRNA and protein level.

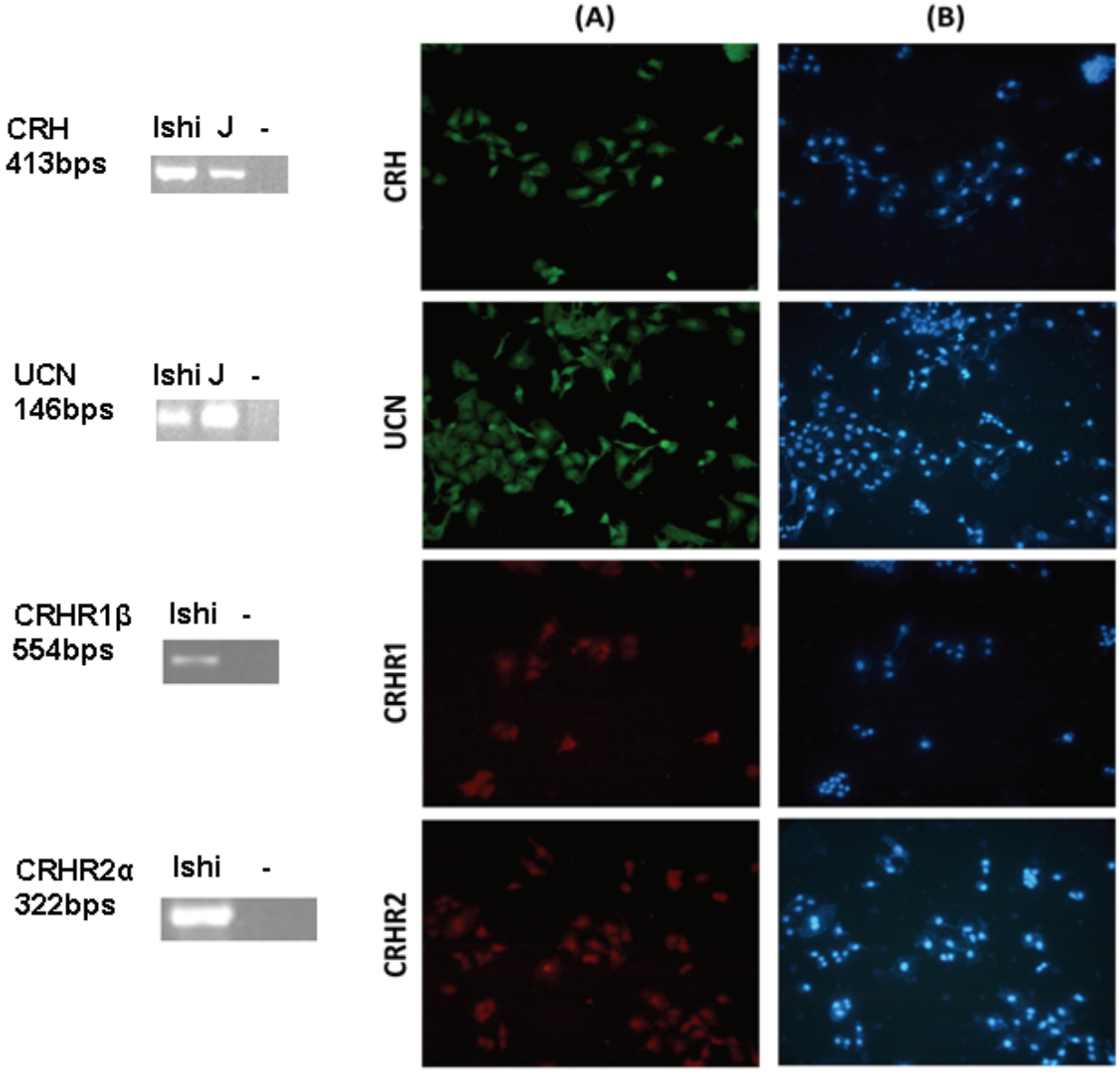


Figure 2: mRNA and protein expression of CRH, UCN, CRHR1 and CRHR2 in Ishikawa cell line.
RT-PCR: mRNA expression of CRH, UCN, CRHR1β and CRHR2α in Ishikawa cell line. Ishi: Ishikawa cell line, J: JEG3 cell line used as positive control, -: negative control. A. Immunofluorescence detection of CRH, UCN, CRHR1 and CRHR2 in Ishikawa cells. B. presentation of the corresponding DAPI stainings.

❖ 9.3. Higher expression levels of CRHR1 and CRHR2 in eutopic endometrium of endometriotic women compared to eutopic endometrium of healthy women.

Evaluation and comparison of the expression of CRHR1 and CRHR2 in eutopic endometrium of both healthy and endometriotic women, at mRNA and protein level were performed. mRNA extracts of eutopic endometrium of endometriotic women (16 patients) and healthy women (10 patients) used to perform RT-PCR for both receptors. It was found that CRHR1β and CRHR2α are more highly expressed in eutopic endometrium of endometriotic women compared to eutopic endometrium of healthy women (CRHR1β:1.204±0.012 a.u. in endometriotic women vs 0.458± 0.020 a.u. in healthy women, which corresponds to 2.62 fold increase when eutopic endometrium of healthy women is set as control , $p<0.01$; CRHR2α: 2.518 ±0.012 a.u in endometriotic women vs 1.895± 0.016a.u. in healthy women, which corresponds to 1.32 fold increase when eutopic endometrium of healthy women is set as control, $p<0.01$)) (Figure 3A). This was further corroborated at protein level (CRHR1: 2.926±0.048 a.u in endometriotic women vs 2.187±0.034 a.u in healthy women, which corresponds to 1.33 fold increase when eutopic endometrium of healthy women is set as control, $p<0.01$; CRHR2: 1.087±0.021 a.u in endometriotic women vs 0.685± 0.017 a.u in healthy women, which corresponds to 1.58 fold increase when eutopic endometrium of healthy women is set as control, $p<0.01$) by performing western blotting for CRHR1 and CRHR2 (Figure 3B) using protein extracts from the same material. Both receptors showed an excessive expression in eutopic endometrium of endometriotic women compared to healthy women. GAPDH was used as a house keeping gene for both RT-PCR and Western Blotting.

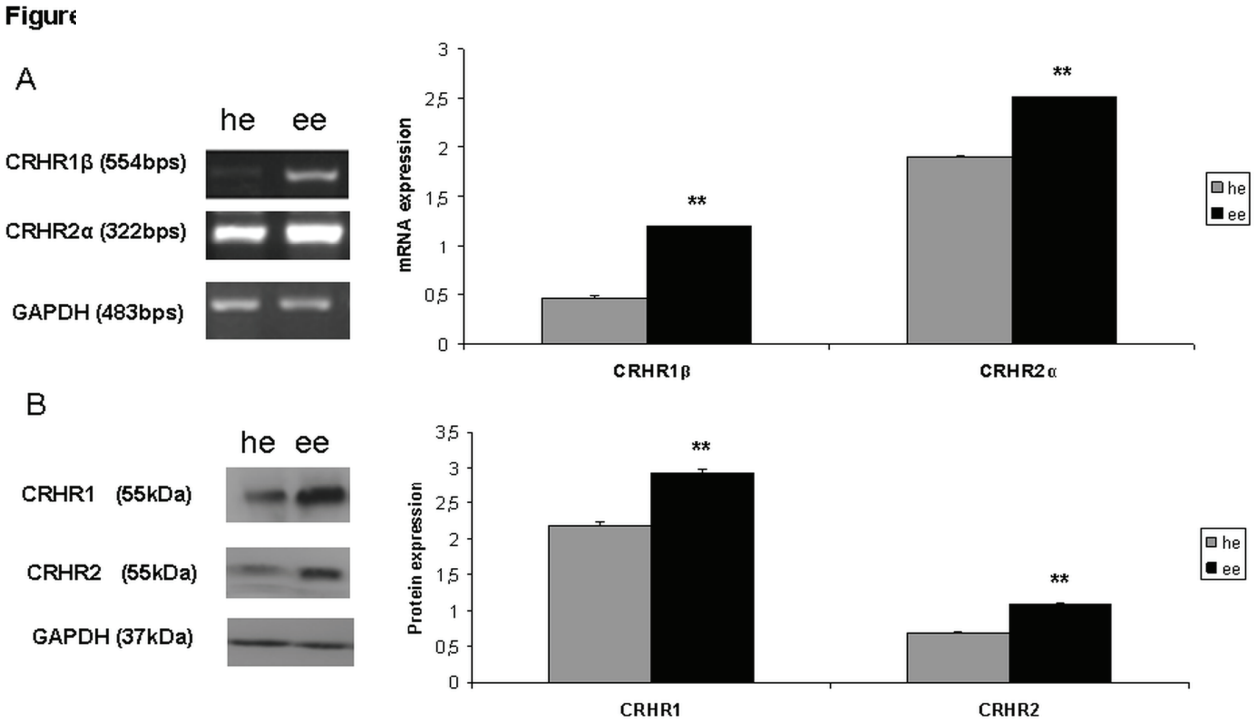


Figure 3. Higher CRHR1 and CRHR2 expression in eutopic endometrium of endometriotic women at an mRNA and protein level. A: mRNA expression of CRHR1β(554bps) and CRHR2α(322bps) is higher in eutopic endometrium of endometriotic women(ee) compared to eutopic endometrium of healthy women(he). B: protein expression of CRHR1 (55kDa) and CRHR2 (55kDa) is higher in ee compared to he. (**p<0,01) (representative data)

❖ 9.4. CRH, UCN, CRHR1β and CRHR2α molecules are more abundant in endometriotic tissues (ectopic endometrium) compared to the corresponding eutopic endometrium.

The expression levels of CRH, UCN, CRHR1b and CRHR2a in the endometriotic lesions and the corresponding eutopic endometrium of the same women (16 patients) were compared. Total RNA was extracted from the eutopic and ectopic endometrial samples (obtained from 16 patients), cDNA was synthesized and was semi-quantitavely assessed for the presence of CRH, UCN, CRHR1β and CRHR2α transcripts. It was found that in endometriotic foci CRH, UCN, CRHR1β and CRHR2α mRNA expression was significantly higher compared to the respective CRH, UCN, CRHR1β and CRHR2α mRNA content in the eutopic endometrium of the same patients (CRH: 4.101±0.582 a.u in ectopic vs 1.741±0.580 a.u in eutopic endometrium, 2.35 fold increase, $p<0.01$; UCN: 0.956±0.136 a.u in ectopic vs 0.282±0.075 a.u in eutopic endometrium, 3.38 fold increase, $p<0.01$; CRHR1β: 1.134±0.410 a.u in ectopic vs 0.186±0.046 a.u in eutopic endometrium, 6.07 fold increase, $p<0.05$; CRHR2α: 11.615±1.837 a.u in ectopic vs 5.023±1.723 a.u in eutopic endometrium, 2.31 fold increase, $p<0.01$. In all cases eutopic endometrium was set as control) (Figure 4a,A, 4b,A). The above findings were further verified by evaluating the protein expression levels in eutopic endometrium and endometriotic sites of the same women performing immunohistochemistry and western blot analysis. The signal of CRH and UCN in western blot analysis was significantly low and could not be augmented because of the small size of the protein molecules, thus formalin-fixed paraffin-embedded tissue sections obtained from 16 patients were used for the detection of CRH and UCN by immunohistochemistry. According to IRS Score calculations (Table 1), CRH and UCN were found to be expressed more intensely in ectopic endometrium (Figure 4a,B(D) and Figure 4a,B(E) respectively) compared to eutopic endometrium (Figure 4a,B(A) and Figure 4a,B(B) respectively) of the same women (Figure4b, B,) (CRH: 7.2±0.489 a.u in ectopic vs 6.1±0.378 a.u in eutopic endometrium, 1.18 fold increase, $p<0.05$; UCN: 6.9±0.458 a.u in ectopic vs 5.5±0.5 a.u in eutopic endometrium, 1.25 fold increase, $p<0.05$. In both cases eutopic endometrium was set as control). In order to identify the protein expression levels of the receptors CRHR1 and CRHR2 between eutopic and ectopic endometrium, western blot analysis was performed in 16 patients samples and showed that both receptors are more highly expressed in ectopic than eutopic endometrium of the same women (Figure 4a, C, Figure 4b, C) (CRHR1: 3.323±0.053 a.u in ectopic vs 2.926±0.048 a.u in eutopic endometrium, 1.13 fold increase, $p<0.01$; CRHR2: 2.657±0.040 a.u in ectopic vs 1.087±0.021a.u in eutopic endometrium, 2.44 fold increase, $p<0.01$. In both cases eutopic endometrium was set as control). GAPDH was used as a house keeping gene for both RT-PCR and western blotting analysis.

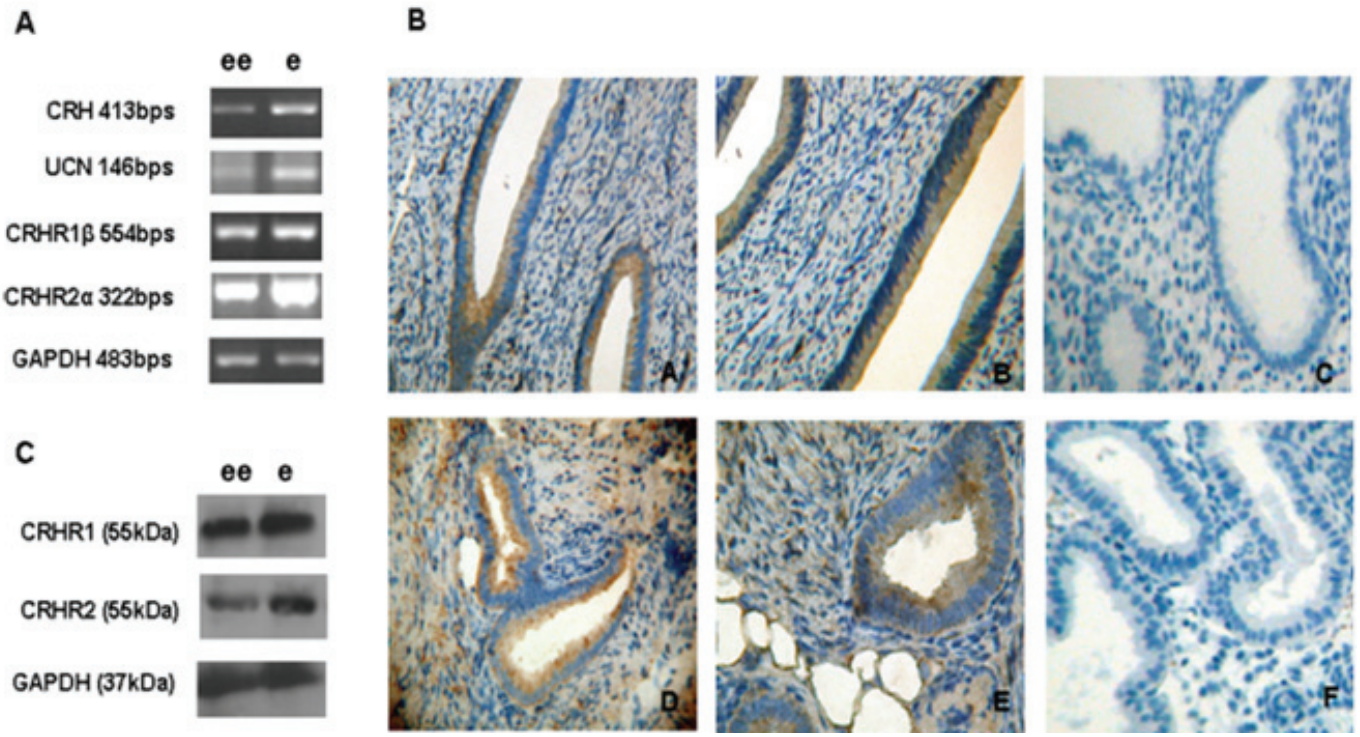
Table 1. IRS Score results.

Intensity of staining	Percentage of positive cells	IRS points-classification
0=no reaction	0=no positive	0-1= negative
1=weak staining	1=<25% of the cells	2-3= mild
2=moderate staining	2=25–50% of the cells	4-8= moderate
3=strong staining	3=51–75% of the cells	9-12=strongly positive
	4=>75% of the cells	

IRS points-classification	CRH expression endometrium	CRH expression endometriosis	UCN expression endometrium	UCN expression Endometriosis
0-1= negative	-	-	-	-
2-3= mild	-	-	-	-
4-8= moderate	14/16 (87,5%)	9/16 (69,2%)	15/16 (93,75%)	11/16 (68,75%)
9-12=strongly positive	2/16 (12,5%)	7/16 (43,75%)	1/16 (6,25%)	5/16 (31,25%)

It was found that 7/16(43,75%) ectopic endometrium samples showed strong CRH expression and 9/16(69,2%)ectopic endometrium samples showed moderate CRH expression compared to 2/16(12,5%) eutopic endometrium samples of strong CRH expression and 14/16(87,5%) eutopic endometrium samples of moderate CRH expression. Concerning the UCN expression in eutopic and ectopic endometrium of the same patients, it has been found that 5/16(31,25%) ectopic endometrium samples showed strong UCN expression and 11/16(68,75%) ectopic endometrium samples showed moderate UCN expression compared to 1/16(6,25%) eutopic endometrium samples of strong UCN expression and 15/16(93,75%) eutopic endometrium samples of moderate UCN expression.

4a


A
ee
e
CRH 413bps
UCN 146bps
CRHR1β 554bps
CRHR2α 322bps
GAPDH 483bps
C
ee
e
CRHR1 (55kDa)
CRHR2 (55kDa)
GAPDH (37kDa)
B
A
B
C
D
E
F

4b

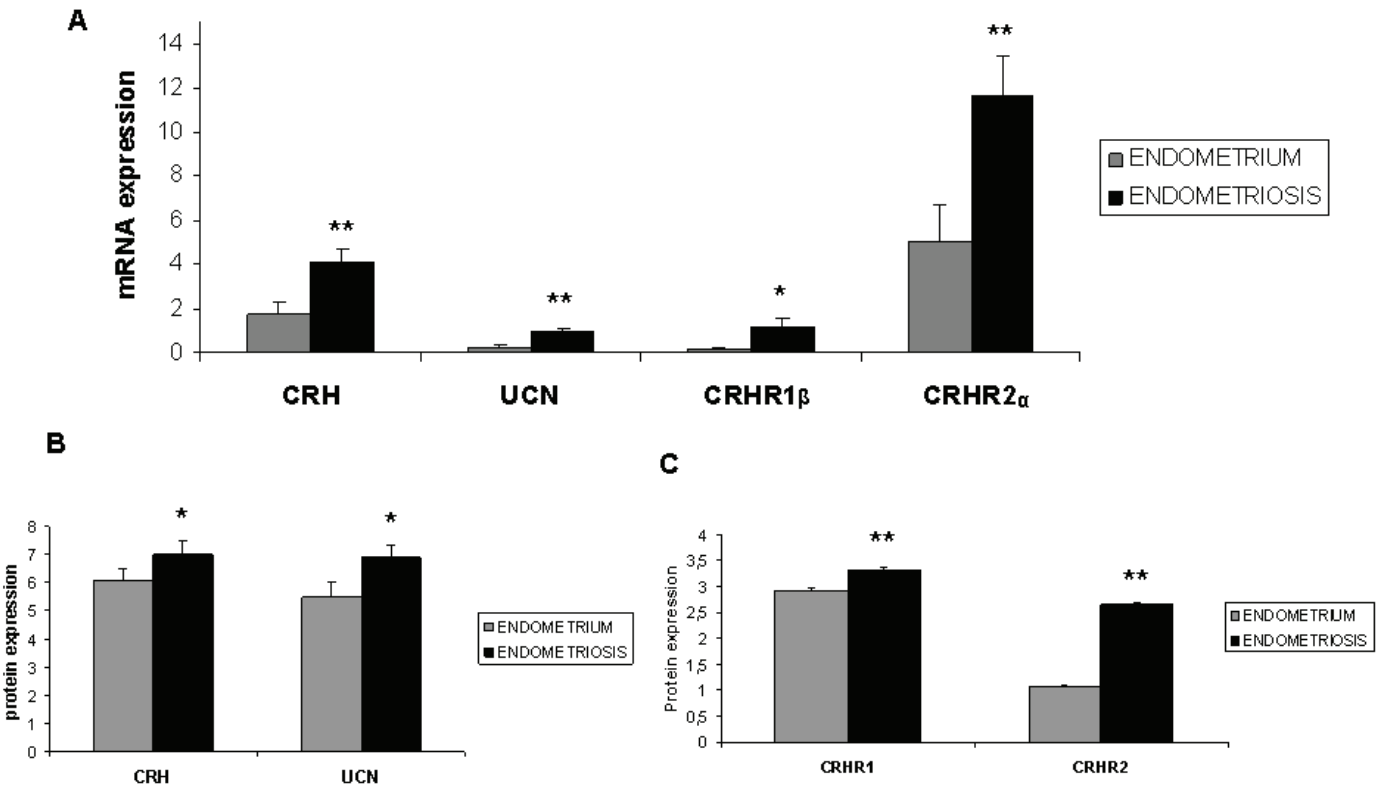

A
mRNA expression
14
12
10
8
6
4
2
0
**
**
*
**
CRH
UCN
CRHR1β
CRHR2α
ENDOMETRIUM
ENDOMETRIOSIS
B
protein expression
8
7
6
5
4
3
2
1
0
*
*
CRH
UCN
ENDOMETRIUM
ENDOMETRIOSIS
C
Protein expression
4
3,5
3
2,5
2
1,5
1
0,5
0
**
**
CRHR1
CRHR2
ENDOMETRIUM
ENDOMETRIOSIS

Figure 4. CRH, UCN, CRHR1 and CRHR2 excessive expression in endometriotic sites (ectopic endometrium) compared to eutopic endometrium of endometriotic women

4a) A: mRNA expression of CRH (413bps), UCN (146bps), CRHR1β (554bps), CRHR2α (322bps), GAPDH (483bps) in endometriotic tissue (e) – ectopic endometrium and the corresponding eutopic endometrium (ee). B: Immunohistochemical expression of CRH (A,D) and UCN(B,E) in eutopic(A,B) and ectopic endometrium(D,E). Both CRH and UCN are mainly expressed in endometriotic lesions.(C: negative eutopic endometrium, F: negative ectopic endometrium). C: Western blot immunodetection of CRHR1 (55KDa) and CRHR2 (55KDa), in endometriotic tissue(e) and eutopic endometrium(ee) of endometriotic women.GAPDH (37kDa) used as a house keeping gene. CRH, UCN, CRHR1 and CRHR2α are significantly more expressed at an mRNA and protein level in endometriotic tissue compared to the corresponding eutopic endometrium. (representative data)

4b) Presentation of the increased CRH, UCN, CRHR1 and CRHR2 expression in the study population at mRNA level(A) and protein level(B: CRH and UCN protein levels, C: CRHR1 and CRHR2 protein levels) (*, $p<0,05$, **,$p<0,01$)

❖ 9.5. Galectin-1 expression in eutopic endometrium of healthy women

In order to investigate the galectin-1 expression in eutopic endometrium of healthy women immunohistochemical analysis in eutopic endometrium tissue sections of healthy women (10 patients) was performed in three different phases of the menstrual cycle (proliferative, early secretory and late secretory phase). As shown in the figure 5, galectin-1 expression is higher in late secretory phase (Figure 5C). According to IRS Score calculations galectin-1 expression is higher at the late secretory phase (proliferative phase: 5.125±0.25 a.u, early secretory phase 6.375±0.43 a.u, late secretory phase: 7.6875±0.38 a.u., $p<0.05$ and 1.24 fold of gal-1 expression in early secretory phase endometrium when setting the proliferative phase endometrium as control and 1.20 fold of gal-1 expression in the late secretory phase when setting the early secretory phase endometrium as control.

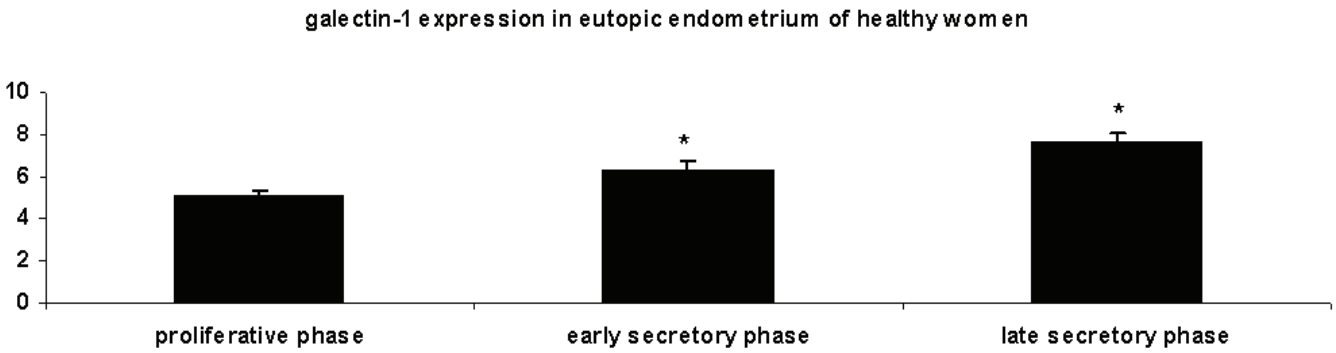
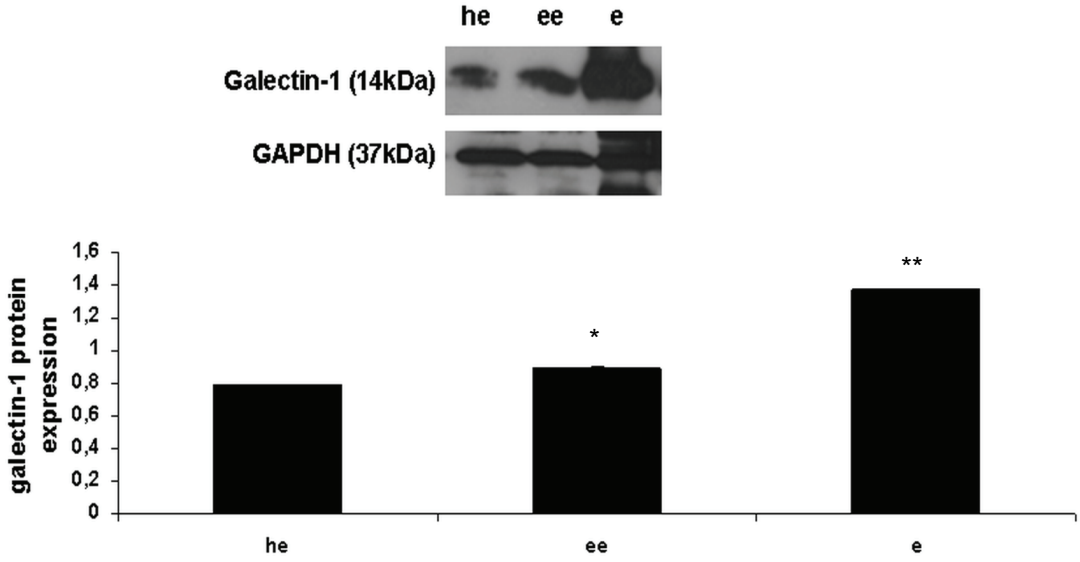


Figure 5: Galectin-1 overexpression in late secretory phase of eutopic endometrium. Galectin-1 expression increases through the three different phases of the menstrual cycle of the endometrium (A: proliferative, B: early secretory phase, C: late secretory phase) by showing a higher expression pattern at the late secretory phase of the endometrium(C) $p<0.05$.

❖ 9.6. Galectin-1 is overexpressed in endometriotic tissue.

In order to investigate the galectin-1 expression levels in eutopic endometrium of healthy women (n=3), in eutopic and ectopic endometrium of endometriotic women (n=3), western blotting (Figure 6A) and immunohistochemistry (Figure 6B) were performed. As a result, when performing western blotting, it was shown that galectin-1 is overexpressed in ectopic endometrium (e) compared to eutopic endometrium of endometriotic women (ee) and eutopic endometrium of healthy women (he)(Figure 6A). Concerning Western blotting analysis: Galectin-1: 0.887±0.007 a.u in eutopic endometrium of endometriotic women vs 0.786±0.005 a.u in eutopic endometrium of healthy women, 1.12 fold increase, $p<0.05$, when setting eutopic endometrium of healthy women as control. Galectin-1: 1.378±0.003 a.u in ectopic endometrium vs 0.887±0.007 a.u in eutopic endometrium of endometriotic women, 1.55 fold increase, $p<0.01$, when setting eutopic endometrium of endometriotic women as control. As far as the immunohistochemical analysis (Figure 6B) and IRS score calculations, eutopic (n=16, Figure 6Ba) and ectopic endometrium (n=16, Figure6Bb, c) of the same endometriotic women has been used and galectin-1 showed an abundant expression in ectopic endometrium when compared to eutopic endometrium of the same women. Galectin-1 expression in eutopic endometrium of endometriotic women: 4.875±0.25 a.u., galectin-1 expression in ectopic endometrium of endometriotic women: 7.375±0.43 a.u., $p<0.01$, having a 1.51 fold expression of galectin-1 when setting the eutopic endometrium of endometriotic women as control.

A



B

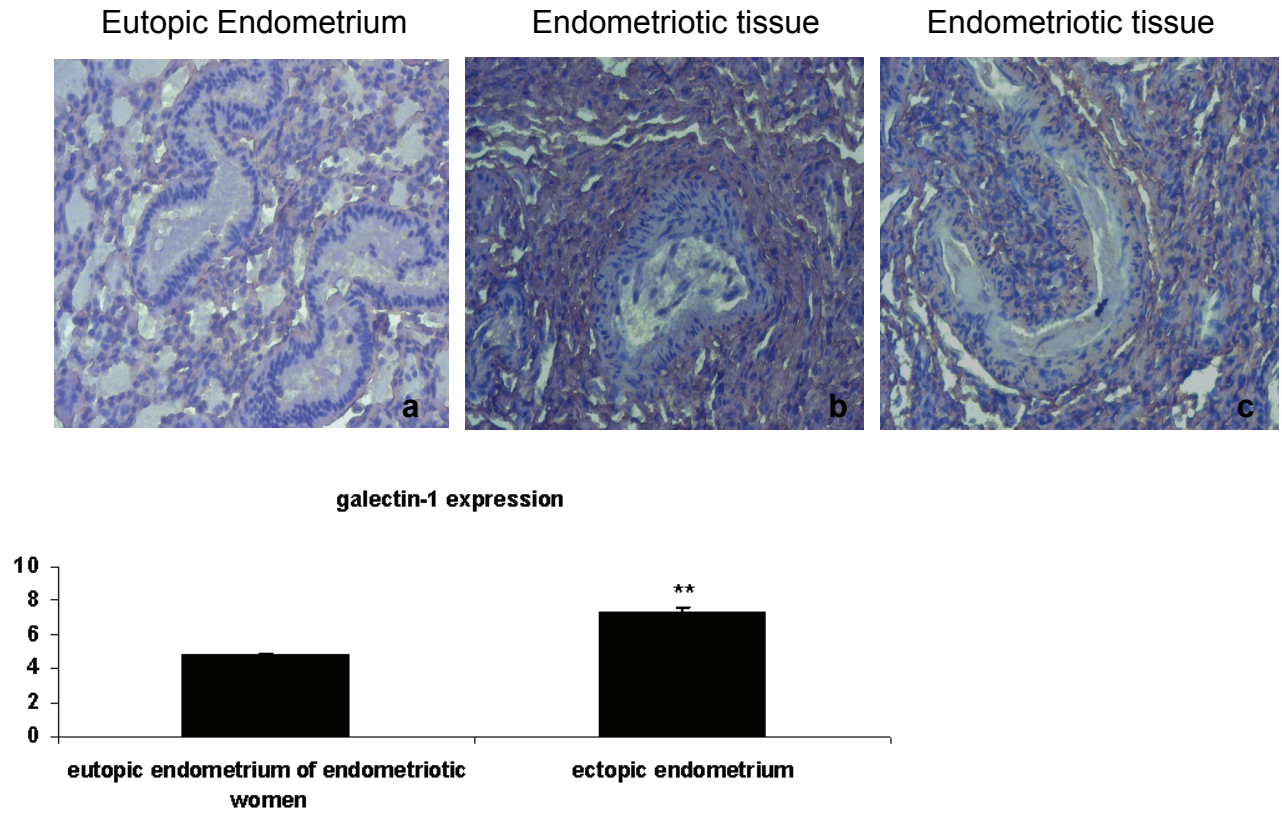


Figure 6: Galectin-1 overexpression in ectopic endometrium: Galectin-1 is overexpressed in eutopic endometrium of endometriotic women (ee) when compared to eutopic endometrium of healthy women(he), $p<0.05$ and galectin-1 shows an abundant expression in ectopic endometrium(e) when compared to eutopic endometrium of the same endometriotic women(ee), $p<0.01$. **B:** Galectin-1 overexpression in ectopic endometrium (b,c) compared to eutopic endometrium(a) of the same endometriotic women, $p<0.01$.

9.7. Higher co-localization of galectin-1 and macrophages in endometriosis

As to investigate if galectin-1 is expressed at the same location where macrophages accumulate in eutopic and ectopic endometrium, double immunofluorescense using galectin-1 (green staining) and CD14 (red staining) antibodies in eutopic and ectopic endometrium of the same endometriotic women (n=16) was performed. As shown in figure 7 there is a higher co-localization (yellow staining) effect of galectin-1 and CD-14 expression in ectopic endometrium (Figure 7F) compared to eutopic endometrium (Figure 7C) of the same endometriotic women.

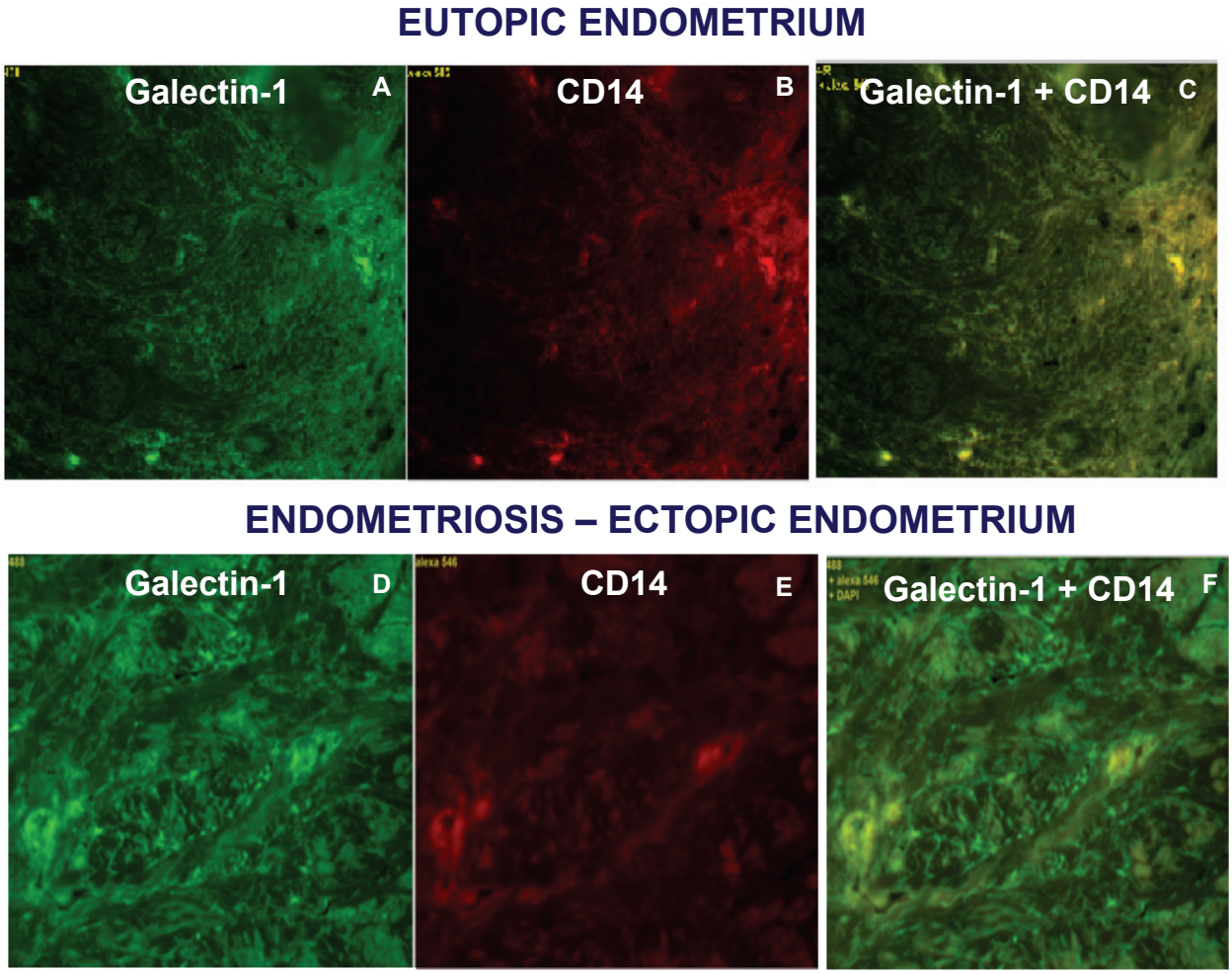


Figure 7: Higher co-localization in ectopic endometrium of endometriotic women.
Galectin-1 expression (A, D- green staining), CD-14 expression (B, E- red staining) and co-localization of both the antibodies(C, F- yellow staining) was investigated in eutopic endometrium(A, B, C) and ectopic endometrium(D, E, F) of the same endometriotic women.

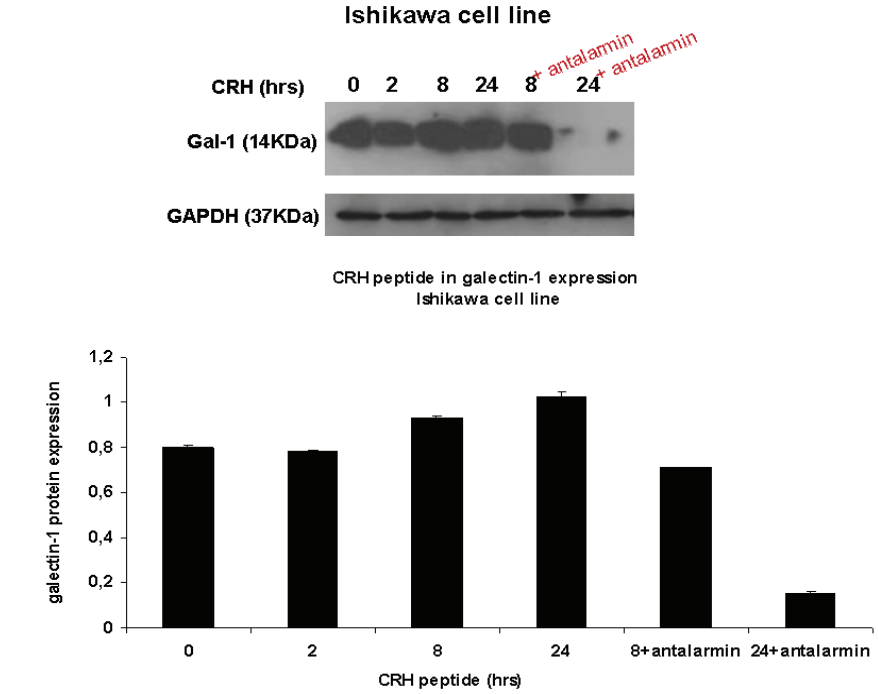
❖ 9.8. CRH upregulates galectin-1 expression in Ishikawa cell line and macrophages through CRHR1.

In order to investigate how galectin-1 expression is regulated in Ishikawa cell line and macrophages upon stimulation of these cells by CRH peptide, western blotting and immunofluorescence (in Ishikawa cell line) were performed. All types of cells where treated with CRH peptide for 0, 2, 8, 24hrs. Antalarmin, which is an endogenous antagonist of CRHR1, had been used at the time points where higher expression of galectin-1 was shown (8hrs and 24hrs of CRH peptide + Antalarmin). As a result, it is shown that CRH upregulates galectin-1 expression in Ishikawa cell line (Figure 8a,A) mostly at 8hrs and 24hrs of stimulation and in macrophages at 24hrs of stimulation(Figure 8a,B) time dependently in both cases and this is mediated by CRHR1. Antalarmin blocked the upregulative effect of CRH in galectin-1 expression in a higher way at 24hrs stimulation both in Ishikawa cell line and macrophages. The experiment had a three time replication. (Galectin-1 expression in Ishikawa cell line – CRH peptide stimulation for 0hrs: 0.8±0.007, 2hrs: 0.78±0.004 a.u, 8hrs: 0.9±0.006 a.u, 24hrs: 1.02±0.018 a.u, 8hrs + antalarmin: 0.7±0.004, 24hrs+antalarmin: 0.15±0.009). (Galectin-1 expression in macrophages - CRH peptide stimulation for 0hrs: 0.05±0.0008, 2hrs: 0.21±0.001 a.u, 8hrs: 0.4±0.001 a.u, 24hrs:

0.47±0.008 a.u, 8hrs + antalarmin: 0.04±0.0006, 24hrs+antalarmin: 0.01±0.001). Immunofluorescense has been performed in Ishikawa cell line as well. As Figure 8bA shows galectin-1 expression (Alexa488, green staining) was upregulated when the cells where stimulated for 8hrs and 24hrs by CRH and when antalarmin was added (Figure 8bB), mostly for 24hrs, this effect was minimised indicating the role of CRHR1 in CRH upregulative effect in galectin-1 expression. DAPI had been used for nuclear staining.

(a)

A



B

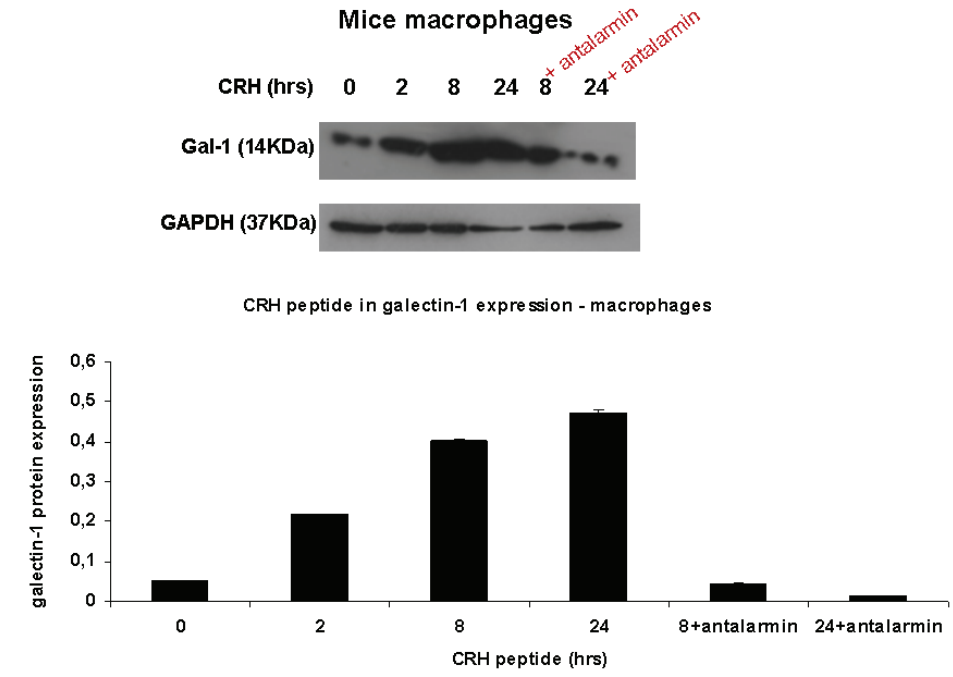
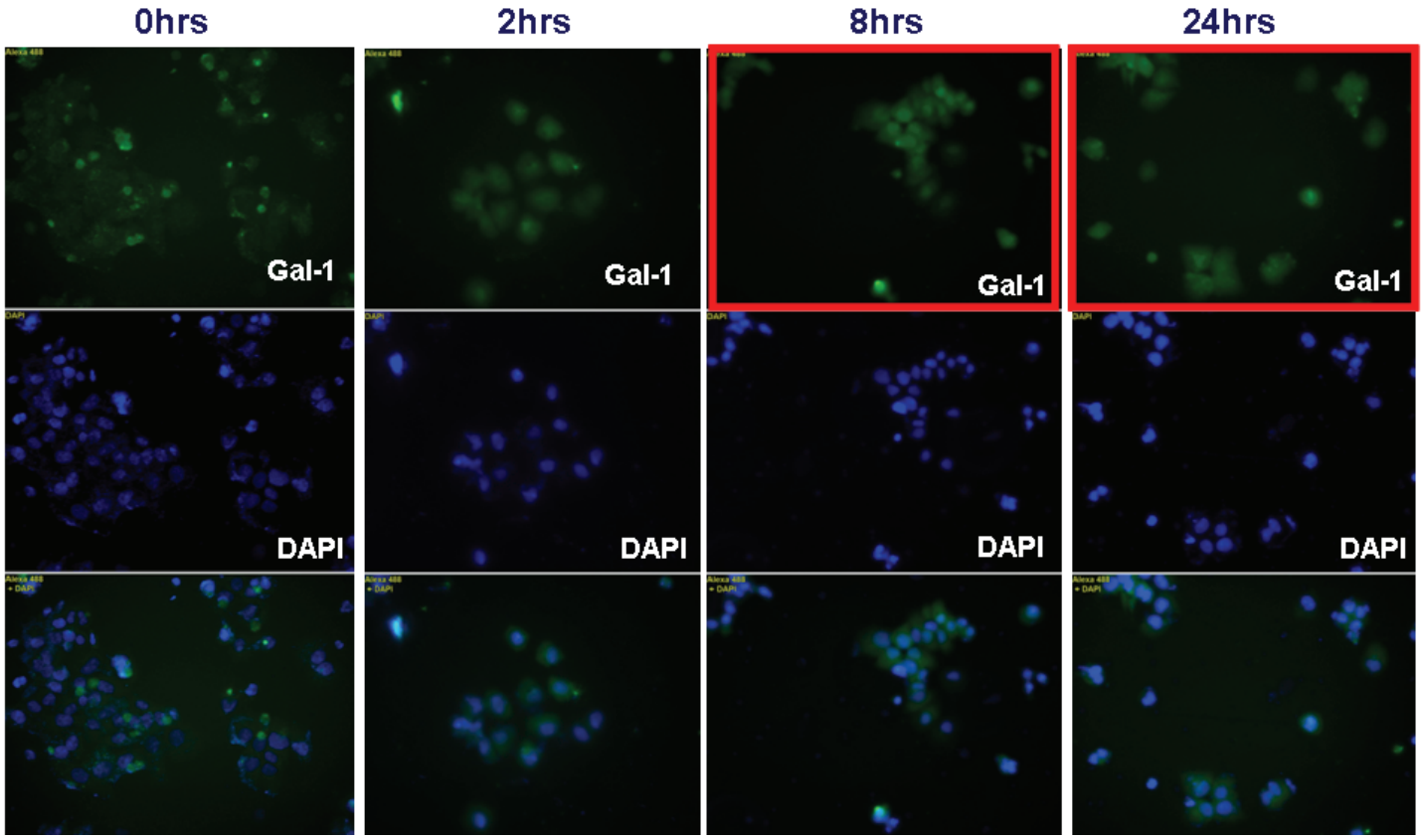


Figure 8a: Galectin-1 is upregulated in Ishikawa cell line and macrophages upon CRH stimulation and this is mediated by CRHR1 – western blotting. A: In Ishikawa cell line, galectin-1 is upregulated by CRH, showing a higher expression at 8hrs and 24hrs of stimulation. When antalarmin is added, galectin-1 shows an impaired expression mostly at 24hrs of stimulation.B: In mice macrophages, galectin-1 expression is upregulated by CRH mostly at 24hrs and antalarmin's effect is stronger at 24hrs.

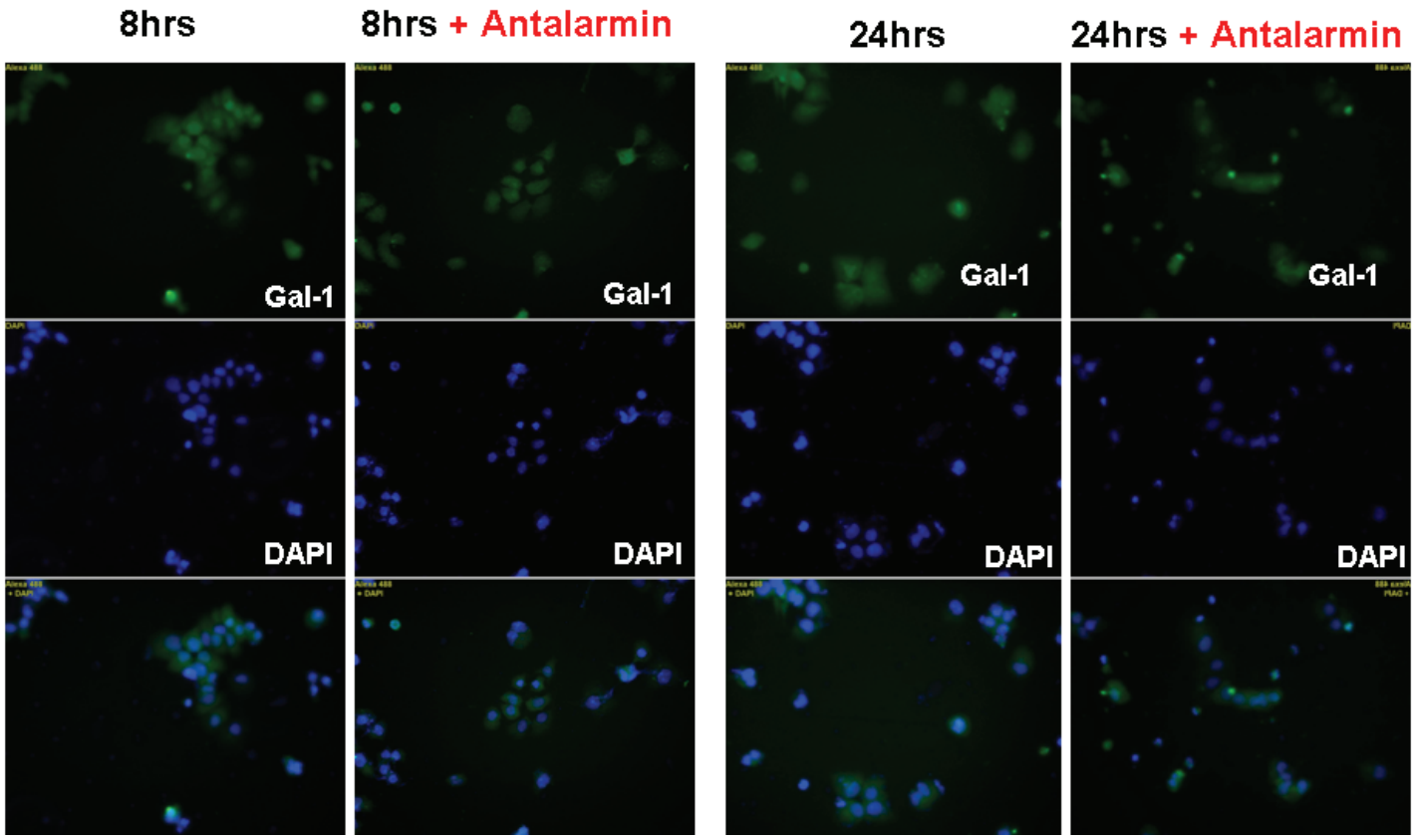
Figure 8b: Galectin-1 is upregulated in Ishikawa cell line upon CRH stimulation and this is mediated by CRHR1 – immunofluorescense. (A)Galectin-1 expression (Alexa488,green staining) is higher upon 8hrs and 24hrs of CRH peptide stimulation and it is impaired when antalarmin is added, more specifically in 24hrs CRH peptide+antalarmin(B). DAPI was used for nuclear staining.

(b)

A



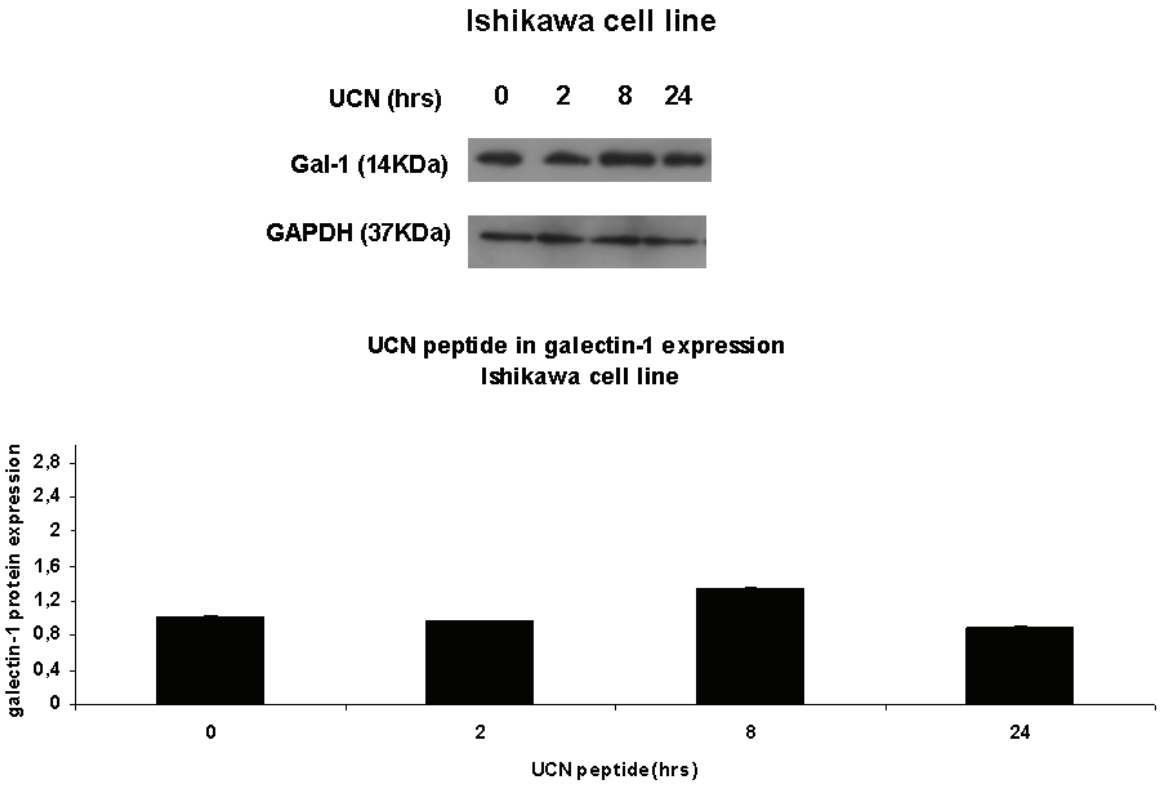
B



❖ 9.9. UCN upregulates galectin-1 expression in Ishikawa cell line and macrophages.

After showing that CRH has an upregulative effect in galectin-1 expression in Ishikawa cell line and macrophages, UCN peptide effect at galectin-1 expression has been tested at the same time points of stimulation (0, 2, 8, 24hrs) in both cell types. UCN peptide caused an increase in galectin-1 expression mostly at 8hrs of stimulation in Ishikawa cell line and at 24hrs of stimulation in macrophages (Figure 9aA, B). The experiment had a three time replication. (Galectin-1 expression in Ishikawa cell line – UCN peptide stimulation for 0hrs: 1.01±0.006, 2hrs: 0.97±0.003 a.u, 8hrs: 1.33±0.02 a.u, 24hrs: 0.88±0.03 a.u,). (Galectin-1 expression in macrophages – UCN peptide stimulation for 0hrs: 0.7±0.01, 2hrs: 1.21±0.02 a.u, 8hrs: 1.37±0.018 a.u, 24hrs: 1.81±0.03 a.u). Immunofluorescense has been performed in Ishikawa cell line as well. As Figure 9bA shows galectin-1 expression (Alexa488, green staining) was upregulated when the cells where stimulated for 8hrs by UCN. DAPI had been used for nuclear staining.

(a)
A



B

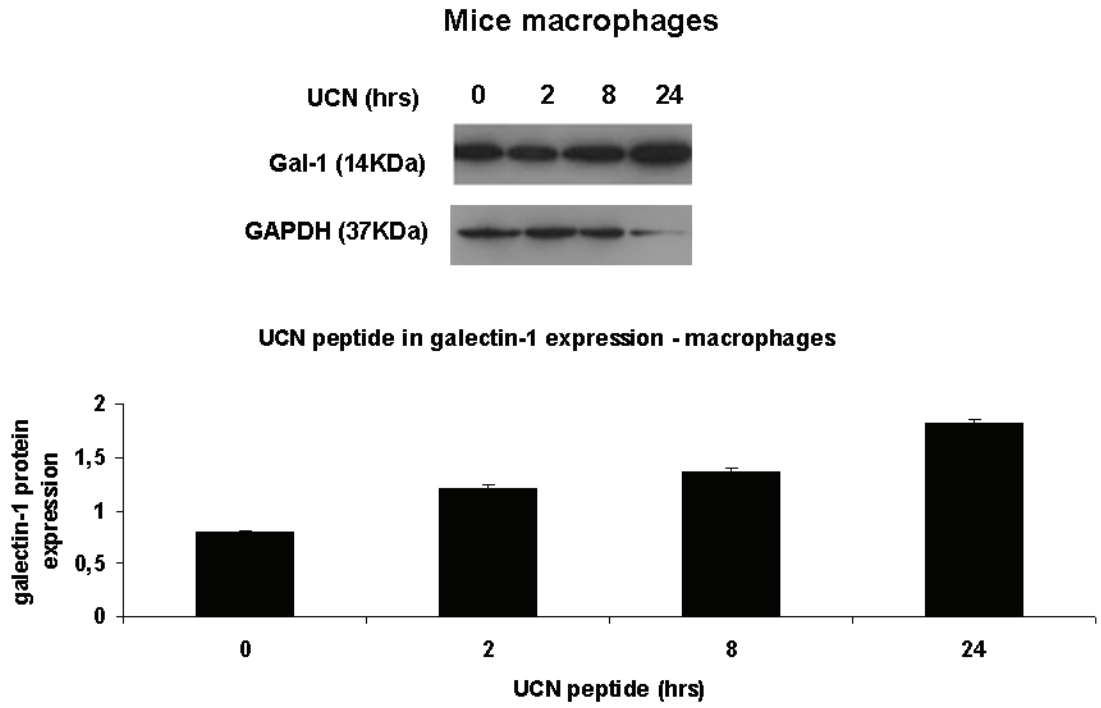
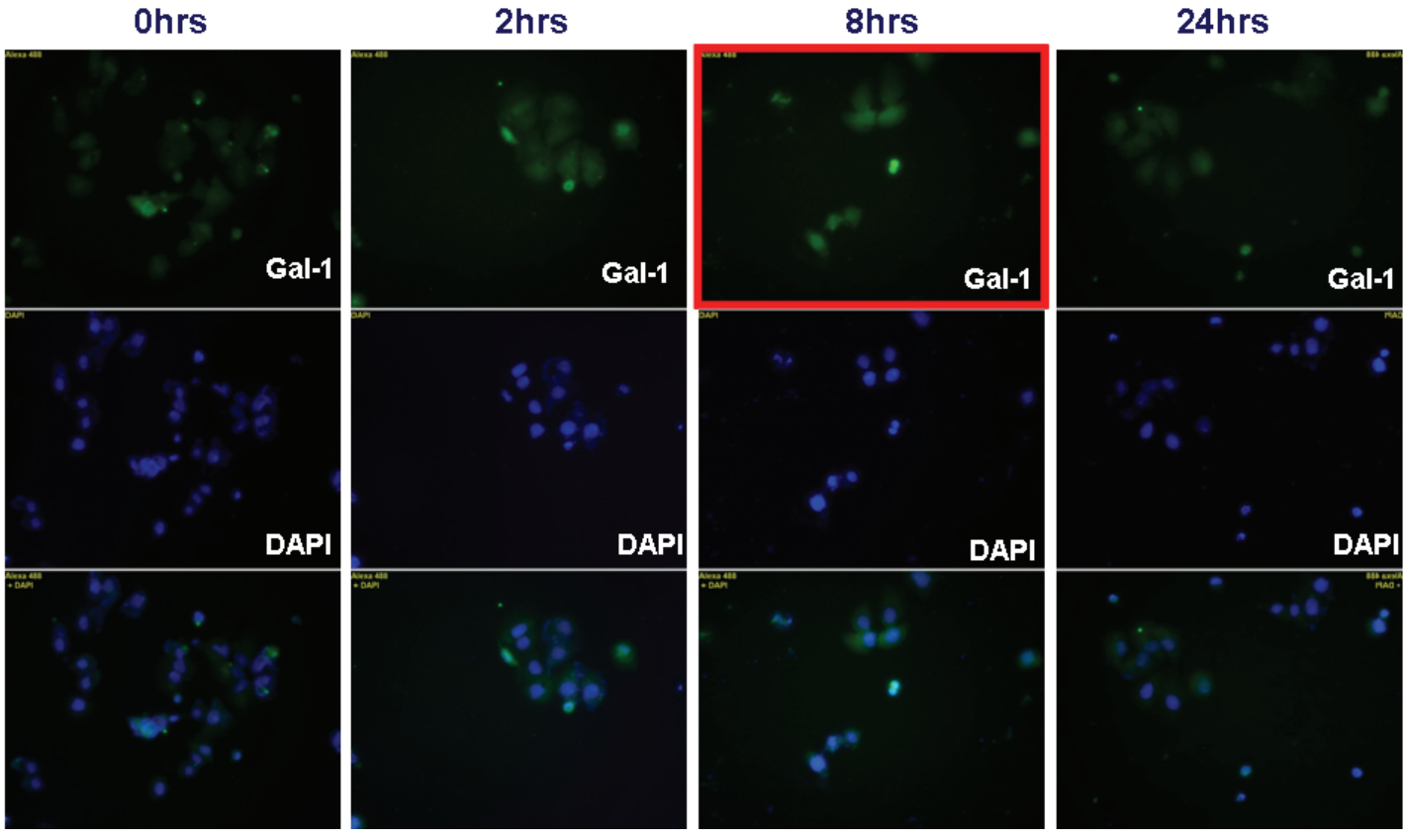


Figure 9a: Galectin-1 is upregulated in Ishikawa cell line and macrophages upon UCN stimulation– western blotting. A: In Ishikawa cell line, galectin-1 is upregulated by UCN, showing a higher expression at 8hrs of stimulation. B: In mice macrophages, galectin-1 expression is mostly upregulated by UCN at 24hrs. **Figure 9b: Galectin-1 is upregulated in Ishikawa cell line upon UCN stimulation –immunofluorescense.** Galectin-1 expression (Alexa488,green staining) is higher upon 8hrs of UCN peptide stimulation. DAPI was used for nuclear staining.

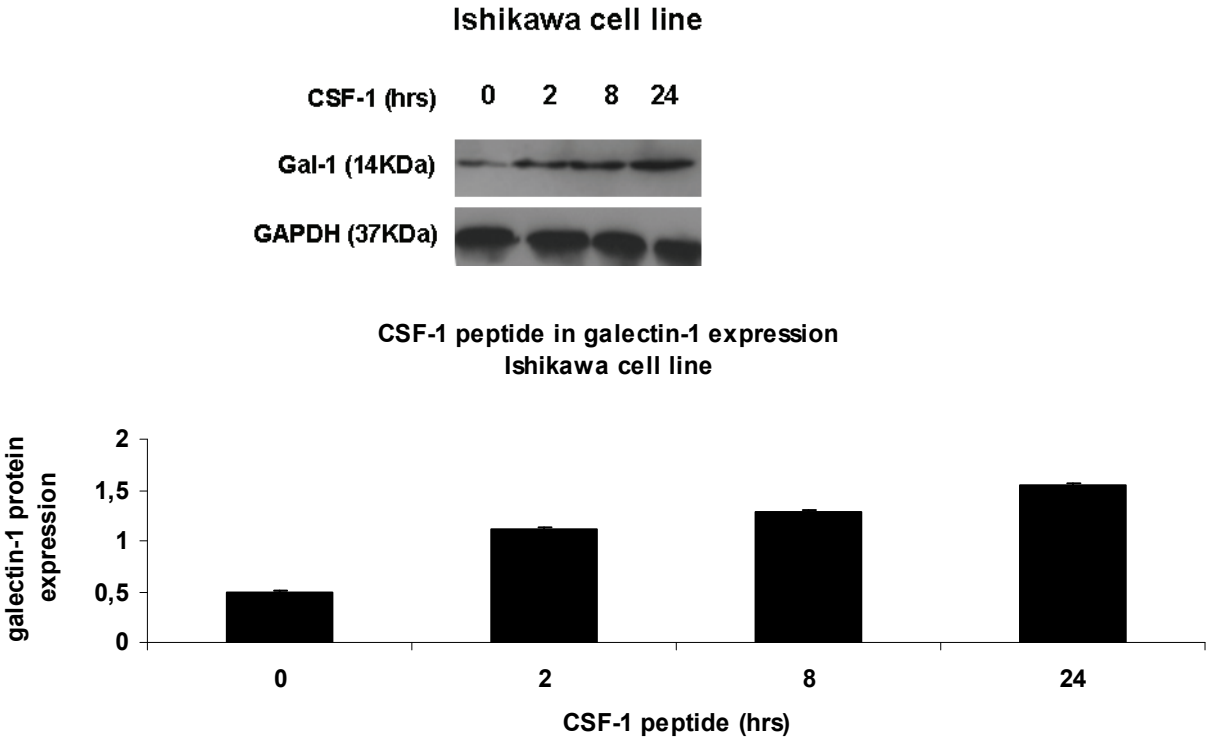
(b)



❖ 9.10. CSF-1 upregulates galectin-1 expression in Ishikawa cell line and macrophages.

Ishikawa cell line and macrophages were treated with CSF-1 peptide as well for the specific time points of 0hrs, 2hrs, 8hrs, 24hrs. Upon CSF-1 stimulation of Ishikawa cells, galectin-1 expression increased time dependently, the more CSF-1 stimulated the cells, the higher the galectin-1 expression was, reaching its highest expression level at 24hrs of stimulation (Figure 10aA). Concerning galectin-1 expression in macrophages upon CSF-1 stimulation, galectin-1 showed a higher expression levels at 24hrs stimulation by CSF-1. (Figure 10aB). The experiment had a three time replication. (Galectin-1 expression in Ishikawa cell line – CSF-1 peptide stimulation for 0hrs: 0.49±0.01, 2hrs: 1.01±0.02 a.u, 8hrs: 1.27±0.01 a.u, 24hrs: 1.54±0.01 a.u,). (Galectin-1 expression in macrophages – CSF-1 peptide stimulation for 0hrs: 1.24±0.01, 2hrs: 1.50±0.05 a.u, 8hrs: 1.7±0.01 a.u, 24hrs: 2.08±0.01 a.u). Immunofluorescense has been performed in Ishikawa cell line as well. As Figure 10bA shows that galectin-1 expression (Alexa488, green staining) was mostly upregulated when the cells where stimulated for 24hrs by CSF-1. DAPI had been used for nuclear staining.

(a)
A



B

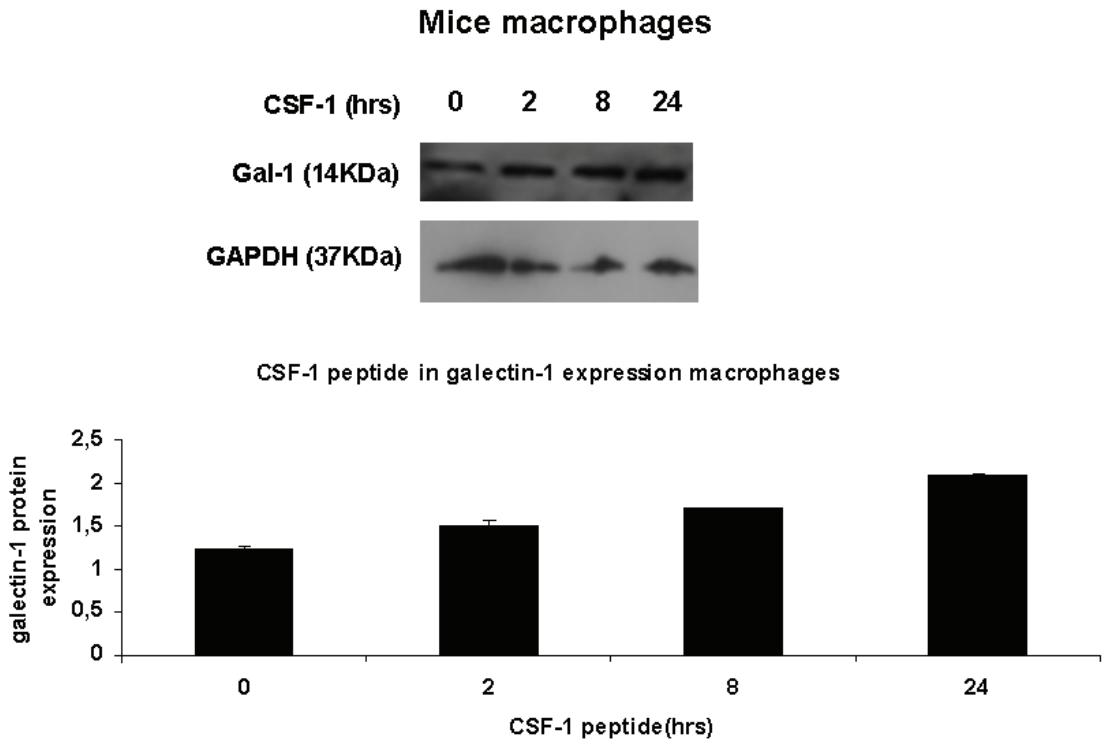
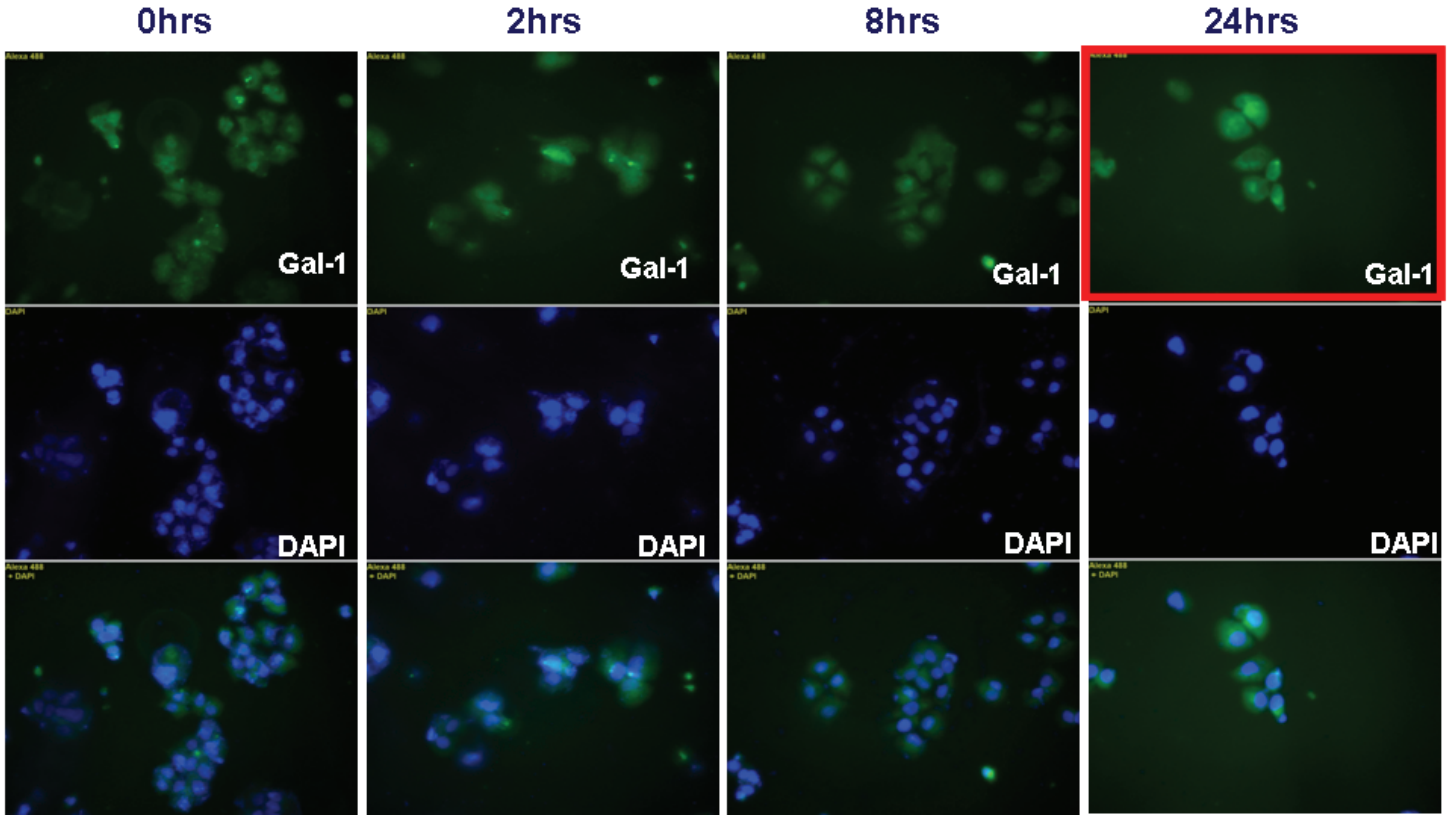


Figure 10a: Galectin-1 is upregulated in Ishikawa cell line and macrophages upon CSF-1 stimulation– western blotting. A: In Ishikawa cell line, galectin-1 is upregulated by CSF-1, showing a time dependently increasing expression, by having an abundant expression at 24hrs of stimulation. B: In mice macrophages, galectin-1 expression is upregulated by CSF-1 peptide mostly at 24hrs. **Figure 10b: Galectin-1 is upregulated in Ishikawa cell line upon CSF-1 stimulation–immunofluorescense.**Galectin-1 expression (Alexa488,green staining) is higher upon 24hrs of CSF-1 peptide stimulation. DAPI was used for nuclear staining.

(b)



❖ 9.11. CSF-1 is overexpressed in endometriotic tissue

In order to investigate the CSF-1 expression levels in eutopic endometrium of healthy women (n=3), in eutopic and ectopic endometrium of endometriotic women (n=3), western blotting (Figure 11) was performed. As a result, when performing western blotting, it was shown that CSF-1 is overexpressed in ectopic endometrium (e) compared to eutopic endometrium of endometriotic women(ee) and eutopic endometrium of healthy women(he)(Figure 11). Galectin-1 expression: 1.15±0.02 a.u in eutopic endometrium of endometriotic women vs 0.77±0.01 a.u in eutopic endometrium of healthy women, 1.48 fold increase, $p<0.01$, when setting eutopic endometrium of healthy women as control. Galectin-1: 1.65±0.02 a.u in ectopic endometrium vs 1.15±0.02 a.u in eutopic endometrium of endometriotic women, 1.43 fold increase, $p<0.01$, when setting eutopic endometrium of endometriotic women as control.

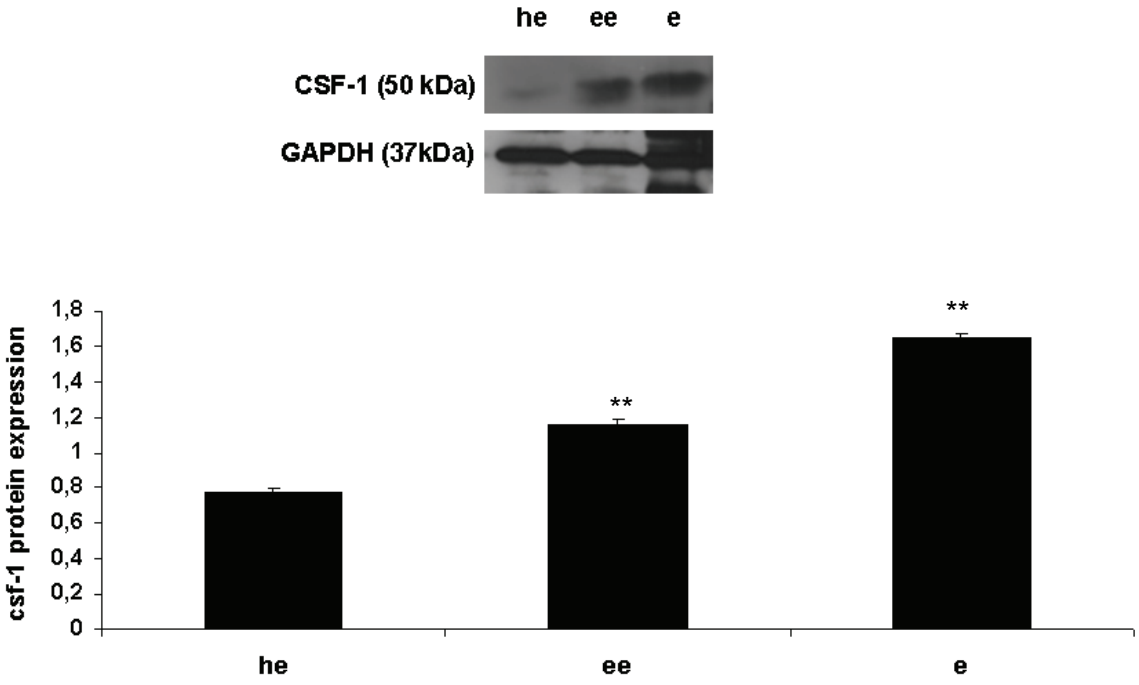
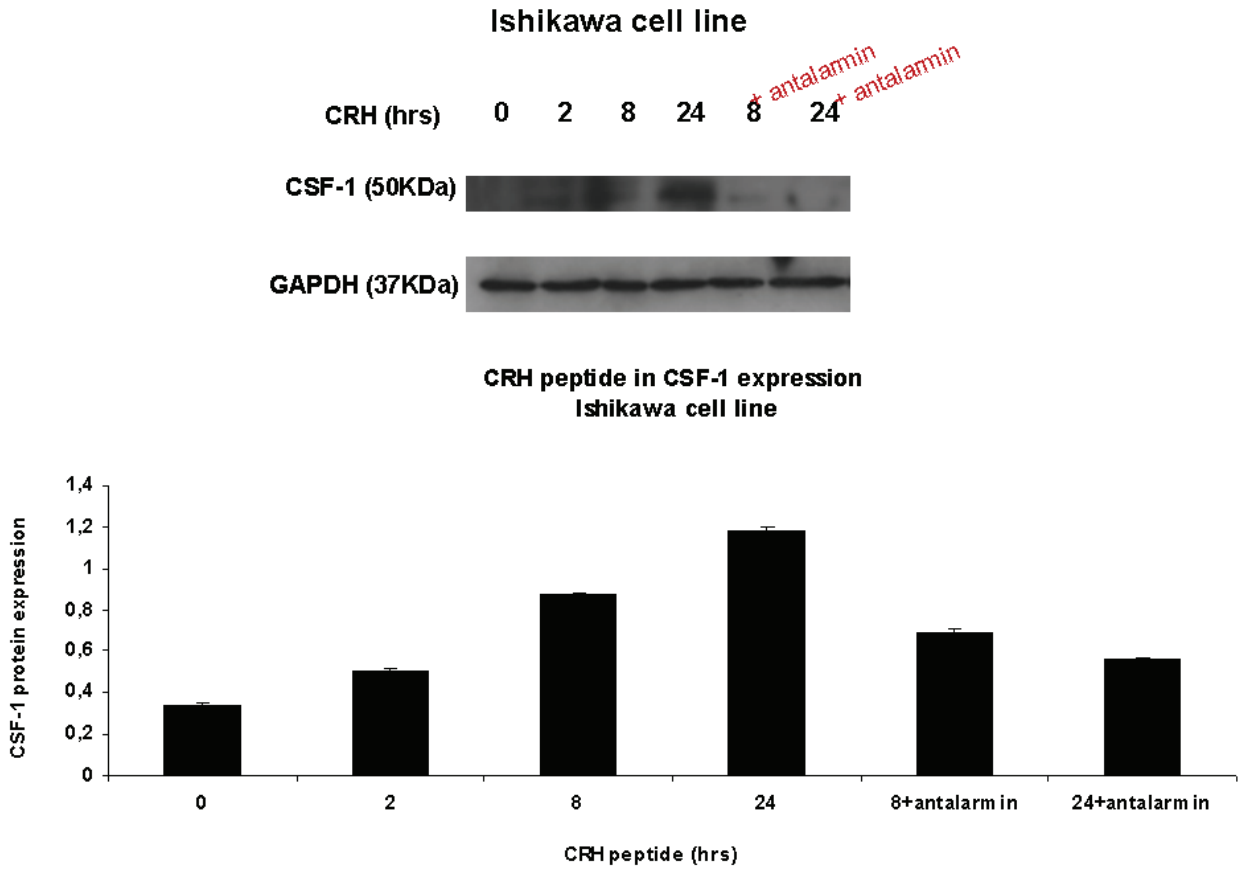


Figure 11: CSF-1 overexpression in ectopic endometrium.
CSF-1 is overexpressed in eutopic endometrium of endometriotic women(ee) when compared to eutopic endometrium of healthy women(he), $p<0.01$ and CSF-1 shows an abundant expression in ectopic endometrium(e) when compared to eutopic endometrium of the same endometriotic women(ee), $p<0.01$.

❖ 9.12. CRH upregulates CSF-1 expression in Ishikawa and macrophages through CRHR1.

After examining the galectin-1 expression levels in Ishikawa cell line and macrophages upon CRH, UCN and CSF-1 stimulation, the same experiments were performed in order to investigate how CSF-1 expression is regulated in Ishikawa cell line and macrophages upon stimulation by these peptides. Firstly, all types of cells where treated with CRH peptide for 0, 2, 8, 24hrs. Antalarmin had been used at the time points where higher expression of CSF-1 was shown (8hrs and 24hrs of CRH peptide + Antalarmin). As a result, it was shown that CRH upregulates CSF-1 expression in Ishikawa cell line (Figure 12A) mostly at 24hrs and in macrophages as well (Figure 12B) time dependently in both cases and this is mediated by CRHR1. Antalarmin blocked the upregulative effect of CRH in CSF-1 expression in a higher way at 24hrs of stimulation in Ishikawa cell line and macrophages. The experiment had a three time replication. (CSF-1 expression in Ishikawa cell line – CRH peptide stimulation for 0hrs: 0.34±0.006, 2hrs: 0.5±0.008 a.u, 8hrs: 0.87±0.009 a.u, 24hrs: 1.17±0.01 a.u, 8hrs + antalarmin: 0.69±0.01, 24hrs+antalarmin: 0.56±0.005). (CSF-1 expression in macrophages - CRH peptide stimulation for 0hrs: 0.26±0.01, 2hrs: 0.26±0.008 a.u, 8hrs: 0.34±0.01 a.u, 24hrs: 0.73±0.006 a.u, 8hrs + antalarmin: 0.15±0.009, 24hrs+antalarmin: 0.05±0.005).

A



B

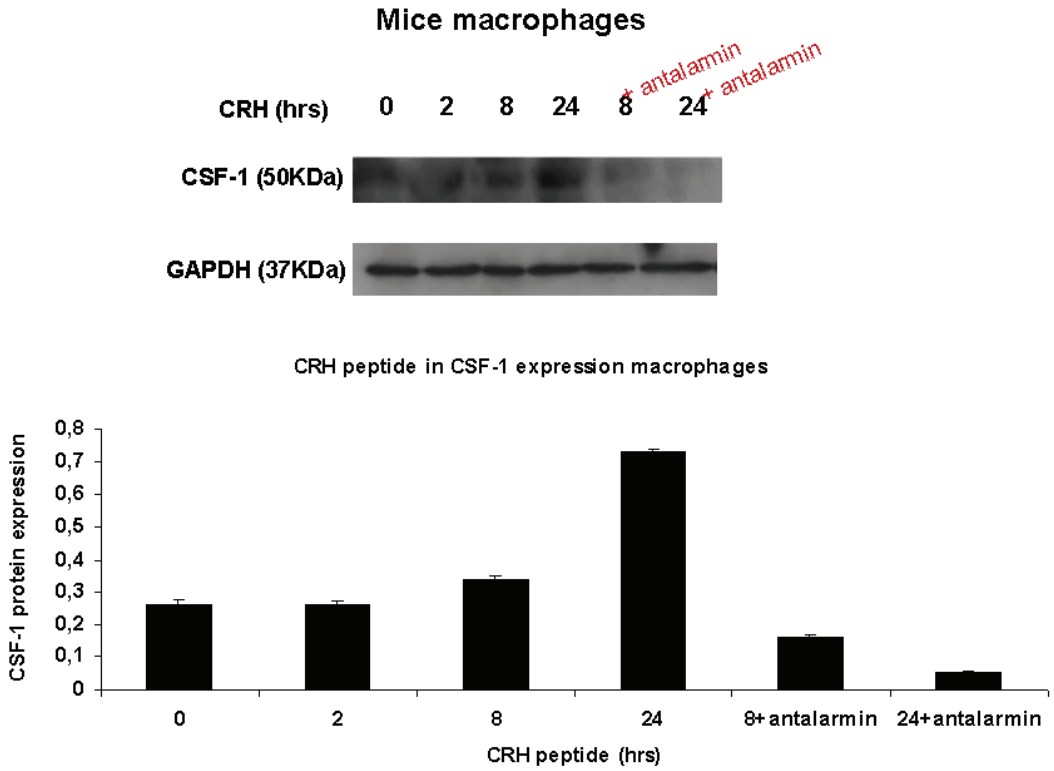
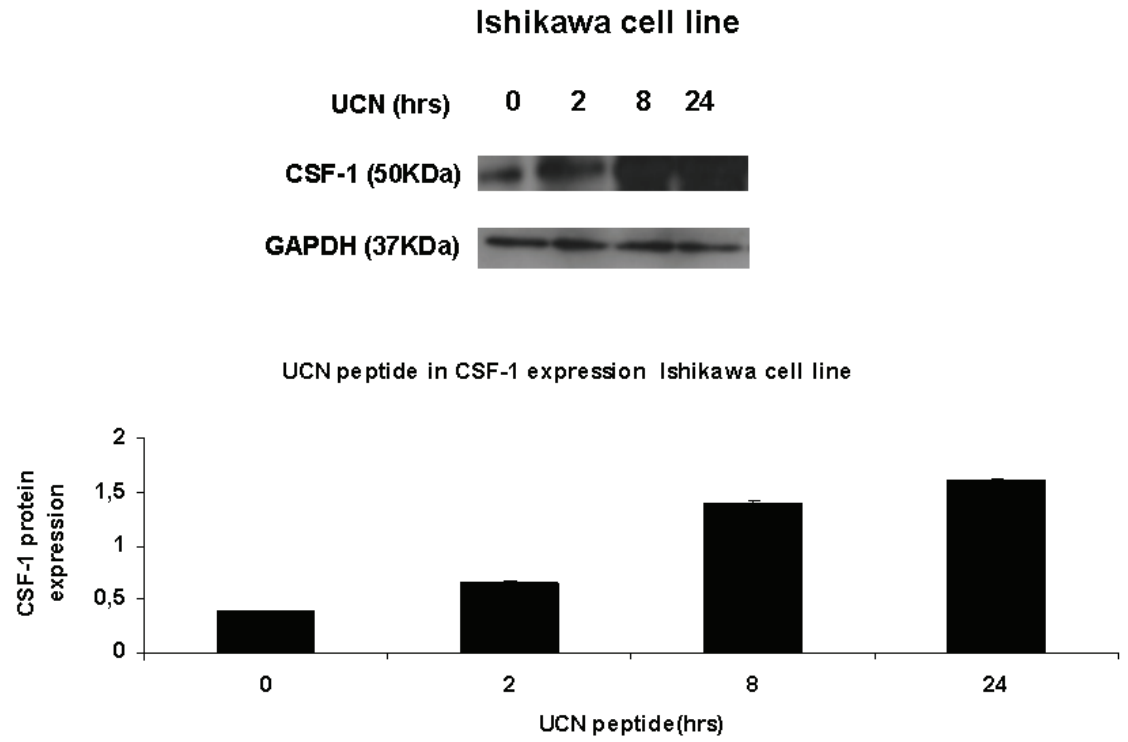


Figure 12: CSF-1 is upregulated in Ishikawa cell line and macrophages upon CRH stimulation– western blotting. A: In Ishikawa cell line, CSF-1 is upregulated by CRH, showing a higher expression at 24hrs of stimulation. B: In mice macrophages, CSF-1 expression is upregulated by CRH mostly at 24hrs of stimulation and antalarmin blocked this upregaltive effect in both cell lines at 8hrs and 24hrs of stimulation.

❖ 9.13. UCN upregulates CSF-1 expression in Ishikawa and macrophages

After showing that CRH has an upregulative effect in CSF-1 expression in Ishikawa cell line and macrophages, UCN peptide has been tested at the same time points of stimulation(0,2,8, 24hrs) in both cell types in order to identify if this peptide will have the same effect in CSF-1 expression. Indeed, UCN peptide caused an increase in CSF-1 expression levels time dependently in Ishikawa cell line and macrophages (Figure 13A, B). The experiment had a three time replication. (CSF-1 expression in Ishikawa cell line – UCN peptide stimulation for 0hrs: 0.38±0.007, 2hrs: 0.65±0.02 a.u, 8hrs: 1.4±0.007 a.u, 24hrs: 1.6±0.01 a.u,). (CSF-1 expression in macrophages – UCN peptide stimulation for 0hrs: 0.12±0.007, 2hrs: 0.48±0.01 a.u, 8hrs: 0.68±0.007 a.u, 24hrs: 2.05±0.03 a.u).

A



B

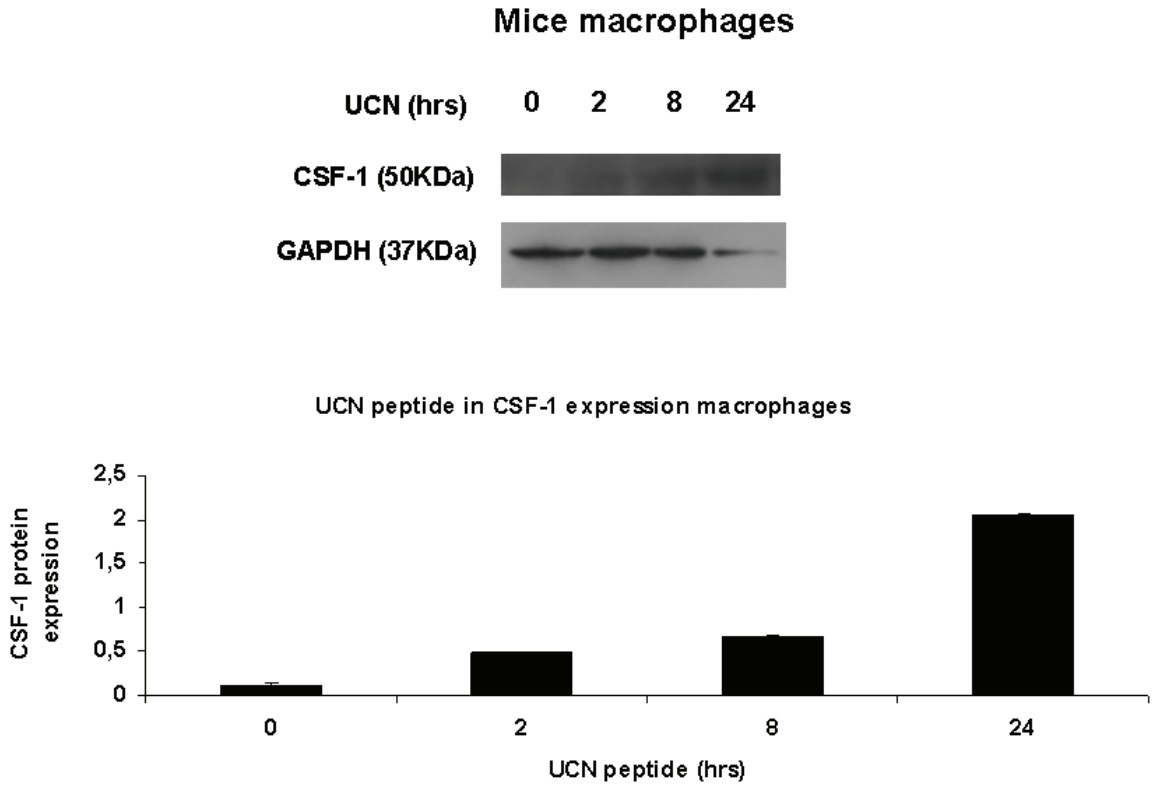
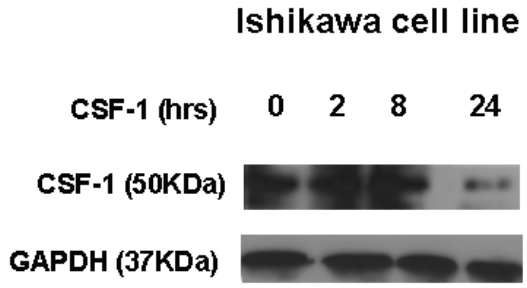


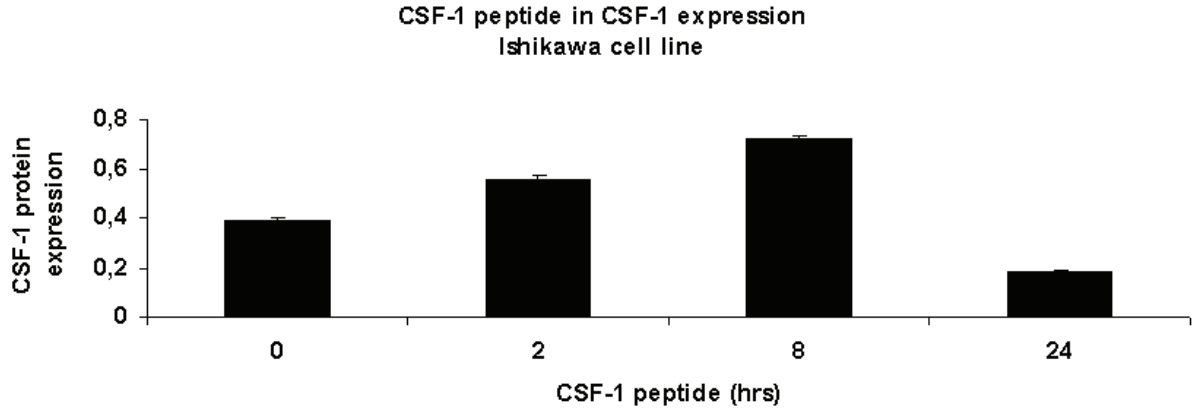
Figure 13: CSF-1 is upregulated in Ishikawa cell line and macrophages upon UCN stimulation– western blotting. (A): In Ishikawa cell line and in (B): In mice macrophages, CSF-1 is upregulated by UCN peptide, time dependently, showing a higher expression at 24hrs.

❖ 9.14. CSF-1 expression is upregulated in Ishikawa cell line and macrophages when these cells are stimulated by CSF-1.

Ishikawa cell line and macrophages were treated with CSF-1 peptide for specific time points of 0hrs, 2hrs, 8hrs, 24hrs. When Ishikawa cells where treated with exogenously added CSF-1, CSF-1 expression presented some fluctuations, by increasing at 2 and 8hrs of stimulation and showing an impaired expression at 24hrs (Figure 14A). Concerning CSF-1 expression in macrophages upon CSF-1 stimulation exogenously added, CSF-1 expression was increased time dependently, the longer CSF-1 stimulated the cells, the more elevated the CSF-1 expression was, reaching its higher expression level at 24hrs (Figure14B). The experiment had a three time replication. (CSF-1 expression in Ishikawa cell line – CSF-1 peptide stimulation for 0hrs: 0.39±0.007, 2hrs: 0.56±0.01 a.u, 8hrs: 0.72±0.007 a.u, 24hrs: 0.18±0.008 a.u,). (CSF-1 expression in macrophages – CSF-1 peptide stimulation for 0hrs: 0.06±0.002, 2hrs: 0.23±0.01 a.u, 8hrs: 0.49±0.01 a.u, 24hrs: 0.9±0.007 a.u).

A





B

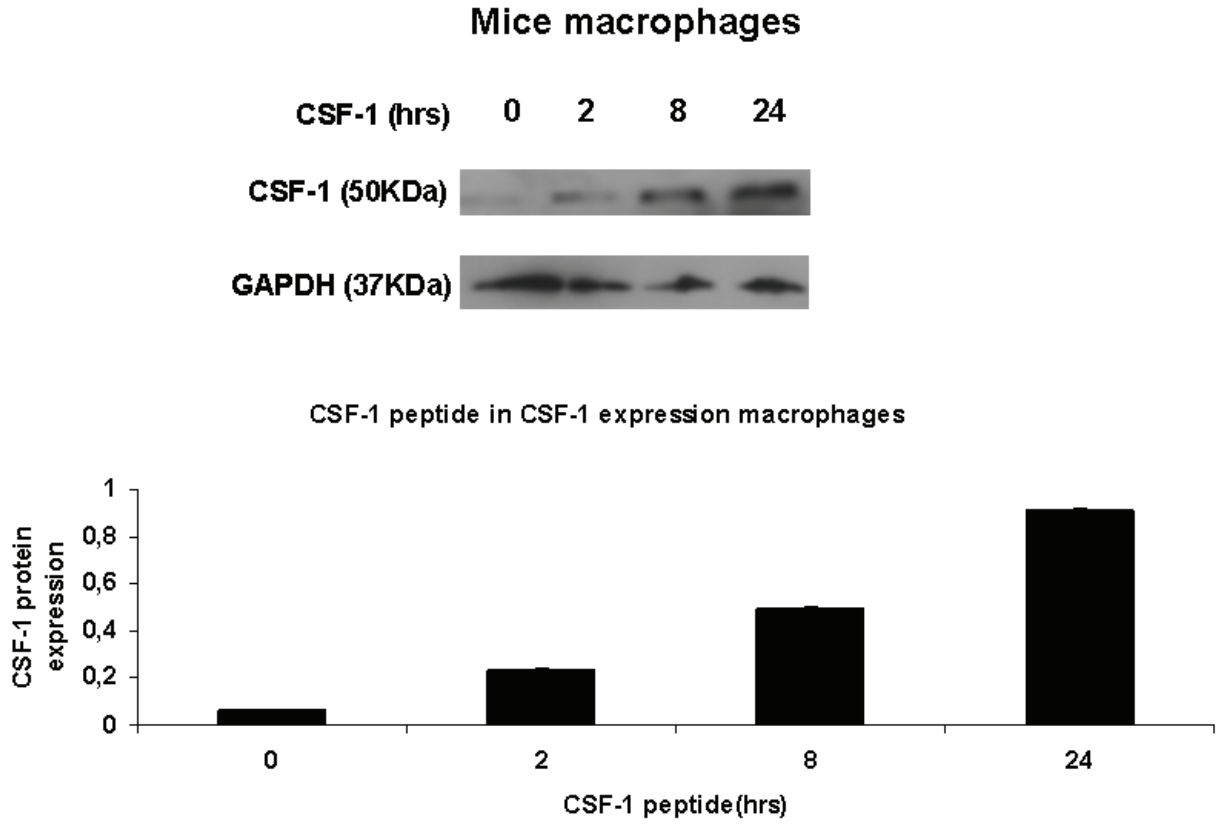


Figure 14: CSF-1 is upregulated in Ishikawa cell line and macrophages upon CSF-1 stimulation– western blotting. A: In Ishikawa cell line, CSF-1 is upregulated by CSF-1, showing a time dependently increasing expression up to 8hrs and having its expression decreased at 24hrs of stimulation. B: In mice macrophages, CSF-1 expression is upregulated by CSF-1 peptide time dependently reaching its higher point at 24hrs.

❖ 9.15. CRH upregulates FasL expression in Ishikawa cell line and macrophages through CRHR1.

In order to investigate how CRH regulates FasL expression in Ishikawa cell line and macrophages, treatment of these cells was performed with CRH peptide at different time points and antalarmin had been used in order to identify if CRHR1 is implicated in this regulative effect. So, it was noticed that CRH upregulates FasL expression in Ishikawa cell line mostly at 24hrs. Antalarmin's effect at 8hrs and 24hrs of incubation showed an impaired FasL expression, which indicates CRHR1 important role in CRH upregulative effect in FasL expression (Figure 15A). In macrophages, CRH increased FasL expression at 2 and 8hrs of stimulation but FasL expression was decreased at 24hrs. Antalarmin diminished FasL expression in macrophages at both 8hrs and 24hrs of stimulation (Figure 15B). The experiment had a three time replication. (FasL expression in Ishikawa cell line – CRH peptide stimulation for 0hrs: 0.70±0.01, 2hrs: 0.79±0.02 a.u, 8hrs: 0.77±0.01 a.u, 24hrs: 1.12±0.006 a.u, 8hrs + antalarmin: 0.2±0.008, 24hrs+antalarmin: 0.1±0.01). (FasL expression in macrophages - CRH peptide stimulation for 0hrs: 0.21±0.01, 2hrs: 0.43±0.03 a.u, 8hrs: 1.04±0.06 a.u, 24hrs: 0.15±0.01 a.u, 8hrs + antalarmin: 0.03±0.006, 24hrs+antalarmin: 0.01±0.002).

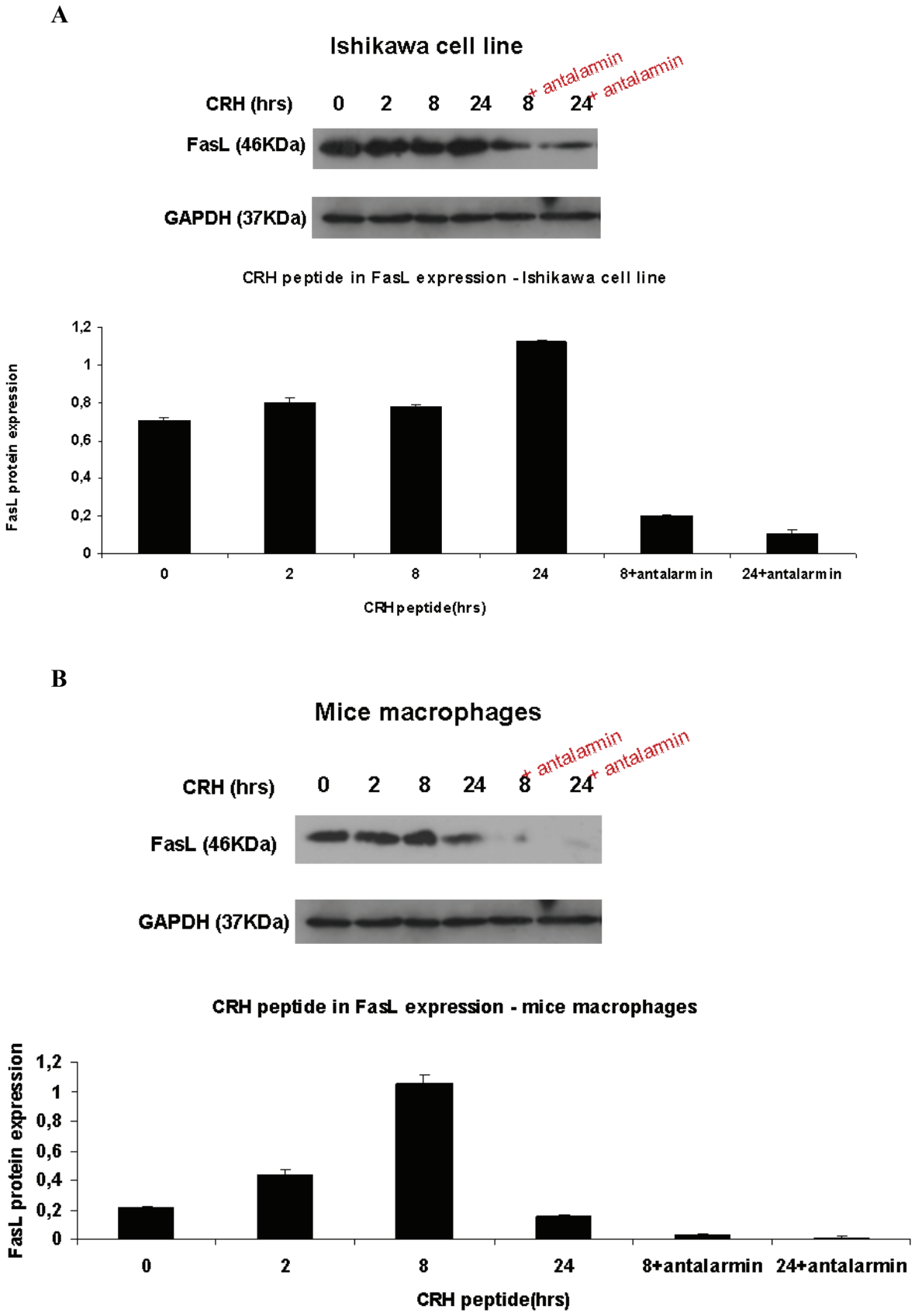
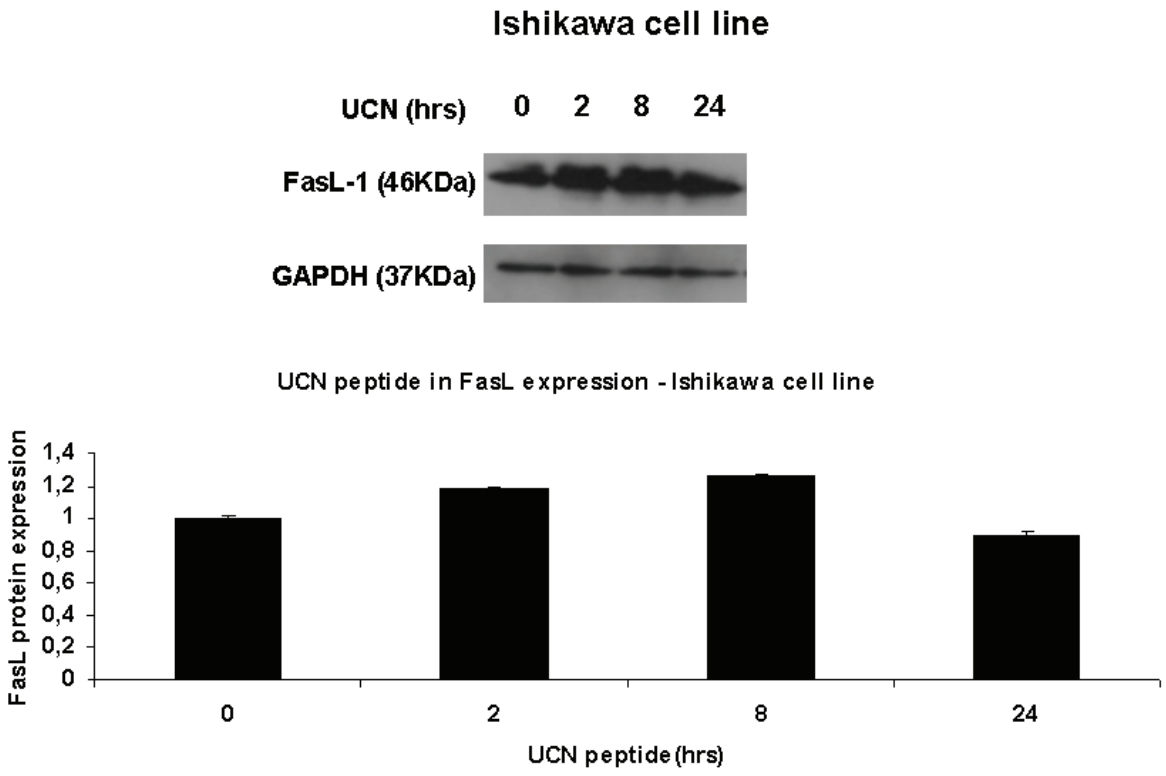


Figure 15: FasL expression is upregulated in Ishikawa cell line and macrophages upon CRH stimulation– western blotting.A: In Ishikawa cell line, FasL expression is upregulated by CRH, showing a higher expression at 24hrs of stimulation. B: In mice macrophages, FasL expression is upregulated by CRH time dependently up to 8hrs but then is decreased at 24hrs of stimulation and antalarmin blocked this upregulative effect in both cell types.

❖ 9.16. UCN upregulates FasL expression in Ishikawa cell line and macrophages

After showing that CRH has an upregulative effect in FasL expression in Ishikawa cell line and macrophages, UCN peptide has been tested at the same time points of stimulation(0,2,8, 24hrs) in both cell types in order to identify if this peptide has the same effect in FasL expression. Indeed, UCN peptide increased FasL expression levels up to 8hrs of stimulation but at 24hrs, FasL expression decreased (Figure 16A). In macrophages, FasL expression fluctuated when the cells were stimulated by UCN by being increased at 2hrs of stimulation and then its expression decreased at 8 and 24hrs (Figure 16B). The experiment had a three time replication. (FasL expression in Ishikawa cell line – UCN peptide stimulation for 0hrs: 1±0.009, 2hrs: 1.18±0.01 a.u, 8hrs: 1.26±0.008 a.u, 24hrs: 0.9±0.02 a.u,). (FasL expression in macrophages – UCN peptide stimulation for 0hrs: 0.78 ±0.01, 2hrs: 1.22±0.006 a.u, 8hrs: 1.1±0.01 a.u, 24hrs: 0.6±0.01 a.u).

A



B

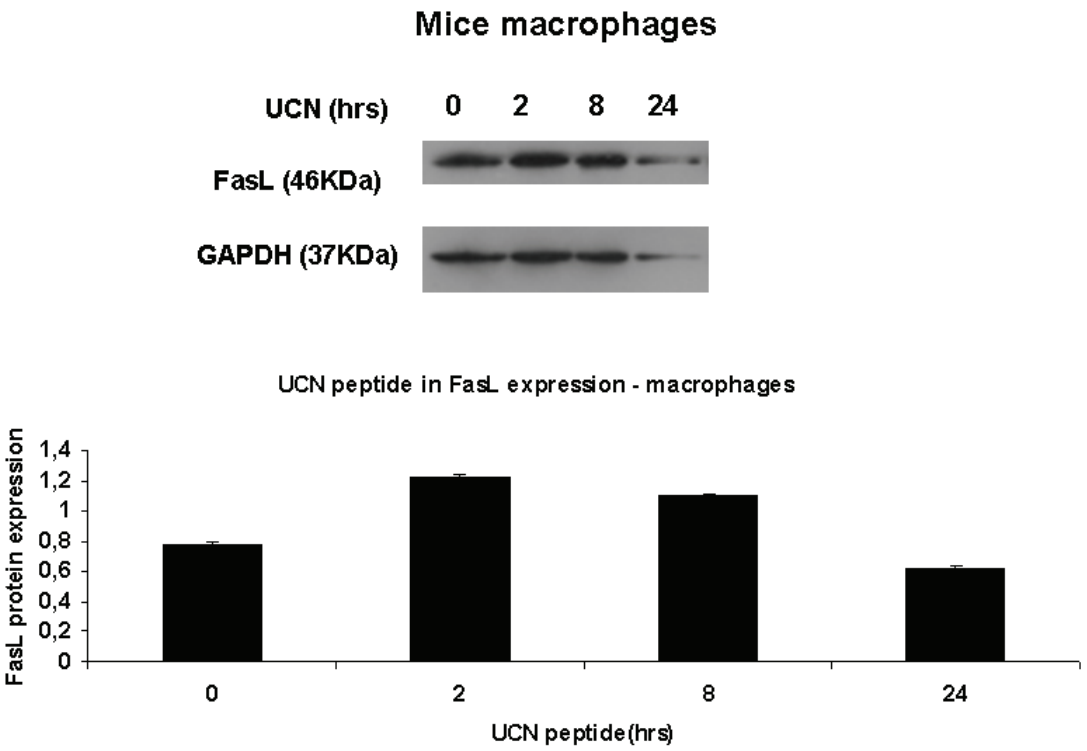
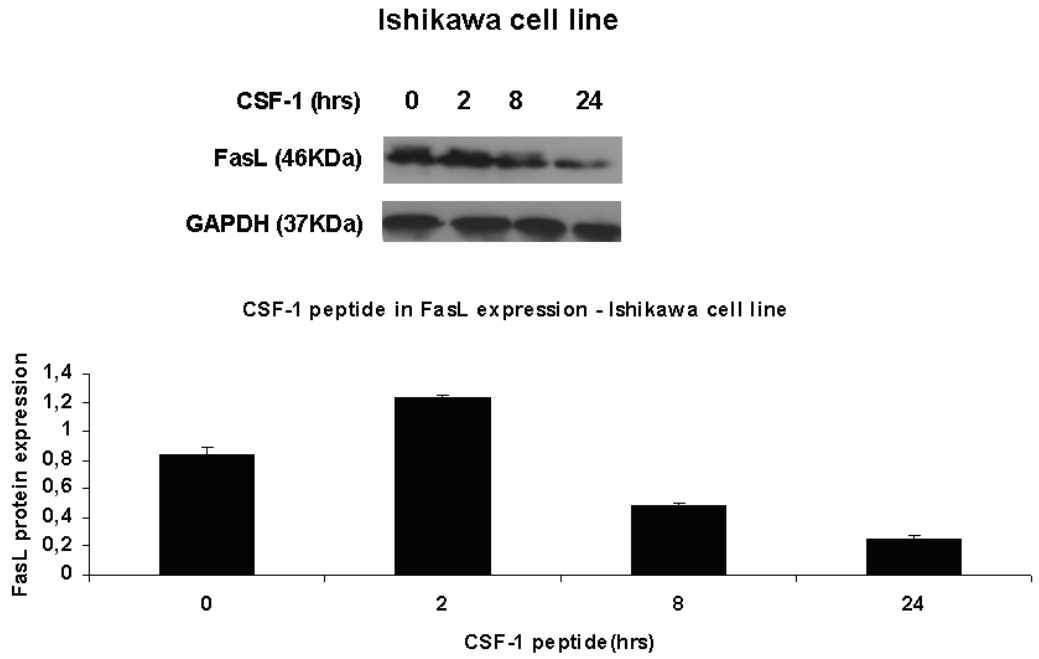


Figure 16: FasL expression is upregulated in Ishikawa cell line and macrophages upon UCN stimulation–western blotting. In Ishikawa cell line(A), FasL expression is increased at 2 and 8hrs of UCN stimulation and in macrophages(B) is increased at 2hrs of stimulation and then decreased till 24hrs of stimulation.

❖ 9.17. CSF-1 upregulates FasL expression in Ishikawa cell line and macrophages.

Ishikawa cell line and macrophages were treated with CSF-1 peptide for specific time points of 0hrs, 2hrs, 8hrs, 24hrs. In both cell types, Ishikawa cell line (Figure17A) and macrophages (Figure17B), CSF-1 peptide had an upregulative effect in FasL expression. So, when CSF-1 was added for 2hrs increased FasL expression but under 8 and 24hrs of CSF-1 incubation, FasL expression was decreased. The experiment had a three time replication. (CSF-1 expression in Ishikawa cell line – CSF-1 peptide stimulation for 0hrs: 0.84±0.05, 2hrs: 1.24±0.009 a.u, 8hrs: 0.49±0.008 a.u, 24hrs: 0.24±0.02 a.u,). (CSF-1 expression in macrophages – CSF-1 peptide stimulation for 0hrs: 1.08±0.01, 2hrs: 1.42±0.009 a.u, 8hrs: 0.84±0.008 a.u, 24hrs: 0.42±0.02 a.u).

A



B

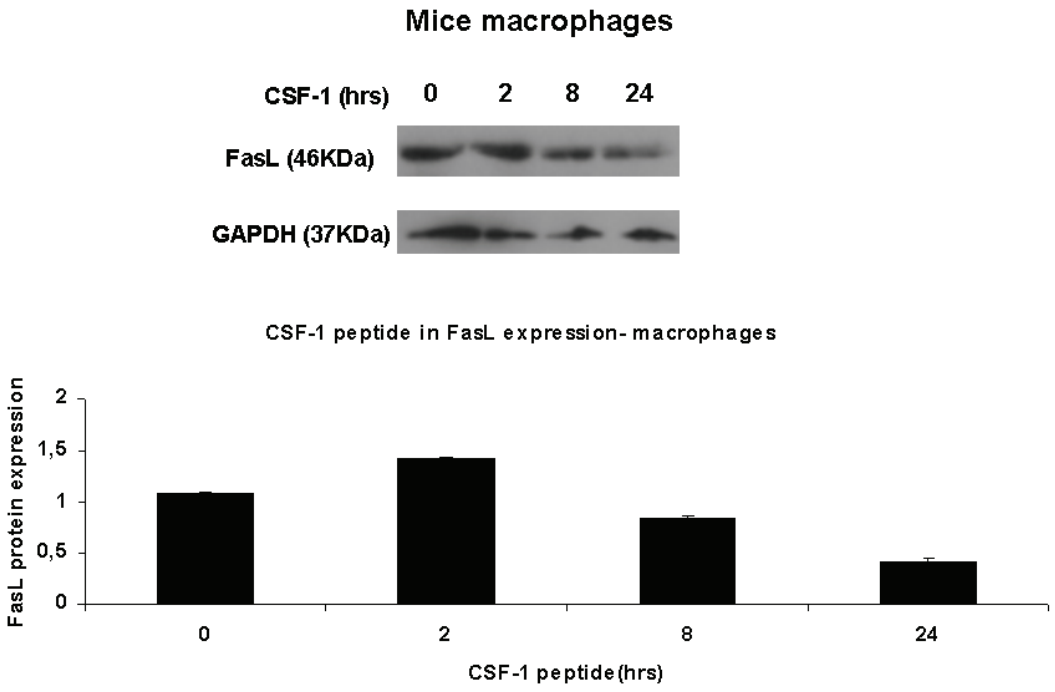


Figure 17: FasL expression is upregulated in Ishikawa cell line and macrophages upon CSF-1 stimulation– western blotting.
A: In Ishikawa cell line and macrophages, CSF-1 induced higher FasL expression at 2hrs of stimulation and then FasL was decreased at 8 and 24hrs of CSF-1 stimulation.

DISCUSSION

CHAPTER 10

Discussion

In this study, it is shown that CRH, UCN, CRHR1, CRHR2 and the receptor subtypes CRHR1β and CRHR2α are expressed in endometrium and endometriotic sites at mRNA and protein level. This is the first time that the receptor subtype expression is identified in endometriotic sites. Moreover, this study shows for the first time that CRHR1 and CRHR2 are more highly expressed in eutopic endometrium of endometriotic women compared to endometrium of healthy women. Interestingly, this study shows that the expression of CRH, UCN and their receptors, CRHR1 and CRHR2 is stronger in ectopic endometrium compared to that in eutopic endometrium of women with endometriosis. Moreover, as endometriosis is an inflammatory disease and it is characterised by cytokine expression **[61]** such as galectins and an increased amount of macrophages and growth factors such as CSF-1, combined with the fact that Ishikawa cell line is a well studied model for endometrium endocrinology, those two experimental models (Ishikawa cell line and macrophages) have been used to identify how galectin-1, CSF-1 and FasL are regulated by CRH, UCN and CSF-1. In this current study, it was found that there was an upregulative effect concerning the molecules mentioned above. It is very important that the CRH upregulative effect in gal-1, CSF-1 and FasL expression is mediated through CRHR1.

Women suffering from endometriosis are characterized by increased stress. Both chronic pelvic pain and inflammation, commonly seen in endometriosis, act as stressors, supporting a deregulation of homeostasis towards a state of increased stress. Corticotropin releasing hormone (CRH) is the main hormone of stress, being expressed in different sites in the human body **[130,170]**. Since endometriosis is a stress condition, it would be anticipated that reproductive CRH may play a key-role in pathophysiology of endometriosis, especially when pelvic pain or infertility are associated with the disease.

10.1. CRH, UCN, CRHR1 and CRHR2 expression in the endometriotic sites.

CRH and UCN are neuropeptides expressed in several sites of the female reproductive system including human endometrium **[130,170]** and their highest mRNA expression is reached in the secretory phase **[125,158,159]**. The main source of endometrial CRH is epithelial cells whereas stromal cells express CRH only after the decidualization process has begun **[125,158,159,258]**. CRH/UCN could activate mast cells triggering thus inflammation and adhesion formation, indicating an immune role of these molecules in eutopic endometrium. Moreover, these neuropeptides have been recently identified in endometriotic lesions **[64,116]**. Concerning the CRH-related inflammatory profile of endometriosis, progestins could inhibit the

CRH-induced inflammation of peritoneal cells *in vitro* as it has been found that TNF/IL-10 ratio in peritoneal cells of endometriotic women was increased by CRH and was dampened when the progesterone derivative dydrogesterone was added in the culture. So CRH could have an inflammatory effect on peritoneal cells of endometriotic women and low levels of progesterone in the follicular phase could be responsible for the progression of endometriosis and the related pain **[259]**. Based on this published data, this study firstly examined and confirmed the CRH and UCN mRNA expression in endometriotic lesions of endometriotic women.

Given that CRH and UCN expression is mediated through CRHR1 and CRHR2 and these receptors are expressed in several sites of the female reproductive system **[122]**, their expression in endometriotic lesions is investigated. Despite the fact that CRH/UCN have been implicated in endometriosis – a fact also verified by this study's results, no data has been reported so far concerning the expression of CRHR1 and CRHR2, the CRH and UCN receptors. This is the first time a study shows that CRHR1 and CRHR2 and specifically the CRHR1β and CRHR2α receptor subtypes are expressed at mRNA and protein level, not only in the endometrium but also at endometriotic sites indicating a potential crucial role of CRH and UCN in endometriosis.

10.2. Higher CRHR1 and CRHR2 expression in eutopic endometrium of endometriotic women compared to eutopic endometrium of healthy women.

The expression levels of CRHR1 and CRHR2 were compared in eutopic endometrium of endometriotic women with that in eutopic endometrium of healthy women to further examine the implication of these molecules in endometriosis and the infertility profile of endometriotic women. It is shown, for the first time, that CRHR1 and CRHR2 are significantly more expressed in the eutopic endometrium of endometriotic women compared to eutopic endometrium of healthy women. As previously described, CRH, UCN and CRHR1 are expressed by human epithelial and stromal endometrial cells. Endometrial stromal decidualization is a process taking place in the luteal phase of the menstrual cycle, where CRH expression is higher and leads to the induction of endometrial stroma decidualization, stimulated by progesterone in a cAMP – dependent manner **[16,20,157-159,258].** Upon decidualization, CRH inhibits the production of PGE2 and stimulates IL-1 and IL-6 production in human endometrial stromal cells **[19].** Endometriosis is an aseptic inflammatory process accompanied by altered immune-related cell functions such as accumulation of macrophages and increased expression of growth factors, cytokines and specifically interleukins IL-1 and IL-6 **[56,61,260,261].** Additionally, the expression of CRH in endometriosis has been correlated with proinflammatory responses influencing thus innate and acquired immune responses **[139,161]**. These data indicate that CRH and UCN may be of importance in maintaining chronic inflammation and thus local stress. Such stress could be further correlated to the local symptoms often seen in endometriosis as increased infertility rates, affected by modulation of the decidualization process and improper function of endometrium in women with endometriosis. A recent study **[116]** has proposed that there is an impaired CRH and UCN

expression in eutopic endometrium of endometriotic women compared to healthy women endometrium. In this PhD study, the CRHR1 and CRHR2 mRNA and protein expression levels in eutopic endometrium of endometriotic and healthy women is evaluated and it is shown for the first time that the expression of both receptors is more elevated in eutopic endometrium of endometriotic compared to that of healthy women. It could be hypothesized, as it has been shown by previous studies, that the impaired expression of CRH and UCN in eutopic endometrium of endometriotic women **[116]** and the reduced capacity of CRH and UCN to induce *in vitro* decidualization of endometriotic women stromal cells **[116]** may contribute to further expression of their receptors to keep a proper function of endometrium in endometriotic women. Unidentified endometrial defects in endometriosis could also affect the expression of CRH and UCN resulting in increased expression of CRHR1 and CRHR2 acting as a regulatory mechanism to compensate for the reduced efficiency of proper endometrial function of endometriotic women. Despite the fact that little is known so far for the expression patterns of the CRH receptors in intrauterine tissue, these findings could be in cohort with other studies showing that long term stimulation of pituitary **[262]** and myometrial cells **[263]** with CRH can down-regulate its own CRHR1 receptor. Further studies are needed to further elucidate this controversial expression pattern between CRH, UCN and their receptors expression in eutopic endometrium of endometriotic women.

CRH and UCN exert an important role not only in decidualization but also in blastocyst implantation **[157].** Invasion of the blastocyst in the decidualized endometrium needs to take place for a successful embryo implantation **[11]**. CRH is produced by maternal decidual cells and embryonic trophoblast and is implicated in the maternal- blastoyst immune ‘’cross-talk’’ by stimulating the expression of FasL in invasive extravillous trophoblast and maternal decidual cells and by increasing the apoptosis of T-lymphocytes through FasL induction. Thus the graft vs host reaction from the maternal immune system to the fetus is prevented and this is mediated through CRHR1 **[20].** Studies in mice models have also shown that blocking of CRHR1 results in implantation dysfunctions **[264]** reinforcing thus the role of CRH in embryo implantation. In this current study, it has been shown that CRHR1 and CRHR2 are more abundantly expressed in eutopic endometrium of endometriotic women and this might explain the fact that endometriotic women can be fertile, characterised as hypofertile but not completely sterile, as there is excessive expression of CRHR1 and CRHR2 overcoming thus the low levels of CRH and UCN leading to implantation.

10.3. CRH, UCN, CRHR1β and CRHR2α molecules are more abundant in endometriotic tissues compared to the corresponding eutopic endometrium.

In this current study, it has also been examined the expression of CRH, UCN, CRHR1 and CRHR2 in ectopic endometrium of endometriotic women compared to their eutopic endometrium. For the first time it is found that all these molecules are more highly expressed in ectopic rather than eutopic endometrium of the same patients at mRNA and protein level. The fact that these molecules are more highly expressed in ectopic

endometrium indicates that their function outside the uterus might be strengthened, which may potentially contribute to implantation and pregnancy maintenance problems of women with endometriosis.

Given also that high levels of stress are correlated with the progression of endometriosis followed by adhesion formation and infertility symptoms [265] combined with the fact that CRH is activated by high levels of stress [156,259,266], these results may explain the neuroendocrine vicious circle of stress, mediated by CRH and UCN which is expected to maintain a chronic inflammatory profile as well as infertility.

10.4. Expression of CRH UCN, CRHR1 and CRHR2 in Ishikawa cell line

Ishikawa cell line is a well differentiated endometrial adenocarcinoma cell line and is characterized by high levels of expression of estrogen and progesterone receptors **[256]**.Ishikawa cell line expresses almost all steroid hormone receptors and represents an extensively used in vitro model for the study of steroid hormone effects on human endometrium **[127-129].** Furthermore, Ishikawa cell line is an excellent in vitro model for studying endometrial epithelial functions. Apart from the fact that maintains both estrogen and progesterone receptor expressions, it is quite responsive to ovarian steroids as well **[267,268]**. Moreover, this cell line expresses functional enzymes, integrins and structural proteins, the same as endometrial cells **[268]**. CRH and CRHR1 have already been detected in human endometrial adenocarcinoma Ishikawa cell line **[126].** In this study the expression of CRH, UCN, CRHR1 and CRHR2 were further confirmed at an mRNA and protein level in Ishikawa cell line. It is already known that the colony stimulating factor, CSF-1, is expressed in Ishikawa cell line and stimulates Ishikawa cell line proliferation **[269,270]**. Moreover, galectin-1 is expressed in this adenocarcinoma cell line **[271]** as well. So, this was a motivation, in order to investigate how molecules such as CRH, UCN and CSF-1 that are expressed in Ishikawa cell line, eutopic and ectopic endometrium could regulate important immunomodulatory molecules such as galectin-1, CSF-1 and FasL which are also expressed at the same cell type and tissues.

10.5. Galectin-1 is expressed in endometriotic sites and is overexpressed in ectopic sites compared to eutopic endometrium of endometriotic women and eutopic endometrium of healthy women

Firstly, the expression of galectin-1 in endometrium of healthy women was tested and so the already established notion **[212]** that galectin-1 shows a higher expression at the late secretory phase of the menstrual cycle was confirmed. Furthermore, in this PhD thesis, for the first time, it is presented that galectin-1 is expressed in endometriotic tissue- ectopic endometrium. Moreover, galectin-1 expression is found to be higher in ectopic rather than eutopic endometrium of endometriotic women. These findings can be correlated with a previous study showing that galectin-3 is overexpressed in ectopic endometrium and much more highly expressed in eutopic endometrium of endometriotic women compared to endometrium of healthy

women **[236]**. As endometriosis is characterized by the release of several proteins including growth factors and the expression of integrins, cadherins and lectins that regulate cell migration, invasion, angiogenesis, immune functions and apoptosis**[60]**, galectins should play an important role in this inflammatory disease. Galectin-1 plays a major role in initiation, activation and resolution of inflammatory responses and can act as a pro-inflammatory or an anti-inflammatory cytokine. Gal-1 induces inhibition of cell growth and cell-cycle arrest and promotes the apoptosis of activated immune cells. So, galectin-1 is involved in cell adhesion, chemotaxis, antigen presentation and apoptosis, functions that characterize inflammatory situations as endometriosis as well **[218]**. The finding that the galectin-1 is expressed in endometriotic sites, it of high importance as it may regulate several immune functions concerning the pathogenesis of the disease and by being highly expressed in endometriotic sites, promoting T cell apoptosis, it could be hypothesized that gal-1 favors the persistence ,establishment and immune escape of endometriotic tissue in ectopic sites. In addition, galectin-1 stimulates proliferation of vascular endothelial cells **[205]**, so by being expressed in ectopic sites , it could hypothesized that it could facilitate the endometriotic tissue vascularogenesis, favoring the persistence of the disease.

Furthermore, considering that galectin-1 is expressed widely throughout the nervous system, that both reduced and oxidized forms of gal-1 participate in the process of Wallerian degeneration and that it has been suggested that gal-1-1/Ox may be useful as a novel therapeutic agent for functional restoration after peripheral nerve injury **[272]**,plus the fact that galectin-1 plays an important role in survival, differentiation and synaptic plasticity of neurons, preventing neuronal loss in case of injury in the central nervous system **[224]**, it is of high importance the hypothesis that galectin-1 by being expressed in endometriotic sites, might play a very important role in nerve generation and pain in women with endometriosis.

Moreover, given that it is found that galectin-1 is much more highly expressed in ectopic rather than eutopic endometrium, could indicate an important role of galectin-1 in the infertility profile of women with endometriosis. Galectin-1 is expressed in endometrium at the late secretory phase and is expressed by decidual stromal cells as well. Additionally, galectin-1 is involved in immune- endocrine cross-talk, maternal – decidual expression of gal-1 is regulated by progesterone and galectin-1 increases progesterone concentrations, suggesting its role in the maintenance of pregnancy **[227]**. Galectin-1 is synthesized in the trophoectoderm of blastocysts, suggesting a role in the attachment of the embryo in the uterine epithelium. Galectin-1 has been implicated in some of the pathological conditions such as early pregnancy loss, preeclampsia and trophoblastic malignant disease. Galectin-1 has also been recently qualified as a member of human trophoblast cell invasion machinery**[231]**. Moreover galectin-1 is under the control of ovarian steroids during blastocyst implantation **[232]** and influences trophoblast immune invasion **[234]**. Galectin-1 plays an important role in the organization of extracellular matrix and the regulation of trophoblast differentiation and cell motility during placentation **[231,233]**. Moreover, in stress induced abortions, galectin-1 expression was decreased **[77]**. Galectin-1 induces generation of tolerogenic dendritic cells and regulatory T cells, so knock out mice of galectin-1 led to a higher rate of fetal loss in allogenic mating.

Circulating gal-1 levels could serve as a predictive factor for pregnancy success in early human gestation as compared with normal pregnant women, circulating gal-1 levels were significantly decreased in patients who suffered a miscarriage **[234].** So, taking everything into consideration, it could be hypothesized that galectin-1 decreased expression in eutopic endometrium of women with endometriosis compared to ectopic endometrium plays a possible important role in implantation and decidualization defects of endometriotic women. The fact that galectin-1 is more highly expressed in ectopic endometrium indicates that its function outside the uterus might be strengthened, which may potentially contribute to implantation and pregnancy maintenance problems of women with endometriosis.

As far as the comparison of the galectin-1 expression in eutopic endometrium of endometriotic and healthy women, in this current study, it is found that galectin-1 expression is higher in eutopic endometrium of endometriotic women rather than eutopic endometrium of healthy women. The same expression pattern has been noticed in a previous study concerning only gal-3 **[236]**. This finding is in accordance with the established theory suggesting that there are several differences between eutopic endometrium of healthy women and endometriotic women including differences in structure, proliferation, immune components, adhesion molecules, proteolytic enzymes and inhibitors, steroid and cytokine production and responsiveness, gene expression and protein production**,** in order to permit the 'altered endometrium' of endometriotic women to adhere, grow, and spread outside the uterine cavity more easily **[86,112].**

10.6. Higher co-localization of macrophages with galectin-1 in endometriotic sites.

Endometriosis is an inflammatory disease characterised by increased concentrations of cytokines, growth factors and macrophages. Macrophages are implicated in innate immune system and are responsible for secreting growth factors, cytokines and complement components, prostagladins and hydrolytic enzymes. Apart from the growth stimulatory effect of macrophages to endometriotic implants, macrophage products are connected with pathophysiology of endometriosis, referring to pain and infertility **[49,50]**. The concentration of peritoneal macrophages is highly increased in women with endometriosis compared to healthy **[51-54]**. In this PhD thesis, it was further confirmed that ectopic endometrium is characterised by higher expression of macrophages compared to eutopic endometrium of healthy women (data not shown). As it has been previously mentioned by recent studies, peritoneal macrophages are increased in number, concentration and activation mode (reviewed by [**56]**). Increased macrophages are also related to the stage of endometriosis **[57]**. Macrophages play also a very important role in growth and development of endometriotic lesions and in generation of pain through interaction with nerve fibres as well. So macrophages and their products had been found to play important role in stimulation, growth and repair of nerve fiber, inducing pain in endometriosis.

Macrophages secrete cytokines and growth factors and concerning endometriosis, these macrophage products can promote stimulation of endometrial cell proliferation, implantation of endometrial cells or tissue, increased tissue remodeling through regulation of matrix metalloproteinase and increased

angiogenesis of the ectopic endometrial tissue **[56,61]**. Increased number of macrophages in endometriotic sites equals increased number of cytokine production as well. Cytokines can act in an autocrine or paracrine way and have a role in initiation, propagation and regulation of immune and inflammatory responses and can have proliferative, cytostatic, chemoattractant or differentiative effects. All these cytokines are not only produced by macrophages but from endometriotic lesions as well and so it is galectin-1 which is not only expressed by macrophages **[205]** but it is found in this study to be overexpressed in ectopic endometrium as well. Moreover, it is shown here, that there is a co-localization of macrophages and galectin-1 in endometriotic tissue, so that was a motivation, in order to investigate how galectin-1 is regulated, by neuropeptides present in endometriosis and macrophages and by a macrophage stimulatory factor.

10.7. CSF-1 overexpression in ectopic endometrium- compared to eutopic endometrium of endometriotic women and CSF-1 higher expression in eutopic endometrium of endometriotic patients compared to healthy ones.

The colony stimulating factor- 1, is also known as macrophage colony stimulating factor and it is a secreted cytokine, characterized as a hematopoetic growth factor which influences hematopoietic stem cells to differentiate into macrophages or other related cell types. CSF-1 regulates the survival, proliferation and differentiation of mononuclear phagocytes to macrophages **[237,238]**. M-CSF1 is produced by fibroblasts, endothelial cells, monocytes and macrophages. CSF-1 has immunomodulatory roles in tumours and inflammation.

As endometriosis is an inflammatory disease, there are several cytokines and growth factors that are implicated in this immune-disequilibrium status. As secretory cells, monocytes and macrophages are vital to the regulation of immune responses and the development of inflammation. These cytokines are produced by peritoneal macrophages, lymphocytes, mesothelial cells and endometriosis implants **[251]**. Furthermore, women and female baboons with endometriosis are characterised by increased number of peritoneal macrophages **[252]**. Women with endometriosis are characterised by increased expression of CSF-1 compared to healthy ones **[53]**. Although, it is unclear whether increased levels of CSF-1 in patients with endometriosis originate from inflammation associated macrophages or it is produced by the endometriotic lesions. Therefore, one study supports that CSF-1 and its receptor are involved in the genesis of early endometriotic lesions and that endometriotic lesions contribute to elevated CSF-1 levels in the peritoneal fluid of women with endometriosis and that elevated levels of CSF-1R in endometrial cells from women with endometriosis after co-culture with peritoneal mesothelial cell suggests that endometrial tissue is highly responsive to CSF-1 signalling **[253,254]**.Moreover, endometrial tissue from CSF-1 knockdown mice develop significantly fewer endometriotic lesions than control tissue in a syngeneic mouse model of endometrial tissue transplantation into the pelvic cavity **[255]**. Peripheral blood monocytes from women with

endometriosis promote proliferation of cocultured autologous endometrial cells and apart from the growth stimulatory effect of macrophages to endometriotic implants, macrophage products are connected with pathophysiology of endometriosis, referring to pain and infertility **[49,50]**. The concentration of peritoneal macrophages are highly increased in women with endometriosis compared to healthy ones and this can be linked to increased levels of CSF-1 deriving from endometrial/endometriotic cells or peritoneal macrophages respectively **[51-54]**. It has been found that peritoneal environment controls the differentiation of macrophage precursors, leading them to an alternatively activated status. The alternatively activated macrophages are quite important for ectopic tissue to vascularize and grow **[55].** So it was quite important for this PhD thesis, firstly, to examine and then to evaluate the CSF-1 expression in endometriotic sites, and it was found and further confirmed the already known notion that CSF-1 is overexpressed in ectopic endometrium of women with endometriosis.

Moreover, it was found that CSF-1 expression is higher in eutopic endometrium of endometriotic women compared to healthy ones. As previously mentioned there are several differences between eutopic endometrium of healthy women and endometriotic women including differences in structure, proliferation, immune components, adhesion molecules, proteolytic enzymes and inhibitors, steroid and cytokine production and responsiveness, gene expression and protein production, in order to permit the 'altered endometrium' of endometriotic women to adhere, grow, and spread outside the uterine cavity more easily **[86,112].** So, the differential expression of CSF1-1 between eutopic endometrium of endometriotic and healthy women could further promote this notion.

Concerning the fact that CSF-1 expression is found in this study to be higher in ectopic rather than eutopic endometrium of the same women, this could possibly show new insights in the pathophysiology of endometriosis and the infertility profile of women with endometriosis. Macrophages and other cells of the female reproductive tract are also regulated by locally produced CSF-1. CSF-1 plays an important role in placental growth and differentiation. CSF1 and its receptor are expressed by endometrial stromal cells, endometrial epithelial cells and peritoneal mesothelial cells. It has also been detected in endometrial tissue, amniotic fluid and placenta. The concentration of CSF-1 in uterus increases during pregnancy. Progesterone and estradiol regulate CSF-1 synthesis by the luminal and glandular secretory epithelial cells of the uterus. Also, CSF-1 stimulates endometrial epithelial cells in an autocrine mechanism. Knock down of CSF-1 in endometrial epithelial cells results in decreased proliferation and transmethothelial invasion of endometrial epithelial cells **[244]**. Levels of colony-stimulating factor (CSF)-1 synthesized by the uterine epithelium increase at the time of implantation and continues to elevate dramatically throughout the process of placentation. This CSF-1 is the major regulator of the mononuclear phagocytic lineage and controls the proliferation, migration, viability, and function of DCs and macrophages and affects decidual cells and trophoblasts **[245]**. A role for CSF-1 in implantation is indicated by studies in osteopetrotic mice, which lack functional CSF-1. Homozygous females with a mutated CSF-1 gene (op/op) were consistently infertile when

mated with males with the same genotype **[247]**. Moreover, it has been indicated that CSF-1 increases the rate of preimplantation mouse embryo development and the number of trophoectodermal cells in the blastocyst **[248]**. In humans, there is an increased local production of CSF-1 in tissues found at the maternal-fetal interface during the time of implantation and early pregnancy. As CSF-1R is expressed in these tissues, CSF-1 might have an important role in decidual function and placental growth **[249]**. Low CSF-1 serum levels have been correlated with unexplained recurrent spontaneous abortion in both the preconceptional and conceptional phases. Moreover, decidual T cells clones from women suffering from unexplained recurrent abortion display decreased production of CSF-1**[250]** . So, the fact that CSF-1 is found to be decreased in eutopic endometrium of women with endometriosis compared to ectopic endometrium could possibly indicate the important role of CSF-1 in altered eutopic endometrium and fertility defects of women with endometriosis.

10.8. CRH, UCN and CSF-1 upregulate galectin-1, CSF-1 and FasL expression in Ishikawa cell line and macrophages. The CRH upregulative effect is mediated through CRHR1.

Taking everything into consideration, it was quite important to examine how CRH, UCN and CSF-1 regulate galectin-1, CSF-1 and FasL expression in an endometrial adenocarcinoma cell line, mimicking eutopic endometrium, as to further elucidate in the role of these molecules in eutopic endometrium and infertility problems of women with endometriosis and in an important modulatory factor of inflammation such as macrophages, as endometriosis is a benign inflammatory disease.

10.9. Galectin-1 and CSF-1 upregulation by CRH, UCN, CSF-1

Galectin-1 can act as pro- or anti-inflammatory cytokine and participates in cell growth, cell-cycle arrest and promotes apoptosis of activated T cells **[205]**. Moreover, galectin-1 is expressed by cancer cells and promotes tumour progression by inducing T cell apoptosis **[208,214]**. Galectin-1 is expressed by Ishikawa cell line as well **[213]**. Moreover, Galectin-1 has been found in the female reproductive system, in immune privileged sites such as the placenta and endometrium **[204]**. It is expressed in the endometrium at the late secretory phase as shown by this current study as well and is found, in this current study, to be overexpressed in ectopic endometrium as well. Taking everything into consideration, the finding that CRH, UCN and CSF-1 upregulate galectin-1 expression in Ishikawa cell line is of high importance concerning its regulation in endometrium, as Ishikawa cell line, is an in vitro model for endometrium endocrinology. So, galectin-1 is upregulated by neuropeptides that are expressed in eutopic and ectopic endometrium as well and by a very important inflammatory factor such as CSF-1. CSF-1 is found to be upregulated in Ishikawa cell line as well, under stimulation of CRH, UCN and exogenously added CSF-1. CSF-1 is a hematopoetic growth factor regulating macrophage growth, differentiation and survival. Moreover, CSF-1 overexpression has been noticed in ovarian, cervical and endometrial carcinomas. It is a very important factor in

inflammatory processes as it is secreted by macrophages and plays a vital role in placental growth and differentiation and in implantation **[238,245]**. Moreover, as inflammatory molecules, CRH, CRHR1 **[126]** and UCN are both expressed in cancer cell line, such as the endometrial adenocarcinoma cell line. In this study the expression of CRH, UCN, CRHR1 and CRHR2 were further confirmed at an mRNA and protein level in Ishikawa cell lline. Moreover, galectin-1 showed to be overexpressed in endometriotic tissue and CSF-1 as well. Both galectin-1 and CSF-1 by playing important role in cancer and eutopic endometrium, are expressed by Ishikawa cell line as well **[271]** and the last one stimulates Ishikawa cell line proliferation **[269,270].** CRH, UCN and CSF-1 have been found to be less expressed in eutopic endometrium of endometriotic women compared to ectopic endometrium and galectin-1 as well, plus the fact that there is a co-localization effect of macrophages and galectin-1 in ectopic endometrium.So, it could probably hypothesised that there is an autocrine or paracrine regulative effect between these molecules in endometrium. According to this finding, less CRH, UCN and CSF-1 in endometrium could lead to less galectin-1 and CSF-1 expression, which could have severe implications in reproductive functions. All above could be a possible hypothesis as galectin-1 plays a major role in pregnancy maintenance and a recent study reported that galectin-1 knock out mice led to a higher rate of fetal loss **[77]** plus the fact that galectin-1 has been involved in several pathological conditions involving trophoblast such as early pregnancy loss, preeclampsia and trophoblastic malignant disease **[231]**. Moreover, CSF-1 stimulates endometrial epithelial cells in an autocrine mechanism. Knock down of CSF-1 in endometrial epithelial cells results in decreased proliferation and transmethothelial invasion of endometrial epithelial cells. Levels of colony-stimulating factor (CSF)-1 synthesized by the uterine epithelium increase at the time of implantation and continue to elevate dramatically throughout the process of placentation **[244]** and low CSF-1 serum levels have been correlated with unexplained recurrent spontaneous abortion in both the preconceptional and conceptional phases **[250]**.

Moreover, galectin-1 is expressed by a variety of cells in central and peripheral immune sites including T cells, macrophages and activated B cells. Galectin-1 has been characterised as modulator of monocyte/macrophage functions. Moreover galectin-1 induces apoptosis of activated T cells **[205,220]** and participates in acute and innate inflammation processes, such as endometriosis, as it is shown by this study, that galectin-1 is overexpressed in endometriotic sites and co-localizes with macrophages. So, the fact that galectin-1 and CSF-1 expression is upregulated in macrophages under stimulation of CRH, UCN and CSF-1 could reveal new immunomodulatory roles of these cytokine in macrophages, accumulating in endometriosis. Moreover, CRH is secreted at inflammatory sites and exerts proinflammatory properties both in innate and acquired immune processes. Locally secreted CRH promotes local inflammation and acts as an autocrine or paracrine inflammatory cytokine which is also secreted by macrophages. UCN has been found to be increased in inflammatory situations and also to be expressed by spleen, thymus, macrophages and lymphocytes as well **[21,152]**. CSF-1 is a modulatory factor of growth, differentiation and survival of macrophages and it is secreted by macrophages as well **[238]** .CSF1R plays a vital role in the accumulation

of macrophages in tumor sites. So the upregulative effect of CRH, UCN and CSF-1 in galectin-1 and CSF-1 expression in macrophages could possibly indicate a new local immunomodulatory effect. Moreover, this upregulative effect could be explained as macrophages secrete cytokines and growth factors and concerning endometriosis, these macrophage products can promote stimulation of endometrial cell proliferation, implantation of endometrial cells or tissue, increased tissue remodeling through regulation of matrix metalloproteinase and increased angiogenesis of the ectopic endometrial tissue **[56,61]**. Increased number of macrophages in endometriotic sites equals increased number of cytokine production as well. So, it could be hypothesized that the more upregulation of galectin-1 and CSF-1 expression exists, the more activated T cells become apoptotic and the ectopic tissue persists.

10.10. FasL is upregulated in Ishikawa cell line and macrophages under stimulation by CRH, UCN and CSF-1

A very important apoptotic factor such as FasL has been shown to play vital role in decidualization and implantation and as FasL is implicated in inflammatory processes, Harada *et al*. (1996) found that Fas is expressed randomly in both eutopic and ectopic endometrial tissues **[36]**. Fas when bound to its receptor induces apoptosis and Fas/FasL interactions regulate immune responses and progression of cancer **[174]**. Ishikawa cell line is an endometrial adenocarcinoma cell line and an in vitro model concerning endometrium endocrinology as previously mentioned. So, as in this current study it is shown that CRH, UCN and CSF-1 upregulate FasL expression and the upregulative effect of the former one is regulated by CRHR1, this could be a further hypothesis contributing to the fact that CRH regulates and stimulates FasL expression having an important role in crucial endometrial processes such as decidualization and implantation. As it has already been found by previous studies, Fas ligand plays an important role in leukocyte trafficking between the mother and the fetus. Fas and FasL are expressed in human endometrium throughout the menstrual cycle. CRH induces Fasl expression in both maternal deciduas and trophoblast cells through CRHR1 in order to potentiate the apoptosis of activated maternal T-lymphocytes and prevent the graft versus host reaction and rejection of the embryo **[187]**. The presence of CRH, both in the maternal (decidua) and fetal (trophoblast) sites suggests that locally produced CRH regulates FasL production, affecting the invasion process through a local auto/paracrine way in cytotrophoblast cells, regulating their own apoptosis. Inadequate CRH- mediated induction of FasL in extravillous trophoblasts might be involved in the pathophysiology of infertility and recurrent fetal absorption or miscarriage **[187].** Moreover, aberrant expression of CRH in pre-eclampsia may activate the FasL-positive decidual macrophages, impair the physiological turnover of EVT and eventually disturb placentation **[188]**. So, the finding of this current study that Fasl is upregulated by CRH, UCN and CSF-1 in Ishikawa cell line can further contribute to its important role and interaction with CRH in endometrial functions.

As previously mentioned, Fas is expressed randomly in both eutopic and ectopic endometrial tissues **[36].**Higher expression of FasL by endometriotic tissues contributes to their survival and the development of endometriosis. FasL is also expressed by macrophages as macrophage-conditioned media induced FasL expression by endometrial stromal cells in a dose-dependent manner **[179]**. The sources of the elevated levels of soluble FasL in the peritoneal cavity were endometriotic lesions and peritoneal fluid leukocytes. Endometrial glandular and stromal cells express FasL, at both the mRNA and protein levels. Peritoneal fluid leukocytes are another plausible source for high levels of soluble FasL in women with endometriosis, because human‐activated peripheral blood mononuclear cells were shown to express FasL messenger RNA **[193]**. So, in this study it was very important to evaluate how FasL expression is regulated in macrophages, as there is an increased amount of macrophages in endometriotic sites and an increased expression of Fasl. As a result, it is shown that CRH, UCN and CSF-1 upregulate FasL expression in macrophages, indicating that FasL has an important role in the persistence of the disease as T cell apoptosis is increased and higher levels of soluble FasL in the peritoneal fluid of women with endometriosis may contribute to increased apoptosis of Fas‐bearing immune cells in the peritoneal cavity, leading to their decreased scavenger activity **[179]**.FasL produced by macrophages can play a vital role in the immune surveillance of the endometriotic tissue. Moreover, it is quite important that this upregulative effect of FasL expression in macrophages is regulated by CRH and UCN, strengthening further the regulatory role of these neuropeptides to FasL expression. Furthermore, it is of quite notice that CSF-1, this macrophage stimulatory factor upregulates FasL expression as it is in accordance with other previous studies suggesting that macrophage‐conditioned media containing PDGF and TGF‐β, which are macrophage‐derived growth factors like CSF-1 and they are elevated in women with endometriosis like CSF-1, induced FasL expression by endometrial stromal cells, suggesting that peritoneal macrophages in endometriosis might stimulate a Fas‐mediated apoptosis of immune cells. Taking into account all these, it could be hypothesized a very important role of FasL and its regulation by these specific neuropeptides and this macrophage factor in endometriosis participating in the persistence and immunosurveillance of the disease.

10.11. Role of CRHR1 in upregulative effect of CRH in galectin-1 expression, CSF-1 expression and FasL expression in Ishikawa cell line and macrophages

As it is shown in this current study, CRH upregulates galectin-1, CSF-1 and FasL expression in Ishikawa cell line and peripheral macrophages and this effect was reduced or omitted when antalarmin was added, indicating that this upregulative effect of CRH was mediated by CRHR1. So, taking everything above into consideration, it could be proposed that Antalarmin could be used for therapeutic interventions in order to regulate galectin-1, CSF-1 and FasL expression in endometrium and macrophages leading to a more balanced immune response concerning proper endometrial function and immunology of endometriosis.

CONCLUSIONS

CHAPTER 11

CONCLUSIONS

In conclusion, this current study shows for the first time that not only CRH and UCN but also CRHR1 and CRHR2 are expressed in endometriotic lesions and that CRH, UCN, CRHR1 and CRHR2 are significantly more abundant in endometriotic lesions than the corresponding eutopic endometrium of endometriotic women. It was also shown that these receptors are more highly expressed in eutopic endometrium of endometriotic women compared to healthy individuals. These findings point to a new inflammatory modulator pathway in which CRH, UCN, CRHR1 and CRHR2 are involved acting by an autocrine/paracrine pathway in eutopic and ectopic endometrium potentially affecting the pathogenesis of this benign disease and infertility profile of endometriotic women. These results suggest that a new therapeutic intervention could potentially based on blockage of CRH, UCN and their receptors leading to the improvement of the quality of endometriotic women's life. Furthermore, it is the first time that galectin-1 has been shown to be expressed in endometriotic tissue. Galectin-1 showed an abundant expression in ectopic endometrium rather than in eutopic endometrium of endometriotic women suggesting that this protein could play a vital role in the pathogenesis of the disease. CRH, UCN and CSF-1 have been found to upregulate galectin-1, CSF-1 and FasL expression in Ishikawa cell line and macrophages. As all the peptides causing these upregulative effects and the molecules that are regulated by them are implicated in inflammatory procedures such as endometriosis and in reproductive functions, these results could possibly set new light to the immune disequilibrium of endometriosis and the infertility profile of endometriotic women. Finally, based on these results, showing that the CRH upregulative effect in galectin-1, CSF-1 and FasL is mediated by CRHR1, the potential use of Antalarmin could be reinforced in accessing the immune disequilibrium noticed in eutopic and ectopic endometrium of women with endometriosis.

Future perspectives

- Further mechanistic experiments and experiments on appropriate models needs to be done in order to clarify the role of CRH, UCN and their receptors' in endometriosis and highlight a potential use of anti-CRHR1 and anti-CRHR2 treatment in endometriosis

- Further mechanistic experiments would be appropriate as to further clarify the role of galectin-1, CSF-1 and FasL in endometriosis, eutopic and ectopic endometrium

- The use of astressin as an inhibitor of CRHR2 could be used in order to examine if the upregulative effect of UCN in galectin-1, CSF-1 and FasL is mediated by CRHR2.

- Further mechanistic and excessive experiments could be done concerning the important role of galectin-1, CSF-1 and FasL in more cancer cell lines and tumour macrophages.

REFERENCES

CHAPTER 12

References

1. Palmer R, Thiery G (1950) [Endometrial biopsy]. Ann Endocrinol (Paris) 11: 183-186.
2. Buckley CH, H.Fox (1989) biopsy pathology of the endometrium. In: Gottlieb LS, a.m.neville, r.walker, editors. biopsy pathology series: chapman& hall medica.
3. Murphy CR (2000) The plasma membrane transformation of uterine epithelial cells during pregnancy. J Reprod Fertil Suppl 55: 23-28.
4. de Ziegler D, Fanchin R, de Moustier B, Bulletti C (1998) The hormonal control of endometrial receptivity: estrogen (E2) and progesterone. J Reprod Immunol 39: 149-166.
5. Castro-Rendon WA, Castro-Alvarez JF, Guzman-Martinez C, Bueno-Sanchez JC (2006) Blastocyst-endometrium interaction: intertwining a cytokine network. Braz J Med Biol Res 39: 1373-1385.
6. Maruyama T, Yoshimura Y (2008) Molecular and cellular mechanisms for differentiation and regeneration of the uterine endometrium. Endocr J 55: 795-810.
7. Dunn CL, Kelly RW, Critchley HO (2003) Decidualization of the human endometrial stromal cell: an enigmatic transformation. Reprod Biomed Online 7: 151-161.
8. Maruyama T, Yoshimura Y, Sabe H (1999) Tyrosine phosphorylation and subcellular localization of focal adhesion proteins during in vitro decidualization of human endometrial stromal cells. Endocrinology 140: 5982-5990.
9. Maruyama T, Yoshimura Y, Yodoi J, Sabe H (1999) Activation of c-Src kinase is associated with in vitro decidualization of human endometrial stromal cells. Endocrinology 140: 2632-2636.
10. Guzeloglu A, Michel F, Thatcher WW (2004) Differential effects of interferon-tau on the prostaglandin synthetic pathway in bovine endometrial cells treated with phorbol ester. J Dairy Sci 87: 2032-2041.
11. Makrigiannakis A, Minas V, Kalantaridou SN, Nikas G, Chrousos GP (2006) Hormonal and cytokine regulation of early implantation. Trends Endocrinol Metab 17: 178-185.
12. Cavagna M, Mantese JC (2003) Biomarkers of endometrial receptivity--a review. Placenta 24 Suppl B: S39-47.
13. Psychoyos A (1986) Uterine receptivity for nidation. Ann N Y Acad Sci 476: 36-42.
14. Norwitz ER, Schust DJ, Fisher SJ (2001) Implantation and the survival of early pregnancy. N Engl J Med 345: 1400-1408.
15. Ma WG, Song H, Das SK, Paria BC, Dey SK (2003) Estrogen is a critical determinant that specifies the duration of the window of uterine receptivity for implantation. Proc Natl Acad Sci U S A 100: 2963-2968.
16. Makrigiannakis A, Zoumakis E, Margioris AN, Stournaras C, Chrousos GP, et al. (1996) Regulation of the promoter of the human corticotropin-releasing hormone gene in transfected human endometrial cells. Neuroendocrinology 64: 85-92.
17. Makrigiannakis A, Margioris AN, Chatzaki E, Zoumakis E, Chrousos GP, et al. (1999) The decidualizing effect of progesterone may involve direct transcriptional activation of corticotrophin-releasing hormone from human endometrial stromal cells. Mol Hum Reprod 5: 789-796.
18. Makrigiannakis A, Margioris AN, Zoumakis E, Stournaras C, Gravanis A (1999) The transcription of corticotropin-releasing hormone in human endometrial cells is regulated by cytokines. Neuroendocrinology 70: 451-459.
19. Zoumakis E, Margioris AN, Stournaras C, Dermitzaki E, Angelakis E, et al. (2000) Corticotrophin-releasing hormone (CRH) interacts with inflammatory prostaglandins and interleukins and affects the decidualization of human endometrial stroma. Mol Hum Reprod 6: 344-351.
20. Makrigiannakis A, Zoumakis E, Kalantaridou S, Coutifaris C, Margioris AN, et al. (2001) Corticotropin-releasing hormone promotes blastocyst implantation and early maternal tolerance. Nat Immunol 2: 1018-1024.
21. Bamberger AM, Minas V, Kalantaridou SN, Radde J, Sadeghian H, et al. (2006) Corticotropin-releasing hormone modulates human trophoblast invasion through carcinoembryonic antigen-related cell adhesion molecule-1 regulation. Am J Pathol 168: 141-150.

22. Dey SK, Lim H, Das SK, Reese J, Paria BC, et al. (2004) Molecular cues to implantation. Endocr Rev 25: 341-373.
23. Minas V, Loutradis D, Makrigiannakis A (2005) Factors controlling blastocyst implantation. Reprod Biomed Online 10: 205-216.
24. Kliman HJ, Feinberg RF, Haimowitz JE (1990) Human trophoblast-endometrial interactions in an in vitro suspension culture system. Placenta 11: 349-367.
25. Dimitriadis E, White CA, Jones RL, Salamonsen LA (2005) Cytokines, chemokines and growth factors in endometrium related to implantation. Hum Reprod Update 11: 613-630.
26. Chen JR, Cheng JG, Shatzer T, Sewell L, Hernandez L, et al. (2000) Leukemia inhibitory factor can substitute for nidatory estrogen and is essential to inducing a receptive uterus for implantation but is not essential for subsequent embryogenesis. Endocrinology 141: 4365-4372.
27. Dimitriadis E, Robb L, Salamonsen LA (2002) Interleukin 11 advances progesterone-induced decidualization of human endometrial stromal cells. Mol Hum Reprod 8: 636-643.
28. von Rango U, Alfer J, Kertschanska S, Kemp B, Muller-Newen G, et al. (2004) Interleukin-11 expression: its significance in eutopic and ectopic human implantation. Mol Hum Reprod 10: 783-792.
29. Linjawi S, Li TC, Tuckerman EM, Blakemore AI, Laird SM (2004) Expression of interleukin-11 receptor alpha and interleukin-11 protein in the endometrium of normal fertile women and women with recurrent miscarriage. J Reprod Immunol 64: 145-155.
30. Bulun SE (2009) Endometriosis. N Engl J Med 360: 268-279.
31. Sampson JA (1927) Metastatic or Embolic Endometriosis, due to the Menstrual Dissemination of Endometrial Tissue into the Venous Circulation. Am J Pathol 3: 93-110 143.
32. Ferguson BR, Bennington JL, Haber SL (1969) Histochemistry of mucosubstances and histology of mixed mullerian pelvic lymph node glandular inclusions. Evidence for histogenesis by mullerian metaplasia of coelomic epithelium. Obstet Gynecol 33: 617-625.
33. Sasson IE, Taylor HS (2008) Stem cells and the pathogenesis of endometriosis. Ann N Y Acad Sci 1127: 106-115.
34. Laschke MW, Giebels C, Menger MD (2011) Vasculogenesis: a new piece of the endometriosis puzzle. Hum Reprod Update 17: 628-636.
35. Signorile PG, Baldi A (2010) Endometriosis: new concepts in the pathogenesis. Int J Biochem Cell Biol 42: 778-780.
36. Signorile PG, Baldi F, Bussani R, D'Armiento M, De Falco M, et al. (2009) Ectopic endometrium in human foetuses is a common event and sustains the theory of mullerianosis in the pathogenesis of endometriosis, a disease that predisposes to cancer. J Exp Clin Cancer Res 28: 49.
37. Houston DE (1984) Evidence for the risk of pelvic endometriosis by age, race and socioeconomic status. Epidemiol Rev 6: 167-191.
38. Giudice LC (2010) Clinical practice. Endometriosis. N Engl J Med 362: 2389-2398.
39. Anger DL, Foster WG (2008) The link between environmental toxicant exposure and endometriosis. Front Biosci 13: 1578-1593.
40. Rier SE, Martin DC, Bowman RE, Dmowski WP, Becker JL (1993) Endometriosis in rhesus monkeys (Macaca mulatta) following chronic exposure to 2,3,7,8-tetrachlorodibenzo-p-dioxin. Fundam Appl Toxicol 21: 433-441.
41. Evian Annual Reproduction Workshop G, Fauser BC, Diedrich K, Bouchard P, Dominguez F, et al. (2011) Contemporary genetic technologies and female reproduction. Hum Reprod Update 17: 829-847.
42. Falconer H, D'Hooghe T, Fried G (2007) Endometriosis and genetic polymorphisms. Obstet Gynecol Surv 62: 616-628.
43. Stratton P, Berkley KJ (2011) Chronic pelvic pain and endometriosis: translational evidence of the relationship and implications. Hum Reprod Update 17: 327-346.
44. (1997) Revised American Society for Reproductive Medicine classification of endometriosis: 1996. Fertil Steril 67: 817-821.
45. Tariverdian N, Theoharides TC, Siedentopf F, Gutierrez G, Jeschke U, et al. (2007) Neuroendocrine-immune disequilibrium and endometriosis: an interdisciplinary approach. Semin Immunopathol 29: 193-210.

46. Practice Committee of the American Society for Reproductive M (2006) Endometriosis and infertility. Fertil Steril 86: S156-160.
47. Kennedy S, Bergqvist A, Chapron C, D'Hooghe T, Dunselman G, et al. (2005) ESHRE guideline for the diagnosis and treatment of endometriosis. Hum Reprod 20: 2698-2704.
48. Kokcu A (2013) Possible effects of endometriosis-related immune events on reproductive function. Arch Gynecol Obstet.
49. Seli E, Arici A (2003) Endometriosis: interaction of immune and endocrine systems. Semin Reprod Med 21: 135-144.
50. Seli E, Berkkanoglu M, Arici A (2003) Pathogenesis of endometriosis. Obstet Gynecol Clin North Am 30: 41-61.
51. Akoum A, Lemay A, McColl S, Turcot-Lemay L, Maheux R (1996) Elevated concentration and biologic activity of monocyte chemotactic protein-1 in the peritoneal fluid of patients with endometriosis. Fertil Steril 66: 17-23.
52. D'Hooghe TM, Hill JA, Oosterlynck DJ, Koninckx PR, Bambra CS (1996) Effect of endometriosis on white blood cell subpopulations in peripheral blood and peritoneal fluid of baboons. Hum Reprod 11: 1736-1740.
53. Fukaya T, Sugawara J, Yoshida H, Yajima A (1994) The role of macrophage colony stimulating factor in the peritoneal fluid in infertile patients with endometriosis. Tohoku J Exp Med 172: 221-226.
54. Haney AF, Muscato JJ, Weinberg JB (1981) Peritoneal fluid cell populations in infertility patients. Fertil Steril 35: 696-698.
55. Bacci M, Capobianco A, Monno A, Cottone L, Di Puppo F, et al. (2009) Macrophages are alternatively activated in patients with endometriosis and required for growth and vascularization of lesions in a mouse model of disease. Am J Pathol 175: 547-556.
56. Nothnick WB (2001) Treating endometriosis as an autoimmune disease. Fertil Steril 76: 223-231.
57. Khan KN, Masuzaki H, Fujishita A, Kitajima M, Sekine I, et al. (2004) Differential macrophage infiltration in early and advanced endometriosis and adjacent peritoneum. Fertil Steril 81: 652-661.
58. Tran LV, Tokushige N, Berbic M, Markham R, Fraser IS (2009) Macrophages and nerve fibres in peritoneal endometriosis. Hum Reprod 24: 835-841.
59. Na YJ, Jin JO, Lee MS, Song MG, Lee KS, et al. (2008) Peritoneal fluid from endometriosis patients switches differentiation of monocytes from dendritic cells to macrophages. J Reprod Immunol 77: 63-74.
60. Lebovic DI, Mueller MD, Taylor RN (2001) Immunobiology of endometriosis. Fertil Steril 75: 1-10.
61. Harada T, Iwabe T, Terakawa N (2001) Role of cytokines in endometriosis. Fertil Steril 76: 1-10.
62. Tsudo T, Harada T, Iwabe T, Tanikawa M, Nagano Y, et al. (2000) Altered gene expression and secretion of interleukin-6 in stromal cells derived from endometriotic tissues. Fertil Steril 73: 205-211.
63. Gomez-Torres MJ, Acien P, Campos A, Velasco I (2002) Embryotoxicity of peritoneal fluid in women with endometriosis. Its relation with cytokines and lymphocyte populations. Hum Reprod 17: 777-781.
64. Kempuraj D, Papadopoulou N, Stanford EJ, Christodoulou S, Madhappan B, et al. (2004) Increased numbers of activated mast cells in endometriosis lesions positive for corticotropin-releasing hormone and urocortin. Am J Reprod Immunol 52: 267-275.
65. Anaf V, Chapron C, El Nakadi I, De Moor V, Simonart T, et al. (2006) Pain, mast cells, and nerves in peritoneal, ovarian, and deep infiltrating endometriosis. Fertil Steril 86: 1336-1343.
66. Cao WG, Morin M, Metz C, Maheux R, Akoum A (2005) Stimulation of macrophage migration inhibitory factor expression in endometrial stromal cells by interleukin 1, beta involving the nuclear transcription factor NFkappaB. Biol Reprod 73: 565-570.
67. Vercellini P, Trespidi L, Colombo A, Vendola N, Marchini M, et al. (1993) A gonadotropin-releasing hormone agonist versus a low-dose oral contraceptive for pelvic pain associated with endometriosis. Fertil Steril 60: 75-79.
68. Xue Q, Lin Z, Cheng YH, Huang CC, Marsh E, et al. (2007) Promoter methylation regulates estrogen receptor 2 in human endometrium and endometriosis. Biol Reprod 77: 681-687.
69. Fujishita A, Nakane PK, Koji T, Masuzaki H, Chavez RO, et al. (1997) Expression of estrogen and progesterone receptors in endometrium and peritoneal endometriosis: an immunohistochemical and in situ hybridization study. Fertil Steril 67: 856-864.

70. Wieser F, Schneeberger C, Tong D, Tempfer C, Huber JC, et al. (2002) PROGINS receptor gene polymorphism is associated with endometriosis. Fertil Steril 77: 309-312.
71. Attia GR, Zeitoun K, Edwards D, Johns A, Carr BR, et al. (2000) Progesterone receptor isoform A but not B is expressed in endometriosis. J Clin Endocrinol Metab 85: 2897-2902.
72. Fazleabas AT, Brudney A, Chai D, Langoi D, Bulun SE (2003) Steroid receptor and aromatase expression in baboon endometriotic lesions. Fertil Steril 80 Suppl 2: 820-827.
73. Fang Z, Yang S, Gurates B, Tamura M, Simpson E, et al. (2002) Genetic or enzymatic disruption of aromatase inhibits the growth of ectopic uterine tissue. J Clin Endocrinol Metab 87: 3460-3466.
74. Sakamoto Y, Harada T, Horie S, Iba Y, Taniguchi F, et al. (2003) Tumor necrosis factor-alpha-induced interleukin-8 (IL-8) expression in endometriotic stromal cells, probably through nuclear factor-kappa B activation: gonadotropin-releasing hormone agonist treatment reduced IL-8 expression. J Clin Endocrinol Metab 88: 730-735.
75. Vliagoftis H, Dimitriadou V, Boucher W, Rozniecki JJ, Correia I, et al. (1992) Estradiol augments while tamoxifen inhibits rat mast cell secretion. Int Arch Allergy Immunol 98: 398-409.
76. McLaren J, Prentice A, Charnock-Jones DS, Millican SA, Muller KH, et al. (1996) Vascular endothelial growth factor is produced by peritoneal fluid macrophages in endometriosis and is regulated by ovarian steroids. J Clin Invest 98: 482-489.
77. Blois SM, Joachim R, Kandil J, Margni R, Tometten M, et al. (2004) Depletion of CD8+ cells abolishes the pregnancy protective effect of progesterone substitution with dydrogesterone in mice by altering the Th1/Th2 cytokine profile. J Immunol 172: 5893-5899.
78. Joachim R, Zenclussen AC, Polgar B, Douglas AJ, Fest S, et al. (2003) The progesterone derivative dydrogesterone abrogates murine stress-triggered abortion by inducing a Th2 biased local immune response. Steroids 68: 931-940.
79. Orsal AS, Blois S, Labuz D, Peters EM, Schaefer M, et al. (2006) The progesterone derivative dydrogesterone down-regulates neurokinin 1 receptor expression on lymphocytes, induces a Th2 skew and exerts hypoalgesic effects in mice. J Mol Med (Berl) 84: 159-167.
80. Katsuki Y, Takano Y, Futamura Y, Shibutani Y, Aoki D, et al. (1998) Effects of dienogest, a synthetic steroid, on experimental endometriosis in rats. Eur J Endocrinol 138: 216-226.
81. Quereda F, Barroso J, Acien P (1996) Individual and combined effects of triptoreline and gestrinone on experimental endometriosis in rats. Eur J Obstet Gynecol Reprod Biol 67: 35-40.
82. Schor E, Baracat EC, Simoes MJ, de Freitas V, Giannotti Filho O, et al. (1999) Effects of conjugated estrogens and progestogen in surgically induced endometriosis in oophorectomized rats. Clin Exp Obstet Gynecol 26: 158-161.
83. Vercellini P, Fedele L, Pietropaolo G, Frontino G, Somigliana E, et al. (2003) Progestogens for endometriosis: forward to the past. Hum Reprod Update 9: 387-396.
84. Crosignani P, Olive D, Bergqvist A, Luciano A (2006) Advances in the management of endometriosis: an update for clinicians. Hum Reprod Update 12: 179-189.
85. Schweppe K-W, Rabe T, Langhardt M, Woziwodzki J, Petraglia F, et al. (2013) Endometriosis – Pathogenesis, Diagnosis, and Therapeutic Options for Clinical and Ambulatory Care. J Reproduktionsmed Endokrinol 2013 10.
86. Braun DP, Dmowski WP (1998) Endometriosis: abnormal endometrium and dysfunctional immune response. Curr Opin Obstet Gynecol 10: 365-369.
87. Meola J, Rosa e Silva JC, Dentillo DB, da Silva WA, Jr., Veiga-Castelli LC, et al. (2010) Differentially expressed genes in eutopic and ectopic endometrium of women with endometriosis. Fertil Steril 93: 1750-1773.
88. Sharpe-Timms KL (2001) Endometrial anomalies in women with endometriosis. Ann N Y Acad Sci 943: 131-147.
89. Stilley JA, Birt JA, Sharpe-Timms KL (2012) Cellular and molecular basis for endometriosis-associated infertility. Cell Tissue Res 349: 849-862.
90. Cahill DJ, Wardle PG, Maile LA, Harlow CR, Hull MG (1995) Pituitary-ovarian dysfunction as a cause for endometriosis-associated and unexplained infertility. Hum Reprod 10: 3142-3146.
91. Cheesman KL, Ben N, Chatterton RT, Jr., Cohen MR (1982) Relationship of luteinizing hormone, pregnanediol-3-glucuronide, and estriol-16-glucuronide in urine of infertile women with endometriosis. Fertil Steril 38: 542-548.

92. Bancroft K, Vaughan Williams CA, Elstein M (1992) Pituitary-ovarian function in women with minimal or mild endometriosis and otherwise unexplained infertility. Clin Endocrinol (Oxf) 36: 177-181.
93. Tummon IS, Maclin VM, Radwanska E, Binor Z, Dmowski WP (1988) Occult ovulatory dysfunction in women with minimal endometriosis or unexplained infertility. Fertil Steril 50: 716-720.
94. Williams CA, Oak MK, Elstein M (1986) Cyclical gonadotrophin and progesterone secretion in women with minimal endometriosis. Clin Reprod Fertil 4: 259-268.
95. Ronnberg L, Kauppila A, Rajaniemi H (1984) Luteinizing hormone receptor disorder in endometriosis. Fertil Steril 42: 64-68.
96. Cunha-Filho JS, Gross JL, Bastos de Souza CA, Lemos NA, Giugliani C, et al. (2003) Physiopathological aspects of corpus luteum defect in infertile patients with mild/minimal endometriosis. J Assist Reprod Genet 20: 117-121.
97. Cunha-Filho JS, Gross JL, Lemos NA, Brandelli A, Castillos M, et al. (2001) Hyperprolactinemia and luteal insufficiency in infertile patients with mild and minimal endometriosis. Horm Metab Res 33: 216-220.
98. Bergqvist A, D'Hooghe T (2002) Mini symposium on pathogenesis of endometriosis and treatment of endometriosis-associated subfertility. Introduction: the endometriosis enigma. Hum Reprod Update 8: 79-83.
99. Cahill DJ, Hull MG (2000) Pituitary-ovarian dysfunction and endometriosis. Hum Reprod Update 6: 56-66.
100. Nakahara K, Saito H, Saito T, Ito M, Ohta N, et al. (1997) The incidence of apoptotic bodies in membrana granulosa can predict prognosis of ova from patients participating in in vitro fertilization programs. Fertil Steril 68: 312-317.
101. Muscato JJ, Haney AF, Weinberg JB (1982) Sperm phagocytosis by human peritoneal macrophages: a possible cause of infertility in endometriosis. Am J Obstet Gynecol 144: 503-510.
102. Harada T, Yoshioka H, Yoshida S, Iwabe T, Onohara Y, et al. (1997) Increased interleukin-6 levels in peritoneal fluid of infertile patients with active endometriosis. Am J Obstet Gynecol 176: 593-597.
103. Yoshida S, Harada T, Iwabe T, Taniguchi F, Mitsunari M, et al. (2004) A combination of interleukin-6 and its soluble receptor impairs sperm motility: implications in infertility associated with endometriosis. Hum Reprod 19: 1821-1825.
104. Mansour G, Abdelrazik H, Sharma RK, Radwan E, Falcone T, et al. (2009) L-carnitine supplementation reduces oocyte cytoskeleton damage and embryo apoptosis induced by incubation in peritoneal fluid from patients with endometriosis. Fertil Steril 91: 2079-2086.
105. Iwabe T, Harada T, Terakawa N (2002) Role of cytokines in endometriosis-associated infertility. Gynecol Obstet Invest 53 Suppl 1: 19-25.
106. Jana SK, K NB, Chattopadhyay R, Chakravarty B, Chaudhury K (2010) Upper control limit of reactive oxygen species in follicular fluid beyond which viable embryo formation is not favorable. Reprod Toxicol 29: 447-451.
107. Aghajanova L, Hamilton AE, Giudice LC (2008) Uterine receptivity to human embryonic implantation: histology, biomarkers, and transcriptomics. Semin Cell Dev Biol 19: 204-211.
108. Donaghay M, Lessey BA (2007) Uterine receptivity: alterations associated with benign gynecological disease. Semin Reprod Med 25: 461-475.
109. Giudice LC, Telles TL, Lobo S, Kao L (2002) The molecular basis for implantation failure in endometriosis: on the road to discovery. Ann N Y Acad Sci 955: 252-264; discussion 293-255, 396-406.
110. Attar E, Bulun SE (2006) Aromatase and other steroidogenic genes in endometriosis: translational aspects. Hum Reprod Update 12: 49-56.
111. Bulun SE, Cheng YH, Yin P, Imir G, Utsunomiya H, et al. (2006) Progesterone resistance in endometriosis: link to failure to metabolize estradiol. Mol Cell Endocrinol 248: 94-103.
112. Giudice LC, Kao LC (2004) Endometriosis. Lancet 364: 1789-1799.
113. Wu Y, Strawn E, Basir Z, Halverson G, Guo SW (2006) Promoter hypermethylation of progesterone receptor isoform B (PR-B) in endometriosis. Epigenetics 1: 106-111.
114. Arici A, Oral E, Bukulmez O, Duleba A, Olive DL, et al. (1996) The effect of endometriosis on implantation: results from the Yale University in vitro fertilization and embryo transfer program. Fertil Steril 65: 603-607.

115. Bazer FW, Spencer TE, Johnson GA, Burghardt RC, Wu G (2009) Comparative aspects of implantation. Reproduction 138: 195-209.
116. Novembri R, Borges LE, Carrarelli P, Rocha AL, De Pascalis F, et al. (2011) Impaired CRH and urocortin expression and function in eutopic endometrium of women with endometriosis. J Clin Endocrinol Metab 96: 1145-1150.
117. Tomassetti C, Meuleman C, Pexsters A, Mihalyi A, Kyama C, et al. (2006) Endometriosis, recurrent miscarriage and implantation failure: is there an immunological link? Reprod Biomed Online 13: 58-64.
118. Mahutte NG, Arici A (2001) Endometriosis and assisted reproductive technologies: are outcomes affected? Curr Opin Obstet Gynecol 13: 275-279.
119. Chrousos GP, Gold PW (1992) The concepts of stress and stress system disorders. Overview of physical and behavioral homeostasis. JAMA 267: 1244-1252.
120. Chrousos GP (1992) Regulation and dysregulation of the hypothalamic-pituitary-adrenal axis. The corticotropin-releasing hormone perspective. Endocrinol Metab Clin North Am 21: 833-858.
121. Seong E, Seasholtz AF, Burmeister M (2002) Mouse models for psychiatric disorders. Trends Genet 18: 643-650.
122. Hillhouse EW, Grammatopoulos DK (2006) The molecular mechanisms underlying the regulation of the biological activity of corticotropin-releasing hormone receptors: implications for physiology and pathophysiology. Endocr Rev 27: 260-286.
123. Kalantaridou SN, Zoumakis E, Weil S, Lavasidis LG, Chrousos GP, et al. (2007) Reproductive corticotropin releasing hormone, implantation, and fetal immunotolerance. Crit Rev Clin Lab Sci 44: 461-481.
124. Zoumakis E, Kalantaridou SN, Makrigiannakis A (2009) CRH-like peptides in human reproduction. Curr Med Chem 16: 4230-4235.
125. Di Blasio AM, Pecori Giraldi F, Vigano P, Petraglia F, Vignali M, et al. (1997) Expression of corticotropin-releasing hormone and its R1 receptor in human endometrial stromal cells. J Clin Endocrinol Metab 82: 1594-1597.
126. Graziani G, Tentori L, Portarena I, Barbarino M, Tringali G, et al. (2002) CRH inhibits cell growth of human endometrial adenocarcinoma cells via CRH-receptor 1-mediated activation of cAMP-PKA pathway. Endocrinology 143: 807-813.
127. Gravanis A, Gurpide E (1986) Effects of estradiol on deoxyribonucleic acid polymerase alpha activity in the Ishikawa human endometrial adenocarcinoma cell line. J Clin Endocrinol Metab 63: 356-359.
128. Holinka CF, Hata H, Kuramoto H, Gurpide E (1986) Responses to estradiol in a human endometrial adenocarcinoma cell line (Ishikawa). J Steroid Biochem 24: 85-89.
129. Makrigiannakis A, Margioris A, Markogiannakis E, Stournaras C, Gravanis A (1992) Steroid hormones regulate the release of immunoreactive beta-endorphin from the Ishikawa human endometrial cell line. J Clin Endocrinol Metab 75: 584-589.
130. Florio P, Arcuri F, Ciarmela P, Runci Y, Romagnoli R, et al. (2002) Identification of urocortin mRNA and peptide in the human endometrium. J Endocrinol 173: R9-14.
131. Wetzka B, Sehringer B, Schafer WR, Biller S, Hor C, et al. (2003) Expression patterns of CRH, CRH receptors, and CRH binding protein in human gestational tissue at term. Exp Clin Endocrinol Diabetes 111: 154-161.
132. Chatzaki E, Margioris AN, Gravanis A (2002) Expression and regulation of corticotropin-releasing hormone binding protein (CRH-BP) in rat adrenals. J Neurochem 80: 81-90.
133. Iavazzo C, Baka S, Malamitsi-Puchner A (2009) The role of urocortin in gynecological and obstetrical conditions. Arch Gynecol Obstet 279: 613-619.
134. Donaldson CJ, Sutton SW, Perrin MH, Corrigan AZ, Lewis KA, et al. (1996) Cloning and characterization of human urocortin. Endocrinology 137: 3896.
135. Vaughan J, Donaldson C, Bittencourt J, Perrin MH, Lewis K, et al. (1995) Urocortin, a mammalian neuropeptide related to fish urotensin I and to corticotropin-releasing factor. Nature 378: 287-292.
136. Kehne JH, Cain CK (2010) Therapeutic utility of non-peptidic CRF1 receptor antagonists in anxiety, depression, and stress-related disorders: evidence from animal models. Pharmacol Ther 128: 460-487.
137. Broadbear JH, Winger G, Rice KC, Woods JH (2002) Antalarmin, a putative CRH-RI antagonist, has transient reinforcing effects in rhesus monkeys. Psychopharmacology (Berl) 164: 268-276.

138. Chatzaki E, Minas V, Zoumakis E, Makrigiannakis A (2006) CRF receptor antagonists: utility in research and clinical practice. Curr Med Chem 13: 2751-2760.
139. Webster EL, Lewis DB, Torpy DJ, Zachman EK, Rice KC, et al. (1996) In vivo and in vitro characterization of antalarmin, a nonpeptide corticotropin-releasing hormone (CRH) receptor antagonist: suppression of pituitary ACTH release and peripheral inflammation. Endocrinology 137: 5747-5750.
140. Deak T, Nguyen KT, Ehrlich AL, Watkins LR, Spencer RL, et al. (1999) The impact of the nonpeptide corticotropin-releasing hormone antagonist antalarmin on behavioral and endocrine responses to stress. Endocrinology 140: 79-86.
141. Nielsen DM, Carey GJ, Gold LH (2004) Antidepressant-like activity of corticotropin-releasing factor type-1 receptor antagonists in mice. Eur J Pharmacol 499: 135-146.
142. McCarthy JR, Heinrichs SC, Grigoriadis DE (1999) Recent advances with the CRF1 receptor: design of small molecule inhibitors, receptor subtypes and clinical indications. Curr Pharm Des 5: 289-315.
143. Webster EL, Barrientos RM, Contoreggi C, Isaac MG, Ligier S, et al. (2002) Corticotropin releasing hormone (CRH) antagonist attenuates adjuvant induced arthritis: role of CRH in peripheral inflammation. J Rheumatol 29: 1252-1261.
144. Gabry KE, Chrousos GP, Rice KC, Mostafa RM, Sternberg E, et al. (2002) Marked suppression of gastric ulcerogenesis and intestinal responses to stress by a novel class of drugs. Mol Psychiatry 7: 474-483, 433.
145. Greenwood-Van Meerveld B, Johnson AC, Cochrane S, Schulkin J, Myers DA (2005) Corticotropin-releasing factor 1 receptor-mediated mechanisms inhibit colonic hypersensitivity in rats. Neurogastroenterol Motil 17: 415-422.
146. Martinez V, Tache Y (2006) CRF1 receptors as a therapeutic target for irritable bowel syndrome. Curr Pharm Des 12: 4071-4088.
147. Kalantaridou SN, Makrigiannakis A, Zoumakis E, Chrousos GP (2004) Stress and the female reproductive system. J Reprod Immunol 62: 61-68.
148. Kalantaridou SN, Makrigiannakis A, Zoumakis E, Chrousos GP (2004) Reproductive functions of corticotropin-releasing hormone. Research and potential clinical utility of antalarmins (CRH receptor type 1 antagonists). Am J Reprod Immunol 51: 269-274.
149. Kalantaridou S, Makrigiannakis A, Zoumakis E, Chrousos GP (2007) Peripheral corticotropin-releasing hormone is produced in the immune and reproductive systems: actions, potential roles and clinical implications. Front Biosci 12: 572-580.
150. Kalantaridou SN, Zoumakis E, Makrigiannakis A, Godoy H, Chrousos GP (2007) The role of corticotropin-releasing hormone in blastocyst implantation and early fetal immunotolerance. Horm Metab Res 39: 474-477.
151. Florio P, Vale W, Petraglia F (2004) Urocortins in human reproduction. Peptides 25: 1751-1757.
152. Baigent SM (2001) Peripheral corticotropin-releasing hormone and urocortin in the control of the immune response. Peptides 22: 809-820.
153. Webster EL, Torpy DJ, Elenkov IJ, Chrousos GP (1998) Corticotropin-releasing hormone and inflammation. Ann N Y Acad Sci 840: 21-32.
154. Mastorakos G, Pavlatou MG, Mizamtsidi M (2006) The hypothalamic-pituitary-adrenal and the hypothalamic- pituitary-gonadal axes interplay. Pediatr Endocrinol Rev 3 Suppl 1: 172-181.
155. Theoharides TC, Singh LK, Boucher W, Pang X, Letourneau R, et al. (1998) Corticotropin-releasing hormone induces skin mast cell degranulation and increased vascular permeability, a possible explanation for its proinflammatory effects. Endocrinology 139: 403-413.
156. Chrousos GP, Torpy DJ, Gold PW (1998) Interactions between the hypothalamic-pituitary-adrenal axis and the female reproductive system: clinical implications. Ann Intern Med 129: 229-240.
157. Makrigiannakis A, Margioris AN, Le Goascogne C, Zoumakis E, Nikas G, et al. (1995) Corticotropin-releasing hormone (CRH) is expressed at the implantation sites of early pregnant rat uterus. Life Sci 57: 1869-1875.
158. Ferrari A, Petraglia F, Gurpide E (1995) Corticotropin releasing factor decidualizes human endometrial stromal cells in vitro. Interaction with progestin. J Steroid Biochem Mol Biol 54: 251-255.
159. Mastorakos G, Scopa CD, Kao LC, Vryonidou A, Friedman TC, et al. (1996) Presence of immunoreactive corticotropin-releasing hormone in human endometrium. J Clin Endocrinol Metab 81: 1046-1050.

160. Hillhouse EW, Grammatopoulos D, Milton NG, Quartero HW (1993) The identification of a human myometrial corticotropin-releasing hormone receptor that increases in affinity during pregnancy. J Clin Endocrinol Metab 76: 736-741.
161. Karalis K, Sano H, Redwine J, Listwak S, Wilder RL, et al. (1991) Autocrine or paracrine inflammatory actions of corticotropin-releasing hormone in vivo. Science 254: 421-423.
162. Makrigiannakis A, Psychoyos A, Zoumakis E, Margioris AN, Stournaras C, et al. (1997) Endometrial corticotropin-releasing hormone: expression, regulation, and potential physiological implications. Ann N Y Acad Sci 816: 116-128.
163. Frank GR, Brar AK, Jikihara H, Cedars MI, Handwerger S (1995) Interleukin-1 beta and the endometrium: an inhibitor of stromal cell differentiation and possible autoregulator of decidualization in humans. Biol Reprod 52: 184-191.
164. Gravanis A, Makrigiannakis A, Zoumakis E, Margioris AN (2001) Endometrial and myometrial corticotropin-releasing hormone (CRH): its regulation and possible roles. Peptides 22: 785-793.
165. Muglia L, Jacobson L, Dikkes P, Majzoub JA (1995) Corticotropin-releasing hormone deficiency reveals major fetal but not adult glucocorticoid need. Nature 373: 427-432.
166. McLean M, Bisits A, Davies J, Woods R, Lowry P, et al. (1995) A placental clock controlling the length of human pregnancy. Nat Med 1: 460-463.
167. Li W, Challis JR (2005) Corticotropin-releasing hormone and urocortin induce secretion of matrix metalloproteinase-9 (MMP-9) without change in tissue inhibitors of MMP-1 by cultured cells from human placenta and fetal membranes. J Clin Endocrinol Metab 90: 6569-6574.
168. Makrigiannakis A, Semmler M, Briese V, Eckerle H, Minas V, et al. (2007) Maternal serum corticotropin-releasing hormone and ACTH levels as predictive markers of premature labor. Int J Gynaecol Obstet 97: 115-119.
169. Minas V, Jeschke U, Kalantaridou SN, Richter DU, Reimer T, et al. (2007) Abortion is associated with increased expression of FasL in decidual leukocytes and apoptosis of extravillous trophoblasts: a role for CRH and urocortin. Mol Hum Reprod 13: 663-673.
170. Petraglia F, Tabanelli S, Galassi MC, Garuti GC, Mancini AC, et al. (1992) Human decidua and in vitro decidualized endometrial stromal cells at term contain immunoreactive corticotropin-releasing factor (CRF) and CRF messenger ribonucleic acid. J Clin Endocrinol Metab 74: 1427-1431.
171. Torricelli M, De Falco G, Florio P, Rossi M, Leucci E, et al. (2007) Secretory endometrium highly expresses urocortin messenger RNA and peptide: possible role in the decidualization process. Hum Reprod 22: 92-96.
172. Gravanis A, Margioris AN (2005) The corticotropin-releasing factor (CRF) family of neuropeptides in inflammation: potential therapeutic applications. Curr Med Chem 12: 1503-1512.
173. Florio P, Busacca M, Vignali M, Vigano P, Woods RJ, et al. (1998) Peritoneal fluid levels of immunoreactive corticotropin-releasing factor (CRF) and CRF-binding protein (CRF-BP) in healthy and endometriosic women. J Endocrinol Invest 21: 37-42.
174. Florio P, Reis FM, Torres PB, Calonaci F, Toti P, et al. (2007) Plasma urocortin levels in the diagnosis of ovarian endometriosis. Obstet Gynecol 110: 594-600.
175. Harada T, Kaponis A, Iwabe T, Taniguchi F, Makrydimas G, et al. (2004) Apoptosis in human endometrium and endometriosis. Hum Reprod Update 10: 29-38.
176. Andersen MH, Schrama D, Thor Straten P, Becker JC (2006) Cytotoxic T cells. J Invest Dermatol 126: 32-41.
177. Villa-Morales M, Fernandez-Piqueras J (2012) Targeting the Fas/FasL signaling pathway in cancer therapy. Expert Opin Ther Targets 16: 85-101.
178. Taliouri E, Vrekoussis T, Vergetaki A, Agorastos T, Makrigiannakis A (2013) Corticotropin-releasing hormone (CRH) is expressed in the human cervical carcinoma cells (HeLa) and upregulates the expression of Fas ligand. Tumour Biol 34: 125-130.
179. Garcia-Velasco JA, Arici A, Zreik T, Naftolin F, Mor G (1999) Macrophage derived growth factors modulate Fas ligand expression in cultured endometrial stromal cells: a role in endometriosis. Mol Hum Reprod 5: 642-650.
180. Song J, Rutherford T, Naftolin F, Brown S, Mor G (2002) Hormonal regulation of apoptosis and the Fas and Fas ligand system in human endometrial cells. Mol Hum Reprod 8: 447-455.
181. Yamashita H, Otsuki Y, Matsumoto K, Ueki K, Ueki M (1999) Fas ligand, Fas antigen and Bcl-2 expression in human endometrium during the menstrual cycle. Mol Hum Reprod 5: 358-364.

182. Otsuki Y (2001) Apoptosis in human endometrium: apoptotic detection methods and signaling. Med Electron Microsc 34: 166-173.
183. Watanabe H, Kanzaki H, Narukawa S, Inoue T, Katsuragawa H, et al. (1997) Bcl-2 and Fas expression in eutopic and ectopic human endometrium during the menstrual cycle in relation to endometrial cell apoptosis. Am J Obstet Gynecol 176: 360-368.
184. Selam B, Kayisli UA, Mulayim N, Arici A (2001) Regulation of Fas ligand expression by estradiol and progesterone in human endometrium. Biol Reprod 65: 979-985.
185. Tanaka M, Suda T, Haze K, Nakamura N, Sato K, et al. (1996) Fas ligand in human serum. Nat Med 2: 317-322.
186. Salamonsen LA, Woolley DE (1996) Matrix metalloproteinases in normal menstruation. Hum Reprod 11 Suppl 2: 124-133.
187. Gravanis A, Makrigiannakis A, Chatzaki E, Zoumakis E, Tsatsanis C, et al. (2002) Stress neuropeptides in the human endometrium: paracrine effects on cell differentiation and apoptosis. Hormones (Athens) 1: 139-148.
188. Petsas G, Jeschke U, Richter DU, Minas V, Hammer A, et al. (2012) Aberrant expression of corticotropin-releasing hormone in pre-eclampsia induces expression of FasL in maternal macrophages and extravillous trophoblast apoptosis. Mol Hum Reprod 18: 535-545.
189. Harada M, Suganuma N, Furuhashi M, Nagasaka T, Nakashima N, et al. (1996) Detection of apoptosis in human endometriotic tissues. Mol Hum Reprod 2: 307-315.
190. Das H, Koizumi T, Sugimoto T, Chakraborty S, Ichimura T, et al. (2000) Quantitation of Fas and Fas ligand gene expression in human ovarian, cervical and endometrial carcinomas using real-time quantitative RT-PCR. Br J Cancer 82: 1682-1688.
191. Fauvet R, Poncelet C, Hugol D, Lavaur A, Feldmann G, et al. (2003) Expression of apoptosis-related proteins in endometriomas and benign and malignant ovarian tumours. Virchows Arch 443: 38-43.
192. Powell WC, Fingleton B, Wilson CL, Boothby M, Matrisian LM (1999) The metalloproteinase matrilysin proteolytically generates active soluble Fas ligand and potentiates epithelial cell apoptosis. Curr Biol 9: 1441-1447.
193. Suda T, Okazaki T, Naito Y, Yokota T, Arai N, et al. (1995) Expression of the Fas ligand in cells of T cell lineage. J Immunol 154: 3806-3813.
194. Oosterlynck DJ, Meuleman C, Waer M, Koninckx PR (1994) Transforming growth factor-beta activity is increased in peritoneal fluid from women with endometriosis. Obstet Gynecol 83: 287-292.
195. Gearing AJ, Beckett P, Christodoulou M, Churchill M, Clements J, et al. (1994) Processing of tumour necrosis factor-alpha precursor by metalloproteinases. Nature 370: 555-557.
196. Kayagaki N, Kawasaki A, Ebata T, Ohmoto H, Ikeda S, et al. (1995) Metalloproteinase-mediated release of human Fas ligand. J Exp Med 182: 1777-1783.
197. Ueda M, Yamashita Y, Takehara M, Terai Y, Kumagai K, et al. (2002) Gene expression of adhesion molecules and matrix metalloproteinases in endometriosis. Gynecol Endocrinol 16: 391-402.
198. Selam B, Kayisli UA, Garcia-Velasco JA, Arici A (2002) Extracellular matrix-dependent regulation of Fas ligand expression in human endometrial stromal cells. Biol Reprod 66: 1-5.
199. Iwabe T, Harada T, Tsudo T, Nagano Y, Yoshida S, et al. (2000) Tumor necrosis factor-alpha promotes proliferation of endometriotic stromal cells by inducing interleukin-8 gene and protein expression. J Clin Endocrinol Metab 85: 824-829.
200. Iwabe T, Harada T, Tsudo T, Tanikawa M, Onohara Y, et al. (1998) Pathogenetic significance of increased levels of interleukin-8 in the peritoneal fluid of patients with endometriosis. Fertil Steril 69: 924-930.
201. Leuenroth S, Lee C, Grutkoski P, Keeping H, Simms HH (1998) Interleukin-8-induced suppression of polymorphonuclear leukocyte apoptosis is mediated by suppressing CD95 (Fas/Apo-1) Fas-1 interactions. Surgery 124: 409-417.
202. Kwak JY, Park SW, Kim KH, Na YJ, Lee KS (2002) Modulation of neutrophil apoptosis by plasma and peritoneal fluid from patients with advanced endometriosis. Hum Reprod 17: 595-600.
203. Cooper DN (2002) Galectinomics: finding themes in complexity. Biochim Biophys Acta 1572: 209-231.
204. Rabinovich GA, Baum LG, Tinari N, Paganelli R, Natoli C, et al. (2002) Galectins and their ligands: amplifiers, silencers or tuners of the inflammatory response? Trends Immunol 23: 313-320.

205. Rabinovich GA, Rubinstein N, Toscano MA (2002) Role of galectins in inflammatory and immunomodulatory processes. Biochim Biophys Acta 1572: 274-284.
206. Leffler H (2001) Galectins structure and function--a synopsis. Results Probl Cell Differ 33: 57-83.
207. Gruson D, Ko G (2012) Galectins testing: new promises for the diagnosis and risk stratification of chronic diseases? Clin Biochem 45: 719-726.
208. Toscano MA, Ilarregui JM, Bianco GA, Campagna L, Croci DO, et al. (2007) Dissecting the pathophysiologic role of endogenous lectins: glycan-binding proteins with cytokine-like activity? Cytokine Growth Factor Rev 18: 57-71.
209. Kabir-Salmani M, Fukuda MN, Kanai-Azuma M, Ahmed N, Shiokawa S, et al. (2008) The membrane-spanning domain of CD98 heavy chain promotes alpha(v)beta3 integrin signals in human extravillous trophoblasts. Mol Endocrinol 22: 707-715.
210. Rabinovich GA (1999) Galectins: an evolutionarily conserved family of animal lectins with multifunctional properties; a trip from the gene to clinical therapy. Cell Death Differ 6: 711-721.
211. Shimizu Y, Kabir-Salmani M, Azadbakht M, Sugihara K, Sakai K, et al. (2008) Expression and localization of galectin-9 in the human uterodome. Endocr J 55: 879-887.
212. von Wolff M, Wang X, Gabius HJ, Strowitzki T (2005) Galectin fingerprinting in human endometrium and decidua during the menstrual cycle and in early gestation. Mol Hum Reprod 11: 189-194.
213. Jeschke U, Mayr D, Schiessl B, Mylonas I, Schulze S, et al. (2007) Expression of galectin-1, -3 (gal-1, gal-3) and the Thomsen-Friedenreich (TF) antigen in normal, IUGR, preeclamptic and HELLP placentas. Placenta 28: 1165-1173.
214. Camby I, Le Mercier M, Lefranc F, Kiss R (2006) Galectin-1: a small protein with major functions. Glycobiology 16: 137R-157R.
215. Symons A, Cooper DN, Barclay AN (2000) Characterization of the interaction between galectin-1 and lymphocyte glycoproteins CD45 and Thy-1. Glycobiology 10: 559-563.
216. Wells V, Davies D, Mallucci L (1999) Cell cycle arrest and induction of apoptosis by beta galactoside binding protein (beta GBP) in human mammary cancer cells. A potential new approach to cancer control. Eur J Cancer 35: 978-983.
217. Harvey KJ, Lukovic D, Ucker DS (2001) Membrane-targeted green fluorescent protein reliably and uniquely marks cells through apoptotic death. Cytometry 43: 273-278.
218. Almkvist J, Karlsson A (2004) Galectins as inflammatory mediators. Glycoconj J 19: 575-581.
219. Paclik D, Werner L, Guckelberger O, Wiedenmann B, Sturm A (2011) Galectins distinctively regulate central monocyte and macrophage function. Cell Immunol 271: 97-103.
220. Rabinovich GA, Modesti NM, Castagna LF, Landa CA, Riera CM, et al. (1997) Specific inhibition of lymphocyte proliferation and induction of apoptosis by CLL-I, a beta-galactoside-binding lectin. J Biochem 122: 365-373.
221. He J, Baum LG (2004) Presentation of galectin-1 by extracellular matrix triggers T cell death. J Biol Chem 279: 4705-4712.
222. Gabius HJ (2001) Probing the cons and pros of lectin-induced immunomodulation: case studies for the mistletoe lectin and galectin-1. Biochimie 83: 659-666.
223. Baum LG, Blackall DP, Arias-Magallano S, Nanigian D, Uh SY, et al. (2003) Amelioration of graft versus host disease by galectin-1. Clin Immunol 109: 295-307.
224. Egnaczyk GF, Pomonis JD, Schmidt JA, Rogers SD, Peters C, et al. (2003) Proteomic analysis of the reactive phenotype of astrocytes following endothelin-1 exposure. Proteomics 3: 689-698.
225. Rabinovich GA, Toscano MA (2009) Turning 'sweet' on immunity: galectin-glycan interactions in immune tolerance and inflammation. Nat Rev Immunol 9: 338-352.
226. Rabinovich GA (2005) Galectin-1 as a potential cancer target. Br J Cancer 92: 1188-1192.
227. Than NG, Erez O, Wildman DE, Tarca AL, Edwin SS, et al. (2008) Severe preeclampsia is characterized by increased placental expression of galectin-1. J Matern Fetal Neonatal Med 21: 429-442.
228. Froehlich R, Hambruch N, Haeger JD, Dilly M, Kaltner H, et al. (2012) Galectin fingerprinting detects differences in expression profiles between bovine endometrium and placentomes as well as early and late gestational stages. Placenta 33: 195-201.
229. Jeschke U, Reimer T, Bergemann C, Wiest I, Schulze S, et al. (2004) Binding of galectin-1 (gal-1) on trophoblast cells and inhibition of hormone production of trophoblast tumor cells in vitro by gal-1. Histochem Cell Biol 121: 501-508.

230. Walzel H, Brock J, Pohland R, Vanselow J, Tomek W, et al. (2004) Effects of galectin-1 on regulation of progesterone production in granulosa cells from pig ovaries in vitro. Glycobiology 14: 871-881.
231. Kolundzic N, Bojic-Trbojevic Z, Kovacevic T, Stefanoska I, Kadoya T, et al. (2011) Galectin-1 is part of human trophoblast invasion machinery--a functional study in vitro. PLoS One 6: e28514.
232. Choe YS, Shim C, Choi D, Lee CS, Lee KK, et al. (1997) Expression of galectin-1 mRNA in the mouse uterus is under the control of ovarian steroids during blastocyst implantation. Mol Reprod Dev 48: 261-266.
233. Jeschke U, Toth B, Scholz C, Friese K, Makrigiannakis A (2010) Glycoprotein and carbohydrate binding protein expression in the placenta in early pregnancy loss. J Reprod Immunol 85: 99-105.
234. Tirado-Gonzalez I, Freitag N, Barrientos G, Shaikly V, Nagaeva O, et al. (2013) Galectin-1 influences trophoblast immune evasion and emerges as a predictive factor for the outcome of pregnancy. Mol Hum Reprod 19: 43-53.
235. Than NG, Abdul Rahman O, Magenheim R, Nagy B, Fule T, et al. (2008) Placental protein 13 (galectin-13) has decreased placental expression but increased shedding and maternal serum concentrations in patients presenting with preterm pre-eclampsia and HELLP syndrome. Virchows Arch 453: 387-400.
236. Noel JC, Chapron C, Borghese B, Fayt I, Anaf V (2011) Galectin-3 is overexpressed in various forms of endometriosis. Appl Immunohistochem Mol Morphol 19: 253-257.
237. Stanley E, Lieschke GJ, Grail D, Metcalf D, Hodgson G, et al. (1994) Granulocyte/macrophage colony-stimulating factor-deficient mice show no major perturbation of hematopoiesis but develop a characteristic pulmonary pathology. Proc Natl Acad Sci U S A 91: 5592-5596.
238. Stanley ER, Berg KL, Einstein DB, Lee PS, Yeung YG (1994) The biology and action of colony stimulating factor-1. Stem Cells 12 Suppl 1: 15-24; discussion 25.
239. Stanley ER, Berg KL, Einstein DB, Lee PS, Pixley FJ, et al. (1997) Biology and action of colony--stimulating factor-1. Mol Reprod Dev 46: 4-10.
240. Rickard AJ, Young MJ (2009) Corticosteroid receptors, macrophages and cardiovascular disease. J Mol Endocrinol 42: 449-459.
241. Naito M, Umeda S, Takahashi K, Shultz LD (1997) Macrophage differentiation and granulomatous inflammation in osteopetrotic mice (op/op) defective in the production of CSF-1. Mol Reprod Dev 46: 85-91.
242. Roussos ET, Condeelis JS, Patsialou A (2011) Chemotaxis in cancer. Nat Rev Cancer 11: 573-587.
243. Pollard JW (2004) Tumour-educated macrophages promote tumour progression and metastasis. Nat Rev Cancer 4: 71-78.
244. Aligeti S, Kirma NB, Binkley PA, Schenken RS, Tekmal RR (2011) Colony-stimulating factor-1 exerts direct effects on the proliferation and invasiveness of endometrial epithelial cells. Fertil Steril 95: 2464-2466.
245. Lee JY, Lee M, Lee SK (2011) Role of endometrial immune cells in implantation. Clin Exp Reprod Med 38: 119-125.
246. Pollard JW (2008) Macrophages define the invasive microenvironment in breast cancer. J Leukoc Biol 84: 623-630.
247. Pollard JW, Hunt JS, Wiktor-Jedrzejczak W, Stanley ER (1991) A pregnancy defect in the osteopetrotic (op/op) mouse demonstrates the requirement for CSF-1 in female fertility. Dev Biol 148: 273-283.
248. Bhatnagar P, Papaioannou VE, Biggers JD (1995) CSF-1 and mouse preimplantation development in vitro. Development 121: 1333-1339.
249. Kauma SW, Aukerman SL, Eierman D, Turner T (1991) Colony-stimulating factor-1 and c-fms expression in human endometrial tissues and placenta during the menstrual cycle and early pregnancy. J Clin Endocrinol Metab 73: 746-751.
250. Katano K, Matsumoto Y, Ogasawara M, Aoyama T, Ozaki Y, et al. (1997) Low serum M-CSF levels are associated with unexplained recurrent abortion. Am J Reprod Immunol 38: 1-5.
251. Oral E, Arici A (1996) Peritoneal growth factors and endometriosis. Semin Reprod Endocrinol 14: 257-267.
252. Sukhikh GT, Sotnikova NY, Antsiferova YS, Posiseeva LV, Veryasov VN, et al. (2004) Cytokine production by immunocompetent cells of peritoneal fluid in women with external genital endometriosis. Bull Exp Biol Med 137: 568-571.

253. Budrys NM, Nair HB, Liu YG, Kirma NB, Binkley PA, et al. (2012) Increased expression of macrophage colony-stimulating factor and its receptor in patients with endometriosis. Fertil Steril 97: 1129-1135 e1121.
254. Mettler L, Schmutzler AG, Koch K, Schollmeyer T, Salmassi A (2004) Identification of the M-CSF receptor in endometriosis by immunohistochemistry and RT-PCR. Am J Reprod Immunol 52: 298-305.
255. Jensen JR, Witz CA, Schenken RS, Tekmal RR (2010) A potential role for colony-stimulating factor 1 in the genesis of the early endometriotic lesion. Fertil Steril 93: 251-256.
256. Nishida M (2002) The Ishikawa cells from birth to the present. Hum Cell 15: 104-117.
257. Stanley ER (1985) The macrophage colony-stimulating factor, CSF-1. Methods Enzymol 116: 564-587.
258. Makrigiannakis A, Zoumakis E, Margioris AN, Theodoropoulos P, Stournaras C, et al. (1995) The corticotropin-releasing hormone (CRH) in normal and tumoral epithelial cells of human endometrium. J Clin Endocrinol Metab 80: 185-189.
259. Tariverdian N, Rucke M, Szekeres-Bartho J, Blois SM, Karpf EF, et al. (2010) Neuroendocrine circuitry and endometriosis: progesterone derivative dampens corticotropin-releasing hormone-induced inflammation by peritoneal cells in vitro. J Mol Med (Berl) 88: 267-278.
260. Garcia-Velasco JA, Mulayim N, Kayisli UA, Arici A (2002) Elevated soluble Fas ligand levels may suggest a role for apoptosis in women with endometriosis. Fertil Steril 78: 855-859.
261. Minici F, Tiberi F, Tropea A, Miceli F, Orlando M, et al. (2007) Paracrine regulation of endometriotic tissue. Gynecol Endocrinol 23: 574-580.
262. Pozzoli G, Bilezikjian LM, Perrin MH, Blount AL, Vale WW (1996) Corticotropin-releasing factor (CRF) and glucocorticoids modulate the expression of type 1 CRF receptor messenger ribonucleic acid in rat anterior pituitary cell cultures. Endocrinology 137: 65-71.
263. Karteris E, Goumenou A, Koumantakis E, Hillhouse EW, Grammatopoulos DK (2003) Reduced expression of corticotropin-releasing hormone receptor type-1 alpha in human preeclamptic and growth-restricted placentas. J Clin Endocrinol Metab 88: 363-370.
264. Athanassakis I, Farmakiotis V, Aifantis I, Gravanis A, Vassiliadis S (1999) Expression of corticotrophin-releasing hormone in the mouse uterus: participation in embryo implantation. J Endocrinol 163: 221-227.
265. Lima AP, Moura MD, Rosa e Silva AA (2006) Prolactin and cortisol levels in women with endometriosis. Braz J Med Biol Res 39: 1121-1127.
266. Chrousos GP (1999) Reproductive placental corticotropin-releasing hormone and its clinical implications. Am J Obstet Gynecol 180: S249-250.
267. Hata H, Kuramoto H (1992) Immunocytochemical determination of estrogen and progesterone receptors in human endometrial adenocarcinoma cells (Ishikawa cells). J Steroid Biochem Mol Biol 42: 201-210.
268. Lessey BA, Ilesanmi AO, Castelbaum AJ, Yuan L, Somkuti SG, et al. (1996) Characterization of the functional progesterone receptor in an endometrial adenocarcinoma cell line (Ishikawa): progesterone-induced expression of the alpha1 integrin. J Steroid Biochem Mol Biol 59: 31-39.
269. Croxtall JD, Pollard JW, Carey F, Forder RA, White JO (1992) Colony stimulating factor-1 stimulates Ishikawa cell proliferation and lipocortin II synthesis. J Steroid Biochem Mol Biol 42: 121-129.
270. Takeda S, Soutter WP, Dibb NJ, White JO (1996) Biological activity of the receptor for macrophage colony-stimulating factor in the human endometrial cancer cell line, Ishikawa. Br J Cancer 73: 615-619.
271. Jeschke U, Walzel H, Mylonas I, Papadopoulos P, Shabani N, et al. (2009) The human endometrium expresses the glycoprotein mucin-1 and shows positive correlation for Thomsen-Friedenreich epitope expression and galectin-1 binding. J Histochem Cytochem 57: 871-881.
272. Sango K, Tokashiki A, Ajiki K, Horie M, Kawano H, et al. (2004) Synthesis, localization and externalization of galectin-1 in mature dorsal root ganglion neurons and Schwann cells. Eur J Neurosci 19: 55-64.

Internet Links

- (http://www.yalemedicalgroup.org/stw/Page.asp?PageID=STW022188)
- (http://www.nelsonginecologia.med.br/localmechmenstr_engl.htm)
- http://quizlet.com/6841533/familiarize/embedv2?&m
- (http://www.yalemedicalgroup.org/stw/Page.asp?PageID=STW029029)
- (http://dentistryandmedicine.blogspot.gr/2011/07/menstrual-cycle-gynecology-lecture.html)
- http://www.rockymountainfertility.com/diagnosis/endometriosis/#
- http://www.glycoforum.gr.jp/science/word/lectin/LEA01E.html