# Estrogen receptors and inflammation in colorectal cancer. Role in prognosis and therapeutic opportunities.

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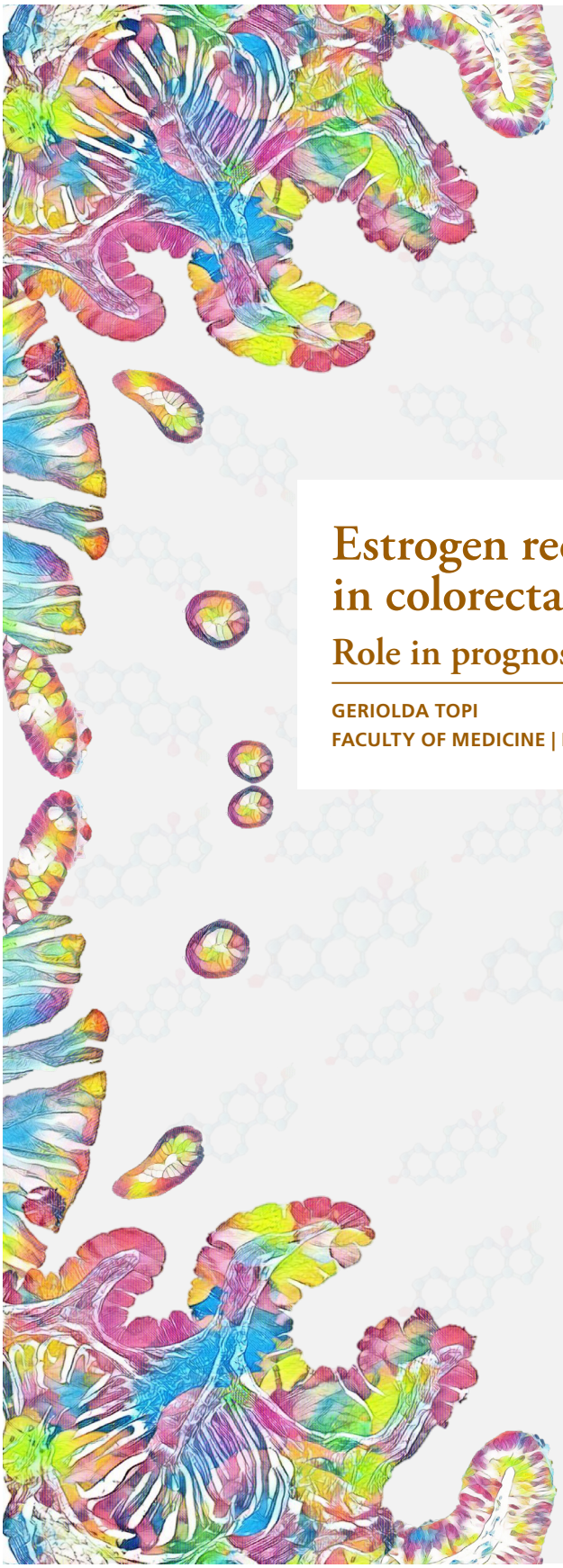

# Estrogen receptors and inflammation in colorectal cancer

## Role in prognosis and therapeutic opportunities

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**Geriolda Topi** graduated as medical doctor in Tirana, Albania (2012); defended master thesis in Clinical Epidemiology in Rotterdam, The Netherlands (2013) and worked as a physician and lecturer at the Medical Faculty in Tirana until she moved to Malmö, Sweden to conduct her PhD studies at the Department of Translational Medicine, Lund University, Skåne University Hospital. The main focus of her research work is to investigate the role of estrogen receptors as prognostic markers and therapeutic opportunities in patients with colorectal cancer.


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# Estrogen receptors and inflammation in colorectal cancer

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Geriolda Topi

LUND UNIVERSITY

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**Abstract:** •
Colorectal cancer (CRC) is the third most common cause of cancer-related deaths globally. Inflammation, pro-inflammatory mediators and different immune cells have been linked with CRC development and progression. Mast cells (MCs), among the earliest immune cells recruited during tumorigenesis, have a controversial role in CRC.

On the other hand, gender is associated with CRC risk, progression and localization. For the same age, men have a higher incidence of CRC than women. Right-sided colon cancer is more common in women and rectal cancer more common in men. Furthermore, the use of hormone replacement therapy (HRT) is associated with a lower risk for CRC among women. All these findings implicate a prognostic role of estrogen signaling in CRC.

The overall aim of this research work was to investigate the prognostic role of estrogen receptors, ERα and ERβ, in CRC, both as predictive markers for patient's survival and as possible target therapies. An additional aim was to explore the role of mast cells density (MCD) in patients with CRC. We investigated the role of ERα and ERβ in normal and cancer patient's tissues, colon cancer cell lines, three different mouse models of colon cancer and an *in-vivo* zebrafish xenograft metastasis model; whereas the role of MCs was investigated in normal and cancer patient's tissues and in a colitis-associated colon cancer (CAC) mouse model with a *Cysltr1* gene disruption.

There was a significant decrease of ERβ and an increase of ERα in the cancerous tissues compared with the normal colonic mucosa in CRC patients. High ERβ expression was associated with better prognosis and reduced risk of cancer recurrence in CRC patients, while the opposite pattern was observed for patients with positive ERα expression. High ERβ expression in CRC tissue was associated with shorter breastfeeding time and long-term use of HRT, but not with life style indicators.
In addition, treatment of colon cancer cells with the ERβ-selective agonist ERB-041, or ERα-selective antagonist AZD9496 in addition to ERα induction or in combination with ERB-041 increased the expression of antitumorigenic proteins, increased the phosphorylation of β-catenin and decreased its translocation into the nucleus, and reduced the metastatic burden in the *in-vivo* zebrafish xenograft model. Furthermore, concomitant expression of ERβ and ERα improved the predicting ability for the risk of cancer recurrence, when added to the standard model adjusted for age and TNM stage, compared with other models where only one of the estrogen receptors was taken into consideration. Finally, high MCD in the cancer tissue was independently associated with longer overall survival in CRC patients. All these findings were confirmed using publicly available databases with mRNA data for CRC patients and in mouse models of colon cancer.

If these findings would be further validated, they could be beneficial to create new treatment opportunities and combine treatment therapies, with the aim to improve the prognosis of colorectal cancer patients towards a more individualized treatment.



**Key words:** colorectal cancer, estrogen receptor beta, estrogen receptor alpha, mast cells, prognosis, overall survival, disease-free survival, zebrafish, cysteinyl leukotriene receptors, metastasis, ERB-041, PPT, AZD9496

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*To my parents, my husband and my daughter Aria*
*and all who believed in me.*

*Dedikuar prindërve, bashkëshortit dhe vajzës time Aria*
*dhe gjithë atyre që besuan tek unë.*

# Table of Contents

# List of papers

*This thesis is based on the following papers, which will be referred to in the thesis by their Roman numerals I-V*:

I. Association of the estrogen receptor beta with hormone status and prognosis in a cohort of female patients with colorectal cancer. **Topi G**, Ehrnström R, Jirström K, Palmquist I, Lydrup ML, Sjölander A. Eur J Cancer. 2017 Sep;83:279-289.

II. Tumor-suppressive effect of estrogen receptor β in colorectal cancer patients, colon cancer cells and a zebrafish model. **Topi G**, Satapathy SR, Dash P, Mehrabi SF, Olsson R, Ehrnström R, Lydrup ML, Sjölander A. Accepted. Journal of Pathology. DOI: 10.1002/path.5453

III. Positive expression of estrogen receptor alpha correlates with poor prognosis and metastasis in colorectal cancer. **Topi G**, Satapathy SR, Lindö C, Ehrnström R, Lydrup ML, Sjölander A. Manuscript.

IV. Prognostic relevance of concomitant expression of ERβ and ERα in female colorectal cancer patients. **Topi G**, Ehrnström R, Lydrup ML, Sjölander A. Manuscript.

V. High tumor mast cell density is associated with longer survival of colon cancer patients. Mehdawi L, Osman J, **Topi G**, Sjölander A. Acta Oncol. 2016 Dec;55(12):1434-1442.

The complete papers have been reproduced with the kind permission of the publishers and are enclosed at the end of the thesis.

# Abbreviations

| | |
|---|---|
| AF-1/2 | Activation function-1/2 |
| AOM | Azoxymethane |
| AP1 | Activator protein 1 |
| APC | Adenomatous polyposis coli |
| AUC | Area under the curve |
| CAC | Colitis associated cancer |
| CC | Colon cancer |
| CIN | Chromosomal instability |
| CK1 | Casein kinase 1 α |
| COX-2 | Cyclooxygenase - 2 |
| CRC | Colorectal cancer |
| CysLTR1 | Cysteinyl leukotriene receptor 1 |
| CysLTR2 | Cysteinyl leukotriene receptor 2 |
| DFS | Disease-free survival |
| DSS | Dextran Sulfate Sodium |
| EGFR | Epidermal growth factor receptor |
| ERE | Estrogen response element |
| ERα | Estrogen receptor alpha |
| ERβ | Estrogen receptor beta |
| FAP | Familial adenomatous polyposis |
| GDP | Guanosine diphosphate |
| GPER | G-protein coupled estrogen receptor 1 |
| GSK-3β | Glycogen synthase kinase 3β |
| HC | Hormonal contraception |

| | |
|---|---|
| HNPCC | Heriditary non-polyposis colon cancer |
| HRT | Hormone replacement therapy |
| IBD | Inflammatory bowel disease |
| IECs | Intestinal epithelial cells |
| IGFR1 | Insulin-like growth factor receptor 1 |
| IHC | Immunohistochemistry |
| IRS | Immuno-reactive score |
| MAPK | Mitogen activated protein kinase |
| MCs / MCD | Mast cells /Mast cell density |
| MSI | Microsatellite instability |
| MSS | Microsatellite stable |
| OS | Overall survival |
| PI3K | Phosphoinositide 3-kinase |
| PLA2 | Phospholipase A2 |
| SP1 | Stimulating protein 1 |
| TCF/LEF | T-cell factor/ Lymphoid enhancer factor |
| TGF-β | Transforming growth factor-β |
| TMA | Tissue microarray |
| TNM | Tumor-lymph node-metastasis |
| ZO-1 | Zonula occludens-1 (tight junction protein 1) |
| 15-PGDH | 15-hydroxy prostaglandin dehydrogenase |
| 5-LOX | 5 - Lipoxygenase |

# Definitions of terms

**Overall survival** - The length of time that a patient with cancer lived from the date of diagnosis until the date of death.

**Disease-free survival** - The length of time that a patient lived without any signs of cancer.

**KRAS, BRAF, SMAD4 -** Proto-oncogenes. Genes that in healthy physiological conditions are silent and when get activated, due to a mutation, lead to cancer formation.

**ERB-041** - ERβ -selective agonist; a component that activates in a selective way the estrogen receptor beta.

**PPT** - ERα-selective agonist; a component that activates in a selective way the estrogen receptor alpha.

**PHTPP** - ERβ -selective antagonist; a component that inhibits in a selective way the function of estrogen receptor beta.

**AZD9496** - ERα-selective antagonist; a component that inhibits in a selective way the function of estrogen receptor alpha.

**Zebrafish** - a freshwater fish that in its embryonal phase is transparent in color. It is commonly used in experimental research to investigate biological mechanism.

**Xenograft model** – the injection of human cells, for example cancer cells, into a non-human living organism, such as a mouse or a zebrafish.

**Prognostic markers** – a biological parameter or factor that provides information related to the likely course of a disease in a group of patients.

**Predicting ability** – the ability of a parameter or factor to identify a subgroup of patients who most likely will have a certain course of the disease or most likely will respond to a certain treatment.

# Summary

Cancer is defined as a malignant disease characterized by uncontrolled cell growth that leads to formation of abnormal (atypical) cells, different from their origin and capable to invade and migrate to other organs. Colorectal cancer (CRC) is a type of cancer that raises from the large intestine and is one of the most common malignant diseases worldwide. In Sweden, every year around 6 500 people are diagnosed with CRC. About 75% of these cases are over 65 years old with a 5-year survival of approximately 65%. CRC is the third most common cause of cancer-related deaths globally. Important risk factors for developing CRC are age above 50 years, genetic and hereditary factors, a daily diet rich in red meat and low in vegetables and fruits, obesity, and excessive consumption of alcohol and smoking.

In addition to these, gender plays an important role in CRC risk and prognosis. Women before menopause have a lower risk to develop CRC compared to men of the same age. Also, the survival after CRC surgery is better in young women than in your men, but the opposite is seen in older patients. All these findings implicate an important role of estrogen hormone in CRC development.

Estrogen is a dominant hormone in females but is also produced in males and plays important physiological roles in both genders. This hormone expressed its function in the body through specific proteins that are found inside the cell and are referred as receptors. There are two main estrogen receptors: alpha (ERα) and beta (ERβ). This thesis shows the implication of ERα and ERβ in CRC prognosis. In the colorectal cancer tissues of patients included in this research work, there was a significant decrease of ERβ and a significant increase of ERα compared to the normal colon tissues. Also, CRC patients with high levels of ERβ in their tumors had lower risk of death and cancer recurrence. In the contrary, CRC patients with high levels of ERα in their tumors had higher risk of death and cancer recurrence. These findings indicate that the expression of ERα and ERβ can be beneficial to predict the prognosis of CRC patients.

Despite the surgical treatment alone or in combination with chemotherapy, the majority of patients with CRC have a recurrence of the disease within the first three years. Also, current target therapies are beneficial for small groups of patients, based on the mutations and molecular profile of the cancer. This raises the need for new therapeutic treatments. Experimental work with colon cancer cell lines that is provided in this thesis, showed that the induction of ERβ and the blocking of ERα

reduced the proliferation, migration and metastasis of cancer cells. These results can have an important clinical relevance because they implicate the possible use of ERβ and ERα as target therapies in CRC in combination with current therapies. These findings open new treatment opportunities that can lead toward a more individualized treatment of CRC patients. Because of no difference in the levels of estrogen receptors in the colon cancer tissues between men and women, the results of this research work can be beneficial for all CRC regardless their gender.

Finally, the work of this thesis provides important information about the prognostic role of mast cells in CRC prognosis. The development of CRC occurs in close interaction with immune cells. Mast cells are among the earliest immune cells recruited at the tissue where the tumor develops. The research work included in this thesis book shows that patients with CRC that have high mast cells density in their tumor tissues have longer survival compared with patients that have a low mast cell density. This is an important finding that indicates the anti-tumor role of mast cells in colorectal cancer.

# Populärvetenskaplig sammanfattning (Summary in Swedish)

Cancer definieras som en allvarlig sjukdom som kännetecknas av okontrollerad celltillväxt som leder till bildning av onormala (atypiska) celler, som skiljer sig från dess ursprung. Dessa onormala/förändrade celler kan invadera och vandra till andra organ. Tjocktarmscancer och ändtarmscancer eller kolorektalcancer är en av de vanligaste maligna sjukdomar i Sverige. Varje år får ca 6 500 personer i Sverige denna cancersjukdom. Där ca 75% är över 65 år med en 5-årsöverlevnad på ca 65%. Kolorektalcancer är den tredje vanligaste orsaken till cancerrelaterade dödsfall i Sverige och globalt. Viktiga riskfaktorer för att utveckla tjocktarmscancer är ålder över 50 år, genetiska och ärftliga faktorer, en daglig diet rik på rött kött, låg fiberhalt och fetma, hög konsumtion av alkohol samt rökning.

Utöver dessa riskfaktorer spelar kön en viktig roll för risk och prognos av kolorektalcancer. Kvinnor före klimakteriet har en lägre risk att utveckla tjocktarmscancer jämfört med män i samma ålder. Överlevnaden efter tjocktarmscanceroperation är också bättre hos unga kvinnor än hos unga män, men motsatsen ses hos äldre patienter. Alla dessa fynd pekar på att östrogenhormon kan ha en viktig roll i utvecklingen av kolorektalcancer.

Östrogen är det dominerande könshormon hos kvinnor men produceras också hos män och har en viktig fysiologiska roller i båda könen. Detta hormon uttrycker sin funktion i kroppen genom specifika proteiner som finns i cellen som kallas receptorer. Det finns två huvudsakliga östrogenreceptorer, alfa (ERα) och beta (ERβ). Avhandling visar på en viktig roll av både ERα och ERβ för kolorektalcancerprognos. I kolorektalacancervävnader hos patienter som ingår i detta forskningsprojekt fanns en signifikant minskning av ERβ och en signifikant ökning av ERα jämfört med normal kolonvävnaderna. Kolorektalcancerpatienter med höga nivåer av ERβ i sin cancervävnad hade också lägre risk för återfall av cancer och mortalitet. Tvärtom, kolorektalcancerpatienter med höga nivåer av ERα i sina tumörer hade en högre risk för återfall av cancer och mortalitet. Dessa fynd indikerar att uttrycket av ERα och ERβ kan vara viktigt för att förutsäga prognosen för patienter med kolorektalcancer.

Trots den kirurgiska behandlingen, enskild eller i kombination med kemoterapi, har majoriteten av patienterna med avancerad kolorektalcancer återfall i sjukdomen under de tre första åren. De aktuella målterapier som finns är alltså fördelaktiga för

endast en mindre grupp av patienter, baserat på cancers mutation och molekylprofil. Detta väcker behovet av nya terapeutiska behandlingar. Experimentellt arbete med tjocktarmscancercellinjer som används i avhandlingsarbetet, visade att aktivering av ERβ och blockering av ERα minskar celltillväxt, cellrörelse (migration) samt metastasering av cancerceller. Mina resultat kan få en viktig klinisk relevans och innebär en möjlig användning av ERβ och ERα som målterapier för dessa patienter i kombination med nuvarande terapier. Dessa fynd öppnar nya behandlingsmöjligheter som kan leda till en mer individualiserad behandling av patienter med kolorektalcancer. Då det inte fanns någon skillnad av östrogenreceptorernivåer mellan män och kvinnor, kan resultatet från dessa studier vara gynnsamt för alla patienter med kolorektalcancer oavsett kön.

Slutligen, arbetet i denna avhandling ger också en viktig information om mastcells prognostiska roll vid kolorektalcancer. Utvecklingen av kolorektalcancer sker i nära interaktion med immunceller. Mastceller är bland de tidigaste immunceller som rekryteras till vävnaden där tumören utvecklas. Forskningsarbetet som ingår i denna avhandlingsbok visar på att patienter med kolorektalcancer som har hög mastcellstäthet i sin tumörvävnad har en bättre överlevnad jämfört med patienter som har en låg mastcellstäthet. Detta är ett viktigt fynd som visar på en antitumörroll hos mastceller vid kolorektalcancer.

# Përmbledhje (Summary in Albanian)

Kanceri është një nozologji që përfshinë rreth 200 sëmundje të ndryshme malinje, të cilat karakterizohen nga një rritje e pakontrolluar qelizore që çon në formimin e qelizave jonormale (atipike), të ndryshme nga origjina e tyre dhe të afta për të pushtuar dhe migruar në organe të tjera. Kanceri kolorektal është një lloj kanceri që prek zorrën e trashë dhe përfaqëson një nga sëmundjet malinje më të shpeshta në të gjithë botën. Kjo sëmundje përbën shkakun e tretë më të shpeshtë të vdekjeve nga kanceri në nivel global. Ndër faktorët më të rëndësishëm të riskut për zhvillimin e kancerit të zorrës së trashë janë mosha mbi 50 vjeç, predispozita gjenetike dhe hereditare, konsumi i tepërt i mishit të kuq dhe një diet e varfër në perime e fruta, obeziteti, konsumi i tepërt i alkoolit si dhe duhanpirja.

Krahas këtyre, gjinia luan një rol të rëndësishëm në riskun dhe prognozën e kancerit kolorektal. Gratë para menopauzës janë më pak të rrezikuara për tu prekur nga kanceri kolorektal në krahasim me burrat e së njëjtës moshë. Gjithashtu, jetëgjatësia e pacientëve me kancer kolorektal është më e lartë tek femrat e moshave të reja krahasuar me meshkujt, por e kundërta shihet tek pacientët e moshave të vjetra. Të gjitha këto të dhëna flasin për një rol të rëndësishëm të hormonit estrogjen në zhvillimin e kancerit kolorektal.

Estrogjeni është një hormon mbizotërues tek femrat, por që gjithashtu prodhohet në sasi të vogla edhe tek meshkujt duke luajtur një rol të rëndësishëm fiziologjik në të dyja gjinitë. Ky hormon e realizon funksionin e tij në organizëm përmes proteinave specifike që gjenden brenda qelizave dhe që njihen si receptorë. Ekzistojnë dy lloje kryesore të receptorëve të estrogjenit: alfa (ERα) dhe beta (ERβ).

Ky studim tregon ndikimin e ERα dhe ERβ në prognozën kancerit kolorektal. Në materialin indor të marrë nga pacientët me kancer kolorektal të përfshirë në këtë punë kërkimore, u vu re një rënie e ndjeshme e ERβ dhe një rritje e konsiderueshme e ERα, krahasuar me indin e shëndoshë të zorrës së trashë. Gjithashtu, pacientët me kancer kolorektal me nivele të larta të ERβ në tumoret e tyre, kishin risk më të ulët të vdekshmërisë dhe përsëritjes së sëmundjes. E kundërta u vu re tek pacientët që kishin nivele të larta të ERα indin kancerogjen, të cilët kishin risk më të lartë të vdekshmërisë dhe përsëritjes së kancerit. Këto rezultate tregojnë se nivelet e ERα dhe ERβ ne indin kanceroz mund të jenë të dobishme për të parashikuar ecurinë dhe prognozën e pacientëve me kancer të zorrës së trashë.

Pavarësisht trajtimit kirugjikal të kombinuar ose jo me kemioterapi, shumica e pacientëve me kancer të zorrës së trashë kanë një rikthim të sëmundjes brenda tre viteve të para. Gjithashtu, terapitë më të avancuara siç janë imunoterapitë dhe terapitë target, janë efektive vetëm në grupe të vogla pacientësh, duke u bazuar në mutacionet dhe profilin molekular të kancerit. Kështu shtrohet nevoja për të zbuluar trajtime të reja terapeutike. Puna eksperimentale me linjat qelizore të kancerit të zorrës së trashë që është realizuar në këtë studim, tregoi se induksioni i ERβ dhe bllokimi i ERα zvogëluan proliferimin, migrimin dhe metastazimin e qelizave kancerogjene. Këto rezultate mund të kanë një domethënie klinike të rëndësishme pasi sugjerojnë përdorimin e mundshëm të agonistëve selektivë të ERβ dhe antagonistëve selektivë të ERα si terapi target në kancerin kolorektal në kombinim me terapitë aktuale. Këto përfundime krijojnë mundësi te reja terapuetike, të cilat mund të çojnë drejt një trajtimi më të individualizuar të pacientëve me kancer kolorektal. Duke qënë se nuk ka ndryshime në nivelin e receptorëve të estrogjenit midis meshkujve dhe femrave përsa i përket indeve të shendosha dhe atyre kanceroze të zorrës së trashë, rezultatet e këtij studimi janë të vlefshme për të gjithë të sëmuret me kancer kolorektal, pavarësisht gjinisë.

Së fundmi, puna e këtij studimi shkencor siguron informacione të rëndësishme lidhur me rolin prognostik të mastociteve në kancerin kolorektal. Zhvillimi i kancerit kolorektal ndodh në bashkëveprim të ngushtë me qelizat imune. Mastocitet janë ndër qelizat e para imune që rekrutohen në indet ku zhvillohet tumori. Puna hulumtuese e këtij studimi tregoi se pacientët me kancer kolorektal që kanë përqëndrim të lartë të mastociteve në indet e tyre tumorale, kanë një mbijetesë më të gjatë krahasuar me pacientët që kanë një përqëndrim të ulët të këtyre qelizave në indin tumoral. Ky është një përfundim i rëndësishëm që tregon rolin antitumoral të mastociteve në kancerin kolorektal.

# Introduction

Colorectal cancer (CRC) is the third most common cause of cancer-related deaths globally [1]. It is established that people with chronic inflammatory bowel diseases (IBD), such as ulcerative colitis and Crohn's disease have higher risk to develop colon cancer in comparison with the general population [2]. Many inflammatory and pro-inflammatory mediators have been linked with CRC formation and progression. Different immune cells have been the center of research for their role in tumor microenvironment, cancer progression and treatment opportunities [3]. Mast cells, among the earliest immune cells recruited during tumorigenesis, have a controversial role in cancer [4].

On the other hand, gender is associated with CRC risk, progression and localization [5-7]. For the same age, men have a higher incidence of CRC than women. Also, right-sided colon cancer is more common in women and rectal cancer more common in men. A better survival is observed in young women compared to young men after CRC surgery, but in older patients the opposite pattern exists [8,9]. Furthermore, the use of hormone replacement therapy (HRT) has a protective role in the prevention of CRC [10,11]. All these findings implicate a prognostic role of estrogen signaling in CRC.

Estrogen acts via two main receptors, the receptor alpha (ERα) and beta (ERβ) both members of nuclear receptor family [12]. The colon mucosa has a dominance of ERβ expression, which declines with CRC progression [13]. The recent years, ERβ has gain popularity as a possible target for treatment of many cancers, including CRC [14]. Since ERα is expressed to a very little extent in the normal colonic mucosa, its role in CRC is very little studied [13].

However, the role of ERs in CRC prognosis and their interaction with inflammatory cells and mediators in the tumor microenvironment requires further investigations.

The papers included in this thesis aimed to analyze the prognostic role of both ERα and ERβ in CRC and their correlations with inflammatory proteins and cells, providing new evidence for their possible role as therapeutic opportunities to improve the outcome in patients with CRC. Also, the prognostic role of mast cells in survival of CRC patients is evaluated.

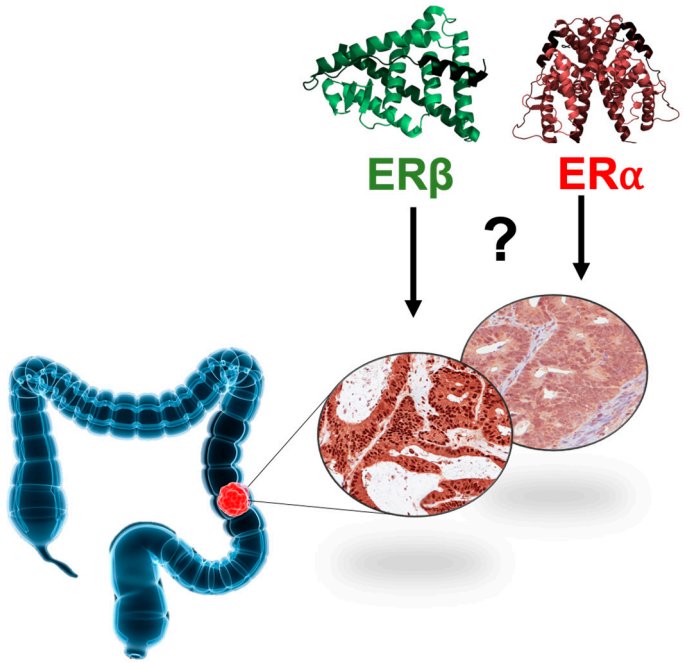


**Figure 1**. Colorectal cancer stained for the nuclear expressions of ERβ and ERα. Do estrogen receptors influence the cancer prognosis?

# Estrogen hormones

Estrogen hormones are the dominant sex hormones in women, but their levels are also present in men. They are cholesterol-derived steroid hormones produced via aromatization of androgens mainly in the ovaries (in women), but also in other tissues such as adipose, muscle, nervous tissue, liver, breast (in both women and men) and Leydig cells of the testes (in men) [15].

There are three main forms of circulating estrogens in women: estrone (E1), which is dominant in postmenopausal women and is mainly produced via aromatization of androgens in the adipose tissue; 17-β estradiol (E2), which is the most potent form of estrogen and predominant in premenopausal women; estriol (E3), which is the weakest form of estrogen and predominant during pregnancy [16].

Estrogens play a key role in the reproductive function, development of primary and secondary sexual characteristics and sexual behavior in women. Additionally, estrogen hormones are important for many physiological functions in both men and women, such as cell growth and differentiation, as well as maintaining a normal function of bone metabolism, cardiovascular, nervous and immune system [15-17]. On the other hand, estrogens are implicated in various pathological conditions including cancers of reproductive tissues, such as ovary, uterus and breast cancer in women and prostate cancer in men, but also cancers of non-reproductive tissues including gastrointestinal tract [12,18]. All these actions of estrogens in the human body are mediated via specific proteins inside the cell known as estrogen receptors (ER).

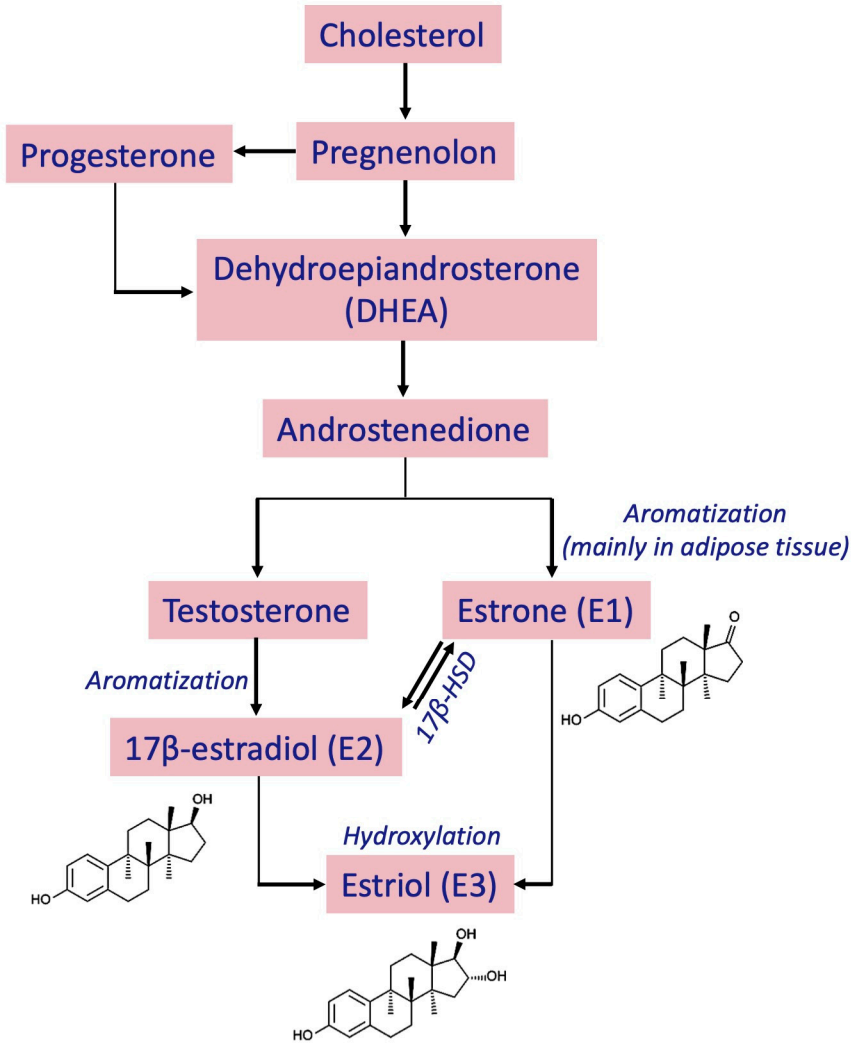


**Figure 2**. Schematic representation of estrogen synthesis.

# The nuclear receptor superfamily

Nuclear receptors are proteins inside the cells that have transcriptional effects. When they get activated by their ligands they are transported into the nucleus regulating the expression of their target genes [19-21]. In the human, the nuclear receptor superfamily includes 48 members, which are involved in regulating many important physiological activities such as cell metabolism, reproduction, inflammation, immune response, cell proliferation and electrolyte balance [22]. The endogenous ligands for nuclear receptors include small and lipophilic molecules such are steroid hormones (estrogen, progesterone, androgens, glucocorticoids, mineralocorticoids), thyroid hormones, vitamin A and D. Furthermore, exogenous compound found in plants or in environment, referred as xenobiotics, can activate the nuclear receptors disturbing in this way with the normal endocrine system of the body. This phenomenon is known as endocrine disruption [23]. Those nuclear receptors that have an unknown or unidentified endogenous ligand are named as "orphans" nuclear receptors [22].

Even though the nuclear receptors have different target genes and different functions, they all share a similar structure [22]. They are composed of five domains and have an N-terminal (domain A/B), a DNA-binding domain (C domain) and a C-terminal (E/F) which is lipophilic in nature and holds the binding site of the ligand (Ligand-binding domain). The D domain connects the DNA-binding domain with ligand-binding domain.

Nuclear receptors are involved in many pathological processes such as cardio-metabolic diseases, cancer, diabetes, hormone-resistance syndrome, rheumatoid arthritis, asthma, neurological and psychiatric dysregulations [22]. Dues to this fact, but also that nuclear receptors bind to small lipophilic molecules that can easily be modified through drug design, they have been in the focus of pharmaceutical field as possible target for the treatment of different diseases [24].

# Estrogen receptors

Estrogen signaling in human cells is mediated through estrogen receptors (ERs), where two distinct receptors ERα and ERβ are included [25], and a G-protein-coupled estrogen receptor 1 (GPER) [26]. Estrogen receptors belong to the nuclear receptor superfamily, but membrane-associated ERs have also been identified [27,28]. Furthermore, ERs have been identified in cytoplasmic organelles such are mitochondria and endoplasmic reticulum. It is suggested that mitochondrial ERs are involved in apoptotic/anti-apoptotic signaling, however their role in human physiology and pathophysiology is not fully understood [29,30].

## Structure and isoforms

Up to date, two ERs have been identified, estrogen receptor alpha (ERα) and estrogen receptor beta (ERβ). ERα, encoded by gene *ERS1* located on chromosome 6, was the first ER to be identified in 1958 and the corresponding gene was cloned in 1985 [31]. ERβ was discovered many years later in 1996 from prostate and ovary tissues of rat and is coded by *ESR2* gene located on chromosome 14 [32]. Both ERα and ERβ are members of nuclear receptor family. Their structure consists of five domains organized in three main compartments [12,25]. The N-terminal compartment involves domain A/B and includes the activation function 1 (AF1) which acts on a ligand-independent way. The DNA-binding domain (DBD) is formed by C domain and is the compartment which binds to a specific region of the promotor of target genes. The C-terminal compartment, which involves the domain E and F, is called the ligand-binding domain (LBD) and includes the activation function 2 (AF2) that plays an important role in the receptor dimerization after ligand binding [33]. The D domain, also called the hinge domain, connects DBD with LBD and included amino acid sequences important for post-translational modification [25]. ERα receptor is longer than ERβ, however the receptors share a high homology between domains, especially in the DNA-binding domain (Figure 3).

The original sequence of the ERs can be modified due to alternative splicing of mRNAs. Therefore, several isoforms of ERs are identified [12,34]. In humans, three ERα isoforms exist: ERαΔ3 where the C domain is absent, ERα46 where the AF1

region is absent, and ERα36 with both AF1 and AF2 regions missing. Regarding ERβ, at least four different isoforms are identified. All ERβ isoforms have a modified C-terminal compartment which does not allow them to function in a ligand-dependent way. Even though it is demonstrated that ERβ1 is the only functional isoform [35], the physiological role of ERs isoforms is still unclear [34].

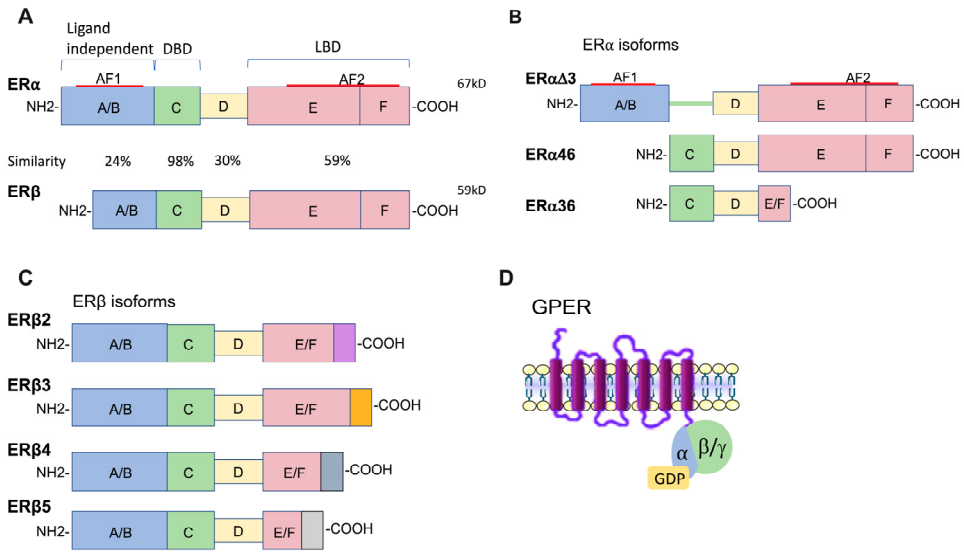


**Figure 3**. The structure of ERs and GPER. **A** ERα and ERβ composed of five main domains: A/B domain (ligand-independent domain), C domain (DNA-binding domain, DBD), D domain (hinge domain), E and F domain (ligand-binding domain). **B** ERα isoforms. **C** ERβ isoforms. **D** G-protein-coupled estrogen receptor1 (GPER).

# Membrane-associated estrogen receptors

Apart the classical nuclear ERs, extranuclear ERs have been identified in the plasma membrane. These receptors are called membrane-associated ERs, and studies have shown that these receptor are identical to the classical nuclear ERs and coded by the same genes [28]. Mice cells with a double knockout of ERα and ERβ also lack the presence of plasma membrane ERs [36]. Membrane-associated ERs compose approximately 10% of all ERs in a cell [28]. The activation of these receptors mediates rapid estrogen actions by generating secondary messengers in the cells such are cyclic adenosine monophosphate (cAMP) and calcium, activating protein kinase cascades such as the mitogen-activated protein kinase (MAPK) and the phosphoinositide 3-kinase (PI3K) pathways and can lead to both transcriptional and non-transcriptional effects [16,28,37]. Furthermore, some of membrane-associated

ERs attach to extracellular sites or lipid raft domain in the plasma membrane, thus interacting with other transmembrane receptors such as epidermal growth factor receptor (EGFR), human epidermal growth factor receptor 2 (HER2) and insulin-like growth factor receptor I (IGFR1) [27].

But what is the mechanism by which ERs translocate to the plasma membrane? Studies suggest that palmitoylation of ERs, as well as the transporter Caveolin-1, are necessary for the localization of ERs at the plasma membrane the cells [28].

# G-protein-coupled estrogen receptor1 (GPER)

GPER is a transmembrane receptor with seven domains that belongs to the group of G protein-coupled receptor family (Figure 3). The G-protein coupled receptors (GPCRs) are the largest family of the cell surface receptors, which pass across the cell membrane seven times and transduce most of the signaling in our body [38]. The heteromeric G protein consist on three main subunits: alpha (Gα), beta (Gβ) and gamma (Gγ) with several subtypes described for each subunit [39].The β and γ subunit form a an inseparable β/γ complex. Upon ligand activation, Gα binds and hydrolyzes guanosine triphosphate (GTP) releasing guanosine diphosphate (GDP), which in its inactive state is connected to the Gα subunit of the receptor. The released GDP binds to the β/γ complex which separates form Gα when the receptor gets activated. All these changes lead to the activation of several cascades of signaling pathways [38,39].

The GPER was first discovered as an orphan G protein-coupled receptor localized at the plasma membrane or endoplasmic reticulum, referred as GPR30, which mediated rapid effects of 17-β estradiol in human cells [40]. Activation of GPER by estrogen or estrogen-agonists stimulates intracellular calcium release, production of cAMP, cyclin D2 and PI3K activation, together with a transactivation of EGFR [26,36]. Even though it is reported that high levels of 17-β estradiol are required to activate this receptor [41], GPER-mediated estrogen effects are implicated in many physiological responses in different tissues and organs of the human body, such as reproductive, nervous, immune and cardiovascular system, as well as in cancer progression and metastasis [26].

# Organ distribution

ERs are widely expressed in reproductive and non-reproductive organs and systems of the human body, regulating various physiological processes. In the majority of these tissues both ERs subtypes, ERα and ERβ, are expressed almost to the same

extent. However, in some tissues such as liver, lungs and colon only one ER subtype is dominant [25,42-44]. The distribution and physiological function of ERs subtypes are presented in Table 1.

**Table 1:** Distribution and function of ERs subtypes in the human body.

| ORGAN/SYSTEM | ER SUBTYPE | FUNCTION |
|---|---|---|
| Brain | ERα, ERβ | Adjustment of libido and body temperature; memory function |
| Breast | ERα, ERβ | Breast development at puberty; breastfeeding function |
| Bone | ERα, ERβ | Increase of bone strength and density |
| Cardiovascular | ERα, ERβ | Protection from atherosclerosis; modulation of vascular function |
| Gastrointestinal | ERβ dominant | Decrease of gastrointestinal motility |
| Liver | ERα dominant | Regulation of cholesterol production |
| Lungs | ERβ dominant | Involvement in bronchial reactivity and inflammation response |
| Urinary bladder | ERβ dominant | Regulation of bladder contraction |
| Uterus | ERα dominant | Monthly preparation for pregnancy or menstrual cycle |
| Ovary | ERα, ERβ | Stimulation of menstruation |
| Prostate | ERβ dominant | Normal development and growth |

Even in those organs where both ERs are expressed, one of the receptors has the prominent role. For example, ERα has a more prominent role in preserving the bone density, while ERβ has a more prominent role in the brain. Additionally, ERβ opposes the ERα hyperproliferative effects in breast and ovary [42]. However, there are recent reports that suggest a bi-faceted role of ERβ in cancer tissues that have a stabile expression of both estrogen receptors [45]. For example, in ERα/ERβ-positive breast cancer cells, ERβ instead of opposing ERα-mediated hyperproliferative effects, forms heterodimers with ERα in presence and absence of 17β-estradiol, inducing transcriptional changes that do not include anti-proliferative effects [45].

# Signaling pathways

Activation of ERs leads to genomic and non-genomic effects. These actions can be dependent or independent on ERs and ER-ligands, therefore four different pathways are identified [16,25].

*Pathway 1: Nuclear ERs signaling, genomic effects*

This is the main signaling pathway which leads to genomic effects through activation of nuclear ERs in a ligand-dependent manner (Figure 4, light pink color). Binding of the ligand activates the ERs and changes their conformation. Depending in the type and concentration of the ligand and the number of the receptors present at the moment, ERs can form homodimers (ERα-ERα or ERβ-ERβ), heterodimers

(ERα-ERβ) or a combination of the two. After ligand binding, the ER-ligand complexes translocate from the cytoplasm into the nucleus, where they bind to the so-called estrogen responsive elements (EREs) and regulate the expression of ERE-containing genes. Since ERα and ERβ share 98% similarity of the DNA-binding domain (DBD), they have the same affinity and selectivity to bind to EREs [16].

Apart binding to ERE-promoters, ER-ligand activated complex can bind to other gene promoters in a non-classical manner, via ER-DNA indirect way by regulating the activity of transcriptional factors such as stimulating protein 1 (SP1), activator protein 1 (AP1). This ER-DNA indirect transcriptional activity composes around 35% of the pathway 1 [16].

Studies conducted using the breast cancer cell line MCF7, which expresses to a very high extent ERα, have shown several genes that contain ERE-promoters such as, MYC, BCL2, pS2, SIAH2 and GREB1[46,47]. Genes that are altered through the interaction of ERs with AP1 transcription factor are cyclin D1, IGF-I, ovalbumin and collagenase, which get activated, and choline acetyltransferase gene which gets repressed [37]. Additionally, the interaction of ERs with SP1 activates LDL-R (low-density lipoprotein receptor), c-fos and cyclin D1 [37]. The repressive effect of 17β-estradiol on interleukin 6 (IL-6) gene is mediated via interaction of ERs with two transcription factors, nuclear factor κβ (NF-κB) and CCAAT/enhancer binding protein β (C/EBPβ) [37]

*Pathway 2: Membrane-associated ERs signaling*

This pathway is responsible for the rapid and acute estrogen effects generated by membrane-associated ERs and GPER (Figure 4, orange color), both described in more details in the sections above. Ligand-dependent activation of membrane-associated ERs or GPER leads to non-genomic effects by regulating the secondary messengers such as cAMP, calcium and potassium, interacting with tyrosine kinase receptors (EGFR and IGFR1) in the cytoplasmic membrane and activating protein kinase cascades such are MAPK and PI3K [16,28,37]. Furthermore, activation of cAMP and protein kinase cascades via this pathway can lead to genomic effects by phosphorylating AP1 and SP1 transcriptional factors of certain target genes. The rapid effects of estrogen mediated through ligand activation of membrane-associated ERs and GPER play an important role in liver, bones and nervous system [16,26].

*Pathway 3: ER-independent signaling*

Being a steroid hormone, estrogen can easily cross the cell membrane and enter into the cytoplasm where it can interact with enzymatic activities without binding to ERs (Figure 4, blue color). Estrogen has a phenolic A ring, which allows it to regulate redox activities providing anti-oxidant effects [16]. Studies report that estrogen reduces oxidative stress by preventing mitochondrial release of reactive oxygen species (ROS) [48]. Additionally, the ER-independent effect of estrogen has proven in mouse models of breast cancer, where estradiol could promote breast cancer even in mice with an endogenous deletion of ERα and ERβ [49].

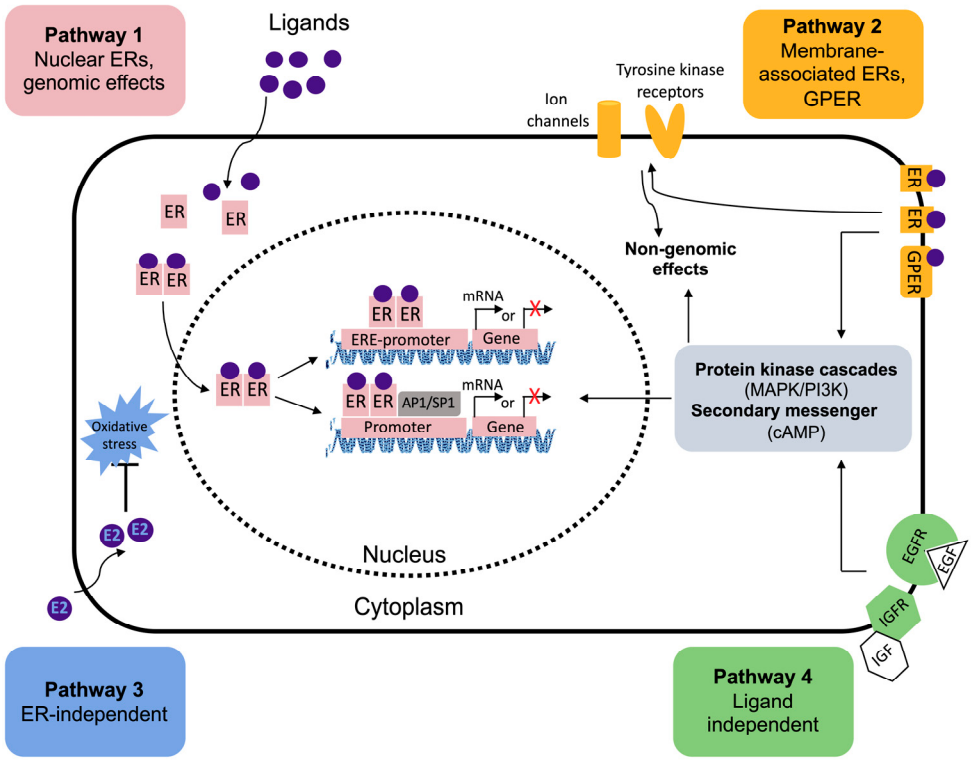


**Figure 4**. Signaling pathways of estrogen and estrogen receptors. Pathway 1 (light pink) represents the ligand activation of nuclear ERs, leading to genomic effects. Pathway 2 (orange) represents the ligand activation of membrane-associated Era and GPER, which leads to non-genomic and genomic effects. Pathway 3 (blue) represents the anti-oxidative effects of estrogen in a receptor-independent way. Pathway 4 (green) represent the activation of ERs in a ligand-independent way. Adapted from Cui *et al.*, 2013 [16].

### *Pathway 4: Ligand-independent activation of ERs*

In addition to estrogen, ERs can be activated by other factors as well (Figure 4, green color). Dopamine (a neurotransmitter), epidermal growth factors (EGF), insulin-like growth factor-1 (IGF-1), protein kinase C and A can phosphorylate the ERs and activate them in a ligand-independent way [16]. It is reported that EGF can phosphorylate Ser 118 in the AF-1 domain of ERα and activate the transcriptional activity of the receptor [50]. In the same way, protein kinase A can phosphorylate ERα in the Ser 236 in the DNA binding domain and regulate the receptor dimerization. Also, activation of MAPK and PI3K can activate ERs in a ligand-independent manner by phosphorylating them [16].

# Ligands

Estrogen is the endogenous ligand for the activation of ERs. However, ERs can be activated by many other compounds which can fit into their ligand-binding domain. These compounds are grouped into synthetic estrogens, phytoestrogens (natural estrogen coming from plants) and environmental estrogens [34]. Based on their function, these ligands are grouped into agonists, antagonists, and selective estrogen receptor modulators (SERMs) [25,34,51]. Agonists bind to ERs and activate them, while antagonists block ERs activation after binding to them.

SERMs are a group of synthetic nonsteroidal compounds that modulate the effect of ERs. SERMs are grouped in two categories: non-selective (classical) and selective SERMs [51].

Classical SERMs bind to both ERα and ERβ with the same affinity but different efficacy. A very good example of classical SERMs are tamoxifen and raloxifene [52]. Tamoxifen has anti-estrogenic effects in breast and is used in clinic for treatment of ER+ breast cancer, but it has estrogen-like effects in other tissues especially in bone, where it preserves the bone density protecting from osteoporosis [52]. Likewise, raloxifene has the same anti-estrogenic effects in breast and pro-estrogenic effects in bone as tamoxifen, but it's estrogen-like properties in other tissues are weaker [52].

The other class of SERMs binds selectively to only one of ERs subtypes. In the studies included in this thesis the ERα-selective agonist 4,4',4"-(4-Propyl-1H - pyrazole-1,3,5-triyl) trisphenol (PPT) and the ERβ-selective agonist ERB-041 have been used. PPT has 410-fold selectivity for ERα over ERβ, while ERB-041 bind to ERβ with more than 200-fold selectivity over ERα.

Also, selective antagonists for each ER subtype exist. They can be silent antagonist (full antagonist is another term used) or partial agonists. A silent antagonist is a competitive ER antagonist that has zero intrinsic activity for activating the receptor [53]. An example is the ERβ-selective antagonist 4-(2–phenyl-5,7–bis (trifluoromethyl) pyrazolo (1,5-a)-pyrimidin-3-yl) phenol (PHTPP), which possesses full antagonistic properties with 36-fold selectivity for ERβ over ERα. In addition, a partial agonist is a compound that in a presence of a full agonist acts as a competitive antagonist, but if its concentration is too high it can activate the receptor as well [53].

A specific group are the antagonists that belong to the group of selective estrogen receptor degrader (SERD). When they bind to ER, they cause conformational changes which result in receptor degradation and downregulation [51].

An example of SERD are the compounds named fulvestrant and AZD9496 which bind selectively to ERα [54]. Fulvestrant is an approved medication for breast cancer patient administered with monthly intramuscular injections. AZD9496 is the first oral SERD that has shown higher bioavailability compared to fluvestrant and has been successfully tested in clinical trials of breast cancer patients [54,55]

# Anatomy and physiology of the large intestine

The large intestine is the last part of the gastrointestinal (GI) track and includes appendix, cecum, colon, rectum and anal canal (Figure 5). In comparison to small intestine, the large intestine is shorter, only 1.5 meter (one fifth of the whole length of GI tact) but has a wider and larger lumen [56]. It starts with a blind pouch named cecum, which connects with the end of ileum (the last part of the small intestine) via the ileocecal valve. The cecum continues proximally with a vermiform part called appendix, and distally with colon. The appendix is a blind formation rich in lymphoid tissue and with a free ending which floats in the peritoneal cavity [57]. Colon is composed of the following parts: ascending part, hepatic (right) flexure, transverse colon, splenic (left) flexure and descending colon. The two last parts of the large intestine are rectum and anal canal.

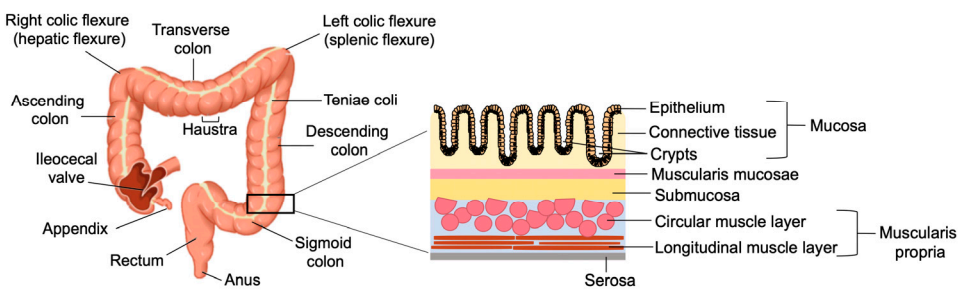


**Figure 5**. Schematic illustration of large intestine anatomy and histology.

Three separate longitudinal ribbons of smooth muscle, referred as teniae coli, go along the large intestine length. Since the length of teniae coli is shorter, it causes the large intestine to contract and form regular segments called haustra [56].

The wall of the large intestine is formed of five main layers, which are mucosa, muscularis mucosae, submucosa, muscle layer (muscularis propria) and serosa (Figure 5).

Mucosa is the most inner layer and contains the intestinal epithelial cells (IECs) and connective tissue. The connective tissue underneath the epithelium is referred as lamina propria and is rich in stromal cells and immune cells such as dendritic cells, macrophages and lymphocytes [58]. The immune cells in lamina propria together with immunoglobulin A (IgA) protect the intestinal epithelium from different pathogens. Epithelial cells form a layer with many deep and narrow invaginations called crypts. As shown in Figure 6, the large intestine epithelium contains four types of differentiated cells: enterocytes, Goblet cells, enteroendocrine cells and Paneth cells [59]. Enterocytes are columnar absorptive epithelial cells with brush border microvilli in the apical surface. Goblet cells produce mucin-2 and mucin 5AC [60]. Paneth cells reside at the bottom of the crypt and produce mainly antimicrobial peptides and proteins such as α-defensins and angiogenin-4. Enteroendocrine cells produce and release hormones, such are vasoactive intestinal peptide, enteroglucagon, secretin, motilin and neurotensin, in response to different stimuli. In the basis of the crypts are located the intestinal stem cells (ISC), which have the ability to maintain themselves (self-renewal capacity) and at the same time to produce all the differentiated cells of the intestinal epithelium [61]. The renewal capacity of the large intestine is very high. ISC divide every 12-16 h generating around 300 cells/crypt. Expect the Paneth cells, which stay at the base of the crypt for 3-6 weeks, all the other intestinal epithelial cells move continuously upward to reach the top of the crypt (Figure 6). In this way, the epithelial turnover occurs approximately every 5 days.

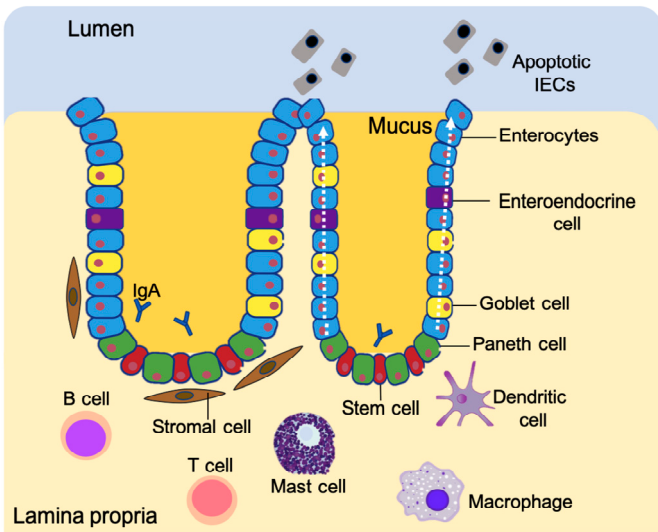


**Figure 6.** Schematic illustration of the histological composition of the large intestine mucosa. The white dotted arrows indicate the turnover of the epithelial cells.

Muscularis mucosae is a very thin layer of smooth muscles that divides mucosa from the submucosa layer. Submucosa is dense layer of irregular connective tissue that contains nerves, blood and lymphatic vessels, and the submucosal plexus of Meissner [59]. The muscle layer is the thickest layer of the large intestine wall and contains circular and longitudinal muscles. In this layer is located the myenteric plexus of Auerbach, which is composed of both parasympathetic and sympathetic nerves which provide the necessary motoric function for the muscle layer to move the indigestible materials along the large intestine until they are eliminated via anal canal [59]. The outer layer of the large intestine wall is serosa.

The main functions of large intestine are to absorb the remaining water and electrolytes from indigestible materials before eliminating them via rectum, and production of vitamins [59]. More than 90% of water and nutrients from the digested food is absorbed in the small intestine. When indigestible materials reach the colon, its role is to absorb the remaining water and electrolytes, solidifying more the materials to form feces, which will further be eliminated via rectum and anal canal. The colon microbiota is composed a large variety of bacteria which colonize the colon and, among many other functions, form different vitamins such as vitamin K, B1 (thiamine) and B2 (riboflavin), through the process of fermentation [59,62]. These vitamins are then absorbed from colonic mucosa into the blood and used in different metabolic processes in the body.

The intestine has its own immune system known as gut-associated lymphoid tissue (GALT), which protected the intestine from different infections. Studies have shown that the dysfunction of GALT can predispose people to develop inflammatory bowel disease (IBD) due to unbalanced inflammatory response in the intestine [63].

# Inflammation and cancer

Inflammation is a physiological and defensive response of the body against a harmful stimulus that can be damaged tissue, invading pathogen or toxic factors. It is a complex response that includes blood vessels, the immune system and many molecular mediators which are referred as inflammatory mediators [64]. Vasodilation and recruitment of immune cells by the inflammatory mediators produced are responsible for the five major signs of the inflammatory response: pain, swelling, redness, heat, and loss of tissue function [64]. If the harmful stimuli persist for a long time a chronic inflammatory response is caused.

Cancer refers to any disease that is characterized by an abnormal and uncontrolled cell growth, which leads to formation of immature cells that lose their main function and instead gain the ability to invade and spread to other tissues and organ. The first to hypothesize a direct relationship between chronic inflammation and cancer was the German physician Rudolf Virchow in 1863. To date, inflammation is an established and very important hallmark of cancer development [65]. Inflammation contributes to CRC by increasing oxidative and metabolic stress, pro-tumorigenic inflammatory mediators and changing in the intestinal microbiota [66]. Other important hallmarks for CRC are immune evasion, signaling pathways that escape apoptosis and growth suppressors and promote replicative immortality, and microsatellite instability (MSI) which leads to mismatch repair protein expression and DNA damage [67]. The hallmarks of CRC are summarized in Figure 7.

## Inflammatory bowel disease and colitis-associated colon cancer

Inflammatory bowel disease (IBD) is a condition characterized by chronic inflammation of the colon and small intestine due to the impaired immune response in the tissue [68]. IBD includes two main entities: Crohn's disease (CD) and Ulcerative colitis (UC). Crohn's disease can affect the whole GI tract, from mouth to anus but is more common in the ileum part of small intestine. Instead, UC affects only the large intestine with a preference for sigmoid and rectal part. The inflammation pattern in UC is localized in the mucosa, whereas in CD is mostly transmural [69]. With the highest prevalence in the industrialized countries,

Caucasian population and higher socio-economic groups, the etiology of IBD includes an interaction of environmental factors, genetic predisposition and unbalanced gut microbiota and immunoregulatory factors [70]. Both CD and UC have a bimodal age distribution with the highest frequency in young individuals. Gender differences are also reported for IBD: UC is more common in men while CD occurs more frequently in women [70]. As a chronic condition IBD requires a lifetime care. Current treatment involves the use of 5-aminosalicylic acid (5-ASA), high doses of steroids and immunomodulators (azathioprine). In very difficult cases where the inflammation cannot be controlled, surgery remains the last option. Emerging therapies are focusing to reduce the inflammation by controlling the T helper 1 (TH1) and T helper 2 (TH2) cell response and blocking NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) which is a protein complex that controls DNA transcription, cytokine production and cell survival [70].

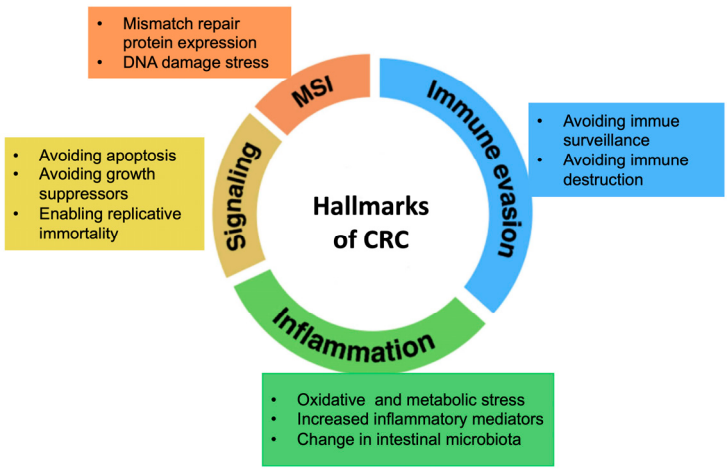


**Figure 7**. Important factors that contribute to CRC development summarized as hallmarks of CRC. Adapted from Caiazza *et al.*, 2015 [13]

The link between IBD and CRC risk has been established since long ago [2,71]. It is estimated that patients with IBD has a 2-fold increased risk to develop CRC compared to the general population, depending on the duration and severity of the disease. The link between inflammation and colitis-associated colon cancer (CAC) is established also in animal mouse models. The carcinogen azoxymethane (AOM) can induce multiple colonic tumors even in single and small doses if combined with the colon irritant Dextran Sulfate Sodium (DSS). DSS causes colon inflammation when administered daily in the drinking water of mice. But high doses and multiple injections of AOM were required to induce tumorigenesis in mice where DSS was not given [72,73].

# Colorectal cancer

Colorectal cancer (CRC) is one of the most common malignant diseases worldwide. Around eight hundred thousand people are diagnosed with CRC each year. Regarding the incidence it is the fourth most common malignant tumor after lung, breast and prostate cancer, but it is ranked the third regarding the cancer mortality [1]. Respective to the gender it is ranked second among women and third among men. The incidence has increased in the last decades with the highest rates in USA, Western Europe and Australia and the lowest rates in China, India and some parts of Africa.

In Sweden, around 6 500 cases of CRC are diagnosed each year, ranking it fourth after prostate, breast and skin cancer (excluding malignant melanoma and basal cell carcinoma). The risk to develop CRC before the age of 75 is 2.2% for men and 2% for women [74].

## Risk factors

Several risk factors have been pointed out for CRC which are divided in two major groups: non-modifiable and modifiable risk factors [67]. The non-modifiable risk factors have to do with the individual predisposition to develop CRC, where age is the most important one. More than 30% of the patients are 80 years of age or older at the time of diagnosis, whereas cases before the age of 50 compose only 4% of the total patients [75]. Other non-modifiable risk factors are IBD [71] as well as genetic and hereditary factors such as familial adenomatous polyposis (FAP) and Lynch syndrome (hereditary nonpolyposis colorectal cancer - HNPCC). Almost 20% of the patients with CRC have a hereditary component. The risk is increased with 2-fold for those who have a first-degree relative with CRC. The risk is even higher for those who have two first-degree relatives, or a close relative diagnosed with CRC before the age of 50 years [76,77].

The modifiable risk factors have to do with lifestyle and diet and include high intake of red meat and a diet low in fibers, obesity and sedentary life, high consume of alcohol and smoking [67,75]. The importance of lifestyle and dietary pattern is stressed with the fact that those individuals who migrate from a low incidence area to a high incidence country have an increased risk to develop CRC [67]. Studies

suggest that 20-30% of CRC cases could be prevented through changes in lifestyle and dietary pattern [78]. The risk factors for CRC are summarized in Figure 8.

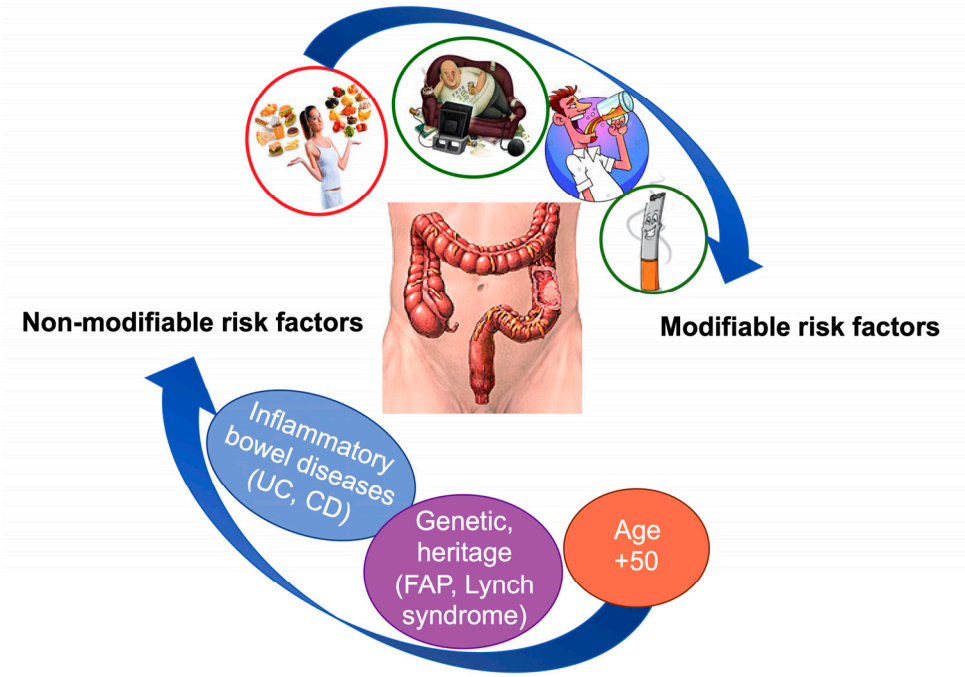


**Figure 8**. Modifiable and non-modifiable risk factors for colorectal cancer.

# Etiology and carcinogenesis

CRC arises from the epithelial cells of the mucosa. The majority of CRC, about 70-75%, is sporadic and occurs without any family history or genetic predisposition. The theory behind the CRC formation is that the site of origin is a stem or stem-like cell that resides at the base of the crypts [79]. As suggested by the multistep model of colorectal carcinogenesis, the loss of genomic and epigenomic stability in the cancer stem cell leads to the accumulation of different mutations, alteration of oncogenes and tumor suppressor genes and initiation of the tumorigenic process [67,80]. There are currently two accepted models for the transition from normal colon mucosa to adenoma and then to cancer [67].

Adenomas are benign tumors, quite common in the general population and more frequent in men than women. The risk to develop CRC is associated with the size of adenomas: the bigger the adenoma the higher is the risk to develop CRC [81].

Several histological types of adenomas exist such as tubular, tubulovillous, villous, and sessile serrated. Only 10% of the adenomas are transformed into a cancer and this process requires 10-15 years to occur, but it can occur much faster if there is a genetic predisposition such as in patients with FAP or Lynch syndrome [67]. Adenomas that rise in the right part of the large intestine (cecum, ascending colon and transverse colon) have more microsatellite instability (MSI), while adenomas from the left part of the large intestine (descending colon, sigmoid and rectum) are typically microsatellite stable (MSS) [67]. Tubulovillous and tubular adenomas follow a separate pathway to transform in CRC compared to serrated adenomas. However, both pathways share common mutation pathways [67,82].

The classic pathways, which is the most common one, includes the transformation of tubular and tubulovillous adenomas into cancer (Figure 9). One of the earliest mutations that occurs to transform the normal epithelium into dysplastic epithelium is the inactivation of the tumor suppressor gene adenomatous polyposis coli (*APC*). The *APC* mutation is one of the most common and accurse in approximately 85% of all CRC cases [67,82]. When inherited as a germline mutation, *APC* is responsible for the familiar adenomatous polyposis coli (FAP). The rest of CRC cases that don't have an *APC* mutation, do have a mutation in the β-catenin gene (*CTNNB1*) [67]. This includes the non-classic pathway of transformation adenoma-carcinoma, which is also referred as serrated-methylated pathway (Figure 9) [82]. Other important alterations that occur in the adenoma-carcinoma process are the activation of proto-oncogenes *KRAS* and *BRAF*, inactivation of tumor suppressor genes *TP53* and *SMAD4,* and alterations in the genes that control phosphatidylinositol 3-kinase (PI3K) and transforming growth factor-β (TGF-β), respectively *PIK3CA* and *TGFBR2* [67].

KRAS mutation, which accounts for approximately 45% of all CRC, is very frequent in tubular and tubulovillous adenomas, but rarely found in serrated lesions [82,83]. Meanwhile, *BRAF* mutation is found in 5-10% of CRC, more common in serrated adenomas and is associated with a more aggressive phenotype [83]. The p53 gene, *TP53*, plays an important role in controlling cell cycle, proliferation and apoptosis. Mutation of *TP53* accrues in 40-50% of CRC cases and correlates with disease aggressiveness and metastasis [84].In healthy individuals, the TGF-β is activated and provides growth inhibitory signals in the normal intestinal epithelium and its function is controlled from *SMAD4* gene [82].

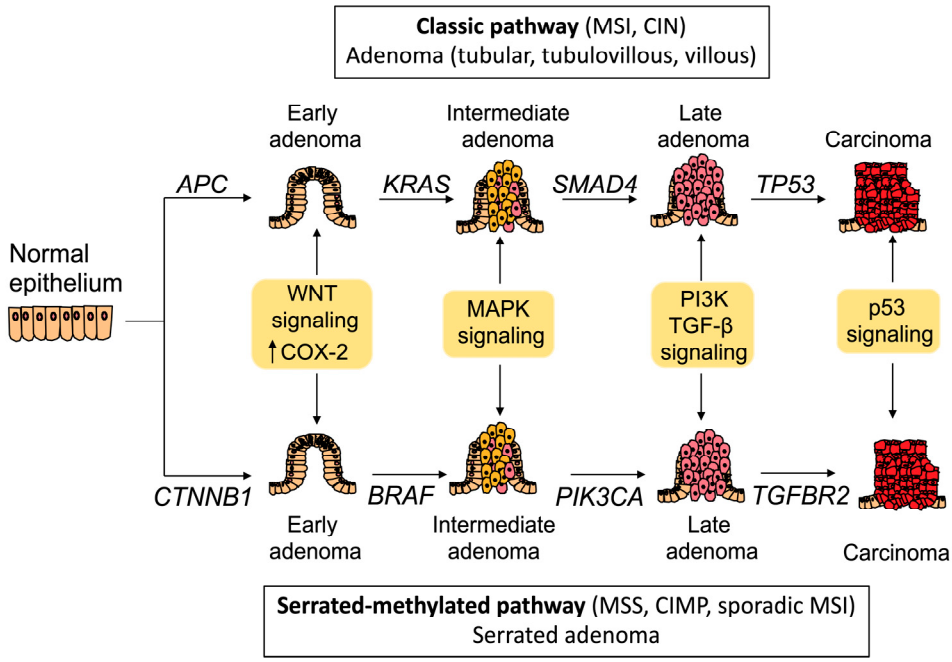


**Figure 9.** The multistep process of adenoma to colorectal carcinoma transformation. The classic pathway has mostly microsatellite instability (MSI) and chromosomal instability (CIN). The serrated-methylated pathway has more microsatellite stability (MSS), CpG Island Methylator Phenotype (CIMP), and few sporadic MSI. Adapted from Kuipers *et al.*, 2015 [67].

# Diagnosis, classification and staging

CRC can be diagnosed after the symptoms have started or by a random screening. Symptoms depend of the cancer size and localization, but the most common symptoms are blood in stool, alteration of bowel transit with prolonged constipation and alteration with diarrhea, considerable weight loss and loss of appetite [85]. The diagnosis is confirmed by colonoscopy, CT of abdomen and chest and tumor biopsy [86].

Based on the morphology of cancer cells, as set by the histopathological examination of the biopsy specimen, CRC can be classified as well, moderate and poorly differentiated [87]. The majority of CRC are adenocarcinomas that don't produce mucus. If more than 50% of cancer cells produce and secrete mucus, then the cancer is classified as mucinous adenocarcinoma; if this parentage is less than 50% then the adenocarcinoma is classified as partly mucinous [87]. Mucinous adenocarcinomas can be classified further based on the molecular type of the mucus: mucin 2 and mucin 5AC. Mucin 2 levels, which are reduced in CRC tissues in

comparison to the normal colon mucosa, are linked with the cancer differentiation: the higher the mucin 2 levels the better differentiated is the cancer [88,89].

The molecular classification of CRC is based on the presence or absence of mutations, such as *APC*, *KRAS, BRAF, TP53*, chromosomal instability (CIN), CpG Island Methylator Phenotype (CIMP), as well as microsatellite instability (MSI) [90,91]. Based on MSI status, CRC are classified as high MSI (MSI-H), low MSI (MSI-L) or microsatellite stable (MSS). Colorectal cancer patients with MSI-H have a better prognose compared to patients with MSI-L or MSS [92].

The staging for CRC, randomly used in the clinic, is done by the American Joint Committee on Cancer (AJCC) based on the TNM (tumor-node-metastasis) classification system, where T stands for the size of the primary tumor, N for the spread of the tumor in the regional lymph nodes and M for presence of distant metastasis (Table 2) [93].

**Table 2.** Staging of colorectal cancer based on TNM classification [93]

| AJCC STAGE | TNM | CRITERIA |
|---|---|---|
| Stage 0 | Tis N0 M0 | Carcinoma *in situ* (Tis): tumor localized in the mucosa layer |
| Stage I | T1/T2 N0 M0 | Tumor localized in submucosa (T1) or muscularis propria (T2) |
| Stage IIA | T3 N0 M0 | Tumor has invaded the whole colon wall up to serosa |
| Stage IIB | T4a N0 M0 | Tumor has grown through the colon wall without invading nearby tissues |
| Stage IIC | T4b N0 M0 | Tumor has grown through the colon wall invading nearby tissues |
| Stage IIIA | T1/T2 N1 M0 or T1 N2a M0 | Metastasis to: 1-3 regional lymph nodes (N1),T1 or T2; 4-6 regional lymph nodes (N2a), T1 |
| Stage IIIB | T3/T4 N1 M0 or T2/T3 N2a M0 or T1/T2 N2b M0 | Metastasis to: 1-3 regional lymph nodes (N1), T3 or T4; 4-6 regional lymph nodes (N2b), T2 or T3; 7 or more regional lymph nodes (N2b), T1 or T2 |
| Stage IIIC | T4a N2a M0 or T3/T4a N2b M0 or T4b N1/N2 M0 | Metastasis to: 4-6 regional lymph nodes (N2b), T4a; 7 or more regional lymph nodes (N2b), T3 or T4a; at least one regional lymph node, T4b |
| Stage IVA | Any T, any N, M1a | Metastasis to one distant organ but not in the abdominal cavity (M1a) |
| Stage IVB | Any T, any N, M1b | Metastasis to more than one distant organ but not in the abdominal cavity (M1b) |
| Stage IVC | Any T, any N, M1c | Metastasis to the abdominal cavity (M1c) |

The schematic representation for the progression of CRC from stage 0 to IV is shown in Figure 10.

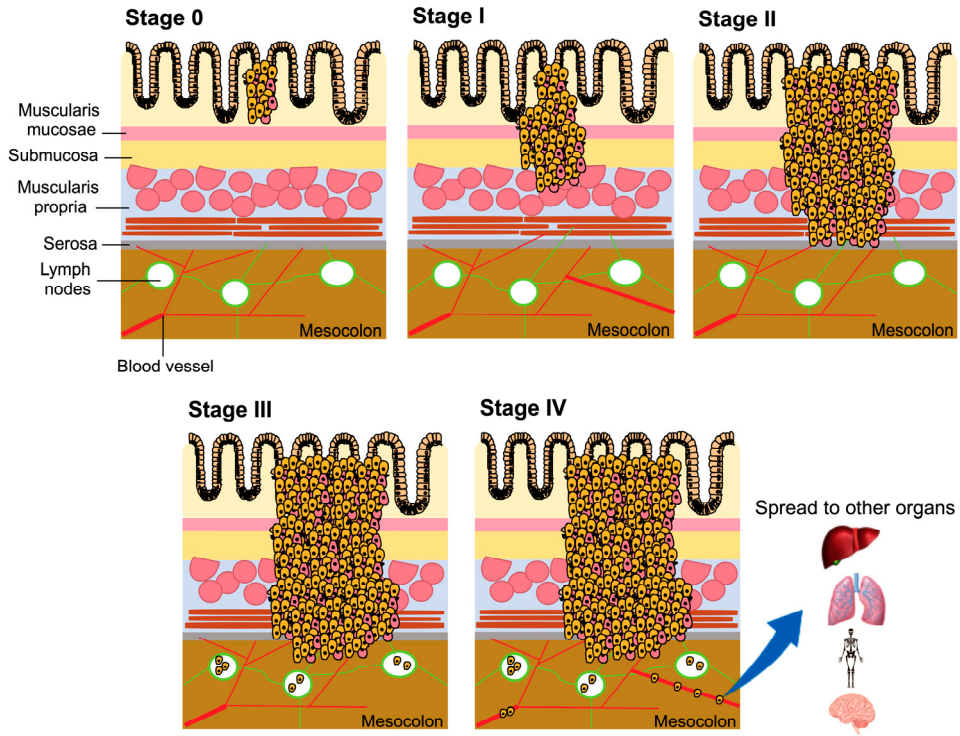


**Figure 10**. Schematic representation of CRC progression from stage 0 to IV.

# Treatment

The main treatment for non-metastatic CRC is the surgery to remove the tumor. The efficacy of the treatment is strongly related to the quality of the surgery (if the tumor is removed in the safe margins or not), the cancer stage, age of the patient and comorbidities [67].

Two other approaches are neoadjuvant and adjuvant treatment. Neoadjuvant treatment has to do with the management of patient before surgery. Neoadjuvant radiotherapy or chemotherapy is given to patients with rectal cancer, in order to shrink the tumor facilitating the surgery, but also to reduce the risk of local recurrence [67,94]. Adjuvant chemotherapy is usually given after CRC surgery to reduce the risk of cancer recurrence and is a standard treatment for patients with stage III of cancer. The most common combination in adjuvant chemotherapy is 5-fluorouracil plus oxaliplatin [67,86]. However, the cancer relapse after surgery, in combination or not with chemotherapy, occurs usually within 3 years [86].

Target therapies using monoclonal antibodies against tyrosine kinase receptors are a very good approach to treat patients based on the mutation profile that they have. Such therapies are anti-EGFR (epithelial growth factor receptor), given only to patients that lack a *KRAS* or *NRAS* mutation, and anti-VEGF (vascular endothelial growth factor). The use of bevacizumab (anti-VEGF) or cetuximab (anti-EGFR) in combination with standard chemotherapy has increased the survival rate of the patients with 30 months [95]. A novel and recent target therapy is the so called immune checkpoint inhibitor using antibodies against programmed cell death 1 (PD1), such as pembrolizumab and nivolumab, and cytotoxic T lymphocyte antigen 4 (CTLA4) such as ipilimumab, which are proven to be very beneficial in CRC patients with MSI-H [96].

The management of metastatic colorectal cancer remains a challenge. The treatment of these patients requires a multidisciplinary team for decision making, and the combination of standard chemotherapy with the above-mentioned target therapies [67,96,97].

# Prevention and screening

Primary prevention has to do with the modification of lifestyle factors and dietary patterns in order to reduce the risk for developing CRC [98]. The use of aspirin in low doses in people over 50 years of age, apart reducing the risk for cardiovascular diseases, is shown to prevent CRC development [99].

Secondary prevention for CRC implies the screening programs which are offered to the group of population that is in high risk to develop CRC [100,101]. The screening is usually offer to the individuals of age 50-75 years, however different countries have different age cut-off based on the screening program that they use [102]. Part of the screening programs are also the individuals that have a genetic predisposition to develop CRC such as those with FAP and Lynch syndrome. These individuals should be monitored regularly to detect the pre-malignant/malignant lesions quite early [101].

Current screening methods include: non-invasive examinations such as fecal occult blood test (gFOBT) and fecal immunochemical test (FIT), invasive examinations (flexible sigmoidoscopy and total colonoscopy), as well as genomic approaches such as multi-target fecal DNA test to detect for mutations (*KRAS, APC; TP53*) and biomarkers in blood (carcinoembryonic antigen - CEA) [67,102].

In Sweden, since 2014, a screening program is being applied in elderly population 60-74 years of age with the aim to detect CRC at early stages [74,103].

# Signaling pathways in colorectal cancer

## Wnt/β-catenin pathway

The Wnt signaling is composed of a group of proteins with a molecular size around 40 kDa and rich in cysteine. This signaling play an important role in embryonic development by controlling organogenesis, cell division and tissue regeneration [104]. The Wnt signaling expresses its functions either in a paracrine way, through nearby cell-cell communication, or in an autocrine way through the same cell communication, and plays a critical role in cancer as well [105].

Wnt signaling activates several intracellular downstream pathways, where the most important one is the interaction with the β-catenin pathway. This is also called the canonical pathway [105,106]. The non-canonical Wnt signaling, which is independent from the β-catenin pathway, and the Wnt/$Ca^{2+}$ pathway also exist [107,108]. The Wnt5A is among the most studied non-canonical pathways and is involved in cancer cell metabolism, migration, invasion and cell metastasis, being a focus for target therapies in melanoma, prostate and ovaria cancer [109].

In CRC, Wnt/β-catenin pathway plays a pivotal role. Wnt signaling is a key factor for the normal function of intestine and mostly for the self-renewal of stem cells located at the base of intestinal crypts [110]. On the other hand, β-catenin is a protein with multiple subcellular localizations and multiple functions, encoded by the gene *CTNNB1* in chromosome 3. It is localized in the cell membrane where plays an important role in maintaining cell to cell adhesion; in the cytoplasm where β-catenin levels are strongly controlled by a complex of proteins known as "β-catenin destruction complex"; and in the nucleus where it interacts with transcription factors and activates target genes such are *CCND1* (cyclin D1), *MYC* (c-Myc) and *PTGS2* (COX-2) affecting mainly cell proliferation, survival and migration [111-114].

The "β-catenin destruction complex is formed from a group of proteins, where the tumor suppressor gene *APC* plays an important role in keeping the complex together (Figure 11) [115]. In physiological condition, Wnt signaling is "OFF" and the destruction complex formed by Axin, APC, casein kinase 1α (CK1) and glycogen synthase kinase 3β (GSK3β) surrounds the cytoplasmic β-catenin and

phosphorylates it, inducing its proteasomal degradation (Figure 11A) [106]. In the presence of an *APC* mutation, the Wnt signaling is "ON", GSK3β gets phosphorylated becoming inactive and the destruction complex disintegrates. Since β-catenin does not get phosphorylated, it gets stabile and accumulates in the cytoplasm. Cytoplasmic accumulation leads to translocation of β-catenin into the nucleus, where it interacts with T-cell factor/lymphoid enhancer factor (TCF/LEF) transcription factors to activate target genes that increase cell proliferation, survival and migration (Figure 11B) [106,114].

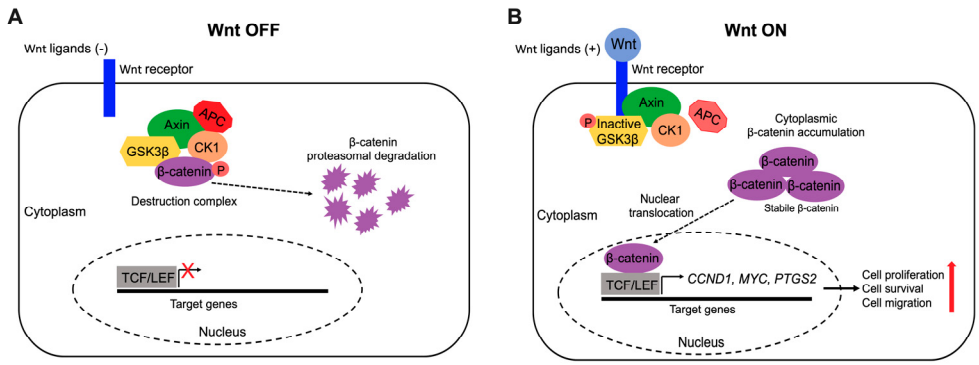


**Figure 11**. Wnt/β-catenin pathway. **A** In the absence of an *APC* mutation, Wnt is "OFF" and β-catenin undergoes degradation due to phosphorylation. **B** In the presence of a *APC* mutation, Wnt signaling gets activated leading to accumulation of stabile β-catenin in the cytoplasm and its translocation into the nucleus increasing transcriptional activity of genes that control cell proliferation, survival and migration.

# The COX pathway

Cyclooxygenase (COX) is the key enzyme to produce prostanoids, which include prostaglandins (PG) and thromboxane A2 ($TXA_2$). This enzyme transforms that arachidonic acid, that is formed from the conversion of membrane phospholipids through phospholipase A2 ($PLA_2$) into prostanoids (Figure 12) [116]. Two isoforms of COX enzyme exist: COX-1 and COX-2. COX-1 is expressed in a huge variety of cells and produces cytoprotective prostaglandins especially important to maintain the integrity of gastric mucosa as well as maintains the homeostatic levels of prostaglandins. Whereas COX-2 is induced by inflammatory stimuli and is the major source of prostaglandin production in inflammation and cancer [117]. Apart their role in inflammation, prostaglandins are involved in wound healing, immune response, bone metabolism, kidney function and blood coagulation ($TXA_2$) [118].

Additionally, prostaglandins play an important role in colorectal cancer (CRC) development, prognosis and microenvironment [119]. COX-2 levels, encoded by

gene *PTGS2*, are significantly elevated in human colorectal adenocarcinomas and are associated with a worse prognose [120,121]. The regular use of low doses (80-100 mg) of nonsteroidal anti-inflammatory drugs (NSAIDs), such as aspirin, inhibits the COX-2 activity and reduces the incidence of CRC [122].

$PGE_2$ is among the most important prostaglandin produced via COX-2 pathway in cancer, due to its potent protumor effects. Evidence shows that COX-2-mediated $PGE_2$ synthesis increases proliferation, angiogenesis and migration of cancer cells, but also expands the number of colon cancer stem cells and promotes their metastasis [123,124]. The cytoplasmic enzyme 15-hydroxyprostaglandin dehydrogenase (15-PGDH) has a pivotal role in $PGE_2$ degradation by converting it into inactive metabolites. The levels of this enzyme are expressed to high extend in the normal colon mucosa, but its expression is significantly decreased in colon cancer tissue [125]. Encoded by gene *HPGD*, 15-PGDH is considered to be a tumor suppressor in CRC, as upregulation of its levels inhibit the colon cancer cell activity by promoting cell differentiation and improving the disease progression [119,126].

# The 5-Lipoxygenase pathway

The enzyme 5-lipoxygenase (5-LOX) is the key for production of leukotrienes (LTs). The main leukotriene produces directly by 5-LOX activity is $LTA_4$, which is later metabolized into $LTB_4$ or into cysteinyl leukotrienes (CysLTs), $LTC_4$, $LTD_4$ and $LTE_4$ (Figure 12). Apart their well-studied role in allergic diseases such as asthma and d allergic rhinitis [127], leukotrienes are implicated in cancer as well [128].

Cysteinyl leukotrienes $LTC_4$ and $LTD_4$ are the most studied for their role in cancer, especially in CRC. They act via two distinguished receptors, named $CysLT_1R$ and $CysLT_2R$, which are G-protein coupled receptors [129]. The family of G-protein coupled receptors and their structure is introduced in a previous section “G-protein-coupled estrogen receptor1”.

$CysLT_1R$ is the receptor with the highest affinity for cysteinyl leukotrienes. It preferably binds to $LTD_4$ and has a lower affinity for $LTC_4$. In the contrary, $CysLT_2R$ has an equal affinity for both $LTC_4$ and $LTD_4$, but a lower overall affinity compared to $CysLT_1R$ [129]. There is a lot of evidence that links $CysLT_1R$ with a bad phenotype and $CysLT_2R$ with a better phenotype in CRC. High expression levels of $CysLT_1R$ are observed in colon adenocarcinomas and associated with a worse prognosis [130]. Additionally, induction of $CysLT_1R$ in colon cancer cells upon $LTD_4$ stimulation increases translocation of β-catenin into the nucleus thus increasing cell proliferation and migration and promotes colon cancer stem cells in both cell lines and xenograft mouse model [131-133]. All these effects are antagonized when blocking the activity of $CysLT_1R$ with the selective-antagonist

Montelukast [134,135]. Furthermore, *Cysltr1*$^{-/-}$ female mice, which lack $CysLT_1$ receptor, have significantly reduced tumor burden in comparison to wild type mice that have an intact $CysLT_1R$ [136].

On the other hand, activation of $CysLT_2R$ through $LTC_4$ treatment has anti-tumor effects by increasing 15-PGDH levels and inducing differentiation of colon cancer cells [137,138]. It is reported that CRC patients that express high levels of $CysLT_2R$ and low levels of $CysLT_1R$ in their tumors have a better over survival, which stresses even more the opposite effects that these CysLT receptors have in CRC [139].

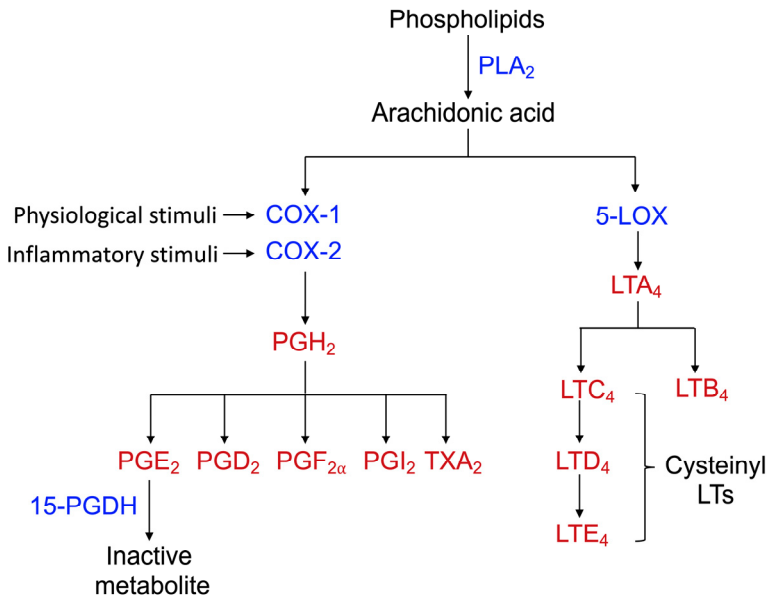


**Figure 12**. COX and 5-Lipoxygenase (5-LOX) pathways.

# Estrogen signaling in colorectal cancer

In addition to age and other risk factors mentioned in the sections above, gender is associated with CRC incidence and progression, but also with CRC localization [5-7]. For the same age, men have higher incidence of CRC than women. Right-sided colon cancer is more common in women and rectal cancer more common in men. Furthermore, a better survival is observed in young women compared to young men after CRC surgery, but in older patients the opposite pattern exists [8,9]. The use of hormone replacement therapy (HRT) has a protective role in the prevention of CRC

[10,11,140]. The biggest trial to investigate the role of HRT on CRC incidence was the Women's Health Initiative (WHI) trial with 16,608 postmenopausal women with a five-year follow-up time. The trial concluded that the short-term use of combined (estrogen + progestin) HRT decreased the incidence of CRC. However, for those women who were assigned to HRT and developed CRCs, the diagnose was made at a more advanced stage compared to women in placebo group [140]. All these findings implicate a prognostic role of estrogen signaling in CRC.

The normal colon mucosa expresses dominantly ERβ, while ERα is very little expressed [141]. However, the ERβ expression declines with CRC progression and this decline in expression correlates with tumor stage and differentiation [142,143]. Furthermore, CRC patients with high expression of ERβ in their tumors have significantly better overall (OS) and disease-free (DFS) survival [144]. A large body of evidence has reported the anti-tumor properties of ERβ induction in *in-vitro* experiments and *in-vivo* mouse models [13,145]. Over expression of ERβ in colon cancer cells reduced cell proliferation, survival and induced cell-cycle arrest and apoptosis [146] [147], down-regulated the levels of interleukin 6 (IL-6) which is a potent pro-tumorigenic inflammatory mediator [148], and reduced colon cancer metastasis by repressing *PROX1* oncogene expression [149]. Additionally, $ER\beta^{-/-}$ mice, where the receptor was knock-down, had changes in their colons characterized by hyperproliferation, dedifferentiation, decreased apoptosis and disruption of epithelial tight junctions compared with mice that had an intact ERβ [150,151]. Moreover, treatment of $Apc^{Min/+}$ mice with an ERβ-selective agonist diarylpropionitrile (DPN) reduced the number of polyps in the small intestines of both male and female mice [152]. The use of dietary phytooestrogens, which have higher affinity for ERβ than ERα, is linked to a reduced CRC incidence among populations that consume daily soy products [153] [154]. Based on all these findings, ERβ has gain popularity as a possible target for treatment of CRC [14].

Transfection of colon cancer cells to overexpress ERα resulted in activation of Wnt/β-catenin pathway. The effect was antagonised when blocking ERα [155]. Few studies have evaluated ERα expression in colon cancer tissue and reported that ERα expression has an inverse relationship with patient's survival [156,157].

# Immune cells and colorectal cancer

The development of colorectal cancer (CRC) involves a close interaction with the tumor microenvironment and the host immune response. Different immune cells have been the focus of research for decades for their role in CRC development, prognosis and treatment [3]. Depending on their subtype and the tumor microenvironment conditions, immune cells can be anti- or pro-tumorigenic [158]. T-lymphocytes play a crucial role in fighting the tumor, but if they undergo the so-called process of T-cell exhaustion they lose they anti-tumor capacity [159] [160]. In addition, tumor-associated macrophages (TAM) that have a M2-like phenotype promote colon cancer cell migration and metastasis [161]. The role of mast cells in CRC, which are among the earliest immune cells recruited during tumorigenesis, remains controversial [4].

## Mast cells

Mast cells (MCs) are granular immune cells that derive from myeloid stem cells and contain granules rich in histamine, heparin, tryptase, chymase and many other inflammatory mediators [162]. After formation, MCs leave the bone marrow and throw themselves into blood circulation to complete their maturation in mucosal or connective tissues. With a capacity to live long, up to twelve weeks, MCs are considered multifunctional as they play an important role in allergic reactions and anaphylaxis, wound healing, angiogenesis, immune tolerance and immune response against pathogens, as well as in the normal function of blood–brain barrier [163]. In human, MCs are divided in two main groups: tryptase containing MCs ($MC_T$) and tyrptase and chymase containing MCs ($MC_{TC}$). However, both types contain the all the other granules with heparin, histamine and can produce inflammatory mediators. While in mice, MCs are divided into mucosal MCs, that contain mostly chymase, very low levels of histamine and no heparin, and connective tissue-like MCs that have high concentrations of tryptase, chymase, histamine and heparin [162].

When MCs recognize their specific IgE receptors, they get activated and degranulate, releasing their content in the microenvironment. Furthermore, they also produce and release lipid mediators, such as prostaglandins and cysteinyl leukotrienes, and different cytokines and chemokines. In this way they affect cells present in the microenvironment and also recruit other immune cells (Figure 13) [163,164].

The role of MCs in CRC is controversial with studies reporting a pro-tumor effect [165-167] and others showing an anti-tumor effect [168,169]. The pro-tumor effect of MCs in CRC is mostly linked with the induction of angiogenesis due to trypsin release [165], meanwhile the anti-tumor effects are linked to production of anti-tumorigenic cytokines and activation of nuclear peroxisome proliferator-activated receptor gamma (PPAR-γ), which is associated with a better prognose in CRC patients [170,171].

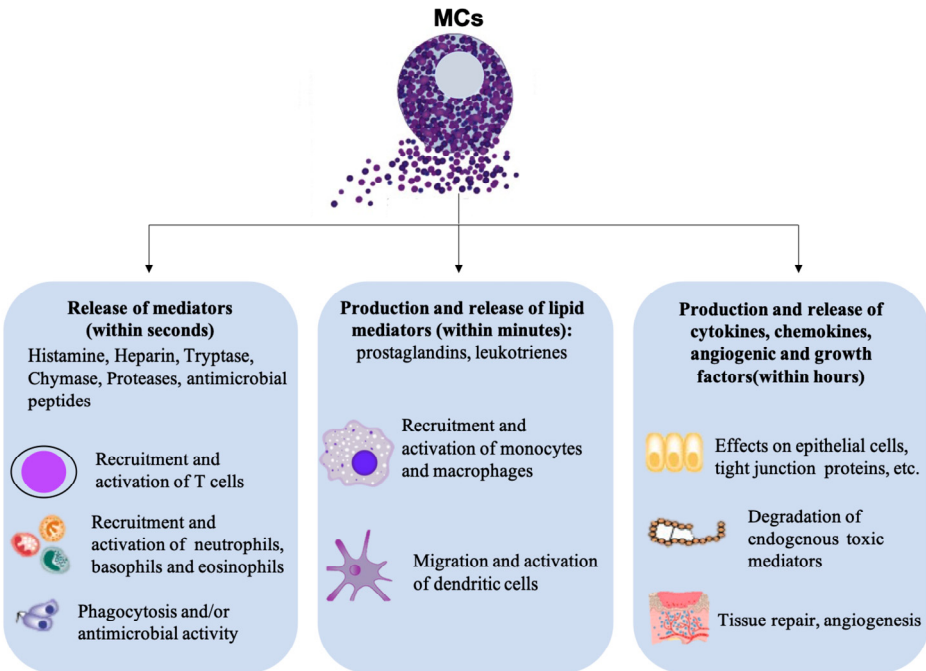


**Figure 13**. Effects of mast cells (MCs) after they get activated. Figure adapted from Metz *et al.*, 2007 [167].

# Aims and objectives

The overall aim of this doctoral thesis was to investigate the prognostic role of estrogen receptors, ERα and ERβ, in CRC, both as predictive markers for patient's survival and as possible target therapies. An additional aim was to explore the role of mast cells in patients with CRC.

In order to achieve these aims, five studies were conducted, each with the following specific objectives:

I. Paper I: To investigate the role of ERβ in a female cohort of CRC patients and its association with hormone status and lifestyle indicators.

II. Paper II: To investigate the correlations of ERβ with specific proteins (CysLT1R, CysLT2R, β-catenin, COX-2 and 15-PGDH) important for CRC development and prognosis and to explore the anti-tumor effects of the ERβ-selective agonist ERB-041.

III. Paper III: To explore the role of ERα expression in CRC prognosis; to investigate the pro-tumor effects of inducing ERα expression in colon cancer cells using the ERα-selective agonist PPT and antagonizing ERα-mediated pro-tumor effects by blocking ERα with the selective antagonist AZD9496.

IV. Paper IV: To investigate the role of concomitant expression of ERβ and ERα in predicting the prognosis of female patients with CRC and to correlate this concomitant expression with hormonal status.

V. Paper V: To explore the relevance of mast cells in CRC patients and verify the results in a colitis-associated colon cancer mouse model.

# Material and Methods

In this section will be discussed the general principles of the methods used in the research work included in this thesis. Specific details about each method are provided in the "Material and methods" section of each individual paper included at the end of this thesis.

## Study population

The patients included in this thesis belong to two different cohorts: The Female cohort and the Malmö cohort, both approved by the Ethical Committee at Lund University.

Patients from the Female cohort are included in papers I-IV. Female patients from five different cities of southern Sweden, operated for primary CRC between 1st January 2008 and 30th June 2012, physically and mentally able to participate and effectively communicate in Swedish were eligible for the study. Written informed consent was obtained from all study participants. In all the papers I-IV the following patients were excluded from the analysis: patients operated previously for CRC (n=5), carcinoid tumors (n=3), cancer stage 0 (n=3) and patients with no available pathology record (n=1). In papers III and IV, where ERα is evaluated, patients operated and treated for breast cancer before CRC surgery were excluded from the analysis due to the alteration of ERα expression (n=14).

The Malmö cohort includes both male and female patients operated for primary CRC during 1990 at the Malmö hospital. Patients form Malmö cohort are included in paper III and paper V.

## Data collection and follow-up

For all the study participants the pathology records and medical charts were collected to verify the diagnosis and collect information regarding patients and tumors characteristics. For the Female cohort, a standardized questionnaire regarding the hormonal status was sent to each participant approximately one year

after the operation. The follow-up time started at the day of diagnosis and ended at the date of death or cancer recurrence. Censoring occurred on 31st December 2000 (for the Malmö cohort) and 31st August 2016 (for the Female cohort).

# Tissue microarray and Immunohistochemistry

Tumor samples received from patients were incorporated into tissue microarray (TMA) blocks. From each tumor block two different 1.5 mm tissue cores were placed in a paraffin block. An experienced pathologist chose the tumor area for TMA production. The normal areas were chosen from the distal parts of the safe margins for all the patients included in Malmö cohort and only for 19 random patients in the Female cohort.

The tissue form TMA blocks was stained for the expression of estrogen receptors, proteins of interest and mast cells using specific antibodies which are mentioned in each individual paper. The staining intensity was evaluated using immunohistochemistry (IHC).

# The immune-reactive score (IRS)

The scoring of estrogen receptors and other proteins of interest (paper II) was performed based on the IRS (range 0 to 9) with the following formula: IRS = SI (staining intensity) × PP (percentage of positive nuclei/cells), where SI was scored as 0 = negative, 1 = weak, 2 = moderate and 3 = strong, and PP was scored as 1, <10%, 2, 11 - 50% and 3, >50%. The scoring was done by two blinded independent investigators. To determine the scoring intensity, the rules reported by Konstantinopoulos *et al*., [143] were applied as follow:

- Negative if less than 10% of positively stained cells/nuclei.
- Weak if either more than 50% of cells/nuclei were weakly stained or if 11 - 50% of cells/nuclei were moderately stained.
- Moderate if either more than 50% of cells/nuclei were moderately stained or if 11 - 50% of cells/nuclei were strongly stained.
- Strong if more than 50% of the cells/nuclei were strongly stained.

For ERα and ERβ the nuclear expression was taken in consideration. ERβ expression was grouped as low (negative and weak) and high (moderate and strong), while ERα expression was grouped as negative and positive (if more than 10% of the nuclei were stained regardless the intensity).

For all the other proteins of interest in paper II, $CysLT_1R$, $CysLT_2R$, COX-2 and 15-PGDH the cytoplasmic staining was evaluated; for β-catenin the nuclear and membrane staining was taken in consideration. In all the papers, cores with tissue loss or with only stromal tissue were considered unreadable and therefore excluded from the analysis.

# Mast cell density (MCD)

Two independent investigators evaluated blindly the MCD for each patient's tissue core form the Malmö cohort. MCD was defined as the total number of tryptase - and chymase - positive MCs in the whole tissue core (ø 1.5mm). Patients were grouped in low and high MCD based on the mean MCD and the frequency of distribution in the $25^{th}$, $50^{th}$ and $75^{th}$ percentiles; cutoff was 10 cells for tryptase-positive MCs and 50 cells for chymase - positive MCs. For the muse tissue, MCD was calculated as the number of tryptase - positive MCs per hotspot and the average of two hotspots per mouse was considered.

# Colon cancer mouse models

*Colitis-associated colon cancer*

Colitis-associated colon cancer (CAC) mouse model was used in paper V to investigate the presence of MCs in the tissue of wild-type mice (C57BL/6N) and mice that have a Cysltr1 gene disruption (*Cysltr1*$^{-/-}$). This chemically induced colon cancer model is based on the peritoneal injection of the carcinogen azoxymethane (AOM), 10 mg/kg body in 6 to 8-week-old female mice, followed by three cycles of the colon irritant dextran sodium sulfate (DSS) in the drinking water for five days. The experiment lasted for 90 days and after that the mice were sacrificed, their colons were dissected, fixed in formalin, embedded in paraffin and sectioned. The colon tissues from this model were also used in paper II and III to evaluate the expression of ERβ and ERα.

The CAC model was also used for mice with a *Cysltr2* gene disruption, *Cysltr2*$^{-/-}$, and the colon tissues were stained for the ERβ expression (paper II).

*Apc*$^{Min/+}$ *colon cancer mice model*

In the *Apc*$^{Min/+}$ model the mice develop spontaneous polyps in the small intestine and tumors in the colon. Due to the *Apc* mutation, the Wnt/β-catenin signaling is 'ON', preventing the phosphorylation and degradation of β-catenin and promoting its

translocation into the nucleus. Therefore, the tissue from this model was used in paper II and III to evaluate the expression of ERβ and ERα in order to validate their correlation with nuclear β-catenin.

## *In-vitro* experiments

With the purpose to investigate the effects of ERβ and ERα induction on colon cancer cells, *in-vitro* experiments were conducted in paper II and III. SW-480, HCT-116, HT-29 and Caco-2 colon cancer cell lines were treated with an ERβ-selective agonist (ERB-041) and antagonist (PHTPP), and ERα-selective agonist (PPT) and antagonist (AZD9496). The treatment was done twice per day for 48 up to 72 h depending on the experiment. To prove the specificity of the treatment the receptors were knock-down using specific *siRNAs*. Treated cells were compared with untreated/vehicle cells and cell proliferation, migration and survival were evaluated, as well as mRNA and protein expression of $CysLT_1R$, $CysLT_2R$, COX-2,15-PGDH and β-catenin. The different techniques used for evaluates of protein expression, cell proliferation, migration and survival are written in details in paper II and III.

## *In-vivo* zebrafish xenograft model

With the aim to investigate the role of ERβ and ERα in colon cancer cell metastasis, an *in-vivo* zebrafish xenograft metastasis model was conducted. The method is described in detail in paper II and III. Briefly, untreated and ERB-041-treated (paper II) and PPT-and AZD9496-treated (paper III) colon cancer cells were microinjected into the perivitelline space of the zebrafish. Cells were let to migrate for 48 h and after that, pictures of the zebrafish tail, which was considered as the distant metastatic site, were taken using a fluorescence microscope and compared with the corresponding pictures at the time of injection. Before microinjection into the zebrafish, cells were labeled with Vybrant-DiI (red color) to facilitate their identification into the zebrafish.

## Statistical analysis

Mann-Whitney or t-test for continuous variables, or $\chi^2$ or Fisher's test for categorical variables were used as indicated in each of the papers. The Cox regression model was used to compute hazard ratios (HRs) for the probability of death or cancer recurrence. Survival curves, adjusted for age, TMN stage and tumor

intravascular invasion were generated using the Kaplan-Meier method and compared with log-rank test. Correlations between variables were assessed by Spearman or Pearson correlation coefficient. The predictive ability of the final models for OS and DFS in paper I, III and IV was evaluated using receiver-operating characteristic (ROC) curves and the area under the ROC curves (AUC) was calculated for each model. All tests were two-sided, and differences with p values $<0.05$ were considered statistically significant.

# Public databases

To validate and support the findings observed in patients, in paper II, III and IV, publicly available mRNA data were used form TCGA database [172], a CRC cohort with 688 patients from Budinska *et al* [173] and from Sieber et *al*., with 286 patients [174].

# Results

*Main findings paper I*

ERβ expression was significantly reduced in CRC tissues compared with matched normal tissues. Patients with high ERβ expression had 50% reduced risk for overall mortality (HR, 0.50; CI, 0.30-0.83) and 76% for cancer recurrence (HR, 0.24; CI, 0.11-0.52) after adjusting for age, TNM stage and tumour intravascular invasion. Furthermore, high ERβ expression significantly correlated with shorter breastfeeding time and longer use of hormone replacement therapy. No association was found between ERβ expression and lifestyle indicators.

*Main findings paper II*

Patients with high ERβ expression had significantly higher levels of $CysLT_2R$, membrane-associated β-catenin, and 15-PGDH, all have anti-tumor effects, and additionally lower levels of $CysLT_1R$, COX-2 and nuclear β-catenin, all with tumor-promoting effects. These correlations were supported by publicly available databases with mRNA data from CRC patients, and by inducing ERβ expression in colon cancer cell lines using the ERβ-selective agonist ERB-041. Furthermore, ERB-041-treated cells showed significantly decreased migration, survival and colony formation, and increased apoptotic activity evaluated by CASPASE-3 activity and formation of apoptotic blebs. Finally, ERB-041-treated cells showed significantly lower tail metastasis in the *in-vivo* zebrafish xenograft model compared to vehicle-treated cells.

*Main findings paper III*

ERα expression was significantly higher in cancer tissues compared to the matched normal tissues. Positive ERα expression was independently associated with worse OS (HR=0.42; 95% CI: 0.26-0.51) and DFS (HR=0.32; 95% CI: 0.18-0.56), after adjustment for age and TNM stage, and with worse tumor outcome (higher TNM stages, more metastasis in regional lymph nodes and distant organs). Moreover, patients with positive ERα expression had higher levels of $CysLT_1R$ and nuclear β-catenin, both with potent tumor-promoting effects. *In-vitro*, treatment with ERα-selective agonist PPT significantly increased cell migration and invasion, $CysLT_1R$ and nuclear β-catenin levels, and decreased the phosphorylation of β-catenin and protein expression of ZO-1, an important cell adhesion protein, compared to vehicle-treated cells. These tumor-promoting effects of PPT were reduced when

adding to the cells the ERα-selective antagonist AZD9496. The effects of AZD9496 treatment were much stronger when combined with ERB-041 treatment, especially for increasing the expression of ZO-1 at the cell membrane and reducing the protein levels of nuclear β-catenin. The zebrafish xenograft metastasis model showed that fish injected with PPT-treated cells had significantly more tail metastasis compared to fish injected with vehicle-treated cells (control group); meanwhile fish injected with AZD9496-treated cells in addition to PPT treatment showed drastically less tail metastasis compared to both PPT and control groups.

*Main findings paper IV*

Concomitant expression of high ERβ and negative ERα in CRC tissues correlated with longer OS and DFS, better tumor outcome (smaller tumor extends, less metastases in the regional lymph nodes and distant organs, predominantly stage I and II), higher expression of anti-tumorigenic proteins and lower expression of pro-tumorigenic proteins compared with tumors with a concomitant expression of low ERβ and positive ERα. Most importantly, concomitant expression of ERβ and ERα improved the predicting ability for the risk of cancer recurrence, when added to the basic model adjusted for age, TNM stage and tumor vasal invasion, compared with extended models were only ERβ or ERα were taken into consideration. Interestingly, tumors with high ERβ and negative ERα expressions were associated more with the mucinous type of colon adenocarcinoma and a never smoking status. Additionally, female patients with high ERβ and negative ERα expressions had lower number of pregnancies, shorter breastfeeding time, never use of HC and long-term use of HRT, both estrogen monotherapy and combined HRT, compared with female patients with low ERβ and positive ERα expression levels.

*Main findings paper V*

Colon cancer tissues had a reduced number of MCs, predominantly chymase-positive cells, in comparison with normal colon tissues. Patients with high MCD in their cancer tissues showed significantly longer overall survival compared to those with a low MCD (HR, 0.539; 95% CI, 0.302-0.961), independently of gender and cancer stage. In addition, a negative correlation was found between cytoplasmic $CysLT_1R$ expression levels and number of MCs. In support of this finding, in the CAC mouse model, *Cysltr1*$^{-/-}$ mice showed significantly higher MCs in their polyp/tumor areas compared with wild-type mice.

# General discussion

In this research work I have investigated the role of estrogen receptors and mast cells in colorectal cancer patients, cell lines, mouse models and *in-vivo* zebrafish xenograft model. I found that high ERβ expression levels in CRC tissues significantly correlated with better tumor profile, longer patient's OS and DFS, higher expression levels of anti-tumorigenic proteins and lower expression levels of tumor-promoting proteins. These anti-tumor effects of high ERβ expression in CRC were further supported by publicly available databases with mRNA data for CRC patients, CAC-mouse models with *Cysltr2* and *Cysltr1* gene disruption and $Apc^{Min/+}$ mouse model. The beneficial role of high ERβ expression in CRC patients is reported by previous studies conducted in cohorts with both males and females [144,175]. Even though the criteria used for scoring ERβ expression were comparable to ours, Fang *et al.* used a different antibody [175], whereas the same anti-ERβ antibody was used by Rudolph and colleagues [144].

Furthermore, I investigated the anti-tumor effects of ERβ induction using the ERβ-selective agonist ERB-041 in different colon cancer (CC) cell lines and in an *in-vivo* zebrafish xenograft metastasis model and concluded that ERB-041 has important anti-tumor effects in CRC by inducing specifically the expression of ERβ. To the best of our knowledge, this research work is the first to report a direct correlation between ERβ, $CysLT_1R$, $CysLT_2R$ and 15-PGDH. Furthermore, by knocking-down $CysLT_2R$ in CC cells we showed that ERB-041-mediated effects on cell migration and colony formation were mediated via $CysLT_2R$. Our findings suggested that ERB-04 acts on transcriptional levels to downregulate or upregulate the expression of these genes. These multitarget effects of ERB-041 are complex and further mechanistic studies are required in order to prove if the effects on gene expressions are direct or indirect. However, these findings provide important prognostic information about the possible use of ERB-041 as a treatment opportunity in CRC patients. Previous studies reported valuable and important information about the possible underlying mechanisms of anti-tumor effects of ERβ induction in CC cells, such as repression of micro-RNA 17 (miR-17) to influence cell death upon DNA damage [176], downregulation of interleukin-6 (IL-6) and downstream networks such as MAPK pathway [148] and reduction of CC metastasis by repression of *PROX1* gene which is linked to poor prognose in CRC [149]. Additionally, we reported that ERB-041 reduced CC cell metastasis in an *in-vivo* zebrafish xenograft model. The treatment of $Apc^{Min/+}$ mice with the ERβ-selective agonist DPN reduced

the number of polyps in the small intestines by modulation of the TGFβ pathway [152]. It would be to huge interest to validate this finding by using ERB-41 as a future prospective.

Next, I investigated the role of ERα expression in CRC tissues, its role in patient's prognosis and the pro-tumorigenic effects of ERα induction in CC cells. The results revealed that CRC tissues had significantly higher expression levels of ERα compared with the matched normal tissues based on the IHC analysis, a finding which was further confirmed by ERα protein expression in the paired normal and cancer tissues from 6 CRC patients. In contrast to this finding, a previous report showed that ERα mRNA levels were not significantly different between paired tumor and mucosa samples in either males or females, and generally ERα mRNA levels in tumor tissues were lower than in normal mucosa [177]. However, as mentioned, the authors investigated mRNA levels of ERα which are not always translated in protein expression. In addition, we found that positive ERα expression is associated with worse tumor outcome, worse OS and DFS, higher levels of $CysLT_1R$ and nuclear β-catenin, further supported by publicly available mRNA data for CRC patients and mouse model data. Differently from us, Lopez et al., reported no correlation between ERα expression and clinicopathological features, but instead showed a correlation between high ERα expression with older age and higher glucose levels [156]. However, the authors of this study took in consideration the cytoplasmic expression of ERα while we evaluated only ERα nuclear expression. In cell line experiments PPT treatment, increased migration and invasion, also reduced the protein levels of ZO-1, an important tight junction protein to maintain cell to cell contact. This could possibly explain, among other factors, increased tail metastasis in the zebrafish xenograft injected with PPT-treated CC cells. Very few studies have evaluated the prognostic role of ERα in CRC, with a common conclusion that increased ERα expression had a negative impact in CRC prognosis and survival [156,157]. While previous report has showed the activation of Wnt/ β-catenin by overexpression of ERα in CC cells [155], our study is the first to demonstrate a correlation between ERα and $CysLT_1R$ expression levels.

Finally, I investigated the prognostic role of MCD in CRC patients and found that patients with high MCD in their cancer tissues had independently longer OS compared with patients with low MCD. This result was supported by the finding of higher number of MCs in polyp/tumor areas of *Cysltr1*$^{-/-}$ mice, which are reported to exhibit a less aggressive phenotype [178], compared with wild-type mice. In contrast to our finding, other studies propose that high MCD in the CRC tissue promotes tumor development and progression by promoting angiogenesis [165,166]. However, these studies took in consideration tryptase-postive MCs, whereas in our study we had to a high extend chymase -positive MCs and very few tryptase-positive cells were identified in both normal and cancer tissues. On the other hand, our results go in line with several reports that showed an anti-tumor effect of MCs due to chymase release that possesses proapoptotic effects [170],

production and release of prostaglandin D2 $PGD_2$, which promotes apoptosis and cell differentiation and inhibits angiogenesis [119]. A reversal shift between chymase- and tryptase-positive MCs is related with the capacity of MCs to promote cancer progression [4,164]. This is an important finding, as in our study we did not observe a shift between the MCs phenotypes and chymase-positive MCs were dominantly expressed in all patients in both normal and cancer tissues. High levels of $CysLT_1R$ in CRC patients are linked to a poor prognosis [138] and in our study we found a significant negative correlation between cytoplasmic expression of $CysLT_1R$ and number of MCs, which further supports our results for an anti-tumor effect of MCs in CRC.

# Translational relevance

Despite the current treatment therapies, cancer recurrence remains a major risk for CRC patients [179]. This creates the need for more prognostic markers, to identify patients with an increased risk for relapse, and targeted therapies to help establish individualized treatment for patients with CRC. In a recent report that investigated twenty-six cancer-related pathways, inactivation of the ER pathway was a common finding in CRC [180], which highlights even more the need for ERβ-selective agonist treatments to improve the progression of disease and patient outcomes.

Our findings, which of course need to be further validated using *in-vivo* mouse models of colon cancer, provide a valuable information about the possible use of ERB-041 as a target therapy in CRC patients. Additionally, we showed that even patients who are considered to have a poor prognosis, such as patients with high $CysLT_1R$, nuclear β-catenin and COX-2 expression levels, still have significantly better DFS rates as long as they have high ERβ expression. Based on this finding, we suggest that target therapies aimed to induce ERβ expression can be beneficial even for CRC patients with poor prognosis and limited treatment opportunities.

Since our findings suggest that ERα expression is significantly increased in CRC tissues and patients with a negative ERα expression have better OD and DFS, the use of ERα-selective antagonists can be beneficial in CRC patients with positive ERα, in combination with current therapies. In addition, we found that the majority of patients with positive ERα expression had higher frequency of activated KRAS mutation, whereas the majority of patients with negative ERα expression had a wild-type KRAS. This result was further supported by mRNA data from TCGA COAD database, where we found a significant positive correlation between mRNA levels of *ESR1* and *KRAS*. This is an important and can open new treatment opportunities for patients with activated KRAS mutation since they do not benefit from anti-EGFR therapy [181].

All the pro-tumorigenic effects of ERα in CC cells induction by PPT were antagonized by the ERα-selective antagonist AZD9496, which in addition to PPT or in combination with ERb-041 significantly reduced tail metastasis in the zebrafish xenograft model, decreased nuclear β-catenin and increased β-catenin phosphorylation and ZO-1 expression in CC cells compared to both PPT-treated and vehicle-treated groups. AZD9496, which degrades ERα when binds to it, is the first oral estrogen antagonist that has shown higher bioavailability compared to fluvestrant and has been successfully tested in clinical trials of breast cancer patients [54,55]. Based on our findings, AZD9496 treatment can be very beneficial for female patients, who apart breast cancer, have developed CRC or vice versa. An example are female patients with Lynch syndrome. In our Female cohort we had 14 patients that were operated and treated for breast cancer before CRC surgery.

Finally, we showed that the concomitant expression of estrogen receptors, when added to the basic model, gave the best predicting ability, especially for DFS, compared to other models where only the expression of one estrogen receptor was taken in consideration. These findings propose that concomitant expression of ERβ and ERα could be beneficial to predict the risk of cancer recurrence in CRC patients; however, validations using other cohorts of patients are needed.

# Methodological considerations

*Paper I*

The study had certain strength and limitation. The events of interests were verified by medical records; therefore, misclassification is unlikely. All CRC samples were chosen randomly from 5 different cities of Sweden, which lowers the risk of selection bias.

The sample size was considerable with a study power of 80%. We had a very low proportion of samples with unsuccessful measurement of ERβ (only 1.9 %). However, for the association with life style indicators the sample size was quite small, due to unavailable information for patients operated between 2008-2009.

We did a sensitivity analysis excluding patients older than 85 years to control for the risk of recall bias, but no change was seen regarding the association between ERβ and hormonal status. The questionnaire was self-reported; therefore, the risk of information bias cannot be excluded, especially for information regarding alcohol consumption, smoking and physical activity.

The use of TMAs in cancer research raises the concern whether the punched tissue is representative of the whole cancer. However, the use of two cores to represent the tumor has shown sufficient concordance for many cancer types, including CRC [182].

Radiotherapy neoadjuvant treatment given in case of rectal cancer can affect the epithelial cells and the tumor microenvironment. There were 52 patients (17%) in our Female cohort who underwent radiotherapy treatment before tumor resection via surgery. It should be noted that radiotherapy treatment before resection, affects the tissue, as indicated by differentiation reported in the pathology record after surgical removal, and might also affect ERβ expression. That could possibly explain why we did not observe a significant association between OS or DFS with ERβ expression in the subgroup of rectal cancer patients.

The antibody that we used, 14C8, recognizes apart wild-type ERβ, most of ERβ variants, and is shown to be useful for assessment of ERβ expression in paraffin-embedded tissues [183]. A recent publication that validated three different antibodies in 44 different tissues, showed that the best and most specific antibody for detection of ERβ in IHC and especially protein expression is PPZ0506 [184]. However, for colon tissue 14C8 antibody showed in IHC the same intensity band as PPZ0506, and that correlated with ERβ mRNA levels detected in the tissue (Figure 3 of ref. [184]). Moreover, the 14C8 antibody used in this study was from GeneTex (Irvine, California, USA), while we have used the one from Abcam (Cambridge, UK).

*Paper II* and *III*

Charcoal-stripped bovine serum (CSS) is reported to play an important role when studying steroid hormones, especially in breast and prostate cancer [185]. CSS is also used many studies with colon cancer cell lines to study the role of ERs. For all experiments in paper II and III the treatments with agonists/antagonists are done in 1.5% fetal bovine serum (FBS). Before the treatment, cells were put in serum-free (0% FBS) media overnight and treatment was done next morning. We also repeated the experiments using CSS; 24 h before treatment cells were left in 5% CSS for hormone-deprivation, and after that the treatment was done using 5%, 1.5% and 1% CSS (based on previous studies). We observed no significant change between the results achieved using 1.5% FBS media and CSS media (regardless the percentage of CSS used). This could be related with the fact that hormone-deprivation is important in case of breast or prostate cancer, because the development of this cancers is strongly related with the hormonal microenvironment and cells get sensitized towards the respective hormone, which can mask the effect of the treatment [185]. On the other hand, serum-free media is reported to be an alternative medium to mimic hormonal-deprivation in human prostate cancer cell lines [186]. Furthermore, if any additional effect of 1.5% FBS media during the treatment, it was present also in vehicle-treated control cells; therefore, the effect is normalized.

In all our experiments, we used the basal levels of ERα and ERβ in colon cancer cell lines and used selective agonists to induce the expression of the receptors, which we think mimics better the physiological conditions; whereas in other studies have overexpressed ERα and/or ERβ were overexpressed.

To investigate the effects of ERα and ERβ in colon cancer cell metastasis, we used an *in-vivo* zebrafish xenograft metastasis model. Both embryonic and adult zebrafish express ERα and ERβ [187]. However, the treatment of colon cancer cells with ERB-041, PPT and AZD9496+PPT was done in vitro, and then after 48h the cells were injected into zebrafish embryos. This normalized any possible effect of zebrafish endogenous ERs in both the control and experimental groups. A question raised could be that *in-vivo* nude mouse xenograft model would have been better to study the effects of ERB-041on tumor reduction and metastasis. The efficacity of ERB-041 in reducing tumor growth has recently been reporter, mice treated with ERB-041 had fewer and smaller skin tumors compared to the control group [188]. Regarding the effects of ER agonists/antagonists in colon cancer metastasis, we believe that the zebrafish is a very good, elegant and popular *in-vivo* model to study cancer metastasis due to the convincing visualization, the ability to monitor the marker of interest and observe cellular and molecular processes *in-vivo* in real time. Furthermore, the experiment time is shorter using the zebrafish xenograft model, only 48 h, whereas in a mouse xenograft model it is a minimum of 3 weeks that the metastases develop in the liver or other organs. Additionally, the facilities to carry out a zebrafish xenograft model, compared to a mouse model, are better. Zebrafish can produce a large number of offspring at low cost, allowing performance of a larger number of experiments compared to mouse models.

The antibody used in IHC to evaluate ERα expression is a cocktail antibody (1D5 + 6F11) that is created by mixing two monoclonal antibodies that target ERα. To validate the IHC staining, another ERα monoclonal antibody D-12 (sc-8005) was used to randomly stain two TMA blocks. The same tissues that were found positive for ERα expression using the cocktail antibody, were also stained positive with D-12 antibody but the staining intensity was weaker.

*Paper V*

The discrepancy between previous and our findings, in terms of high MCD and patient's survival in CRC, could perhaps be affected by the method of staining and the patient cohort. In the present study we used a patient cohort from 1990. Indeed, the sample size was small, only 72 patients, but the cohort is very specific, in terms that CRC patients were rarely given at that time neoadjuvant or adjuvant radio/chemotherapy [189]. Therefore, this cohort is well suited for analyzing the importance of any biomarker, including MCs, for the survival of these patients. Some studies used Giemsa blue staining or toluidine blue while we used anti-chymase and anti-tryptase antibodies.

Chymase-positive MCs were predominant in both normal and cancer tissues, compared to tryptase-postive MCs. A possible explanation can be that tryptase is more difficult to be captured in the IHC due to either short life or easily destruction during tissue fixation. Also, the characteristics of the tissue microenvironment that influence the reversal shift between chymase- and tryptase-positive MCs, can be another factor. However, no association was found between OS and tryptase-positive.

# Final conclusions

- There was a significant decrease of ERβ and an increase of ERα in the cancerous tissues compared with the normal colonic mucosa in CRC patients.
- In female patients with CRC, high ERβ expression is independently associated with a better prognosis and reduced risk of cancer recurrence. ERβ expression in female CRC tissues was associated with hormone status but not with life style indicators.
- ERβ-selective agonist ERB-041 increased the expression of antitumorigenic proteins, decreased the translocation of β-catenin into the nucleus and reduced the metastatic burden of colon cancer cells.
- CRC patients with positive ERα expression have a poor prognosis and increased risk of cancer recurrence. The use of ERα-selective antagonist AZD9496 in addition to PPT or ERB-041 treatment significantly increased β-catenin phosphorylation and ZO-1 expression and reduced nuclear β-catenin and metastatic burden of colon cancer cells.
- Concomitant expression of ERβ and ERα improved the predicting ability for the risk of cancer recurrence, when added to the basic model adjusted for age, TNM stage and tumor vasal invasion, compared with other models where only one of the estrogen receptors was taken into consideration.
- High chymase-positive MCD in the tumor tissue is independently associated with longer overall survival in CRC patients.

Taken together, these results provide valuable information to create new treatment opportunities and combine treatment therapies, aiming to improve the prognosis of colorectal cancer patients towards a more individualized treatment.

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