Between cancer and therapy: Studies of the colon
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CHAPTER 2

Sexual dimorphism in colon cancer development

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\textit{Review in preparation}
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ABSTRACT
Male gender is one of the most important risk factors for the development of colorectal cancer (CRC), suggesting that this disease behaves in a sex hormone dependent manner. Here we highlight the role of sex hormones in the development of CRC both from a clinical and experimental point of view. The available data suggest that sex hormones may be an interesting target for preventive or therapeutic treatment of this disease.
INTRODUCTION

Men have a twofold increased risk for developing colorectal cancer (CRC) compared to women and this risk is apparent throughout all stages of CRC development including benign precursor lesions. Next to male sex, a large number of other risk factors are recognized (Table 1). Patients with active inflammatory disease of the colon such as ulcerative colitis or Crohn’s disease have a relative risk of 5.6 and are subjected to repeated screening colonoscopies. Recent advances in treatment modalities for inflammatory bowel diseases may have reduced this risk considerably. Patients that have a family history of CRC have a relative risk of 2.4. These patients often harbor mutations that cause hereditary cancer syndromes. Since CRC development of both hereditary cancer and inflammatory disease occurs through distinct molecular mechanisms they are in fact regarded as distinct diseases. Therefore, for the remaining 75% of sporadic CRC (e.g. those cancers that develop in the absence of overt risk factors), male sex is one of the strongest associated risk factors and arguably the most important contributor epidemiologically due to the rarity of the other risk factors. It has however received little scientific attention thus far.

Table 1. Risk factors for CRC

<table>
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<th>Risk factors for CRC</th>
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<th>Reference</th>
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<tr>
<td>Ulcerative Colitis</td>
<td>5.7</td>
<td>Ekbom, NEJM 1990</td>
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<tr>
<td>M. Crohn (colonic disease)</td>
<td>5.6</td>
<td>Ekbom, Lancet 1990</td>
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<tr>
<td>First degree relatives</td>
<td>2.4</td>
<td>Johns, Am J Gastr 2001</td>
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<td>High social class</td>
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<td>Teppo, SJWEH 1984</td>
</tr>
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<td>Obesity</td>
<td>1.3</td>
<td>Larsson, Am J Clin Nutr 2007</td>
</tr>
<tr>
<td>Acromegaly</td>
<td>2.5</td>
<td>Baris, CCC 2002</td>
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<td>Red meat</td>
<td>2.2</td>
<td>Willet, NEJM 1990</td>
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<td>Smoking</td>
<td>1.1</td>
<td>Terry, Int J Canc 2001</td>
</tr>
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<td></td>
<td>1.3</td>
<td>Nyren, J Nat Canc Inst 1996</td>
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<td>2.0</td>
<td>Ferlitsch, JAMA 2011</td>
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<td></td>
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Long apparent sexual dimorphism in CRC development has been contributed to environmental, behavioral and toxicological differences. Mounting evidence over the last decades suggests however that adenoma and cancer formation are critically regulated by sex hormones but how tumorigenesis is influenced by sex hormones remains largely elusive. In this review we will discuss evidence pointing towards sex hormones as major determinant of the risk to develop CRC.
PART 1: GENDER DIFFERENCES IN CRC IN HUMANS

The notion that men are at an increased risk of developing colorectal cancer (CRC) already dates from the middle of the previous century and has repeatedly been corroborated\textsuperscript{2,3,13,16}. Men do not only exhibit higher incidence rates of colorectal cancer compared to women, but disease presentation in men is at a more progressed stage (patients diagnosed with Dukes’ stage D disease in men vs. women is 22 vs. 16.1 percent) and men have worse survival outcomes than women (overall survival in men vs. women is 52 vs. 57.8 months)\textsuperscript{17,18}. Furthermore, whereas the relative risk for CRC development in men increases with age, this is mainly noted for tumors that occur on the left side of the colon\textsuperscript{19-21}. Large screening studies that employed colonoscopy in asymptomatic individuals have corroborated male sex as a risk factor in development of CRC\textsuperscript{3,13}. These and other studies additionally showed that CRC development in men is more prevalent in all age groups\textsuperscript{3}. Colorectal carcinomas develop from adenomatous precursor lesions and adenomas that harbor high risk for malignant transformation. These high-risk lesions are collectively called ‘advanced neoplasias’ and include adenomas larger than 1 cm, adenomas with villous histology and adenomas with high-grade dysplasia. Men are not only at increased risk for the development of colorectal carcinomas but also have an increased incidence of adenomas and advanced neoplasias compared to women\textsuperscript{3,22}. Thus, compared to women, men have an increased risk of developing colorectal cancer and this sex difference is apparent at all stages of colon cancer development. This argues that the factors that promote colon cancer development in men act at an early stage of tumor development.

Female hormones

In an attempt to explain gender disparity in CRC development by effects of a hormonal factor, the observation that nuns experience excess not only of known hormonal cancers (breast, ovary and endometrium), but also of colon cancer, was among the first reported evidence\textsuperscript{23}. Later, analysis of reproductive data of colon cancer patients confirmed protection by reproductive and hormonal factors\textsuperscript{24-26}. It must be noted however that parity does not only alter circulating female hormones but also effects levels of other hormonal substances that may be involved in carcinogenesis such as growth hormones\textsuperscript{27}.

A number of studies next examined protective effects of female hormone replacement therapy (HRT). In a large population based study mainly focusing on estrogen treatment, Calle et al. found that ever use of estrogen replacement therapy, either as monotherapy or in combination with a progestin, was associated with a 29% reduced risk of developing fatal CRC\textsuperscript{28}. Later, analyses of subgroups in a Swedish cohort exposed that not estrogen monotherapy but a combination of estradiol and the synthetic progestin levonorgestrel protected women from development and mortality of CRC\textsuperscript{29}. These studies differed in the aspect that the latter included postmenopausal women exclusively whereas the former included both pre- and postmenopausal women. Additional studies corroborated protective effects that emanated from a combination of estradiol and a progestin but also of estrogen monotherapy. Additionally, it was shown that longer use of HRT
was associated with increased protection\textsuperscript{30-32}. Lastly, HRT in women already diagnosed with CRC improved survival outcomes\textsuperscript{33}. Next to differences in tumor development, estrogen use results in reduced incidence of microsatellite instable (MSI) tumors\textsuperscript{34}. These tumors are a distinct subtype of CRC that bear a more beneficial prognosis\textsuperscript{35}. The retrospective observational studies thus demonstrate protective effects of estrogens, but more likely a combination of estrogens and a progestin. Probably, protective effects are largest in postmenopausal women lacking endogenous production of these hormones.

The first, and to date only, prospective intervention studies were performed a decade later. Two double blind randomized controlled trials examining HRT in postmenopausal women exclusively, were initiated by the Women’s Health Initiative (WHI). A first study showed that treatment with equine estrogen plus the progestin medroxyprogesterone acetate (MPA) substantially reduced the overall risk of developing colorectal cancer compared to placebo (odds ratio = 0.63) after five years follow-up\textsuperscript{36}, confirming previous cohort data. No such protection was found in the second RCT in which women, that had previously undergone hysterectomy, were treated with equine estrogen alone (odds ratio = 1.08)\textsuperscript{37}. Thus a combination of estrogen and a progestin protect against CRC. Although retrospective data suggest that estrogen monotherapy may exert some protective effects before menopause, it does not influence tumorigenesis in postmenopausal women.

Furthermore, in intestinal epithelium, the 5' promoter region of the ERβ gene increasingly undergoes CpG island methylation with age. This epigenetic feature, closely correlated to silencing of gene transcription was found more frequently in cancer tissue compared to patient matched normal mucosa\textsuperscript{38}. Similarly, ERβ expression was found to be lost in cancers\textsuperscript{39,40}.

\textbf{Progestins}

The WHI studies showed that a combination of estrogen and the synthetic progestin medroxyprogesterone acetate (MPA) exhibited marked reduction in CRC prevalence in postmenopausal women, whereas estrogen monotherapy was unsuccessful\textsuperscript{36,37}, advocating a critical role for progestins or progesterone signaling during intestinal tumorigenesis. Progesterone, most known for its functions in female fertility signals through the progesterone receptor (PR), of which at least two splice variants with distinct functions are known\textsuperscript{41}. Expression of the progesterone receptor has been shown in normal colonic mucosa and colon cancer using binding studies and techniques that quantified mRNA\textsuperscript{42-45}. Immunohistochemical studies however showed only one out of 156 colon cancer samples expressing PR\textsuperscript{46}. Likely, cells that express PR in colorectal tumors and normal tissue are of non-epithelial mesenchymal or hematological origin. Therefore involvement of the progesterone receptor in CRC development is likely to occur in an independent manor. It must be stated however that in contrast to ovary-derived progestins, the synthetic progestin MPA has the capacity to disrupt androgen signaling by competitive binding to the androgen receptor\textsuperscript{47}. This leaves the option open of involvement of the AR instead of the PR in protection against CRC by MPA.
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Male hormones: androgens

In human disease, to date no clinical intervention studies have been performed that show a link between androgens and CRC and data of patients that were treated with anti-androgenic agents are biased since these patients virtually all suffer from prostate cancer.

PART 2: ANIMAL MODELS OF CRC

Experimental challenges in studying the relationship between sexual dimorphism and colon cancer development

Animal models for cancer

Many animal models are used for studies on human colorectal cancer of which only few model invasion, metastasis and death. These models are of complex genetic makeup and often require colonic treatment with abrasive substances such as dextrane sodium sulfate (DSS). Instead of developing full blown cancers, the most frequently used animal models become moribund already upon development of premalignant adenomas and therefore do not develop invasive disease frequently and do not metastasize. The discrepancy between adenomas and cancer has been a matter of debate in the interpretation of rodent studies. In the human situation however, it was importantly shown that gender disparity is not only limited to full-blown CRC, but commences at the smallest visible lesion found when using endoscopic procedures. Therefore, models that examine adenomatous precursor lesions may yield valuable information on development of human CRC.

Ovariectomy versus menopause

Another dilemma encountered in experimental studies on the effect of sex hormones, most particular ovarian hormones, is the fact that most experimental animals do not enter menopause at advanced age. Most cancer in humans develops at advanced age and most often, women in this age are in the postmenopausal period. The most frequently used method to mimic this phase experimentally is making use of ovariectomies. The most important clinical studies in humans on CRC and hormone replacement have exclusively studied postmenopausal women that lacked production of endogenous ovarian hormones due to physiological postmenopausal ovarian failure, a situation that is hormonally distinct from the post-ovariectomy state. After removal of ovaries, GnRH and subsequently LH and FSH are upregulated highly as a result of reduced feedback of estrogens on the hypothalamus and pituitary. In postmenopausal women, this feedback mechanism is found to a similar extent at first, but tends to attenuate over time. Furthermore, although ovaries in postmenopausal women do not produce female sex hormones, they may produce other substances that have oncogenic effects, such the androgen androstenedione. Although murine models for induction of ovarian failure and therefore induction of the menopause have been described, they have yet to be used in investigation of hormonal influences on intestinal tumorigenesis.
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Hormonal feedback in receptor knockout animals
Upon measurement of estrogen levels in mice that genetically lacked the estrogen receptor alpha (Erα⁻/⁻), it was found that these animals had highly increased levels of estrogens and as a result had increased signaling of the estrogen receptor beta isoform. To correct for feedback effects in production of ovarian hormones when using animals that genetically lack a specific hormone receptor or in which hormonal balance is shifted by supplementation, it is thus necessary to block endogenous hormone production (most often by performing ovariectomies) and supplement ovarian hormones to desired levels. Lack of ovariectomies and replacement has rendered a large volume of experiments difficult to interpret and in the current review, we refer solely to those articles in which hormonal status of experimental animals has been controlled for by ovariectomies and supplementation of steroids.

Sexual dimorphism in animal models of CRC
We have recently identified two genetic animal models of colonic adenoma development that show clear sexual dimorphism with male tumor predominance. The identification of these models argues that sex differences in colon cancer development in humans may be a phenotypic difference between the sexes that is related to differences in circulating sex hormones rather than differences in diet, smoking behavior or other environmental exposure factors. One of the two models with a clear male predominance in adenoma development is the ApcPirc/⁺ rat. This rat harbors a germline truncating mutation in the Apc gene (ApcPirc/⁺), a tumor suppressor gene that is mutated in the majority of human CRC. As a consequence of having a single functional Apc allele, ApcPirc/⁺ rats develop multiple tumors throughout the small intestine and colon. Similar to humans, male Apc mutant rats have increased tumor burden at all polyp stages and develop polyps at an earlier age than female rats. It was later found that ApcMin/⁺ mice, the most frequently used animal in the study of intestinal tumorigenesis also show male predominance in adenoma development, however this is restricted to the relatively rare adenomas in the colon whereas the majority of the adenomas in these mice develop in the small intestine. Therefore the group size required to investigate the underlying mechanism would be too large for it to be used as an experimental model.

In addition to these genetic models, we recently reported that male mice were more susceptible to chemical induction of colorectal tumors with the carcinogen azoxymethane compared to female mice. Similar findings were reported by another study in which colorectal tumors were induced in rats with the carcinogen dimethylhydrazine.

Thus, a biological difference of reproductive and hormonal origin underlies gender disparity in development of CRC. This biological effect is conserved in humans and rodents. Since gender differences are seen at the adenoma stage in both mice, rats and humans, it is likely that these effects take place at one of the earlier steps of carcinoma development.
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Female hormones

In an effort to implicate female hormones in animal models of CRC, it was shown that ovariectomies increase tumorigenesis in Apc\(^{Min/+}\), but lack of sham operations in control animals may have confounded these results\(^{60}\). In the rat counterpart of Apc\(^{Min/+}\) mice developed in the same lab (Apc\(^{Pirc/+}\)), no changes in polyp numbers were found after ovariectomies although these animals exhibited a strong gender difference in tumorigenesis\(^{55}\). Additionally, OVX did not alter the number of early adenoma precursor lesions that evolved after injection with the carcinogen azoxymethane (AOM)\(^{55,61}\). Thus CRC development exhibits gender disparity in both humans and rodents and in postmenopausal women, hormone substitution containing estrogens and a progestin seems to reduce CRC. To date however no animal studies have shown protective effects from either endogenous or exogenous female hormones in development of sporadic CRC.

Estrogens

In animal studies, effects of estrogens in intestinal tumorigenesis have been ambiguous. Whereas tumor numbers in ovariectomized Apc\(^{Min/+}\) mouse could be reduced by supplementation of estrogen\(^{62}\), no effect of estrogen supplementation was found in Apc\(^{Pirc/+}\) rats. Moreover, a protective role for ER\(\beta\) was found using ER\(\beta\) knockout mice\(^{63}\). Treatment with the ER\(\beta\) selective agonist diarylpropionitrile (DPN) corroborated these results\(^{64}\). In both models, reduced enterocyte proliferation was observed, potentially explaining protection from tumor formation. Thus, in the case of sporadic colorectal carcinogenesis, rodent experiments have shown that there may be beneficial effects of estrogens. Human studies however have not shown protective effects of estrogen.

Progestins

In experimental studies, the strong evidence that was formed by the first WHI study supporting a role of progesterone signaling in intestinal tumorigenesis, could not be corroborated. Using Apc\(^{Min/+}\) mice crossed into mice that lacked the progesterone receptor (PRKO), no differences were found between groups\(^{45}\). Additionally, in both Apc\(^{Min/+}\) rats and rats that were injected with AOM, supplementation of medroxyprogesterone acetate (MPA), the progestin used in the WHI, did not alter tumorigenesis or prevalence of ACFs\(^{55,15}\). Potential discrepancy of clinical and experimental data depends on duration of treatment (years versus weeks), tumor location (small intestine versus colon) or on tumor stage, since tumors in mice rarely progress beyond the adenoma level and no metastasis is observed in either mice or rats. Alternatively, progesterin signaling is distinct in rodents of fertile age that may have undergone ovariectomy versus women that have entered a postfertile, postmenopausal physiology. Perhaps, studies using the murine model of ovarian failure will explain protective effects of progestins in humans.

Male hormones: androgens

Gender differences in intestinal tumors in Apc\(^{Min/+}\) rats could not be explained by protective effects of female hormones, but were reconstructed by castration of male rats instead and supplementation of these animals with testosterone caused increased tumorigenesis to the sham-operated wild type.
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levels. This was the case for AOM induced polyps in mice as well\textsuperscript{55}. Gender disparity in experimental animals is thus explained by tumor promoting effects of male hormones instead of by protective effects of female hormones. Of interest it was found in the mouse model for menopause, that postmenopausal ovaries lose the capacity to produce female hormones but retain the potential to generate androgens\textsuperscript{65}. Potentially, protective effects of HRT in humans result from reduced androgen signaling, but more research is warranted to confirm this hypothesis.

Perspective
Colorectal cancer behaves in a sex dependent fashion, but to date the precise mechanism behind this disparity remains incompletely understood. The strongest evidence in humans, generated by the \textit{womens health initiative}, points towards protective effects of progesterone, either alone or in combination with estrogen. Biological differences however, as were replicated in rats that develop polyps, point towards androgens as tumor promoters, being responsible for gender differences. Future experiments, that are aimed at reconciling these two findings and further explaining tumorigenic roles of androgens on a mechanistic level have yet to be performed.
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Box 1: The adenoma to carcinoma sequence

The majority of colorectal cancers develops in the absence of overt predisposing factors and is referred to as sporadic CRC. However, disease may arise in the context of conditions that are recognized to modulate this risk. These include hereditary CRC syndromes and inflammatory bowel disease (IBD)\cite{4,5,14,15}. Inheritable syndromes may contribute to 20% of CRC development with an estimated 5-10% of disease being Mendelian in nature\cite{15}. CRC that develops in the context of IBD contributes to approximately 2% of all cases and is referred to as colitis-associated cancer (CAC). Of all cases, approximately 80% thus develops sporadically, constituting the vast majority of cases.

With the exception of IBD-associated tumorigenesis and a small percentage of hereditary CRC, the majority of tumors derives from adenomatous polyps, which in turn develop from dysplastic crypts. Following a specific sequence known as the adenoma to carcinoma sequence (Figure 1), designated mutations accumulate in specific stages of a neoplasia\cite{66}. Most frequently, mutations in the tumor suppressor gene APC constitute a first step in dysplastic transformation of colonic crypts and as a result, aberrant crypts and subsequently small polyps develop. In humans, approximately 90% of all colorectal cancer harbors a mutation in APC causing activation of the canonical WNT-signaling pathway. Those tumors that do not harbor APC mutations often have a mutation in CTNNB1 which, equal to APC mutations, results in WNT-signaling activation\cite{66}. When disease progresses to larger polyps, oncogenic mutations in RAS frequently occur\cite{68}. Later, additional mutations, among which those in SMAD4 and eventually p53 promote further progression into invasive and eventually metastasizing malignancies\cite{66}.

Although not all polyps progress into invasive adenocarcinomas, they are widely accepted as premalignant lesions and they have served as readout for screening and chemoprevention studies\cite{69}. In animal models that are used for the study of CRC, tumors are often studied in early stages. Mostly, genetic models are used with animals that harbor mutations in the Apc gene, which genetically and phenotypically mimics the first step of CRC development. These animals develop large numbers of polyps, but invading carcinomas are rare and metastasis has not been reported to date. Alternatively, using injections with alkylating agents such as azoxymethane (AOM), a large amount of aberrant crypts may be induced, but after a number of months, only a small proportion hereof eventually develop into polyps. Reportedly, AOM induced ACFs mostly harbor mutations in the K-Ras oncogene, whereas polyps that later evolve from ACFs have Apc or Ctnnb1 mutations predominantly\cite{70,71}. Carcinomas thus develop from polyps that derive from dysplastic crypts following the adenoma to carcinoma sequence. Most used animal models that mimic this development harbor mutations in the Apc gene, analogous to the human situation. Judging animal studies, it is important to realize that studies on CRC in animals are limited by lack of development past the polyp stage and no metastasis.

![Figure 1. Adenoma to carcinoma sequence](image-url)
Box 2: Sex hormone signaling

Peripherally produced sex hormones include estradiol, progesterone and testosterone. These steroids are secreted from gonads during fertile age. Signaling occurs primarily through distinct hormone receptors that all belong to the nuclear hormone receptors superfamily (Figure 2). These receptors all signal in a similar fashion. In contrast to progesterone and testosterone, estradiol can bind to two distinct estrogen receptors (ER), ERα and ERβ that are transcribed from distinct genes localized on human chromosome 6 and 14 respectively. Progesterone and testosterone signal through a single receptor (progesterone receptor (PR) and androgen receptor (AR)). Sex hormones are steroidal and thus lipophilic and diffuse through the plasma membrane freely. In the cytosol they bind to their specific receptor that undergoes a conformational change and dimerizes subsequently before translocating to the nucleus. In the case of estrogen receptors, either hetero- or homodimers are formed. In the nucleus, these hormone receptors exert active roles in transcription.

Figure 2. Hormone signaling
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