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Estrogen Receptor beta in Colorectal Cancer Prevention: Do we have Conclusive Proof?

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Abstract

Familial Adenomatous Polyposis (FAP) and Lynch syndrome are hereditary conditions that lead to Colorectal Cancer (CRC). FAP represents the ideal model for studying colorectal carcinogenesis since, in the same subject; "normal" mucosa coexists with low and high-grade dysplastic lesions as well as adenocarcinoma, offering the opportunity to compare different parameters in the various stages of the carcinogenetic process, free from individual variations. Epidemiological studies on women in pre-or postmenopausal age assuming oral contraceptive or hormone replacement therapy, respectively, strongly suggest a protective role of estrogens on CRC and colonic adenomatous polyps. Such findings have been confirmed by studying the behavior of the two high affinity Estrogen Receptors (ERs), estrogen receptors alpha (ER-α) and estrogen receptors beta (ER-β), both in humans with sporadic CRC, FAP and sporadic polyps. ERs reduction has also been associated to microsatellite instability, a DNA mutation encountered in Lynch syndrome and in 15-25% of sporadic CRC.

ER-β, abundantly expressed in the normal colon, progressively decreases in adenomas and CRC in relation to the disease aggressiveness. A similar behavior is encountered in FAP, where ER-β levels and ER-β/ER-α ratio progressively decrease in pre-neoplastic and neoplastic tissues. Finally, ER-β deficiency enhances intestinal tumorigenesis in $Apc^{Min/+}$ mice, an animal model that represents the equivalent of human FAP. ER-β would act by promoting apoptosis and inhibiting the stimulatory effect of ER-α on proliferation.

Recently, the use of selective ER-β agonists, such as phytoestrogens, has been suggested in primary CRC prevention. These natural compounds would represent an ideal therapeutic approach, since their use is not associated to the classic side effects encountered with either estrogens (active on both α and β estrogen receptors) or cyclooxygenase-2 (COX2) inhibitors. Moreover, they could be indifferently used in men and women since estrogen sexual activity is related to ER-α pathway.

Keywords: Cancer prevention; Phytoestrogens; Sex steroid hormones

Abbreviations: FAP: Familial Adenomatous Polyposis; APC: Adenomatous Polyposis Coli; CRC: Colorectal Cancer; ERs: Estrogen Receptors; COX2: Cyclooxygenase-2 Enzyme

Genetic Bases of Colorectal Cancer (CRC)

Intestinal carcinogenesis is the final outcome of a multi-step process resulting from genetic alterations that are influenced by environmental factors (especially dietary components) and host related factors (cytokines and hormones including sex steroid hormones). However, variations in cancer incidence among and within populations with similar dietary patterns suggest that the predominant pathogenetic factor is represented by the gene mediated individual response, through the expression of different protein and metabolite patterns [1].

Colorectal Cancer (CRC) is one of the most frequent malignant neoplasms in humans, being the second cause of death in men and the third in women [2]. It is mainly triggered by a mutation of the tumor suppressor gene *Adenomatous Polyposis Coli* (APC). The APC mutation provides the genetic background for the onset of the tumor process, making intestinal cells susceptible to tumor progression and promotion through the accumulation of further mutations as a result of epigenetic phenomena largely influenced by environmental (especially dietary) factors [3]. The APC mutation is present in 80% of sporadic CRCs and 100% of cases of *Familial Adenomatous Polyposis* (FAP) [4,5]. FAP is a pre-cancerous condition that invariably leads to CRC and represents the most frequent type of hereditary Polyposis with a prevalence of approximately 1 in 8,000 people (Figure 1). In particular, this hereditary disease arises from a germ-line mutation of the APC gene and displays an autosomal dominant inheritance with 100% penetrance [6,7].

The main mechanism, by which APC would act as tumor suppressor gene, overseeing intestinal epithelial homeostasis, is the control of cytoplasmic cellular levels of β-catenin, the central activator of transcription in the Wnt signaling pathway. At the molecular level, APC regulates the destruction of a multiprotein complex, composed of the tumor suppressor Axin and the protein kinases GSK3b and CK1, which promotes the phosphorylation and subsequent ubiquitin-mediated degradation of β-catenin [8]. The lack of APC protein allows nuclear import of β-catenin, followed by the formation of nuclear β-catenin/T-Cell Factor complexes that activate target gene transcription [9,10].

In addition to the regulation of proliferation and differentiation through Wnt/β-catenin signaling, APC controls other β-catenin-

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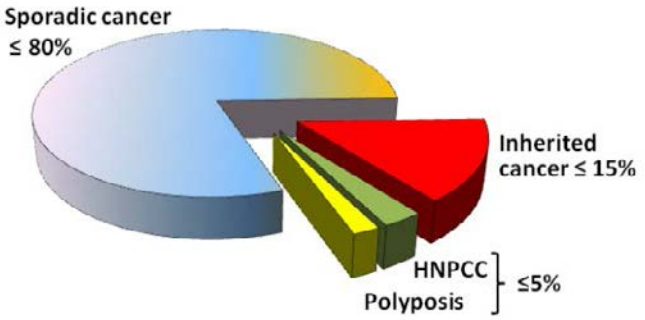


HNPCC: Hereditary Nonpolyposis Colorectal Cancer; Polyposis include FAP and other inherited syndromes

Figure 1: Different incidence of sporadic, familial and inherited colorectal cancer.

independent fundamental cellular activities, as demonstrated by the embryonic lethality of homozygous Apc-knock-out mutations [11-13].

Transgenic mice with a mutation of Apc gene (official designation: $Apc^{Min/+}$ mice) show molecular events similar to those observed in FAP patients in the small intestine and involving β-catenin downregulation [14]. The high frequency of intestinal tumor formation in these mice arises from loss of the wild-type allele (LOH) and the following stabilization and accumulation of active β-catenin [11]. In this model, the nature of the germline mutation in Apc determines the type of somatic mutation that occurs in the second allele. As a consequence, the resulting Wnt pathway activity enhances tumor formation [14,15]. Since the $Apc^{Min/+}$ mouse model represents the equivalent of FAP in humans, it is considered the most suitable model for experimental CRC studies [16].

Notably, germline as well as sporadic single amino acid substitutions (missense mutations) in APC predispose to the development of colorectal adenomas [17]. The majority of these missense mutations are located outside the regions required for binding β-catenin and were reported in extra-intestinal tumors [18].

Another important hereditary CRC is represented by Lynch syndrome, also known as Hereditary Nonpolyposis Colorectal Cancer (HNPCC). This pathological condition is clinically characterized by the development of CRC in almost all patients, followed by endometrial and other cancers. It is caused by a mutation in one of the mismatch repair (MMR) genes MSH2, MLH1, MSH6 and PMS2 and by a constitutional 3'end deletion of EPCAM, which is immediately upstream of the MSH2 gene and may act through an epigenetic silencing of MSH2 [19]. Such a mutation is mainly represented by Microsatellite Instability (MSI), the major characteristic of Lynch syndrome patients, also found in 15-28% of sporadic CRC [20]. MSI consists of the accumulation of insertions or deletions in the microsatellites, which are short DNA repeats. Accumulation of such mutations or point mutations in tumor suppressor genes and oncogenes, are thought to be key events in the development of tumors [20].

Estrogen Receptor Pathways and Functions

Two types of estrogen receptors mediate the biological activity of estrogens, namely estrogen receptor alpha (ER-α) and beta (ER-β). They belong to the steroid/thyroid hormone receptor super family of nuclear receptors [21]. They are localized predominantly in the nucleus and mediate gene transcription, both in ligand independent and dependent fashion. However, evidence also supports the presence of functional membrane-localized estrogen receptors [22].

Genes encoding for ER-β and ER-α are located on different chromosomes, the former located on Chromosome 14 (14q22-24) and the latter on the long arm of Chromosome 6 (6q25.1) [23,24]. ER-α and beta ER-β also differ as regards their distribution throughout the various organs and apparatuses: ER-β is the prevalent form in the gut while ER-α is essentially expressed in the breast, bone, cardiovascular tissue, urogenital tract and central nervous system and is also responsible for sex related functions/activities [25].

In both ERs we can recognize three main regions:

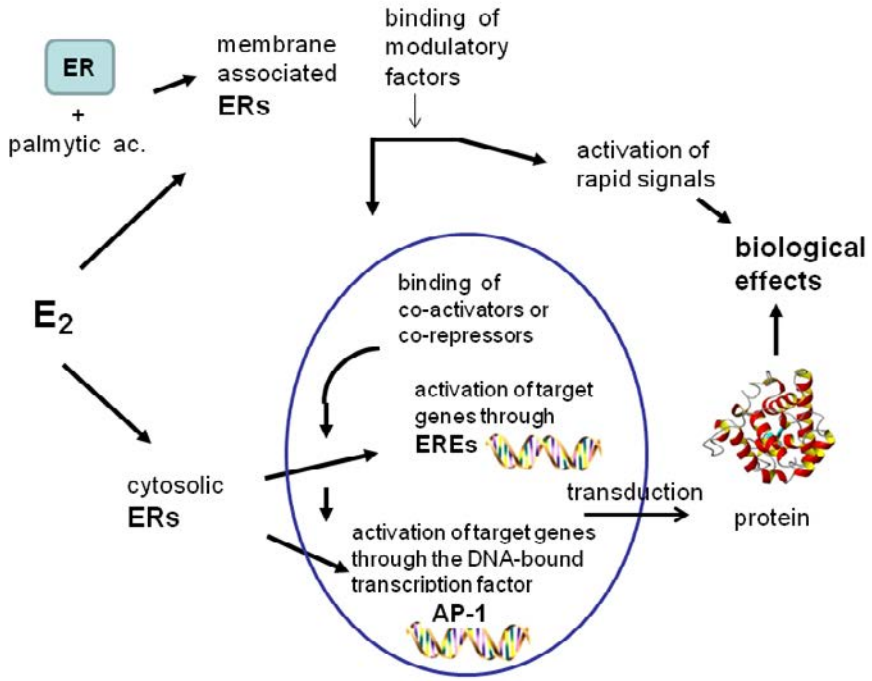
- A hypervariable N-terminal, that contributes to the transactivation function
- A highly conserved DNA-binding domain, responsible for specific DNA-binding and dimerization
- A C-terminal domain involved in ligand-binding, nuclear localization, and ligand-dependent transactivation functions [26,27].

In mammals both ER-α and ER-β have conserved DNA binding domains (96%) but they differ in their C-terminal domain showing only 58% homology [28]. ER-α has two distinct transcriptional Activation Functions (AF). AF-1 and AF-2. AF-1, located at the N-terminal region, is ligand-independent and constitutively active while the AF-2 domain is under the control of ligands. ER-β contains the AF-2 transcriptional activation function that is under the control of ligands [29,30].

After binding to ligands, ER-mediate signaling can be transmitted by different pathways (Figure 2). ERs directly interact with cis-regulatory elements of target genes through Estrogen-Response Elements (EREs). On the other hand, ERs can indirectly modulate target gene expression through their interaction with DNA-bound transcription factors such as activator protein 1 (AP-1) and specificity protein 1 (Sp1) a modality that accounts for the transcriptional activation of approximately one-third of all estrogen-responsive genes [31-33]. Finally, an alternative pathway consists of non-genomic mechanisms. In this case, plasma membrane-localized classical sex steroid receptors can activate intracellular kinase cascade(s) [34,35].

Other important aspects to consider in ER-mediated signaling transmission are the possibility that ERs may form homodimers (ERαα, ERββ) or heterodimers (ERαβ) and the modulatory effect of a set of different co-activators, enzymes and co-repressors on the assembly of the transcriptional complex and the subsequent transcription of the ER-responsive genes [36].

As far as RE non-genomic mechanisms, it has long been known that estrogens are able to activate very rapid signals (in seconds) to generate calcium flux and cyclic AMP in vitro and in vivo through plasma membrane-localized binding sites [34]. Palmitoylation and other enzymatic lipid modifications drive membrane localization of many integral membrane proteins. Previous studies have shown that palmitoylation occurs on a conserved cysteine as part of a nine amino acid motif in the ligand binding domains of all sex steroid receptors [37]. Palmitoylation promotes the physical interaction of ERs with the caveolin-1 protein and consequently the transport of ERs from cytoplasm to the plasma membrane [38,39]. On estrogen stimulation, ER-α is de-palmitoylated and dissociated from caveolin-1, stimulating signals of cell proliferation. On the contrary, after binding to ER-β, estrogens increase the association of the receptor complex



E_2: Estradiol; ERs: Estrogen Receptors; EREs: Estrogen-response Elements; AP-1: Activator Protein 1

Figure 2: Estrogen receptors pathways .

with caveolin-1 and p38 (a member of the MAPK family), in order to promote apoptosis [40].

ER-α and ER-β activate different pathways in physiological conditions [25]. The development of the ER-β knock out mouse model has confirmed that ER-β modulates ER-α activity [41,42]. Since it is able to reverse the effects of ER-α including stimulation of proliferation [31,43]. These differences are also evident in tumor cells as demonstrated by biologically opposite patterns activated by ER-α and ER-β in the mouse mammary cell line HC11 that expresses both ERs [44]. All the above mentioned differences together with the opposite effect as regards proliferation and apoptosis describe a type of interaction between the two estrogens receptors defined as "ying yang" relationship [45].

Role of Estrogen Receptors in Colon Carcinogenesis

The existence of a possible link between female sex steroid hormones and CRC first came up in the early 80's, when reviewing epidemiological, metabolic, and animal data McMichael et al. proposed that reproductive events and endogenous or exogenous female sex hormones affect carcinogenesis in the large bowel [46].

In 1987, our studies provided a demonstration on the possible relationship between ERs and CRC, using radiolabeled estradiol. In fact, we demonstrated that well differentiated forms of CRC express higher levels of ERs as compared to undifferentiated colorectal cancers [47]. Subsequently, we demonstrated by immunoenzymatic assay that ER levels are lower in neoplastic mucosa than in normal surrounding tissues and that polyamine (polycationic compounds normally implicated in cell proliferation) reached higher levels in ER negative colorectal carcinomas as compared to ER-positive colorectal carcinomas [48]. Later on, these findings were confirmed in patients with CRC, demonstrating not only that the normal colonic mucosa contains abundant ER expression as compared to tumor tissue, but also that ER-β is clearly the prevalent form and its expression progressively declines paralleling the grade of dedifferentiation of adenocarcinomas [49].

Ten years later numerous epidemiological studies appeared on the relationship between parity or use of oral contraceptives and CRC [50]. Since then, several epidemiological studies have been published on the protective effect of estrogens and/or oral contraceptive in CRC but a single study highlighted a lack of this protective effect for rectal cancer [51-56].

Finally, we find in the literature various attempts to investigate the mechanism(s) linking estrogens and/or oral contraceptive and CRC prevention. So far, as CRC development in humans is concerned, a markedly reduced ER-β expression seems to be related to the worsening of CRC stage and grade [57-59]. It has been suggest that the protective effect of estrogens would depend, at least in part, on ER-β mediated up regulation, at the transcriptional level of Cyclin-Dependent Kinase Inhibitor 1A (also known as CDKN1A or p21 or Cip1), responsible of a G(1) phase cell cycle arrest and inhibition of cell proliferation [60]. Finally, ER-β stimulation is reported to be responsible of G2/M cell cycle arrest in DLD-1 human colon adenocarcinoma cells through a down-regulation of cyclic expression and up-regulation of p21 expressions, two activities abolished by ER-β gene silencing [61].

Since the development of adenocarcinoma mostly involves the formation of polyps, considered pre-cancerous lesions and good intermediate biomarkers of CRC, numerous investigations have been also conducted on the relationship between estrogens and colorectal polyps [62-64]. Most of these studies are retrospective and were performed in pre o postmenopausal women using oral contraceptives or hormonal replacement therapy, respectively.

Two studies related obesity, menopause and use of hormonal

replacement therapy, reporting an increased risk for colorectal adenomas in obese premenopausal women, but a decreased risk in postmenopausal women, especially in the case of postmenopausal hormone use [64,65].

Murff et al. report that estrogen replacement therapy users have a reduced adjusted odd for adenomas when compared to non-users. However, they did not observe any beneficial effect in women <56 years [66]. In our opinion, this could be explained either by a long-lasting protective effect of endogenous estrogens or by the fact that the process leading to polyp formation requires a long period of time. The latter hypothesis implies that increased risk of adenoma becomes evident only after some years after menopause and therefore the protective effect of estrogen replacement therapy would be evident only at this time. Finally Giardiello et al. report a regression of colorectal adenomas with the use of estrogen/progesterone compounds restricting their positive findings to distal colonic adenomas [67].

We recently demonstrated that ER-β expression was significantly reduced in human adenomatous sporadic polyps as compared to normal mucosa, determining a striking increase in ER-α/ER-β ratio since ER-α expression remained unmodified. This condition well correlated with the increased proliferative activity that was not counterbalanced by an augmented apoptosis, and this would explain the progressive increase in polyp size [68]. Moreover, we observed a similar reduction in ER-β expression in adenomatous polyps of patients with FAP. This reduced ER-β expression was significantly correlated with an increased proliferative activity and inversely correlated to apoptosis [69]. The novelty of our latter study resides in the possibility of studying the natural history of colorectal cancer, in the same subject, free from individual and environmental variability factors. This condition makes it possible to determine possible correlations between ER-β expression and cell proliferation or apoptosis evaluated in the "normal" mucosa and the different evolutive steps encountered in CRC development (low- and high-grade dysplasia and adenocarcinoma) simultaneously present in the same patient [69].

Finally, we reported a reduced ER-β/ER-α in patients with ulcerative colitis. This reduction reached statistical significance (p=0.03) when colonic mucosa from normal controls was compared with colonic mucosa with dysplastic lesions from patients with ulcerative colitis [70]. These findings were also observed in the azoxymethane/dextran sodium sulfate-induced colitis-associated colorectal cancer model using ER-β knockout (βERKO) mice [71]. Other *in vivo* experimental studies confirming the relationship between ER expression and intestinal polyps demonstrate that ER-β deficiency enhances small intestinal tumorigenesis in $Apc^{Min/+}$ mice. Giroux et al. addresed this relationship directly showing that ER-β deficiency induced by ER-β knockout in female ApcMin/+ mice led to enhanced intestine tumorigenesis. As a proof of concept, the administration of estrogens or β-selective agonists abolished the increased intestinal neoplastic development in ovariectomized $Apc^{Min/+}$ mice [72,73]. Finally, for the first time in intact male $Apc^{Min/+}$ mice, we demonstrated that an enhanced ER-β activity induced by the administration of estrogens or β-selective agonists significantly reduced intestinal polyp number and size [74].

Recently, Tu et al. have reported that ER-β affects cell migration and invasion in HCT-116 colon cancer cells in vivo and potentiates the anti-proliferative activity of raloxifene on HCT-116 cells in vitro [75]. In addition, in vitro studies using COLO205, SW480 and MCF-7 cell lines demonstrate that estrogens are able to regulate microRNA expression and mismatch repair gene activity via ER-β, suggesting that these mechanisms might be the basis for the anti-cancer effect in colorectal cells [76]. These recent findings confirm our previous data describing a significant association between microsatellite instability and ER status in colorectal tumors [77]. Interestingly, we verified that Microsatellite Instability (MSI) tumors harbored low levels of ER expression. Moreover, the withdrawal of estrogens also resulted in an increasing risk of MSI CRC tumors. On the basis of these findings, an interesting hypothesis could link estrogens to gender differences in CRC through a mechanism involving MSI which is not only the major characteristic of Lynch syndrome patients, but is found in 15-28% of sporadic CRC [20,78].

In conclusion, it seems reasonable to affirm that there is a substantial body of evidence suggesting that the level of ER-β expression and/or the reduction in the ER-β/ER-α ratio are related to colonic carcinogenesis in both humans and animal models of CRC. ER-β is abundantly expressed in the normal colon but its expression is progressively decreased in adenomas and CRC in relation to the disease aggressiveness [58,68,79,80]. Similarly, familial adenomatous polyposis shows progressively lowered ER-β levels and a reduced ER-β/ER-α ratio in pre-neoplastic and neoplastic tissue [69].

Phytoestrogens and Colorectal Cancer

Phytoestrogens (heterocyclic non-steroid phenols) are plant-derived compounds that structurally and functionally act as estrogen agonists in mammals [81]. Their binding affinity to ERs is different from estradiol, being higher for ER-β (even higher than estradiol itself) and lower for ER-α [82]. For this reason phytoestrogens can act as estrogen agonists or antagonists according to the type of ER present in the tissue, its expression and the level of endogenous circulating hormones [83,84]. As proposed for estrogens, genomic and non-genomic mechanisms have also been suggested for phytoestrogens to explain their biological activities [85]. Non-estrogen-receptor-based mechanisms may also account for some of the proposed health benefits of phytoestrogens and their metabolites such as their anti-oxidant properties and ability to inhibit enzymes such as aromatase tyrosine kinase and DNA topoisomerase [86-89].

Phytoestrogens can be grouped into three classes of compounds on the basis of their different molecular structure and biological activities: isoflavones, coumestans and lignans [90]. Isoflavones are strongly represented in soy products, such as soy milk, soy meat and soy energy bars while cereals, grains, vegetables and fruits are common sources of lignans [90]. The lignans of major interest are those that can be metabolized by the intestinal microflora to enterolactone and enterodiol, which are more physiologically active than their precursors [91]. Soybean sprout, tofu, regular beans and soyabean are the major source of coumestrol [92].

Several epidemiological studies have reported a reduction in CRC risk associated with the consumption of isoflavones (found in legumes such as soy) and lignans (found in grains, seeds, nuts, fruits, and vegetables) [93,94]while two case–control studies suggest that lignans may be protective against polyps [95,96].

On the other hand, several experimental studies have been performed in this field, leading to the conclusion that phytoestrogens do reduce colorectal cancer development. The administration of a diet enriched with a potent ER-β agonist such as cumestrol in ovariectomized $Apc^{Min/+}$ female mice induced a reduction in the number of polyps and increased enterocyte migration compared to control animals [72]. In another study a diet enriched with silymarin, which consists of a family of flavonoids (silybinin, isosilybinin, silydianin, silychristin and taxifolin) with a selective ER-β agonist significantly

reduced azoxymethane-induced intestinal carcinogenesis in male mice [97]. This effect was dose dependent and determined a reduction in the number of cryptic adenomas that are known to anticipate the development of colic adenocarcinoma [98]. More recently, a reduced azoxymethane-induced intestinal carcinogenesis was also observed with a genistein- or soya-enriched diet. This treatment prevented up-regulation of WNT/β-catenin signaling determining a repression of the two WNT target genes Cyclin D1 and c-Myc [99]. We tested, in intact $Apc^{Min/+}$ male mice, the effect of a silymarin/lignin-enriched diet on intestinal tumor development. In this experimental setting we not only evaluated the relationship between intestinal polyp development and ER-β expression but assessed epithelial cell proliferation, apoptosis, and cell migration. The addition of silymarin or lignin to the diet and even more a specific combination of these two compounds significantly counteracted intestinal tumorigenesis by increasing ER-β mRNA and protein levels. Cell proliferation and apoptosis were rebalanced and cell migration accelerated, restoring levels similar to those observed in wild-type animals [74]. As recently reported by Bulzomi et al. in ERβ-transfected HeLa and in ERβ-containing DLD-1 colon cancer cell lines treated with the flavonoid quercetin, the apoptotic effect can be induced by the activation of p38, responsible for pro-apoptotic activation of caspase-3 and the cleavage of poly(ADP-ribose) polymerase, without influencing the expression of survival kinases AKT and ERK1/2 or Bcl-2. On the contrary, quercetin inhibited ERβ-dependent cyclin D1 promoter activity [100]. Finally Agarwal et al. suggest that silymarin can suppress the proliferation of a variety of tumor cells (including human colon carcinoma HT-29 cells) by different mechanisms including the down-regulation of cyclin D1 and the induction of cyclin-dependent kinase inhibitors (p21 and p27), which are inversely regulated by estradiol, and the induction of apoptosis [101-103].

Very recently, we performed the first randomized, double-blind, placebo-controlled study to determine whether short term administration of dietary phytoestrogens and insoluble fibers, can modulate ER expression in the colonic mucosa of patients (60 pts, men and/or post-menopausal women) undergoing surveillance colonoscopy after a previous polypectomy [104]. Our final intent was to demonstrate that dietary supplements are able to increase the levels of ER-β, an intermediate biomarker of CRC risk. A dietary supplement containing a combination of insoluble fibers and dietary phytoestrogens (silymarin and lignans) was given, without dietary restrictions, for 60 days before performing colonoscopy. We first analyzed pts receiving dietary supplement *vs.* placebo, and then a further analysis was performed considering 4 subgroups: supplement *vs.* placebo, with or without polyp recurrence. Our results confirmed our hypothesis, demonstrating a significant increase in ER-β in all treated patients and a general reduction in ER-α which was statically significant in treated patients without polyp recurrence. These changes were associated to a reduced proliferative activity and increased apoptosis. As suggested by others our findings represent a further step towards the concept that ER-β should be considered a target for CRC prevention and/or therapy [105].

Conclusions

Numerous *in vivo* and *in vitro* studies clearly demonstrate an involvement of ER-β in experimental colorectal cancer. In mice predisposed to CRC ($Apc^{Min/+}$) or receiving chemical-carcinogens, estrogen deprivation increases while ER-β selective agonists inhibit intestinal tumor formation. ER-β selective agonists also counteract CRC in intact male $Apc^{Min/+}$ mice. ER-β activity is due to a reduction of proliferative activity and an increase of apoptosis through well described molecular mechanisms. In humans, there are cohort and case control studies, showing a reduction of CRC in women assuming oral contraceptive or hormonal replacement therapy, and correlation studies on ER-β expression and progression of the disease. However, with regard to human CRC, we still have to wait for the final proof which should consists of a double-blind, randomized controlled trial on the prevention (secondary prevention) of intestinal polyps (in patients with sporadic polyps and/or with polyposis) through the use of selective β agonists.

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