

Role of anabolic agents in colorectal carcinogenesis: Myths and realities (Review)

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Abstract. Colorectal cancer (CRC) is one of the four leading causes of cancer-related mortality worldwide. Even though over the past few decades the global scientific community has made tremendous efforts to understand this entity, many ques-

tions remain to be raised on this issue and even more to be answered. Epidemiological findings have unveiled numerous environmental and genetic risk factors, each one contributing to a certain degree to the final account of new CRC cases. Moreover, different trends have been revealed regarding the age of onset of CRC between the two sexes. That, in addition to newly introduced therapeutic approaches for various diseases based on androgens, anti-androgens and anabolic hormones has raised some concerns regarding their possible carcinogenic effects or their synergistic potential with other substances/risk factors, predisposing the individual to CRC. Notably, despite the intense research on experimental settings and population studies, the conclusions regarding the majority of anabolic substances are ambiguous. Some of these indicate the carcinogenic properties of testosterone, dihydrotestosterone (DHT), growth hormone and insulin-like growth factor (IGF) and others, demonstrating their neutral nature or even their protective one, as in the case of vitamin D. Thus, the synergistic nature of anabolic substances with other CRC risk factors (such as type 2 diabetes mellitus, metabolic syndrome and smoking) has emerged, suggesting a more holistic approach.

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Abbreviations: CRC, colorectal cancer; DHT, dihydrotestosterone; IGF, insulin-like growth factor; FAP, familial adenomatous polyposis; HNPCC, hereditary non-polyposis colorectal cancer; CAC, colitis-associated cancer; TAMs, tumor-associated macrophages; STAT3, signal transducer and activator of transcription 3; IECs, intestinal epithelial cells; GH, growth hormone; DHEAS, dehydroepiandrosterone sulfate; DHEA, dehydroepiandrosterone; GnRH, gonadotropin-releasing hormone; ARs, androgen receptors; AAS, anabolic-androgenic steroids; SARMs, selective androgen receptor modulators; IR, insulin receptor; EIR, epithelial insulin receptor; VIR, vascular insulin receptor; IGF-R, insulin-like growth factor receptor; IGF-BPs, insulin-like growth factor binding proteins; VDR, vitamin D receptor; VEGF, vascular endothelial growth factor; EGF, epidermal growth factor; RXR, retinoid X receptor; VDREs, vitamin D response elements

Key words: anabolic agents, androgens, colorectal cancer, testosterone, dehydroepiandrosterone, vitamin D, insulin, IGF-1, growth hormone

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1. Introduction

Colorectal cancer (CRC) is the fourth most common type of malignancy after breast, lung and prostate cancer, accounting for 49,190 deaths annually in the USA alone (1). Numerous

risk factors have been identified, such as age, a family history of CRC, ethnic background (individuals of African descent), a high carbohydrate diet, poor physical activity, obesity and metabolic syndrome, smoking and alcohol abuse (2-5). Originating from epithelial cells at the base of intestinal crypts, the current model of carcinogenesis is that of the adenoma-carcinoma sequence, first described in the 1990s by Bert Vogelstein and Kenneth Kinzler [Fearon and Vogelstein (6) and Kinzler and Vogelstein (7)]. This model proposed a sequential transformation of the normal colorectal epithelium to an adenoma that could further transform into an invasive and metastatic tumor (carcinoma). Mutations in key regions begin to aggregate, turning normal mucosa to an early adenoma and, after a certain point of no return, the accumulated genetic alterations transform it to a carcinoma. Chromosomal instability, microsatellite instability, CpG island methylation and activating oncogenic mutations in genes such as *adenomatous polyposis coli* (*APC*), *K-ras* and *p53* are found to play a key role in this sequence (7-9).

However, as it was found thereafter, the Vogelstein model could explain 90-95% of CRC cases. The remaining 5-10% of cases were found to be germline-inherited cancers, such as familial adenomatous polyposis (FAP) and hereditary non-polyposis colorectal cancer (HNPCC). Notably, 2-3% of all CRC cases are associated with pre-existing inflammation and are referred to as colitis-associated cancer (CAC) (10). In these cases, the activation of nuclear factor (NF)- κ B signaling in tumor-associated macrophages (TAMs) leads to the indirect activation of *signal transducer and activator of transcription* (*STAT*)3 in pre-malignant intestinal epithelial cells (IECs) (11,12). Even though epidemiologic studies have witnessed a shift towards younger age groups over the past decade, the age group most commonly affected remains that of the middle-aged (>50 years of age) (13), a finding closely related to the Vogelstein model (the accumulation of mutations) (14,15).

Moreover, although CRC is not considered a sex-related malignancy per se, sex differences in incidence rates do exist (16-21). As far as the male population is considered, cancer incidence exhibits two peaks; the first one appears before the age of 35 and the second after the age of 55. On the other hand, in the female population, there is a single peak trend, between 35 and 54 years of age (22,23). Taking into consideration that physical activity performed before or after cancer diagnosis is related to a reduced mortality risk among CRC survivors (24) and is therefore recommended, along with the high prevalence of the use/misuse and abuse of anabolic agents with hormonal activity, such as testosterone, dihydrotestosterone (DHT), finasteride, insulin, insulin-like growth factor-1 (IGF-1) and growth hormone (GH) in the sports community over the past decades (25), a great concern of any possible carcinogenic properties or synergistic effects of the anabolic agents with the already well-studied and identified CRC risk factors has emerged (5). Nonetheless, the data are not consistent: An increasing body of evidence indicates that adequate levels of vitamin D, structurally related to a number of anabolic agents, can indeed protect against carcinogenesis via genomic and non-genomic mechanisms. In addition, the general population experiences uncontrolled multi-chemical exposure from several different sources at doses around or well

below regulatory limits (pesticides, food additives, lifestyle products components) (5,15,26) that can contribute to genotoxicity, endocrine disruption, target organ toxicity (3,4,27-29) by affecting systemic mechanistic pathways, such as oxidative stress and cell aging (14,30-32). These, along with the finding that human colorectal adenocarcinomas express specific steroid hormone receptors (33-40), has sparked the interest of the scientific community to unveil any possible pathogenetic mechanisms. Nonetheless, an increasing body of evidence indicates that adequate levels of vitamin D, structurally related to a number of anabolic agents, can indeed protect against carcinogenesis via genomic and non-genomic mechanisms.

2. Androgens

An androgen is considered any molecule capable of inducing and maintaining the male phenotype in an organism (male primary and secondary sexual characteristics and fertility) and taking part in the universal outgrowth of the musculoskeletal system and the anabolic shift of the metabolic status (41). Generally, the androgen-producing endocrine glands are able to synthesize five androgens via a sole pathway: Testosterone, dehydroepiandrosterone sulfate (DHEAS), dehydroepiandrosterone (DHEA), androstenedione and androstenediol, the latter of which has both androgenic and estrogenic properties. The molecules that prevail in this category are testosterone (the principal androgen in mammals) and DHT (potent metabolite of testosterone). In fact, they are the only androgens with direct androgenic activity. Other molecules, such as DHEA, due to their inferior potency, have received less attention. In an adult male organism, testosterone is primarily produced by Leydig cells in the testes. In addition, the extra-gonadal synthesis of testosterone and DHT by the adrenal testosterone precursor, DHEA, also occurs (42). Although adrenal androgens represent a minor fraction of the circulating testosterone for an adult male with an intact androgen biosynthesis cascade, they can be the main androgens in a female or a pre-puberty male (43). In the majority of cases, the classical mode of action of androgen superfamily is mediated by the androgen receptor.

Anabolic androgenic steroids (AAS) are used in the treatment of several disorders, such as hypogonadism, cachexia of various etiologies, hypercalcemia, hypercalciuria, in oncology as a supportive treatment and other chronic diseases (44). Since the early 1930s, AAS have been extensively used by amateur and professional athletes and the general public for the improvement of their physical condition and athletic performance (45-49). When used for ergogenic or recreational purposes, the dose levels are usually 5ement of physical condition and athletic performance (47,50,51). At such supraphysiological levels, AAS can cause a number of severe side-effects, including liver dysfunction, renal disorders, cardiotoxicity and potentially, stroke (52). In addition, anti-androgen therapy is also relatively common, having a wide variety of applications ranging from severe conditions (such as the treatment of prostate cancer and polycystic ovary syndrome) to more benign or even aesthetic conditions (such as acne and male pattern hair-loss). Thus, given their wide use in modern society, it is reasonable to scrutinize whether imbalanced androgen levels may possibly have a direct or indirect connection with CRC (41). In the following paragraphs, the

current data regarding androgens and CRC will be presented according to the clinical significance of the studied molecule.

Natural androgens

Testosterone. Testosterone, being the most clinically important androgen, has attracted scientific interest from as early as 1986. At that time, studies advocated that androgens played a protective role against CRC. In detail, Izbicki *et al* (1986) conducted experiments on 40 male rats. They found that chemical castration increased colonic tumor incidence, while testosterone administration following surgical castration produced a borderline statistically significant reduction in tumor incidence ($P < 0.053$), particularly in the right colon (22). In recent years, hypotestosteronemia (defined as levels of testosterone < 11 nmol/l or 320 ng/dl) was found to contribute to the development of CRC (53). Further data indicating the protective role of testosterone came from studies on patients who received androgen deprivation treatment for prostate cancer. In detail, it was found that the group with the higher risk of developing CRC was that of the orchiectomized male patients followed by patients receiving gonadotropin-releasing hormone (GnRH) agonist therapy (particularly if the treatment was prolonged) (54). On the contrary, there is evidence to suggest that androgens may act as promoters of colon carcinogenesis (2,55-57). Experiments using genetically modified mice, found that in orchiectomized males, the tumor load was lower when there was no administration of male hormone replacement therapy (2). Other researchers have indicated that, at an early stage, androgens may play an active role in the transition from adenoma to carcinoma (6). In contrast to both previous statements, a large study on 4,165 males aged 70-88 years demonstrated that increased testosterone levels were associated neither with an increased nor with a decreased risk of colon cancer risk (58). The same conclusion was achieved by a prospective study of 8,771 males and females from the general population of Denmark, who were followed-up for > 30 years (59). Whichever the case may be, androgens can be related to CRC either through direct mechanisms (mediated through androgen receptors), indirect mechanisms (smoking and alcohol habits, metabolic syndrome and type 2 diabetes mellitus, altered gut microbiota and increased stress hormones) or even a combination of both (60). Worthy of mention is the fact that regardless of the nature of the study (*in vitro*, *in vivo* or epidemiological), a common ground has yet to be found given the opposing data derived from these studies. Thus, in order to reach a consensus on the possible carcinogenetic properties of testosterone and the circumstances under which they appear, and to provide the grounds for a safe comparison between studies, research teams will have to adopt a common methodology as the basis of their experiments (27).

DHEA. Despite being less extensively studied, opposing results have been obtained on this less potent molecule as well. In an observational study on 170 individuals, the plasma levels of dehydroepiandrosterone sulfate were shown to be inversely associated with the risk of developing colon cancer (with a borderline statistical significance) (61). Moreover, another study proposed that DHEA strongly blunts serum deprivation-induced apoptosis. The anti-apoptotic effects of DHEA have been found to be completely reversed by testosterone through the blockade of DHEA receptors, thereby antagonizing its actions (62).

Mode of action. There are namely two modes of action, direct action and indirect action. These are discussed below.

a) Direct action: Androgen receptors (ARs). Steroid hormones, and thus androgens, exert their effects mainly through interactions with specific receptor proteins (37,56). The presence of ARs in human colonic tumors was first shown by Alford *et al* (34). The gene encoding the AR is located on the X chromosome. It contains two polymorphic trinucleotide repeat segments that encode polyglutamine (CAG) and polyglycine (GGC) (normally, ranging from 6 to 39 repeats). Surprisingly, only the number of CAG repeats has been found to be associated with misbalanced androgen levels. In fact, studies have demonstrated an inverse association between the number of repeats and the risk of prostate cancer (63,64). According to Hoque *et al*, the number of these repeats is gradually reduced in prostate cancers (65). Existing data advocate that fewer CAG repeats result in a higher transcription activity of AR, a finding positively associated with prostate cancer (66,67). In parallel, a greater number of repeats are associated with increased serum androgen levels, indicating a protective role of these against CRC (61,66).

Notably, Westberg *et al* came across a sex-related difference in the association between the number of the CAG repeats and the risk of developing CRC. The association was found to be direct in males, but inverse in females. They concluded that the inverse association observed in females would imply a stimulatory rather than inhibitory influence of AR on androgen production. A possible explanation could be found in the implication of both the AR and the estrogen receptor b (ERb) in the regulation of serum androgen levels (68). The above-mentioned hypothesis was reinforced by the similar findings of Slattery *et al*, who analyzed two large case-control studies of CRC. They further found a greater chance of *microsatellite instability (MSI)* or *p53* mutated tumors in the male population when more CAG repeats were present in the AR gene. Their concern was the lack of a common accepted parameter to determine the regulation of androgen levels. It has not yet been established whether the CAG repeat (as suggested by haplotype analysis of the AR gene) or the functionality of the receptor for the specific polymorphism is the most informative variant (64).

The above-mentioned results are in discordance with a following larger scale study of 1,798 CRC cases and 1,810 controls. In that study, the implication of the AR CAG repeat polymorphism in colorectal cancer prognosis was investigated for the first time. Of note, no association was found between the above-mentioned polymorphism and CRC overall or the disease-specific survival rate. As outlined by the authors, the genotyping error rate calculated from the duplicated samples was relatively high for the AR CAG repeat polymorphism (69). A genotyping error occurs when the genotype of an individual observed in the laboratory does not correspond to the individual's true genotype (70). The causes could be categorized as a variation of the DNA sequence, the low quantity or quality of DNA, biochemical artifacts (low quality reagents, poor equipment precision or reliability, Taq polymerase errors, the lack of specificity, electrophoresis artifact) and human error (sample manipulation, experimental error, data handling) (71). Since the main cause of genotyping

error is human error (71), Rudolph *et al* concluded that further studies are required to extract solid results (69).

Further attempts to unveil a possible prognostic value of the AR CAG repeat sequence with regards to sex differences have yielded some interesting findings. A recent case-control study of 550 CRC cases and 540 healthy controls concluded that long CAG repeats confer an increased risk of developing CRC in both sexes along with a poor 5-year survival, while fewer CAG repeats seem to protect against CRC (72). Previous studies have indicated that the CAG repeat length of AR inversely affects its transactivation potential, either through directly altering the receptor's function (73) or indirectly reducing AR messenger in RNA and protein levels (74). Either way, long CAG repeat sequences lead to absence of AR expression, which, in turn, has been linked to tumor size >5 cm in diameter and moderate to poor differentiation (at T3-T4 and N1-2 stages). These patients have also demonstrated a great risk of recurrence or metastasis. Notably, the above are found less frequently when there are short CAG repeat sequences and, thus, the normal expression of AR (72).

It should be pointed out that the trans-activating function of AR is dependent on its ligands, the androgens. The receptor participation in controlling cellular differentiation and proliferation in hormone-dependent tissues does not always occur in the same manner (75). Catalano *et al* shed some light onto this phenomenon. They mentioned two isoforms of the AR; androgen receptor A (AR-A) (87 kDa) and androgen receptor B (AR-B) (110 kDa). In the healthy colonic mucosa, both receptors are present; however, in the neoplastic colonic mucosa, only AR-A could be detected. The loss of expression of AR-B and the continuous expression of AR-A was proposed to indicate a loss of cell differentiation (76).

Another category of AR was also discovered; the membrane androgen receptors (mARs). Normally, the actions of the androgens are mediated through intracellular receptors (iARs). mARs seem to mediate opposite actions than iARs, inducing tumor regression (77). Furthermore, the affinity and selectivity of mARs differ among specific androgens (78). Through this receptor, testosterone exerts pro-apoptotic effects in both prostate and colon cancer cells. In human colon cancer cell lines, the activation of the ligand-bound androgen receptor suppresses the transcription of β -catenin. Consequently, there is a decreased expression of β -catenin target oncogenes, including *cyclin D1* (79).

The long-term activation of mAR by testosterone-albumin conjugated (TAC) treatment has also been linked to the dephosphorylation of protein Kinase B (Akt) both *in vitro* and *in vivo* (77). It is closely connected with the invasiveness of colon cancer cells in response to a variety of stimuli [heregulin, P21 (RAC1) activated kinase 1 (PAK1), Sprouty-2 etc.] (80-82), a finding supported through the examination of the upstream regulators of Akt, in particular PI3K. Upon long-term TAC treatment, it is dephosphorylated, leading to reduced cell motility in colon cancer and, consequently, invasiveness. Of note, although testosterone seems to induce p-Akt downregulation when it binds to mARs, iAR bondage induces p-Akt upregulation, even within the same cells. The main target of mAR activation that may regulate cell motility is thought to be vinculin; a cytoskeletal protein which links integrin adhesion molecules to actin. The inhibition or silencing of vinculin via

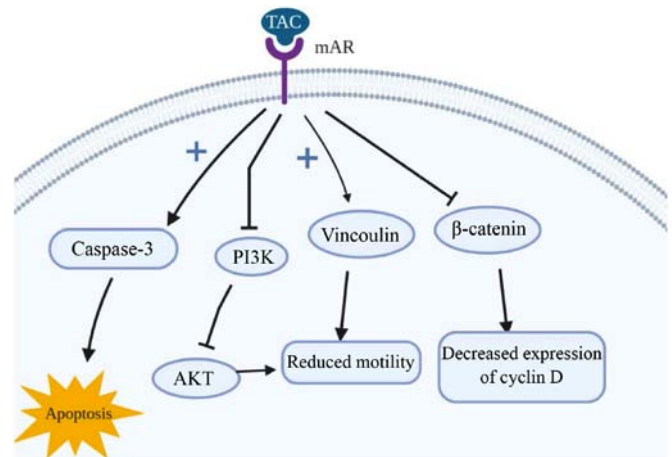


Figure 1. Actions of membrane androgen receptors (mARs). mARs have been found to mediate opposing actions to the intracellular androgen receptors. They induce apoptosis through the activation of caspase-3. Furthermore, they phosphorylate vinculin in cancer cells, leading to the inhibition of their migration. The suppression of β -catenin transcription leads to decreased expression of β -catenin target oncogenes, including cyclin D1. Finally, the long-term activation of mAR has been linked to the dephosphorylation of PI3K. As a result, Akt is downregulated and the motility of the colon cancer cells and their invasiveness are reduced. IGF-R, insulin-like growth factor receptor; IRS-1, insulin receptor substrate 1; IGF-BPs, insulin-like growth factor binding proteins; ERK, extracellular signal-regulated kinase; VEGF, vascular endothelial growth factor.

the phosphorylation by specific inhibitors, as is the case with activation of mARs, largely reverses both actin reorganization and the inhibition of migration (77). An illustrated representation of the mode of action of mAR is presented in Fig. 1.

Upstream in the AR activation pathway, the role of co-activator-associated arginine methyltransferase 1 (CARM1) is crucial. CARM1 is a protein with arginine-specific histone methyltransferase activity. It primarily binds to the histone and *p160* co-activators, leading to the activation of nuclear receptors, ARs included. Thus, it promotes nuclear receptor activity and acts as a molecular switch for gene-specific transcription factors including p53, NF- κ B, lymphoid enhancer-binding factor 1 (LEF1)/transcription factor 4 (TCF-4) and E2Fs (83,84). Taking the above into consideration, it is clear that the role of CARM1 is of utmost importance for cell proliferation and survival (85,86). Kim *et al* reported the overexpression of CARM1 in CRC specimens, but a weak expression in normal mucosal cells. They demonstrated that CARM1 inhibits the p53 response and instead promotes the NF- κ B response in Caco2 cells (87). However, the mechanisms involved remain to be fully clarified (87,88).

b) Indirect mode of action. Decreased levels of androgens seem to lead to a net increase in stress hormone levels, such as cortisol, which affect the tumor environment (89). The involvement of the innate immune system in the development of CRC has been demonstrated as well. The neutrophil count is reduced, as found by Chuang *et al* in castrated males, although it can be restored to normal levels through androgen supplementation (90). Androgens also seem to affect the production and excretion of biliary steroids and bile, compounds that are suggested to act as co-carcinogens (17,38). Lastly, an induction of insulin resistance following androgen deprivation therapy (91) has been linked to increased risk of developing

CRC (92). The connection between insulin and CRC will be further discussed.

Synthetic anabolic agents. Synthetic anabolic agents are categorized into two categories: Anabolic-androgenic steroids (AAS) and selective androgen receptor modulators (SARMs) (93). The AAS molecules that have thus far been approved as therapeutic agents are testosterone, nortestosterone, dihydrochloromethyltestosterone (DHCMT), metenolon, metandienone, methyltestosterone, oxandrolone, fluoxymesterone, stanozolol, formestane, 5 α -metandienone (94). SARMs are non-steroidal alternatives to AAS with the selective activation of the AR in either muscle tissue or bones (93).

A number of adverse effects of these compounds have been described when used either as medicine or as doping agents. As far as AAS's relation to cancer is concerned, a positive association with hepatocellular carcinoma (95), renal cancer (96-98), soft tissue carcinoma (99), adenocarcinoma (100,101) and lymphosarcoma (102) has been found, along with a case report hypothesizing their involvement in leiomyosarcoma (103). SARMs have also been linked to prostate cancer (104). However, to the best of our knowledge, there are currently no data available in the current literature associating any of these compounds with CRC.

3. Anabolic hormones

Insulin and insulin receptors (IRs). Insulin is one of the principal anabolic hormones in the majority of animals since it regulates the metabolism of almost all key energy points in favor of their synthesis and storage. The target substances of insulin are namely carbohydrates, lipids and proteins. Acting on adipocytes, hepatocytes and muscle cells, induces and maintains to a certain extent, an anabolic state which is described by the synthesis of carbohydrates, fatty acids and proteins, while reducing their degradation. Although a basic requirement is the prior balance between the circulating levels of the target substances and the intracellular ones, for a short period of time, it can overcome the concentration gradient of a substance and induce its endocytosis (105). The role of insulin in CRC was first introduced by the observation that obesity was associated with an increased risk of developing CRC in males. Subsequently, hyperinsulinemia and insulin resistance were linked to obesity and CRC development (106). In a large epidemiological study of almost 25,000 patients with type 2 diabetes mellitus (107) and in a large meta-analysis of 16 studies (108), a direct association between long-term insulin therapy and type 2 diabetes mellitus with an increased risk of developing CRC was found. It has also been established that CRC survivors with excess amounts of blood insulin have a greater risk of recurrence (109). By contrast, a large registry-based study in Connecticut that included 9,395 patients with CRC (110) and a smaller Norwegian study of 1,194 hospitalized patients with CRC (111), failed to find an association between diabetes and CRC-specific death. It must be stated though, that the latter studies included patients with metastatic CRC as well.

Schoen *et al* found a statistically significant association between insulin and adenoma status. The association was even stronger with advanced adenomas. The association with adenomatous polyps, the precursor of CRC, confirms a link between insulin and early neoplasia (112). It must be kept in mind that

high insulin levels increase serum IGF-1 levels (113,114), a factor which is closely related to carcinogenesis.

To date, two isoforms of IR have been described, differing at the short exon 11 (encodes 12 amino acids). The absence of exon 11 transcripts the IR-A (short isoform), while its presence the IR-B (long isoform) (115). Existing evidence supports that the two IR isoforms play different biological roles. IR-A mostly exerts mitogenic effects and IR-B modulates cell metabolism (116). Abbruzzese *et al* found a strong IR expression in adenomas and low-grade adenocarcinomas (117). The expression of IR-A has been found in cells that have lost their differentiation, a finding which is in accordance with the presence of this receptor in cancer (118,119).

The expression of IR is mainly considered to be present in epithelial tumor cells [epithelial insulin receptor (EIR)]. However, Heckl *et al* found the expression of the receptor in other tumor compartments, e.g., the tumor-vasculature [vascular insulin receptor (VIR)]. When 1,580 cases of CRC were examined concerning the expression of the IR, differential expression patterns of the IR in tumor cells (EIR) and endothelial cells of tumor vessels (VIR) have been observed. EIR expression is strongly associated with distant metastasis, lymphatic invasion, lymph node metastasis, tumor-specific survival (TSS) and overall survival (OS). Moreover, EIR has been found almost exclusively in the cytoplasm of tumor cells, whereas a (simultaneous) membranous IR expression was less prevalent (120). These findings were further clarified by *in vitro* experiments by Morcavallo *et al*, who demonstrated that insulin or IGF-II stimulation induced the phosphorylation-activation of IR-A, which was then internalized from the cell surface. The underlying mechanism was speculated to be a sustained phosphorylation of the receptor, leading to prolonged activation (121).

On the other hand, VIRs are thought to contribute to neovascularization following abduction by elevated insulin levels. VIRs are frequently found in CRC, particularly in left-sided CRCs, and they are significantly associated with tumor invasiveness (120).

From another point of view, insulin resistance in vascular endothelial cells can promote tumor formation, possibly through mechanisms involving chronic inflammation (122). This resistance is characteristic of endothelial dysfunction in obesity and type 2 diabetes (123-126) and was found to promote tumor development. By contrast, there was almost no effect of insulin signaling on intestinal carcinogenesis through epithelial receptors (122).

In order to fully understand the role of insulin in the tumor cascade, special reference must be made to extracellular vesicles (EVs). These are vesicles found in the extracellular space of various cell types. They can be found under normal and pathological conditions (127,128). EVs package biologically active content (including proteins, mRNA and miRNA), which they further transfer to the recipient cells. Due to their action they are considered as mediators of signaling cascades (129,130). EVs are known to mediate various biological cascades relative to cancer, such as the activation of Wnt signaling and the activation of PI3K/Akt signaling (129).

Insulin, similar to other growth factors, induces PI3K/Akt signaling (131-133). PI3K/Akt-positive CRC cells react to PI3K/Akt signaling by producing EVs which, in turn, amplify

the proliferative signal in other CRC cells in the close environment. The deeper in the proliferative core the EVs reach, the greater the benefit for the tumor, as in these areas the transfer of nutrients/growth factors would have been otherwise impossible. Thus, EVs amplify the proliferative signal and aid cancer progression (134).

IGF and its receptor (IGF-R). IGF is a hormone that serves as the mediator of growth hormone (GH)-stimulated somatic growth, as well as a mediator of GH-independent anabolic responses in a number of cells and tissues, while it is also associated with mitogenesis, cell survival and differentiation (135-142). In detail, IGF-1 promotes cell cycle progression and inhibits apoptosis either by triggering other growth factors or by interacting with pathways which have an established role in carcinogenesis and cancer promotion (142). Ma *et al* stated that there is an increase in IGF-1 levels in patients with CRC (143), while other studies have advocated the overexpression of IGF-R, as well (144-147). In accordance with this, several studies have linked elevated plasma IGF-1 and IGF-1R downstream signaling to an enhanced risk of colorectal neoplasia and a poor survival (148-152). Furthermore, Peters *et al* found that IGF-1 was closely related to the expression of the proliferation marker Ki-67 (153). Ki-67 is a nuclear protein that is active only in dividing cells and absent in cells locked in G0 phase. This is a logical outcome when taking into consideration that IGF-1 can stimulate the expression of cyclin D1, a molecule that accelerates the progression of the cell cycle from G1 to S phase (154). However, there is no prognostic relevance of Ki-67 in CRC, regardless of the stage of disease (155,156). Through a reverse line of thought, octreotide, a molecule that lowers the IGF-1 concentration, has been shown to attenuate the growth rate of tumor cells *in vivo* (157).

The expression of *IGF-2* is also highly associated with tumor stage. The association was speculated when it was found that autocrine IGF-2 production and consequent IGF-R activation increased tumor growth and reduced apoptosis (153). Thus, it is plausible that, when IGF-1 or IGF-2 is present, an IGF-1R self-stimulation will produce an autocrine/paracrine loop in CRC. However, no prognostic effect of IGF-1 and IGF-2 has been proven (153).

A family of six circulating proteins termed insulin-like growth factor binding proteins (IGFBPs) has been found to interfere with the action of IGF. Thus, their involvement in CRC must be investigated, as well. They act either as tumor suppressors by limiting IGFs activity (158) or as inhibitors of cancer growth through IGF-independent mechanisms (159). High IGFBP2 plasma levels were found by Liou *et al* to be independently associated with a reduced overall survival (OS) of patients with CRC (160). By contrast, IGFBP3 has been shown to be inversely associated with CRC, as its plasma levels were found to be lower in those patients (143). Notably, IGFBP-3 can either oppose or enhance the biologic action of IGF-I through direct bondage to IGF-I or indirectly to IGF-R (161).

In a prospective cohort study of 210 patients with CRC, IGF-1 expression was shown to be closely associated with tumor size and the depth of invasion. However, it was stated that a shift of investigation towards the IGF-1/IGFBP-3 ratio is warranted, as it better describes the biological effects of IGF-1 (152). A nested case control study of males in the

Physician's Health Study demonstrated an increased risk of CRC in subjects with high IGF-I levels. The risk was decreased when high IGFBP-3 levels were measured (143). A study of 460 patients was carried out to further examine the association between IGF-I and the IGF-I/IGFBP-3 ratio with colorectal adenomatous polyps. A statistically significant positive association was found, with greater odds ratio when the case group was limited to advanced adenomas. This finding indicates a possible stimulation of non-advanced adenomas towards advanced adenomas (112).

Closer attention must be paid to the IGF-R, as it has been stated that it contributes to resistance to cytotoxic (162), radiation (163) and targeted therapies (164-166). Indeed, the silencing of the receptor increases the intracellular drug concentration (such as oxaliplatin and vincristine); an effect mediated via the PI3K/Akt pathway (167). A progressive increase in *IGF-1R* expression occurs in normal colonic mucosa, while it transits to adenomatous, as well as in the transition from adenomatous to carcinomatous tissue (147). Peters *et al* have confirmed a strong expression of the IGF-1 receptor in >99% of all CRC cell lines of their experiments (153).

Following its activation, IGF-R induces multiple intracellular mechanisms, as shown in Fig. 2. It induces the transcription of the *vascular endothelial growth factor (VEGF)* gene (168,169), upregulates the anti-apoptotic protein, Bcl-xL (170) and inhibits the action of β -catenin (through PI3K/Akt activation) (171). From another perspective, Nahor *et al* demonstrated that the tumor-suppression genes *p63* and *p73* inhibit the IGF-1R promoter, reducing the endogenous IGF-1R levels in a dose-dependent manner. Through this mechanism, it was proposed that they control colon cancer proliferation (172).

The pro-oncogenic activities of IGF-1R are solely mediated through its proximal downstream effectors: Insulin receptor substrate 1 (IRS-1) and 2 (IRS-2) (173,174). IRS-1 expression appears to be inversely associated with CRC differentiation. However, it may be upregulated in both primary and metastatic human CRC, a finding that has not been observed in normal colonic epithelium (175). It has been further supported that the upregulation of IRS-1 can occur directly from androgens (64). IRS-2 mRNA and protein expression have a positive association with the transition from normal colorectal epithelium to adenoma and adenocarcinoma. Furthermore, *IRS-2* overexpression promotes the invasiveness of CRC cells. It activates the oncogenic PI3K/Akt pathway and at the same time reduces cell adhesion (176). Finally, IRS-1 and IRS-2 polymorphisms have been independently associated with the risk of developing CRC in a direct manner (177).

The above-mentioned findings are further supported by the action of NT157 in murine and human CRC cells. NT157 is a molecule that, through bondage to an allosteric site of the IGF-1R, it induces a conformational change. As a result, the receptor is dissociated from IRS1 and IRS2 proteins. Consequently, IGF-1R stronger interacts with the adaptor protein Shc, leading to an enhanced activation of extracellular signal-regulated kinase (ERK). Indeed, experiments have confirmed that NT157 activates ERK1/2, without activating Akt (178).

Vitamin D. Vitamin D regulates cellular functions, such as differentiation and proliferation in normal and malignant

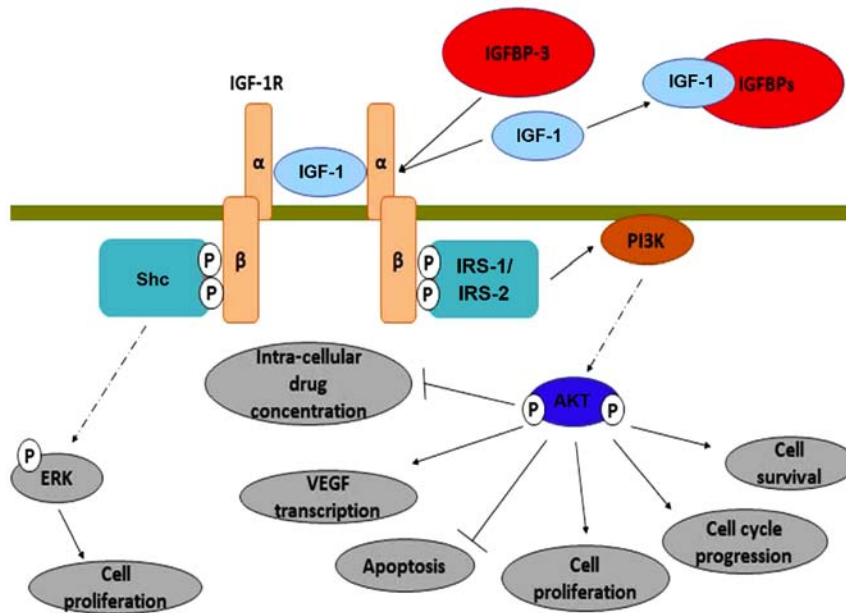


Figure 2. Mechanisms mediated through IGF-1/IGF-1R. The activation of IGF-1R takes place following the bonding of IGF-1 (or IGF-2) to the extracellular part of the receptor. IGF-1 plasma levels, and thus IGF-1R activation, are reduced by the IGFBPs. However, IGFBP-3 can act as an enhancer of the IGF-1 activity when it is bonded to the receptor and not directly to IGF-1. Following its activation, IGF-1R induces the phosphorylation of IRS-1,2 molecules, which they further activate the PI3K-Akt pathway. Consequently, there is induction of cell survival, cell cycle progression, cell proliferation and VEGF transcription. At the same time, apoptosis is inhibited and the cell gains resistance against cancer therapies. Even when IRS-1,2 molecules are silenced, the activation of IGF-1R leads to increased cell proliferation through the activation of Shc-ERK pathway. IRS-1, insulin receptor substrate 1; IGFBPs, insulin-like growth factor binding proteins; ERK, extracellular signal-regulated kinase; VEGF, vascular endothelial growth factor.

tissues. It also regulates cell adhesion in tumor cells and modifies tumor angiogenesis, invasion and metastasis along with decreasing oxidative DNA damage (179). Vitamin D deficiency has been associated with various cancer types (180,181).

Vitamin D first attracted scientific attention after an inverse association was observed between solar UV-B exposure and CRC incidence in both sexes (182). As it has been well-established, UV-B radiation is essential for the production of vitamin D₃, which after two steps becomes 1,25-(OH)₂-vitamin D (calcitriol), the most active component (183,184).

The study by Boscoe and Schymura on 3.1 million individuals from the northern part of the USA supported that low levels of vitamin D can induce the progression of CRC, although no association was found with disease onset. Their proposal was based upon a higher death rate which occurred during the winter months (when levels of vitamin D are markedly reduced) (182). Feskanich *et al* found an inverse association of 25-OH-vitamin D and CRC in observation in the female population, although only in areas where high levels of UV-B are available (185). In fact, levels of 25-OH-vitamin D >20 ng/ml have been advocated to provide protection against CRC (186). If levels of 25-OH-vitamin D are >82 ng/ml, then it is estimated that the cancer incidence is decreased by 50% (187). However, no association has been found between 1,25-OH₂-vitamin D and CRC (185); a finding disputing the results of two previous studies (188,189).

Calcitriol has been found to reduce tumorigenesis in rats (190,191) and proliferation in both normal and premalignant human rectal epithelioma (192), as well as in human colorectal cell lines, while it stimulates their differentiation (193-196). Vandewalle *et al* proposed that an increased expression of *vitamin D receptor (VDR)* may lead to cell differentiation

and growth inhibition either through calcitriol or through non-calcemic analogs (195). The mechanisms behind the protective role of vitamin D against CRC are multiple and can be categorized into genomic and non-genomic mechanisms, as discussed below.

Genomic mechanisms. The genomic mechanisms are mediated through the VDR as is shown in Fig. 3. Following the bonding of 1,25-OH₂-vitamin D to the receptor, the complex is dimerized with another receptor, the retinoid X receptor (RXR). This heterodimer targets specific areas in genes called *vitamin D response elements (VDREs)* (197). As a result, several miRNAs are affected through up- or down-regulation. These miRNAs have been speculated to suppress oncogenes or enhance the expression of tumor suppressor genes. For example, vitamin D can induce the promoter of miR-627. This gene has been inversely linked with CRC, as its decreased expression (an aftermath of reduced levels of vitamin D) has been found to promote cancer (198).

Further action of vitamin D in human colon tumor cells leads to the upregulation of the potent anti-angiogenic factor, thrombospondin 1 (199). The upregulation of the transcription of the Wnt-inhibitors, the *DICKKOPF-1* and *DICKKOPF-4* genes, has also been linked with the action of calcitriol (200). There are data to suggest that calcitriol regulates apoptosis as well (201). Following treatment of colorectal cell lines with 1,25-OH₂-vitamin D, it was found that apoptosis was triggered through secreted protein acidic and rich in cysteine (SPARC)-induced VDR synthesis (202). In another cell line, treatment with calcitriol induced the mRNA expression of the pro-apoptotic protein, G0S2 (G0/G1 switch gene) (203). Furthermore, WAF1 and KIP1 were found to be up-regulated, leading to cell-cycle arrest in G1 phase (201,204,205).

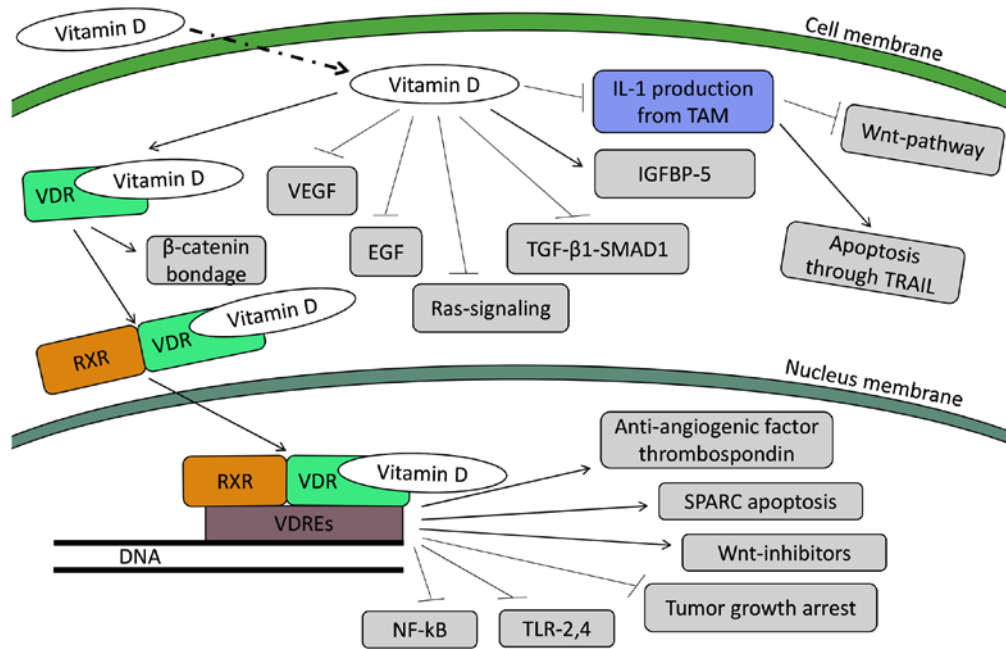


Figure 3. Mechanisms mediated through vitamin D. The insertion of vitamin D into a colorectal cell triggers multiple pathways; both in the cytoplasm (non-genomic actions of vitamin D) and in the nucleus (genomic actions of vitamin D). Through its non-genomic actions, vitamin D halts tumor development through the inhibition of VEGF, EGF, Ras-signaling and TGF- β . At the same time it induces IGFBP-5 production, diminishing this way the cancer-promoting effects of IGF-1,2. Also of importance is the reprogramming of TAMs, which results in lower levels of IL-1 production. Consequently, the Wnt-pathway is halted, while apoptosis through the TRAIL pathway is triggered. Following its binding with the VDR it can either bind β -catenin (inhibiting its translocation to the nucleus) or, after being heterodimerized with the RXR, translocates to the nucleus. In the nucleus, the complex vitamin D-VDR-RXR binds to VDREs gene sequences. As an aftermath, the induction of the anti-angiogenic factor thrombospondin 1 occurs, as well as apoptosis through the SPARC pathway and transcription of Wnt-inhibitors. Finally, the inhibition of NF- κ B and TLR-2,4, while tumor growth is halted (through inhibition of CDK-Is, cyclin D1, c-myc, etc). ERK, extracellular signal-regulated kinase; VEGF, vascular endothelial growth factor; TAMs, tumor-associated macrophages; VDR, vitamin D receptor; RXR, retinoid X receptor; VDREs, vitamin D response elements; SPARC, secreted protein acidic and rich in cysteine.

Other studies have demonstrated the effects of calcitriol on tumor growth. Through the induction of cyclin-dependent kinase inhibitors, such as p21, p27 and cystatin D and the inhibition of pro-proliferative genes, including *c-myc* and *cyclin D1*, tumor growth is halted (198). *NF- κ B* is another target family of genes (fundamental for the cancer cell survival) which are downregulated by vitamin D (206). Finally, the reduction of colon cancer cell lines has been suggested following the decreased expression of *Toll-like receptor (TLR)2* and *4* on human monocytes (207).

Non genomic mechanisms. Apart from the genomic action through the nuclear receptor, calcitriol can bind to membrane receptors in certain tissues (including the intestines), leading to non-genomic, non-nuclear actions (208-211). It has been well documented that in CRC, APC mutation is by far one of the most common, allowing various downstream pro-oncogenic pathways to upshift. One of these pathways is the Wnt pathway, where β -catenin acts as a transcriptional co-regulator, cooperating with transcription factors of the T-cell factor (TCF) family to determine gene expression (212,213). Pendás-Franco *et al* described the protective action of calcitriol in colon cancer cells, according to which it induces VDR to bind with β -catenin and restrain it from translocating to the nucleus and inducing the expression of pro-carcinogenic genes (200). Furthermore, vitamin D-related compounds have been found to induce the production of IGFBP-5. These molecules bind both with IGF-1 and IGF-2, suppressing the stimulating effect of these molecules (214).

Furthermore, vitamin D seems to be able to reprogram the tumor-associated macrophages (TAMs) in a manner that halts their tumor-promoting actions (215), probably by inhibiting STAT1 activity. As a result, there is no production of interleukin (IL)-1 from the latter, rendering the tumor colon cells sensitive to apoptosis through the TRAIL pathway (216). In parallel, due to the lack of *IL-1 β* expression, the Wnt pathway is deactivated (215).

Further observations of a crosstalk between vitamin D and TGF- β 1/SMAD1 signaling in the growth inhibition of human colon cancer-derived cells has shown that this interaction halts tumor growth by blocking the expression of cell cycle proteins and inhibiting the action of cyclins D1, D2, D3 and E (217). It has also been found to inhibit mitogenic Ras signaling, as well as the epidermal growth factor (EGF) (218-220). Furthermore, Ben-Shoshan *et al* exhibited an inhibition of vascular endothelial growth factor (VEGF) in colon cancer cell lines by vitamin D (221).

Last but not least, the association of vitamin D with calcium must be examined. It seems they exert a synergistic effect in reducing CRC incidence. This was first described in Apcmin mouse models by Harris and Go (222) and later on by Lappe *et al* who carried a clinical trial on post-menopausal women in Nebraska. They concluded that although calcium alone reduced the all-cancer incidence by 44%, when accompanied by vitamin D the reduction reached 77% (223).

Table I. Association between anabolic hormones and CRC.

Substance	Relation to CRC	Author (year)/(Refs.)	Nature of the study	
Testosterone	Physiological testosterone levels induce reduction in tumor incidence especially in right colon	Izbicki <i>et al</i> (1986) (22)	<i>In vivo</i>	
	Hypotestosteronemia contributes to CRC development	Gould and Petty (53) Gillesen <i>et al</i> (54)	<i>In vivo</i> Epidemiological	
	Androgens may act as promoters of CRC	Amos-Landgraf <i>et al</i> (2) Izbicki <i>et al</i> (1983) (55) Mehta <i>et al</i> (56) Moon and Fricks (57)	<i>In vivo</i>	
	Androgens may play an active role in the adenoma => carcinoma sequence	Fearon and Vogelstein (6)	<i>In vivo</i>	
	Increased testosterone levels have no connection with CRC	Hyde <i>et al</i> (58) Orsted <i>et al</i> (59)	Epidemiological	
	Dehydroepiandrosterone (DHEA)	DHEA levels are inversely associated with CRC risk	Alberg <i>et al</i> (61) Anagnostopoulou <i>et al</i> (62)	Epidemiological <i>In vitro</i>
Androgen receptors (ARs)	Number of CAG repeats is linearly correlated with CRC in men and inversely in women	Slattery <i>et al</i> (2005) (64)	Epidemiological	
	Number of CAG repeats is independent from CRC survival rate	Rudolph <i>et al</i> (69)	Epidemiological	
	Long CAG repeats	Huang <i>et al</i> (72)	Epidemiological	
	Increased risk for CRC in both sexes Poor 5-year survival High T and N stage Few CAG repeats protect against CRC			
Synthetic anabolic agents (AAS and SARMs)	mARs seem to mediate opposite actions than iARs	Gu <i>et al</i> (2011) (77)	<i>In vitro</i>	
	AASs positive correlation with a variety of cancers (among which is adenocarcinoma)	Watanabe and Kobayashi (95)	Epidemiological	
	SARMs are linked with prostate cancer but not with CRC	Rosner and Khan (96) Martorana <i>et al</i> (97) Bryden <i>et al</i> (98) Zahm and Fraumeni (99) Bronson and Matherne (102) Froehner <i>et al</i> (103) Chacon and Monga (104)	<i>In vivo</i>	
	Insulin	Long-term insulin therapy/ high blood insulin levels	Yang <i>et al</i> (107) Larsson <i>et al</i> (108) Flood <i>et al</i> (109)	Epidemiological
		Increased risk of CRC Greater risk of CRC recurrence		
		No connection between diabetes and CRC-specific death	Polednak (110) Jullumstrø <i>et al</i> (111)	Epidemiological
Epithelial insulin receptor (EIR)	EIR expression results to distant metastasis, lymphatic invasion, lymph node metastasis, tumor specific survival and overall survival	Heckl <i>et al</i> (120) Morcavallo <i>et al</i> (121)	Epidemiological <i>In vitro</i>	
Vascular insulin receptor (VIR)	VIR is frequently found in CRC, especially in left-sided CRCs, and associated with tumor invasiveness	Heckl <i>et al</i> (120)	Epidemiological	
Insulin-like growth factor 1 (IGF-1)	High levels of IGF-1 are related with CRC	Soubry <i>et al</i> (149) Lee <i>et al</i> (148) Ma <i>et al</i> (143) Ollberding <i>et al</i> (150) Giovannucci (2001) (151) Shiratsuchi <i>et al</i> (152)	<i>In vivo</i> <i>In vitro</i> Epidemiological	
	IGF-1 expression is associated with tumor size and depth of invasion	Shiratsuchi <i>et al</i> (152)	Epidemiological	
	IGFBPs	High IGFBP2 positively associated with reduced overall survival in CRC	Liou <i>et al</i> (160)	Epidemiological

Table I. Continued.

Substance	Relation to CRC	Author (year)/(Refs.)	Nature of the study
Vitamin D	Low levels of vitamin D can induce the progression of CRC	Boscoe and Schymura (182)	Epidemiological
	Levels of 25-OH-vitamin D >20ng/ml can protect against CRC	Braun <i>et al</i> (186) Gorham <i>et al</i> (187)	Epidemiological
	Levels of 25-OH-vitamin D >82ng/ml cancer incidence is decreased by 50%		
	Vitamin D can induce the promoter of onco-protective miR-627	Padi <i>et al</i> (198)	<i>In vivo</i>
	Vitamin D in human colon tumor cells up-regulates the potent anti-angiogenic factor thrombospondin 1	Fernandez-Garcia <i>et al</i> (199)	<i>In vitro</i>
	Vitamin D up-regulates the transcription of the Wnt-inhibitors DICKKOPF-1 and DICKKOPF-4	Pendás-Franco <i>et al</i> (200)	<i>In vitro</i>
	Vitamin D regulates apoptosis through SPARC	Ylikomi <i>et al</i> (201) Taghizadeh <i>et al</i> (202)	<i>In vitro</i>
	Vitamin D down-regulates NF-κB, TLR2 and TLR4	Liu <i>et al</i> (206) Kim and Brasitus (2001) (208)	<i>In vitro</i>
Vitamin D may be able to reprogram the tumor-associated macrophages (TAM) and halt their tumor-promoting actions	Kaler <i>et al</i> (215)	<i>In vitro</i>	
Growth hormone (GH)	Growth hormone suppresses p53, PTEN, and APC	Brown-Borg <i>et al</i> (231)	Epidemiological
		Chesnokova <i>et al</i> (232)	<i>In vivo</i>
		Morin <i>et al</i> (233)	<i>In vivo</i>
		Clevers and Nusse (234)	

CRC, colorectal cancer; IGF, insulin-like growth factor; IGFBP, insulin-like growth factor binding protein; SPARC, secreted protein acidic and rich in cysteine; TLR, Toll-like receptor; PTEN, phosphatase and tensin homolog; APC, adenomatous polyposis coli.

Growth hormone (GH). Human growth hormone (hGH) or somatotropin, is a peptide-hormone secreted mainly by somatotrophic cells within the lateral wings of the anterior pituitary. After entering the bloodstream it reaches its target organs (namely the liver, muscles, bones and adipose tissue) binding to its receptor [growth hormone receptor (GHR)] and thus inducing its anabolic properties through the activation of the mitogen activated protein kinase (MAPK)/ERK and JAK/STAT pathways (224,225). It has been well documented that GH plays a key role in longitudinal growth during childhood, while maintaining various important metabolic functions throughout life (promoting lipolysis, protein synthesis and gluconeogenesis, while reducing glucose uptake from the liver) (226). Of note though, GH action is slightly more sophisticated. Apart from its direct action, an indirect one through the production of IGF-1 also takes place, representing an important part of GH physiology. In fact, a potent stimulus of IGF-1 production is the GH per se. Moreover, the tissues producing IGF-1 (the liver 75% and the peripheral tissues) are indeed the target organs of GH (227). However, it has been proven that GH is not only synthesized in the pituitary, but also in various other tissues, such as the large intestine, prostate and breast (228,229). In this case however, GH lacks the endocrine potential and its action is mainly restricted to an autocrine or paracrine manner (230).

Due to its proliferative properties, GH has attracted reasonable attention for its carcinogenic potential. In fact, various studies have demonstrated that GH is indeed able to create a favorable microenvironment for tumor cells. In detail, GH overexpression is linked to an increased risk of malignancies (231), while its downregulation is linked to a carcinoprotective state. As for CRC risk per se, GH has been proven to act as a tumor promoter in colon tissue by suppressing p53 (232), *phosphatase and tensin homolog (PTEN)* and *APC* (8), while it has also been proven that colon cancer cells overexpress *GHR* (232). In fact, upregulated GH has been exhibited to increase ERK phosphorylation and to decrease *APC* expression (232). It is known that a decreased *APC* expression promotes the nuclear accumulation of β-catenin, which in turn increases Wnt signaling through the activation of pro-proliferative genes (233,234). Thus, even though it is difficult to estimate the exact concentration for any given age above which GH poses its carcinogenic effect on colon cells, there is enough evidence supporting that the ability of GH to change the microenvironment of tumor cells can have a synergistic effect with other CRC risk factors (such as intestinal dysbiosis, smoking etc.), shifting the balance towards tumor survival and proliferation (235).

4. Conclusions

After 30 years of intense research on CRC and its biological behavior, only few facts have withstood the test of time, all revealing the impressive complexity and diversity of this entity. In fact, this is the case for the association between CRC and anabolic substances. Driven by epidemiological observations on both sexes, a time pattern of CRC onset has been found. However, studies thus far have failed to reach a consensus regarding the direct connection between androgens and the risk of developing CRC, as some studies have indicated a negative effect, while others have pointed out a neutral or even a protective one (Table I). In the case of androgens, the androgen receptor (the principal mediator of their action) has been proven to be altered in CRC in contrast to healthy individuals. Moreover, anabolic substances are also put to scrutiny given the intense presence of such substances in patients with CRC (namely IGF-1). However, no direct or well-studied indirect mode of action on CRC pathogenesis has been found for both classes. Thus, more studies are needed that will focus on both epidemiologic data (that will try to investigate how the use of anabolic agents, androgens included, alters CRC statistics) and the elucidation of molecular pathways implicated in CRC, in order to allow the extraction of solid conclusions. In addition, as passive everyday life exposure to hazardous chemicals could affect traditional clinical risk factors and act synergistically, the patterns of living and consumers' trends should also be taken into consideration when evaluating CRC.

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Authors' contributions

JT conceived and designed the study. TK and TKN researched the literature, performed analysis of data and drafted the manuscript. DAS, CT and AT made substantial contributions to the conception of the study and critically revised the article for important intellectual content. MS, JS, TMS made substantial contributions to the design of the study and critically revised the article for important intellectual content. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

DAS is the Editor-in-Chief for the journal, but had no personal involvement in the reviewing process, or any influence in terms of adjudicating on the final decision, for this article. The other authors declare that they have no competing interests.

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