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**EDITORIAL**

- 4093** Interleukin 12/interleukin 23 pathway: Biological basis and therapeutic effect in patients with Crohn's disease
Aggeletopoulou I, Assimakopoulos SF, Konstantakis C, Triantos C

REVIEW

- 4104** Role of microRNAs in alcohol-induced liver disorders and non-alcoholic fatty liver disease
Torres JL, Novo-Veleiro I, Manzanedo L, Alvela-Suárez L, Macías R, Laso FJ, Marcos M
- 4119** Calcium-sensing receptor in colorectal inflammation and cancer: Current insights and future perspectives
Iamartino L, Elajnaq T, Kallay E, Schepelmann M
- 4132** Production of extracellular lysophosphatidic acid in the regulation of adipocyte functions and liver fibrosis
Yang F, Chen GX

MINIREVIEWS

- 4152** Ten years of sorafenib in hepatocellular carcinoma: Are there any predictive and/or prognostic markers?
Marisi G, Cucchetti A, Ulivi P, Canale M, Cabibbo G, Solaini L, Foschi FG, De Matteis S, Ercolani G, Valgiusti M, Frassinetti GL, Scartozzi M, Casadei Gardini A

ORIGINAL ARTICLE**Basic Study**

- 4164** Differential expression of mucin 1 and mucin 2 in colorectal cancer
Kasprzak A, Siodla E, Andrzejewska M, Szmaja J, Seraszek-Jaros A, Cofta S, Szaflarski W
- 4178** Mechanism of combined use of vitamin D and puerarin in anti-hepatic fibrosis by regulating the Wnt/ β -catenin signalling pathway
Huang GR, Wei SJ, Huang YQ, Xing W, Wang LY, Liang LL

Retrospective Study

- 4186** Frequency, types and treatment of anemia in Turkish patients with inflammatory bowel disease
Bengi G, Keyvan H, Durmaz SB, Akpınar H

Prospective Study

- 4197** Low-dose spectral insufflation computed tomography protocol preoperatively optimized for T stage esophageal cancer - preliminary research experience
Zhou Y, Liu D, Hou P, Zha KJ, Wang F, Zhou K, He W, Gao JB

CASE REPORT

- 4208** Novel methionyl-tRNA synthetase gene variants/phenotypes in interstitial lung and liver disease: A case report and review of literature
Abuduxikuer K, Feng JY, Lu Y, Xie XB, Chen L, Wang JS

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Calcium-sensing receptor in colorectal inflammation and cancer: Current insights and future perspectives

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Abstract

The extracellular calcium-sensing receptor (CaSR) is best known for its action in the parathyroid gland and kidneys where it controls body calcium homeostasis. However, the CaSR has different roles in the gastrointestinal tract, where it is ubiquitously expressed. In the colon, the CaSR is involved in controlling multiple mechanisms, including fluid transport, inflammation, cell proliferation and differentiation. Although the expression pattern and functions of the CaSR in the colonic microenvironment are far from being completely understood, evidence has been accumulating that the CaSR might play a protective role against both colonic inflammation and colorectal cancer. For example, CaSR agonists such as dipeptides have been suggested to reduce colonic inflammation, while dietary calcium was shown to reduce the risk of colorectal cancer. CaSR expression is lost in colonic malignancies, indicating that the CaSR is a biomarker for colonic cancer progression. This dual anti-inflammatory and anti-tumourigenic role of the CaSR makes it especially interesting in colitis-associated colorectal cancer. In this review, we describe the clinical and experimental evidence for the role of the CaSR in colonic inflammation and colorectal cancer, the intracellular signalling pathways which are putatively involved in these actions, and the possibilities to exploit these actions of the CaSR for future therapies of colonic inflammation and cancer.

Key words: Calcium-sensing receptor; Colon; Cancer; Inflammation; Calcimimetics; Calcilytics

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Core tip: The extracellular calcium-sensing receptor (CaSR) is best known for its roles in maintaining body calcium homeostasis, but it is also expressed in the intestines where it is assumed to be involved in pathologies such as inflammatory bowel disease and colorectal cancer. It has been suggested to act as a tumour suppressor in colorectal tumourigenesis. In this review we highlight the evidence for the anti-inflammatory and anti-tumourigenic roles of the CaSR, its signalling pathways, and its potential for future use as a drug target in the context of inflammatory bowel disease and colorectal cancer.

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INTRODUCTION

Extracellular calcium-sensing receptor

The extracellular calcium-sensing receptor (CaSR) was first identified in bovine parathyroid cells. It is a G protein-coupled receptor (GPCR) that is activated by extracellular calcium (Ca^{2+}), which acts as a first messenger of the CaSR signalling cascade^[1]. The main physiological role of the CaSR is to control serum Ca^{2+} levels through regulating the synthesis and secretion of parathyroid hormone (PTH), which acts directly on the kidneys, bones and indirectly on the intestines to maintain normocalcaemia^[2]. Therefore, the CaSR acts as a "calciostat" which maintains serum Ca^{2+} concentration within a tight range (1.1-1.3 mmol/L free ionised Ca^{2+}) and is expressed in calcitropic tissues, such as parathyroid glands, kidneys and bone. In addition to its pivotal role in maintaining serum Ca^{2+} homeostasis, the CaSR also regulates non-calcitropic functions, such as gene expression, smooth muscle contraction, differentiation, proliferation, inflammation, and ion channel activity in other tissues, such as the colon, liver, vasculature, lung, pancreas, brain and the placenta^[3-6]. The CaSR is also expressed along the entire gastrointestinal (GI) tract and regulates various functions in the intestines. These include dual regulation of fluid transport, where it stimulates Cl^- and short chain fatty acid-dependent HCO_3^- secretion, but inhibits cyclic adenosine monophosphate (cAMP)-dependent HCO_3^- secretion^[7]. In addition, the CaSR is expressed in the myenteric plexi where it regulates gut motility^[8]. It also acts as a nutrient sensor for digestion products^[9], such as amino acids. Additionally, it plays a role in intestinal inflammation and in the maintenance of gut microbiota and immune homeostasis^[10].

The CaSR is a multifaceted GPCR that couples to several heterotrimeric G proteins. It modulates signalling pathways downstream of $\text{G}_{q/11}$, $\text{G}_{i/o}$, $\text{G}_{12/13}$ ^[11] and in specific cell contexts G_s ^[12]. Signalling output by the CaSR is also ligand-dependent as well as cell-type specific, thus adding to the diversity of the CaSR-mediated signalling pathways. Table 1^[5,16-26] shows examples of both naturally occurring and synthetic CaSR ligands and their reported direct effects on inflammation and cancer *in vivo*.

Mutations in the *CASR* gene result in Ca^{2+} homeostasis-related diseases, including familial hypocalciuric hypercalcaemia (FHH1) and neonatal severe hyperparathyroidism (NSHPT), both of which are caused by inactivating mutations, as well as autosomal dominant hypocalcaemia (ADH1), which is caused by activating mutations (for review see^[27]). Such disease causing mutations result in altered signalling output by the receptor and/or reduced cell surface expression^[28]. In the intestines, more focus is directed towards the CaSR as a therapeutic target for intestinal diseases including diarrhoea, inflammatory bowel disease and colorectal cancer. In the colon, loss of CaSR expression is associated with colonic tumourigenesis^[29]. In addition, clinical trials show that Ca^{2+} intake can favourably modulate normal colon tissue and circulating inflammation biomarkers for risk of colorectal neoplasms in sporadic colorectal adenoma patients^[17]. This has led to the hypothesis that the CaSR plays a role in cancer prevention. In the following sections, we highlight the role of the CaSR in intestinal inflammation and colorectal cancer.

ROLE OF THE CaSR IN INFLAMMATION

The CaSR is expressed in a wide range of inflammation-associated cell types where it regulates various functions. It is expressed in immune cells including macrophages, eosinophils and monocytes^[5,30,31]. In these CaSR-expressing human and murine circulating monocytes, extracellular Ca^{2+} induces a chemokinetic effect^[32]. The CaSR is also implicated in immune regulation where it plays a dual role: as a responder to inflammatory cytokine release on the one hand, and as a promoter of inflammation on the other. The link between the CaSR and inflammation has been explored in several studies. *In vitro*, inflammatory cytokines upregulate the CaSR expression in various cell types through defined response elements on the *CASR* gene^[33,34]. *In vivo* studies also suggest a link between inflammatory cytokines and the CaSR, as intraperitoneal injection of IL-1 β and IL-6 reduced PTH and 1,25(OH) $_2\text{D}_3$ levels followed by a decrease in serum Ca^{2+} ^[33,35]. Furthermore, clinical studies show that hypocalcaemia occurs in critically ill patients where plasma inflammatory cytokines levels are increased^[36]. In addition, the expression of the CaSR is increased in monocytes from rheumatoid arthritis patients with severe coronary artery calcification^[37].

Table 1 Examples of orthosteric agonists and allosteric modulators of the calcium-sensing receptor^[13-15]

Ligand type	Class and examples	Reported effects on inflammation	Reported effects on cancer	Ref.
Orthosteric agonists	Inorganic divalent and trivalent cations: Zn ²⁺ , Ca ²⁺ ; Mg ²⁺ ; Gd ³⁺	Reduces inflammation in mouse models of colitis Intake is correlated with reduced inflammation	High Ca ²⁺ intake: Associated low risk for CRC	[16-18]
	Polyamines: Spermine spermidine, putrescine	Increase airway inflammation and hyperresponsiveness	Reduce pancreatic cancer growth in mice	[5,19]
	Aminoglycoside antibiotics: Neomycin, gentamycin, tobramycin	-	-	
	Basic polypeptides: poly-L-arginine, L-poly-L-lysine, and amyloid β -peptides	Induces airway inflammation Reduces inflammation in mouse models of colitis	-	[5,20]
Combined orthosteric and allosteric modulators	D-amino-acid polypeptides: Etelcalcetide	-	-	
	L-amino acids: Phenylalanine, tryptophan	-	-	
	Glutamyl dipeptides: L-Glu-Val, L-Glu-Cys	Reduces inflammation in mouse models of colitis	-	[21]
Allosteric modulators (calcimimetics and calcilytics)	Small molecule calcimimetics: Sensipar (L-Cinacalcet HCl), NPS-R568, GSK3004774	Increases airway hyperresponsiveness	Treatment of parathyroid tumours Inhibits neuroblastoma tumour growth Reduces hypercalcaemia of malignancy	[5,22-24]
	Small molecule calcilytics: NPS-2143, Calhex, Ronacalaret, AXT-914	Reduces pulmonary inflammation and airway hyperresponsiveness in rodents	-	[5,25,26]

¹Indicates the compounds for which the *in vivo* effects were reported. While many of these modulators have been reported to have *in vitro* effects on (cancer) cell lines, evidence of their *in vivo* activity has remained scarce. The table summarises their known (putatively) CaSR-mediated direct effects on inflammation and cancer in humans or animals. CRC: Colorectal cancer.

Inflammatory pathways regulated by the CaSR

The CaSR regulates diverse and intricate signalling networks and this regulation is tissue-, and ligand-dependent. In murine macrophages, the CaSR activates the NACHT, LRR and PYD domains-containing protein 3 (NLRP3) inflammasome through a mechanism that involves increased intracellular Ca²⁺ and decreased cAMP levels^[38]. Moreover, the CaSR regulates polymorphonuclear neutrophil function through a mechanism that likely involves the NF κ B pathway^[39]. The mechanism by which L-tryptophan, L-valine and glutamyl dipeptides mediate the CaSR-dependent inhibition of pro-inflammatory cytokine secretion in colonocytes appears to require β -arrestin 2^[20,21]. Moreover, in the thick ascending limb of the kidneys, the CaSR has been shown to induce TNF- α -dependent cyclooxygenase 2 expression and prostaglandin E₂ synthesis *via* a Gi-dependent mechanism^[40]. However, the exact mechanism by which the CaSR regulates inflammation is still unclear and needs further investigation.

Tissue-specific roles of the CaSR in inflammation

Interestingly, regulation of inflammation by the CaSR appears to be tissue-dependent. One example is the pivotal role of the CaSR in airway hyperresponsiveness and inflammation in allergic asthma. Studies on mice show that the calcilytic NPS-2143 ameliorates the severity of allergen-induced airway hyperresponsiveness^[5]. In agreement with that, NPS-2143 was also shown

by an independent group to be protective against lipopolysaccharide-induced pulmonary inflammation^[26] and against inflammation caused in cigarette smoke extract-stimulated airway epithelial cells^[25]. The CaSR plays a pro-inflammatory role also in human adipose cells and adipose tissue, where it induced the expression of inflammatory cytokines^[41]. Paradoxically, in the intestines the CaSR has been suggested by several studies to play an anti-inflammatory role. Below, we highlight the evidence for the anti-inflammatory effects of the CaSR in the intestines and the potential to exploit it for nutraceutical and pharmaceutical intervention.

CaSR IN INTESTINAL INFLAMMATION

In vivo anti-inflammatory effects of the intestinal CaSR

Evidence supporting the role of the CaSR in intestinal inflammation comes from a study in an intestinal epithelial cell-specific CaSR knock-out mouse model. This study showed that deletion of the CaSR from the intestinal epithelial cells diminished intestinal barrier integrity, altered the composition of the gut microbiota and induced stimulatory inflammatory responses^[42]. These intestine specific CaSR knock-out mice were more susceptible to dextran sulphate sodium (DSS)-induced inflammation leading to colitis, which is a model for chemically induced inflammation in rodents. *Ex vivo* assessment of intestinal permeability revealed that in the knock-out mice the paracellular transport pathway

was impaired. Consistent with that observation, the colonic expression of tight junction proteins, particularly claudin-2, was reduced in knock-out mice, while the expression of myosin light-chain kinase-1, an enzyme that controls contractility of the perijunctional actomyosin rings and epithelial permeability, was significantly increased^[42]. No significant differences were seen between the overall richness and diversity of the gut microbiota of knock-out and wild type littermates, yet the bacterial composition was significantly changed. Moreover, intestine specific CaSR knock-out mice had significantly lower epithelial expression of Reg3 β and Reg3 γ that encode secreted C-type lectins which bind and protect against translocation and dissemination of bacteria. Furthermore, gene array analysis revealed increased expression of inflammatory cytokines including IL-1R in the distal colons of the intestinal epithelium-specific CaSR knock-out mice, as well as in their colonic CD4⁺ and CD8⁺ T lymphocytes. In addition, a marked increase in NF κ B-dependent genes was observed in the knock-out mice. The expression of programmed cell death protein 1 (PD-1) was significantly enhanced in colonic CD4⁺ and CD8⁺ T cells^[42].

Similarly, recent studies support the anti-inflammatory role of the CaSR in a DSS-colitis mouse model, where poly-L-lysine and glutamyl dipeptides, orthosteric agonists of the CaSR, reduced inflammation. These anti-inflammatory effects were suggested to be dependent on the CaSR, as their effect was reduced by the intravenous administration of the calcilytic NPS-2143^[20,21]. Whether this inhibition of the anti-inflammatory effects was due to the systemic actions of the calcilytic or due to a direct action of the drug at the inflamed tissue is yet unknown. Studies assessing whether the expression level of the CaSR is affected by the chronic inflammation of the intestine in human patients suffering from inflammatory bowel disease are still outstanding.

Mechanisms by which the CaSR putatively modulates colonic inflammation

Studies on colon cancer cell lines using CaSR agonists and allosteric modulators suggested that the CaSR influences the production of inflammatory cytokines induced by tumour necrosis factor α (TNF- α). L-tryptophan and L-valine inhibited interleukin 8 (IL-8) secretion in both Caco-2 and HT-29 colon cancer cell lines. This effect was reversed by the calcilytic NPS-2143^[20]. In addition, glutamyl dipeptides inhibited pro-inflammatory cytokines and chemokines including IL-8, IL-6, and IL-1 β , while increasing the expression of the anti-inflammatory IL-10 in Caco-2 cells^[21]. However, it was reported that the CaSR is not detectable in colon cancer cell lines, such as HT-29, which is also supported by evidence from independent studies indicating the scarcity of the CaSR in colon cancer tissue and cell lines^[43]. Therefore, further validation is needed to confirm whether these anti-inflammatory effects are actually mediated *via* the CaSR. Of note, inflammatory cytokines, such as TNF- α , IL-1 β and IL-6, increased the

expression of the CaSR at the mRNA and protein level in some colon cancer cell lines^[34]. This was suggested to be a defence mechanism against inflammation in the intestines. However, this explanation will have to be carefully validated, as *e.g.*, in lung epithelium, CaSR expression is also increased in the inflamed tissue. There however, the increase (and indeed the CaSR itself) represent a rather pro-inflammatory mechanism, as inhibition of the CaSR markedly reduced airway inflammation and hyperresponsiveness^[5].

Given that inflammation is a high risk factor for colorectal cancer, it is imperative to ask the following question: is there a causal relationship between activation of the CaSR, reduced inflammation and the prevention of colorectal cancer? As of yet, this question remains unanswered. It is unclear whether dietary or pharmacological activation of the CaSR in the GI tract prevents inflammation in humans. It is also still unclear whether loss of the CaSR in colorectal tumours correlates with loss of its proposed anti-inflammatory effects. Moreover, it is noteworthy that the presence of inflammatory cytokines in the GI tract and their effect on the expression and/or function of the CaSR add to the complexity of the scenario *in vivo*. Nonetheless, inflammation is a key risk factor for colorectal cancer^[44,45], thus targeting the CaSR for mitigating inflammation may very well contribute to colorectal cancer prevention in one fell swoop. Below, we summarise the evidence for the involvement of the CaSR in cancer and specifically colorectal cancer as well as its potential as a therapeutic target.

ROLE OF THE CaSR IN CANCER

The CaSR plays a ying-yang role in tumours: while it is suggested to be an oncogene in breast and prostate tumours, in parathyroid, neuroblastoma and colorectal cancers it acts as a tumour-suppressor^[46-48]. The CaSR signals *via* multiple signalling pathways and is sensitive to many ligands, the bioavailability of which varies among tissues. The different ligands and different signalling pathways can generate a tissue-specific CaSR response, justifying this dual behaviour during cancer development. Table 2^[3,19,29,49-72] summarises the different roles of the CaSR in various types of cancer.

CaSR acts both as oncogene and tumour suppressor

The CaSR was implicated in the promotion of metastases from breast, prostate, and kidney tumours, thus acting as an oncogene in these tissues. Its oncogenic role is often mediated by parathyroid hormone related peptide (PTHrP).

Breast cancer has a tendency to form metastases in particular in the bones^[73]. Metastases originated from breast tumours promote bone resorption which, in turn, causes the release of trophic factors (*e.g.*, TGF- β and IGF1) that stimulate tumour cell growth, thus forming a vicious cycle. Osteolysis is driven by osteoclasts that are activated by PTHrP, which is synthesised and

Table 2 Dual function of the calcium-sensing receptor as tumour suppressor and oncogene in various cancers and the affected calcium-sensing receptor-coupled signalling pathways

Cancer type	CaSR	Expression of the CaSR	Detection	Proposed signalling pathway	Ref.
Gastric	Oncogene	Increased	mRNA, protein	TRPV4	[49]
Prostate	Oncogene	Increased	mRNA, protein	PTHrP <i>via</i> trans-activation of the EGFR and ERK1/2 phosphorylation	[50-52]
Breast	Oncogene	Increased in breast primary tumours and in bone metastases	mRNA, protein	AKT phosphorylation PTHrP <i>via</i> cAMP ERK1/2 and TRPC1 Inhibition of OPG <i>via</i> epiregulin	[53-57]
Renal carcinoma	Oncogene	Increased in bone metastasising tumours	mRNA, protein	AKT phosphorylation	[58]
Colorectal	Tumour suppressor	Reduced	mRNA, protein	Canonical and non-canonical Wnt/ β -catenin pathway and EMT	[3,29,59-62]
Endometrial	Tumour suppressor	Reduced	Protein	Apoptosis Wnt/ β -catenin VEGFR3	[63]
Parathyroid	Tumour suppressor	Reduced	mRNA, protein	Caveolin-1 and Gαq Cyclin D1 and RGS5	[64-69]
Neuro-blastoma	Tumour suppressor	Reduced	mRNA, protein	Apoptosis <i>via</i> ERK1/2 Cancer testis antigens (CTAs)	[22,70,71]
Pancreatic	Unknown	Reduced	mRNA, protein	NCX1/Ca ²⁺ / β -catenin	[19,72]

released from breast cancer cells^[3,74,75]. The CaSR, highly expressed in metastatic breast cancer cells^[53], stimulates PTHrP release, contributing thereby to bone degradation^[54]. A recent study revealed that cancer cells overexpressing the CaSR had a higher osteolytic potential compared with untransfected cells^[57]. Therefore, the CaSR could be a predictive marker for bone metastasis and for the patient's poor prognosis.

Like breast cancers, prostate neoplastic lesions have a high capacity to form metastasis in the bone. Highly aggressive prostate cancer cells, such as PC-3, express the CaSR^[50] while there is no evidence of CaSR expression in normal prostate tissue^[3]. A cohort study, analysing 1241 prostate cancer patients, found that expression of the CaSR correlated positively with tumour lethality^[76].

Although dietary calcium has been suggested to have beneficial effects on the digestive tract as being preventative against colorectal cancer, a recent study pointed out a controversial effect of calcium on gastric cancer development. Xie *et al.*^[49], have shown that calcium-activated CaSR promoted gastric cancer cell proliferation and metastasis. Thus, CaSR is suggested to act as an oncogene in the upper part of the gastro-intestinal tract, whereas it seems to act as a tumour suppressor in the lower gastro-intestinal tract (see below) although further studies are required to confirm this hypothesis.

In other cancers like parathyroid cancers, neuroblastoma and colorectal cancer the receptor acts as a tumour suppressor. In parathyroid tumours CaSR expression is inversely correlated with tumour development. CaSR mRNA expression is reduced in parathyroid adenomas and hyperplasias as compared

with normal parathyroid tissue and it is lost in parathyroid carcinoma^[65]. In the nervous system, the CaSR is expressed during the differentiation of neurons and glial cells^[77,78]. In neuroblastoma, CaSR expression is positively correlated with neuroblast differentiation and low clinical risk, while undifferentiated and malignant neuroblastomas are CaSR-negative^[79]. Indeed, ectopic re-expression of the CaSR in MYCN-amplified neuroblastoma cells, which are normally CaSR negative, reduced xenograft growth^[71]. In addition, treatment with cinacalcet, a positive allosteric modulator of the CaSR, was able to induce the expression of differentiation markers, to inhibit cell proliferation *in vitro* and the growth of mouse tumour xenografts *in vivo*^[22].

Another organ in which the CaSR acts as tumour suppressor is the colon.

CaSR IN COLORECTAL CANCER

The physiological role of the colon is to process and absorb undigested nutrients, absorb electrolytes and water, and to excrete waste products *via* the rectum. As it is a highly renewable tissue, it is prone to malignant transformation. Colorectal cancer (CRC) is one of the most recurrent types of malignancies in the western countries and accounts for over 1,2 million of new cases per year^[80]. Colorectal tumorigenesis is a complex mechanism developing from the alteration of different molecular processes that control gene expression, cell cycle and apoptosis, which are affected by genetic (*e.g.*, APC mutation), environmental (*e.g.*, diet, alcohol abuse, cigarette smoking, *etc.*), microbial and inflammatory cues that either activate oncogenes or repress tumour suppressors leading then to tumour

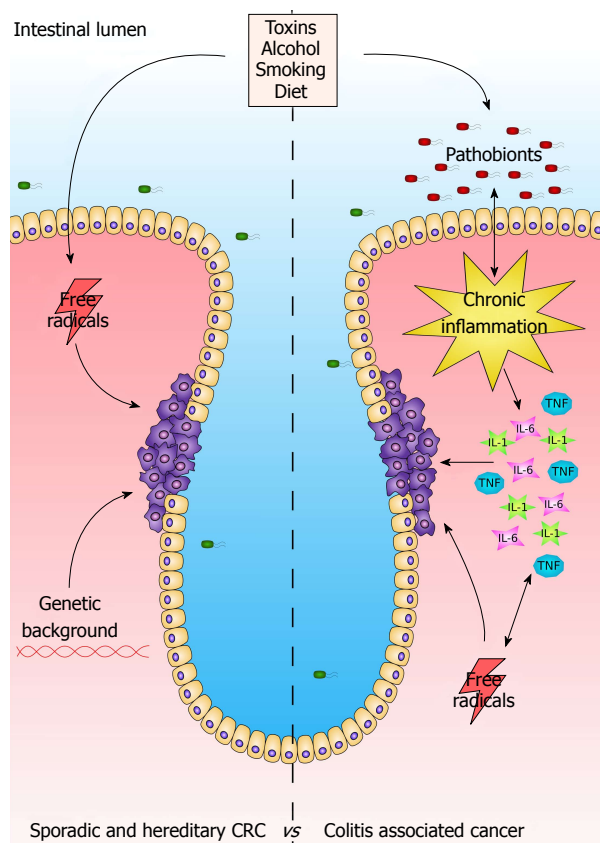


Figure 1 Scheme of colonic carcinogenesis. Left: Environmental cues such as toxins, alcohol, smoke and diet can produce free radicals (such as reactive oxygen and nitrogen species) that can damage genomic DNA. Accumulating mutations, in particular in genes that encode for mitogenic, cell cycle or apoptosis factors such as APC, BRAF, KRAS, EGF and p53 can then eventually lead to colon carcinogenesis. Genetic background such as inborn APC mutations (hereditary familial adenomatous polyposis) or other hereditary mutations also predispose towards colon tumourigenesis, although hereditary CRC is rare^[81]. Right: In addition to their direct noxious effect on the tissue, environmental cues can also alter the microbiotic population of the intestine, promoting the proliferation of pathogenic bacteria (pathobionts). Pathobionts and chronic inflammation are closely related and both induce the expression of pro-inflammatory cytokines that accumulate in the mucosa. Persistent inflammation interferes with cell proliferation and apoptosis processes leading to tumourigenesis and in particular in colitis associated cancer. Inflammation itself also induces the production of free radicals that hamper genome stability and can thus cause tumour development^[82]. TNF: Tumor necrosis factor; IL: Interleukin; CRC: Colorectal cancer.

development (Figure 1^[81,82]).

As mentioned above, colonic inflammation is a risk factor for developing colorectal cancer. Chronic intestinal inflammatory diseases such as Crohn's disease and ulcerative colitis often lead to colorectal cancer through a process called colitis-associated carcinogenesis (CAC). Similarly to (spontaneous) CRC, CAC leads to genome instability, targeting tumour suppressors and DNA repair mechanisms. However, CRC and CAC differ for prevalence and sequential-timing of the changes in biomarkers during their pathogenesis^[83].

CAC is often accompanied by the alteration of the gut microbiota (dysbiosis). Commensal bacteria (eubionts) help to metabolise undigested food, modulating also the immune system of the digestive tract. On the other hand,

pathogenic bacteria can trigger an immune response that, in the worst case, can lead to chronic colitis and other inflammatory bowel diseases^[84]. As detailed above, the CaSR has been implicated in affecting gut microbiota, and the expression of inflammatory cytokines and thus might play a protective role against the development of CAC by protecting from the deleterious effects of inflammation.

Epidemiology

In 1985, a small trial demonstrated for the first time that calcium regulates colonocyte proliferation^[85]. In the same year, Garland *et al.*^[16] published a retrospective study showing that diets with high calcium content lower the risk of developing colorectal tumours. In the following years, several cohort studies and animal experiments supported the theory that diets rich in calcium and vitamin D prevent the development of colon hyperplasia and cancer - in contrast to western diets with high fat and low calcium and fibre content^[86-88]. Meta-analyses have since reported that high Ca^{2+} intake (more than 1400 mg/d), independent of its source, lowers the risk of CRC, in particular in the distal colon^[89,90]. Indeed, the evidence for the protective actions of high levels of dietary calcium intake (dairy products) or calcium supplements was rated to be "probably strong" by the World Cancer Research Fund in its most recent update of 2017^[91].

Numerous studies have suggested that there is a close interaction between the CaSR and calcium and its protective action against CRC. A randomised clinical trial found that dietary calcium supplementation increased the expression of the CaSR in the colonic mucosa^[92]. In a meta-analysis, Yang *et al.*^[93] showed that while dietary calcium reduced the risk of developing CaSR positive tumours, the risk for CaSR negative ones remained unchanged, suggesting that dietary calcium exerts its anti-tumourigenic properties *via* CaSR. A recent study demonstrated that CaSR expression in the tumours correlated with a reduced risk of mortality, indicating that CaSR expression might be a biomarker for positive prognosis^[94].

CaSR localisation in the intestine

A common agreement on the pattern of CaSR localization in the intestine is still missing. Whitfield suggested that the Ca^{2+} concentration is unevenly distributed along the colonic crypts, with low levels found at the bottom of the crypts and higher levels at the top. In this way, Ca^{2+} could exert its pro-differentiating and anti-proliferative effects only in the upper part of the crypts where the post-differentiated mature colonocytes are localised. CaSR activation would follow this concentration gradient along the crypts. Stronger activation of the CaSR at the top and weaker activation at the bottom could thus provide a physiological rationale for why the CaSR would inhibit proliferation on the differentiated top but allow proliferation at the rapidly dividing bottom of the

crypts^[95]. It was also suggested that this Ca^{2+} gradient influences CaSR expression itself, in addition to the receptor's activation^[46]. This theory is supported by the studies of Chakrabarty *et al.*^[96], who have found CaSR protein to be expressed only in the upper half of the crypts of human colon cancer biopsies. However, the actual expression pattern of the CaSR in the colon is still under debate. Contrary to the findings by Chakrabarty *et al.*^[96], Sheinin *et al.*^[97] have found CaSR expression only in the enteroendocrine cells of human colonic mucosa^[97], whereas Cheng *et al.*^[8] have found the CaSR in the enteric nervous system and in the apical and basolateral side of the crypts of rat colons^[8,98]. Further studies are therefore required to determine accurately the location of the CaSR in the colon and whether this expression pattern is dependent on factors like diet, age, *etc.*

We know that CaSR expression is lost in tumour cells. While it is still found in pre-neoplastic lesions, expression of the CaSR is lost in poorly differentiated tumours^[29,96,99,100]. However, whether this loss is cause or effect of the tumourigenesis is still unknown.

CaSR down-regulation in CRC

Epigenetic aberrancies play a major role in tumour malignancy in general and thereby also in colorectal cancer^[101]. CaSR expression is affected by repressive epigenetic marks in malignant colorectal lesions. The promoter region of the CaSR contains a large CpG island which is highly methylated in colorectal tumours. CaSR expression could be partially restored in colorectal cancer cell lines by the administration of 5-aza-2'-deoxycytidine, an inhibitor of DNA methylation. This effect was further enhanced with the addition of histone deacetylase inhibitors, suggesting that in the CaSR promoter regions the acetylation of histones is reduced and, therefore, the chromatin has a less permissive structure that hinders the recruitment of the transcription machinery^[29,102]. The level of CaSR methylation increases from hyperplastic polyps and adenomas to lymph node metastases in parallel with the reduction of the receptor's expression^[102]. However, this is not a general mechanism, as in parathyroid tumours no hypermethylation of the CaSR locus was found^[64,103].

Non-coding RNAs, such as miRNAs, also regulate CaSR expression in colorectal tumours. Different studies found that miR-21, miR-135a, miR-135b, miR-145, miR-146b and miR-503 inhibited CaSR expression in CRC cell lines and therefore constitute potential targets for restoring CaSR mRNA level^[61,104,105].

So far, no CaSR mutations have been found that would promote tumour development in the intestine^[48], although several SNPs (*e.g.*, Q1011E, A986S, R990G) might increase colorectal cancer susceptibility although their contribution is controversial^[106-109].

It is important to fully understand the molecular mechanisms that drive CaSR loss during colorectal carcinogenesis and whether this loss could be reverted or prevented and whether such an action would be

beneficial for patient prognosis, pointing towards the CaSR as potential therapeutic target for a novel anti-CRC therapy or prevention.

Evidence and molecular pathways for the anti-tumourigenic actions of the CaSR

Mouse models of systemic CaSR knock-out are not viable or die shortly after birth due to severe hyperparathyroidism and hypercalcemia^[110]. However knocking out PTH rescues the lethal CaSR^{-/-} phenotype in the PTH double knock-out (PTH^{-/-} CaSR^{-/-}) mouse model^[111]. The colonic mucosa of the PTH^{-/-} CaSR^{-/-} mice as well as that of the intestinal epithelium-specific CaSR knock-out mouse model show signs of hyperproliferation. These mice develop pre-malignant intestinal lesions and are highly susceptible to the carcinogen azoxymethane (AOM)^[112]. The intestines of these mice are often inflamed and express pro-inflammatory markers. Furthermore, PTH^{-/-} CaSR^{-/-} mice are highly sensitive to DSS induced inflammation as well, suggesting a possible role of the CaSR as an anti-inflammatory factor^[42,112].

Overexpression of the exogenous CaSR in colon cancer cell lines induced cellular differentiation and apoptosis, and inhibited proliferation and invasion capacity in these transfected cells. Presence of the CaSR repressed expression of stem cells markers, re-established the expression of E-cadherin and inhibited epithelial to mesenchymal transition, a process exploited by cancer cells to form metastases^[47,59].

Ca^{2+} exerts its anti-tumourigenic function not only by binding and precipitating toxic agents such as secondary bile acids and fatty acids but also by modulating different cellular mechanisms such as proliferation, differentiation and apoptosis, potentially *via* the CaSR^[3,100,113,114]. The mechanism involves inhibition of c-myc, upregulation of E-cadherin and inhibition of the canonical wnt-signalling pathway^[99,100,115]. A recent study reported that Ca^{2+} inhibited the expression of replication-licensing factors in a CaSR dependent manner^[60].

Both colorectal cancer cell lines and CaSR-deficient mice show that loss of the CaSR causes a higher recruitment of β -catenin into the nucleus, thus sustaining a proliferative Wnt pathway^[59,112,116]. However, some studies have discovered that the CaSR is able to activate the non-canonical Wnt pathway involving the interaction between Wnt5a and its receptor, Ror2 (receptor tyrosine kinase-like orphan receptor 2). Wnt5a/Ror2 counteracts the proliferative signalling of Wnt/ β -catenin, recruiting the ubiquitin ligase Siah2, which, in turn, degrades β -catenin. In myofibroblasts, CaSR activation induces the secretion of Wnt5a, while, in colonic epithelia CaSR increases the expression of Ror2^[62]. Thus, the CaSR might stimulate the Wnt5a/Ror2 paracrine pathway which inhibits colonic proliferation, interfering with Wnt/ β -catenin, and seems to promote the expression of colonic differentiation markers such as sucrase-isomaltase, caudal type homeobox 2 and villin^[62,117,118].

CaSR pathways could potentially interact with many cellular processes in preventing or counteracting

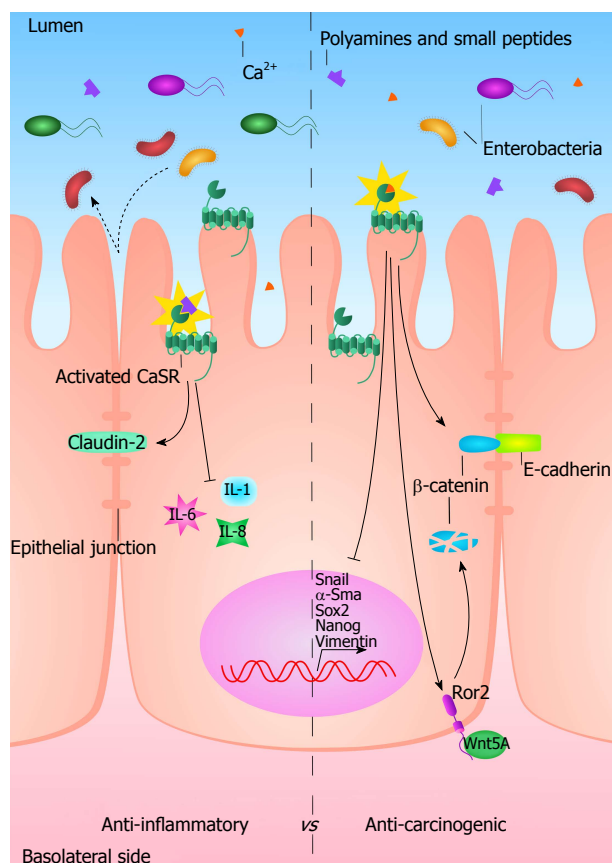


Figure 2 Protective function of the calcium-sensing receptor against inflammation and carcinogenesis in the colon. Left: The CaSR promotes intestinal barrier integrity, potentially by promoting claudin-2 expression, and inhibits the expression of pro-inflammatory cytokines, thus preventing inflammation. Right: The CaSR exerts an anti-tumourigenic effect by counteracting the mitogenic Wnt pathway, preventing β -catenin translocation into the nucleus, which is either sequestered by E-cadherin at the cell junctions or it is degraded by the non-canonical Wnt signalling (Ror2-Wnt5A) and inhibits the expression of mesenchymal and stem cells markers. IL: Interleukin; CaSR: Calcium-sensing receptor.

tumour development and progression. In this context, the existence of a cross talk between the CaSR and the vitamin D system has been suggested. It seems that both pathways converge in the modulation of the Wnt signalling to control colonocyte proliferation. Moreover, vitamin D seems to regulate CaSR transcription^[119,120] through regulatory elements present in the CaSR promoter, which are recognised by the transcription factor vitamin D receptor. Indeed, a high vitamin D (2500 IU/kg) diet over 5 weeks more than doubled the expression of the CaSR in the colon mucosa of mice^[121,122].

As of yet, a detailed description of CaSR signalling in the intestine is still missing. Given the fact that the CaSR is able to sense not only Ca^{2+} , but also polyamines and amino acids, which are highly abundant in the intestinal lumen through the food, and that ligand biased signalling is a known feature of the CaSR^[3], it is possible that the CaSR could activate different down-stream signals depending on these specific ligands also in the colon. Potential mechanisms by which the CaSR could affect

inflammation and CRC are summed up in Figure 2 but a detailed map of the molecular pathways that the CaSR activates in the gut is still missing. This would allow researchers to discover potential therapeutic targets for counteracting intestinal tumourigenesis.

FUTURE PERSPECTIVES - THE CaSR AS A DRUGGABLE TARGET IN THE COLON

The CaSR is considered to prevent or counteract intestinal carcinogenesis and inflammation. Thus, the CaSR might constitute a promising therapeutic target for the treatment of colorectal cancer and of inflammatory bowel diseases. Dietary Ca^{2+} supplementation reduces the risk for developing colorectal cancer and studies have shown the beneficial effects of CaSR agonists, such as dipeptides, polyamines for preventing colonic inflammation and cancer. As chronic inflammation is a risk factor for colorectal cancer, the CaSR might actually be a link that connects the beneficial effect of Ca^{2+} in preventing both inflammation and cancer in the colon. Indeed, these roles of the CaSR indicate that activating the CaSR, or in the case of CRC also restoring CaSR expression - or preventing its loss - might be an important way for treating or preventing colonic inflammation, CRC, and, especially, CAC. However, a direct pharmacological intervention study targeting the CaSR in colonic inflammation or colorectal cancer is still missing.

Further research will be required for finding and evaluating means to restore or prevent the loss of the expression of the CaSR during carcinogenesis. One such mean could be the use of pharmacological CaSR activators, the calcimimetics. In addition to their action as allosteric agonists of the CaSR, calcimimetics also act as so called "pharmacochaperones" for the CaSR. They stabilise the expression of the CaSR, preventing the receptor's degradation. At the same time, they increase trafficking of the CaSR from its intracellular reservoirs into the cell membrane^[123,124]. As of yet, there are no data for the efficacy of calcimimetics for the prevention / treatment of CRC or CAC.

As chronic inflammation is posing a high risk for developing CAC, preventative measures should be administrable over long periods of time and should therefore ideally elicit few or no systemic side effects. Recently a novel calcimimetic, GSK3004774, which is non-resorbable and thus has gut restricted effects, has been published^[125]. This compound could be useful for testing whether locally acting calcimimetics can elicit a preventive effect against intestinal inflammation, CRC and CAC without affecting systemic calcium homeostasis.

Known side effects of the FDA-approved calcimimetic cinacalcet treatment include hypocalcaemia and, notably, nausea^[126]. Whether these gastrointestinal tract-related side effects are elicited *via* the systemic actions of the drug or a direct effect of the drug on the gastrointestinal

organs is unclear. In addition, calcimimetics have been shown to actually enhance inflammation in other epithelial tissues, *e.g.*, the lung, while calcilytics, antagonists of the CaSR, ameliorated the inflammation. In this context, the CaSR also promoted the activation of the immune system and showed a general pro-inflammatory action^[5]. Whether these *in vivo* effects - in the complex context of immune-cells, inflamed tissue and cytokines - are tissue specific or related to a ubiquitous activation of CaSR-bearing lymphocytes is unclear. Taken together, these considerations do not allow a definite conclusion for a potential treatment of colonic inflammation or cancer with pharmacological CaSR modulators alone or in combination with conventional or targeted chemotherapies. Extensive future studies will be required to satisfactorily answer all these questions.

CONCLUSION

The CaSR emerges as a direct player in colonic inflammation and cancer development. Current evidence suggests that activation of the CaSR reduces the risk for both diseases, the strongest evidence being that dietary Ca²⁺ reduces the risk for CRC and that this effect is apparently mediated by the CaSR while expression of the CaSR is lost during tumourigenesis and progression of CRC. Making direct use of the CaSR as a drug target to reduce or prevent colonic inflammation and at the same time prevent colonic tumourigenesis seems a promising strategy, especially for CAC, where a dietary or pharmacological intervention could hit two birds with one stone, as it were. Future studies will be needed to address where exactly the receptor is expressed in the colonic microenvironment, which signalling pathways are mediated by the CaSR in the settings of inflammation and cancer *in vivo*, and whether these actions of the CaSR can be exploited for therapy and prevention.

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