Role of prostaglandins in colorectal tumorigenesis: Localization and expression of COX-1, COX-2, microsomal Prostaglandin E Synthase-1 and the EP2 receptor

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ABSTRACT

Background: Prostaglandans, in particular prostaglandin E2 (PGE2), are elevated in adenomas and colorectal cancers (CRC). Experimental and epidemiological studies have demonstrated reduced incidence of adenomas and CRC by inhibitors of prostanoid synthesis (NSAIDs). This study aimed to characterize the expression and localization of key enzymes/receptors for PGE2 synthesis in adenomas and CRC in comparison to normal colon.

Methods: Immunoblotting and immunohistochemistry were used for semi-quantitative and qualitative analysis of COX-1, COX-2, mPGES-1 and the EP2 receptor in biopsies from patients undergoing resection of adenomas or surgery for CRC (Dukes’ A-C). Normal colon served as control for the corresponding tumor in each of the CRC patients.

Results: COX-1 was decreased significantly in all groups of CRC (Dukes’ A-C) compared to normal colon. In contrast, COX-2 was increased, but only in the combined group of CRC. Microsomal PGES-1 was increased in CRC (Duke’s B), and EP2 was augmented in adenomas and CRC. The localization was predominantly epithelial in normal colon and in adenomas, while in CRC both epithelial- and stromal expression was demonstrated.

Conclusions: The results support the PGE2- pathway, with epithelial- stromal interactions, in the evolvement of adenomas and in the progression of CRC. Co-expression of COX-1 and COX-2 is in line with the preventive effects of non-specific NSAIDs on adenoma formation. The decrease of COX-1, in combination with an increase of COX-2, favors the potential use of selective COX-2 inhibitors as an adjunct therapy in CRC.
INTRODUCTION
Colorectal cancer (CRC) ranks as one of the top three malignancies in most Western populations. Earlier studies from the Middle East reported that CRC was less common than in the West. However, the association between diet, a sedentary lifestyle and overweight makes it more likely that the incidence of CRC will increase substantially in Qatar, as well as in other parts of the Middle East. An additional risk factor in this region for CRC is consanguinity that is demonstrated to be strongly associated with the development of CRC. In fact, studies from several countries in the Middle East reports an increasing incidence in CRC, and several characteristics of Western populations, e.g. earlier age of incidence. The present model of colon tumorigenesis involves the conversion of adenomas to the malignant phenotype. Prostaglandins (PGs), in particular PGE2, have been implicated in the growth of both adenomas and cancer. The cyclooxygenases (COX), COX-1 and COX-2, are key enzymes in the synthesis of prostanoids. COX-1 is considered to produce PGs to maintain tissue homeostasis, e.g. integrity of the gastrointestinal mucosa, whereas COX-2 is induced by hormones, growth factors and cytokines, and generates PGs responsible for inflammatory responses, angiogenesis, cell proliferation and apoptosis. The importance of PGs for malignancy is demonstrated by epidemiological studies where the use of non-steroidal anti-inflammatory drugs (NSAIDs) resulted in a substantial reduction of the risk to develop tumors, in particular CRC. Previous studies have demonstrated low or non-detectable levels of COX-2 in normal colon tissue and increased contents in tumors, while COX-1 was expressed at low levels in both normal and malignant cells. Multiple reports have focused on COX-2 in CRC and recently, a trial with a COX-2 specific inhibitor, celecoxib, reduced the frequency of colorectal polyps in patients with an earlier history of colorectal adenomas. The final conversion to PGE2 is mediated by prostaglandin E synthase (PGES). Two microsomal isoforms are described, mPGES-1 and mPGES-2; and both forms were found to be over-expressed in CRC. There are four receptors for PGE2 (EP1–EP4); all demonstrated to participate in experimental carcinogenesis. Interestingly, activation of the EP2 receptor by PGE2 was demonstrated to interact with the axin/β-catenin signaling in human colon cancer cells in vitro. Alteration of this pathway by mutations in the APC gene is a crucial event in colorectal tumorigenesis, both in humans and experimental animal models. An additional role for PGE2 in tumorigenesis is the activation of the cyclic AMP signaling pathway in “tumor associated stromal cells”, e.g. immune cells, where an altered function might result in a defective anti-tumor response.

The aim of this study was to characterize the expression, cellular localization and contents of key enzymes for the synthesis of PGE2 (COX-1, COX-2, mPGES-1), and the EP2 receptor, in normal tissue, in adenomas, and in CRC.

MATERIALS AND METHODS
Tissues of adenomas, normal colon and CRC
Adenomas were from (surgical pathology files, Sahlgrenska University Hospital, Göteborg, Sweden) six patients with sporadic adenomatous polyposis (1 tubular, 5 tubulovillous). Five were of mild to moderate dysplasia and one of severe dysplasia. Biopsies of normal colon and CRC were obtained from patients (surgical pathology files, the University Hospital, Uppsala, Sweden) undergoing routine surgical procedures with colonic or rectal resection. Non-neoplastic tissue adjacent to the cancer was obtained from all patients with CRC and served as control tissue (normal colon) for the corresponding tumor of the same patient. The tumors were classified according to Dukes’ stages. Tissue samples were snap frozen and kept at −70°C until analysis.

Homogenization of tissues and immunoblotting
Preparation of tissue extracts and the procedure for immunoblotting/visualization (Tropix, Bedford, UK) were described previously in detail. The primary antibodies were from Cayman Chemical Co. (Ann Arbor, MI, USA): COX-1 (monoclonal, dilution 1:1,000), COX-2 (rabbit polyclonal, 1:1,000), mPGES-1 (rabbit polyclonal, 1:500), EP2 receptor (rabbit polyclonal, 1:500). Proliferating cell nuclear antigen (PCNA) (monoclonal, 1:1,000) was from Santa Cruz Biotechnology (San Diego, CA, USA). Electrophoresis standards for COX-1 and COX-2 (Cayman Chemical Co.) were used as positive controls. Each blot contained an identical sample (see below) as an internal control in order to compare the levels of expression between blots.
Densitometric scanning
Semi-quantitative measurements of the immunoblots were made by densitometry (Fluor-S™ Multimager, Quantity One ver. 4.1.0., BioRad, Hercules, CA, USA). The optical density (OD) of each band was measured. The internal standard on each blot was set to 100%. A CRC sample (Dukes’ B) was used as an internal standard for COX-2, mPGES-1 and EP2 measurements, while normal colon from a Dukes B patient was used for COX-1. The signal from each band was compared to the standard and the obtained relative value was used for statistical analysis.19

Immunohistochemistry
The immunohistochemical analysis is described in detail elsewhere.19 The primary antibodies COX-1 (1:100), COX-2 (monoclonal, 1:100), mPGES-1 (1:50), EP2 (1:50) were from Cayman Chemical Co. (Ann Arbor, MI, USA) and Cytokeratin 8 (CK8, monoclonal, 1:400, marker of epithelial cells) was from DAKO (Copenhagen, Denmark). Primary antibodies were replaced by equal amounts of TBS as negative controls.

Statistics
Values are given as the mean ± standard error of the mean (SEM). ANOVA followed by Fischer’s LSD post-hoc test was used for the analysis of the immunoblotting data obtained by densitometric scanning. When comparing normal and CRC samples from the same patient, paired students t-test was used. A p-value less than 0.05 was considered significant.

RESULTS

COX-1 in normal colon, adenomas and CRC
A strong expression of COX-1 was noticed in most of the normal colon samples obtained from patients of all Dukes’ stages. The adenocarcinomas of all stages demonstrated a lower expression compared to the corresponding normal colon tissue from each individual patient (Fig. 1A–C). The decrease was significant for all three stages: Dukes’ A tumors (n = 8) 37% (p < 0.05), Dukes’ B tumors (n = 9) 32% (p < 0.05), and in Dukes’ C tumors (n = 10) 31% (p < 0.05) (Fig. 1A–C). When all normal colon samples (n = 27) and the corresponding CRC samples (n = 27) were compared as groups with the content in adenomas (n = 6), the latter content was in between that of normal samples and the adenocarcinomas and did not significantly differ from either group (Fig. 1D).

COX-1 was localized to epithelial cells in the normal colon (Fig. 2A) and in the adenomas (Fig. 2E). No epithelial staining was observed in the CRC, where the expression was confined to the stroma (Fig. 2C). Staining of cells in the stroma was scarce in normal colon and absent in the adenomas (Fig. 2A, E).

COX-2 in normal colon, adenomas and CRC
COX-2 was low in normal colon samples of CRC patients (n = 32), but a trend towards an increased content was observed in the normal colon obtained from Dukes’ C patients. There was no significant difference between the individual Dukes’ stages and the corresponding normal colon (Fig. 3A–C). However, the COX-2 expression in adenocarcinomas (Dukes’ A-C) combined to one group (n = 32), was significantly elevated compared to the normal colon (n = 27) (p < 0.05) (Fig. 3D). The levels in adenomas (n = 6) were not significantly different compared to the normal colon or to the CRC (Fig. 3D).

Only a few epithelial cells and stromal cells stained positive for COX-2 in normal colon tissue (Fig. 2B). The adenomas exhibited staining limited to epithelial cells (Fig. 2F) whereas a majority of the CRC exhibited staining of both epithelial and stromal cells (Fig. 2D).

mPGES-1 in normal colon, adenomas and CRC
A slight increase of mPGES-1, although not significant, was observed for Dukes’ A (n = 7) and C (n = 8) tumors (Fig. 4A, C). The content in Dukes’ B (n = 6) tumors was significantly increased compared to the corresponding normal colon tissue (n = 6) (Fig. 4B). The levels in the combined group of CRC (Dukes’ A-C) (n = 21) demonstrated a trend of increase compared to normal colon (n = 21), but this was not significant (Fig. 4D). Neither did the expression in the adenomas (n = 6) significantly differ from the groups of normal colon or adenocarcinomas (Dukes’ A-C) (Fig. 4D).

The normal colon demonstrated a weak staining of mPGES-1 in some epithelial cells, as well as sporadic staining of stromal cells (Fig. 5A). A distinct, epithelial staining (cytoplasmic) was observed in...
adenomas (Fig. 5E). A positive staining (cytoplasmic), mainly of clusters of stromal cells, was present in the CRC (Fig. 5C).

EP2 receptor in normal colon, adenomas and CRC

The CRC samples showed a strong expression of the EP2 receptor, and the contents were also increased significantly in the Dukes’ A (n = 8), B (n = 9), C (n = 10) stages compared to the corresponding groups of normal colon (p < 0.05) (Fig. 6A, C). An increase, but not significant, was also observed in the Dukes’ B (n = 6) tumors (Fig. 6B). The content of EP2 was significantly increased in adenomas (n = 6) compared to the group of normal colon (p < 0.05) (Fig. 6D). The increase of EP2 was also significant in CRCs (Fig. 6B, C, D). The samples are plotted as percentage of a normal colon tissue from a patient with a Dukes’ B tumor that was used as an internal standard sample on each immunoblot. Paired t-test was used for statistical analysis. An asterisk (*) denotes a statistically significant difference (p < 0.05) between tumor and normal colon of the same Dukes stage. ANOVA followed by Fischer’s LSD post-hoc test was used for statistical analysis. (a) p < 0.05 vs. normal samples. All CRC stages were included in the CRC group.

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significant in the combined group of CRC (n = 21) compared to the corresponding group of control tissue (n = 21) (p < 0.05) (Fig. 6D).

The EP<sub>2</sub> receptor was demonstrated (membrane staining) in both epithelial and stroma cells in normal colon (Fig. 5B). The adenomas exhibited a strong staining (membrane/cytoplasmic) of epithelial cells, while the signal from stromal cells was very weak or undetectable (Fig. 5F). The stromal cells in the adenocarcinomas showed a clear signal (membrane/cytoplasmic), while the staining of epithelial cells was less intense (Fig. 5D).

**PCNA in normal colon, adenomas and CRC**

Proliferating cell nuclear antigen (PCNA), an enzyme involved in DNA synthesis, was used as a marker to estimate proliferation. A significant increase of PCNA content was demonstrated by immunoblotting (data not shown) in CRC compared to the corresponding normal colon (normal 20 ± 3%, tumor 44 ± 9%, p < 0.01). Interestingly, the contents of PCNA in adenomas were increased even more in comparison to normal colon (76 ± 9%, p < 0.001).

**DISCUSSION**

The importance of PGE<sub>2</sub> in colorectal tumorigenesis is supported by experimental and epidemiological studies. This study demonstrates that key enzymes for the synthesis of PGE<sub>2</sub>, i.e. COX-1, COX-2, mPGES-1, and the EP<sub>2</sub> receptor, exhibit cell- and stage specific expression patterns in adenomas and CRC.

The PGs, generated by COX-1, are involved in the maintenance of barrier functions of the intestinal mucosa and the protection against damage, and are also essential for the survival of intestinal stem cells. Our results demonstrated a major localization of COX-1 to epithelial cells of the normal colon, in agreement with earlier studies. The content of COX-2 in the normal epithelium was sparse with only a few positive cells. This finding was expected since the COX-2 expression is commonly associated with inflammation. Microsomal PGES-1 was also localized to the epithelial cells of the normal colon,
similar to the findings by Yoshimatsu and co-workers. The EP₂, EP₃ and EP₄ receptors were demonstrated in normal human colon.

Our study focused on the EP₂ receptor, since previous experimental studies in vivo and in vitro showed that this receptor is important for intestinal polyposis and proliferation of colon cancer.

Fig. 3. COX-2 expression in normal colon, adenomas and CRC. The semi-quantitative measurements were obtained by densitometric scanning of immunoblots. A–C) The graph demonstrates the content of COX-2 in normal colon and CRC (paired values) from each patient. A) Dukes’ A (n = 11), B) Dukes’ B (n = 10), C) Dukes’ C (n = 11). The bars to the left and right hand sides of the figures represent Mean ± SEM of COX-2 content in normal colon and CRC. The samples are plotted as percentage of a normal colon tissue from a patient with a Dukes’ B tumor that was used as an internal standard sample on each immunoblot. Paired t-test was used for statistical analysis. An asterisk (*) denotes a statistically significant difference (p < 0.05) between tumor and normal colon of the same Duke stage. D) Histogram representing samples of normal colon tissues, adenomas (n = 6) and CRC. ANOVA followed by Fischer’s LSD post-hoc test was used for statistical analysis. (a) p < 0.05 vs. normal samples. All CRC were included in the CRC group.
The present study demonstrated EP_2 receptors in the epithelium and in the stroma of normal colon. The stained stromal cells were not further characterized, but were most likely immune cells. COX-1, COX-2, mPGES-1 and the EP_2 receptor were mainly confined to epithelial cells of the adenomas, and the contents of COX-1, COX-2 and mPGES-1 were similar to that of normal colon. In contrast, the EP_2 receptor demonstrated a significant increase. This suggests that the epithelial cells in adenomas have the potential of an enhanced responsiveness to PGE2. The significance of co-expression of COX-1 and COX-2 in adenomas, as demonstrated in the present study, was analyzed in a rodent model for FAP.

**Fig. 4.** mPGES-1 expression in normal colon, adenomas and CRC. The semi-quantitative measurements were obtained by densitometric scanning of immunoblots. A–C) The graph demonstrates the content of COX-1 in normal colon and tumor tissue (paired values) from each patient. A) Dukes’ A (n = 7), B) Dukes’ B (n = 6), C) Dukes’ C (n = 8). The bars to the left and right hand sides of the figures represent Mean ± SEM of mPGES-1 content in normal colon and CRC. The samples are plotted as percentage of a normal colon tissue from a patient with a Dukes’ B tumor that was used as an internal standard sample on each immunoblot. Paired t-test was used for statistical analysis. An asterisk (*) denotes a statistically significant difference (p < 0.05) between tumor and normal colon of the same Dukes’ stage. D) Histogram representing samples of normal colon tissues, adenomas (n = 6) and CRC. ANOVA followed by Fischer’s LSD post-hoc test was used for statistical analysis. (a) p < 0.05 vs. normal samples. All CRC stages were included in the CRC group.
where COX-2 was expressed only in larger polyps/adenomas, while COX-1 was found in polyps/adenomas of any size. The authors proposed that the presence of COX-1 “secured” basal levels of PGE₂ for early growth of polyps to a stage/size when additional expression of COX-2 (and mPGES-1) contributed to an accelerated growth. Indeed, the present study provides support for an accelerated proliferation rate in adenomas, since the expression of PCNA was significantly increased compared to normal colon. A recent study using a human cell line established from early microadenoma of a polyposis patient demonstrated that exogenous PGE₂ could stimulate progression of related genes in these cells, e.g. c-fos, the ERK signaling pathway and COX-2. We did not observe expression of COX-2 in the stroma of adenomas and there are conflicting results regarding the cellular localization of over-expressed COX-2. Some studies reported an increase of COX-2 in cancerous cells in colon adenomas while others demonstrated COX-2 expression predominantly in stromal cells, e.g. macrophages, and only weak expression in epithelial cells. In polyps from patients with FAP, COX-2 was demonstrated predominantly in stromal fibroblasts and endothelial cells, but to a very small extent in bone-marrow derived cells. One study showed active inflammatory signaling in polyps, suggesting that NSAIDs acted, at least in part, on stromal- rather than epithelial cells. Expression of COX-2 was observed in epithelial cells and macrophages in sporadic colorectal polyps as well as in fibroblasts and endothelial cells of polyps from patients with FAP. In addition, COX-2 expression was positively correlated to an increased severity of dysplasia in epithelial cells and in size of colorectal adenomas. The major stromal expression of COX-2 in adenomas or polyps has been attributed to macrophages. This finding suggested that COX-2 was involved in the synthesis of PGs for signaling between macrophages and epithelial cells in polyps in vivo. A paracrine signaling pathway demonstrated in experiments in vitro.

The adenomas examined in the present study, might represent stages of increased growth, but without a phenotype lacking “tumor-antigenicity” to recruit immune cells (macrophages, T-cells) to the stroma. A mechanism for tumor-derived PGE₂ to participate in malignant progression could be to convert adaptive, regulatory T-cells (Treg) into a regulatory phenotype. Treg have been demonstrated to accumulate in tumors where they may impair an effective anti-tumor immune response. In fact, an inverse correlation between the percentage of Treg in peripheral blood and disease prognosis was demonstrated in patients with gastrointestinal malignancies.
Dukes’ stage A demonstrated a reduced content of COX-1 compared to control colon and to adenomas. A similar reduction of the COX-1 protein in CRC, was noted by Kargman and coworkers. They also found similar contents of COX-1 in normal colon and in adenomas. Many studies have focused on the involvement of COX-2 in tumorigenesis since COX-2 expression stimulates growth, cell survival, angiogenesis and tumor cell invasiveness. Furthermore, genes regulating apoptosis, proliferation and

Fig. 6. EP2 expression in normal colon, adenomas and CRC. The semi-quantitative measurements were obtained by densitometric scanning of immunoblots. A–C) The graph demonstrates the content of COX-1 in normal colon and tumor tissue (paired values) from each patient. A) Dukes’ A (n = 7), B) Dukes’ B (n = 6), C) Dukes’ C (n = 8). The bars to the left and right hand sides of the figures represent Mean ± SEM of mPGES-1 content in normal and CRC tissue. The samples are plotted as percentage of a normal colon tissue from a patient with a Dukes’ B tumor that was used as an internal standard sample on each immunoblot. Paired t-test was used for statistical analysis. An asterisk (*) denotes a statistically significant difference (p < 0.05) between tumor and normal colon of the same Dukes’ stage. D) Histogram representing samples of normal colon tissues, adenomas (n = 6) and CRC. ANOVA followed by Fischer’s LSD post-hoc test was used for statistical analysis. (a) p < 0.05 vs. normal samples. All CRC stages were included in the CRC group.
cell-cell communication were affected by COX-2 expression.\textsuperscript{26} Taken together, these studies suggested the involvement of COX-2, but not of COX-1, in carcinogenesis. The present study demonstrated also a significant augmentation of COX-2 in the combined group of CRC (Dukes’ A-C). Interestingly, we observed a trend of increase in the corresponding normal colon samples, in particular in samples obtained from patients with Dukes’ C tumors. This finding is most likely due to an increased systemic inflammatory response, with an infiltration of immune cells also into the surrounding normal colon tissue, adjacent to the growing tumor. Clinical studies have also demonstrated elevated levels of C-reactive protein in patients with CRC, and that this marker of a generalized inflammatory response was associated with cancer-specific survival.\textsuperscript{40} Recent reports have shown the presence of COX-2 expressing immune cells, e.g. macrophages, in the stroma of colon tumors.\textsuperscript{35} We observed that the content of COX-2 varied between individual CRC stages, and similar variations have been reported earlier.\textsuperscript{34,44} Sheehan and co-workers showed a correlation between a greater expression of COX-2 with more advanced Dukes’ stage.\textsuperscript{31} In contrast, Dimberg and collaborators\textsuperscript{41} were unable to find this correlation, similar to our results.

Previous studies have reported an increased content of mPGES-1 in CRC.\textsuperscript{13} In the present study, a significant elevation of mPGES-1 was demonstrated in Dukes’ stage B tumors. Previous immunohistochemical analysis\textsuperscript{13} demonstrated low levels of mPGES-1 localized to epithelial cells in normal colon. An increased expression was observed in adenomas and in adenocarcinomas.\textsuperscript{13,42} All these studies reported an exclusive epithelial localization of mPGES-1. The lack of stromal staining in adenocarcinomas is in contrast to our findings, where mPGES-1 was demonstrated in clusters of stromal cells, a similar pattern to that of COX-2. Two studies\textsuperscript{37,47} observed an increase of COX-2 in conjunction to mPGES-1 in tumors. In support for a stromal localization of mPGES-1 (as well as for COX-2) are clinical,\textsuperscript{35} as well as experimental studies,\textsuperscript{23} demonstrating the importance of macrophages in this compartment.

Experimental models have demonstrated a role for all four EP receptors in experimental carcinogenesis.\textsuperscript{9,23} The present study focused the expression of the EP\textsubscript{2} receptor, which similar to EP\textsubscript{4}, activates the PKA-pathway, but in addition also participates in the $\beta$-catenin/axin/APC pathway.\textsuperscript{9} It was also suggested that the EP\textsubscript{2} and EP\textsubscript{4} receptors were most likely mediators of the paracrine effects of PGE\textsubscript{2} on intestinal epithelial cells, both at early and at late stages of colorectal carcinogenesis.\textsuperscript{8,43} Targeted deletion of the EP\textsubscript{2} receptor gene in $\text{apc}^{-/-}$ knockout mice resulted in a decrease in the number of polyps, and the phenotype resembled the $\text{cox}-2^{-/-}$ mice.\textsuperscript{24} In contrast, targeted deletion of the EP\textsubscript{1} or EP\textsubscript{3} receptors did not affect polyp formation. These studies also showed that COX-2 expression was boosted by PGE\textsubscript{2} through the EP\textsubscript{2} receptor, via a positive feedback loop. In both COX-2 and EP\textsubscript{2} deficient mice, induction of angiogenesis-related factors was suppressed.\textsuperscript{26} Other studies have also reported that angiogenic effects of COX-2 may be mediated by the EP\textsubscript{2} receptor.\textsuperscript{44} These results proposed a role for the EP\textsubscript{2} receptor in early stages of tumorigenesis. Indeed, our result showed that the EP\textsubscript{2} receptor was increased in adenomas and in CRC of all Dukes’ stages, supporting a role in both polypl formation and in the progression of CRC. A study in mice reported an increase of the EP\textsubscript{2} receptor in the intestine after radiation injury.\textsuperscript{21} They could also demonstrate that pro-survival- and anti-apoptotic effects of PGE\textsubscript{2} were mediated by the EP\textsubscript{2} receptor. These experimental findings, and the observed increase of the EP\textsubscript{2} receptor in adenomas and CRC in the present study, suggest participation in cell cycle control and programmed cell death, resulting in an increased growth rate, reflected by elevated levels of PCNA in the present study.

The present findings support a role for PGE\textsubscript{2} signaling in the growth of adenomas and in the progression of CRC. The contents of COX-2, mPGES-1 and EP\textsubscript{2} were significantly elevated in CRC in comparison to the corresponding normal colon. In contrast to the normal colon tissue and adenomas, the level of COX-1 was decreased in CRC. Expression in cells outside of the epithelium, i.e. particular in the stroma of CRC, of COX-2, mPGES-1 and EP\textsubscript{2}, supports previous experimental studies emphasizing stromal-epithelial interactions in tumor progression.\textsuperscript{39} Our observation of co-expression of COX-1 and COX-2 in adenomas provides a plausible explanation to the more efficient reduction of adenomas/CRC observed in epidemiological studies of users of non-specific NSAIDs compared to clinical trials with coxibs,\textsuperscript{9,12} suggesting that non-specific NSAIDs can prevent earlier stages in tumorigenesis.

Acknowledgements

Support was provided by the Swedish Cancer Society, the Swedish Society of Medicine, the Swedish Medical Research Council (13475 to K.S. and L.H.), the Norwegian Cancer Society (to L.H.), the Novo Nordisk Research Foundation (to L.H.), Sysknon Svenssons, Lars Hiertas foundation, Foundations of Assar Gabrielson and Foundation King Gustav V Jubilee Clinic Cancer Research.
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