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An insight of consequences of body weight on altered expression of nuclear receptors and interrelation of cyclooxygenase-2 in human colorectal cancers

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Abstract
Extensive studies on risk factors for colorectal cancer (CRC) have mainly focused on diet, and being overweight is now recognized to contribute significantly to CRC risk. Overweight and obesity are defined as an excess of adipose tissue mass and are associated with disorders in lipid metabolism. Peroxisome proliferator-activated receptors (PPARs) and retinoid-activated receptors (RARs and RXRs) are important modulators of lipid metabolism and cellular homeostasis. Alterations in expression and activity of these ligand-activated transcription factors might be involved in obesity-associated diseases, which include CRC. Cyclooxygenase-2 (COX-2) also plays a critical role in lipid metabolism and alterations in COX-2 expression have already been associated with unfavourable clinical outcomes in epithelial tumors. The objective of this study is to examine the hypothesis questioning the relationship between alterations in the expression of nuclear receptors and COX-2 and the weight status among male subjects with CRC.
Depending multidimentional reviews, findings show that alterations in the pattern of nuclear receptor expression observed in CRC do not appear to be correlated with patient weight status. However, the analysis of COX-2 expression in normal colon mucosa from subjects with a high BMI suggests that COX-2 deregulation might be driven by excess weight during the colon carcinogenesis process.

Keywords: Body weight, colorectal cancer (CRC), cyclooxygenase-2, body mass index (BMI), Peroxisome proliferator-activated receptors (PPARs)

1. Introduction
Approximately 5% of individuals will develop colorectal carcinoma during their lifetime [1]. This disease typically progresses from adenomatous polyps and dysplastic polyps to invasive carcinoma [2, 3]. CRC seems particularly susceptible to specific nutritional factors and dietary habits (for review) [4]. Indeed, excessive consumption of calories from fat is thought to be largely responsible for the increasing incidence of CRC in western countries. Moreover, overweight and obesity status associated with high body mass indices have been correlated with a higher risk of developing CRC [5, 6]. Nevertheless, the mechanisms of why and how excess weight increases cancer risk are only slowly emerging. One proposed mechanism is the rise of insulin resistance resulting in hyperinsulinemia that can cause growth-promoting effects [7].
Hyperinsulinemia as well as hyperlipidemia, hypertension, overweight and type II diabetes are metabolic disorders which might be caused by alterations in the homeostasis of the metabolism of fatty acids [8]. These obesity-related symptoms could affect the integrity of colon tissue homeostasis and therefore be involved in CRC etiology [9]. Anti-diabetic treatments initially used for improving parameters such as insulin sensitivity have been shown to be able to inhibit colon carcinogenesis in rodent models [10, 11] and to promote differentiation of colon cancer cells [12]. Moreover, concomitant suppression of hyperlipidemia and polyp formation were observed in APC-deficient mice treated with insulin-sensitizing drugs called thiazolidinediones (TZD) [13]. In such a context, PPAR family members initially recognized for their involvement in regulating fat metabolism and adipogenesis have emerged as attractive targets for therapeutic approaches for obesity and CRC. Indeed, PPARγ agonists including anti-diabetic agents, polyunsaturated fatty acids as well as non-steroidal anti-inflammatory drugs (NSAID) [14, 15] have been demonstrated to affect proliferation and differentiation in cancer cell lines [16].

Moreover, both anti-proliferative effects of PPARγ observed *in vitro* [12] and inactivating mutations in the PPARγ gene found in colon tumors [17] provide evidence for a tumor suppressor function. This is also supported by the finding that an increased risk of polyp occurrence in colon mucosa was found to be significantly associated with a decrease in PPARγ mRNA expression [18]. Another isotype, PPARδ, may also play an important role in the process of colon carcinogenesis since it has been efficiently targeted by hypolipidemic and hypoglycemic drugs [19]. However, PPARδ might display distinct functions in lipid metabolism and colon carcinogenesis. Indeed and in contrast to PPARγ, PPARδ was found frequently overexpressed in colon cancer cells [20] and tumors of chemically-initiated animals [21]. Nevertheless, the role of PPARδ in colonic epithelium stays unclear due to conflicting evidence [22].

The transcriptional activity of PPAR depends on the presence of the retinoic X receptor (RXR), activated by 9-cis retinoic acid (9-cis RA). Heterodimerization with RXR is essential for the activity of all class II nuclear receptors [23] and explains how fatty acids and retinoids control lipid metabolism [24]. The active forms of vitamin A, 9-cis RA and all-trans retinoic acid (atRA), also exhibit anti-tumoral properties in many tissues mainly through RXR and retinoic acid-activated receptor (RAR) binding. Indeed, retinoids have displayed chemopreventive and chemotherapeutic activities with regard to their capacity to induce cell differentiation and apoptosis (for review) [25]. RXRα is by far the most prevalent isoform in the colon, while RXRβ and RXRγ are expressed at low levels [26]. All three RAR isotypes, α, β, and γ, are expressed and induced by retinoids in colon cancer cell lines [27]. Alterations in retinoid-activated receptor expression and biological activity have been observed both *in vivo* and *in vitro* [28]. However, potential alterations have been poorly investigated in CRC although they may affect the response of target cells to retinoid and lipid derivatives.

COX-2 is a key enzyme in lipid metabolism and is well-known to convert arachidonic acid to growth-regulating molecules such as prostaglandins. COX-2, activated by growth factors and pro-inflammatory cytokines, has been shown to be overexpressed in several epithelial cancers including CRC [29, 30]. This enzyme might mediate the promotion of colon carcinogenesis by metabolic disorders and inflammation and several lines of evidence indicate that COX-2 might be regulated by PPARγ[31] and RARβ activation in cancer cells [32, 33] although the mechanism is unclear. It was also shown that deregulations in nuclear receptor expression might promote COX-2 upregulation [34].

In the current report, our interest was (i) to evaluate alterations in the expression of the different nuclear receptors and COX-2 in several colon cancer specimens from patients undergoing surgery to remove tumors and (ii) to clarify whether or not the expression of nuclear receptors and COX-2 was affected by the weight status of patients with CRC.

RNA extraction and reverse transcription (RT)

Adipose tissue has emerged as a major endocrine organ producing a wide spectrum of hormones and factors that play crucial roles in regulating cell turnover and function, not only locally within the adipose tissue but also in the brain and other key metabolic organ systems. It is known that gene activity is controlled at both transcriptional and post-transcriptional levels. Consequently, one of the most important means by which the activity of a gene is assessed is through the determination of levels of the corresponding messenger ribonucleic acid (mRNA). This process involves the isolation of total cellular RNA and subsequent analysis of the mRNA of interest. Given the unique nature of adipose tissue and adipocytes (i.e., containing high amounts of lipid), special RNA isolation techniques that have been tested in both white adipose tissue and isolated mature adipocytes from rats and mice will be presented. Although several methods are available for mRNA quantitation, we will describe a real-time quantitative reverse transcription polymerase chain reaction protocol because of its superior sensitivity and reliability.

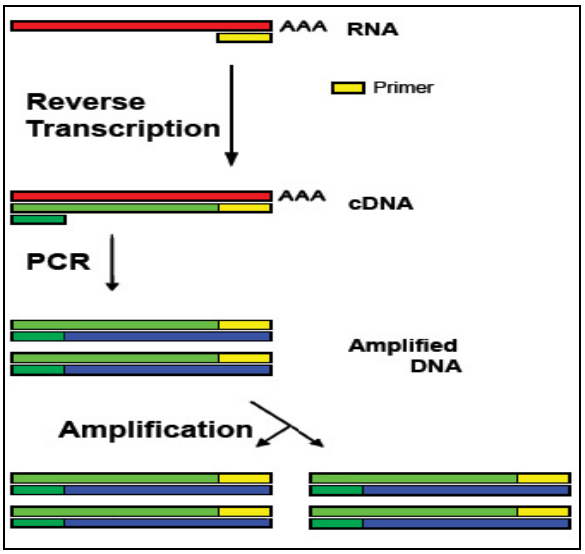

Fig 1: Reverse Transcription process

Generally, total RNA used to extract from colon tissue samples using the RNA –gents. Total RNA Isolation System kit is correlated to the manufacturer's instructions. Reverse transcription was as follows: 2 µg of total RNA was mixed with RNasin (1 U/µL, Promega, Charbonnières, France) and DNase I (0.5 U/µL, Roche Diagnostics, Meylan, France) and incubated 15 min at 37°C. Reverse primers (0.75 µM of each) were added and incubated for 10 min at 70°C. ImProm-II™ 5× reaction buffer (1×, Promega, Charbonnières, France), $MgCl_2$ (2.5 mM, Promega, Charbonnières, France), dNTP (0.5 mM each one, Roche Diagnostics, Meylan, France) and ImProm-II™ Reverse Transcriptase (Promega, Charbonnières, France) were added for 1 hr at 42°C. The total volume was 20 µL and each target mRNA was co-reverse transcribed with β2-microglobulin mRNA.

Real-time Polymerase Chain Reaction (PCR)

The scientific, medical, and diagnostic communities have been presented the most powerful tool for quantitative nucleic acids analysis: real-time PCR [Bustin, S.A., 2004. A-Z of Quantitative PCR. IUL Press, San Diego, CA]. This new technique is a refinement of the original Polymerase Chain Reaction (PCR) developed by Kary Mullis and coworkers in the mid 80:ies [Saiki, R.K., *et al*., 1985. Enzymatic amplification of beta-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia, Science 230, 1350], for which Kary Mullis was

awarded the 1993 year's Nobel prize in Chemistry. By PCR essentially any nucleic acid sequence present in a complex sample can be amplified in a cyclic process to generate a large number of identical copies that can readily be analyzed. This made it possible, for example, to manipulate DNA for cloning purposes, genetic engineering, and sequencing. But as an analytical technique the original PCR method had some serious limitations. By first amplifying the DNA sequence and then analyzing the product, quantification was exceedingly difficult since the PCR gave rise to essentially the same amount of product independently of the initial amount of DNA template molecules that were present. This limitation was resolved in 1992 by the development of real-time PCR by Higuchi *et al*. [Higuchi, R., Dollinger, G., Walsh, P.S., Griffith, R., 1992. Simultaneous amplification and detection of specific DNA-sequences. Bio-Technology 10(4), 413-417]. In real-time PCR the amount of product formed is monitored during the course of the reaction by monitoring the fluorescence of dyes or probes introduced into the reaction that is proportional to the amount of product formed, and the number of amplification cycles required to obtain a particular amount of DNA molecules is registered. Assuming a certain amplification efficiency, which typically is close to a doubling of the number of molecules per amplification cycle, it is possible to calculate the number of DNA molecules of the amplified sequence that were initially present in the sample. With the highly efficient detection chemistries, sensitive instrumentation, and optimized assays that are available today the number of DNA molecules of a particular sequence in a complex sample can be determined with unprecedented accuracy and sensitivity sufficient to detect a single molecule. Typical uses of real-time PCR include pathogen detection, gene expression analysis, single nucleotide polymorphism (SNP) analysis, analysis of chromosome aberrations, and most recently also protein detection by real-time immuno PCR.

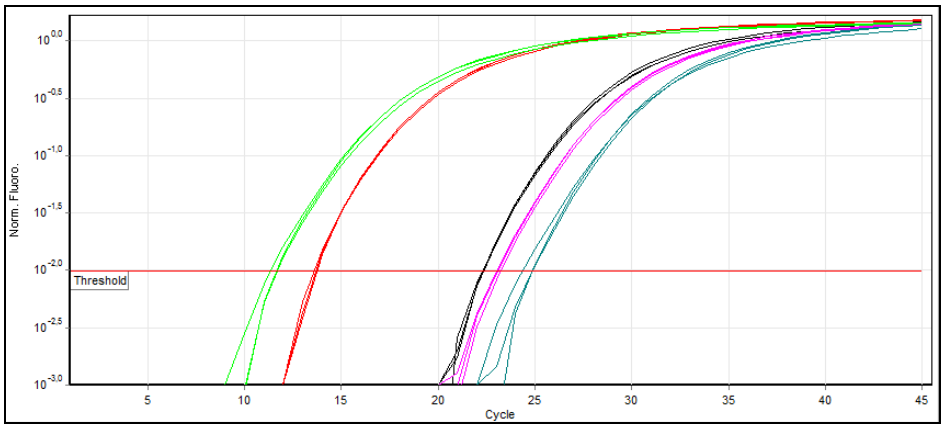

Fig 2: SYBR Green fluorescence chart produced in real-time PCR.

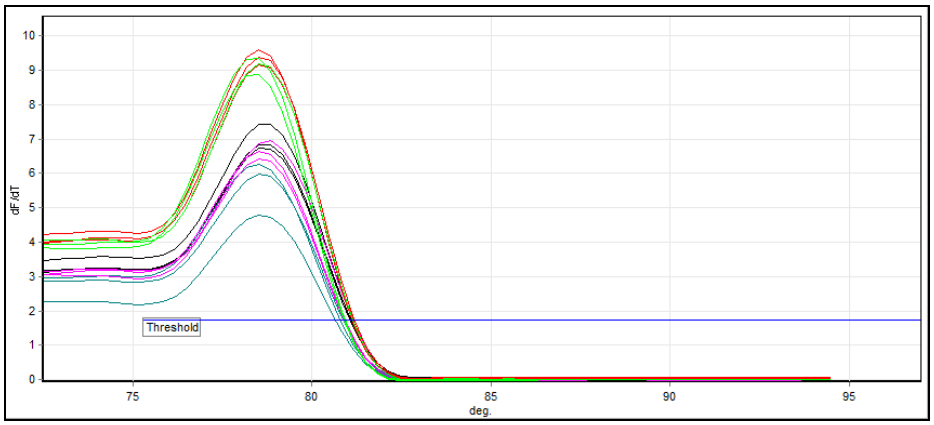

Fig 3: Melting curve produced at the end of real-time PCR.

The forward and reverse primer sequences and the probes were as follows:
β2-microglobulin: sense 5' CTTGGGCTGTGACAAAGTC 3', antisense 5' GTCTTTC-AGCAAGGACTGG 3', Taqman probe 5' (6-Fam)TGGTTCACTCGGCAGGCATAC-TC(Tamra) 3';
PPARδ: sense 5'GGGAGAGGTCTGTGTAGCTGCTG 3', antisense 5' ATGGAGCA-GCCACAGGAGGAAGCC 3';
PPARγ: sense 5' CGGATGGCCACCTCTTTGCTC 3', antisense 5' GGCGAGGGCG-ATCTTGACAGG 3';
RARα: sense 5' ACGTTGTTCTGAGCTGTTGTTCGTA 3', antisense 5' CTGCCAGT-ACTGCCGACTGC 3';
RARβ: sense 5' AG-GCTTGCTGGGTCGTCTTT 3', antisense 5' CCTTCTCAGTGC-CATCTGCTTAAT 3', Taqman probe 5' (6-Fam)AGACCGCCAGGACCTTGAGGA-ACCGA(Tamra) 3';
RARγ: sense 5' GCAAAGACAAGGTCTGT-GAG 3', antisense 5' GACCAGATCAC-TCTGCTCAAAGC 3', Taqman probe 5' (6-

Fam)TATCCTGATGCTGCGTATCTGC-ACAAGGT(Tamra);
RXRα: sense 5' GAGCAGCTTATTCCAGCCTGCC 3', antisense 5' CGACCCTGTC-ACCAACATTTGC 3';
COX-2: sense 5'TGGTGCCTGGTCTGATGAT 3', antisense 5' GCCTGCTTGTCTG-GAACAAC 3'

2. Statistical methods

In most of the cases, Statistical analyses were carried out using the Windows SPSS® 9.0 software package. Associations between clinicopathological variables (age, TNM and Dukes stage (Dukes B *vs.* C *vs.* D), BMI (BMI < 25 *vs.* BMI ≥ 25), tumor site (descendant/sigmoid colon/rectum *vs.* ascendant colon)) were assessed by Spearman's correlation coefficient test. Associations between mRNA expression levels were tested for correlation by Spearman's test and mRNA levels were compared with regard to clinicopathological features. Specifically, comparison of mRNA expression levels in healthy tissue with regard to BMI and tumor site was performed using the Mann-Whitney U test. The Kruskal-Wallis test was used to assess for significant differences in mRNA expression with regard to Dukes stage (Dukes B *vs.* C *vs.* D). The significance of differences in mRNA expression levels between healthy mucosa and tumor tissue was evaluated using the Wilcoxon-test. A P value < 0.05 was considered as significant.

3. Results

From our review found that, samples of CRC specimens and adjacent non-neoplastic colonic mucosa were collected from patients undergoing surgery. Pertinent clinical and pathological data are listed accordingly. All patients were men with a median age of 72 years old (range 41–87). Six patients (30%) had Dukes' B tumors, nine patients (45%) were classified as Dukes' C or as Dukes' D (25%). Eleven patients (55%) had a BMI greater than 25, and were designated as overweight. Four of these had a BMI value above 30, corresponding to obesity status. No correlation was found between age, BMI, and tumor classification.

Nuclear receptors and COX-2 were detectable by quantitative real-time RT-PCR in all normal-looking tissue and tumor samples. The median mRNA expression values are also well mentioned. The median relative PPARγ-expression level in tumors remained unchanged as compared to normal mucosa. Indeed, among 20 investigated cancer tissue samples, PPARγ increased between 1.5- and 4-fold in 35% (n = 7), while we noted a 1.5- to 6-fold decrease in 25% (n = 5). No changes were observed in the remaining 40% (n = 8). In contrast, the expression of PPARδ in tumors was significantly upregulated (1.54 *vs.* 1.30, P = 0.001) relative to normal mucosa. All retinoid nuclear receptors were also upregulated in tumor tissues compared to healthy mucosa (n = 20, P<0.001). Expression levels were increased by the following percentages: RXRα 26.7%, RARα 27.4%, RARβ 54.9%, and RARγ 149.6%. COX-2 mRNA expression was multiplied by 8.5 between normal and tumor tissues (P<0.001). Relationships between nuclear receptor and COX-2 mRNA expression were also tested statistically and listed. Further combined analysis of receptor and COX-2 mRNA expression levels with regard to Dukes' stages and tumor localisations did not display any significant statistical difference. COX-2 expression was not correlated with tumor stages and localisations (data not shown), but was associated with RARα and RARβ mRNA expression in tumor tissue.

Differences in expression of nuclear receptors and COX-2 between normal and tumor tissues were also observed when patients were segregated into groups with low and high BMI (BMI < 25 *vs.* BMI ≥ 25). We also compared the expression of nuclear receptors and COX-2 in healthy mucosa regarding the BMI of patients. Statistics revealed that COX-2 expression is significantly increased in the normal-looking mucosa from patients with the highest BMI.

4. Discussion

Nuclear receptors are involved in many cellular processes from embryonic development to cell death. Dysfunction of nuclear receptor signaling can lead to proliferative and metabolic diseases such as cancer and obesity. In the current report, we assessed the mRNA expression levels of nuclear receptors and COX-2 in 20 CRC specimens and sought a possible relationship with patient's weight status defined by BMI ranging from 18.7 to 38.7.

PPARγ constitutes the most extensively studied of the three PPAR subtypes (α, β, γ) since its function relates to lipid metabolism as well as cell differentiation, apoptosis and cancer. PPARγ can be activated by certain lipids and derivatives and by anti-diabetic agents. Activated PPARγ has been shown able to stimulate differentiation and apoptosis in cancer cells from various origins [35-38]. Nevertheless, in contrast with results generated *in vitro*, data concerning PPARγ expression in human cancer specimens raised questions about the anti-neoplastic activity of the receptor *in vivo*. For example, PPARγ was found highly expressed in ovarian carcinoma [39] and its overexpression in pancreatic carcinoma was associated with poor prognosis [40]. By contrast, our data, in agreement with others [41], showed PPARγ expression globally unchanged in CRC compared with adjacent normal tissues, although Dubois *et al*. [42] found a marked increase of PPAR mRNA expression in four CRC samples and in different colon cancer cell lines. Discrepancies might be attributed to germline mutations in the adenomatous polyposis gene (APC). Indeed, PPARγ has been involved in increasing resistance towards carcinogens by preventing the accumulation of β-catenin, which is regulated by APC. However, PPARγ functions are lost when *APC* is mutated [43]. Another report has shown that deregulated APC/β-catenin indirectly induced aberrant PPARγ overexpression [44], explaining previous experimental data in APC^{Min}/+ mice showing a promoting effect of PPARγ on carcinogenesis [45]. This has relevance for humans because mutations in the tumor suppressor gene *APC* are the initiating event in about 85% of sporadic CRC. Therefore, APC status could dramatically affect expression and function of PPARγ and the steady-state levels of PPARγ reported here do not exclude loss of PPARγ transcriptional activity due to somatic mutations [17], alterations in intracellular distribution [46], post-translation modifications [47] or inhibition by PPARδ [48].

Like PPARγ, PPARδ gene expression is detected in the colon and the receptor can be activated by fatty acids and derivatives. Herein, we reported an elevated level of PPARδ (~18%) in CRC. Upregulation of PPARδ gene expression might be attributed to deregulation in the APC/β-catenin pathway since PPARδ is considered to be a downstream target gene [20]. Increased levels of PPARδ expression have already been observed in rodent colorectal tumors and in

primary human colorectal adenocarcinomas [20, 21]. Nevertheless, PPARδ function remains elusive, with data showing that PPARδ was dispensable for polyp formation [49]. Our data and others suggested a contribution of PPARδ in the carcinogenesis process [16, 50] while Marin *et al*. [22] described that agonist-activated PPARδ protects against cancer development. As for PPARγ, the integrity of the tumor suppressor APC might be essential to guarantee PPARδ normal function.

Critical to the transcriptional activity of PPARs is the ability to form a complex with RXR and bind to DNA. Synthetic ligands of RXRα were shown to exhibit insulin-sensitizing activity [51, 52] and to act synergistically with PPARγ ligands to enhance PPARγ/RXRα-mediated transactivation [31]. In addition, a positive correlation in healthy mucosa was found between RXRα and PPARγ supporting the idea of a tight relationship in the regulation of the expression of these receptors. While no change in RXRα expression level was previously noted in 17 patients with CRC [41], our data revealed a significant increase in tumor versus normal tissue. A similar upregulation was also observed in human esophageal [53], breast [54] and hepatocellular carcinomas [32]. However, little is known about the function of RXRα in colon tumorigenesis and we propose the possibility of alterations in RXRα functions due to altered localization [55] or inhibitory effect of unliganded RXRα on PPARγ transactivation [56].

Recent data have also suggested that PPARγ anti-tumor activity required a functional RARβ [57]. This implies that PPARγ function may be affected by alterations in the retinoid pathway. To our knowledge, very few reports have examined the expression of retinoid receptors in CRC. Therefore, we described here the first detailed analysis of nuclear receptor RARα, β and γ mRNA expression in CRC. RARβ has been extensively studied in cancer cells and human carcinomas, and several studies have suggested that it may play a role as a tumor suppressor gene [58-60]. However, our results showed a significant upregulation in the expression of all three RAR isotypes in CRC specimens compared to adjacent normal mucosa. Furthermore, we mentioned a complex association between the expression of mRNA for RXRα, RARs, and PPARs in cancer tissue, suggesting interactions and cross-talk between these receptors in tumorigenesis. These results demonstrated that alterations are not restricted to a single receptor. Instead, we observed a profound dysregulation of the retinoid pathway in this CRC. Down-regulation of mRNA expression of RARβ often observed in cancer cells has been considered as a cellular mechanism to prevent retinoid-induced growth arrest [61, 62]. On the other hand, while elevated levels of RAR mRNA expression has also been described in breast, liver and esophageal tumors [53, 63, 64], mechanism and significance are unknown. Nevertheless, if the expression of RAR correlates with tissue sensitivity to retinoids, our results should be confirmed within a larger number of samples and both the mechanism leading to inappropriate RXR and RAR expression and the response of CRC to retinoids should be investigated.

There are strong correlations between the intake of fatty acids, the establishment of metabolic disorders and an increased risk of developing CRC [65, 66]. This suggests the involvement of PPARs and retinoid receptors, activated by fatty acids and derivatives [21] and modulated by metabolic disorders [67] in establishing a link between overweight prevalence and CRC pathogenesis. In the current report, we aimed to clarify whether aberrations in the expression of nuclear receptors may contribute to associate high BMI and CRC. However, alterations in nuclear receptor expression observed in tumors were similar in both patients with low or high BMI. We also investigated COX-2 expression which is involved in cellular responses to lipids and inflammatory processes that favourtumorigenesis by stimulating cell proliferation and angiogenesis [68]. Interestingly, while COX-2 was greatly expressed in CRC as previously shown [69], we also found a significantly increased level of COX-2 expression in normal mucosa from patients with high BMI compared to low BMI patients. Recently, it has been reported that patients with a high risk of developing CRC presented an upregulation of the COX-2 gene in normal-looking colon mucosa [70]. This supports the idea that COX-2 deregulation might be an early event in the process of prevention of carcinogenesis. Nevertheless, although previous reports showed COX-2 regulation by nuclear receptors [31, 71], very few associations were found in our study between COX-2 and nuclear receptor expression. In conclusion, our study described altered expression of nuclear receptors in CRC specimens. Further studies are warranted in order to determine the underlying mechanism leading to altered expression of PPARs and retinoid-activated receptors and the significance of such alterations. Moreover, alterations in nuclear receptor expression were independent of the weight status of patients. Nevertheless, COX-2 might be one early target influenced by excess weight and associated metabolic disorders and consequently might affect nuclear receptor expression and activation.

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