




# Increased Expression of Type-I Collagen in Malignant Colorectal Lesion and Positive Correlations with Clinical Factors\*

## *Expressão aumentada do colágeno tipo I na lesão colorretal maligna e correlações positivas com fatores clínicos*

Monique Pinheiro Santos<sup>1</sup> Izabela Silva Sinara Alves<sup>1</sup> Willian Grassi Bautz<sup>2</sup>  
 Flavya da Silva Souza Ribeiro<sup>3</sup> Luciano Pinto Nogueira da Gama<sup>4</sup> Raquel Spinassé Dettogni<sup>2</sup>  
 Letícia Nogueira da Gama-de-Souza<sup>2</sup> 

<sup>1</sup> Faculdade de Medicina, Centro de Ciências da Saúde, Universidade Federal do Espírito Santo, Vitória, ES, Brazil

<sup>2</sup> Department of Morphology, Centro de Ciências da Saúde, Universidade Federal do Espírito Santo, Vitória, ES, Brazil

<sup>3</sup> Laboratório PAT, Vitória, ES, Brazil

<sup>4</sup> Department of Coloproctology, Vitória Apart Hospital, Serra, ES, Brazil

Address for correspondence Letícia Nogueira da Gama-de-Souza, Ph.D., Av. Marechal Campos, 1468. Maruípe, Vitória, ES, 29043-910, Brazil (e-mail: leticia.souza@ufes.br).

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### Abstract

**Objective** Type-I collagen (Col-I) is one of the main macromolecules of the extracellular matrix, and it is involved in the desmoplastic stromal reaction, an indicator of worse prognosis in cases of colorectal cancer (CRC). The purpose of the present study was to investigate Col-I expression in cases of CRC and adenoma and to correlate with the clinical data and the data regarding the lifestyle of the patients.

**Methods** A retrospective study including 22 patients with adenoma and 15 with CRC treated at a coloproctology service. The clinical and lifestyle data were obtained through medical records, and Col-I expression was investigated by immunohistochemistry.

**Results** Women represented most cases of adenoma (63.64%), whereas CRC was found mainly in men (73.33%) ( $p = 0.0448$ ). Immunoexpression of Col-I showed a basement membrane thickening in areas of lining of epithelium and around the glands in both lesions. The cases of CRC had a quite evident fibrosis process in the stroma. The quantitative analysis demonstrated a higher protein expression in CRCs compared to adenomas ( $p = 0.0109$ ), as well as in female patients ( $p = 0.0214$ ), patients aged  $\geq 50$  years ( $p = 0.0400$ ), and in those with a positive family history of colorectal disease ( $p = 0.0292$ ). These results suggested a remodeling of the microenvironment of the

### Keywords

- colorectal cancer
- colorectal adenoma
- type-I collagen
- extracellular matrix

\* Worked developed at the Department of Morphology, Centro de Ciências da Saúde, Universidade Federal do Espírito Santo, ES, Brazil.

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tumor in CRC carcinogenesis. Importantly, the clinicopathologic positive correlations showed a plausible link between the patient's profile and the immunohistochemical findings, which indicate a possible form of patient stratification.

**Conclusion** The immunohistochemical analysis encourages the performance of more comprehensive studies to ascertain if our results could be a tool for the diagnosis and monitoring of the patients.

## Resumo

**Objetivo** O colágeno tipo I (Col-I) é uma das principais macromoléculas da matriz extracelular, e está envolvido na reação desmoplástica estromal, um indicador de pior prognóstico em casos de câncer colorretal (CCR). O objetivo foi investigar a expressão do Col-I em casos de CCR e adenoma, e correlacioná-la com dados clínicos e de estilo de vida dos pacientes.

**Metodologia** Foi realizado um estudo retrospectivo com 22 pacientes com adenoma e 15 com CCR tratados em um serviço de coloproctologia. Os dados dos pacientes foram obtidos dos prontuários médicos, e a expressão do Col-I foi investigada por imunohistoquímica.

**Resultados** As mulheres representaram a maioria dos casos de adenomas (63,64%), enquanto o CCR (73,33%) ( $p = 0,0448$ ) foi mais comum entre os homens. A imunoexpressão de Col-I mostrou espessamento da membrana basal em áreas de revestimento do epitélio e em volta de glândulas em ambas as lesões. O CCR apresentou fibrose no estroma. As análises quantitativas demonstraram maior expressão proteica no CCR ( $p = 0,0109$ ), assim como em mulheres ( $p = 0,0214$ ), pacientes com idade  $\geq 50$  anos ( $p = 0,0400$ ), e em pacientes com histórico positivo de doença colorretal na família ( $p = 0,0292$ ). Estes resultados sugerem a remodelação do microambiente tumoral na carcinogênese do CCR. As correlações clínico-patológicas positivas mostram uma ligação plausível entre o perfil do paciente e os achados imunohistoquímicos, o que indica uma possível forma de estratificação dos pacientes.

## Palavras-chave

- câncer colorretal
- adenoma colorretal
- colágeno tipo I
- matriz extracelular

**Conclusão** As análises imunohistoquímicas estimulam a execução de estudos mais abrangentes para confirmar se nossos resultados poderão ser uma ferramenta para o diagnóstico e o monitoramento dos pacientes.

## Introduction

Colorectal cancer (CRC) is the third most common cancer and the third cancer-related cause of death worldwide.<sup>1</sup> Although survival rates have improved in recent years, approximately 30% of CRC patients still develop local recurrence, probably resulting from micro-metastatic dissemination or distant metastases.<sup>2</sup> This aspect is related to the behavior of the disease, which usually does not present symptomatology in the initial stages. Factors such as age and family history, as well as genetic and environmental factors, have a positive correlation with CRC.<sup>3-5</sup>

Most lesions follow a specific sequence of mutations, the "adenoma-carcinoma sequence":<sup>4</sup> initially, the normal epithelium is transformed into a hyperproliferative tissue; later, it loses organization and structure and acquires the capacity to form adenomatous polyps, which can evolve to CRC.<sup>3</sup>

The microenvironment of the tumor has demonstrated an important regulatory role in CRC pathogenesis.<sup>6-8</sup> During the progression of the disease, excessive remodeling of the extracellular matrix (ECM) is characterized by increased collagen deposition (desmoplasia) and by its crosslinking,

which promotes tissue stiffening.<sup>7,9</sup> Yue et al.<sup>10</sup> have shown that cancerous stroma interactions are highly dependent on matrix stiffness, interfering in processes such as the migration of cancerous cells.<sup>10</sup>

The desmoplastic stromal reaction is rich in type-I collagen (Col-I) and is considered an indicator of worse prognosis in primary CRC.<sup>9,11</sup> The progressive increase in Col-I expression occurs due to abnormal recruitment of fibroblasts and myofibroblasts, a phenomenon associated with tumor-cell proliferation, angiogenesis and metastasis.<sup>12,13</sup> Type-I collagen is highly expressed in infiltrative growth tumors.<sup>14</sup> Its production during the remodeling of the ECM in carcinogenesis results in a precarcinogenic niche, which works as a tumor escape.<sup>12</sup> Furthermore, the high expression of Col-I in the stroma is related to the ability of CRC cells to exhibit increased proliferation, chemoresistance, and survival.<sup>8,9</sup> This fact contributes to the increased invasiveness and manifestation of a malignant phenotype in the colorectal region, reducing cell adhesion and differentiation as an early event in tumorigenesis.<sup>15</sup>

Therefore, the study of Col-I expression by immunohistochemistry in benign and malignant human samples from

the colorectal region is important to clarify the local alterations that occur in the ECM throughout the progression of carcinogenesis. Moreover, the association between clinical and histopathological features may help select the best actions in the medical monitoring routine.

## Methods

### Sample Selection

The present is a retrospective study, and the patients were selected from the Coloproctology Service at Vitória Apart Hospital, in the state of Espírito Santo, Brazil, between 2015 and 2017. All patients underwent a colonoscopy procedure, and no change in the care protocol was performed for the present study. The samples were obtained from excisional and incisional biopsies of colonic lesions with a suspicion of adenoma or CRC. Slides were prepared and assigned by the Pathological Anatomy (PAT) Laboratory, which is linked to the Coloproctology Service. The clinical profile was obtained from the medical records, and it included: gender, age, family history of benign or malignant colorectal diseases, smoking, and alcohol consumption. The following inclusion criteria were adopted: completion of the questionnaire on clinical profile, availability of data regarding the lesion (histopathological diagnosis), and sufficient tissue for analysis. The present research was approved by the Ethics in Research Committee of Vitória Apart Hospital (under process number 406.002). All patients agreed to participate and signed the consent form.

### Immunohistochemistry

Rabbit polyclonal anti-Col I antibody (Ab34710) (Abcam, Cambridge, MA, US) diluted 1:400 in phosphate-buffered saline (PBS) was used for the detection of Col-I. After deparaffinization and rehydration, the slices were pretreated with citrate buffer (pH: 6.0) for antigen retrieval. Then, the sections were treated with a protein block solution (DPB-125, Spring Bioscience Corp., Pleasanton, CA, US) for 10 minutes at room temperature and incubated with the respective primary antibody in a humidified chamber overnight at 4°C. Endogenous peroxidases were blocked with hydrogen peroxide block (DHP-125, Spring Bioscience Corp.) for 10 minutes. Immunodetection was performed with a biotin-free immunoenzymatic antigen detection system (Reveal System, SPB-999, Biogen, São Paulo, SP, Brazil). The sections were briefly incubated with a complement reagent (DCMT-999, Spring Bioscience Corp.) for 10 minutes, and then, with horseradish peroxidase (HRP) conjugate for 15 minutes. Immunoreactivity was visualized using a 3,3'-diaminobenzidine (DAB) (DABC-004 and DABS-125, Spring Bioscience Corp.) as the chromogen. Finally, the slides were lightly counterstained with Mayer hematoxylin. After being washed with water and dehydrated in alcohol series, the slides were cleaned with xylene and mounted with dibutylphthalate polystyrene xylene (DPX) (Sigma-Aldrich, St. Louis, MO, US). The slides were washed thoroughly with PBS during the immunohistochemistry process, and negative controls were performed omitting the primary antibody.

### Col-I Quantitative Analysis

The immunostained sections were analyzed and scored blindly for clinical information by a single investigator using a PrimoStar microscope with a Zeiss AxioCam ERC5s digital camera (Carl Zeiss Vision GmbH, Aalen, Germany) and the Axio Vision 4.2 Release 4.8.2 image software (Carl Zeiss Vision GmbH). For each slide, two images of the hot spots of the ECM were obtained and imported to the Image J (public domain, available in: <https://imagej.nih.gov/ij/download.html>) software. Then, the area of interest was defined: area and fraction of the ECM. The following segmentation plugin was applied to the expression analysis: color deconvolution, with the vector HDAB. The bottom coloration, the hematoxylin and the DAB were separated. The settings of the limits for the positive DAB were made with the threshold tool adjust, and the analyses were concluded with the measure tool. For each section, a measurement of the two evaluated areas was defined. Lastly, the data were stored in Microsoft Excel 2016 (Microsoft Corp. Redmond, WA, US) spreadsheets.

### Statistical Methods

The analyses were performed with the Graphpad Prism (GraphPad Software, Inc., San Diego, CA, US) software, version 6.0. The Fisher exact test was applied to evaluate the clinical data. The Mann-Whitney test was applied to analyze Col-I expression. The associations among the clinical data, the lesion and the profile expression were analyzed by the two-way analysis of variance (ANOVA) and the Kruskal-Wallis test. In all tests, values of  $p < 0.05$  were considered statistically significant.

## Results

The study sample consisted of 22 patients diagnosed with colorectal adenoma histopathology and 15 patients with CRC. Some patients with adenoma ( $n = 11$ ) had multiple lesions, so the total number of surgical specimens was 48 (33 adenomas and 15 CRCs). Regarding the clinical data, we observed that women were the majority in the adenoma group (63.64%), whereas CRC cases were found mainly in men (73.33%) and this difference was statistically significant ( $p = 0.0448$ ). Individuals aged  $\geq 50$  years represented most cases of adenoma (90.91%) and CRC (66.67%). Family history was found in about 40% of the CRC cases. Finally, smoking was reported by 36.6% of the patients diagnosed with adenoma and by 26.67% of the CRC patients, while alcoholism was mentioned by 59.09% of adenoma cases and by 40% of CRC patients. ►Table 1 shows all clinical data.

Regarding Col-I analysis, we could observe in different areas of the ECM that all samples had some degree of expression, with important variation between the two lesions. The normal aspect of loose connective tissue was visualized only in adenoma lesions (►Fig. 1A). In interface areas between lining epithelium and underlying conjunctiva, it was possible to detect collagen deposition in the basement membrane (►Fig. 1B), which was also observed around the glands (►Fig. 1C). In CRC cases, the fibrosis process in the stroma due to increased Col-I was quite evident (►Fig. 1D).

**Table 1** Clinical data and data on the lifestyle of the patients

		Adenoma	Colorectal cancer	p-value
		Relative frequency: n (%) (n = 22)	Relative frequency: n (%) (n = 15)	
Gender	Female	14 (63.64)	4 (26.67)	0.0448*
	Male	8 (36.36)	11 (73.33)	
Age (years)	< 50	2 (9.09)	5 (33.33)	0.0953
	≥ 50	20 (90.91)	10 (66.67)	
Family history	Yes	9 (40.91)	6 (40.00)	1.0000
	No	13 (59.09)	9 (60.00)	
Smoking	Yes	8 (36.36)	4 (26.67)	0.7235
	No	14 (63.64)	11 (73.33)	
Alcoholism	Yes	13 (59.09)	6 (40.00)	0.3245
	No	9 (40.91)	9 (60.00)	

Notes: \*Statistically significant; Fisher exact test.

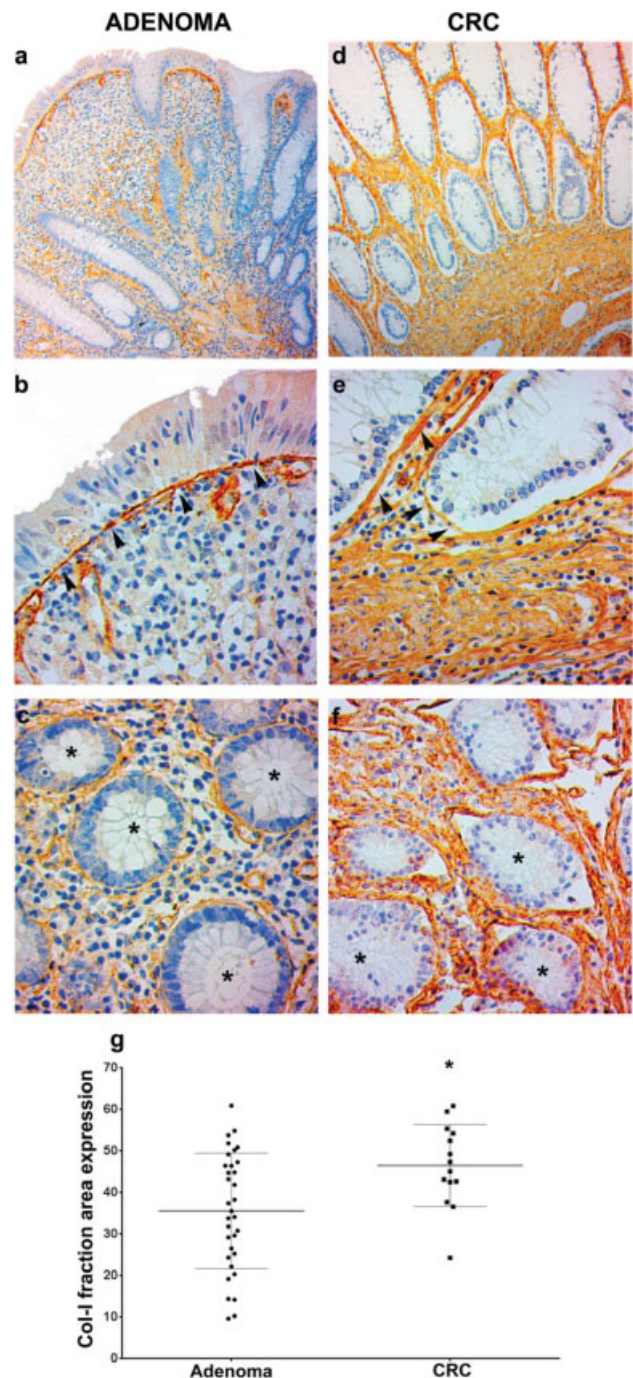
A basement membrane thickening, in the lining (►Fig. 1E) and in the glandular epithelium (►Fig. 1F) was also observed in these lesions. In the quantitative analysis of the labeled area fraction, higher Col-I expression was confirmed in CRC cases in relation to adenomas ( $p = 0.0109$ ). ►Table 2 presents the means and standard errors of the mean (SEM) of the expression of the Col-I fraction area.

Finally, association analyses between the clinicopathologic data and immunohistochemical findings resulted in positive relationships regarding gender, age, and family history and Col-I expression. Higher protein levels were observed in individuals with a positive family history of adenoma or carcinoma (►Fig. 2A,  $p = 0.0325$ ), women diagnosed with CRC (►Fig. 2B,  $p = 0.0251$ ), and patients aged  $\geq 50$  years (►Fig. 2C,  $p = 0.0446$ ). Furthermore, there was a tendency towards higher levels of Col-I labeling among alcohol consumers with adenoma (►Fig. 2D,  $p = 0.0761$ ) and smokers with adenoma or CRC (►Fig. 2E,  $p = 0.0572$ ).

## Discussion

In the present study, we analyzed Col-I expression in patients diagnosed with CRC and adenoma, and its correlation with clinical data and data on the lifestyle of the patients. Positive correlations between factors related to the patients and the immunohistochemical findings were observed regarding age, gender, and family history. The levels of Col-I were significantly elevated in the CRC stroma compared with benign lesions.

The onset an development of CRC are induced by a combination of genetic and environmental factors, whose study is essential for the establishment of new prevention strategies.<sup>5</sup> Although most genes involved in CRC have been well characterized, the influence of additional environmental factors on this disease remains unclear.<sup>4</sup> When analyzing the patients' clinical profile, we observed a higher frequency of individuals



**Fig. 1** The immunoexpression of type-I collagen (Col-I) in adenomas and colorectal cancer (CRC) shows increased protein deposition in malignant lesions. The stroma of the adenoma preserving the aspect of loose connective tissue (A). At a higher magnification, it is possible to observe a collagen deposit in the basement membrane at the interface between the lining epithelium and the connective tissue (arrowheads) (B), which was also observed around the glands (the asterisks show the gland lumens) (C). In CRC lesions, stroma fibrosis was observed, with the entire tissue being taken by the Col-I deposit (D). In addition, thickening of the basement membrane occurred in interface areas of the lining epithelium (E) and glandular epithelium (the asterisks show theb gland lumens) (F). Magnifications: A and D: 100X; C, E, and F: 400X; immunohistochemical method.

aged  $\geq 50$  years, which is consistent with the literature.<sup>4,16</sup> Age is one of the main risk factors for CRC, and after the sixth decade of life, the chance of developing CRC is markedly



**Table 2** Mean and standard error of the mean of type-I collagen fraction area in cases of adenoma and colorectal cancer

	Adenoma (n = 33)	CRC (n = 14)	p-value
Median ± SEM	35.50 ± 2.432	46.21 ± 2.647	0.0148*
95.24% confidence interval	-19.01 to -2.14		

Abbreviations: CRC, colorectal cancer; SEM, standard error of the mean. Note: \*Statistically significant; Mann-Whitney test.

increased.<sup>4</sup> There was a predominance of CRC cases among men, which is also in agreement with a previous study.<sup>17</sup> Family history of colorectal disease was reported by about 40% of the patients. It has already been shown that having one or two first-degree relatives with CRC is associated, respectively, with a 2.26- and a 3.76-fold greater risk of developing cancer.<sup>3</sup> This aspect may be related to inherited mutations or to the environment into which the individual is inserted.<sup>4</sup>

Regarding lifestyle, reports of drinking and smoking were found in cases of adenoma and CRC. Prospective-cohort and case-report studies indicate that high alcohol intake increases the risk of developing CRC, although some of the data have shown to be inconsistent or with only modest associations.<sup>18</sup> Smoking, in turn, can increase the chances of developing CRC by up to 10.8%. Cigarettes, for example, release metabolites that can easily reach the intestine and cause the formation of polyps.<sup>4</sup>

In the process of carcinogenesis, it was described that tumor cells release factors before and upon arrival at the metastatic niche in order to convert an unfavorable environment of a distant site into favorable surroundings.<sup>7,19</sup> In this context, the ECM emerged as an active participant in the carcinogenic process. Deregulation of the biochemical and physical characteristics of the ECM, such as composition, architecture, or rigidity, are associated with loss of stem-cell differentiation, epithelial-mesenchymal transformation and abnormal modulation of cell migration, differentiation, and proliferation, thus supporting the onset and progression of the cancer.<sup>13,20</sup>

Previous data demonstrated that Col-I is highly expressed in CRC with an invasion phenotype,<sup>15</sup> and its expression has been associated with increased tumor-cell proliferation, angiogenesis, and metastasis.<sup>12</sup> A myofibroblast-mediated desmoplastic stromal reaction rich in fibrillar collagens (types I and III) is said to be an indicator of worse prognosis in primary CRC.<sup>12</sup> It has also been observed that the ECM deposition, as well as the release of cytokines and growth factors, stimulate angiogenesis and alter the immune function, enabling the occurrence of tumor growth and metastasis.<sup>21</sup> The analysis of Col-I expression demonstrated increased expression in cases of CRC in comparison to adenomas; still, the stroma of the malignant condition was filled by collagen, involving cancer cells. Moreover, its deposition was not restricted to deep stroma areas; thickening of the basement membrane at the interface of the conjunctival-lining epithelium, as well as around the glands, has also been

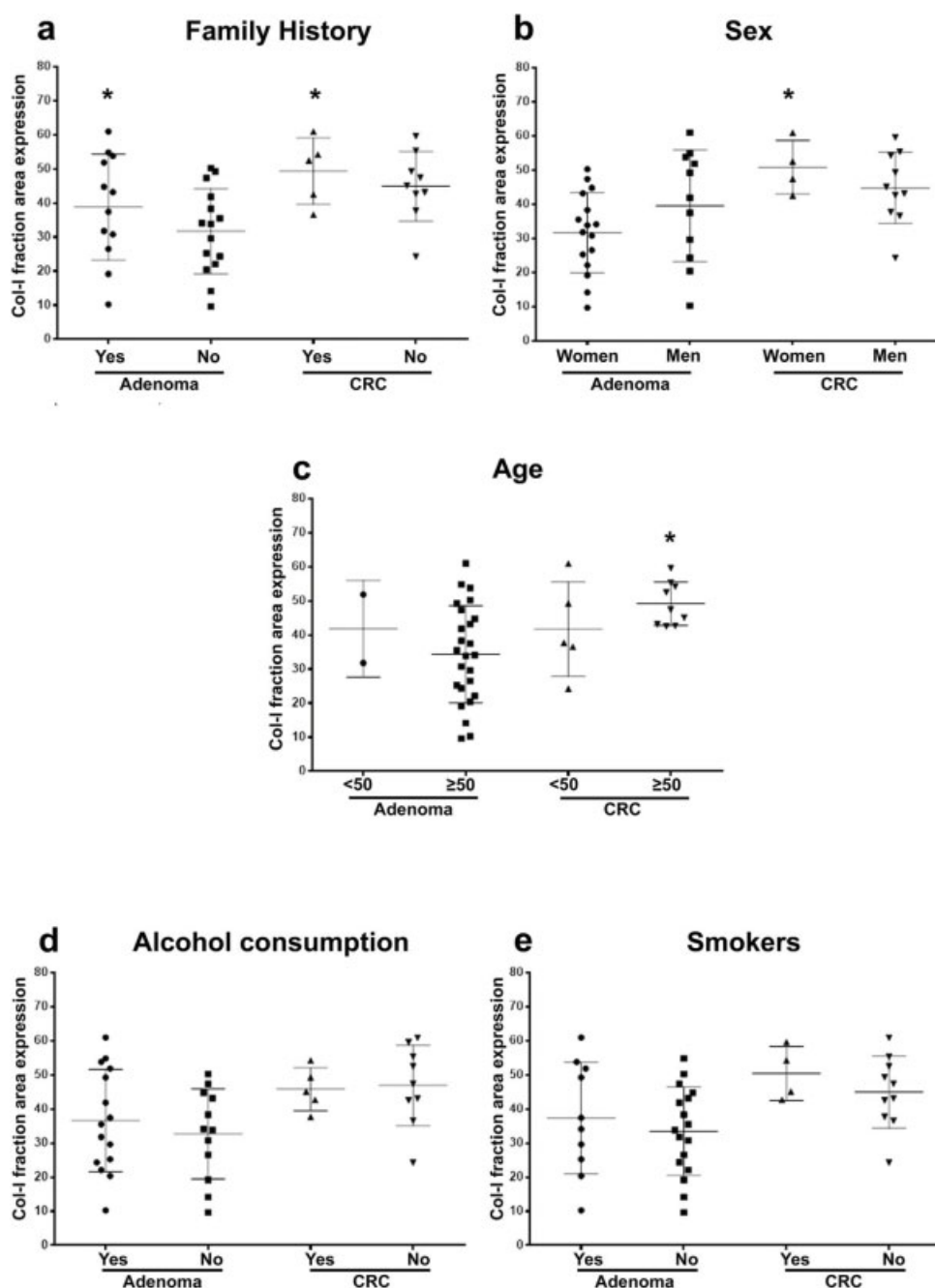
observed. Zou et al.<sup>15</sup> observed similar findings. Alves et al.<sup>16</sup> also described thickening of the basement membrane and fibrosis around glands in adenomas, and around vessels in high-grade dysplasia adenomas and CRC. The authors<sup>16</sup> also mentioned that in dense areas of CRC, a diffuse disorganization of the ECM was observed.

Increasing Col-I expression, even in benign stages, indicates a possible role in tumor onset and progression.<sup>12</sup> This protein activation may be an early event in carcinogenesis by facilitating tumor invasion.<sup>15</sup> Kehlet et al.<sup>7</sup> demonstrated higher serum levels of Col-I-derived fragments in patients with stage-IV tumors compared to all other CRC stages, showing that collagen remodeling is part of the progression of this cancer. Still, a review<sup>9</sup> of 679 stage-II CRC patients demonstrated that the categorization of the desmoplastic reaction could be a new useful prognostic factor in these patients due to the fact that it outperformed other conventional prognostic factors.

Finally, positive associations found between the clinical data and Col-I immunoreactivity reinforce the multifactorial aspect present in the development of colorectal lesions. Higher levels of Col-I have been found in women with CRC, and in patients aged ≥ 50 years also diagnosed with cancer. The relationship between family history and higher Col-I expression was found in both adenoma and CRC. These data indicate a possible form of stratification of the patients, which, according to the clinical profile, could be at a higher risk of developing more aggressive behavioral lesions. This strategy clearly depends on future broader and more comprehensive studies. The link between the profile of the patient and the immunohistochemical findings has been discussed in the context of other diseases, such as oral cancer, in which the expression of ECM molecules has been positively related to the gender, age, and smoking habit of the patients.<sup>22</sup>

The search for possible molecules that really represent biomarkers for cancer progression is a relevant field that can impact directly in the prognosis and treatment efficacy. But larger longitudinal studies are needed in order to better comprehend the cellular events that lead to ECM deregulation of biochemical and physical features. However, our preliminary findings suggest that an increase in the Col-I expression and consequent alteration of the tumor microenvironment occur in the pathway progression colorectal carcinogenesis. The knowledge of events that affect the colon, from benign stages to the establishment of cancer, will facilitate the definition of potential biomarkers to be used in the management of CRC patients. In addition, a link between the clinical and pathological findings, as found in the present study, may play an important role in the prognosis of CRC.

The most obvious limitations of the present study are the sample size, in part due to the design of the study and the decision to select patients from a single coloproctology service, and to the use of a single technique. Thus, any statement herein made requires a complementary study, and the findings are only suggestive. On the other hand, the main idea was to compare Col-I deposition on the stroma



**Fig. 2** Analysis of the correlations of the clinical factors of the patients and Col-I expression resulted in positive data regarding gender, age, and family history in cases of adenomas and colorectal cancer (CRC). This suggests that higher levels of expression are present in patients with adenoma or CRC and positive family history (A;  $p = 0.0325$ . Median/standard error of the mean (SEM): 40.31/4.493; 33.78/3.224; 52.50/4.341; 45.05/3.406), female with CRC (B;  $p = 0.0251$ . Median/SEM: 32.80/2.937; 41.88/4.964; 49.94/3.923; 44.09/3.310), and age  $\geq 50$  years with CRC (C;  $p = 0.0446$ . Median/SEM: 41.85/10.04; 34.09/2.850; 37.68/6.216; 47.37/2.125). A trend towards higher levels of immunostaining was observed among alcoholic patients with adenoma (D;  $p = 0.0761$ . Median/SEM: 35.50/3.883; 33.94/3.805; 45.05/2.823; 47.37/3.930) and smokers with adenoma or CRC (E;  $p = 0.0572$ . Mean/SEM: 35.75/5.186; 33.78/3.131; 49.65/3.937; 45.25/3.342). Two-way analysis of variance (ANOVA) and Kruskal-Wallis test.

between adenoma and CRC lesions, an important phenomenon of connective tissue accumulation caused by neoplastic processes together with host response. In this context, we could affirm that our results were capable not only to significantly demonstrate the increase in fibrous component in CRC cases, but also to establish positive correlations with the clinical data.

## Conclusion

Col-I expression was found to be increased in CRC lesions compared to adenomas, benign stage, and cancer precursor, suggesting that the development of the most aggressive phenotype in the colorectal region is accompanied by important changes in the biochemical and physical aspects

of the ECM. The clinicopathological positive correlations represented a relevant finding. These results may encourage more studies with the aim of finding biomarkers of CRC progression. Moreover, they demonstrate how comprehensive the clinical analysis of the patient may need to be in order for physicians to provide a suitable follow-up.

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#### Conflict of Interests

The authors have no conflict of interests to declare.

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