

RESEARCH ARTICLE

Colon cancer modulation by a diabetic environment: A single institutional experience

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Abstract

Background

Multiple observational studies suggest an increased risk of colon cancer in patients with diabetes *mellitus* (DM). This can theoretically be the result of an influence of the diabetic environment on carcinogenesis or the tumor biologic behavior.

Aim

To gain insight into the influence of a diabetic environment on colon cancer characteristics and outcomes.

Material and methods

Retrospective analysis of clinical records in an academic tertiary care hospital with detailed analysis of 81 diabetic patients diagnosed of colon cancer matched with 79 non-diabetic colon cancer patients. The impact of streptozotocin-induced diabetes on the growth of colon cancer xenografts was studied in mice.

Results

The incidence of DM in 1,137 patients with colorectal cancer was 16%. The diabetic colon cancer cases and non-diabetic colon cancer controls were well matched for demographic and clinical variables. The ECOG Scale Performance Status was higher (worse) in diabetics (ECOG ≥ 1 , 29.1% of controls vs 46.9% of diabetics, $p = 0.02$), but no significant differences were observed in tumor grade, adjuvant therapy, tumor site, lymphovascular invasion,

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Abbreviations: ADA, American Diabetic Association; ECOG, Eastern Cooperative Oncology Group; BMI, Body Mass Index; pT, Invasion depth or thickness of the tumor; AJCC, American Joint Committee on Cancer; STZ, streptozotocin; CEA, carcinoembryonic antigen; FBS, Fetal Bovine Serum.

stage, recurrence, death or cancer-related death. Moreover, no differences in tumor variables were observed between patients treated or not with metformin. In the xenograft model, tumor growth and histopathological characteristics did not differ between diabetic and nondiabetic animals.

Conclusion

Our findings point towards a mild or negligible effect of the diabetes environment on colon cancer behavior, once cancer has already developed.

Background

Diabetes *mellitus* and cancer are among the most frequent causes of death worldwide [1–3]. Epidemiologic evidence suggests an association between diabetes *mellitus* and an increased risk of many forms of cancer [4–6]. Colon cancer is the third -in men- and the second -in women- most common cancer in the global population [2]. The results of multiple observational studies, but not all, have suggested that diabetes may significantly increase the risk of colon cancer [7–11]. However, data from specific studies have been disputed as based on heterogeneous groups, with unreliable ascertainment of diabetes, and using sources with poorly rigorous data such as Surveillance, Epidemiology, and End Results, which may have led to inconclusive or misleading results [9, 12–14]. Accordingly, the magnitude of the association may have been potentially influenced by hints and bias [15, 16]. It also remains uncertain whether the putative link between diabetes and colon cancer is direct, related to hyperglycemia; whether diabetes is an indicator of latent biologic factors that modify cancer risk such as insulin resistance and hyperinsulinemia; or whether the diabetes-cancer association is indirect and reflects the influence of common risk factors such as age, lifestyle behaviors, overweight, poor dietary habits or therapeutic agents [17]. Moreover, there is a limited knowledge related to the influence of a diabetic environment on the carcinogenesis process, the biologic behavior of the tumor, the response to different treatments and patient outcomes [5, 18]. *In vitro* and *in vivo* studies have supported a direct role of high glucose concentrations in tumor development and characteristics [19]. In this respect, animal models have addressed the influence of diabetes/insulin resistance on carcinogenesis (e.g. carcinogenic molecules used in Leptin receptor-deficient db/db mice) [20], or of diabetes/hyperglycemia on tumor xenograft progression (e.g. glucagon-induced hyperglycemia [21] or streptozotocin-induced diabetes in mice [22, 23]), although colon cancer had not been previously studied in depth.

We have now aimed at gaining insight into the influence of a diabetic environment on colon cancer characteristics and progression through a combination of clinical studies and a preclinical evaluation of the influence of the hyperglycemia on tumor xenograft progression.

Material and methods

Clinical study

The study is a retrospective patient record analysis carried out at Fundacion Jimenez Diaz Hospital, for patients from January 2009 to December 2013. Clinical data and biopsies were obtained after informed consent from patients underwent surgery for purposes not related to this study. Candidate patients were identified using the specific institutional software named Alcor (Sigesa, 2014). This program works with an encoded system, which matched the specific

requested terms. The first research setting included the variables colorectal cancer and diabetes, and the second diabetes, cancer and colorectal cancer.

Study sample. From the total number of patients found with a diagnosis of colorectal cancer and also diabetes at the institution, only 81 diabetic patients met the inclusion criteria established for this study:

Patients with resection of primary colon cancer, histological type colon adenocarcinoma, colon location (rectal cancer patients were excluded), time from surgery up to 6 months, no neoadjuvant treatment, no other concurrent neoplasia or immunosuppressive treatment, and diabetes diagnosed as a documented registry of diabetes, or historical anti-diabetic medication intake or meeting the American Diabetic Association (ADA) criteria for diabetes at time of reviewing the data. The ADA criteria used to determine if patients had diabetes were: Hemoglobin A1c values $\geq 6.5\%$, or fasting blood glucose levels ≥ 125 mg/dL, with high fasting values recorded 2 or more times or random blood glucose levels ≥ 200 mg/dL, with high random values recorded 2 or more times (S1 Fig).

In parallel, this observational study also included 79 non-diabetic patients with primary diagnosis of colon cancer, who underwent resection during the same period, using equal inclusion criteria except the presence of diabetes, aiming to obtain a well-balanced series.

Baseline variables recorded for all patients were age, gender, metformin intake, clinical debut (acute or subacute symptoms), Eastern Cooperative Oncology Group (ECOG) Scale Performance Status, Body Mass Index (BMI), adjuvant therapy, overall survival, disease free survival, and cause of death if deceased. Other variables recorded from peripheral blood at diagnosis were glucose, triglycerides, cholesterol, white blood cell levels, and carcinoembryonic antigen (CEA) level.

Tumor characteristics were analyzed based on the following criteria:

- Information regarding depth of invasion or thickness of the tumor (pT) was collected from the pathology reports after surgery, as defined by the American Joint Committee on Cancer Criteria for colon and rectal cancer staging (<https://cancerstaging.org/About/Pages/8th-Edition.aspx>).
- Tumors were graded as low grade (G1-G2), and high grade (G3) following the 2010 WHO classification (<http://www.pathologyoutlines.com/topic/colontumorwhoclassification.html>).
- Tumor location was categorized as proximal or right (cecum, hepatic flexure, ascending and transverse colon), and distal or left (splenic flexure and descending colon) [24].
- Information regarding lymphovascular invasion was collected from the pathology report, determined on hematoxylin and eosin staining. Vein invasion was identified as present if tumor cells were observed in an endothelial-lined channel with a smooth muscle wall. Lymphatic vessel invasion was identified as present if tumor cells were observed in an endothelial-lined channel devoid of smooth muscle.
- TNM staging: Data on Classification of Malignant Tumours (TNM) staging was collected from the clinical registries in the computerized system and was assessed based on pathology reports available after surgical resection and results from imaging studies. The information was coded according to the TNM staging described in the American Joint Committee on Cancer (AJCC) eighth edition (<https://cancerstaging.org/Pages/default.aspx>). For analytical purposes, T stages were classified as low T stage (T1-T2) or high T stage (T3-T4), N stages were considered as N0 (absence of node involvement) and N+ (lymph node metastases) and final stage as low stage (0, I, any II) or high stage (any III, IV).

The study was approved by the Institutional Scientific and Ethical Committee at IIS-Fundacion Jimenez Diaz (Madrid, Spain) (CEIC-FJD, approval code 08/13; on October 1st, 2013) in

accordance with the ethical principles stated in the Declaration of Helsinki. Informed consent is included in the clinical history of each participant and recorded by the standard requirements of data protection rules established by the SPANISH DATA PROTECTION AGENCY (LOPD 15/1999).

Tumor xenograft model in streptozotocin-induced diabetic mice

Fifteen male athymic mice NU-Foxn1nu (8-week-old) were purchased from Charles River Laboratories and housed in the specific pathogen-free room of the Animal Model Core Facility of Research Health Institute–Fundacion Jimenez Diaz (ES28079000089). Mice were maintained in individually ventilated cages (IVCs) with a 12:12 h light-dark cycle. 2014 Teklad global 14% protein irradiated diet and autoclaved water was provided *ad libitum*.

All animal procedures and experimental protocols were approved by the Ethical Animal Research Committee at IIS-Fundacion Jimenez Diaz (Madrid, Spain) and were also conducted in accordance with institutional standards (Reference n°: PROEXP 024–15), which fulfilled the requirements established by the Spanish government and the European Community (Real Decreto R.D. 53/2003).

Diabetes induction. Diabetes was chemically induced by a single intraperitoneal injection of 200 mg/Kg body weight streptozotocin (STZ, Sigma-Aldrich) in a total volume of 200 μ l in 50 mM citrate buffer (pH = 4.5) in 10 animals. The control group (5 mice) received 200 μ l citrate buffer. Ten days after the STZ administration, 60% of STZ injected animals presented blood glucose above 200 mg/dl and were considered as the streptozotocin-induced diabetic (STZ-D) group. Blood glucose levels were monitored periodically during the experiment, but animals did not receive insulin or other antidiabetic drug.

Xenograft implantation. The colorectal cancer HT29 cell line, recently classified as “metabolic subtype” [25, 26], was used to generate a xenograft twenty days after streptozotocin or vehicle administration. Cells were cultured in RPMI-1640 medium (Gibco) with 10% fetal bovine serum (FBS) at 37°C in a 5% CO₂ atmosphere. Medium was supplemented with penicillin G (100 U/ml), and streptomycin (0.1 mg/ml). A volume of 200 μ l containing 2x10⁶ cells [1:1 mixture of PBS: Matrigel (BD Biosciences)] was injected subcutaneously into both flanks of the animal. The size of the tumor was measured three times a week using a Vernier caliper along two perpendicular axes and volume was calculated with the formula: volume = (length \times width²)/2 (Fig 1).

Pathological assessment. Fifty-five days after tumor induction, mice were sacrificed by using carbon dioxide (CO₂) euthanasia and tumors were removed and fixed in 10% neutral buffered formalin solution for 24 hours and embedded in paraffin wax. All samples were processed following the same procedure. Tissues were cut into 4- μ m-thick sections, and stained with hematoxylin-eosin stain for morphological examination by light microscopy.

CD31-vascular structures in tumor were also evaluated by immunohistochemistry. Consecutive 4- μ m tissue sections were obtained from formalin-fixed paraffin-embedded samples. Antigen retrieval was performed in PT-Link (Dako) for 20 min at 95°C in high pH buffered solution (Dako). Endogenous peroxidase was blocked by immersing the sections in 0.03% hydrogen peroxide for 5 min. Slides were washed for 5 min with Tris buffered saline solution containing Tween 20 at pH 7.6 and incubated with a primary antibody for CD31 (dilution 1:25, Clone JC70A, Abcam) for 20 min at room temperature, followed by incubation with the appropriate anti-Ig horseradish peroxidase-conjugated polymer (EnVision, Dako). Sections were then visualized with 3, 3'-diaminobenzidine as a chromogen for 5 min and counter-stained with hematoxylin. All stainings were performed in an Autostainer platform (Dako). CD31-vascular structures were counted in ten \times 200 magnification microscopic fields from the area of highest vascular density in the tumor.

Statistical analysis

Data were analyzed by SPSS v.20.0 software. Continuous variables were expressed as mean \pm standard deviation, and categorical variables were expressed as frequencies and percentages. Demographic, clinical characteristics, and tumor pathologic variables were compared between patients with and without diabetes by chi-square or Fisher’s exact test when appropriate, in case of categorical variables and by t-test in case of continuous variables. A *p* value lower than 0.05 was considered statistically significant, in all analyses.

Results

Epidemiological data

Among 1,137 patients diagnosed of incident colorectal cancer from January 2009 to December 2013 found, diabetes was present in 185 (16%); the incidence of any cancer in among 13,873 diabetic patients was 14%, being 1.3% of these cases colorectal cancer (Fig 2).

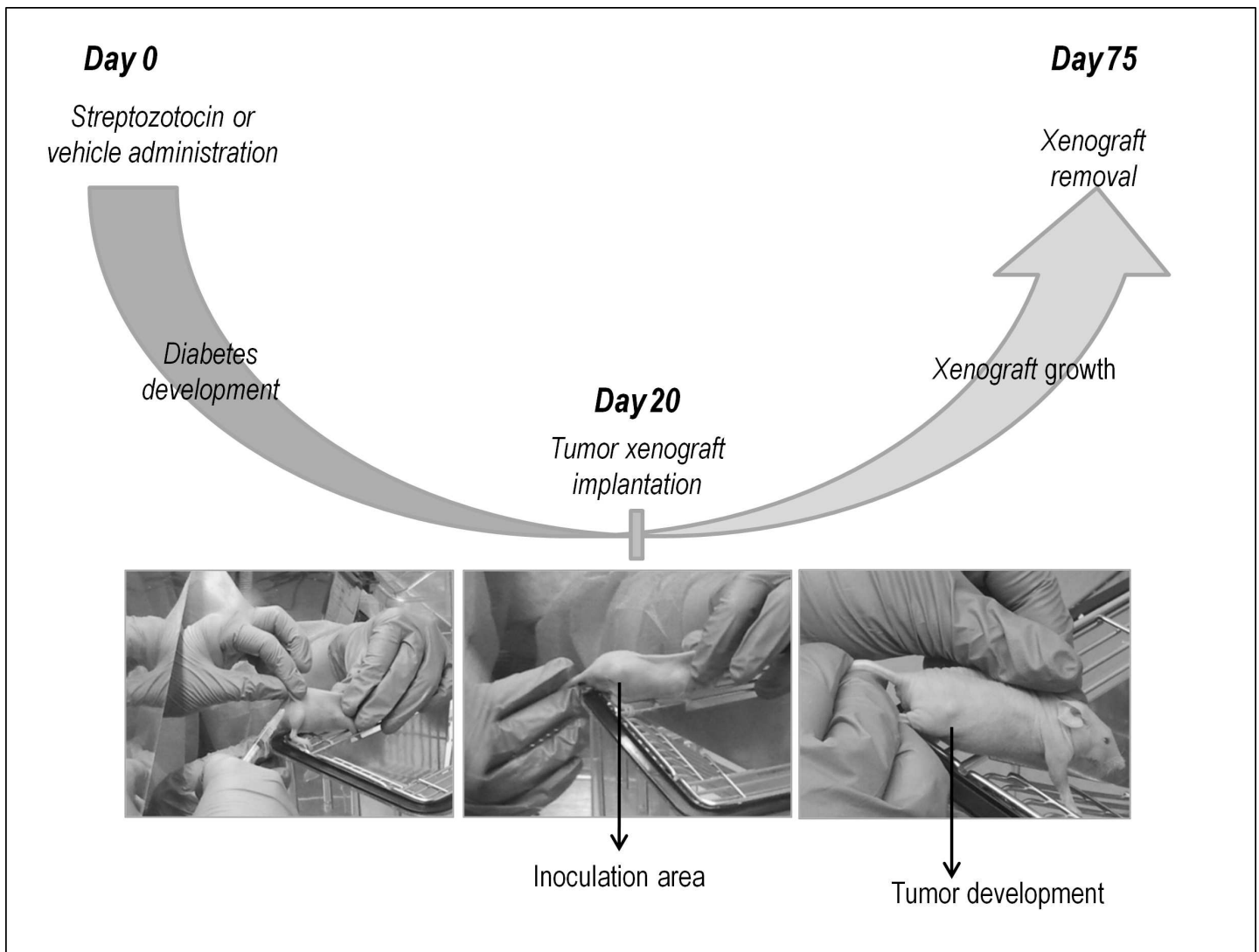


Fig 1. Schematic representation of the cancer xenograft model in mice.

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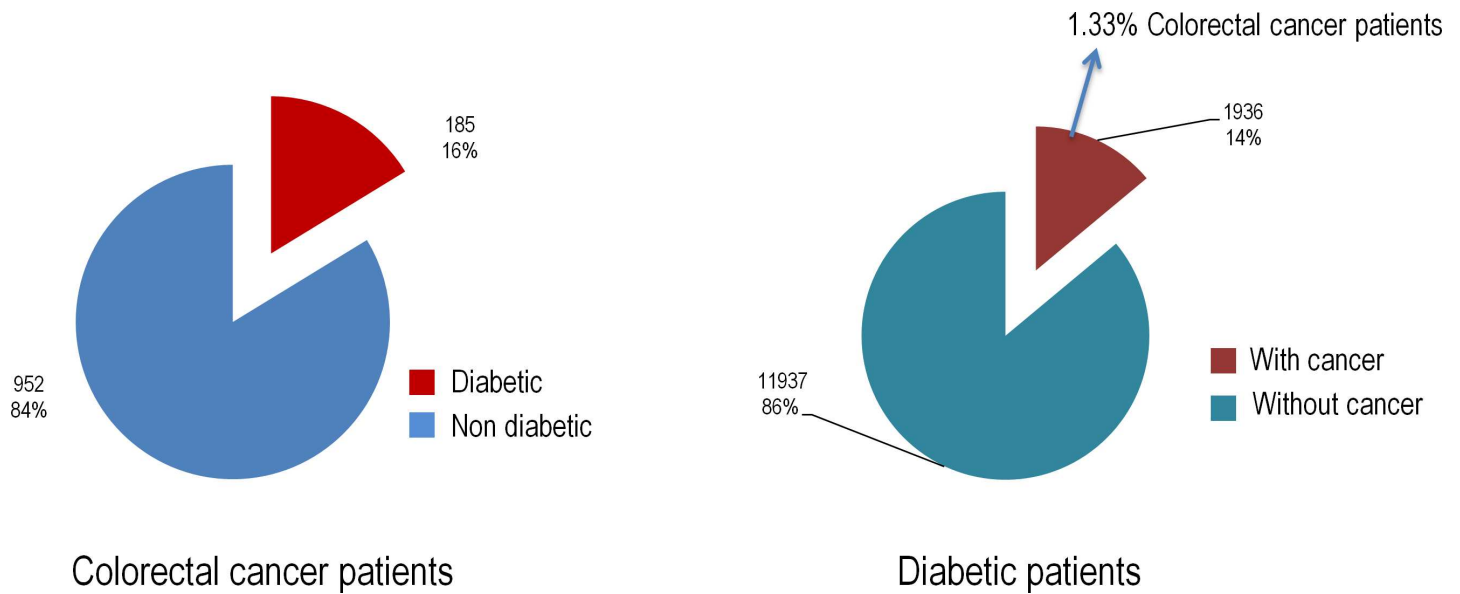


Fig 2. Overview of the stratified data, provided by the medical records of patients at Fundacion Jimenez Diaz.

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Colon cancer characteristics in diabetic versus non-diabetic patients

Descriptive and clinical results and frequencies of variables of diabetic and non-diabetic colon cancer patients that met inclusion criteria are presented in [Table 1](#). Demographic and clinical characteristics in both arms demonstrated the homogeneity of the group. The mean age was 72.9 (range, 45–95 years) for non-diabetic patients and 76.7 (range, 46–91) for diabetic patients.

Results were homogeneous with respect to gender, clinical debut and CEA levels, while serum glucose, triglycerides and circulating neutrophils were significantly higher and serum cholesterol significantly lower in diabetics and there was a non-significant trend towards higher BMI in diabetics.

ECOG Scale Performance Status was 0 in 99 patients (61.9%), and ≥ 1 in the rest of patients (38.1%); with the diabetic group having higher prevalence of high scores (ECOG ≥ 1 29.1% in non-diabetics vs 46.9% in diabetics, $p = 0.02$).

Regarding tumor variables, no significant differences were found between diabetics and non diabetics in the prevalence of high T stage, node involvement, grade of differentiation, presence of lymphovascular invasion, tumor location, recurrence rate, death events or cancer-related deaths.

From the total of diabetic patients, 35 were treated with metformin. No significant differences were observed in tumor-related features between diabetic patients on metformin or not on metformin ([Table 2](#)).

A diabetic environment does not modify tumor xenograft growth in STZ-D mice

Since clinical data did not support a relationship between diabetes and specific tumor features, the influence of diabetes on the growth and histological features of human colon cancer xenografts was assessed in mice, following the experimental protocol represented in [Fig 1](#).

STZ-D mice displayed mean levels of blood glucose >200 mg/dl through the experiment ([Fig 3A](#)). At the end of follow-up no significant differences in tumor volume were observed between control and STZ-D mice ([Fig 3B and 3C](#)).

Table 1. Clinicopathological characteristics of non-diabetic and diabetic colon cancer patients. Data expressed as mean ± SD or N (%).

Variable	Overall (N = 160)	Non-diabetic (N = 79)	Diabetic (N = 81)	P
Age years,	74.8 ± 10.4	72.9 ± 11.3	76.7 ± 9.2	0.02
Glucose levels, mg/dl,	112.4 ± 38.5	93.2 ± 9.0	131.1 ± 46.4	<0.0001*
Triglycerides, mg/dl,	117.8 ± 71.0	95.4 ± 36.1	138.0 ± 87.3	<0.0001*
Cholesterol, mg/dl,	165.8 ± 44.2	180.3 ± 38.9	151.1 ± 40.3	<0.0001*
BMI, kg/m ² ,	24.4 ± 2.8	23.5 ± 2.7	25.9 ± 2.5	0.06
Lymphocytes, x10 ³ µl,	2.1 ± 0.9	2.1 ± 0.8	2.1 ± 0.9	0.70
Neutrophils, x10 ³ µl,	4.7 ± 2.1	4.3 ± 1.9	5.0 ± 2.1	0.02*
Platelets, x10 ³ µl,	283.0 ± 116.0	279.2 ± 111.8	286.1 ± 120.1	0.70
Females, N (%)	67 (41.9%)	37 (46.8%)	30 (37%)	0.21
Clinical debut colon cancer, N (%)				
Subclinical	146 (91.2%)	74 (93.7%)	72 (88.9%)	0.28
Acute	14 (8.8%)	5 (6.3%)	9 (11.1%)	
ECOG, N (%)				
0	99 (61.9%)	56 (70.9%)	43 (53.1%)	0.02*
≥ 1	61 (38.1%)	23 (29.1%)	38 (46.9%)	
CEA (ng/mL), N (%)				
≤ 5	87 (54.4%)	47 (82.5%)	40 (75.5%)	0.39
> 5	23 (14.4%)	10 (17.5%)	13 (24.5%)	
N/A	50 (31.2%)	-	-	
pT, N (%)				
T1-T2	53 (33.1%)	24 (30.4%)	29 (35.8%)	0.61
T3-T4	107 (66.9%)	55 (69.6%)	52 (64.2%)	
pN, N (%)				
N0	101 (63.1%)	48 (60.8%)	53 (65.4%)	0.54
N+	59 (36.9%)	31 (39.2%)	28 (34.6%)	
Grade, N (%)				
Low grade	147 (91.9%)	72 (91.1%)	75 (92.6%)	0.73
High grade	13 (8.1%)	7 (8.9%)	6 (7.4%)	
Adjuvant therapy, N (%)	54 (33.8%)	29 (36.7%)	25 (30.9%)	0.43
Tumor site, N (%)				
Right	75 (46.9%)	32 (40.5%)	43 (53.1%)	0.11
Left	85 (53.1%)	47 (59.5%)	38 (46.9%)	
Lymphovascular invasion, N (%)				
Yes	23 (14.4%)	12 (18.8%)	11 (16.4%)	0.72
No	108 (67.5%)	52 (81.2%)	56 (83.6%)	
N/A	29 (18.1%)	-	-	
Stage, N (%)				
Low	100 (62.5%)	49 (62.0%)	51 (63.0%)	0.90
High	60 (37.5%)	30 (38.0%)	30 (37.0%)	
Recurrence, N (%)	16 (10.0%)	7 (8.9%)	9 (11.1%)	0.63
Death, N (%)	23 (14.4%)	11 (13.9%)	12 (14.8%)	0.87
Cancer-related death, N (%)**	12 (52.1%)	5 (45.4%)	7 (58.3%)	0.53

* p<0.05 denotes statistical significance.

** % of total deaths.

Abbreviations: BMI, Body mass index; ECOG, Eastern Cooperative Oncology Group; CEA, carcinoembryonic antigen; SD, standard deviation; N/A, Not available

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Table 2. Cancer-related clinicopathological characteristics and metformin use in diabetic patients with colon cancer.

Variable	Non Metformin (N = 46)	Metformin(N = 35)	P
ECOG, N(%)			
0	27 (58.7%)	16 (45.7%)	0.24
≥ 1	19 (41.3%)	19 (54.3%)	
pT, N(%)			
T1-T2	18 (39.1%)	11 (31.4%)	0.47
T3-T4	28 (60.8%)	24 (68.5%)	
pN, N(%)			
N0	31 (67.4%)	22 (62.9%)	0.67
N+	15 (32.6%)	13 (37.1%)	
Grade, N(%)			
Low grade	42 (91.3%)	33 (94.3%)	0.69
High grade	4 (8.7%)	2 (5.7%)	
Lymphovascular invasion, N(%)			
Yes	7 (15.2%)	4 (11.4%)	0.75
No	32 (69.6%)	24 (68.6%)	
N/A	7(15.2%)	7 (20%)	
Stage, N(%)			
Low	30 (65.2%)	21 (60.0%)	0.63
High	16 (34.8%)	14 (40.0%)	
Recurrence, N(%)			
Yes	6 (13.0%)	3 (8.6%)	0.75
No	40 (87.0%)	32 (91.4%)	
Death, N(%)			
Cancer-related death, N(%)*	4 (57.1%)	3 (60%)	0.29

* % of total deaths

Abbreviations: ECOG, Eastern Cooperative Oncology Group; N/A, Not available

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The morphological analysis of tumors in control and STZ-D groups revealed identical architectural and cytologic characteristics, showing a homogeneous solid growth pattern with scattered differentiated glands, invasive tumor edge on surrounding soft tissues and focal intratumoral necrosis. Similarly, no differences in proliferation rates were observed (Fig 4A and 4B). The examination of other organs did not demonstrated dissemination of tumor cells.

Analysis of microvascular density in tumors using CD31 as endothelial marker for quantification also demonstrated similar results between control and STZ-D mice (Fig 4C).

Discussion

There are many epidemiological studies addressing the risk of developing cancer in diabetic patients, but few reports have focused on the influence of diabetes on colon cancer behavior once colon cancer has already developed. We now report that no differences in tumor behavior or characteristics were found when colon cancer in diabetic patients was compared with colon cancer in non-diabetic patients or when colon cancer xenografts were implanted in diabetic or control mice.

The main strength of the present study is the homogeneous, well-balanced and well-characterized population of the clinical study that limits the bias that may result from more heterogeneous or less characterized populations [12, 14, 27, 28]. Another strength is a preclinical study that supports the conclusions of the clinical dataset.

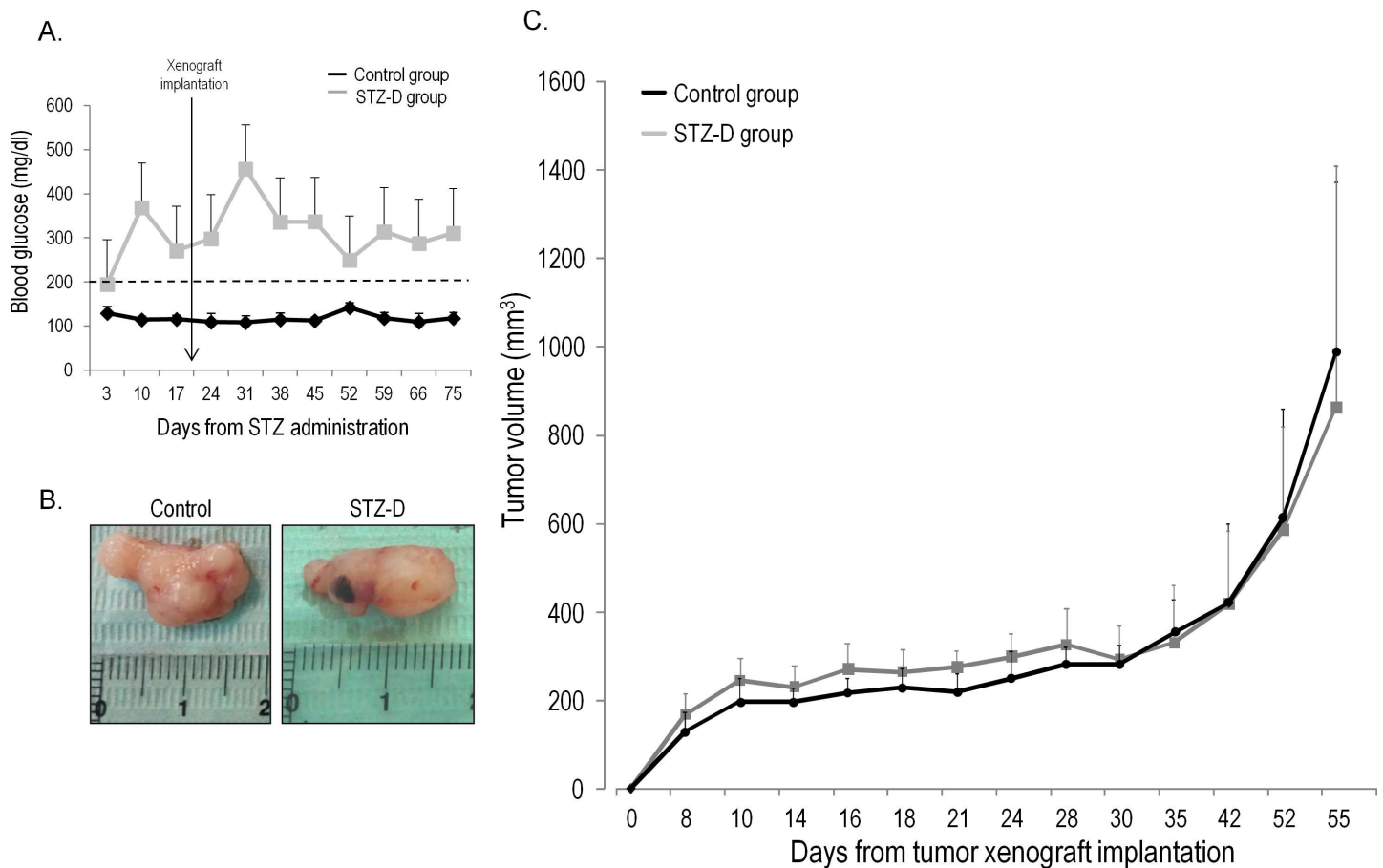


Fig 3. A diabetic environment does not modify tumor xenograft growth in mice. **A)** Blood glucose levels in diabetic and control mice during the experiment. The cutoff point to define development of diabetes was established at 200 mg/dl; **B)** Representative images of tumors from diabetic and control mice; **C)** Tumor growth curves for xenografts in diabetic and control mice.

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Our clinical results are in accordance to those in the “European American” ethnic group, ethnically closer to our cohort, in a multiethnic cohort [12]. Thus, there were no differences between diabetics and controls in terms of anatomical or pathological characteristics as stage or right/left localization. A previous report describing an association between diabetes and worse histopathologic colorectal cancer features may have been limited by excessive patient heterogeneity related to the inclusion of diverse histological types and of patients with rectal cancer [28]. Colon and rectal cancer show multiple biological and clinical differences, probably related to different mechanism of oncogenesis [24, 29, 30]. Furthermore, the standard treatment for rectal cancer shows more inter-institutional variability depending on the surgeon expertise. We excluded rectal cancer from our study in order to better preserve the anatomical structure. Standard rectal cancer treatment includes neoadjuvant chemo-radiation, which essentially modifies cell integrity and the microenvironment and may make the tumor disappear. Thus, rectal cancer patients merit a separate analysis. Adenocarcinoma is the most common colon cancer subtype and the most probably linked with a pro-inflammatory environment. Thus, we excluded other histologic types, since additional oncogenic mechanisms may contribute.

It has been suggested that medication used to control the glucose levels, mainly metformin, may modify the risk of developing cancer [31–34]. For this reason, we analyzed the metformin-treated subgroup in our cohort. We did not observe an effect of metformin on tumor

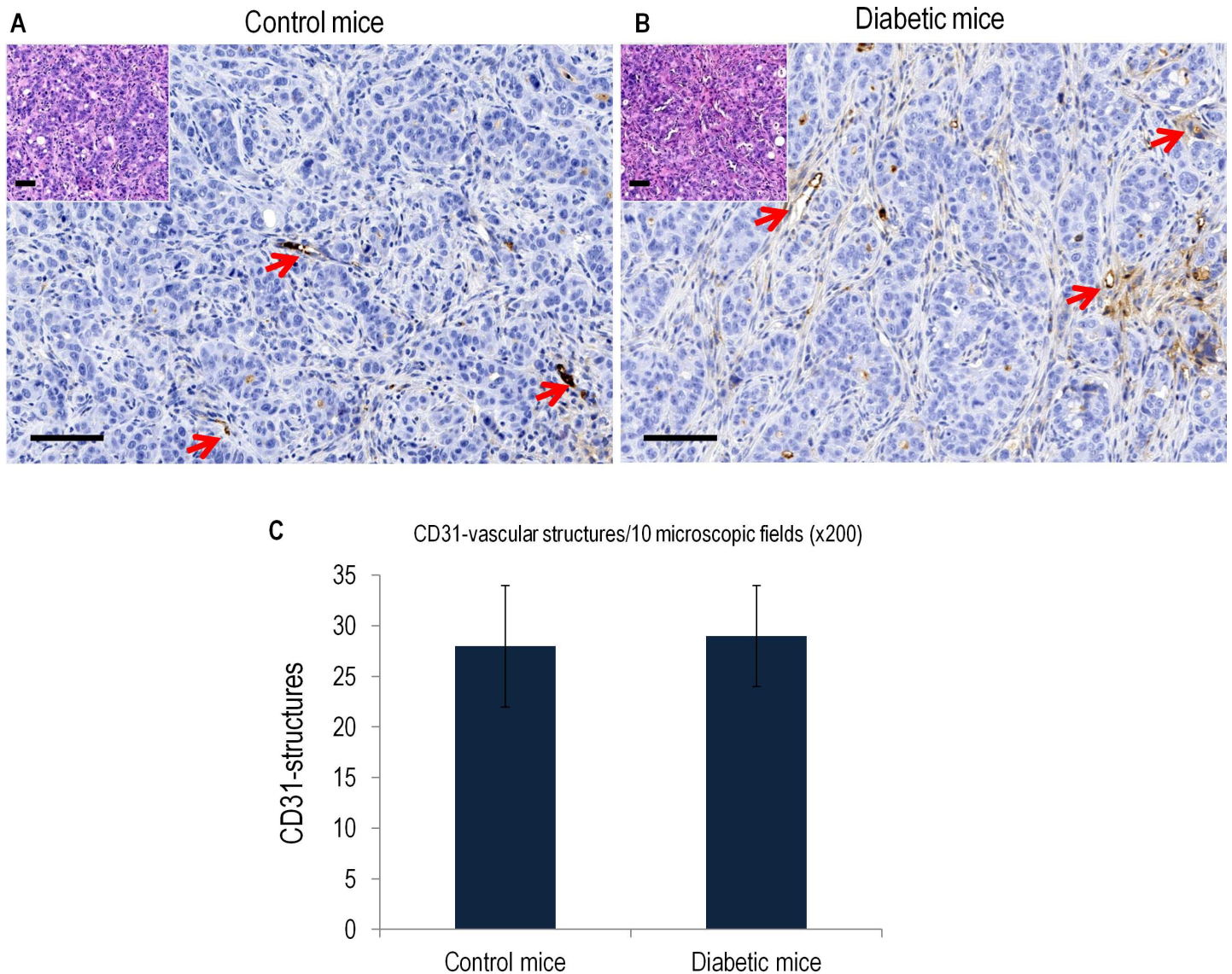


Fig 4. Representative histological images and corresponding quantification of vascular structures in the tumor xenograft model in mice. A) and B) show hematoxylin-eosin staining and CD31 immunohistochemistry images in tumor samples from control and diabetic xenografts. Original magnification x200. Arrows indicate vascular structures. C) Graphical representation of the number of vascular structures/10 microscopic x200 fields in control and diabetic mice. Scale, bar, 100 μ m.

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behavior. However, we are very cautious about this result, since the additional stratification of data resulted in very low number of cases. Nevertheless, despite the few cases analyzed, results were concordant with several reports, which showed that metformin may reduce cancer risk, but its effect on mortality following cancer remains unclear [35, 36], even in other tumor types [37].

The preclinical *in vivo* model is based on published and widely accepted methods [38], yielding an homogeneous and reproducible experimental approach which allows for non-biased robust results. This experimental procedure disclosed no significant differences in tumor growth kinetics or histopathological characteristics between diabetic and control mice. Very few *in vivo* studies have addressed the influence of diabetes on the growth of human

cancer. One report did not observe significant differences in sarcoma xenograft growth kinetics between diabetic and control mice [23]. By contrast, in a similar experimental setting, a protective effect of diabetes in a prostate cancer xenograft model was reported [22]. However, the published patient cohort reports are heterogeneous regarding to the effect of diabetes on prostate cancer patients [39].

Among the weaknesses, we should mention the retrospective nature of the study and the relatively small study population, belonging to a single center. While the smaller size allowed a degree of detail in the analysis that is not possible in large epidemiological studies, the single center nature limits the extrapolation of results to other centers or countries.

To summarize, our findings point towards a mild or negligible influence of diabetes on colon cancer behavior once cancer has already developed. These results serve as the basis to design a larger, multicenter, confirmatory clinical study.

Supporting information

S1 Fig. Schematic representation of the selection of patients who met the inclusion criteria for database development.

(TIF)

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