Pathology - Research and Practice 240 (2022) 154182

Contents lists available at ScienceDirect

Pathology - Research and Practice

journal homepage: www.elsevier.com/locate/prp

Colon tumor CD31 expression is associated with higher disease-free survival in patients with metabolic syndrome

Ana Silva [a,b,c,d,*], Sofia S. Pereira [b,c], José Ricardo Brandão [e], Paulo Brochado [e], Mariana P. Monteiro [b,c,f], António Araújo [g,h], Gil Faria [i,j,k]

[a] Pharmacy Department, Centro Hospitalar Universitário do Porto (CHUPorto), 4099-001 Porto, Portugal
[b] Department of Endocrine & Metabolic Research, UMIB - Unit for Multidisciplinary Research in Biomedicine, ICBAS - School of Medicine and Biomedical Sciences, University of Porto, Porto, Portugal
[c] ITR - Laboratory for Integrative and Translational Research in Population Health, Porto, Portugal
[d] School of Health, Polytechnic Institute of Porto, Polytechnic of Porto, Porto, Portugal
[e] Pathology Department, Centro Hospitalar Universitário do Porto (CHUPorto), 4099-001 Porto, Portugal
[f] Centre for Obesity Research, University College London, London, United Kingdom
[g] Oncology and Oncobiology Research, Unit for Multidisciplinary Biomedical Research (UMIB), University of Porto, Porto, Portugal
[h] Medical Oncology Department, Centro Hospitalar Universitário do Porto (CHUPorto), 4099-001 Porto, Portugal
[i] CINTESIS-Center for Research in Health Technologies and Information Systems, Porto, Portugal
[j] General Surgery, Hospital de Pedro Hispano – Unidade Local de Saúde de Matosinhos, Senhora da Hora, Portugal
[k] Department of Surgery, Faculty of Medicine, University of Porto, Porto, Portugal

ARTICLE INFO

Keywords:
Colon cancer
Angiogenesis
Lymphangiogenesis
Metabolic syndrome
Survival

ABSTRACT

Metabolic syndrome (MS) is recognized as a risk factor for colon cancer (CC). However, how does the interplay between metabolic dysfunction caused by MS and its individual components affect CC microenvironment and prognosis remains unexplored. Angiogenesis and lymphangiogenesis are fundamental processes for tumor progression and dissemination, ensuring oxygen and nutrient delivery and supporting one of the most important pathways of tumor dissemination, contributing to metastasis. Thus, our aim was to evaluate whether the expression of molecular biomarkers involved in angiogenic and lymphangiogenic processes influenced CC clinicopathological features and prognosis in patients with MS. Clinical and pathological data of 300 patients submitted to CC surgical resection at a single tertiary hospital were retrospectively retrieved from hospital records. Tumor tissue microarrays of archived paraffin-embedded blocks were used to assess CD31, VEGF-A and D2–40 tissue expression by immunohistochemistry. The percentage of stained area was quantified by computerized morphometric analysis. No association between tissue expression of angiogenesis and lymphangiogenesis biomarkers and tumor clinical and pathological characteristics was found. However, in subgroup analysis of patients with MS, dysglycemia was associated with lower D2–40 expression ($p = 0.007$) and high waist-circumference was associated with higher D2–40 ($p = 0.0029$) and VEGF-A expression ($p = 0.026$). In an adjusted Cox proportional hazard model CD31 expression was significantly associated with greater disease-free survival (HR=0.62; 95% CI: 0.41–0.95, $p = 0.028$). No association was found between D2–40 and VEGF-A expression and CC prognosis. Our data reinforces previous reports that suggest the potential use of CD31 as a CC prognostic biomarker. Additionally, our data further supports the evidence for an interplay between metabolic dysfunction, tumor microenvironment, and vascularization pathways.

1. Introduction

The incidence of colorectal cancer (CRC) is increasing globally with approximately 2 million new cases diagnosed in 2018 [1]. Although, tumor invasion and lymph node involvement are the main factors that determine disease prognosis, these do not fully predict the response to therapy and individual outcome [2]. Consequently, there is an unmet need to identify additional prognostic markers for treatment

* Corresponding author at: Department of Endocrine & Metabolic Research, UMIB - Unit for Multidisciplinary Research in Biomedicine, ICBAS - School of Medicine and Biomedical Sciences, University of Porto, Porto, Portugal.
E-mail address: analuisap.silva@gmail.com (A. Silva).

https://doi.org/10.1016/j.prp.2022.154182
Received 26 August 2022; Accepted 17 October 2022
Available online 19 October 2022

individualization that will allow to improve healthcare delivery.

Metabolic syndrome (MS) is recognized as a risk factor for colon cancer (CC) [3]. However, how does MS or its individual components affect the tumor microenvironment and disease prognosis remains unclear.

New blood vessels (angiogenesis) and lymphatic vessels (lymphangiogenesis) formation were identified as fundamental processes for tumor progression and dissemination [4,5]. If, on the one hand, tumor growth leads to progressive hypoxia that stimulates the development of new vessels as a survival strategy to ensure oxygen and nutrient supply, on the other hand, lymphatic vessels are one of the most important pathways for metastasis [4,5]. Therefore, the quest towards the identification of molecules that participate in the overall angiogenic process which could act as predictive biomarkers of therapeutic success and survival has been the focus of several research groups over past few the years [6–10]. However, the methodological diversity used across the studies so far conducted, including the variety of molecular biomarkers assessed, different specificities and the use of arbitrary cut-off values, could account for the contradictory results that hamper yielding solid conclusions. The presence of microvessels within the tumor tissue can be identified by the presence of specific molecular patterns and disclosed through histological sections immunohistochemistry staining. Among the molecular targets that allow to identify structures pertaining to the vascular system, platelet-endothelial cell adhesion molecule-1 (PECAM-1) or CD31, a membrane glycoprotein, is one of the most used molecular markers used to evaluate tumor micro vessel density (MVD) as a surrogate for angiogenic activity [8]. In addition, vascular endothelial growth factor A (VEGF-A), which acts as a potent tumor angiogenic factor, by increasing vascular permeability, promoting endothelial proliferation and migration is also widely used in the study of angiogenesis [4]. Assessing lymph angiogenesis, represents a greater challenge, as lymphatic vessels tend to collapse under the presence high interstitial pressure within the tumor. However, identifying microvessels pertaining to the lymphatic linage was rendered possible using LYVE-1 (lymphatic endothelial hyaluronan receptor) and D2–40 (podoplanin) as molecular targets [5]. LYVE-1 and D2–40 specificity for the lymphatic endothelium allows to differentiate lymphatic from blood vessels, enabling the study of neolymphangiogenesis [5,11,12].

The aim of the present study was to evaluate whether the expression of different molecules involved on the angiogenic and lymphangiogenic process, such as CD31, VEGF-A and D2–40 within the tissue of colon tumors could be used as predictive biomarker of CC clinical features and prognosis. Furthermore, we aimed to evaluate whether the expression the aforementioned molecular markers differs according to the MS status or presence of its individual components.

2. Material and methods

2.1. Patients and study protocol

This was a retrospective study that included patients (n = 300) that underwent CC resection surgery at a single tertiary public hospital between January 2010 and December 2015.

Detailed clinical information retrieved from electronic medical records, included clinical presentation, co-morbidities, laboratory findings, and pathological findings required for CC tumor staging.

Patients were excluded from the study if pathology data for accurate diagnosis or tissue specimens were unavailable, when diagnosed with in situ carcinoma or CC with histologic subtypes other than adenocarcinoma, disease staging was incomplete, clinical data that enabled MS classification were incomplete or absent, or if the patients underwent chemotherapy prior to the first assessment and tumor resection. This study protocol including clinical data accession was granted approval by the Institutional Ethics Review Board.

2.2. Metabolic syndrome criteria

Patients were classified as having MS whenever at least 3 out of the 5 individual components of the Harmonized Criteria were present, namely: (i) abdominal obesity (waist circumference (WC) $\geq$ 94 cm (male) or $\geq$ 80 cm (female) (Europid)); (ii) elevated triglycerides (>150 mg/dL) or ongoing treatment with triglyceride lowering drugs; (iii) low HDL-c (<40 mg/dL (males) and < 50 mg/dL (females) or ongoing treatment with HDL-c raising drugs; (iv) high BP (systolic $\geq$130 and/or diastolic $\geq$85 mm Hg) or ongoing treatment with antihypertensive drugs; (v) fasting blood glucose $\geq$ 100 mg/dL or ongoing treatment with glucose lowering drugs [13].

2.3. Colon cancer tumor staging

Data on tumor pathological characteristics including primary tumor location, pathological stage, and presence of lymph, vascular and perineural invasion were retrieved from the hospital electronic clinical records system.

Lymph node ratio (LNR) was calculated as the ratio between the number of metastatic and dissected lymph nodes (LN). In an approach similar to that of other authors, patients with node-positive disease were classified into LNR categories defined according to the 50th percentile of patients with node-positive disease [14]. The LNR threshold was set at 10%, establishing LNR categories according to the extension of LN involvement: LNR= 0%, LNR< 10% and LNR$\geq$ 10%.

Patient follow-up data was retrieved until death or last visit. Overall survival (OS) was defined as the time interval from the date of diagnosis to the date of death. Recurrence-free survival (RFS) was defined as the interval from the date of diagnosis to the date of tumor recurrence or date of last follow-up. Disease-free survival (DFS) was defined as the time interval from the date of diagnosis to date of tumor recurrence, death or date of last follow-up visit.

2.4. Tissue microarray assembly and immunohistochemistry staining

Haematoxylin and eosin (H&E) stained whole sections were analyzed by an experienced pathology consultant (J.R.B.) to identify the morphologically representative regions of the tumor. Then, one tissue core with a diameter of 0.6 mm were harvested from the donor block and re-embedded into recipient tissue microarrays (TMA) blocks. Each TMA was then sectioned into 4–5-μm slices. Immunohistochemistry was performed on a VENTANA BenchMark Ultra (Roche Diagnostics) automated staining instrument using the Optiview DAB IHC Detection Kit (Roche Diagnostics) according to the manufacturer's instructions. Slides were de-paraffinized using EZprep solution (Ventana Medical Systems, Inc., Tucson, AZ) at 72 °C. Epitope retrieval was accomplished on an automated staining station with Cell Conditioning 1 solution (Ventana Medical Systems, Inc.) for 28 min at 97 °C. Endogenous proteins and peroxides were blocked using Ventana pre-peroxidase inhibitor solution. Slides were then incubated with the primary antibody for 20 min at 36ºC for CD31 (1:50; Dako), 16 min at 36ºC for D2–40 (1:100; Sigma-Aldrich) and 20 min at 37ºC for VEGF (1:200; Invitrogen). Counterstaining was done with Hematoxylin (Ventana Medical Systems, Inc.) for 12 min, followed by a post counterstain bluing step (Ventana Medical Systems, Inc.) for 4 min.

2.5. Computerized image analysis

Slides were scanned using the image acquisition software Olympus VS110 virtual slide scanning system. After manual verification of tissue images and elimination of artefacts, such as black ink staining, each image was analyzed using FIJI software (National Institutes of Health, USA) and a color deconvolution plugin (HDab), which allows separating the stained area from the initial image based in the Red-Green-Blue (RGB) system. Using a similar protocol, the total tissue area was also

calculated. The percentage of the stained area for each antibody was assessed by calculating the ratio between the stained area and the total tissue area, as previously described [15].

2.6. Statistical analysis

Normality of the variables was determined by the Shapiro-Wilk test. Continuous variables were expressed as median (interquartile range (IQR)), according to data distribution. Primary outcome measures were used to assess if tissue molecular marker expression were associated with tumor pathology and survival. Differences in baseline clinical and pathologic characteristics of CC according to molecular marker expression were tested for statistical significance using Kruskal-Wallis H test, according to data distribution. Mann-Whitney U-test was used to evaluate differences between groups of MS components in continuous variables, according to data distribution. Statistical analysis for recurrence and survival rates was determined by the Kaplan–Meier method. The log-rank test was used for the comparison of survival between patient groups divided according to the 50th percentile. The prognostic impact of molecular marker expression on survival was quantified with Cox regression, after adjustment for age and staging. Statistical significance was considered for $p < 0.05$. Data was stored and analyzed using SPSS Statistics 27.0 (SPSS Inc., Chicago, IL, USA).

3. Results

Medical records of all patients (n = 300) that underwent surgical resection for CC during a 5-year period at a single tertiary hospital were reviewed for eligibility for study inclusion. A total of 198 patients were excluded for the following reasons: tissue specimen unavailable (n = 87), inadequate/unrepresentative tissue specimen (n = 8), histological diagnosis other than adenocarcinoma, namely mucinous adenocarcinoma or signet-ring cell carcinoma (n = 36), incomplete disease staging (n = 30), carcinoma in situ (n = 5), missing pathology data (n = 6), missing clinical data required for MS classification (n = 2), previous chemotherapy treatment prior to surgical resection (n = 17) or lost to follow-up (n = 7) (Fig. 1).

TMA sections were submitted to IHC staining for CD31 and VEGF-A in order to assess angiogenesis and D2–40 to assess lymphangiogenesis. Fig. 2 shows representative cases of positive staining for CD31, VEGF-A and D2–40. Demographic, clinical and pathological data of patients that fulfilled eligibility criteria for data analysis (n = 102) are listed in Table 1.

Data analysis revealed no association between MS, tumor clinical or pathological characteristics such as tumor location, lymph node invasion or presence of metastasis and expression of tissue molecular markers related to angiogenesis or lymphangiogenesis processes (Table 2).

In the subgroup analysis of patients with MS (n = 48), patients with dysglycemia had lower percentage of D2–40 stained area than patients without dysglycemia (0.02 vs 0.09%, p = 0.007) (Table 3). Moreover, patients with MS and elevated WC revealed higher percentage of D2–40 and VEGF-A stained area when compared to MS patients without elevated WC (0.05 vs 0.01%, p = 0.029 and 0.89 vs 0.11%, p = 0.026, respectively) (Table 3). No association was found between the individual components of MS and the stained area of the evaluated molecular markers in the subgroup analysis of patients without MS (data not shown).

A Kaplan-Meier model was used to estimate the survival probability. During a median follow-up time of 37.9 months, 36 patients experienced disease relapse and 29 patients died. Patients were divided in groups by the 50th percentile expression of CD31, VEGF-A and D2–40. No difference in OS, DFS and RFS (data not shown). In a Cox proportional hazard model for all patients included in the study (n = 102) adjusted for age and tumor staging, CD31 was significantly associated with greater DFS (HR=0.64; 95% CI: 0.41–0.99, p = 0.045) (Table 4).

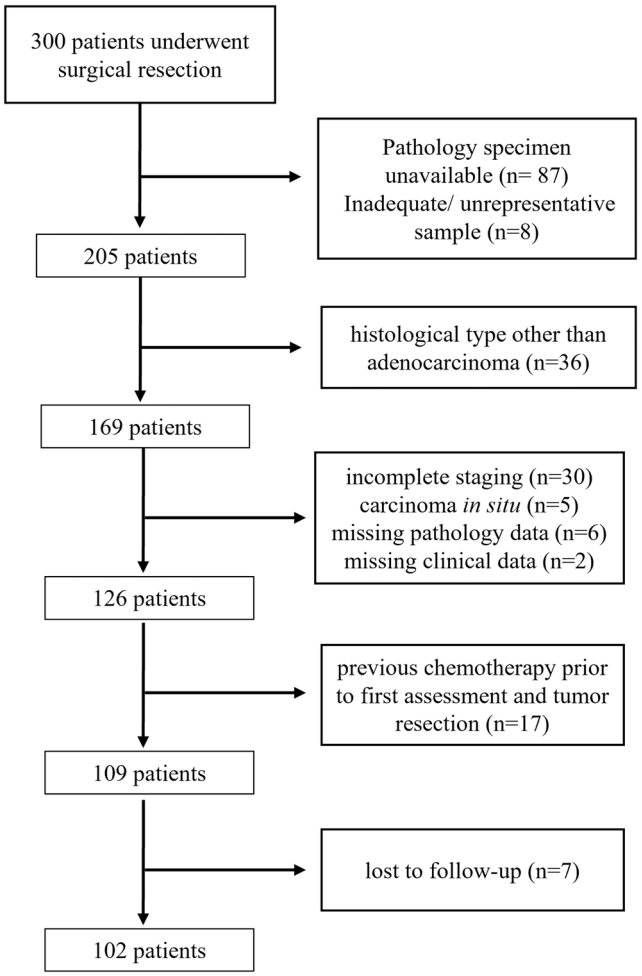

Fig. 1. Flow diagram of patient inclusion.

This favorable DFS trend associated with CD31 was maintained in the subgroup analysis of individuals with MS (HR=0.62; 95% CI: 0.41–0.95, p = 0.028) (Table 5). In individuals without MS, no differences were found (data not shown).

4. Discussion

The role of tissue microenvironment in tumor progression and dissemination has been widely recognized with a particular focus on tumor-derived secreted factors, such as those related to neovascularization [16]. Therefore, the study of molecular pathways potentially involved in cancer dissemination through either blood or lymph vessels formation has been considered increasingly relevant.

In our herein study, no differences were found between tumor tissue molecular markers expression and CC clinical and pathological presentation or MS (Table 2). However, in the subgroups analysis of individuals with MS, dysglycemia was demonstrated to be associated with a lower percentage of D2–40 stained area. In addition, individuals with elevated WC were found to have a higher percentage of D2–40 and VEGF-A stained area (Table 3). It is widely postulated that angiogenesis accompanies the expansion of adipose tissue mass, providing the groundings for its metabolic functions [17]. However, the crosstalk between gut lymphatics and adipose tissue, as well as its relationship with metabolic dysfunction such as dysglycemia is largely unexplored [18]. Obesity is known to be associated with pathological changes within the lymphatic vasculature, while simultaneously a defective lymphatics can promote adiposity deposition [17]. At the same time, individuals with obesity were shown to depict elevated levels of

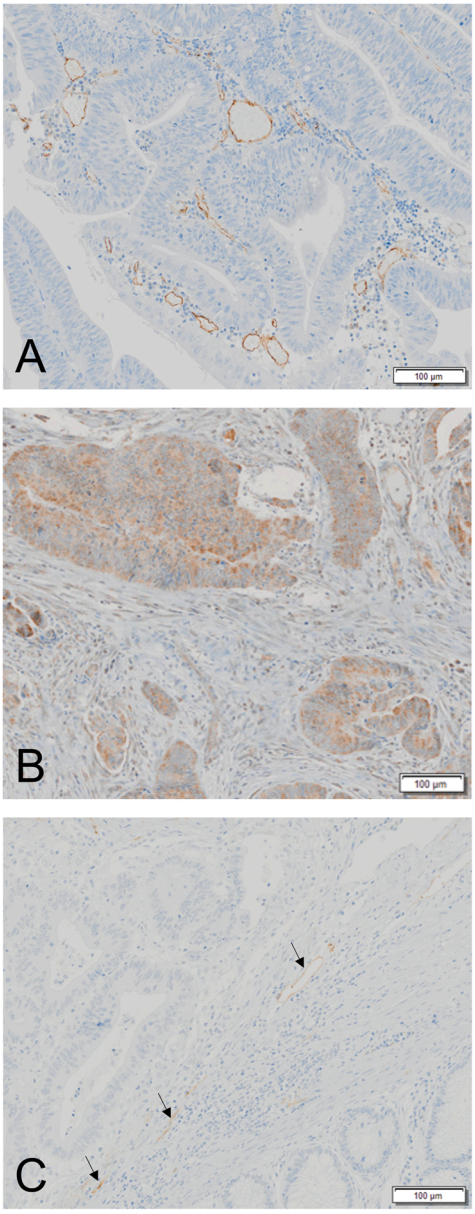

Fig. 2. Colon tumor sections immunohistochemistry stained for CD31 (A), VEGF-A (B) and D2–40 (C). A. CD31 positive staining on vascular endothelium. B. VEGF-A positive staining on adenocarcinoma epithelial cells. C. D2–40 positive staining on lymphatic endothelium, with arrows pointing towards lymphatic vessels.

Table 1 -
Baseline characteristics of patients included in the study.

	All patients (n = 102)
Age (years), mean (SD)	71 (12)
Sex	
Female, n (%)	43 (42.2)
Male, n (%)	59 (57.8)
Tumor size (cm), median (IQR)	4.0 (3.0–5.5)
Dukes Stage	
A	17 (16.7)
B	33 (32.4)
C	31 (30.4)
D	21 (20.6)
Metastatic LN, median (IQR)	0 (0–2)
Retrieved LN, median (IQR)	14 (10–21)

pro-lymphangiogenic factors in response to a pro-inflammatory state, further supporting the hypothesis of a potential role for neo-lymphangiogenesis pathway in metabolically dysfunctional individuals [19].

VEGF-A is the most widely studied angiogenic factor, with numerous studies demonstrating its association with tumor dissemination [20]. However, its prognostic value in CC yielded contradictory results [8,9]. Our data revealed no association between VEGF-A tissue expression and tumor clinical or pathological characteristics (Table 2) nor patient survival (Table 4) including after subgroup analysis (Table 5). There is growing body of evidence suggesting that there is a correlation between VEGF-A genetic polymorphisms and the global outcomes of anti-angiogenic drug treatments [21,22]. In fact, previous studies reported that variable ratios of angiogenic to antiangiogenic VEGF-A isoforms within the tumors as responsible for the heterogeneity of response to anti-angiogenic therapy [23]. Furthermore, hypoxia-driven angiogenesis is described as a complex process with multiple molecular intervenient that can influence the perception of the impact of each individual molecular marker [24,25].

In the present study, no association was found between lymphangiogenesis and features of tumor aggressiveness nor poor prognosis, including after subgroup analysis. Previous studies reported an increased lymphatic MVD in tumor tissue, when compared to its normal counterpart, although its prognostic value remained unclear [5,11]. Authors suggest that lymphangiogenesis may play a paramount role in the initial stage of tumor dissemination, but not at more advanced stages [5]. However, we observed no differences in molecular marker expression across the different tumor stages, nor differences in survival after adjustment for staging.

Our data revealed that CD31 expression has the potential to identify patients with greater DFS, after adjustment for age and tumor staging (Table 4). This trend was maintained in the subgroup analysis in individuals with MS (Table 5). Our results corroborate previous findings of a positive effect of elevated tumor CD31 levels, reflected by increased MVD, on progression-free survival (PFS), but not OS [10]. In a double-blind placebo-controlled randomized phase III trial involving 980 patients, MVD assessed through CD31 + vessels, was demonstrated to have a potential predictive value for PFS, as these patients also experienced greater therapeutic effects when exposed to the anti-angiogenic drug bevacizumab [8].

Controversies regarding the results of the published literature regarding neovascularization and CC outcomes might be accountable to its complex nature and the lack of standardized methods of assessment. For example, despite widely used to assess MVD, CD31 is not always expressed in capillaries, especially in poorly differentiated tumors where the vessels are discontinuous or interrupted. Additionally, there is some evidence that CD31 can also be expressed by tumor-associated macrophages, which could potentially influence immunohistochemical findings [26]. Furthermore, tumor hypoxia that leads to the angiogenic process is dependent on several factors such as initial tissue oxygenation, which may represent an additional confounding factor [27]. The retrospective nature of the study and the fact that it was carried out at a single center, is reflected in the limited sample size. However, despite being representative of the background patient population, some predictive associations may have been missed, which hamper the robustness of our conclusions and limit its generalization. Therefore, our results need to be confirmed in a larger prospective cohort study.

In conclusion, our data reinforces the potential use of CD31 as a molecular marker for CC prognosis. Additionally, our study further supports the need for better understanding the highly complex phenomena underlying angiogenesis and lymphangiogenesis, its implications in the tumor microenvironment and impact on CC patient outcomes.

Table 2
Association between expression of CD31, VEGF and D2–40 and clinical data of patients included in the study (n = 102).

	CD31		VEGF		D2-40	
	Median [IQR]	p value	Median [IQR]	p value	Median [IQR]	p value
Male sex	0.817 (0.420–1.600)	0.119	0.938 [0.486–2.103]	0.842	0.034 [0.014–0.124]	0.909
MS	1.137 (0.612–1.893)	0.426	0.779 (0.325–2.421)	0.525	0.031 (0.012–0.107)	0.389
Tumor Location		0.877		0.396		0.336
Left	1.161 [0.610–1.601]		0.966 [0.490–2.639]		0.043 [0.015–0.143]	
right	1.064 [0.441–1.687]		0.799 [0.185–2.975]		0.030 [0.013–0.090]	
Tumor stage		0.705		0.646		0.760
pT1	1.334 [0.364–2.415]		0.585 [0.092–2.478]		0.020 [0.011–0.151]	
pT2	0.866 [0.391–1.895]		0.793 [0.444–4.514]		0.057 [0.016–0.102]	
pT3	1.228 [0.587–1.603]		0.937 [0.325–1.918]		0.031 [0.014–0.119]	
pT4	0.818 [0.610–1.530]		1.425 [0.463–2.567]		0.075 [0.009–0.161]	
Nodal status		0.286		0.168		0.600
pN0	0.927 [0.442–1.547]		0.915 [0.285–2.515]		0.048 [0.016–0.121]	
pN1	1.125 [0.520–2.050]		1.425 [0.625–3.074]		0.037 [0.017–0.122]	
pN2	1.395 [0.814–1.727]		0.463 [0.144–1.801]		0.031 [0.005–1.127]	
Metastatic stage		0.192		0.821		0.763
M0	1.161 [0.632–1.764]		0.916 [0.380–3.097]		0.041 [0.016–0.121]	
M1	0.812 [0.430–1.395]		1.302 [0.369–1.905]		0.036 [0.006–0.132]	
LNR		0.381		0.889		0.528
LNR = 0%	1.015 [0.443–1.570]		0.936 [0.313–2.788]		0.048 [0.016–0.130]	
LNR < 10%	1.231 [0.512–2.093]		1.330 [0.412–2.354]		0.034 [0.012–0.130]	
LNR ≥ 10%	1.177 [0.814–1.727]		0.835 [0.443–2.515]		0.031 [0.011–0.089]	
Dukes		0.567		0.955		0.980
A	1.322 [0.577–2.103]		0.769 [0.249–4.027]		0.055 [0.015–0.106]	
B	1.215 [0.648–1.562]		0.968 [0.241–4.053]		0.046 [0.015–0.142]	
C	1.084 [0.489–1.770]		0.895 [0.486–3.074]		0.038 [0.020–0.101]	
D	0.812 [0.430–1.395]		1.302 [0.369–1.905]		0.036 [0.006–0.132]	
Lymphatic invasion	1.084 [0.602–1.769]	0.858	0.937 [0.490–2.983]	0.696	0.093 [0.020–0.167]	0.072
Vascular invasion	1.304 [0.610–1.904]	0.261	0.860 [0.369–2.037]	0.403	0.061 [0.013–0.181]	0.214
Perineural Invasion	1.345 [0.812–1.769]	0.147	1.125 [0.490–3.001]	0.435	0.031 [0.013–0.137]	0.873
Death	0.791 [0.357–1.340]	**0.014**	0.799 [0.391–2.417]	0.636	0.031 [0.007–0.098]	0.202
Relapse	0.800 [0.433–1.890]	0.077	1.446 [0.626–2.769]	0.204	0.052 [0.017–0.167]	0.278

Table 3
Association of individual components of MS with CD31, VEGF-A and D2–40 tumor levels in patients with MS (n = 48).

	CD31, median (IQR)	p value	D2-40, median (IQR)	p value	VEGF-A, median (IQR)	p value
Dysglycemia		0.749		**0.007**		0.733
Yes	1.11 (0.39–2.14)		0.02 (0.01–0.06)		1.20 (0.18–2.50)	
No	1.28 (0.39–2.14)		0.09 (0.03–0.15)		0.67 (0.35–2.50)	
Low HDL-c		0.261		0.988		0.775
Yes	1.02 (0.52–1.95)		0.03 (0.01–0.11)		0.77 (0.34–2.56)	
No	1.39 (1.11–1.73)		0.03 (0.01–0.24)		1.71 (0.20–2.42)	
High BP		0.320		0.071		0.436
Yes	1.11 (0.58–1.63)		0.03 (0.01–0.10)		0.89 (0.30–3.15)	
No	1.69 (0.67–2.36)		0.12 (0.04–0.26)		0.66 (0.43–0.90)	
Hypertriglyceridemia		0.393		0.708		0.690
Yes	1.02 (0.56–1.78)		0.03 (0.01–0.13)		0.79 (0.30–3.62)	
No	1.34 (0.69–1.92)		0.03 (0.01–0.12)		0.77 (0.32–1.73)	
Elevated WC		0.260		**0.029**		**0.026**
Yes	1.11 (0.52–1.95)		0.05 (0.02–0.14)		0.89 (0.44–2.71)	
No	1.38 (1.09–1.95)		0.01 (0.00–0.03)		0.11 (0.06–1.05)	

Table 4
Cox-Proportional Regression models for survival outcomes for all patients included in the study adjusted for age and staging (n = 102).

	Overall survival		Relapse-free survival		Disease-free survival	
	HR (95% CI)	p value	HR (95% CI)	p value	HR (95% CI)	p value
CD31	0.73 (0.41–1.31)	0.292	0.74 (0.47–1.16)	0.194	0.64 (0.41–0.99)	**0.045**
VEGF-A	0.93 (0.78–1.11)	0.419	1.04 (0.91–1.18)	0.597	1.03 (0.91–1.16)	0.638
D2–40	0.26 (0.00–17.20)	0.530	9.62 (0.40–230.42)	0.162	5.16 (0.27–97.32)	0.273

CI – Confidence interval; HR – Hazard ratio.

Funding

This project was supported by a grant from Centro Hospitalar e Universitário do Porto (2015/178).

CRediT authorship contribution statement

Ana Silva: Conceptualization, Methodology, Formal analysis, Investigation, Data curation, Writing – original draft preparation, Writing – review & editing. **Sofia S. Pereira:** Methodology, Formal

Table 5
Cox-Proportional Regression models for survival outcomes for patients with MS (n = 48).

	Overall survival		Relapse-free survival		Disease-free survival	
	HR (95% CI)	p value	HR (95% CI)	p value	HR (95% CI)	p value
CD31	0.66 (0.37–1.18)	0.157	0.75 (0.49–1.15)	0.191	0.62 (0.41–0.95)	**0.028**
VEGF-A	0.94 (0.78–1.14)	0.545	1.04 (0.91–1.19)	0.561	1.04 (0.92–1.17)	0.544
D2–40	0.41 (0.01–23.23)	0.663	7.53 (0.32–177.39)	0.210	5.12 (0.30–88.91)	0.262

CI – Confidence interval; HR – Hazard ratio.

analysis, Data curation, Writing – review & editing. **José Ricardo Brandão:** Methodology, Formal analysis, Data curation, Writing – review & editing. **Paulo Brochado:** Methodology, Formal analysis, Data curation, Writing – review & editing. **Mariana P. Monteiro:** Conceptualization, Methodology, Writing – review & editing, Supervision, Project administration. **António Araújo:** Writing – review & editing. **Gil Faria:** Conceptualization, Methodology, Writing – review & editing, Supervision, Project administration.

Competing interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Acknowledgments

UMIB is supported by National Funds through the FCT – Fundação para a Ciência e a Tecnologia (Portuguese national funding agency for Science, Research and Technology) in the frameworks of the UIDB/00215/2020; UIDP/00215/2020; LA/P/0064/2020 – Unit for Multidisciplinary Research in Biomedicine - UMIB/ICBAS/UP.

Availability of data and material

Research data are not publicly available due to privacy and ethical restrictions. However, anonymized data that are required to reproduce results can be made available from the corresponding author upon reasonable request, and upon approval from the Centro Hospitalar Universitário do Porto according to mandatory national law.

References

[1] F. Bray, J. Ferlay, I. Soerjomataram, R.L. Siegel, L.A. Torre, A. Jemal, Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. Ca. Cancer J. Clin. 68 (2018) 394–424, https://doi.org/10.3322/caac.21492.

[2] G. Argilés, J. Tabernero, R. Labianca, D. Hochhauser, R. Salazar, T. Iveson, P. Laurent-Puig, P. Quirke, T. Yoshino, J. Taieb, E. Martinelli, D. Arnold, Localised colon cancer: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up†, Ann. Oncol. 31 (2020) 1291–1305, https://doi.org/10.1016/j.annonc.2020.06.022.

[3] Y.J. Choi, D.H. Lee, K. Do Han, C.M. Shin, N. Kim, Abdominal obesity, glucose intolerance and decreased high-density lipoprotein cholesterol as components of the metabolic syndrome are associated with the development of colorectal cancer, Eur. J. Epidemiol. 33 (2018) 1077–1085, https://doi.org/10.1007/s10654-018-0440-6.

[4] K.K. Auyeung, J.K. Ko, Angiogenesis and Oxidative Stress in Metastatic Tumor Progression: Pathogenesis and Novel Therapeutic Approach of Colon Cancer, Curr. Pharm. Des. 23 (2017), https://doi.org/10.2174/1381612823666170228124105.

[5] C. Huang, Y. Chen, Lymphangiogenesis and colorectal cancer, Saudi Med. J. 38 (2017) 237–244, https://doi.org/10.15537/smj.2017.3.16245.

[6] S.F. Martins, E.A. Garcia, M. Luz, F. Pardal, M. Rodrigues, A. Filho, Clinicopathological Correlation and Prognostic Significance of VEGF-A, VEGF-C, VEGFR-2 and VEGFR-3 Expression in Colorectal Cancer, Cancer Genom. Proteom. 10 (2013) 55–67. ⟨https://cgp.iiarjournals.org/content/10/2/55.long⟩.

[7] V. Medina-Arana, A. Martínez-Riera, L. Delgado-Plasencia, D. Rodríguez-González, A. Bravo-Gutiérrez, H. Álvarez-Arguëlles, A. Alarcó-Hernández, E. Salido-Ruiz, A. M. Fernández-Peralta, J.J. González-Aguilera, Clinicopathological analysis of factors related to colorectal tumor perforation, Med. (U. S.) 94 (2015), https://doi.org/10.1097/MD.0000000000000703.

[8] C. Bais, B. Mueller, M.F. Brady, R.S. Mannel, R.A. Burger, W. Wei, K.M. Marien, M. M. Kockx, A. Husain, M.J. Birrer, Tumor Microvessel Density as a Potential Predictive Marker for Bevacizumab Benefit: GOG-0218 Biomarker Analyses, J. Natl. Cancer Inst. 109 (2017), https://doi.org/10.1093/jnci/djx066.

[9] S.Y. Mohamed, H.L. Mohammed, H.M. Ibrahim, E.M. Mohamed, M. Salah, Role of VEGF, CD105, and CD31 in the Prognosis of Colorectal Cancer Cases, J. Gastrointest. Cancer 50 (2019) 23–34, https://doi.org/10.1007/s12029-017-0014-y.

[10] D. Bianconi, M. Herac, F. Posch, M. Schmeidl, M. Unseld, M. Kieler, R. Brettner, L. Müllauer, J. Riedl, A. Gerger, W. Scheithauer, G. Prager, Microvascular density assessed by CD31 predicts clinical benefit upon bevacizumab treatment in metastatic colorectal cancer: results of the PassionATE study, a translational prospective Phase II study of capecitabine and irinotecan plus bevacizumab followed by capecitabine and oxaliplatin plus bevacizumab or the reverse sequence in patients in mCRC, 1758835920928635, Ther. Adv. Med. Oncol. 12 (2020), https://doi.org/10.1177/1758835920928635.

[11] J. Gao, A. Knutsen, G. Arbman, J. Carstensen, B. Frånlund, X.F. Sun, Clinical and biological significance of angiogenesis and lymphangiogenesis in colorectal cancer, Dig. Liver Dis. 41 (2009) 116–122, https://doi.org/10.1016/j.dld.2008.07.315.

[12] S.S. Pereira, M.M. Costa, S.G. Guerreiro, M.P. Monteiro, D. Pignatelli, Angiogenesis and Lymphangiogenesis in the Adrenocortical Tumors, Pathol. Oncol. Res. 24 (2018) 689–693, https://doi.org/10.1007/s12253-017-0259-6.

[13] K.G. Alberti, R.H. Eckel, S.M. Grundy, P.Z. Zimmet, J.I. Cleeman, K.A. Donato, J. C. Fruchart, W.P. James, C.M. Loria, S.C. Smith Jr., E. International Diabetes Federation Task Force on, Prevention, L. Hational Heart, I. Blood, A. American Heart, F. World Heart, S. International Atherosclerosis, O. International Association for the Study of, Harmonizing the metabolic syndrome: a joint interim statement of the International Diabetes Federation Task Force on Epidemiology and Prevention; National Heart, Lung, and Blood Institute; American Heart Association; World Heart Federation; International, in: Circulation, 120, 2009, pp. 1640–1645, https://doi.org/10.1161/CIRCULATIONAHA.109.192644.

[14] D.G. Priolli, I.A. Cardinalli, J.A. Pereira, C.H. Alfredo, N.F. Margarido, C.A. R. Martinez, Metastatic lymph node ratio as an independent prognostic variable in colorectal cancer: Study of 113 patients, Tech. Coloproctol. 13 (2009) 113–121, https://doi.org/10.1007/s10151-009-0467-5.

[15] S.S. Pereira, M.M. Costa, C.E. Gomez-Sanchez, M.P. Monteiro, D. Pignatelli, Incomplete pattern of steroidogenic protein expression in functioning adrenocortical carcinomas, Biomedicines 8 (2020), https://doi.org/10.3390/BIOMEDICINES8080256.

[16] C. Galindo-Pumariño, M. Collado, M. Herrera, C. Peña, Tumor microenvironment in metastatic colorectal cancer: The arbitrator in patients' outcome, Cancers (Basel) 13 (2021) 1–29, https://doi.org/10.3390/cancers13051130.

[17] N. Escobedo, G. Oliver, The lymphatic vasculature: its role in adipose metabolism and obesity, Cell Metab. 26 (2017) 598–609, https://doi.org/10.1016/j.cmet.2017.07.020.

[18] X. Jiang, W. Tian, M.R. Nicolls, S.G. Rockson, The lymphatic system in obesity, insulin resistance, and cardiovascular diseases, Front. Physiol. 10 (2019) 1402, https://doi.org/10.3389/FPHYS.2019.01402.

[19] H. Wada, S. Ura, S. Kitaoka, N. Satoh-Asahara, T. Horie, K. Ono, T. Takaya, R. Takanabe-Mori, M. Akao, M. Abe, T. Morimoto, T. Murayama, M. Yokode, M. Fujita, A. Shimatsu, K. Hasegawa, Distinct characteristics of circulating vascular endothelial growth factor-A and C levels in human subjects, PLoS One 6 (2011), e29351, https://doi.org/10.1371/journal.pone.0029351.

[20] T.F. Hansen, A. Jakobsen, Clinical implications of genetic variations in the VEGF system in relation to colorectal cancer, Pharmacogenomics 12 (2011) 1681–1693, https://doi.org/10.2217/pgs.11.118.

[21] R. Giampieri, L. Salvatore, M. Del Prete, T. Prochilo, M. Danzeo, C. Loretelli, F. Loupakis, G. Aprile, E. MacCaroni, K. Andrikou, M. Bianconi, A. Bittoni, L. Faloppi, L. Demurtas, R. Montironi, M. Scarpelli, A. Falcone, A. Zaniboni, M. Scartozzi, S. Cascinu, Angiogenesis genotyping and clinical outcome during regorafenib treatment in metastatic colorectal cancer patients, Sci. Rep. 6 (2016), https://doi.org/10.1038/srep25195.

[22] G. Pentheroudakis, L. Mavroeidis, K. Papadopoulou, G.A. Koliou, C. Bamia, K. Chatzopoulos, E. Samantas, D. Mauri, I. Efstratiou, D. Pectasides, T. Makatsoris, D. Bafaloukos, P. Papakostas, G. Papatsibas, I. Bombolaki, S. Chrisafi, H.P. Kourea, K. Petraki, G. Kafiri, G. Fountzilas, V. Kotoula, Angiogenic and Antiangiogenic VEGFA Splice Variants in Colorectal Cancer: Prospective Retrospective Cohort Study in Patients Treated With Irinotecan-Based Chemotherapy and Bevacizumab, in: Clin. Colorectal Cancer, Elsevier Inc, 2019, pp. e370–e384, https://doi.org/10.1016/j.clcc.2019.07.007.

[23] D.O. Bates, P.J. Catalano, K.E. Symonds, A.H.R. Varey, P. Ramani, P.J. O'Dwyer, B. J. Giantonio, N.J. Meropol, A.B. Benson, S.J. Harper, Association between VEGF splice isoforms and progression-free survival in metastatic colorectal cancer patients treated with bevacizumab, Clin. Cancer Res 18 (2012) 6384–6391, https://doi.org/10.1158/1078-0432.CCR-12-2223.

[24] Y.F. Zou, Y.M. Rong, Y.X. Tan, J. Xiao, Z.L. Yu, Y.F. Chen, J. Ke, C.H. Li, X. Chen, X. J. Wu, P. Lan, X.T. Lin, F. Gao, A signature of hypoxia-related factors reveals functional dysregulation and robustly predicts clinical outcomes in stage I/II colorectal cancer patients, Cancer Cell Int 19 (2019) 1–11, https://doi.org/10.1186/s12935-019-0964-1.

[25] J.H. Lee, S. Jung, W.S. Park, E.K. Choe, E. Kim, R. Shin, S.C. Heo, J.H. Lee, K. Kim, Y.J. Chai, Prognostic nomogram of hypoxia-related genes predicting overall survival of colorectal cancer–Analysis of TCGA database, Sci. Rep. 9 (2019) 1–9, https://doi.org/10.1038/s41598-018-38116-y.

[26] J.K. McKenney, S.W. Weiss, A.L. Folpe, CD31 expression in intratumoral macrophages: A potential diagnostic pitfall, Am. J. Surg. Pathol. 25 (2001) 1167–1173, https://doi.org/10.1097/00000478-200109000-00007.

[27] L. Qi, J. Chen, Y. Yang, W. Hu, Hypoxia correlates with poor survival and M2 macrophage infiltration in colorectal cancer, Front. Oncol. 10 (2020) 2491, https://doi.org/10.3389/fonc.2020.566430.