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ORIGINAL ARTICLE

Modulation of rectal cancer stemness, patient outcome and therapy response by adipokines

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Abstract

Response to chemoradiotherapy (CRT) in patients with locally advanced rectal cancer is highly variable. Identification of CRT non-responders and definite accurate biomarkers of response are unmet needs. In turn, adipokines might impact on colorectal cancer development. We hypothesized that imbalance in leptin and adiponectin modulates stemness potential CRT response in rectal cancer. Pre-CRT serum and tissue samples were collected from a cohort of locally advanced rectal cancer patients ($n=33$), submitted to long-course CRT and proctectomy. Adiponectin and leptin were measured by ELISA in serum. In tumour biopsies, mRNA expression of stemness-related genes was evaluated by qRT-PCR and transcription factor STAT3 by immunoblotting. Correlations with clinical data and accuracy of potential CRT response biomarkers were evaluated. Carcinoembryonic antigen (CEA) but not leptin or adiponectin distinguished CRT responders from non-responders ($p<0.05$). However, higher leptin and lower adiponectin serum levels were associated with positive extramesorectal nodes and extramural vascular invasion. mRNA expression of stemness factors was inversely correlated with adiponectin but positively correlated with leptin. STAT3 phosphorylation presented similar results. CEA levels together with STAT3 activation and *OCT4*/*KLF4* expression accurately identified rectal cancer patients, CRT non-responders (AUROC 0.80; $p<0.05$). Adipokines might impact rectal cancer stemness and patient prognosis. The leptin/STAT3 signalling axis provides the rational for a potential biomarker panel that identifies rectal cancer patients who will not benefit from CRT treatment.

Keywords Rectal cancer · Chemoradiotherapy · Adipokines · Stemness · STAT3

Key Points
- To date there are no validated biomarkers of CRT response in rectal cancer.
- Adipokines modulate cancer stemness impacting on patient prognosis and therapy response.
- Leptin/STAT3 signalling axis plays a role in CRT resistance.
- Combination of STAT3, *KLF4*, *OCT4* and CEA allows accurate identification of CRT non-responders.

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Introduction

Colorectal cancer is the third most incident (10.0%) and second most mortal (9.4%) worldwide [55]. Rectal cancer accounts for 35% of all colorectal cancer cases in the European Union [24]. Despite sharing common risk factors and often considered a single entity, colon and rectal cancers present fundamental differences at anatomical and therapeutic levels. While a surgical approach is valid in both cases, neoadjuvant chemoradiotherapy (CRT) is only considered for rectal cancer [58].

Preoperative TNM (T, tumour size; N, lymph node spread; M, metastasis) staging is crucial to identify patients with locally advanced rectal cancer that require neoadjuvant long-course CRT followed by total mesorectal excision [37]. Preoperative CRT offers many advantages, such as tumour downstaging and downsizing, increasing curative resections (R0 resection rates), sphincter-sparing surgery and decreasing local recurrence [34]. Nevertheless, nearly 30% of patients exhibit CRT resistance and are at increased risk

of disease progression, unnecessary toxicity and intestinal and genitourinary morbidity [14, 34].

Pathological response to CRT, assessed as tumour regression grade (TRG), can range from complete or partial response to non-response, in patients resistant to CRT [24]. Accurate and early prediction of CRT response would benefit the selection of the best responders for neoadjuvant treatment and conservative non-surgical approaches [23, 26] and the identification of non-responders sparing patients from unbeneficial CRT and favouring surgical resection. The worldwide incidence of rectal cancer is expected to rise from 0.73 million new cases in 2020 to 1.16 million by 2040, representing a 58.5% change [21]. Concurrently, rectal cancer patients that will be subjected to unbeneficial CRT approaches will also increase, since it is lacking an accurate stratification between responders and non-responders based on each individual clinical staging. To date, CRT response cannot be clinically predicted, but molecular features and signatures have been explored, such as miRNA expression, *KRAS* and *TP53* mutations or carcinoembryonic antigen (CEA) levels [4, 8–10, 12, 46, 49, 52, 53]. The inclusion of molecular markers in a biomarker algorithm would greatly benefit patient stratification.

In the last decades, obesity has emerged as a global epidemic and is considered a risk factor for both colon and rectal cancers [24, 30]. A growing body of evidence supports the role of obesity in colorectal cancer development, progression, response to therapy and outcome [5, 25, 38]. In fact, 5.2% of all rectal cancers are attributable to obesity alone [22], and the relative risk of rectal cancer development is increased with high body-mass index (BMI), particularly in men [42, 44]. Hence, imbalances in metabolism-related hormones such as adipokines play a role in colorectal cancer development. Two of the most well-studied adipokines in the context of colorectal cancer are adiponectin and leptin, which are produced by adipose tissue and are altered in obesity. Yet, they have antagonistic roles in colonic tumorigenesis. While adiponectin has been suggested to be protective [19], leptin is believed to promote cell growth, motility and invasion [29].

Adipokines may also influence cancer stem cell (CSC) biology and response to therapy. Indeed, 5-fluorouracil therapy is offset by higher leptin levels and accompanied by CSC survival [6]. CSCs display high self-renewal capacities, plasticity, resistance to tumour microenvironment stress factors and quiescence, thus being responsible for cancer relapse, metastization and resistance to radio- and chemotherapy [32, 64]. Moreover, in vitro and in vivo studies have shown that obesity can modulate stem cell responsiveness in carcinogenesis [13]. These facts bring forward a potential role for adipokines, like adiponectin and leptin, to modulate not only cancer development but also prognosis and response to therapy.

Here we hypothesized that adipokine imbalance plays a role in rectal cancer development and stemness potential and, ultimately, modulates response to CRT. We explored the influence of adiponectin and leptin on rectal cancer oncogenic pathways, identifying molecular markers and assessing their potential as prognostic biomarkers of response to CRT.

Materials and methods

Patients and sample collection

Tissue and serum samples were collected from 33 patients diagnosed with rectal cancer (stages I–IV, American Joint Committee on Cancer, AJCC) between 2017 and 2019 in the Surgical Department of Hospital Beatriz Ângelo (Loures, Portugal). Patients underwent preoperative staging with thoraco-abdominal-pelvic-computed tomography and pelvic magnetic resonance (MR) or endoanal ultrasound when pelvic MR was not clinically possible. Eligibility criteria consisted of treatment with long-course CRT and proctectomy. The indications for CRT were patients with locally advanced rectal cancer, as per the ESMO guidelines [24], including stage IV patients if they had oligometastatic disease susceptible of resection in a curative intent setting. Patients were excluded if presenting other histological types of rectal malignancy, if not submitted to CRT or proctectomy, if pregnant or if under 18 years old. Patients were subjected to long-course CRT consisting of a total dose of 50.4 Gy of pelvic irradiation, delivered with capecitabine or 5-fluorouracil, and subsequent proctectomy, 10 to 12 weeks later. Tissue samples were collected from all patients, prior to CRT, during pre-therapeutic colonoscopy, and after CRT, from the proctectomy specimens. Samples were directly frozen with CO_2 prior to storage at − 80 °C. Serum samples were also collected at the time of pre-treatment staging colonoscopy. For histopathological analysis, formalin-fixed paraffin-embedded tissue sections were routinely stained with haematoxylin–eosin and post-CRT samples graded by TRG according to the College of American Pathologist guidelines (CAP, TNM 7th edition) by two independent and blinded experienced pathologists. Samples were categorized as TRG0 or complete response (no viable tumour cells), TRG1 or moderate score (single cells or small groups of cancer cells), TRG2 or minimal response (residual cancer outgrown by fibrosis) or TRG3 or poor response (minimal or no tumour killing with extensive residual cancer). Subsequently, two groups of patients were defined: pathological responders (TRG0, 1 and 2; $n = 18$) and pathological non-responders (TRG3; $n = 15$). Patients had up to 47 months of follow-up. Sample collection was carried out after written and signed consent. The study protocol was in accordance with the ethical guidelines of the 1975 Declaration

of Helsinki and was approved by the institutional Human Research Committee and Ethical Committee from Hospital Beatriz Ângelo.

Serum hormone levels

Pre-CRT serum levels of adiponectin and leptin were measured in single determination using specific enzyme-linked immunosorbent assay (ELISA) kits (Mediagnost GmbH, Reutlingen, Germany) according to the manufacturer's instructions. To assess how adiponectin and leptin levels associated with future response to CRT and clinical outcomes, their pre-CRT serum levels were correlated with TGR status, positive extramesorectal nodes (EMN) and extramural vascular invasion (EMVI) and mRNA expression of stemness Yamanaka factors evaluated in pre-CRT tumoral biopsy tissue.

Total RNA extraction and quantitative real-time PCR

For gene expression analysis, total RNA was isolated from fresh-frozen pre-CRT rectal tumour biopsies using TRIzol® reagent (Invitrogen, Thermo Fisher Scientific, Paisley, UK) and reverse-transcribed to cDNA using NZY First-Strand cDNA Synthesis Kit (NZYTech, Lisbon, Portugal). Quantitative real-time PCR (qRT-PCR) was performed on 384-well QS7 Flex Real-Time PCR System (Applied BioSystems, MA, USA) using SensiFAST SYBR Hi-ROX Kit (Bioline, Meridian Bioscience, OH, USA). Primer sequences are listed in Table S1. Gene expression was quantified using the relative standard curve method and normalized to β-actin levels. All procedures were performed according to the manufacturer's instructions. Besides correlation with pre-CRT serum adipokines levels, mRNA expression of Yamanaka factors (*cMYC*, Kruppel-like factor 4 (*KFL4*), octamer-binding transcription factor 4 (*OCT4*) and sex-determining region Y-box 2 (*SOX2*)) was correlated with TRG status and prognostic features (ENM and EMVI).

Total protein isolation and immunoblotting

Total protein extracts were isolated from pre-CRT rectal tumour biopsies. For this purpose, samples were homogenized in lysis buffer containing 10 mM Tris–HCl (pH 7.6), 2.5 mM MgCl2, 0.75 mM KAc, 0.5% Nonidet P-40 (VWR, Pennsylvania, USA), 1 mM dithiothreitol (DTT) and 1 × Halt Protease and Phosphatase Inhibitor Cocktail (Thermo Fisher Scientific) for at least 30 min. Protein lysates were sonicated and then centrifuged at 10,000 × *g*, for 10 min at 4 °C. Protein concentrations were determined using Bio-Rad Protein Assay reagent (Bio-Rad, CA, USA), according to the manufacturer's instructions. Total protein extracts were stored at − 80 °C. To evaluate steady-state protein expression, immunoblot analysis was performed. Briefly, 40 µg of total protein was denatured and separated on 8% sodium dodecyl sulphate polyacrylamide electrophoresis gels (SDS-PAGE) and transferred onto nitrocellulose membranes (RTA Transfer Kit, Bio-Rad) using Trans-Blot Turbo Transfer System (Bio-Rad). After blocking with 5% (w/v) no-fat dry milk in PBS-tween-20 (0.5% v/v), blots were incubated overnight at 4 °C with primary mouse antibody reactive to signal transducer and activator of transcription 3 (STAT3) (124H6; 1:1000; #9139 T; Cell Signaling Technology Inc., MA, USA) or primary rabbit antibody reactive to phosphorylated STAT3 (p-STAT3) (Tyr705; 1:2000; #9145 T; Cell Signaling Technology Inc.). Next, membranes were incubated with horseradish peroxidase-conjugated anti-mouse or anti-rabbit secondary antibodies (all 1:5000; Bio-Rad) for 2 h at room temperature. Lastly, proteins of interest were detected by chemiluminescence using Immobilon Western Chemiluminescent HRP Substrate (Millipore, MA, USA) or SuperSignalTM West Femto Maximum Sensitivity Substrate (Thermo Fischer Scientific) and acquired with a ChemiDoc XRS-imaging system (Bio-Rad). β-actin (1:40,000; #A5441; Sigma-Aldrich) was used as loading control. The relative density of protein bands was analysed with Image Lab version 5.0 densitometric analysis software (Bio-Rad).

Statistical analysis

All data and statistical analysis were carried out with GraphPad Prism 8.4.2 software (California, USA) and IBM SPSS Statistics version 27 (IBM, NY, USA). Normality of value distribution was determined using the Shapiro–Wilk test. Differences in frequencies between responders and non-responders were evaluated using the Qui-square test. Differences in median values (expressed as minimum–maximum values) between responders and non-responders were evaluated with the unpaired t-test or Mann–Whitney U test, according to the normality of value distribution. Two-tailed Spearman correlations were used to correlate hormone serum levels and mRNA expression. Uni-parameter receiver operating characteristic (ROC) curve analysis was used to assess discriminatory power between TRG0 2 and TRG3. Variables with area under the ROC curve (AUROC) superior to 0.6 were selected for binomial logistic regression analysis, and multi-parameter ROC curves were established for relevant parameter combinations. AUROC value superior to 0.8 was considered very good. Cutoff values were retrieved from the Youden Index. Associated sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) were estimated using www.medcalc.org online calculator. A *p*-value inferior to 0.05 was considered significant. GraphPad Prism was used for unpaired t-test, Mann–Whitney U test, Spearman correlation, AUROC analysis and graph production. SPSS was

used for descriptive statistics, Qui-square test and binomial logistic regression.

Results

Patient clinical parameters

Clinical and demographic features of the 33 rectal cancer patients according to their TRG status are summarized in Table 1. Pre-CRT CEA levels were significantly higher in pathological non-responders (TRG3) than in responders (TRG0-2) ($p < 0.05$).

Opposing role of adiponectin and leptin serum levels in clinical prognosis

Although not significant, pre-CRT adiponectin serum values were inversely associated with BMI (Spearman r = −0.21, $p = 0.24$) (Fig. 1A). There were no significant differences in pre-CRT median serum levels of adiponectin between TRG0-2 (8.78 (4.56–13.96) µg/mL) and TRG3 (7.88 (2.26–21.79) µg/mL) patients ($p = 0.64$; Fig. 1B and Fig. S1). However, there was a non-significant increase in adiponectin median levels in patients with negative EMN (9.13 (2.50–21.79) µg/mL) versus positive EMN (6.81 (2.26–20.34) µg/mL) ($p = 0.11$; Fig. 1C and Fig. S1) and in patients without EMVI (8.77 (2.50–21.79) µg/mL) versus those with EMVI (7.36 (2.26–15.13) µg/mL) ($p = 0.59$; Fig. 1D and Fig. S1). Also, higher pre-CRT serum adiponectin levels were associated with lower mRNA expression of stemness Yamanaka factors *OCT4* ($p < 0.05$), *KFL4* and *cMYC* (both $p = 0.08$) in tumour tissue (Fig. 1E).

Conversely, pre-CRT leptin serum levels were positively correlated with BMI (Spearman r = 0.55, $p < 0.001$) (Fig. 2A). In addition, although median pre-CRT serum levels of leptin did not significantly change between TRG0-2 (4.37 (0.42–38.76) ng/mL) and TRG3 patients (2.92 (0.75–24.34) ng/mL) ($p = 0.99$; Fig. 2B and Fig. S1), a non-significant increase in serum leptin levels was observed in patients with positive EMN (11.02 (0.42–38.76) ng/mL) versus negative EMN (2.89 (0.44–24.34) ng/mL) ($p = 0.06$; Fig. 2C and Fig. S1) and again in patients with EMVI (5.80 (0.75–38.76) ng/mL) versus those without EMVI (2.86 (0.42–24.34) ng/mL) ($p = 0.23$; Fig. 2D and Fig. S1). Additionally, a significant positive correlation was found between serum leptin and *OCT4* mRNA levels in tumour tissue ($p < 0.05$; Fig. 2E).

Increased expression of stemness-related genes and STAT3 activation associate with a poor prognosis

Although not significant, mRNA expression of the Yamanaka factors, *cMYC*, *KLF4*, *OCT4* and *SOX2*, were increase in TRG3 patients (Fig. 3A), patients with positive EMN (Fig. 3B) and patients with EMVI (Fig. 3C). STAT3 phosphorylation was slightly increased in TRG3 patients (Fig. 4A), with positive EMN (Fig. 4B) and EMVI (Fig. 4C), although not significant. Additionally, although no correlations were found neither with leptin serum levels nor with patient survival (data not shown), STAT3 activation increased with mRNA expression of Yamanaka factors (Fig. 4D). Thus, STAT3 phosphorylation may contribute to worse prognosis and poor response to CRT.

STAT3 activation, OCT4 and KFL4 in tumour tissue are biomarkers for CRT response

Since STAT3 activation and *OCT4*/*KFL4* mRNA expression protruded in the TRG0-2 versus TRG3 comparison, their performance as biomarkers for the identification of pathological non-responders was evaluated and compared to that of pre-CRT CEA serum levels, the only serological parameter with significant differences between responders and non-responders. ROC curve analysis was carried out (Table 2), and AUROC values were assessed for each parameter individually (Fig. 5). In the identification of pathological non-responders, pre-CRT CEA serum levels presented an AUROC value of 0.78 ($p = 0.02$), with an overall accuracy of 80% and positive predictive value (PPV) of 70%. STAT3 activation ($p = 0.14$), *OCT4* ($p = 0.34$) and *KLF4* ($p = 0.18$) expression levels presented average AUROC values between 0.62 and 0.68. Surprisingly, STAT3 activation levels presented specificity and PPV values of 100% in the identification of non-responders to CRT (Table 2). Still, none of these parameters could produce a satisfactory distinction between pathological responders and non-responders. However, when all parameters were combined in a four-biomarker panel, the AUROC value increased to 0.8 ($p = 0.01$), with an overall accuracy of 80% and very high specificity and PPV values of 93.8% and 83.3%, respectively, for the identification of non-responders (Table 2).

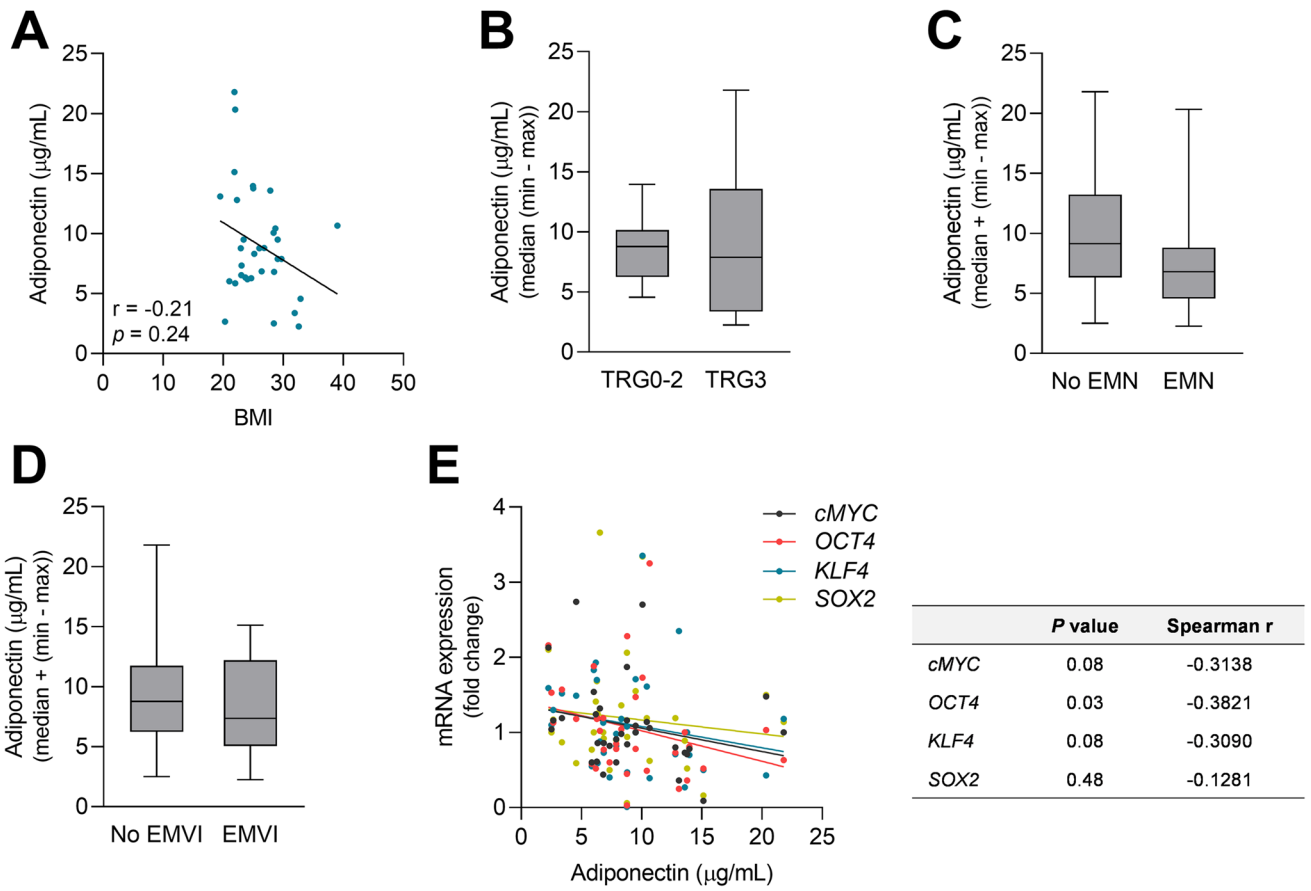
Discussion

In this study, we explored the role of adipokines on rectal cancer stemness, impact on clinical outcome and predictive response to CRT. In that regard, we analysed pre-CRT serum and biopsy tissue samples from a small cohort of rectal cancer patients. Correlations between adipokines, mRNA expression of stemness factors, prognosis factors and response to CRT were explored. This patient cohort includes 16 non-obese rectal cancer patients, 13 overweight, three obese and one severely obese, averaging a median BMI of 25. Nevertheless, the opposed roles of adiponectin and leptin were still observed. Indeed, our results showed that lower

Table 1 Patient clinical and demographic parameters

Parameter	TRG0-2	TRG3
n	18	15
Age (years), median (min–max)	62.5 (48–81)	61.0 (42–88)
Gender, *n* (%)		
Male	11 (61.0%)	11 (73.3%)
Female	7 (39.0%)	4 (21.8%)
BMI (kg/m^2), median (min–max)	25 (21–33)	25 (20–39)
Pre-CRT CEA (mg/mL), median (min–max)	0.70 (0.50–28.30)*	3.50 (0.50–96.20)
ASA score, *n* (%)		
I	0 (0.0%)	0 (0.0%)
II	11 (61.1%)	9 (60.0%)
III	4 (22.2%)	6 (40.0%)
IV	0 (0.0%)	0 (0.0%)
Not discriminated	3 (16.7%)	0 (0.0%)
Tumour grade, *n* (%)		
G1/G2	12 (66.7%)	13 (86.7%)
G3/G4	2 (11.1%)	1 (6.7%)
Not discriminated/determinable	4 (22.2%)	1 (6.7%)
Tumour location, *n* (%)		
1/3 superior	1 (5.6%)	0 (0.0%)
1/3 medium	6 (33.3%)	6 (40.0%)
1/3 inferior	11 (61.1%)	9 (60.0%)
Tumour extension (mm), median (min–max)	50 (19–90)	60 (30–80)
Distance to anal verge (mm), median (min–max)	45 (0–100)	60 (0–90)
cT, *n* (%)		
1	0 (0.0%)	0 (0.0%)
2	5 (27.8%)	1 (6.7%)
3	10 (55.6%)	13 (86.7%)
4	3 (16.7%)	1 (6.7%)
cN, *n* (%)		
0	1 (5.6%)	2 (13.3%)
1	17 (94.4%)	13 (86.7%)
cM, *n* (%)		
0	17 (94.4%)	14 (93.3%)
1	1 (5.6%)	1 (6.7%)
cStage, *n* (%)		
I	0 (0.0%)	0 (0.0%)
II	0 (0.0%)	2 (13.3%)
III	17 (94.4%)	12 (80.0%)
IV	1 (5.6%)	1 (6.7%)
CRM, *n* (%)		
Free	9 (50.0%)	7 (46.7%)
Threatened or invaded	9 (50.0%)	8 (53.3%)
Extramesorectal nodes		
Positive	7 (39.0%)	4 (21.8%)
Negative	11 (61.0%)	11 (73.3%)
EMVI, *n* (%)		
Positive	12 (66.7%)	9 (60.0%)
Negative	6 (33.3%)	6 (40.0%)
CRT, *n* (%)		
5-Fluorouracil based	0 (0.0%)	1 (6.7%)
Capecitabine based	18 (100%)	14 (93.3%)
Weeks post-CRT, median (min–max)	12 (9–37)	15 (11–45)

TRG determined according to the College of American Pathologists guidelines. Values expressed as median (minimum–maximum) values. *ASA*, American Society of Anaesthesiologists; *BMI*, body-mass index; *CEA*, carcinoembryonic antigen; *CRM*, circumferential resection margin; *CRT*, chemoradiotherapy; *EMVI*, extramural vascular invasion; *M*, metastasis; *N*, lymph node spread; *T*, tumour size; *TRG*, tumour regression grade. *$p < 0.05$



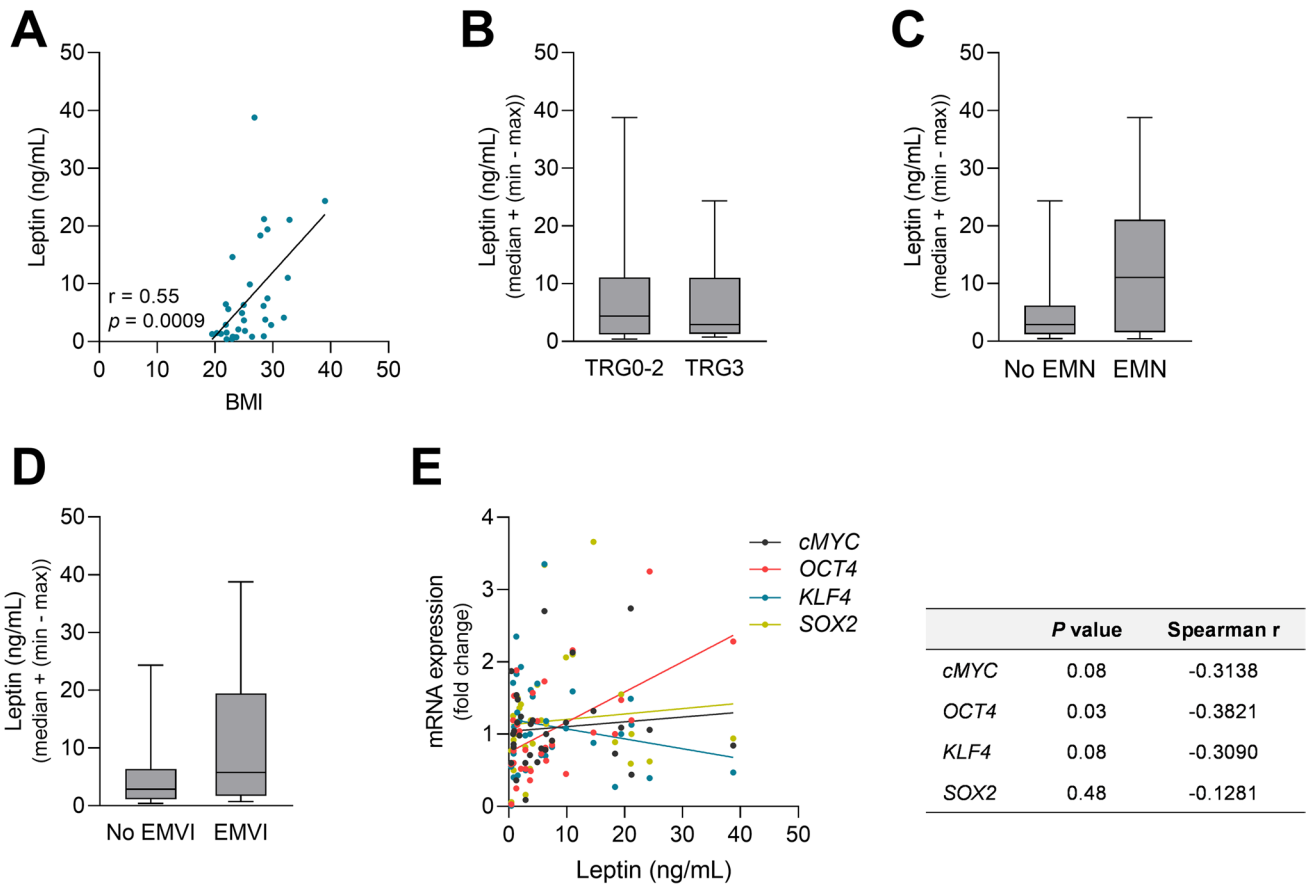
	P value	Spearman r
cMYC	0.08	-0.3138
OCT4	0.03	-0.3821
KLF4	0.08	-0.3090
SOX2	0.48	-0.1281

Fig. 1 Higher levels of pre-CRT serum adiponectin correlate with negative EMN, absence of EMVI and reduced stemness potential in pre-CRT tumour biopsies. **A** Correlation of adiponectin serum levels with BMI. Adiponectin serum levels in **B** pathological responders (TRG0-2; $n=18$) and pathological non-responders (TRG3; $n=15$); rectal cancer patients with and without **C** positive EMN (no EMN, $n=22$; EMN, $n=11$) and **D** EMVI (no EMVI, $n=21$; EMVI, $n=12$). **E** Correlation of adiponectin serum levels with mRNA expression of Yamanaka factors in pre-CRT tumour tissue biopsies (*cMYC*, *OCT4* and *KLF4*, $n=33$; *SOX2*, $n=32$). Data are expressed as median (minimum–maximum) values. BMI, body mass index; EMN, extramesorectal nodes; EMVI, extramural vascular invasion; KFL4, Kruppel-like factor 4; OCT4, octamer-binding transcription factor 4; SOX2, sex determining region Y-box 2; TRG, tumour regression rate

levels of serum adiponectin tended to associate with both the presence of EMVI, a prognostic factor for distance relapse and the presence of positive EMN, an indicator of advanced disease stage and prognostic factor for metastization. In agreement, both in vivo and in vitro studies have suggested a protective role of adiponectin [17, 19, 31, 39], and several clinical studies suggest that decreased levels of circulating adiponectin may be an increased risk factor for colorectal cancer development [11, 36, 45].

In turn, patients with positive EMN and EMVI presented slightly increased leptin serum levels, which correlated with mRNA expression of Yamanaka factors. In fact, several studies have demonstrated an oncogenic role of leptin, by promoting cancer cell growth, motility and invasion [2, 3, 29]. It has been shown a gradual increase in leptin levels during the normal mucosa–adenoma–adenocarcinoma sequence [48], and both leptin and leptin receptor were increased in colorectal tumour tissues when compared to adjacent normal tissues [1]. Still, our results showed that neither adiponectin nor leptin serum levels associated with CRT response. Nonetheless, the observed association between these adipokines and stemness-associated genes prompted us to explore further links that could influence response to CRT.

Several reports have shown that leptin can activate signalling pathways linked to colorectal CSCs and critical for the maintenance of stemness traits [15, 43, 50, 60]. The involvement of leptin in the JAK/STAT3 axis in colorectal cancer development is of particular interest. Upon leptin binding to its cellular membrane receptor, JAK-2 is phosphorylated and prompts STAT3 phosphorylation and translocation into the nucleus, where it regulates gene expression by binding to promoter regions of target genes [18, 41, 56]. In particular, STAT3 activation has been described to induce the expression of the stemness factors *OCT4* and *SOX2*, which in turn would induce the expression of leptin receptor (Ob-R) [16]. Moreover, increased phosphorylation of Ob-R, JAK-2 and STAT3



	P value	Spearman r
cMYC	0.08	-0.3138
OCT4	0.03	-0.3821
KLF4	0.08	-0.3090
SOX2	0.48	-0.1281

Fig. 2 Higher levels of pre-CRT serum leptin correlate with positive EMN, EMVI and increased stemness potential in pre-CRT tumour biopsies. **A** Correlation of leptin serum levels with BMI. Leptin serum levels in **B** pathological responders (TRG0-2; $n=18$) and pathological non-responders (TRG3; $n=15$); rectal cancer patients with and without **C** positive EMN (no EMN, $n=22$; EMN, $n=11$) and **D** EMVI (no EMVI, $n=21$; EMVI, $n=12$). **E** Correlation of leptin serum levels with mRNA expression of Yamanaka factors in pre-CRT tumour tissue biopsies (all $n=33$). Data are expressed as median (minimum–maximum) values. BMI, body mass index; EMN, extramesorectal nodes; EMVI, extramural vascular invasion; KFL4, Kruppel-like factor 4; OCT4, octamer-binding transcription factor 4; SOX2, sex determining region Y-box 2; TRG, tumour regression rate

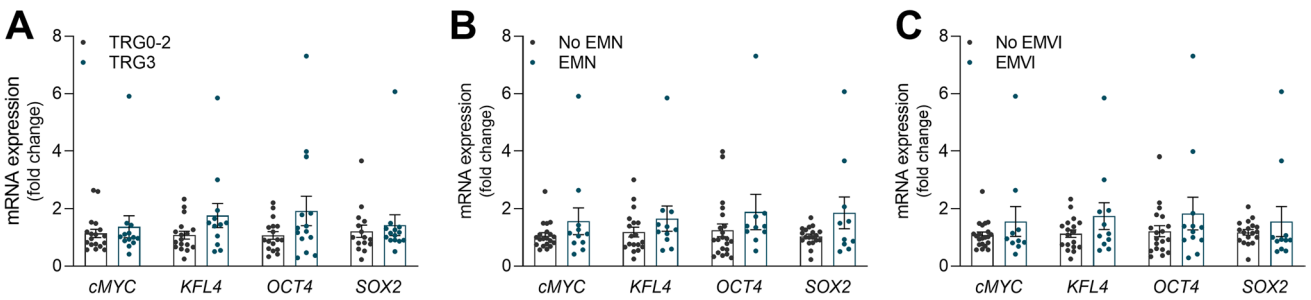
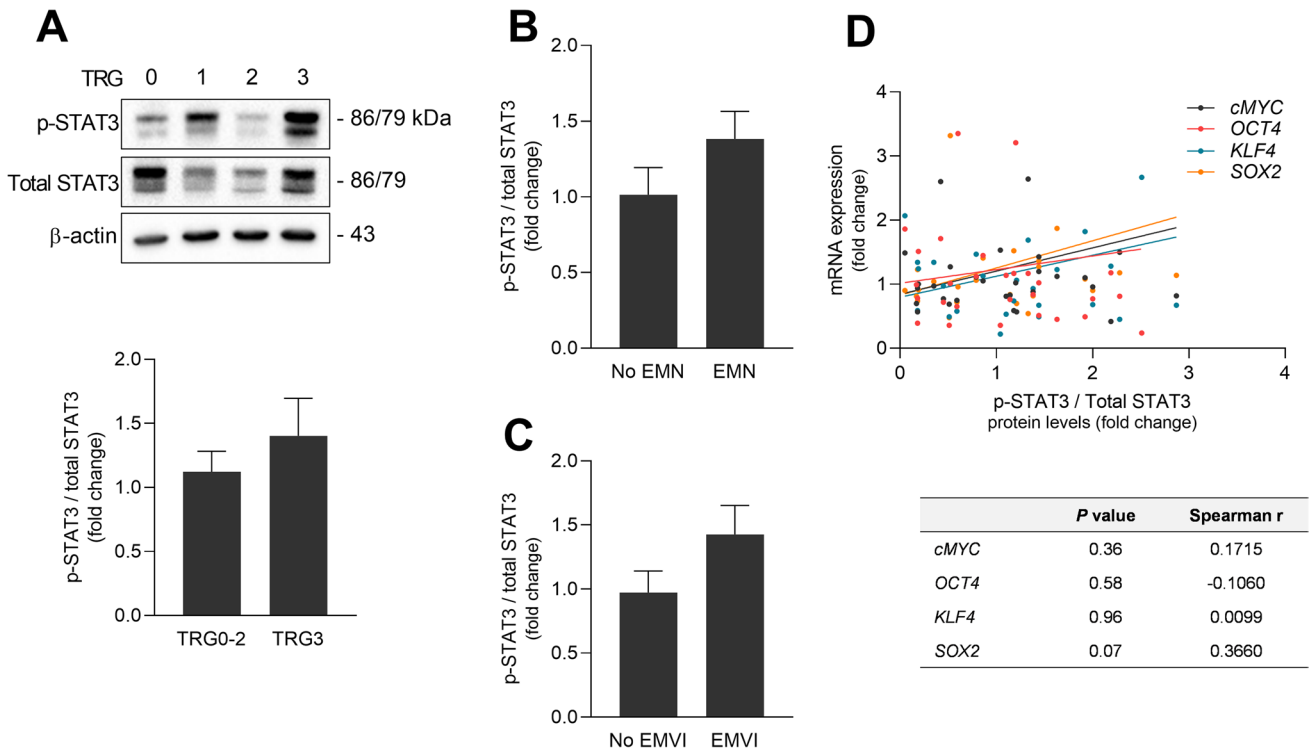


Fig. 3 Increased expression of Yamanaka factors in pre-CRT tumour biopsy tissues associates with poor prognostic factors and lower patient survival. mRNA expression of Yamanaka factors *cMYC*, *KLF4*, *OCT4* and *SOX2* in rectal cancer patients according to **A** TRG (TRG0-2, $n=15$–18; TRG3, $n=12$–14); **B** presence of positive EMN (no EMN, $n=18$–21; EMN, $n=10$–11) and **C** presence of EMVI (no EMVI, $n=18$–21; EMVI, $n=10$–12). mRNA expression data are expressed as mean ± standard error of the mean (SEM) of individual fold change to median. EMN, extramesorectal nodes; EMVI, extramural vascular invasion; KFL4, Kruppel-like factor 4; OCT4, octamer-binding transcription factor 4; SOX2, sex determining region Y-box 2; TRG, tumour regression rate



	P value	Spearman r
cMYC	0.36	0.1715
OCT4	0.58	-0.1060
KLF4	0.96	0.0099
SOX2	0.07	0.3660

Fig. 4 Increased STAT3 activation in pre-CRT tumour biopsy tissues associates with poor prognostic features and increased mRNA expression of Yamanaka factors. **A** Immunoblotting and densitometry of p-STAT3 and STAT3, in pre-CRT tumour biopsy tissues according to TRG (TRG0-2, $n=18$; TRG3, $n=14$). STAT3 activation according to presence of **B** positive EMN (no EMN, $n=22$; EMN, $n=10$) and **C** EMVI (no EMVI, $n=21$; EMVI, $N=11$). **D** Correlation between STAT3 activation and mRNA expression of Yamanaka factors in pre-CRT tumour biopsy tissues. Blots of p-STAT3 were normalized to total STAT3. Data are expressed as mean ± SEM. EMN, extramesorectal ganglia; EMVI, extramural vascular invasion; KFL4, Kruppel-like factor 4; OCT4, octamer-binding transcription factor 4; SOX2, sex determining region Y-box 2; STAT3, signal transducer and activator of transcription 3; TRG, tumour regression rate

Table 2 Performance of CEA levels, STAT3 activation, *OCT4* and *KLF4* mRNA expression, alone and in combination panel in the identification of non-responders to CRT

	CEA	p-STAT3/total STAT3	*OCT4*	*KLF4*	Combination panel
Cut-off	> 1.95 mg/mL	> 2.095	> 0.795	> 1.235	NA
AUROC (95% CI)	0.78 (0.59–0.98)	0.68 (0.43–0.93)	0.62 (0.37–0.87)	0.66 (0.41–0.91)	0.80 (0.61–0.98)
P value	0.02	0.14	0.34	0.18	0.01
Sensitivity (95% CI)	77.8 (40.0–97.2)	44.4 (13.7–78.8)	77.7 (40.0–97.2)	66.7 (29.9–92.5)	55.6 (21.2–86.3)
Specificity (95% CI)	81.3 (54.4–96.0)	100.0 (79.4–100.0)	62.5 (35.4–84.8)	81.3 (54.4–95.6)	93.8 (69.8–99.8)
Accuracy (95% CI)	80.0 (59.3–93.2)	80.0 (59.3–93.2)	68.0 (46.5–85.1)	76.0 (54.9–90.6)	80.0 (59.3–93.2)
PPV (95% CI)	70.0 (44.3–87.3)	100 (NA)	53.6 (36.2–70.6)	66.7 (39.5–86.0)	83.3 (40.7–97.3)
NPV (95% CI)	86.7 (65.2–95.8)	76.2 (64.1–85.2)	83.3 (58.2–94.7)	81.3 (62.6–91.8)	79.0 (64.1–88.7)
TP	7	4	7	6	5
TN	13	16	10	13	15
FP	3	0	6	3	1
FN	2	5	2	2	4

AUROC, area under the ROC curve; *CEA*, carcinoembryonic antigen; *CI*, confidence interval; *FN*, false negative; *FP*, false positive; *KLF4*, Kruppel-like factor 4; *NA*, not applicable; *NPV*, negative predictive value; *PPV*, positive predictive value; *OCT4*, octamer-binding transcription factor 4; *STAT3*, signal transducer and activator of transcription 3; *TN*, true negative; *TP*, true positive

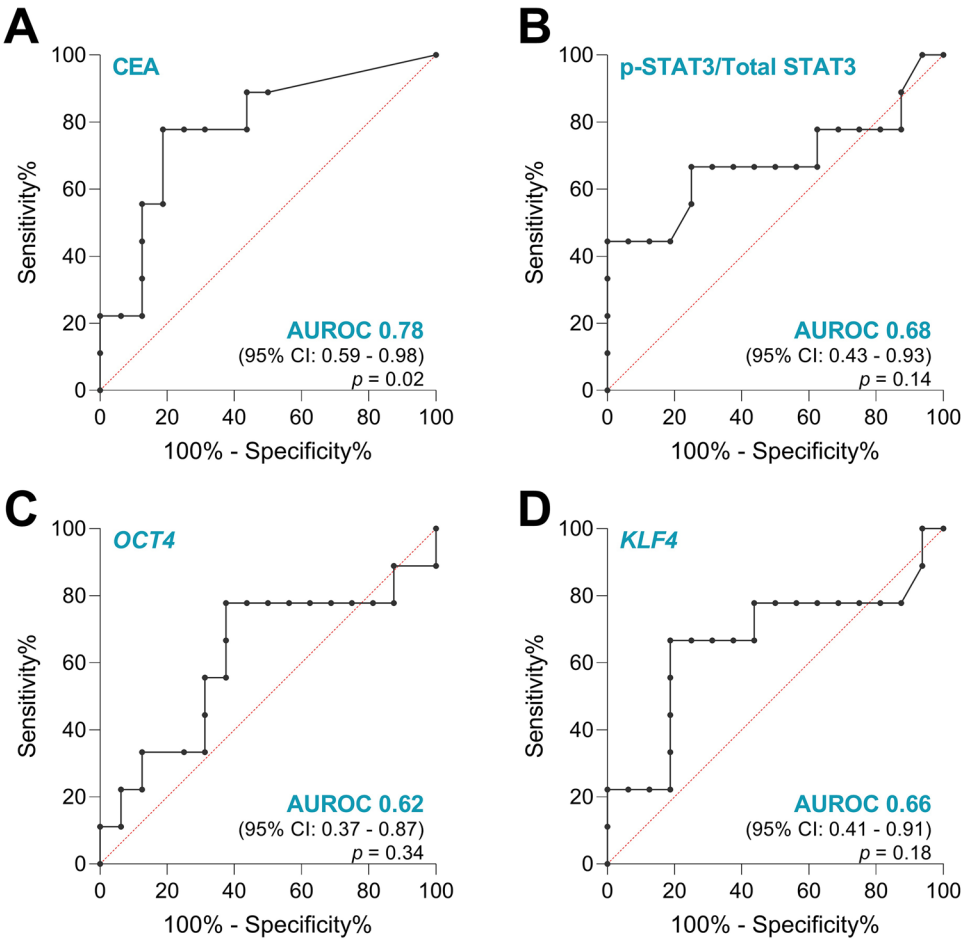


Fig. 5 ROC curves for pre-CRT CEA serum levels (**A**), STAT3 activation (**B**) and mRNA expression of *OCT4* (**C**) and *KLF4* (**D**) as prognostic biomarkers for the identification of pathological non-responders. p-STAT3/STAT3 was evaluated by immunoblotting and *OCT4* and *KFL4* mRNA expression evaluated by qRT-PCR in tissues from pre-CRT tumour biopsies. AUROC, area under the ROC curve; CEA, carcinoembryonic antigen; CI, confidence interval; KLF4, Kruppel-like factor 4; OCT4, octamer-binding transcription factor 4; STAT3, signal transducer and activator of transcription 3

was observed in both colorectal adenoma and carcinoma tissues when compared to normal colorectal tissues [57].

Here, we have observed that despite no significant changes in serum leptin levels, STAT3 activation and mRNA expression of *OCT4* and *KLF4* were slightly increased in non-responders (TRG3) and in patients with EMVI and with positive EMN. Previous reports showed that increased *OCT4* expression in rectal cancer tumour tissues was associated with more severe disease stages and with poor disease-free survival [51, 62]. Similarly, a clinical study has demonstrated that *KLF4* and B lymphoma Mo-MLV insertion region 1 homolog (*BMI1*) levels were positively correlated in rectal cancer and associated with poor therapeutical response. Further in vitro studies showed that *BMI1* deficiency enhanced radiosensitivity, which could be overcome by *KLF4* overexpression [27].

Contrary to our observations, a retrospective study where the prognostic value of phosphorylated STAT3 in rectal cancer patients was evaluated did not find any correlations with clinicopathological features and proposed an improved overall survival for patients with p-STAT3 positive tumours. Nevertheless, albeit having a fairly large patient cohort, this study was based on immunohistochemistry of formalin-fixed paraffin-embedded samples, and their analysis was limited to the 37.5% of p-STAT3-positive samples [40].

Here, we observed that STAT3 activation by immunoblotting was increased in non-responders (TRG3). More recent in vitro and in vivo studies in colorectal cancer models have shown that silencing or de-phosphorylation of STAT3 could suppress cell proliferation and tumour growth [33, 35, 63]. Moreover, it has been demonstrated that STAT3 protein expression positively correlated with CRT resistance in vitro and that STAT3 inhibition in vivo sensitized mice to CRT and delayed tumour regrowth [54]. Hence, STAT3 activation and mRNA expression levels of *KLF4* and *OCT4* seemed plausible biomarker candidates to stratify CRT response. We found that those markers presented moderated AUROC values between 0.62 and 0.68, and overall accuracies in identifying non-responders ranged between 68 and 80% and would benefit from improved sensitivity.

To date, there are no truly validated biomarkers of response to CRT. CEA, as the most widely used tumour marker in colorectal cancer, might be a potential candidate. Here, pre-CRT serum levels alone allowed the distinction between responders and non-responders with an AUROC of

0.78. However, studies have demonstrated that serum CEA is not sensitive enough to be used alone as a biomarker, and its potential has been explored in association with tumour size ratio or by monitorization of its clearance pattern [7, 20, 28]. Moreover, CEA can be affected by smoking status and is more efficient as an adjunct for the assessment of complete response rather than identifying non-responders [28, 59]. Hence, we combined CEA levels with STAT3 activation and *OCT4* and *KFL4* expression levels as a multi-parameter biomarker panel. This strategy allowed to increase the AUROC value to 0.8, with excellent specificity and PPV values of 93.8% and 83.3%.

Other approaches in the literature have identified vascular endothelial growth factor (VEGF) expression or miRNA signatures with AUROC values proximal to 0.6, however focusing on the identification of complete responders [8, 9, 47, 61], while a 13-gene signature with 76% accuracy was proposed for the identification of non-responders [10]. Besides focusing more on the identification of complete responders (TRG0) rather than non-responders (TRG3), most of these studies encompass patients with heterogeneous treatment schedules. Importantly, our study only included patients that followed the same therapeutic approach and scheduling and benefited from access to fresh frozen biopsy samples. However, some limitations are still recognized and shared with other studies, such as the small cohort size from a single hospital centre and the non-randomized retrospective study design in need of prospective validation, with the inclusion of obese patients, whose adipokine levels might be dramatically deregulated. Mechanistic studies of the leptin/STAT3 signalling may clarify its influence on response to CRT, and the role of tissue leptin and its receptor Ob-R still deserve further investigation.

Conclusion

We highlight the role of adipokines on rectal cancer patient prognosis. By exploring the leptin/STAT3 axis and its association with stemness potential in rectal cancer patients, we showed that CEA levels together with STAT3 activation and *OCT4* and *KLF4* mRNA expression levels can be valuable predictors of CRT response and potentially identify non-responders, sparing them from unbeneficial treatment and associated side effects.

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Author contribution VM performed experimental work and statistical analysis and wrote the manuscript. SO collected patient samples, curated clinical data and revised the manuscript. MBA contributed to manuscript writing. CMPR was responsible for funding and revised the manuscript. All authors contributed to the experimental design, revision and approval of the final manuscript. The authors declare that all data were generated in-house and that no paper mill was used.

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Data availability The data that support the current study are available within the article, supplementary materials and from the corresponding author upon reasonable request.

Declarations

Ethics approval The study was conducted in accordance with the Declaration of Helsinki and approved by the institution's Ethical Committee (Comissão de Ética para a Saúde do Hospital Beatriz Ângelo, Project Identification Number 0240) on 13 March 2017. The study was registered in the Portuguese Data Protection Agency (Comissão Nacional de Protecção de Dados) on 27 January 2017.

Informed consent Informed consent was obtained from all subjects involved in the study.

Competing interests The authors declare no competing interests.

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