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A diabetes mellitus és szövődményeinek, valamint a májbetegségek etiológiai és genetikai tényezőinek vizsgálata
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Investigation of paraneoplastic thrombocytosis in colorectal cancer patients with or without type 2 diabetes mellitus

Ph.D. thesis

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List of abbreviations

AJCC	American Joint Committee on Cancer
CrI	Credible interval
CD	Cluster of differentiation
CD40L	CD40 ligand
CI	Confidence interval
CRC	Colorectal cancer
DSS	Disease-specific survival
ECLIA	Electrochemiluminescence immunoassay
ELISA	Enzyme-linked immunosorbent assay
GITR	Glucocorticoid-induced tumor necrosis factor receptor family-related protein
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HbA_{1C}	Glycated hemoglobin
HR	Hazard ratio
IL-1	Interleukin-1
IL-3	Interleukin-3
IL-6	Interleukin-6
IL-11	Interleukin-11
LIF	Leukemia inhibitory factor
NK-cell	Natural killer cell
OS	Overall survival
T2DM	Type 2 diabetes mellitus
TNM	Tumor – Node – Metastasis
TPO	Thrombopoietin

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1. Introduction

1.1. Colorectal cancer

Colorectal cancer (CRC) is a malignant disease, one of the leading causes of mortality in the developed countries. The disease develops in an approximately 10 to 15-year period, usually due to the accumulation of either somatic (acquired) and/or germline (inherited) genetic mutations. CpG hypermethylation, chromosomal instability and mismatch repair are the most commonly occurring changes during the development of CRC. The following developmental stages are known, normal colonic/rectum epithelium, precancerous lesion and ultimately an invasive carcinoma. The importance of CRC is also shown by that it is the third most common type of cancer and second cause of death related to malignant diseases, causing approximately 10% of all tumor-related deaths (1) and with an estimated number of 1.8 million new cases and about 881,000 deaths worldwide per year in the recent years (2). In Hungary, there are about 10,500 new cases annually. Sadly, similar to those of the international trends, the number of new CRC cases shows a rising tendency in Hungary (**Figure 1**): over the past 20 years, it raised from ~8,000 to 10,500 per year (3).

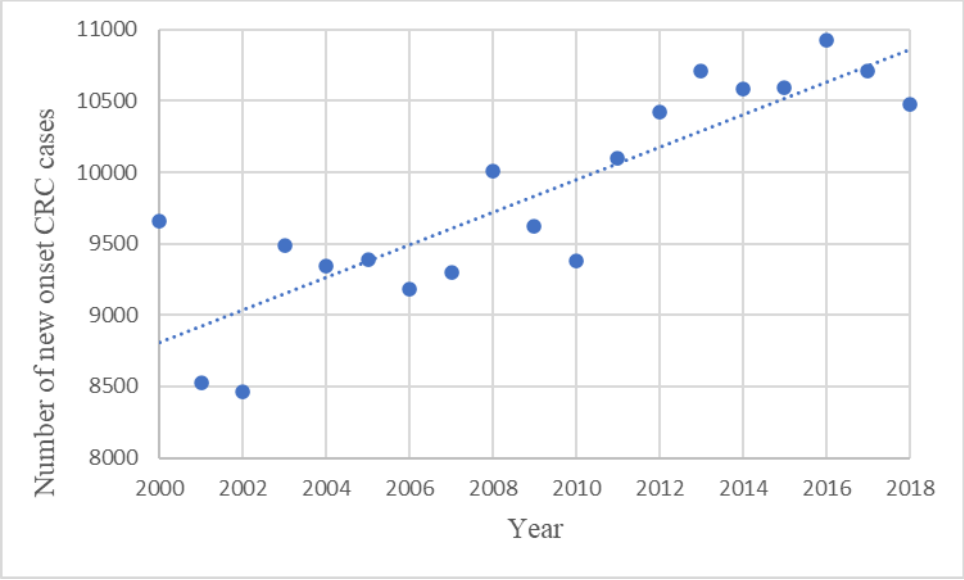

Figure 1: In Hungary between 2000 and 2018, an increasing trend could be observed in the new onset case numbers of colorectal cancer (CRC). Data obtained from National Institute of Oncology. National Cancer Registry: Cancer statistics reports for Hungary (3).

In the colon and rectum, the change of normal epithelial cells to adenocarcinoma follows a predictable progression of histological and concurrent genetic and epigenetic changes. *E.g.*, "in the 'classic' CRC formation model, the vast majority of cancers arise from a polyp originating from an aberrant crypt, which then evolves into an early adenoma (< 1 cm in size, with tubular or tubulovillous histology). The adenoma then progresses to an advanced adenoma (> 1 cm in size, and/or with villous histology), before finally becoming a colorectal cancer. This process is driven by accumulation of mutations and epigenetic alterations and takes 10–15 years to occur but can progress more rapidly in certain settings" (4). The disease is asymptomatic for most of its development; first symptoms are usually bleeding and bloody stools with or without abdominal pain, weight loss, and changes in stool habits. Occult bleeding and anemia of unknown cause can also be alarming symptoms. Colonoscopy followed by imaging studies is required for the diagnosis of CRC (5).

Depth of tumor growth, and the spreading of the disease can be characterized by staging. CRC is known to have several staging systems, of which the most known and used is the so-called Tumor – Node – Metastasis (TNM) system. T (local invasion depth) shows how far the tumor has grown through the bowel wall; N stands for nearby lymph nodes metastasis; while M (metastases) describes whether the cancer has spread (metastasized) to other parts of the body. A combination of T, N, and M status determines a patient's CRC staging as follows:

- T (tumor growth)
    - Tis: It has not grown beyond the (mucosa) of the colon or rectum.
    - T1: The cancer has grown through the muscularis mucosa into the submucosa.
    - T2: It have grown into the muscularis propria.
    - T3: The cancer has grown into the subserosa/serosa of the colon or rectum but has not gone through them.
    - T4a: The cancer has grown through the wall of the colon or rectum but has not grown into other nearby tissues or organs.
    - T4b: The cancer has grown through the wall of the colon or rectum and is attached to or has grown into other nearby tissues or organs.

- N (lymph nodes metastasis)
    - N0: It has not yet spread to nearby lymph nodes.
    - N1: It has spread to 1 to 3 nearby lymph nodes.
    - N2: cancer cells in four or more nearby lymph nodes
- M (distant metastases)
    - M0: It has not spread to distant sites.
    - M1a: It has spread to 1 distant organ or distant set of lymph nodes, but not to distant parts of the peritoneum.
    - M1b: It has spread to more than 1 distant organ or distant set of lymph nodes, but not to distant parts of the peritoneum.
    - M1c: It has spread to distant parts of the peritoneum.

The latest, 8th amended version of the TNM staging was published by the American Joint Committee on Cancer (AJCC) in 2018 (6). Stage I and II, III, and IV CRC are characterized by no distant/lymph node metastases, lymph node metastases, and distant metastases, respectively. In addition, other frequently used staging system are the Dukes (7) and the modified Astler – Coller staging systems (8) (**Table 1**).

Table 1: Dukes and modified Astler – Coller staging systems (7, 8).

Dukes staging system	
Dukes A	invasion into but not through the bowel wall
Dukes B	invasion through the bowel wall but not involving lymph nodes
Dukes C	involvement of lymph nodes
Dukes D	widespread metastases
Modified Astler – Coller staging system	
MAC A	limited to mucosa
MAC B1	extending into muscularis propria but not penetrating through it, nodes not involved
MAC B2	penetrating through muscularis propria, nodes not involved
MAC C1	extending into muscularis propria but not penetrating through it, nodes involved
MAC C2	penetrating through muscularis propria, nodes involved
MAC D	distant metastatic spread

Screening is highly effective in reducing the mortality from CRC. Screening procedures include fecal occult-blood tests, flexible sigmoidoscopy, and colonoscopy. One of these options should be offered to asymptomatic people aged 50 years or older.

Over the past two decades, the range of CRC screening modalities has expanded, and many population-based programs have been implemented. Worldwide, there are two major types of screenings: opportunistic and organized. In 2015, 24 out of 28 European Union countries had established or were preparing nationwide organized or opportunistic CRC screening program (9). Recommendation of screening may be different for certain subsets of patients (*i.e.*, the elderly, women, and ethnic minorities) (10). The most common limitation of the screening programs is the limited endoscopic capacity (11). Although there are some ongoing studies of the natural history of small polyps, evidence-based data will probably take 10 to 20 years to meaningfully translate into clinical-practice recommendations (12).

In Hungary, several pilot programs were conducted for colorectal cancer screening. In 1997–1998, the first Hungarian CRC screening pilot program was performed in Budapest (13). Later, in 2015, another CRC screening pilot program was conducted in Csongrád (14), and the latest one was started in 2019 (15), which was a nationwide complex screening program, in which the goal group was the 50-70 years old Hungarian residents. About 332,000 people had been invited with the possibility to have an occult blood test. About 100,000 tests were performed, of which 8,000 was positive, 3,093 colonoscopy was performed, and a total of 129 histological verified malignancy had been found (15).

CRC treatment can be divided into two major schemes (5). In non-advanced stages endoscopic– or surgical resection is the mainstay curative treatment, which may be followed by chemotherapy. Latest guidelines recommend chemotherapy in the case of poorly differentiated tumors, CRC with vascular, lymphatic or perineural tumor invasion, and in stage III CRC. In advanced stages, the reduction of the size of the tumor is the most important using (neoadjuvant) chemo(radio)therapy, with which the chance of complete resection is significantly higher (16). Usually, a cytotoxic doublet with a biological agent is administered as the first-line and second-line treatments, while irinotecan + cetuximab, panitumumab, and regorafenib or trifluridine/tipiracil are administered as third-line or above (17). Regular imaging and/or colonoscopic follow-up of patients is key in the early detection of tumor recurrence (16). Furthermore, tumor progression may be also detected if the following biomarkers' serum/plasma levels are

elevated: carcinoembryonic antigen, carbohydrate antigen 19-9, and cancer antigens 72-4 and 125 (5, 17, 18).

1.2. Paraneoplastic thrombocytosis and CRC

Thrombocytosis is defined as platelet counts above the upper value of normal range (usually $\geq 400 \times 10^9$/L). In addition to the general similarities, it has been observed that some tumor types have thrombocytosis for different degrees. In CRC, a relatively high proportion of patients have elevated platelet counts (19, 20), moreover, thrombocytosis is described as a poor prognostic sign in CRC. Both pre- and postoperative thrombocytosis is associated with worse patient survival (21, 22). Thrombocytosis may develop for several reasons, 1.) The bleeding of the tumor or 2.) a metabolic change caused by the tumor itself called paraneoplastic. Moreover, platelets may have further roles in CRC as well, most which are still under investigation. *E.g.*, platelets can interact with circulating tumor cells and form metastatic emboli together. Platelets may encapsulate tumor cells protecting them from natural killer cells. And platelets store proteases, growth factors, which promote tumor growth, neoangiogenesis and invasion (23, 24)

1.2.1. Tumor-induced thrombopoiesis

Although platelet production is regulated at several points, the most frequently evaluated and most widely known regulating factor is thrombopoietin (TPO). Elevated TPO levels were found in hepatoblastoma (25), hepatocellular carcinoma (26), ovarian cancer (27), and in CRC (28). In addition to TPO, other cytokines also play a role in the growth of megakaryocytes and the stimulation of platelet production. The most widely known ones are interleukins 1 (IL-1), –3 (IL-3), –6 (IL-6), and –11 (IL-11), leukemia inhibitory factor (LIF), granulocyte-macrophage colony-stimulating factor (GM-CSF), Fms-like tyrosine kinase receptor 3 ligand and fibroblast growth factor (24). Although IL-3 (29) and its receptor (30) contribute to the microvascularization of the tumor, no study is known that would have identified correlation between IL-3 level and tumor stage, prognosis and cancer-related thrombocytosis (24). On the contrary, IL-1 concentration was proposed to correlate with the size of gastrointestinal, esophageal (30), breast (31) and renal cell carcinoma (32), but the expression of IL-1 mRNA did not correlate with

the platelet count in tumor patients (24). In another study (33), elevated plasma granulocyte colony-stimulating factor and GM-CSF levels were observed in different solid tumors in addition to thrombocytosis.

Elevated IL-6 levels have been reported for several malignancies such as gastrointestinal (34), renal cell carcinoma (35), prostate cancer (36), epithelial ovarian cancer (36) and lung cancer (37). Tumor cells can express IL-6 directly (24) and it was found in c-Mpl-deficient mice – the animal model system for thrombocytopenia – that IL-3, IL-6, IL-11 and LIF can restore normal platelet count only in the presence of TPO (38, 39). The strong relationship between IL-6 and TPO was further strengthened by the results of Kaser *et al*. that in the c-Mpl-deficient mice, concomitantly with IL-6 induced thrombocytosis, the mRNA expression of TPO is also increased in the liver resulting in elevated plasma TPO levels (40). Human recombinant IL-6 was also found to accelerate the restoration of platelet count after chemotherapy in different solid tumors (41). Stone *et al.* examined ovarian cancer patients who had elevated platelet count. They found that in addition to TPO, the IL-6 level of patients correlated with the elevated platelet count as well. Based on their evaluation they proposed a paraneoplastic pathway, hence the name paraneoplastic thrombocytosis: the tumor generates an inflammatory microenvironment that also raises the circulating IL-6 level, which stimulates the hepatic TPO production that in turn stimulates megakaryocytes in the bone marrow. The process ultimately results in thrombocytosis (42).

1.2.2. Previous studies investigating paraneoplastic thrombocytosis in CRC

Sasaki *et al*. evaluated the data of 636 patients and confirmed that the elevated platelet count was associated with the presence of distant metastases (43). In another study, the pre- and 1-month postoperative platelet counts in 336 patients with different stages of CRC had been investigated. It was found that the platelet counts significantly decreased following tumor resection, compared to that of the preoperative levels (22). It has to be noted however, that there are only a handful studies that examined the pre- or postoperative thrombocytosis and its prognostic values. Moreover, the change of platelet counts during follow-up is even a less investigated area of research.

1.2.3. Platelets and metastatization

Platelets play a role in the intravascular and extravasation phase of metastatic progression (44). The relationship of platelets and the metastatic potential of tumor cells have been proved with experiments. Correlation was found between the inhibition (45) or depletion (46) of platelets and lower metastatization rate. In platelet-depleted animals the infusion of platelets restored the metastatization potential of intravenously administered tumor cells (47).

The adhesion of migrating tumor cells to platelets has been experimentally verified (48). Platelets can protect the tumor cells from natural killer cells (NK-cells) with immunomodulating proteins expressed on their surface (49), such as the glucocorticoid-induced tumor necrosis factor receptor family-related protein (GITR). Consequently, platelets containing the GITR ligand can protect the tumor cells (50). Furthermore, platelets express a large amount of major histocompatibility complex class I proteins, thus showing a false phenotype and interfering with the recognition of tumor cells by the immune system (50). Studies have shown that platelet-derived growth factor reduces in vitro NK-cell activity (51). Similarly, NK-cell cytotoxicity is ceased by platelet-derived transforming growth factor beta and interferon-γ (52).

During metastatization, the tumor cells have to be attached to the endothelial cells in order to extravasation of the tumor cells to occur. As shearing forces counteract adhesion (53), circulating tumor cells have to slow down as a first step. Stress caused by shearing forces is a well-known platelet-activating factor that considerably increases the adhesion of tumor cells to platelets (54). Glycoprotein IIb/IIIa on the surface of platelets and integrins on tumor cells take part in the adhesion (55). According to experiments, activated platelets attached to tumor cells slow down, and in this way, they facilitate their adhesion to the endothelium (56). In different tumors, metastatization could be decreased by the inhibition of activated platelets. *E.g.*, platelet-deficient NF-E2 (the transcription factor required to the formation of platelets from megakaryocytes) knockout mice are protected from hematogenous metastases (56). Furthermore, platelet depletion caused by the administration of antiplatelet serum decreases the incidence of pulmonary metastases in CRC, while metastases repeatedly appear following the administration of platelets (47).

1.3. The relationship between CRC and CD40 ligand

CD40 ligand (CD40L, synonym: CD154, gp39, or T-B activating molecule) is a type I transmembrane glycoprotein belonging to the tumor necrosis factor-family of cytokines (57, 58). CD40L activates CD40, a transmembrane protein receptor found on antigen-presenting cells (58). The majority of circulating CD40L is assumed to originate from platelets but a membrane-bound CD40L form is also known (58). CD40L has been described to have antitumor effects via the inhibition of tumor cell proliferation and pro-apoptotic features through the activation of apoptotic pathways (58-61). It has been found previously that CRC patients have significantly higher soluble CD40L level than those of healthy control patients, and its possible use as a promising biomarker in CRC was proposed (62-64). The connection between CD40L levels and lymph node involvement or distant metastasis is controversial. One study reported increased CD40L levels in patients with worse disease conditions, while others have found the opposite. Furthermore, neoadjuvant chemoradiotherapy has decreased CD40L levels (28, 62, 63). The role of CD40L in the course of CRC and its relationship with CRC-related thrombocytosis has not been investigated previously.

The CRC cell lines HCT116, Colo320, and Caco-2 have been shown to be positive for CD40, while SW480, HT29, Colo741, and LS174T do not express CD40 (59, 60, 65). The treatment of CD40-positive cell lines with interferon-γ can further increase the expression of CD40 (60). Analysis of resected tumor specimens has shown that approximately every third CRC is moderately or strongly CD40-postive (65). While in another study CD40- and CD40L-positivity has been observed in 79% and 56% of CRC patients, respectively (61). Ex vivo treatment of CD40-positive CRC cell lines with recombinant soluble human CD40L can inhibit tumor growth, induce apoptosis (60), inhibit CRC cell proliferation, stall CRC cells at the G0/G1 state, influence cell adhesion and metastasis, and increase aryl hydrocarbon receptor expression (61). While the T cell membrane bound CD40L can induce enhanced apoptosis of in vitro CRC cells with CD40-positivity (65), less signaling strength has been observed in the case of the soluble form of CD40L. Soluble CD40L can induce apoptosis only following specific pharmacological interventions (65, 66).

1.4. Type 2 diabetes mellitus

1.4.1. A short introduction to type 2 diabetes mellitus

Diabetes mellitus is one of the most prevalent diseases in our time: based on latest estimations – available in the 9th edition of IDF Diabetes Atlas (67) –, the incidence of the disease varies between 4 and 10.4%, with over 460 million diabetes patients around the world (67). It is a complex metabolic disorder with multifactorial etiology. Approximately 90% of the diabetes patients are suffering from type 2 diabetes mellitus (T2DM), which usually develops in later ages (67, 68).

T2DM is thought to be originating from insulin resistance, which leads to elevated insulin and glucose levels in blood, and ultimately to a subsequent beta cell dysfunction (69). As stated above, T2DM has multifactorial etiology, including genetic, epigenetic and lifestyle factors. *E.g.*, obesity, the lack of physical activity, and dietary irregularities (*i.e.*, excessive consumption of carbohydrates) can significantly increase the risk of T2DM development. The importance of genetic predisposition was also confirmed in T2DM by heritability estimates, twin studies, and genome-wide association studies (70). T2DM often develops from metabolic syndrome, which is a complex disease including obesity, dyslipidemia, insulin resistance, and hypertension (71, 72). The clinical relevance of T2DM is due to its high prevalence. Over the age of 60 years, T2DM may occur in every fourth-fifth person (67, 73). Moreover, the associated cardiovascular & other complications (such as nephropathy, neuropathy, retinopathy, etc.) significantly affects patients' quality of life (68, 73).

1.4.2. Type 2 diabetes mellitus and CRC

There are several known mechanisms and factors that have link T2DM and CRC together (74). *E.g.*, older age, the increased incidence related to lifestyle changes and obesity, hyperglycemia, hyperinsulinemia, insulin resistance, oxidative stress, dyslipidemia, and the impairment of insulin-like growth factor pathway(s) (75-78). Overweight is expected to increase cancer mortality with 14-20% (79), moreover, CRC may develop 20-50% more often in the obese (80). Higher levels of serum insulin-like growth factor 1 (IGF-1) are characteristic both for obese patients and insulin resistance, and higher levels of IGF-1 have also been shown in several tumor types (81). A strong

connection between hyperglycemia and the Warburg effect has been also suggested (78, 82).

Compared to the healthy population, an increased occurrence of malignancies is confirmed in T2DM (77, 78, 83). 8-18% of all cancer patients is estimated to suffer from co-existing T2DM, and CRC occurs approximately 1.5-times more often in patients with T2DM (84-86). The co-existence of T2DM is associated with an increased risk for shorter survival in CRC (87, 88). T2DM has been described to have a negative effect on overall-, cancer-specific-, disease-free-, and recurrence-free survival of CRC patients (89, 90).

1.4.3. Platelet irregularities in type 2 diabetes mellitus

Similar to CRC, T2DM can be also described by various platelet abnormalities (91, 92) and an increased TPO production is also known (92-94). The regulation of different signaling pathways of platelets is impaired in diabetes that ultimately results in increased activation, adhesion and aggregation of platelets (95). Platelet volume is somewhat increased in diabetes patients (96), which is associated with an increased reactivity (97). Increased adhesion, aggregation and synthesis of coagulation factors is characteristic for the platelets of T2DM patients. They bind to fibrinogen to a greater extent, and reendothelialization following fibrinolysis is associated with more prolonged and pronounced subendothelial fibrosis. All of these changes are thought to be due to non-enzymatic glycation (93). Glycation may occur directly on the platelet membrane, damaging surface glycoproteins responsible for adhesion, or on membrane-forming lipids by binding to glycoproteins and reducing membrane fluidity (93). In addition to chronically high blood glucose levels, hypoglycemia may also affect platelet function in those with diabetes mellitus. In these cases, higher von Willebrand factor levels have been measured, similarly, with increases platelet activation. Based on *ex vivo* experiments, these changes have persisted after euglycemia has been reached (98).

Intracellular abnormalities characteristic of T2DM have also been observed. In health, thrombin binds to the Gq/11 protein found on the platelet membrane. Initiation of the signal transduction pathway activates phospholipase C, which cleaves to phosphatidylinositol-4,5-bisphosphate (PIP_2), then to inositol-1,4,5-trisphosphate (IP_3), which latter raises intracellular Ca^{2+} levels (99). Both in diabetes patients and in hyperglycemia, phospholipase C can cleave into PIP_2 more frequently, resulting in higher

intracellular free Ca^{2+} levels. Higher intracellular calcium levels lead to phosphorylation of myosin, displacement of actin-myosin filaments, and contraction. This ultimately results in platelet deformation and aggregation, and the release of substances stored in granules (100).

Insulin directly interferes with platelet aggregation: insulin binds to platelet membrane receptor and reduces platelet response to thrombin, ADP, arachidonic acid, collagen and platelet activating factor. Patients with diabetes mellitus are less sensitive to the inhibitory effect of insulin (101). The number and affinity of platelet insulin receptors are decreased in T2DM, therefore, it can be assumed that the decreased insulin sensitivity is responsible for platelet hyperreactivity (102).

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2. Objectives

Although several potential mechanisms link cancers and T2DM together (81), and platelets are affected in both diseases (21, 22, 42, 58, 92-94, 103), the relationship between T2DM, (paraneoplastic) thrombocytosis, CD40L, and CRC was never investigated previously. Therefore, a prospective, real-life observational cohort study was conducted where paraneoplastic thrombocytosis was investigated within CRC patients with and without T2DM through the changes in platelet counts, plasma IL-6, CD40L and TPO levels.

The research objectives were as follows:

1. In CRC patients who attended at the Department of Internal Medicine and Hematology, Semmelweis University, Budapest and the Department of General Surgery, Szent Imre University Teaching Hospital, Budapest
    a. To determine the pre and postoperative platelet counts.
    b. To determine the pre and postoperative plasma IL-6 levels.
    c. To determine the pre and postoperative plasma TPO levels.
    d. To determine the pre and postoperative plasma CD40L levels.
    e. To determine the incidence of T2DM.

2. To examine whether platelet counts, IL-6, TPO and/or CD40L plasma levels correlate with any other routine laboratory parameters examined during routine CRC management, such as complete blood count, liver enzymes, tumor markers or cholesterol levels.

3. To evaluate whether and how platelet counts, IL-6, TPO, and/or CD40L plasma levels change after the primary tumor removal surgery.

4. To observe whether platelet counts, IL-6, TPO, and/or CD40L plasma levels change with the progression of CRC.

5. To determine how platelet counts, IL-6, TPO, and/or CD40L plasma levels affects CRC survival in single-time and longitudinal survival models.

6. To investigate whether T2DM is associated with other routine laboratory parameters, such as complete blood count, liver enzymes, tumor markers or cholesterol levels, that are tested in the routine management of CRC.

7. To observe whether T2DM is associated with changes in platelet count, IL-6, TPO and/or CD40L plasma levels during CRC progression.

8. To determine whether T2DM affects CRC survival in single-time and longitudinal survival models.

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3. Methods

The study was conducted in concordance with the WMA Declaration of Helsinki. It was approved by the Regional and Institutional Committee of Science and Research Ethics, Semmelweis University (SE TUKEB 21-12/1994, approval date of latest modification: February 10, 2017), by the Institutional Review Board of Szent Imre University Teaching Hospital (SZIK IKEB 5/2017), and by the Committee of Science and Research Ethics, Hungarian Medical Research Council (ETT TUKEB 8951-3/2015/EKU). Handling of patient data was in accordance with the General Data Protection Regulation issued by the European Union. Written informed consent was collected from all study participants before any study specific procedures.

3.1. Patients and study design

A prospective, real-life observational cohort study was carried out. A total of 108, histopathologically confirmed CRC patients and 166 voluntary non-CRC subjects were enrolled for the study between 2017 and 2019. Study subjects attended at both the Department of Internal Medicine and Hematology, Semmelweis University, Budapest and the Department of General Surgery, Szent Imre University Teaching Hospital, Budapest. Fifty-one, fifty, and sixty-five of the 166 voluntary non-CRC subjects were healthy young controls (age < 40 years), older controls (age ≥ 40 years), and T2DM patients, respectively. Rationale for the inclusion of healthy young subjects was that some of the measured parameters had no reference values at the time of the study. By investigating the parameters of this group, we hoped to have a more complex view of these factors. The older volunteers included both completely healthy individuals and non-metabolic disease patients. T2DM patients attended at the Metabolic Outpatient Clinic of the Department of Internal Medicine and Hematology, Semmelweis University, Budapest.

Exclusion criteria included age < 18 years, any previous malignancies, known inflammatory bowel- and/or chronic kidney- and/or systemic autoimmune- and/or inadequately controlled thyroid- and/or hematologic- and/or any mental diseases, the usage of erythropoiesis-stimulating agents and/or recent blood transfusion, and patients with an Eastern Cooperative Oncology Group (ECOG) performance status score > 2. In

addition, voluntary non-CRC subjects were excluded in the presence of pre-diabetes or any other metabolic disorders.

Study visits were performed according to the following protocol: 1.) at the diagnosis of CRC and prior to any oncological treatments or the surgical resection of the primary tumor, 2.) at least 6-weeks, 3.) 6-months and 4.) 12-months after tumor removal surgery. Follow-up measurements and data collection were only performed in the case of CRC patients, control subject attended only a single (baseline) visit. At the time of post-operative measurements, study sampling was scheduled for a time when the oncological treatments were stopped/paused for at least 6-weeks prior to sampling, due to the known platelet influencing effects of several cytotoxic regimens (104, 105). For the 108 CRC subjects, a total of 215 measurements were available. 108, 48, 37, and 22 preoperative, postoperative, 6-month, and 12-month measurements were available, respectively.

CD40L measurement was not feasible for 2 CRC patients, due insufficient amount of blood for the CD40L plasma level determination. Moreover, previous literature data suggests that plasma CD40L level might be affected by a number of factors, including age and various diseases (106-110). Therefore, an age and sex matched control group (n = 50) was created using propensity-score matching techniques. In addition, the matching of patients was also adjusted for any bias originating from complete blood count differences.

3.2. Clinical and Laboratory Data Measurements

Anamnestic data including co-morbidities and recent medications, bodyweight and height were collected, and fasting blood samples were drawn at every study visit. Complete blood count, aspartate- and alanine transaminase, gamma-glutamyl transferase, plasma glucose, glycated hemoglobin (HbA_{1C}), and creatinine were measured at the Central Laboratory of Semmelweis University and at the Central Laboratory of Szent Imre University Teaching Hospital. Estimated glomerular filtration rate was calculated using the Chronic Kidney Disease – Epidemiology Collaboration equations (111). Side of CRC was described as right-sided if the tumor was originating from the cecum, ascending colon, and proximal two-third of the transverse colon; and left-sided if originating from the distal one-third of the transverse colon, descending colon, sigmoid colon and rectum (112). Staging was given by histopathological examination of surgical

specimens and imaging studies; the AJCC TNM staging system was used (6). The usage of biological agents was recorded as a dummy variable and chemotherapy was grouped as adjuvant if no metastasis and first-line, second-line etc. if metastases was present. Selection of chemotherapy protocol(s) was based on national and international guidelines (16, 17). Overall– (OS) and CRC-related disease-specific (DSS) survival of patients was defined as the length of time from the date of CRC diagnosis until death from any cause and from the cancerous disease, respectively. Follow-ups of CRC patients was terminated on January 31, 2021. Patients alive at this time point were right censored.

3.3. Measurement of plasma interleukin-6

Plasma IL-6 levels were measured using the ELECSYS® Interleukin-6 ECLIA kit (Roche Diagnostics GmbH, Mannheim, Germany) at the Clinical Genetics and Endocrinology Laboratory, Department of Laboratory Medicine, Semmelweis University. In line with the manufacturer's description, the measurement was performed using a Roche cobas® e411 automated ECLIA analyzer (Roche Diagnostics GmbH, Mannheim, Germany). Total duration of the assay was 18 minutes. The test principle was as follows. During the first incubation 30 µL of sample was incubated with a biotinylated monoclonal IL-6-specific antibody. During a second incubation monoclonal IL-6-specific antibody labeled with a tris(2,2'-bipyridyl)ruthenium(II)-complex and streptavidin-coated microparticles were added to the samples. The antibodies formed a sandwich complex with the antigen of the sample. Then the reaction mixture was aspirated into the measuring cell where the microparticles were magnetically captured onto the surface of the electrode, while unbound substances were removed. Application of a voltage to the electrode then induced the chemiluminescent emission which was measured by a photomultiplier. Results were determined via a calibration curve which was instrument-specifically generated by 2-point calibration and a master curve provided via the reagent barcode or e-barcode.

3.4. Measurement of plasma thrombopoietin

Plasma TPO levels were measured using the Human Thrombopoietin Quantikine® ELISA kit (catalogue number: DTP00B, R&D Systems, Minneapolis, MN, USA) at the Laboratory of the Metabolic Diseases Working Group of the Department of

Internal and Medicine and Hematology, Semmelweis University. In line with the manufacturer's description, TPO levels were measured from platelet-poor plasma. First, samples were centrifugated at 4 °C and 3000 rpm for 10 minutes in a Janetzky K23 refrigerated centrifuge using a swing-out rotor. The supernatant plasma was then transferred to a clean centrifuge tube, and a second centrifugation was performed at 4 °C and 6000 rpm for 10 minutes. Platelet-poor plasma was the resulting supernatant after the second centrifugation.

200 µL of plasma sample with 50 µL buffered protein base with preservatives was placed on the 96-well microplate that was coated with a monoclonal antibody specific for human TPO. After a 180-minute incubation at room temperature, microwell stripes were buffer washed and 200 µL polyclonal antibody specific for human TPO conjugated to horseradish peroxidase with preservatives was added to the samples, followed by a 60-minutes incubation and buffer washing. This was followed by a 30-minute incubation with 200µL stabilized hydrogen peroxide and stabilized chromogen (3,3′,5,5′-tetramethylbenzidine dihydrochloride). After adding 50 µL of 2N sulfuric acid stop solution, the optical densities were determined at a wavelength of 450 nm with a wavelength correction at 570 nm, using a Thermo Scientific Multiskan EX ELISA microplate reader (Thermo Fisher Scientific, Waltham, MA, USA). To determine plasma TPO concentrations from the optical densities, a standard curve obtained from the calibrator samples in the kit were constructed. Samples were applied to the microwell plate in pairs, and the average concentration of pairs was used for evaluating the data.

3.5. Measurement of plasma CD40 ligand

Plasma CD40L levels were measured using the CD40L Human ELISA kit (abcam®, Catalog Number ab99991, Cambridge, MA, USA) at the Laboratory of the Metabolic Diseases Working Group of the Department of Internal and Medicine and Hematology, Semmelweis University. In line with the description of the manufacturer, 100 µL of plasma sample was placed on the 96-well microplate that was coated with a monoclonal antibody specific for human CD40L. After a 150-minute incubation at room temperature, microwell stripes were buffer washed and 100 µL of Biotinylated CD40L Detection Antibody was added to the samples, followed by another 60-minute incubation and buffer washing. This was followed by a 45-minute incubation with and 100 µL

streptavidin-HRP 100, washing, and a 30-minute incubation with 100 µL 3,3′,5,5′-tetramethylbenzidine dihydrochloride substrate. After adding 50 µL of 0.2% sulfuric acid stop solution, the optical densities were determined at a wavelength of 450 nm using a Thermo Scientific Multiskan EX ELISA microplate reader (Thermo Fisher Scientific, Waltham, MA, USA). To determine plasma CD40L concentrations from the optical densities, a standard curve obtained from the calibrator samples in the kit were constructed. Samples were applied to the microwell plate in pairs, and the average concentration of pairs was used for evaluating the data.

3.6. Statistical Analysis

Statistical analysis was performed with R version 4.0.5 (R Core Team, R Foundation for Statistical Computing, 2021, Vienna, Austria). Wilcoxon-Mann-Whitney U-test, Fisher's exact test, Kruskal-Wallis test with p-value corrected pairwise Wilcoxon-Mann-Whitney U-tests as post-hoc, and Spearman rank correlation was used for group comparisons. To detect the changes of various parameters in time, linear mixed effect models were used (R-package *nlme,* developed by Pinheiro, Bates DebRoy, Sarkar and the R Core Team, version 3.1-149). Survival of patients was analyzed for both preoperative and longitudinal data with Cox regression models (R package *survival*, developed by Therneau and Grambsch, version 3.2-10) and Bayesian univariate and multivariate joint survival models (R-package *rstanarm,* developed by Goodrich, Gabry, Ali and Brilleman, version 2.21.1), respectively. Single-time OS and DSS of patients were analyzed with simple Cox regression and cause-specific competing risk survival models, respectively (R package *survival*, developed by Therneau and Grambsch, version 3.2-10). $p < 0.05$ was considered as statistically significant and *p*-values were corrected with the Holm method (113) for multiple comparisons problem. Results were expressed as mean ± standard deviation, the number of observations (percentage), and as hazard ratio (HR) with its 95% confidence interval (95% CI) for continuous, count, and survival data, respectively.

Compared to the "conventional" frequentist methods, Bayesian methods give only a probability distribution for the investigated parameter but no *p*-values. Interpretation of longitudinal survival models, a.k.a. joint model results was as follows. If the Bayesian equivalent to the frequentist confidence interval (CI), the 95% credible interval (95% CrI)

contained the hazard ratio (HR) of 1, the model was considered as clinically not relevant. However, if the 95% CrI was less than or more than HR = 1, the effect of the parameter was considered as a good or bad sign of patient survival, respectively. Naïve Kaplan–Meier and longitudinal survival curves were drawn with the R-package *survminer* (developed by Kassambara, Kosinski, and Biecek, version 0.4.9, 2021) and the built-in methods of the R-package *rstanarm* (developed by Goodrich, Gabry, Ali and Brilleman, version 2.21.1), respectively.

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4. Results

A total of 274 study participants were included in the study: 108 CRC patients and 166 voluntary, non-CRC subjects. CRC patients were divided into two cohorts based on the presence of T2DM. Patients without diabetes (n = 82) were assigned to the 'CRC' group, while patients with a history of T2DM (n = 26) were assigned to the 'CRC+T2DM' group. The average duration of diabetes was 6.88 ± 6.10 years, from which T2DM was diagnosed in four cases around the time of CRC diagnosis. Voluntary non-CRC subjects were divided into three cohorts: 51, 50 and 65 subjects were assigned to the 'Young control', 'Control' and 'T2DM' groups, respectively. Duration of diabetes was 14.91 ± 9.50 years within the T2DM group. The complete list of anamnestic, clinical and laboratory parameters of the study groups are shown in **Table 2**, including the indications of the differences between all five groups.

In most parameters, the two tumor groups did not differ from each other, while plasma glucose was significantly higher (p = 0.0018), and tendentiously higher white blood cell-, neutrophil-, eosinophil- and monocyte counts were observed in the CRC+T2DM group. Platelet aggregation inhibition therapy was more common in CRC+T2DM patients; no difference was found in radiotherapy (neoadjuvant only) while no- or late-line chemotherapy was more common in those CRC patients with T2DM than those of without (p = 0.0560).

The comparisons between the volunteer groups revealed that, as expected, Young controls had the lowest body mass index (p < 0.0001 vs T2DM and Controls), systolic blood pressure (p = 0.0250 vs Controls, p = 0.0410 vs T2DM), mean corpuscular volume (p = 0.0002 vs Controls, p = 0.0041 vs T2DM), red blood cell distribution width (p = 0.0023 vs Controls, p < 0.0001 vs T2DM), fasting plasma glucose (p < 0.0001 vs Controls and T2DM) and the highest heart rate (p = 0.0002 vs Controls, p = 0.0242 vs T2DM) and estimated glomerular filtration rate (p < 0.0001 vs Controls and T2DM). T2DM patients had the highest body mass index (p = 0.0098 vs Controls, p < 0.0001 vs Young controls), white blood cell count (p = 0.0430 vs Controls, p = 0.0008 vs Young controls), gamma-glutamyl transferase (p = 0.0633 vs Controls, p = 0.0003 vs Young controls) and fasting plasma glucose (p < 0.0001 vs Controls and Young controls).

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Table 2: Demographic and clinical characteristics of study participants. Unit of frequency data is the number of observations (percentage).

Parameter	CRC (*n* = 82)	CRC+T2DM (*n* = 26)	Young control (*n* = 51)	Control (*n* = 50)	T2DM (*n* = 65)
Age (years)	67.42 ± 9.33	70.83 ± 7.24	26.13 ± 4.50 [1]	60.31 ± 11.00 [4]	64.68 ± 8.30 [7]
Sex (Male : Female)	51 : 31 (62.2% : 37.8%)	20 : 6 (76.9% : 23.1%)	24 : 27 (47.1% : 52.9%)	22 : 28 [7] (44.0% : 56.0%)	35 : 30 (53.8% : 46.2%)
Body mass index (kg/m^2)	27.26 ± 3.87	27.43 ± 4.64	23.25 ± 3.95 [1]	27.99 ± 5.42 [6]	30.73 ± 5.12 [1]
Systolic blood pressure (mmHg)	144.49 ± 18.48	141.12 ± 19.55	129.82 ± 15.60	138.27 ± 17.34	136.58 ± 16.64
Diastolic blood pressure (mmHg)	78.99 ± 10.19	78.20 ± 9.10	77.27 ± 9.10	80.98 ± 11.85	76.06 ± 10.83
Heart rate (1/min)	77.88 ± 13.76	80.76 ± 16.36	77.59 ± 11.08	67.76 ± 7.95 [1]	73.23 ± 9.18
White blood cell count (10^9/L)	8.14 ± 2.91 *	11.11 ± 7.46	6.48 ± 1.59 [2]	7.04 ± 2.00 [7]	7.97 ± 2.23
- Neutrophil count (10^9/L)	5.60 ± 2.57 *	7.87 ± 5.59	3.66 ± 1.37 [2]	4.35 ± 1.68 [4]	4.93 ± 1.82 [7]
- Eosinophil count (10^9/L)	0.18 ± 0.16 *	0.59 ± 1.73	0.19 ± 0.17	0.18 ± 0.13	0.21 ± 0.13
- Basophil count (10^9/L)	0.06 ± 0.05	0.06 ± 0.04	0.06 ± 0.08	0.06 ± 0.03	0.06 ± 0.05
- Monocyte count (10^9/L)	0.61 ± 0.46 *	0.78 ± 0.51	0.44 ± 0.15 [2]	0.44 ± 0.11 [2]	0.52 ± 0.14
- Lymphocyte count (10^9/L)	1.74 ± 1.11	1.81 ± 0.92	2.09 ± 0.66 [3]	2.15 ± 0.77 [3]	2.25 ± 0.80 [3]
Red blood cell count (10^{12}/L)	4.50 ± 0.58	4.42 ± 0.52	4.97 ± 0.53 [4]	4.92 ± 0.53 [4]	4.81 ± 0.38 [4]
Hemoglobin (g/dL)	12.42 ± 2.14	12.14 ± 2.11	14.60 ± 1.35 [4]	14.69 ± 1.38 [4]	14.07 ± 1.04 [4]
Hematocrit (L/L)	0.38 ± 0.06	0.38 ± 0.06	0.43 ± 0.04 [4]	0.44 ± 0.04 [4]	0.42 ± 0.03 [4]
Mean Corpuscular Volume (fL)	84.84 ± 8.22	84.31 ± 8.54	86.27 ± 3.00 [5]	89.32 ± 4.18 [3]	88.04 ± 3.44
Mean Corpuscular Hemoglobin (pg)	27.30 ± 3.41	27.32 ± 3.81	29.49 ± 1.39 [3]	30.02 ± 1.61 [4]	29.32 ± 1.47 [3]
Mean Corpuscular Hemoglobin Concentration (g/L)	322.35 ± 15.29	324.20 ± 24.53	339.16 ± 9.73 [2]	337.18 ± 8.40 [4]	332.95 ± 8.93 [3]
Red Blood Cell Distribution Width (%)	14.63 ± 3.74	15.53 ± 3.47	12.59 ± 0.79 [1]	13.15 ± 0.92 [7]	13.23 ± 0.83 [7]
Platelet count (10^9/L)	308.38 ± 124.66	339.85 ± 118.86	267.86 ± 50.18	272.18 ± 72.66	263.92 ± 67.23
Aspartate transaminase (U/L)	25.86 ± 21.03	26.37 ± 16.12	27.47 ± 13.96	24.64 ± 6.49	25.34 ± 9.82
Alanine transaminase (U/L)	21.75 ± 12.52	23.10 ± 13.35	25.08 ± 14.77	24.88 ± 11.32 [3]	27.91 ± 14.86
Gamma-glutamyl transferase (U/L)	68.33 ± 117.56	97.24 ± 167.79	25.06 ± 12.56 [6]	32.70 ± 31.24	40.64 ± 29.40

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Table 2 (cont.)

Parameter	CRC (*n* = 82)	CRC+T2DM (*n* = 26)	Young control (*n* = 51)	Control (*n* = 50)	T2DM (*n* = 65)
Plasma glucose (mmol/L)	5.45 ± 0.86	6.62 ± 1.71	4.58 ± 0.45 [1]	4.99 ± 0.57 [1]	8.17 ± 2.63 [1]
Creatinine (μmol/L)	76.99 ± 19.19	82.33 ± 22.41	73.69 ± 12.72	70.20 ± 12.72	75.11 ± 19.05
Estimated glomerular filtration rate (mL/min/1.73 m^2)	82.17 ± 16.81	78.23 ± 18.30	109.33 ± 14.97 [1]	89.66 ± 12.42	83.98 ± 16.05
Known comorbidities					
- Hypertension	48 (58.5%)	20 (76.9%)	0 (0.0%) [1]	16 (32.0%) [1]	56 (86.2%) [3]
- Major cardiovascular event(s) prior first visit	16 (19.5%)	6 (23.1%)	0 (0.0%) [2]	5 (10.0%)	13 (20.0%)
Platelet aggregation inhibition	14 (17.1%) [*]	9 (34.6%)	0 (0.0%) [2]	6 (12.0%) [6]	50 (76.9%) [4]
AJCC Staging (6) [**]					
- Stage I	19 (23.2%)	9 (34.6%)	NA	NA	NA
- Stage II	23 (28.0%)	4 (15.4%)	NA	NA	NA
- Stage III	20 (24.4%)	4 (15.4%)	NA	NA	NA
- Stage IV	20 (24.4%)	9 (34.6%)	NA	NA	NA
Side of CRC (112)					
- Left-sided	60 (73.2%)	15 (57.7%)	NA	NA	NA
- Right-sided	22 (26.8%)	11 (42.3%)	NA	NA	NA
Chemotherapy					
- None	37 (45.1%)	16 (61.5%)	NA	NA	NA
- Adjuvant	21 (25.6%)	2 (7.7%)	NA	NA	NA
- First-line	12 (14.6%)	0 (0.0%)	NA	NA	NA
- Second-line	7 (8.5%)	7 (26.9%)	NA	NA	NA
- Third or Later-line	5 (6.1%)	1 (3.8%)	NA	NA	NA
Radiotherapy	14 (17.1%)	3 (11.5%)	NA	NA	NA
Use of biological therapy	15 (18.3%)	7 (26.9%)	NA	NA	NA

Table 2 (cont.)

Parameter	CRC (*n* = 82)	CRC+T2DM (*n* = 26)	Young control (*n* = 51)	Control (*n* = 50)	T2DM (*n* = 65)
Duration of T2DM (years)	NA	6.88 ± 6.10	NA	NA	14.91 ± 9.50 [7]
HbA_{1C} (%)	NA	6.30 ± 1.04	NA	NA	7.40 ± 1.26 [7]
Treatment used for T2DM [7]					
- Only diet	NA	7 (26.9%)	NA	NA	3 (4.6%)
- Oral Hypoglycemic Medications	NA	18 (69.2%)	NA	NA	32 (49.2%)
- Combination therapy (oral + basal insulin)	NA	1 (3.8%)	NA	NA	14 (21.5%)
- Intensive insulin therapy	NA	0 (0.0%)	NA	NA	16 (24.6%)
Diabetic complications (developed prior CRC)					
- Retinopathy	NA	2 (7.7%)	NA	NA	16 (24.6%)
- Nephropathy	NA	0 (0.0%)	NA	NA	6 (9.2%)
- Neuropathy	NA	5 (19.2%)	NA	NA	14 (21.5%)
- Angiopathy	NA	1 (3.8%)	NA	NA	9 (13.8%)
- Albuminuria	NA	0 (0.0%)	NA	NA	9 (13.8%)
Number of diabetic co-morbidities					
- None	NA	20 (76.9%)	NA	NA	35 (53.8%)
- One	NA	4 (15.4%)	NA	NA	15 (23.1%)
- More than one	NA	2 (7.7%)	NA	NA	15 (23.1%)
Hyperlipidemia	NA	10 (38.5%)	NA	NA	44 (67.7%)

AJCC: American Joint Committee on Cancer; HbA_{1C}: glycated hemoglobin; T2DM: type 2 diabetes mellitus. [1] $p < 0.05$ vs all other 4 groups. [2] $p < 0.05$ vs all diseased groups. [3] $p < 0.01$ vs the colorectal cancer without T2DM group. [4] $p < 0.01$ vs both tumor groups. [5] $p < 0.05$ vs Control and T2DM groups. [6] $p < 0.05$ vs T2DM. [7] $p < 0.01$ vs the colorectal cancer with T2DM group. * Without p-value correction, the differences between the two tumor groups are marginal. ** Without p-value correction, the differences in the distribution of chemotherapy regimens between the two tumor groups are statistically significant (crude $p = 0.0070$).

The duration of diabetes was shorter within the CRC+T2DM group, compared to those of the T2DM group. A higher proportion of oral antidiabetic drug usage and diet-only therapy was observable within the CRC+T2DM group, while the need for insulin therapy was greater in those within the T2DM group ($p = 0.0006$). Furthermore, lower HbA_{1C} level and fewer diabetic complications were found in the CRC+T2DM patients (**Table 2**). The occurrence of hypertension ($p = 0.7790$) and the proportion of previous major cardiovascular event(s) prior the first visit ($p = 0.7004$) did not differ between the two diabetic groups.

4.1. Investigation of paraneoplastic thrombocytosis related parameters

4.1.1. Baseline measurement of paraneoplastic thrombocytosis related parameters

The plasma level of paraneoplastic thrombocytosis parameters of CRC patients was compared to those of all control groups at the time of CRC diagnosis. Highest platelet counts were observed within the two tumor groups: platelet count of CRC patients was significantly higher than those of within the T2DM group ($p = 0.0369$); while platelet count of the CRC+T2DM group was significantly higher than all of the control groups ($p = 0.0369$ vs Young Control and Control, $p = 0.0278$ vs T2DM; **Figure 2A**). Lowest plasma IL-6 levels were observed within the Young controls ($p < 0.0010$ vs. all other cohorts). Subjects of the Control and T2DM groups had similar IL-6 levels; and IL-6 was significantly higher in both tumor groups, compared to all of those observed in control groups ($p < 0.0001$ CRC vs Control & T2DM, $p = 0.0011$ CRC+T2DM vs Control, $p = 0.0069$ CRC+T2DM vs T2DM; **Figure 2B**). TPO level was basically the same in the Young control and Control groups, and a separate cluster was formed by the remaining three groups. The highest TPO levels were observed in the CRC+T2DM group (**Figure 2C**). No further difference could have been justified in any of the study groups if they were further subdivided by the usage of platelet aggregation inhibition therapy, antidiabetic drugs, or in the presence of any diabetic complications.

Highest platelet counts were found if IL-6 was high as well (CRC: Spearman ρ: +0.34, $p = 0.0017$; CRC+T2DM: $p = 0.0786$; CRC groups combined: Spearman ρ: +0.32, $p = 0.0009$). Correlation between platelet count and TPO was only significant in the CRC group (Spearman ρ: –0.24, $p = 0.0332$), while no ($p = 0.8486$) and marginal association

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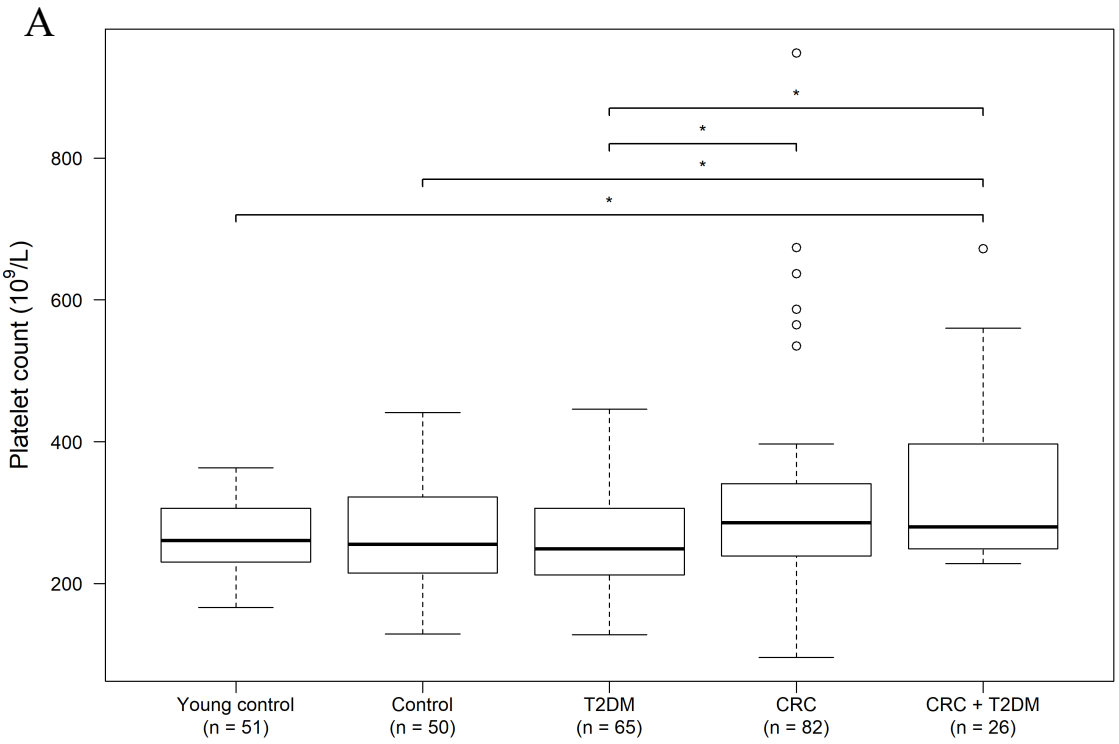
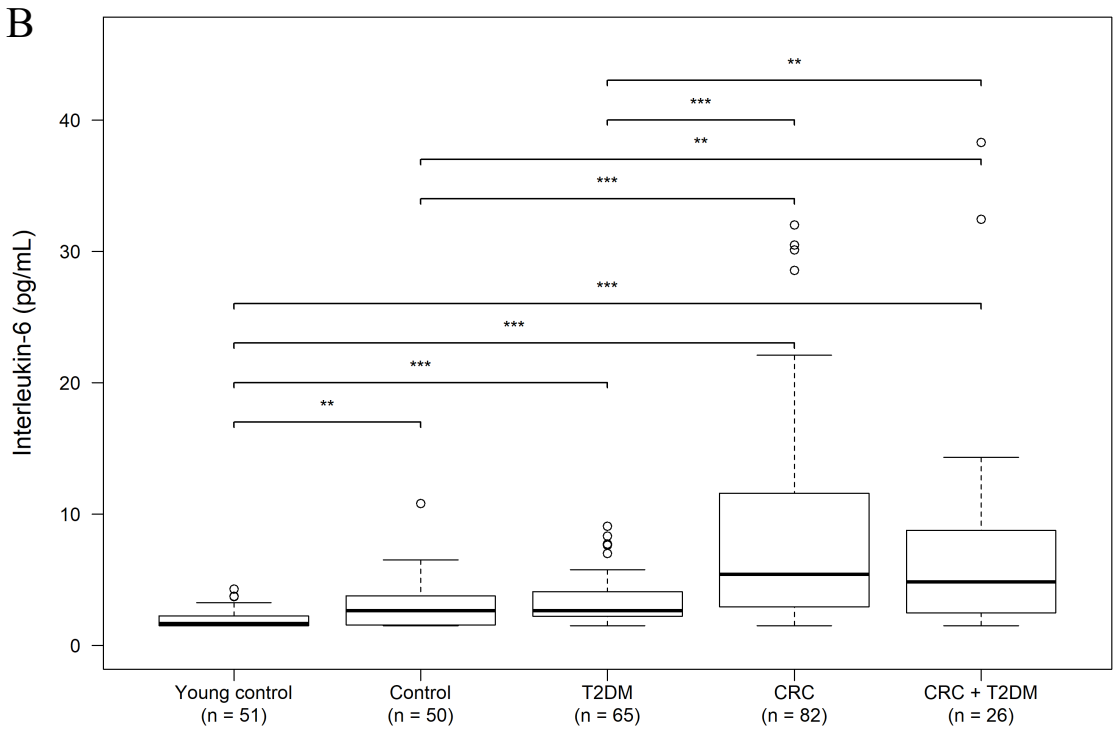
(p = 0.0643) was found in the CRC+T2DM and in the two tumor groups





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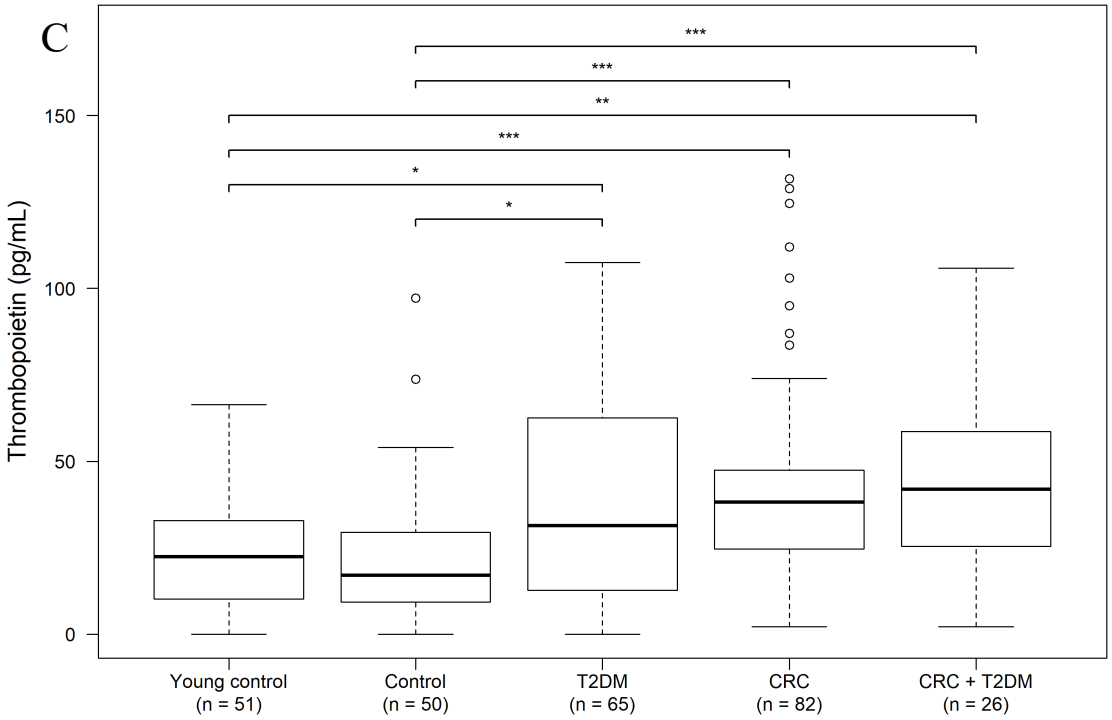

Figure 2: Comparison of paraneoplastic thrombocytosis related parameters within the 5 study groups. Platelet count (**A**) was significantly higher in colorectal cancer (CRC) patients with type 2 diabetes mellitus (T2DM), compared to those in all control groups; and it also differed between the CRC and T2DM groups. Plasma interleukin-6 (**B**) level was significantly higher in all tumor patients, while plasma thrombopoietin (**C**) was significantly lower within the T2DM and CRC, and significantly higher in the CRC+T2DM groups, compared to those of control subjects.

Note: Due to better visibility, on Figure 2B, six and one outlier(s) over 40 pg/mL were not shown in the CRC and CRC+T2DM groups; maximum values were 220.20 pg/mL and 77.19 pg/mL, respectively.

Own figure.

combined, respectively. No correlation was found between IL-6 and TPO levels (CRC: $p = 0.4279$, CRC+T2DM: $p = 0.9921$, tumor groups combined: $p = 0.5383$). No correlation was found between the thrombocytosis related parameters and the duration of T2DM or the preoperative level of HbA_{1C}.

4.1.2. Changes in the parameters of paraneoplastic thrombocytosis with the course of colorectal cancer

For the 108 CRC subjects, a total of 215 measurements were available. 108, 48, 37 and 22 preoperative, postoperative, 6- and 12-month measurements were available, respectively. Significant decrease in later measurements occurred due to the death of patients, disease progression resulting in higher ECOG performance status and patient's unavailability to attend at later visits, the need to initiate chemotherapy earlier than the postoperative visit window, or continuous chemotherapy without drug holiday after the second study visit. Due to the decreasing number of follow-ups, a more robust statistical method not-sensitive to the loss-of follow-up had to be chosen, therefore, it was investigated via age- and stage-corrected linear mixed effect interaction models, how T2DM and worse clinical outcome (death) affects the changes of platelet count, IL-6 and TPO levels with the course of the disease. Diabetes had no significant effect on any of the changes (platelet counts: $p = 0.1190$; IL-6: $p = 0.5571$; TPO: $p = 0.3062$, **Figure 3A, 3C** and **3E**). Platelet counts of all patients decreased, but within those patients who died during the time of the study, the average baseline platelet count was significantly higher (273.70 vs. 353.00 × 10^9/L; $p = 0.0042$) and a faster decrease could have been seen within the first 12 months after the tumor removal surgery, compared to those of who survived (**Figure 3B**). Similarly, increased baseline IL-6 levels (5.76 vs 27.42 pg/mL; $p < 0.0001$) but slowly increasing levels were observed in deceased patients over time, while in those who survived the plasma IL-6 level was constant during our observation ($p = 0.1273$, **Figure 3D**). Initial TPO level did not differ between surviving and deceased patients ($p = 0.5747$) and its change was not affected by the worse clinical outcome ($p = 0.5940$, **Figure 3F**). Usage of radio- and/or chemotherapy did not affect any of the response variables, if included within any of the models.

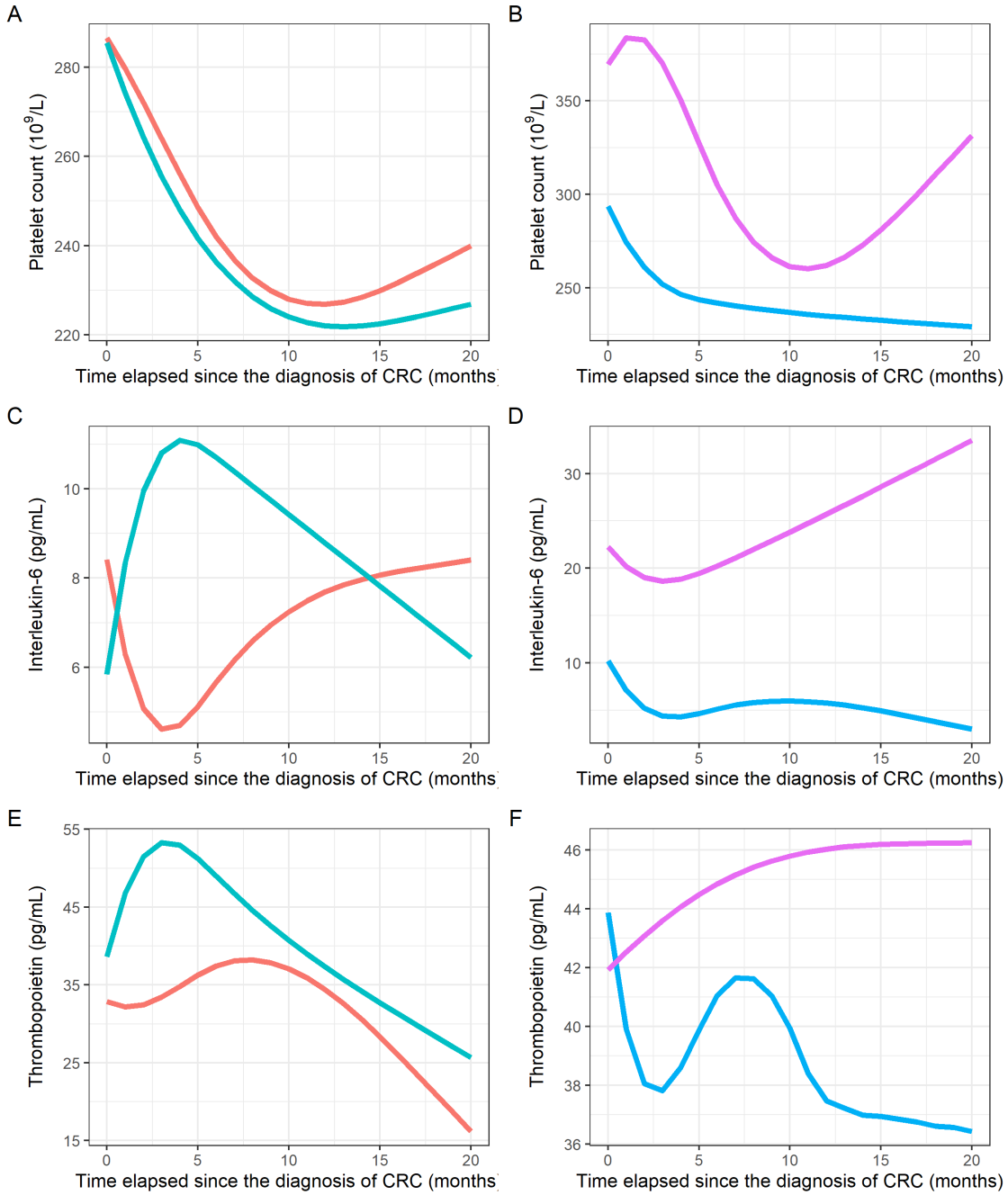

Figure 3: Change in the parameters of paraneoplastic thrombocytosis with the course of the disease (red: CRC; green: CRC+T2DM; blue: survivors; purple: died). No difference was found in any of the parameters (**A, C** and **E**) when stratified with the presence of type 2 diabetes mellitus. Platelet count (**B**) and plasma interleukin-6 (**D**) was significantly higher at the time of colorectal cancer diagnosis in those patients who died during the observation period. Within the first year after tumor removal surgery a significantly faster platelet count decrease was found in those patients who died (**B**), while the significant

difference in plasma interleukin-6 levels remained throughout the whole study between survivors and those patients who died (**D**). No statistical difference was found in plasma thrombopoietin levels of survivors and non-survivors (**F**).
Own figure.

4.1.3. Survival analysis of paraneoplastic thrombocytosis related parameters and T2DM

Thirty of the 108 patients (27.8%) died during the study, from which 20 and 10 belonged to the CRC and CRC+T2DM group, respectively. Despite the higher occurrence of death (24% vs 40%) within the CRC+T2DM group, the univariate Cox model of preoperative data suggested that T2DM had no effect on patient survival (p = 0.1450; **Figure 4**). While IL-6 and TPO had no significant univariate effect, higher preoperative platelet count (HR: 1.0026, 95% CI: 1.0010 – 1.0050, p = 0.0052) could be considered as a poor prognostic sign. However, if combined with T2DM in a multivariate model, higher IL-6 levels had marginal effect (HR: 1.0007, 95% CI: 0.9995 – 1.0140, p = 0.0692) on patient survival. Like in the univariate model, higher platelet counts had the same significant effect in the multivariate model as well (HR: 1.0028, 95% CI: 1.0009 – 1.0050, p = 0.0043). T2DM did not have any effect in either multivariate model. A subgroup analysis within the CRC+T2DM group only revealed that neither the duration of diabetes (p = 0.5590), the use of any antidiabetics (p = 0.2620), the presence of any diabetic complications (p = 0.2860), nor the preoperative level of HbA_{1C} (p = 0.5370) affected patient survival.

To analyze the effect of paraneoplastic thrombocytosis related parameter-changes in time, Bayesian joint models were used. First, three univariate joint models (a single parameter is analyzed within the longitudinal submodel) were constructed: the survival effect of the changes of platelet count, IL-6 or TPO over time was included in the longitudinal submodel and T2DM was included in the survival submodel. An additional multivariate joint model was also constructed, where all three paraneoplastic thrombocytosis parameters were included within the longitudinal submodel, and no change was applied to the survival submodel. Based on the result of the univariate joint models, after the surgical removal of the primary tumor higher platelet count and plasma IL-6 level is a sign of poorer survival. TPO and T2DM did not affect the survival of patients in any of the univariate models. Multivariate joint model results suggested that

IL-6 had the strongest effect on patient survival, while platelet count was marginal; and TPO and T2DM had no clinically relevant effect, similar to those observed in univariate models (**Figure 5**).

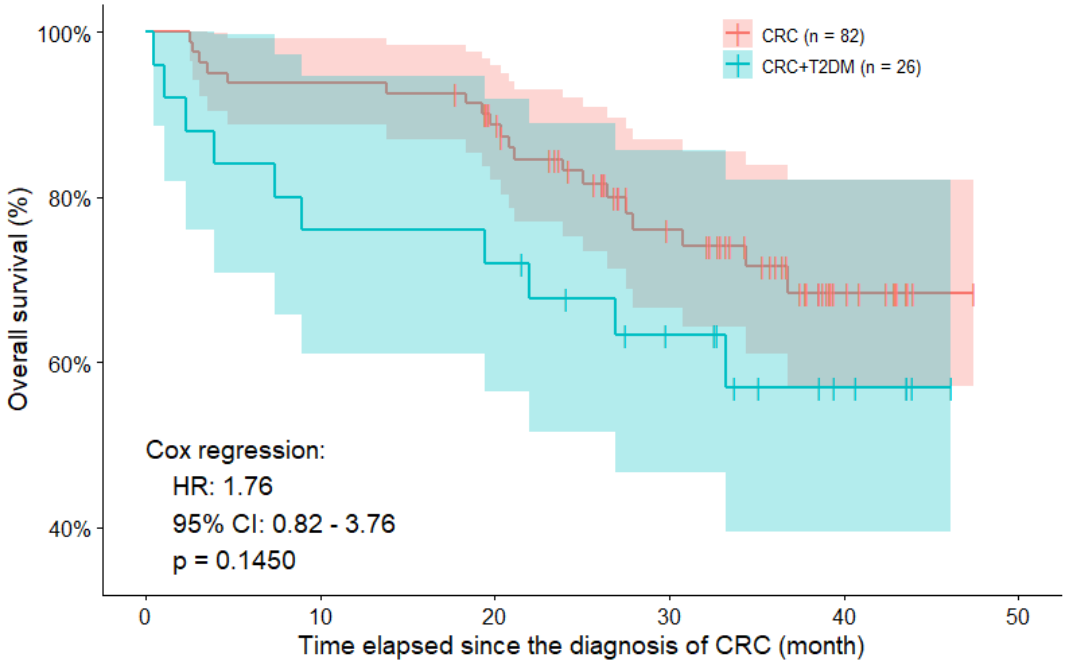

Figure 4: Naïve Kaplan-Meier curves of colorectal cancer (CRC) patients with and without type 2 diabetes mellitus (T2DM). Neither Cox regression (p = 0.1450), nor log-rank test (p = 0.0908) could justify a statistical difference.

Own figure.

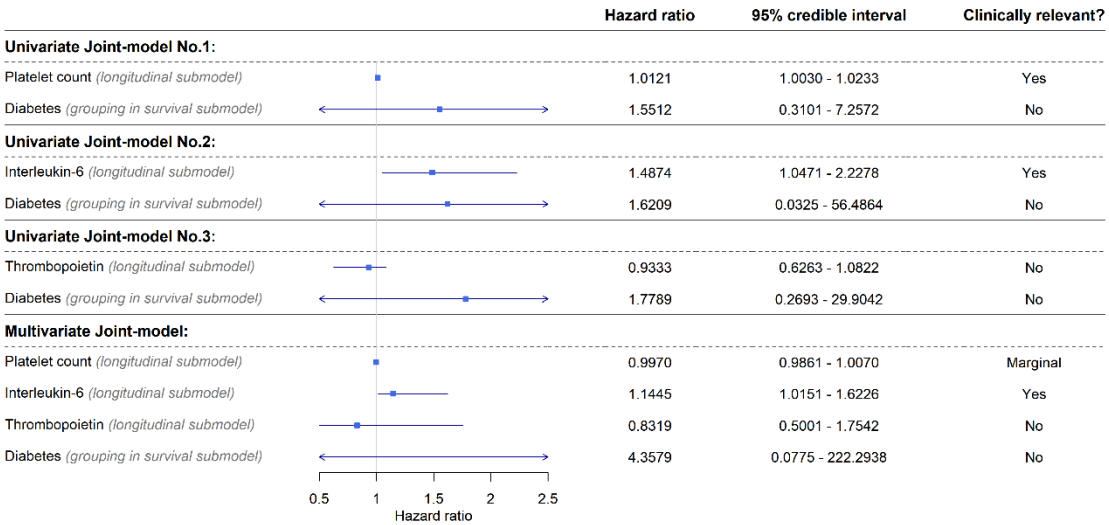

Figure 5: Results of the Bayesian joint survival models. Platelet count and/or plasma interleukin-6 level increasing over time is a poor prognostic factor, while diabetes and plasma thrombopoietin changes did not affect patient survival. Own figure.

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4.2. Investigation of CD40 ligand

4.2.1. Comparisons of baseline CD40 ligand measurements

As detailed in Methods, a total of 106 CRC patients and 50 age and sex-matched voluntary control subjects were enrolled for this part of the study. The two cohorts were well balanced as no significant difference was detected in either of the anamnestic data (**Table 3**). On the contrary, most of the parameters of complete blood count within the CRC group were out of normal range ($p < 0.05$), and 12 of the 106 CRC patients (11.3%) showed signs of thrombocytosis (platelet count > 400 × 10^9/L). CD40L did not differ between the two cohorts (crude P value: 0.2946; **Figure 6**).

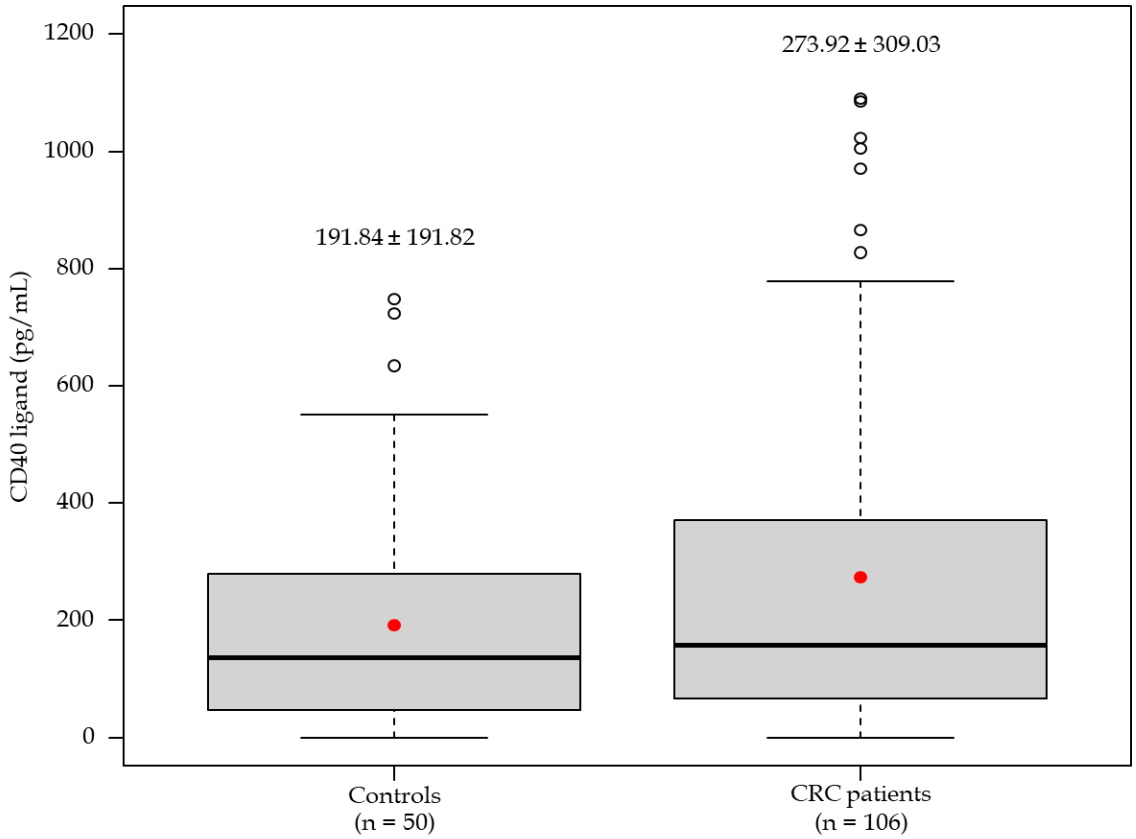

Figure 6: Baseline plasma CD40 ligand level of control subjects and colorectal cancer patients. Crude $p = 0.2946$. The red dot and thick line represent mean and median values, respectively. CRC: Colorectal cancer.

Own figure.

Table 3: Baseline laboratory results and anamnestic data of study subjects.

Parameter	CRC patients (*n* = 106)	Controls (*n* = 50)	*p*-value
Age (year)	68.55 ± 8.64	63.91 ± 10.12	0.2068
Sex (Male : Female)	71 : 35 (67.0% : 33.0%)	35 : 15 (70.0% : 30.0%)	1.0000
Body mass index (kg/m^2)	27.37 ± 4.03	29.26 ± 5.07	0.5995
White blood cell count (10^9/L)	8.76 ± 4.56	7.41 ± 1.99	0.7308
Neutrophil count (10^9/L)	6.05 ± 3.60	4.45 ± 1.64	0.0205
Eosinophil count (10^9/L)	0.28 ± 0.86	0.20 ± 0.15	1.0000
Basophil count (10^9/L)	0.06 ± 0.05	0.06 ± 0.03	1.0000
Monocyte count (10^9/L)	0.65 ± 0.48	0.48 ± 0.12	0.2532
Lymphocyte count (10^9/L)	1.76 ± 1.07	2.22 ± 0.72	0.0002
Red blood cell count (10^{12}/L)	4.48 ± 0.57	4.93 ± 0.51	0.0002
Hemoglobin (g/L)	123.67 ± 21.37	147.04 ± 12.70	0.0001
Hematocrit (L/L)	0.38 ± 0.06	0.44 ± 0.04	< 0.0001
Mean corpuscular volume (fL)	84.69 ± 8.29	89.22 ± 4.06	0.0856
Mean corpuscular hemoglobin (pg)	27.30 ± 3.50	29.93 ± 1.77	0.0001
Mean corpuscular hemoglobin concentration (g/L)	322.94 ± 17.84	335.38 ± 9.44	< 0.0001
Red blood cell distribution width (%)	14.85 ± 3.70	13.13 ± 0.82	0.1911
Platelet count (10^9/L)	315.58 ± 124.55	259.96 ± 73.98	0.0479
Aspartate transaminase (U/L)	25.95 ± 20.22	26.52 ± 6.94	0.0155
Alanine transaminase (U/L)	22.14 ± 12.74	28.62 ± 12.32	0.0047
Gamma-glutamyl transferase (U/L)	75.07 ± 130.37	37.84 ± 31.74	1.0000
Plasma glucose (mmol/L)	5.71 ± 1.23	5.84 ± 1.94	1.0000
Creatinine (μmol/L)	78.26 ± 20.06	74.82 ± 15.28	1.0000
Estimated glomerular filtration rate (mL/min/1.73 m^2)	81.39 ± 17.00	87.21 ± 12.42	0.7308
Known comorbidities			
- Type 2 diabetes mellitus	25 (23.6%)	16 (32.0%)	1.0000
- Hypertension	68 (64.2%)	26 (52.0%)	0.8157
- Major cardiovascular event(s) prior CRC	21 (19.8%)	6 (12.0%)	1.0000
Platelet aggregation inhibition	23 (21.7%)	18 (36.0%)	0.5517

CRC: colorectal cancer.

To test whether other factors, such as age, sex, body mass index, histopathological data, or the presence of comorbidities, affect plasma CD40L levels, further subgrouping within the individual cohorts and correlation analysis was performed. CD40L level of control subjects was affected by the presence of diabetes (without diabetes: 240.41 ± 207.37 pg/mL; with diabetes: 110.09 ± 112.06 pg/mL; p = 0.0313), while no further parameters had any effect on the CD40L level of control subjects. In contrast, the

presence of diabetes had no effect on CD40L levels of CRC patients ($p = 0.7377$). CD40L level was significantly higher in the presence of distant metastasis (M0: 228.27 ± 293.30 pg/mL; M1: 395.11 ± 322.00 pg/mL; $p = 0.0055$; **Figure 7A**) and with thrombocytosis (without thrombocytosis: 248.15 ± 299.20 pg/mL; with thrombocytosis: 475.77 ± 323.43 pg/mL; $p = 0.0294$). Furthermore, a negative correlation was found between CD40L and mean corpuscular volume (Spearman's $\rho = -0.36$, $p = 0.0048$). To further assess the effect of thrombocytosis on CD40L, general linear models were created. TPO did not have any effect on CD40L. Higher platelet count or the presence of thrombocytosis and higher plasma IL-6 levels were strongly correlated with higher CD40L levels (**Table 4**). It should be emphasized that both the individual and combined effect of these parameters only slightly explained the increase in CD40L. The explanatory power of the models, based on adjusted R^2, was at a maximum of 8.1%.

Table 4. Results of general linear models investigating the effect of thrombocytosis on CD40 ligand.

Parameter	Individual effect *p*-value	Multiple effect *p*-value	Multiple effect *p*-value
Interleukin-6 (pg/mL)	0.0130	0.1720	0.0454
Thrombopoietin (pg/mL)	0.1620	0.2393	0.1785
Platelet count (10^9/L)	0.0045	0.0043	-
Presence of thrombocytosis	0.0155	-	0.0138

To further assess whether staging has effect on any of the other parameters, in addition to CD40L, all of the parameters related to paraneoplastic thrombocytosis were also investigated. Similar to those of CD40L levels (**Figure 7A**), higher platelet count was associated with more advanced stages of CRC ($p = 0.0079$; **Figure 7B**). Moreover, higher IL-6 levels were observed in patients with a higher stage range ($p = 0.0025$; **Figure 2C**). Plasma TPO levels were basically equal in all stages, except in Stage II, where decreased TPO levels were observed ($p = 0.0210$; **Figure 7D**).

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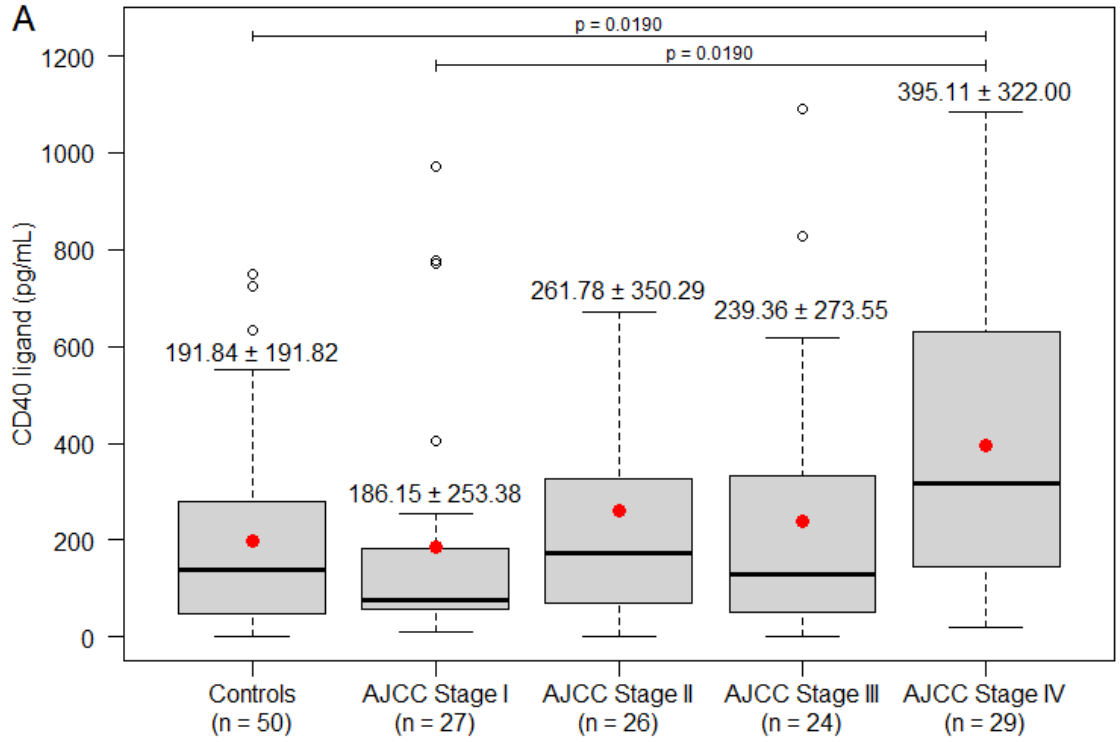

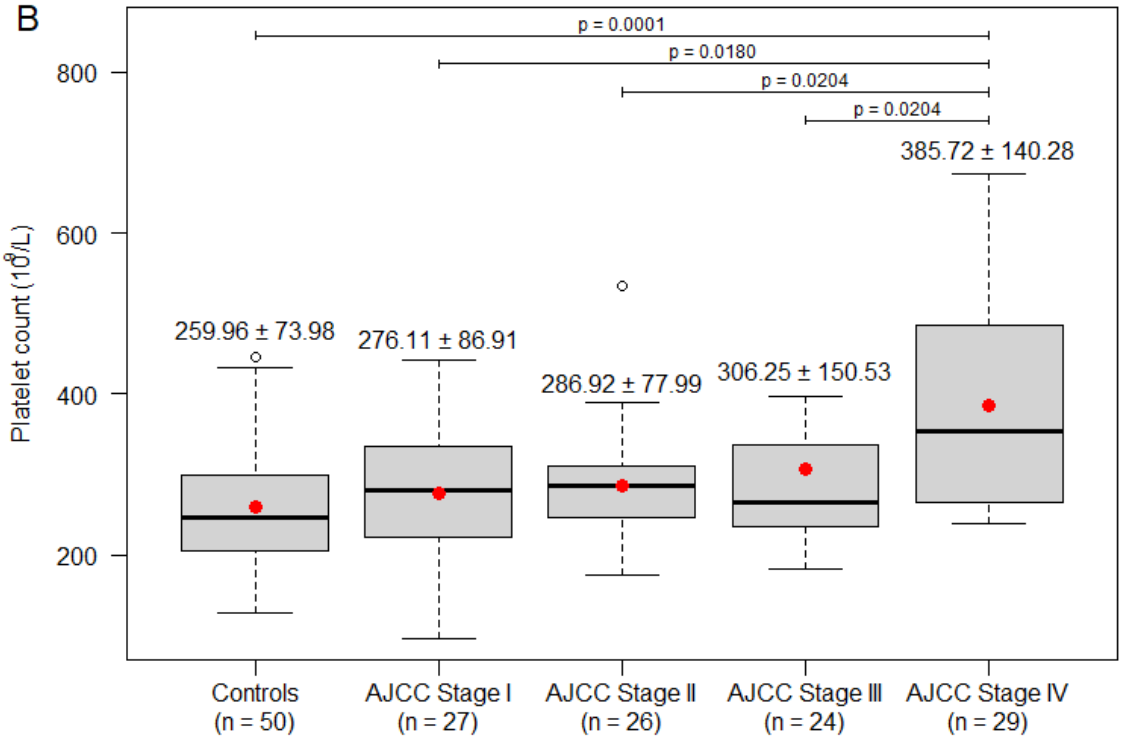

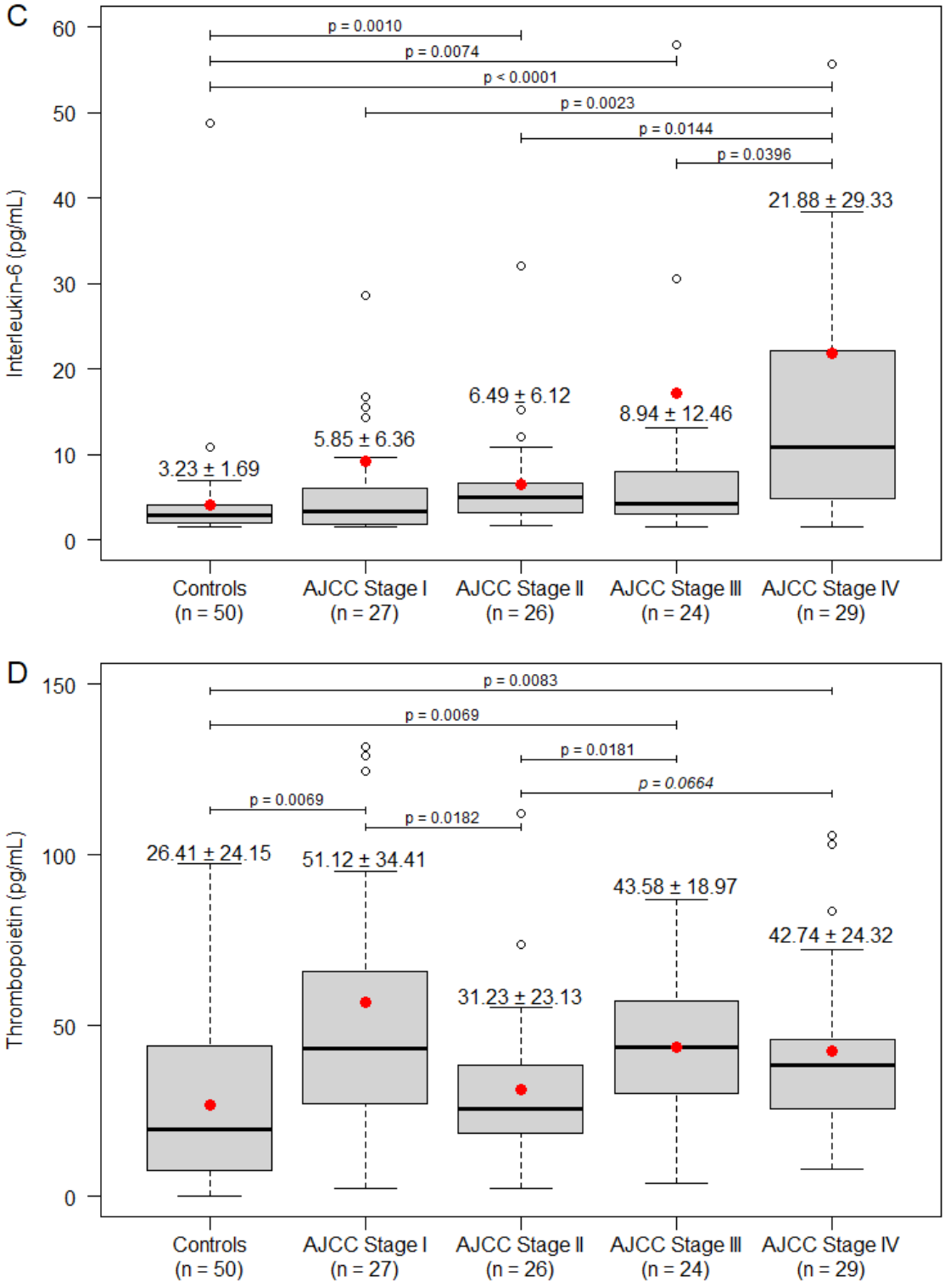

Figure 7: Baseline plasma CD40 ligand (**A**), interleukin-6 (**C**), and thrombopoietin (**D**) level and platelet count (**B**) of study participants (mean ± SD). CD40 ligand level (p = 0.0275) and platelet count (p = 0.0004) was the highest in Stage IV colorectal cancer (CRC) patients. Interleukin-6 ($p < 0.0001$) and thrombopoietin (p = 0.0002) level of the

CRC patient groups, except those in Stage II in the latter, was significantly higher than those of healthy control subjects. The red dot and thick line represent mean and median values, respectively.

Own figure.

4.2.2. Changes in CD40L with the course of the disease

CRC patients were recalled for follow-ups, and 61 of the original 106 patients (call-back rate 57.4%) had at least one repeated measurement of CD40L. A total of 197 measurements were used and 30 CRC patients had all three sets of measurements (**Figure 8**). To determine whether CD40L change with respect to the course of CRC, linear mixed-effects models were constructed. Based on these estimations, no significant changes were observed in the plasma CD40L (p = 0.6813; **Figure 8**) levels of CRC patients.

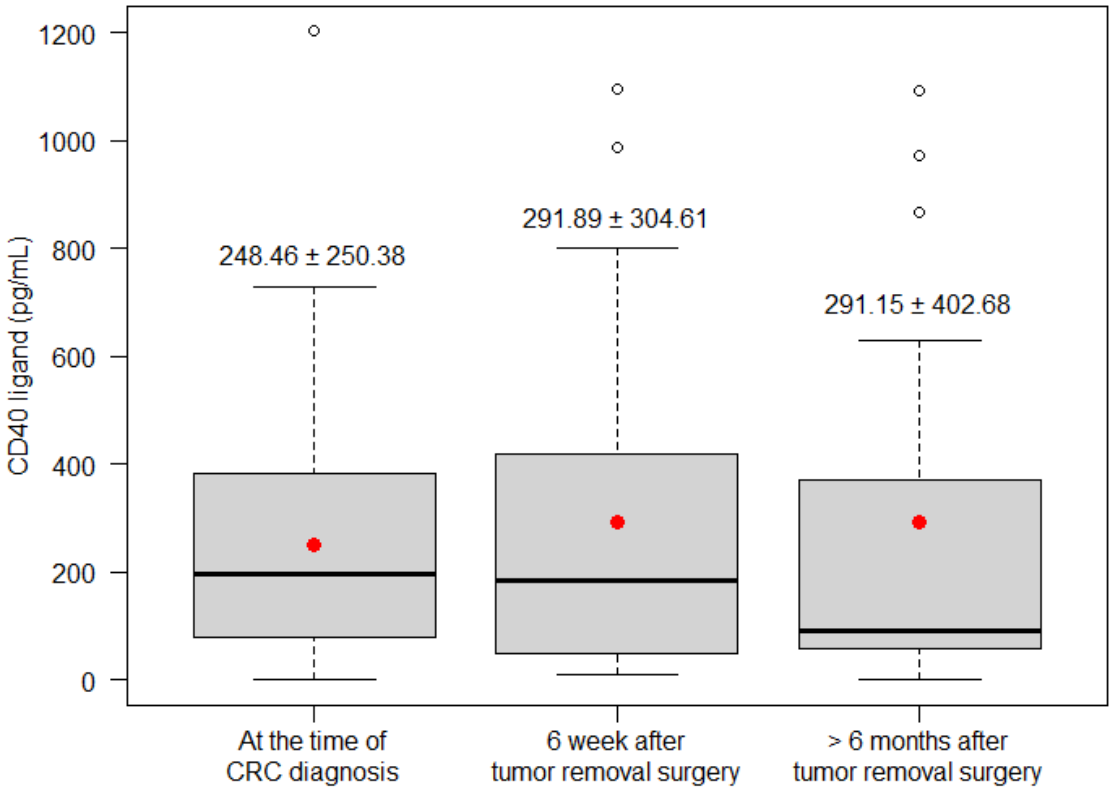

Figure 8: The CD40 ligand level of patients did not change with the course of the disease (p = 0.6813). The red dot and thick line represent mean and median values, respectively. CRC: Colorectal cancer.

Own figure.

The effect of regional metastatic lymph node or distant metastasis and thrombocytosis on plasma CD40L levels within the course of the disease was also assessed. Distant metastasis developed in an additional 14 CRC patients following the surgical removal of the primary tumor. After the surgical resection of the primary tumor, the CD40L level of CRC patients with distant metastasis ($p = 0.6964$) or thrombocytosis ($p = 0.7829$) did not change over time. However, the same increased level could be observed throughout the observation time (stage M0 vs. M1: $p = 0.0326$; with or without thrombocytosis: $p = 0.0008$), as described at the baseline. The strong association between CD40L level and platelet count ($p = 0.0002$) and IL-6 level ($p = 0.0012$), observed at baseline measurements, also persisted throughout the whole time of the study.

4.2.3. Survival analysis of CD40 ligand measurements

Thirty of the 106 patients (28.3%) died during the study. The causes of deaths were postoperative complications, infection, and CRC-related in 4, 1 and 25 cases, respectively. Both pre- and postoperative data had been analyzed, and 106 and 61 cases were used for the calculations, respectively. Patients with higher preoperative plasma CD40L level (HR: 1.001; 95% CI: 1.000-1.002; $p = 0.0159$), plasma IL-6 level (HR: 1.020, 95% CI: 1.010-1.030, $p = 0.0001$), and platelet count (HR: 1.003, 95% CI: 1.001-1.005, $p = 0.0052$) had significantly shorter OS, while preoperative plasma TPO ($p = 0.5550$) did not affect OS in the univariate models. In a multivariate setting, IL-6 had the most prominent effect on OS (HR: 1.024, 95%CI: 1.010-1.039, $p = 0.0012$), while CD40L (95% CI: 0.9995-1.0020) and platelet count (95% CI: 0.9996-1.0040) had marginal effect only. TPO did not affect the OS of patients. The same was observed for preoperative (**Figure 9A**) and postoperative (**Figure 9B**) DSS as well.

Using stratified survival models we could assume different preoperative baseline hazards for patients with or without thrombocytosis (platelet count > 400×10^9/L). In a univariate model, higher preoperative plasma CD40L level indicated poor DSS of CRC patients (HR: 1.001, 95% CI: 1,000-1.002, $p = 0.0332$). However, only a marginal effect was found in multivariate models (HR: 1.001, 95% CI: 0.9998-1.002, $p = 0.1196$). Neither platelet count (univariate: $p = 0.3310$; multivariate: $p = 0.6237$), nor TPO (univariate: $p = 0.9440$; multivariate: $p = 0.5387$) affected patient survival if stratification by thrombocytosis had been applied. The strong effect of IL-6 on survival could be

demonstrated even by the elimination of the effect of thrombocytosis (univariate: p = 0.0016; multivariate: p = 0.0103).

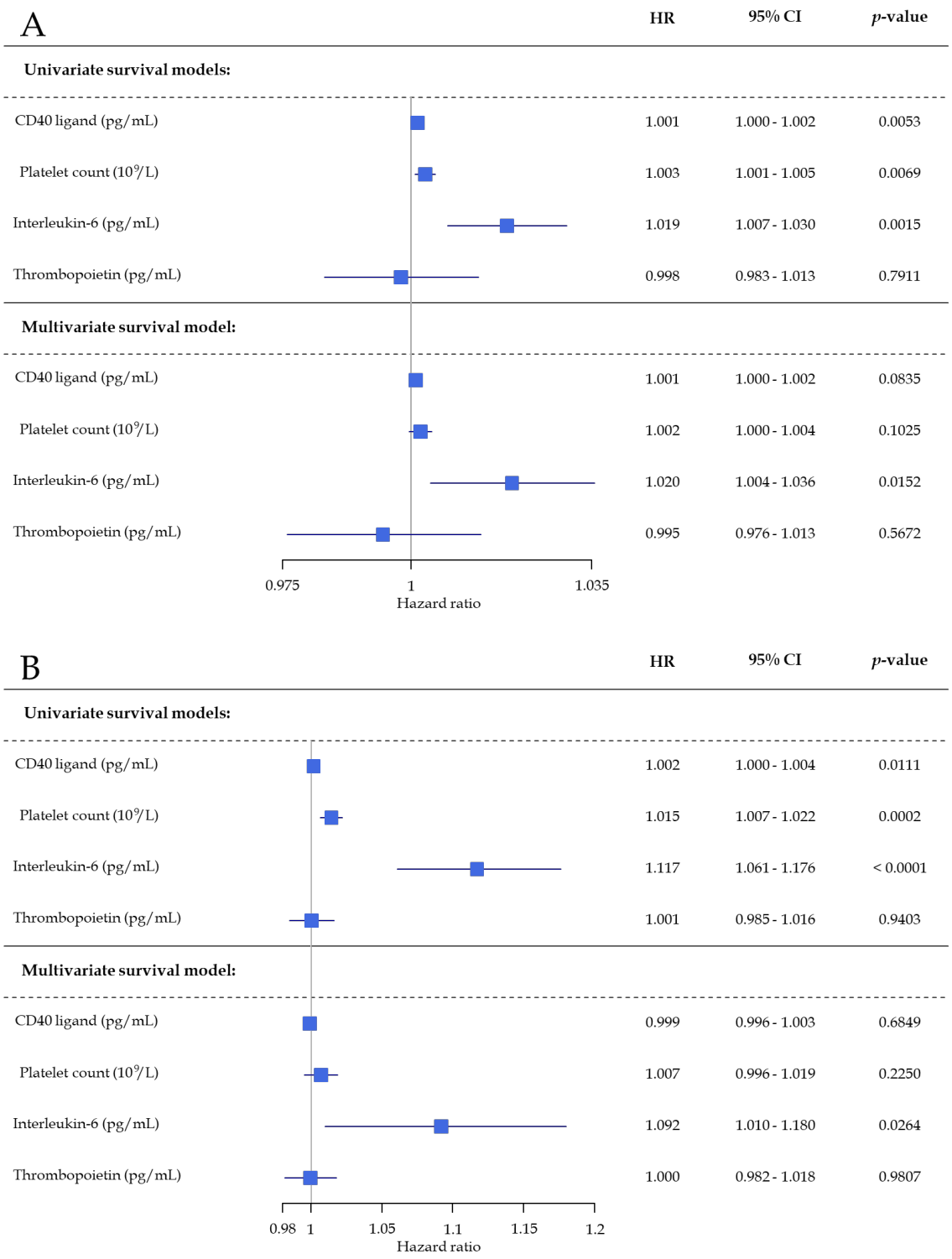

Figure 9: Results of univariate and multivariate analysis on disease-specific survival of colorectal cancer patients concerning preoperative (**A**) and postoperative (**B**) laboratory results. CI: confidence interval; HR: hazard rate. Own figure.

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5. Discussion

5.1. Paraneoplastic thrombocytosis in colorectal cancer

In CRC, the occurrence of thrombocytosis, either prior or after the primary tumor removal surgery, is associated with shorter survival of patients (19, 21, 22, 114-116). The background of thrombocytosis may vary due to several factors, including the bleeding- (reactive thrombocytosis) or metabolic changes (paraneoplastic thrombocytosis) of the tumor. In the latter, the tumor produces cytokines in higher quantities; and those higher cytokine concentrations stimulate hepatic TPO production, which ultimately results in the overproduction of platelets (103, 117). A platelet count of $> 400 \times 10^9$/L or $> 450 \times 10^9$/L are the most common definition of thrombocytosis (21, 42, 118), but several previous studies reported other cut-off values (21) indicating that the definition of thrombocytosis may not be perfect. In a previous work (119) we have reported that a personalized, relative platelet measure can predict disease outcome significantly better than "traditional" thrombocytosis, which further strengthened that the definition of thrombocytosis needs to be revised. Biochemical detection of paraneoplastic thrombocytosis in CRC is a novel research area (120). In the current study, we observed higher IL-6 and TPO level of CRC patients, compared to those of control subjects, supporting the presence of paraneoplastic thrombocytosis, which was further strengthened by the result of correlation and survival analysis, in line with previous findings (19, 21, 22, 115, 116, 120-122).

There is a strong relationship between CRC and various cytokines, including IL-6, which can play significant roles both in the development and progression of the disease (123-125). Specifically, IL-6 is known to for its significant role in tumor proliferation, migration, induction of microsatellite instability, and angiogenesis (126, 127). It has been reported that IL-6 is produced by cancer-associated mesenchymal stem cells (128). When combined with glycoprotein 130 (gp130, synonyms: IL6ST or CD130) IL-6 can regulate disease progression through the Shp2-Ras-ERK, JAK1/2-STAT3 and PI3K-Akt-mTOR pathways and its higher serum levels were associated with larger tumor size, presence of metastases and worse overall- and disease-free survival (123, 124, 129, 130). Due to its strong connection with CRC, IL-6 has been proposed as a good prognostic marker of CRC (131-133). In the current study, we could also confirm the prognostic role of IL-6 over patient survival. The result that preoperative IL-6 did not affected survival was

possibly due to the heterogeneity of the study population, which was somewhat confirmed by the results obtained from the multivariate Cox models, as the predictive effect of IL-6 on survival increased. As a novel result, we found that constantly higher IL-6 level could have been observed in those patients, who died within the first three years after CRC diagnosis.

Research on CRC and TPO is limited. Previous studies have identified that cancer cells can induce circulating TPO production (43), furthermore, higher TPO level is associated with gastrointestinal cancers and a possible relationship between more advanced clinical stages and TPO has been also suggested (28, 134). Similar to the observations above, we found that the TPO level of CRC patients is higher than those of healthy subjects, however, the effect of TPO over survival was negligible and no change with the course of the disease could have been identified in either of our longitudinal models.

5.2. Type 2 diabetes mellitus and colorectal cancer

T2DM, similarly to (paraneoplastic) thrombocytosis, has a known negative effect on CRC (80, 88). Several potential mechanisms link the two diseases together (81), including the increased plasma glucose levels (hyperglycemia), the presence of insulin resistance and hyperinsulinemia, increased insulin-like growth factor-1 levels, increased oxidative stress, higher cytokine concentrations and increased platelet activation (77, 78, 80, 81, 85). CRC is known to have an increased incidence in T2DM patients compared to those of within the healthy population (77, 78), a 1.3 – 1.5-fold increased risk of CRC has been reported (84-86, 135, 136). T2DM has been described to have a negative effect on overall-, cancer-specific-, disease-free- and recurrence-free survival of CRC patients (89, 90). In contrast to previous findings, we found that T2DM did not affected patient survival in the current study statistically, but it has to be noted, that the percentage of patients who died was higher in the CRC+T2DM group (24% vs 40%), and similarly, the survival curves also suggested a tendency toward significant difference. The effect of T2DM on patient survival was further investigated through the presence of diabetic complications, the preoperative HbA_{1C} level and the duration of T2DM. Despite the fact that the duration of diabetes was basically twice as long in patients of the T2DM group, a greater number of diabetic complications were present, and a more significant proportion of patients

required (intensive) insulin therapy, no significant effect could be justified for any of the above-mentioned parameters. We hypothesize, that the reason of the lower HbA_{1C} and serum glucose level of the CRC+T2DM group can be due to the longer duration of T2DM in this group. It is a known fact that the longer the duration of T2DM, the more complication, worse metabolic status will occur (67, 68). It has to be mentioned though that the sample size of CRC+T2DM patients was low, but we could not even prove tendentious differences similar to that of observed in the case of the survival analyses.

5.3. The combined effect of paraneoplastic thrombocytosis and T2DM in CRC

The relationship between CRC-related paraneoplastic thrombocytosis and T2DM through biochemical measurements has not been investigated previously. It has to be mentioned though, that various platelet abnormalities, increased IL-6 and TPO production is known in T2DM (92-94, 137-139). Due to the above-mentioned effects and the high risk of cardiovascular events, the usage of platelet aggregation inhibition therapy is very common in T2DM (140, 141). Based on the available literature, our pre-study hypothesis was that CRC patients who also suffer from T2DM would probably have higher IL-6 and TPO levels than those who are not affected by T2DM. In contrast to our hypothesis, no differences could have been justified in any of the parameters related to paraneoplastic thrombocytosis between the CRC and CRC+T2DM groups. Furthermore, platelet aggregation inhibition also did not have any effect on the parameters of paraneoplastic thrombocytosis even though that the therapy was more common within the CRC+T2DM group. Similarly, the diabetes-related parameters also did not affect the paraneoplastic thrombocytosis related parameters. The fact, that plasma TPO level of non-CRC T2DM patients was more similar to those of CRC patients than those of control subjects was most likely related to the already known fact that TPO levels are higher in T2DM (137), but no previous data is available how similar these values of CRC and T2DM patients should be. The presented data suggests that T2DM do not increases the effect of CRC-related paraneoplastic thrombocytosis, in most probability, and the disease-worsening effect of T2DM, which have been described in previous publications (83), must be carried out through other factors. To identify those factors, further investigations are needed.

5.4. CD40 ligand in colorectal cancer

The role of CD40L in neoplastic diseases is controversial (58). Cellular model studies have revealed that it can significantly contribute to the immunological activity against cancer, while other studies have reported the complete opposite, *i.e.* that CD40L contributes to the progression and growth of the tumor (65, 142). The most promising results and antitumor effects have been observed in melanomas and hematological malignancies (58), including enhanced protection of dendritic cells against apoptosis-inducing factors of tumor cells, enhanced maturation and antigen production of B cells, increased T cell-dependent immune response and the CD40L activation-dependent apoptosis of cancer cells (58). Moreover, CD40- and CD40L-based drugs have been developed recently, and active clinical trials are currently investigating their efficacy (143).

Previous clinical studies revealed that high CD40 expression and higher soluble CD40L concentration are associated with CRC (59, 64), and these elevations are the most prominent with the presence of lymph node metastasis (60, 64, 144), venous invasion (62), and higher TNM stages (60, 64, 144). In vitro stimulation of $CD3^+$, $CD4^+$, and $CD8^+$ T cells of CRC patients resulted in a significantly increased, approximately four-times higher, CD40L expression compared to those of healthy control subjects (145). In contrast, Tada *et al.* (62) and Lima et al. (63) observed lower soluble CD40L levels within those CRC patients with worse clinicopathological features. To our knowledge, no previous study investigated CD40L levels with the course of the disease, and only partial data are available from the study of Tada et al. (62). In that study, rectal cancer patients received neoadjuvant chemoradiotherapy prior to the surgical removal of the tumor, and CD40L was measured before and after the neoadjuvant treatment. They found that the post-treatment CD40L level of patients with a high response rate to the treatment was significantly lower, while no change was observed in those patients with low response rates (62). Results of the current study confirmed the observations of those former studies (60, 64, 144) where circulating CD40L level was tendentiously higher in CRC patients than those of control patients. We also observed the highest measurements in Stage IV cancer and found that the CD40L level of CRC patients is basically constant with the course of the disease. The initial differences in CD40L levels between those patients with or without distant metastasis or thrombocytosis were observable throughout the whole

course of the disease. The latter observation showed, that the CD40L level was the highest in those patients with more advanced disease. This should be the cause behind higher pre- and postoperative CD40L levels associated with shorter survival of patients, with high probability. Similar to our findings, the highest soluble CD40L levels have been observed in patients with distant metastases in squamous cancer or adenocarcinoma of the lung (146), in nasopharyngeal carcinoma (147), and in gastric cancer (148).

Approximately 95% of the soluble form of CD40L is thought to be derived from platelets (149, 150). Soluble CD40L level is strongly correlated with platelet count (151). The highest levels can be measured in reactive thrombocytosis and essential thrombocythemia, while the lowest values can be measured in patients with low platelet count (151). As previously detailed, thrombocytosis is associated with several cancers (21, 22, 42), and the platelet count is usually higher in advanced stages (21). CD40L positively correlates with platelet count in patients with a high response rate to neoadjuvant chemoradiotherapy (62). An assumption was made by Huang *et al.* (144) that in cancer patients soluble CD40L is most probably derived from activated platelets than from T cells. However, this question was never further investigated. Our data showed that CD40L level is positively correlated with several markers of (paraneoplastic) thrombocytosis, in particular with platelet count and IL-6. This strong connection persisted throughout the whole observation period. It has to be mentioned though that the stratification used in our survival models should have fully eliminated the significant effect of CD40L on CRC survival. However, we could not demonstrate this expected effect, which was observed, *e.g.*, in the case of platelet count. This, together with the weaker explanatory powers observed in our linear models suggests that the increase in CD40L levels is possibly not only influenced by (paraneoplastic) thrombocytosis alone. Increased CD40L production is known in various diseases characterized by general inflammation, like atherosclerosis, diabetes, or inflammatory bowel disease (152-155). CRC can also be described as a disease known for its general inflammation (156), high IL-6 level (156), and inadequate T cell activation (144). Furthermore, increased inflammation is also associated with metastasis (157, 158). The strong correlation between CD40L, IL-6, and metastases hints that the answer may be sought in the increased inflammation caused by the tumor or its metastases. To clarify this question, further investigations are needed.

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6. Conclusions

The aim of our study was to better understand the relationship between paraneoplastic thrombocytosis, T2DM, and CRC, through a set of biomarkers, including platelet counts, IL-6, TPO, and CD40L. The following conclusions can be draw from the results:

1. Platelet counts, plasma IL-6, TPO, and CD40L levels are higher in CRC patents, compared to those of control subjects.

2. The highest platelet count, plasma IL-6, TPO, and CD40L level can be observed in those CRC patients with advanced disease.

3. CRC patients having thrombocytosis (platelet count > 400 × 10^9/L) have higher plasma CD40L level.

4. Supporting the presence of paraneoplastic thrombocytosis, there are strong connections between
    a. Platelet count and IL-6,
    b. Platelet count and TPO,
    c. Platelet count and CD40L, and
    d. IL-6 and CD40L.

    These relationships persisted throughout the whole observation period when investigated using longitudinal analysis methods.

5. Constantly higher IL-6 level could be observed in those patients, who died within the first three years after CRC diagnosis.

6. Plasma TPO and CD40L level of study subjects were statistically constant throughout the observation period of the study.

7. The initial differences in CD40L levels between those patients with or without distant metastasis or thrombocytosis are observable throughout the whole course of the disease.

8. Higher platelet counts, and plasma IL-6 and CD40L levels are associated with shorter survival times of CRC patients, of which IL-6 has the strongest effect.

9. Neither TPO, nor T2DM affected the survival of CRC patients.

10. No differences can be justified in any of the parameters related to paraneoplastic thrombocytosis between the CRC and CRC+T2DM groups, suggesting that T2DM do not exert its disease worsening effect through an exacerbation of paraneoplastic thrombocytosis.

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7. Summary

Background: A large variety of factors can affect colorectal cancer (CRC) survival, including type 2 diabetes mellitus (T2DM) and paraneoplastic thrombocytosis. The majority of circulating CD40 ligand (CD40L) is thought to be is produced by platelets, and CD40L level is increased in CRC. The relationship between paraneoplastic thrombocytosis, T2DM and CRC was never investigated previously.

Methods: A prospective, real-life observational cohort study was conducted with the inclusion of 108 CRC patients and 166 voluntary non-CRC subjects. In addition to routine histopathological and laboratory parameters, plasma interleukin-6 (IL-6), thrombopoietin (TPO) and CD40L level was measured at 4 times (baseline, 6 weeks, 6 and 12 months).

Results: Study participants were divided into cohorts based on the presence of T2DM. Platelet count ($p < 0.0500$), IL-6 ($p < 0.0100$) and CD40L ($p = 0.0479$) level was significantly higher in the CRC groups. TPO was higher in the T2DM, CRC and CRC+T2DM groups ($p < 0.0500$). Strong connection was found between platelet counts and IL-6 ($p = 0.0009$), TPO ($p = 0.0332$), and CD40L ($p = 0.0045$), and between IL-6 and CD40L ($p = 0.0130$).

Analysis of parameter changes with the course of the disease revealed that higher platelet counts and IL-6 levels were associated with an increase risk for cancer-related death. TPO and CD40L levels were statistically constant throughout the whole observation period. Survival models revealed that of the investigated parameters IL-6 had the greatest effect over patient survival, followed by platelet counts and CD40L levels, while TPO had no effect on survival times. No differences could be justified when comparing any of the parameters related to paraneoplastic thrombocytosis in CRC patients with and without T2DM. Moreover, no association could be justified between T2DM and CRC survival.

Conclusion: While the independent, disease-worsening effect of paraneoplastic thrombocytosis and T2DM is known, the coexistence of the two did not further impair the survival of CRC patients, suggesting that T2DM has no significant effect over paraneoplastic thrombocytosis. There is a strong relationship between platelet counts, CD40L, IL-6, TPO level, and the thrombocytosis of CRC patients.

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9. Bibliography of the candidate's publications

9.1. Publications Related to the Dissertation

Herczeg, Gy, Somogyi A, Herold M, Fodor Á, Rosta K, Dank M, Lang Zs, Herold Z. (2022) Does diabetes affect paraneoplastic thrombocytosis in colorectal cancer? *Open Med*, 17:160–173. **IF: 2.100**, SJR: Q3.

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Research Article

Gyorgy Herczeg, Aniko Somogyi, Magdolna Herold, Agnes Fodor, Klara Rosta, Magdolna Dank, Zsolt Lang, Zoltan Herold*

Does diabetes affect paraneoplastic thrombocytosis in colorectal cancer?

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Abstract

Background – A large variety of factors can affect colorectal cancer (CRC) survival, including type 2 diabetes mellitus (T2DM) and paraneoplastic thrombocytosis. Although several common factors play a role in their development and platelets are damaged in both diseases, the combined relationship of the three conditions was never investigated previously.

Methods – A prospective, real-life observational cohort study was conducted with the inclusion of 108 CRC patients and 166 voluntary non-CRC subjects. Plasma interleukin-6 and thrombopoietin levels were measured.

Results – Study participants were divided into cohorts based on the presence of T2DM. Platelet count ($p < 0.0500$) and interleukin-6 ($p < 0.0100$) level were significantly higher in the CRC groups. Thrombopoietin level was higher in the T2DM, CRC, and *CRC + T2DM* groups ($p < 0.0500$). Analysis of parameter changes over time and survival models revealed that neither platelet count, interleukin-6, nor thrombopoietin levels were affected by T2DM. Death of patients was associated with higher baseline platelet count ($p = 0.0042$) and interleukin-6 level ($p < 0.0001$).

Conclusion – Although the independent, disease-worsening effect of paraneoplastic thrombocytosis and T2DM is known, the coexistence of the two did not further impair the survival of CRC patients, suggesting that T2DM has no significant effect over paraneoplastic thrombocytosis.

Keywords: colorectal neoplasms, diabetes mellitus, type 2, interleukin-6, thrombocytosis, thrombopoietin

1 Introduction

Thrombocytosis – platelet counts above the upper value of normal range (usually $\geq 400 \times 10^9$/L) – is described as a poor prognostic sign in colorectal cancer (CRC). Both preoperative and postoperative thrombocytoses are associated with worse patient survival [1,2]. Thrombocytosis may develop for several reasons, such as the bleeding of the tumor or a metabolic change caused by the tumor itself called paraneoplastic thrombocytosis [1,3]. The proposed paracrine-signaling pathway of paraneoplastic thrombocytosis [3,4] includes the overproduction of various cytokines (e.g., interleukin-6) by the tumor, which causes increased hepatic thrombopoietin production that modulates the production of platelets within the bone marrow, ultimately resulting in an increased platelet count.

Diabetes mellitus is one of the most prevalent diseases in our time; based on the latest estimations available in the ninth edition of IDF Diabetes Atlas [5], the incidence of the disease varies between 4 and 10.4%, with over 460 million diabetes patients around the world [5]. Approximately 90% of diabetes patients are suffering from type 2 diabetes mellitus (T2DM), which develops in later ages [6], similar to that of CRC [7]. Over the age of 60 years, T2DM may occur in every fourth or fifth person [5,8], and compared to the healthy population, an increased occurrence of malignancies is confirmed in T2DM [9–11]. CRC occurs

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approximately 1.5× more often in patients with T2DM [12,13]; furthermore, the coexistence of T2DM is associated with an increased risk for a shorter survival in CRC [14,15]. Similar to CRC, T2DM can be also described by various platelet abnormalities and an increased thrombopoietin production is also known [16–18].

Although several potential mechanisms link cancers and T2DM together [19], and platelets are affected in both diseases [1–4,16–18], the relationship among T2DM, (paraneoplastic) thrombocytosis, and CRC is poorly investigated. An earlier retrospective study [20] from our group has found that a personalized thrombocytosis measure calculated from preoperative and precancer platelet counts can better predict the patient survival, and it has been associated with various clinicopathological parameters, including T2DM. Although our earlier study has revealed some possible connections between the three conditions, the exact biological effect of T2DM on CRC-related paraneoplastic thrombocytosis could not have been investigated via that retrospective manner. Therefore, a prospective, real-life observational cohort study was conducted where paraneoplastic thrombocytosis was investigated within diabetic and nondiabetic CRC cohorts through the changes in platelet counts, plasma interleukin-6, and thrombopoietin levels.

2 Methods

2.1 Patients and study design

A prospective, real-life observational cohort study was carried out. A total of 108 patients diagnosed with CRC and 166 voluntary non-CRC subjects were enrolled for the study between 2017 and 2019. CRC patients attended at both the Department of Internal Medicine and Hematology, Semmelweis University, Budapest and the Department of General Surgery, Szent Imre University Teaching Hospital, Budapest. Written informed consent was collected from all study participants before performing any study-specific procedures. Exclusion criteria included age <18 years, any previous malignancies, known inflammatory bowel- and/or chronic kidney- and/or systemic autoimmune- and/or inadequately controlled thyroid- and/or hematologic- and/or any mental diseases, the usage of erythropoiesis-stimulating agents and/or recent blood transfusion, and patients with an Eastern Cooperative Oncology Group (ECOG) performance status >2.

Voluntary non-CRC subjects consisted of healthy young controls, older controls, and T2DM patients. Rationale of the inclusion of healthy young subjects was that some of the measured parameters have no reference values and that the older volunteers included both completely healthy individuals and nonmetabolic disease patients (e.g., hypertension). T2DM patients attended at the Metabolic Outpatient Clinic of the Department of Internal Medicine and Hematology, Semmelweis University, Budapest. In addition to the exclusion criteria described by CRC patients above, voluntary nondiseased subjects were excluded in the presence of prediabetes or any other metabolic disorders.

2.2 Clinical and laboratory data measurements

Anamnestic data including comorbidities and recent medications, body weight, and height were collected, and fasting blood samples were drawn according to the following protocol: (1) at the diagnosis of CRC, before any oncological treatments or the surgical resection of the primary tumor, (2) at least 6 weeks, (3) 6 months, and (4) 12 months after the tumor removal surgery. At the time of postoperative measurements, oncological treatments were stopped/paused only as part of the patients' standard of oncological care, and sampling was performed only at least 6 weeks after the last treatment due to the known platelet influencing effects of several cytotoxic regimens [21,22]. If it was not feasible to stop/pause the oncological treatment, in the best interest of the patient, the visit was omitted. Complete blood count, aspartate- and alanine transaminase, gamma-glutamyl transferase, plasma glucose, and creatinine were measured at the Central Laboratory of Semmelweis University and at the Central Laboratory of Szent Imre University Teaching Hospital. Estimated glomerular filtration rate was calculated using the Chronic Kidney Disease-Epidemiology Collaboration equations [23]. Plasma interleukin-6 and thrombopoietin levels were measured using the ELECSYS® Interleukin-6 electrochemiluminescence immunoassay (ECLIA) (Roche Diagnostics GmbH, Mannheim, Germany) and the Human Thrombopoietin Quantikine® enzyme-linked immunosorbent assay (ELISA) (catalog number: DTP00B, R&D Systems, Minneapolis, MN, USA) kits, respectively. Plasma thrombopoietin level was measured from platelet-poor plasma, as per manufacturer description.

Side of CRC was described as right sided if the tumor was originating from cecum, ascending colon, and proximal two-third of the transverse colon and left sided if originating from the distal one-third of the transverse colon, descending colon, sigmoid colon, and rectum [24]. Staging was given by histopathological examination

of surgical specimens and imaging studies; the American Joint Committee on Cancer grouping was used [25]. The usage of biological agents was recorded as a dummy variable and chemotherapy was grouped as adjuvant if no metastasis and first-line, second-line, etc., if metastasis was present. The overall survival of patients was defined as the length of time from the date of CRC diagnosis until death. Follow-up of cancer patients was terminated on January 31, 2021, patients alive at this time point were right censored.

2.3 Statistical analysis

Statistical analysis was performed with R version 4.0.5 [26]. Wilcoxon–Mann–Whitney U test, Fisher's exact test, Kruskal–Wallis test with p-value corrected pairwise Wilcoxon–Mann–Whitney U tests as post-hoc, and Spearman rank correlation were used. To detect the changes of various parameters in time, linear mixed effect models were used (R-package *nlme* [27]). Survival of patients was analyzed for both preoperative and longitudinal data with Cox regression models and Bayesian univariate and multivariate joint survival models (R-package *rstanarm* [28]), respectively. $p < 0.05$ was considered as statistically significant, and p-values were corrected with the Holm method [29] for the problem of multiple comparisons. Compared to the "conventional" frequentist methods, Bayesian methods give only a probability distribution for the investigated parameter but no p-values. Interpretation of joint model results was as follows. If the Bayesian equivalent to the frequentist confidence interval (CI), the 95% credible interval contained the hazard ratio (HR) of 1, the model was considered as clinically not relevant. However, if the 95% credible interval was less than or more than HR = 1, the effect of the parameter was considered as a good or bad sign of patient survival, respectively. Results were expressed as mean ± standard deviation and as the number of observations (percentage) for continuous and count data, respectively. Naïve Kaplan–Meier and longitudinal survival curves were drawn with the R-package *survminer* (Kassambara, Kosinski, and Biecek, version 0.4.9, 2021) and the built-in methods of *rstanarm*, respectively.

Informed consent: Informed consent has been obtained from all individuals included in this study.

Ethical approval: The study was approved by the Regional and Institutional Committee of Science and Research Ethics, Semmelweis University (SE TUKEB 21-12/1994, approval date of latest modification: February 10, 2017), by the Institutional Review Board of Szent Imre University Teaching Hospital (SZIK IKEB 5/2017), and by the Committee of Science and Research Ethics, Hungarian Medical Research Council (ETT TUKEB 8951-3/2015/EKU).

3 Results

A total of 274 study participants were included: 108 CRC patients and 166 voluntary, non-CRC subjects. CRC patients were divided into two cohorts based on the presence of T2DM. Patients without diabetes ($n = 82$) were assigned to the "*CRC*" group, whereas patients with a history of T2DM ($n = 26$) were assigned to the "*CRC + T2DM*" group. Duration of diabetes was 6.88 ± 6.10 years, from which T2DM was diagnosed in four cases around the time of CRC diagnosis.

In most parameters, the two tumor groups did not differ from each other, whereas plasma glucose was significantly higher ($p = 0.0018$), and tendentiously higher white blood cell-, neutrophil-, eosinophil-, and monocyte counts were observed in the *CRC + T2DM* group. Platelet aggregation inhibition therapy was more common in *CRC + T2DM* patients; no difference was found in radiotherapy (neoadjuvant only), whereas no-line or late-line chemotherapy was more common in those CRC patients with T2DM than those of without ($p = 0.0560$; Table 1).

Voluntary non-CRC subjects were divided into three cohorts: 51, 50, and 65 subjects were assigned to the "*Young controls*," "*Control*," and "*T2DM*" groups, respectively. Duration of diabetes was 14.91 ± 9.50 within the *T2DM* group. Anamnestic, clinical, and laboratory parameters of control groups are shown in Table 2. Comparison between the volunteer groups revealed that, as expected, *Young controls* had the lowest body mass index ($p < 0.0001$ vs *T2DM* and *Controls*), systolic blood pressure ($p = 0.0250$ vs *Controls*, $p = 0.0410$ vs *T2DM*), mean corpuscular volume ($p = 0.0002$ vs *Controls*, $p = 0.0041$ vs *T2DM*), red blood cell distribution width ($p = 0.0023$ vs *Controls*, $p < 0.0001$ vs *T2DM*), fasting plasma glucose ($p < 0.0001$ vs *Controls* and *T2DM*), and the highest heart rate ($p = 0.0002$ vs *Controls*, $p = 0.0242$ vs *T2DM*) and estimated glomerular filtration rate ($p < 0.0001$ vs *Controls* and *T2DM*). T2DM patients had the highest body mass index ($p = 0.0098$ vs *Controls*, $p < 0.0001$ vs *Young controls*), white blood cell count ($p = 0.0430$ vs *Controls*, $p = 0.0008$ vs *Young controls*), gamma-glutamyl transferase ($p = 0.0633$ vs *Controls*, $p = 0.0003$ vs *Young*

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Table 1: Metabolic, clinical, and other parameters of CRC patients

Parameter	CRC (n = 82)	*CRC + T2DM* (n = 26)	p-value
Age (years)	67.42 ± 9.33	70.83 ± 7.24	0.3919
Sex (male:female)	51:31 (62.2:37.8%)	20:6 (76.9:23.1%)	0.3778
Body mass index (kg/m^2)	27.26 ± 3.87	27.43 ± 4.64	0.7778
Systolic blood pressure (mmHg)	144.49 ± 18.48	141.12 ± 19.55	0.7654
Diastolic blood pressure (mmHg)	78.99 ± 10.19	78.20 ± 9.10	0.7527
Heart rate (1/min)	77.88 ± 13.76	80.76 ± 16.36	0.7654
White blood cell count (10^9/L)	8.14 ± 2.91	11.11 ± 7.46	0.3919*
Neutrophil count (10^9/L)	5.60 ± 2.57	7.87 ± 5.59	0.3919*
Eosinophil count (10^9/L)	0.18 ± 0.16	0.59 ± 1.73	0.3919*
Basophil count (10^9/L)	0.06 ± 0.05	0.06 ± 0.04	0.7654
Monocyte count (10^9/L)	0.61 ± 0.46	0.78 ± 0.51	0.3919*
Lymphocyte count (10^9/L)	1.74 ± 1.11	1.81 ± 0.92	0.7654
Red blood cell count (10^{12}/L)	4.50 ± 0.58	4.42 ± 0.52	0.7527
Hemoglobin (g/dL)	12.42 ± 2.14	12.14 ± 2.11	0.7654
Hematocrit (L/L)	0.38 ± 0.06	0.38 ± 0.06	0.7778
Mean corpuscular volume (fL)	84.84 ± 8.22	84.31 ± 8.54	0.9471
Mean corpuscular hemoglobin (pg)	27.30 ± 3.41	27.32 ± 3.81	0.9853
Mean corpuscular hemoglobin concentration (g/L)	322.35 ± 15.29	324.20 ± 24.53	0.7527
Red blood cell distribution width (%)	14.63 ± 3.74	15.53 ± 3.47	0.4409
Platelet count (10^9/L)	308.38 ± 124.66	339.85 ± 118.86	0.7527
Aspartate transaminase (U/L)	25.86 ± 21.03	26.37 ± 16.12	0.9853
Alanine transaminase (U/L)	21.75 ± 12.52	23.10 ± 13.35	0.7654
Gamma-glutamyl transferase (U/L)	68.33 ± 117.56	97.24 ± 167.79	0.7527
Plasma glucose (mmol/L)	5.45 ± 0.86	6.62 ± 1.71	0.0018
Creatinine (μmol/L)	76.99 ± 19.19	82.33 ± 22.41	0.7527
Estimated glomerular filtration rate $\left(\frac{\text{mL}}{\text{min} \cdot 1.73\ \text{m}^2}\right)$	82.17 ± 16.81	78.23 ± 18.30	0.7527
AJCC staging [25]			
Stage I	19 (23.2%)	9 (34.6%)	0.4021
Stage II	23 (28.0%)	4 (15.4%)	
Stage III	20 (24.4%)	4 (15.4%)	
Stage IV	20 (24.4%)	9 (34.6%)	
Side of CRC			
Left-sided	60 (73.2%)	15 (57.7%)	0.2987
Right-sided	22 (26.8%)	11 (42.3%)	
Chemotherapy			
None	37 (45.1%)	16 (61.5%)	0.0560**
Adjuvant	21 (25.6%)	2 (7.7%)	
First-line	12 (14.6%)	0 (0.0%)	
Second-line	7 (8.5%)	7 (26.9%)	
Third or later-line	5 (6.1%)	1 (3.8%)	
Radiotherapy	14 (17.1%)	3 (11.5%)	0.7568
Usage of biological therapy	15 (18.3%)	7 (26.9%)	0.4611
Known comorbidities			
Hypertension	48 (58.5%)	20 (76.9%)	0.2851
Major cardiovascular event(s) before CRC	16 (19.5%)	6 (23.1%)	0.7809
Platelet aggregation inhibition	14 (17.1%)	9 (34.6%)	0.2851*

T2DM: type 2 diabetes mellitus. *Without p-value correction, the differences are marginal (white blood cells: p = 0.0843; neutrophils: p = 0.0919; eosinophils: p = 0.0648; monocytes: p = 0.0918; platelet aggregation inhibition therapy: p = 0.0959). **Without p-value correction, the differences in the distribution of chemotherapy regimens is statistically significant (p = 0.0070).

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Table 2: Metabolic, clinical, and other parameters of voluntary control subjects

Parameter	Young controls (n = 51)	Control (n = 50)	T2DM (n = 65)
Age (years)	26.13 ± 4.50[1]	60.31 ± 11.00[4]	64.68 ± 8.30[7]
Sex (male:female)	24: 27 (47.1:52.9%)	22: 28[7] (44.0:56.0%)	35: 30 (53.8:46.2%)
Body mass index (kg/m^2)	23.25 ± 3.95[1]	27.99 ± 5.42[6]	30.73 ± 5.12[1]
Systolic blood pressure (mmHg)	129.82 ± 15.60	138.27 ± 17.34	136.58 ± 16.64
Diastolic blood pressure (mmHg)	77.27 ± 9.10	80.98 ± 11.85	76.06 ± 10.83
Heart rate (1/min)	77.59 ± 11.08	67.76 ± 7.95[1]	73.23 ± `9.18
White blood cell count (10^9/L)	6.48 ± 1.59[2]	7.04 ± 2.00[7]	7.97 ± 2.23
Neutrophil count (10^9/L)	3.66 ± 1.37[2]	4.35 ± 1.68[4]	4.93 ± 1.82[7]
Eosinophil count (10^9/L)	0.19 ± 0.17	0.18 ± 0.13	0.21 ± 0.13
Basophil count (10^9/L)	0.06 ± 0.08	0.06 ± 0.03	0.06 ± 0.05
Monocyte count (10^9/L)	0.44 ± 0.15[2]	0.44 ± 0.11[2]	0.52 ± 0.14
Lymphocyte count (10^9/L)	2.09 ± 0.66[3]	2.15 ± 0.77[3]	2.25 ± 0.80[3]
Red blood cell count (10^{12}/L)	4.97 ± 0.53[4]	4.92 ± 0.53[4]	4.81 ± 0.38[4]
Hemoglobin (g/dL)	14.60 ± 1.35[4]	14.69 ± 1.38[4]	14.07 ± 1.04[4]
Hematocrit (L/L)	0.43 ± 0.04[4]	0.44 ± 0.04[4]	0.42 ± 0.03[4]
Mean corpuscular volume (fL)	86.27 ± 3.00[5]	89.32 ± 4.18[3]	88.04 ± 3.44
Mean corpuscular hemoglobin (pg)	29.49 ± 1.39[3]	30.02 ± 1.61[4]	29.32 ± 1.47[3]
Mean corpuscular hemoglobin concentration (g/L)	339.16 ± 9.73[2]	337.18 ± 8.40[4]	332.95 ± 8.93[3]
Red blood cell distribution width (%)	12.59 ± 0.79[1]	13.15 ± 0.92[7]	13.23 ± 0.83[7]
Platelet count (10^9/L)	267.86 ± 50.18	272.18 ± 72.66	263.92 ± 67.23
Aspartate transaminase (U/L)	27.47 ± 13.96	24.64 ± 6.49	25.34 ± 9.82
Alanine transaminase (U/L)	25.08 ± 14.77	24.88 ± 11.32[3]	27.91 ± 14.86
Gamma-glutamyl transferase (U/L)	25.06 ± 12.56[6]	32.70 ± 31.24	40.64 ± 29.40
Plasma glucose (mmol/L)	4.58 ± 0.45[1]	4.99 ± 0.57[1]	8.17 ± 2.63[1]
Creatinine (μmol/L)	73.69 ± 12.72	70.20 ± 12.72	75.11 ± 19.05
Estimated glomerular filtration rate $\left(\frac{\text{mL}}{\text{min} \cdot 1.73\ \text{m}^2}\right)$	109.33 ± 14.97[1]	89.66 ± 12.42	83.98 ± 16.05
Known comorbidities			
Hypertension	0 (0.0%)[1]	16 (32.0%)[1]	56 (86.2%)[3]
Major cardiovascular event(s) prior visit date	0 (0.0%)[2]	5 (10.0%)	13 (20.0%)
Platelet aggregation inhibition	0 (0.0%)[2]	6 (12.0%)[6]	50 (76.9%)[4]

T2DM: type 2 diabetes mellitus, [1]$p < 0.05$ vs all other four groups, [2]$p < 0.05$ vs all diseased groups, [3]$p < 0.01$ vs the CRC without T2DM group, [4]$p < 0.01$ vs both tumor groups, [5]$p < 0.05$ vs Control and T2DM groups, [6]$p < 0.05$ vs T2DM, and [7]$p < 0.01$ vs the CRC with T2DM group.

controls) and fasting plasma glucose ($p < 0.0001$ vs *Controls* and *Young controls*). Significant differences of comparisons among all five groups have been indicated in Table 2.

The duration of diabetes was shorter within the *CRC + T2DM* group compared to those of the *T2DM* group. A higher proportion of oral antidiabetic drug usage and diet-only therapy was observable within the *CRC + T2DM* group, whereas the need for insulin therapy was greater in those within the *T2DM* group ($p = 0.0006$). Furthermore, lower glycated hemoglobin (HbA_{1C}) level and fewer diabetic complications were found in the *CRC + T2DM* patients (Table 3). The occurrence of hypertension ($p = 0.7790$) and the proportion of previous major cardiovascular event(s) before the first visit ($p = 0.7004$) did not differ between the two diabetic groups. Comparison of diabetes-related parameters is summarized in Table 3.

3.1 Baseline measurement of paraneoplastic thrombocytosis-related parameters

Plasma level of paraneoplastic thrombocytosis parameters of patients was compared to those of all control groups at the time of CRC diagnosis. Highest platelet counts were observed within the two tumor groups: platelet count of *CRC* patients was significantly higher than those of within the *T2DM* group ($p = 0.0369$), whereas the platelet count of the *CRC + T2DM* group was significantly higher than all of the control groups ($p = 0.0369$ vs *Young Control* and

Table 3: Diabetes-related parameters of T2DM and *CRC + T2DM* patients

Parameter	T2DM (n = 65)	*CRC + T2DM* (n = 26)	p-value
Duration of T2DM (years)	14.91 ± 9.50	6.88 ± 6.10	0.0015
HbA_{1C} (%)	7.40 ± 1.26	6.30 ± 1.04	0.0012
Treatment used for T2DM			
Only diet	3 (4.6%)	7 (26.9%)	0.0006
Oral hypoglycemic medications	32 (49.2%)	18 (69.2%)	
Combination therapy (oral + basal insulin)	14 (21.5%)	1 (3.8%)	
Intensive insulin therapy	16 (24.6%)	0 (0.0%)	
Diabetic complications[1]			
Retinopathy	16 (24.6%)	2 (7.7%)	0.4203
Nephropathy	6 (9.2%)	0 (0.0%)	0.5319
Neuropathy	14 (21.5%)	5 (19.2%)	1.0000
Angiopathy	9 (13.8%)	1 (3.8%)	0.5420
Albuminuria	9 (13.8%)	0 (0.0%)	0.3330[2]
Number of diabetic comorbidities			
None	35 (53.8%)	20 (76.9%)	0.4203
One	15 (23.1%)	4 (15.4%)	
More than one	15 (23.1%)	2 (7.7%)	
Hyperlipidemia	44 (67.7%)	10 (38.5%)	0.1210[3]

HbA_{1C}: glycated hemoglobin, [1]All developed prior CRC, [2]Without p-value correction p = 0.0555, and [3]Without p-value correction p = 0.0173.

Control, p = 0.0278 vs *T2DM*; Figure 1a). Lowest plasma interleukin-6 levels were observed within *Young controls* (p < 0.0010 vs all other cohorts). Subjects of the *Control* and *T2DM* groups had similar interleukin-6 levels; and interleukin-6 was significantly higher in both tumor groups, compared to all of those observed in control groups (p < 0.0001 *CRC* vs *Control* and *T2DM*, p = 0.0011 *CRC + T2DM* vs *Control*, and p = 0.0069 *CRC + T2DM* vs *T2DM*; Figure 1b). Thrombopoietin level was basically the same in the *Young control* and *Control* groups, and a separate cluster was formed by the remaining three groups. The highest thrombopoietin levels were observed in the *CRC + T2DM* group (Figure 1c). No further difference could have been justified in any of the study groups if they were further subdivided by the usage of platelet aggregation inhibition therapy, antidiabetic drugs, or in the presence of any diabetic complications.

Highest platelet counts were found if interleukin-6 was high as well (*CRC*: Spearman ρ: +0.34, p = 0.0017; *CRC + T2DM*: p = 0.0786; CRC groups combined: Spearman ρ: +0.32, p = 0.0009). Correlation between platelet count and thrombopoietin levels was only significant in the *CRC* group (Spearman ρ: −0.24, p = 0.0332), whereas no (p = 0.8486) and marginal association (p = 0.0643) was found in the *CRC + T2DM* and in the two tumor groups combined, respectively. No correlation was found between interleukin-6 and thrombopoietin levels (*CRC*: p = 0.4279, *CRC + T2DM*: p = 0.9921, tumor groups combined: p = 0.5383). No correlation was found between the thrombocytosis-related parameters and the duration of T2DM or the preoperative level of HbA_{1C}.

3.2 Changes in the parameters of paraneoplastic thrombocytosis with the course of CRC

For the 108 CRC subjects, a total of 215 measurements were available. 108, 48, 37, and 22 preoperative, postoperative, 6-month, and 12-month measurements were available, respectively. Significant decrease in later measurements occurred due to the death of patients, disease progression resulting in higher ECOG performance status and patient's unavailability to attend at later visits, the need to initiate chemotherapy earlier than the postoperative visit window, or continuous chemotherapy without drug holiday after the second study visit. Due to the decreasing number of follow-ups, a more robust statistical method not sensitive to the loss of follow-up had to be chosen; therefore, it was investigated via age-corrected and stage-corrected linear mixed effect interaction models as to how T2DM and worse clinical outcome (death) affect the changes of platelet count, interleukin-6, and thrombopoietin levels with the course of the disease. Average survival time: 16.96 ± 11.43 months, all within the first 3 years after CRC diagnosis. Diabetes had no significant effect on any of the changes (platelet count: p = 0.1190; interleukin-6: p = 0.5571; thrombopoietin: p = 0.3062, Figure 2a, c, and e). Platelet counts of all patients decreased, but among those patients who died during the time of the study, the average baseline platelet count was significantly higher (273.70 vs 353.00 × 10^9/L; p = 0.0042) and a faster decrease could have been seen within the first 12 months after the tumor removal surgery compared to those of who survived (Figure 2b). Similarly, increased baseline interleukin-6 levels (5.76 vs 27.42 pg/mL; p < 0.0001) and marginally faster decreasing levels (p = 0.0613) were observed in deceased patients over time, whereas in those who survived the plasma interleukin-6 level was constant during our observation (p = 0.1273, Figure 2d). The initial thrombopoietin level did not differ between surviving and deceased patients (p = 0.5747), and its change was not affected by the worse

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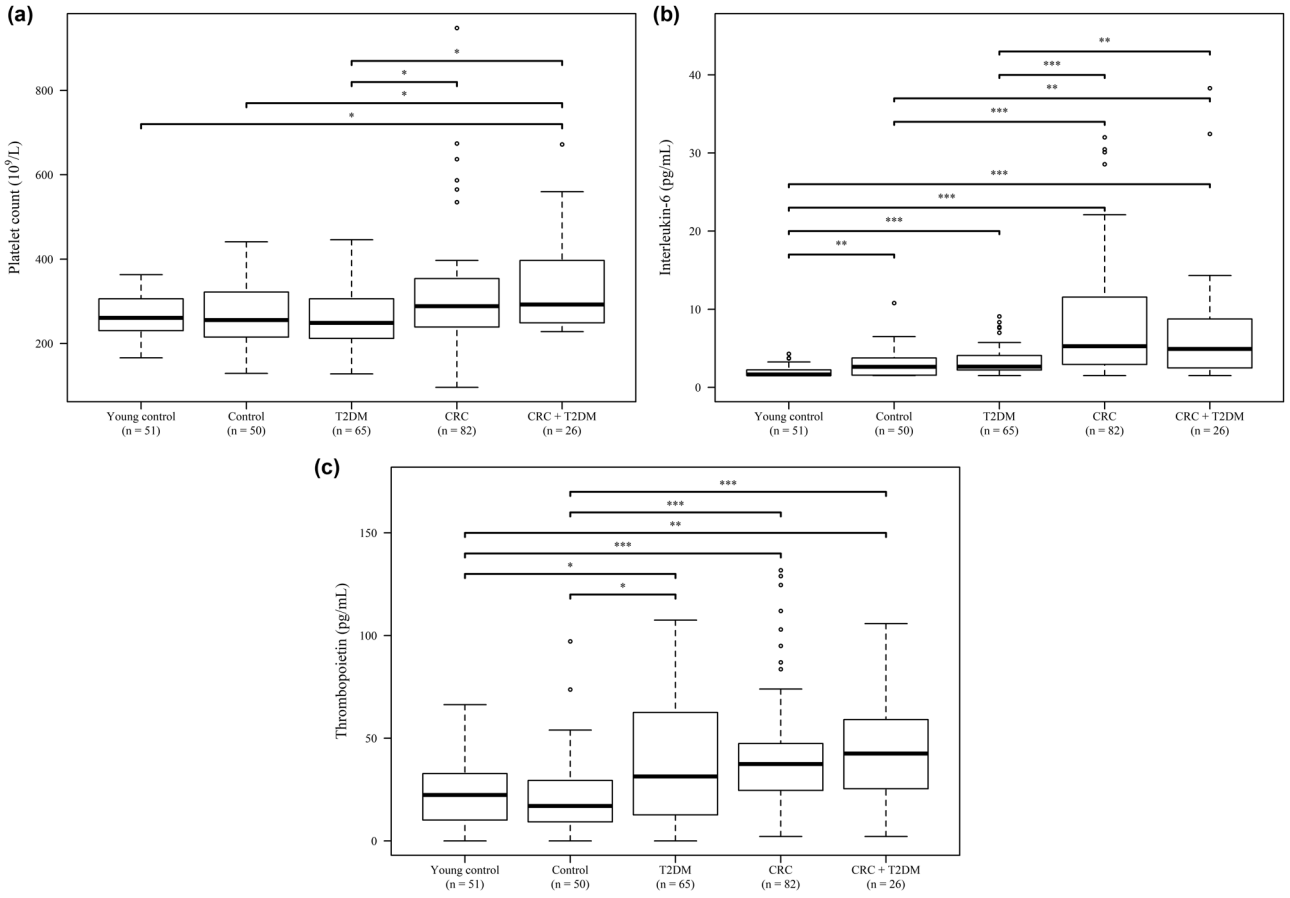

Figure 1: Comparison of paraneoplastic thrombocytosis-related parameters within the five study groups. (a) The platelet count was significantly higher in CRC patients with T2DM, compared to those in all control groups, and it also differed between the *CRC* and *T2DM* groups. (b) The plasma interleukin-6 level was significantly higher in all tumor patients[1], whereas (c) the plasma thrombopoietin level was significantly lower within the *T2DM* and *CRC*, and significantly higher in the *CRC* + *T2DM* groups, compared to those of control subjects. Note: *p*-value correction was used for all between-groups comparisons. [1]Due to better visibility, six and one outlier(s) over 40 pg/mL were not shown in the *CRC* and *CRC* + *T2DM* groups; maximum values were 220.20 and 77.19 pg/mL, respectively.

clinical outcome ($p = 0.5940$, Figure 2f). Usage of radiotherapy and chemotherapy did not affect any of the response variables, if included within any of the models.

3.3 Survival analysis of paraneoplastic thrombocytosis-related parameters and T2DM

Thirty of the 108 patients (27.8%) died during the study, from which 20 and 10 belonged to the *CRC* and *CRC* + *T2DM* group, respectively. Patients were followed up no later than January 31, 2021. The survival analysis was performed on both the preoperative (single-time) and longitudinal data. Despite the higher occurrence of death (24 vs 40%) within the diabetic tumor group and the difference that appears to be significant on the naïve Kaplan–Meier figure (Figure 3), the univariate Cox model of preoperative data suggested that T2DM had no effect on patient survival ($p = 0.1450$). A higher preoperative platelet count (HR: 1.0026, 95% CI: 1.0010–1.0050, $p = 0.0052$) could be considered as a poor prognostic sign. Interleukin-6 and thrombopoietin levels had no significant univariate effect; however, if combined with T2DM in a multivariate model, higher interleukin-6 levels had marginal effect (HR: 1.0007, 95% CI: 0.9995–1.0140, $p = 0.0692$) on patient survival. Similar to the univariate model, higher platelet counts had the same significant effect in the multivariate model as well (HR: 1.0028, 95% CI: 1.0009–1.0050, $p = 0.0043$). T2DM did not have any effect in either multivariate model. A subgroup analysis within the *CRC* + *T2DM* group only revealed that neither the duration of diabetes ($p = 0.5590$), the use of

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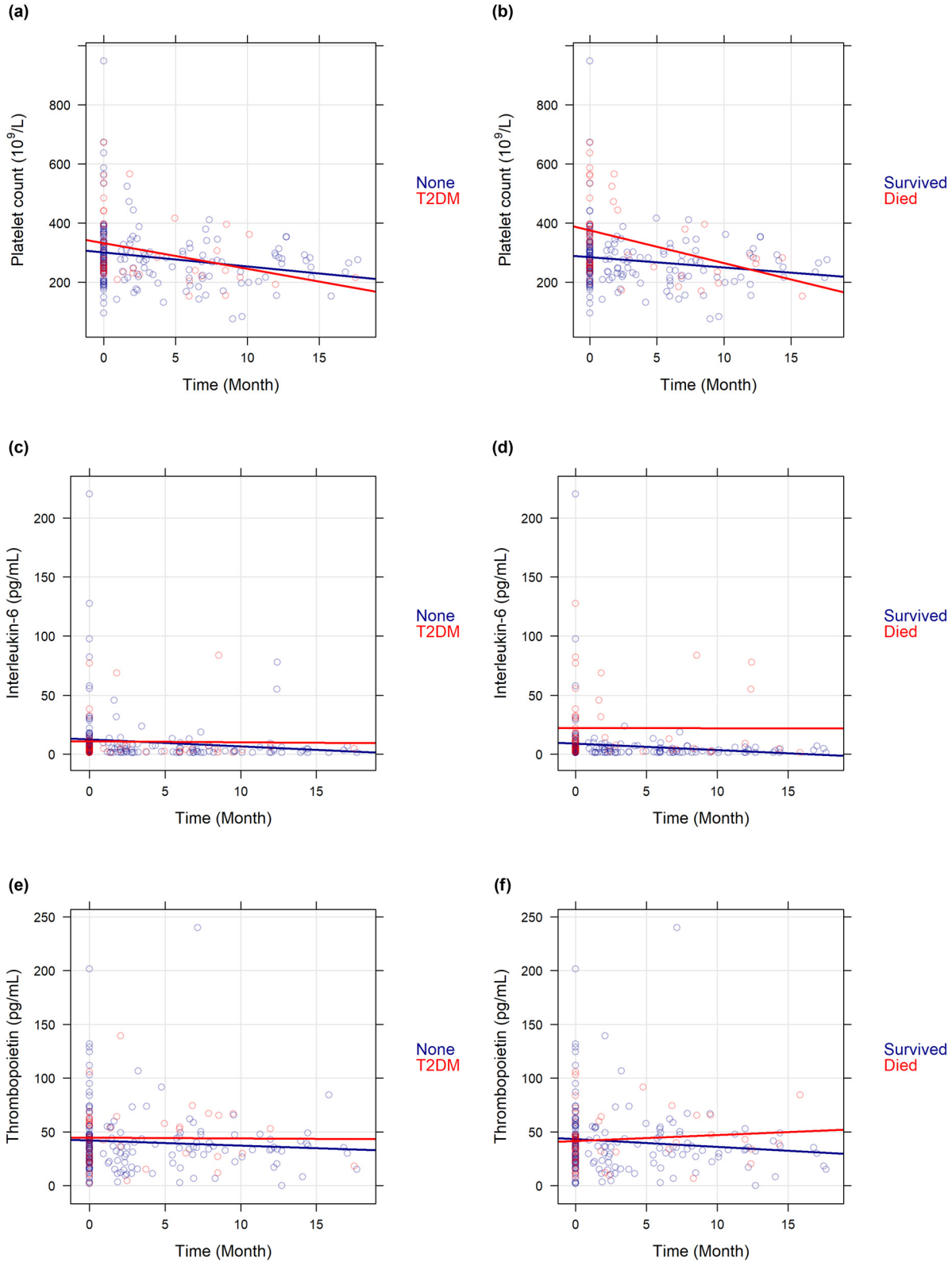

Figure 2: Change in the parameters of paraneoplastic thrombocytosis with the course of the disease. No difference was found in any of the parameters (a, c, e) when stratified with the presence of T2DM. (b) Platelet count and (d) plasma interleukin-6 were significantly higher at the time of CRC diagnosis in those patients who died during the observation period. Within the first year after tumor removal surgery, a significantly faster platelet count decrease was found in those patients who died (b), whereas the significant difference in plasma interleukin-6 levels remained throughout the whole study between survivors and those patients who died (d). No statistical difference was found in plasma thrombopoietin levels of survivors and nonsurvivors (p = 0.5940, f).

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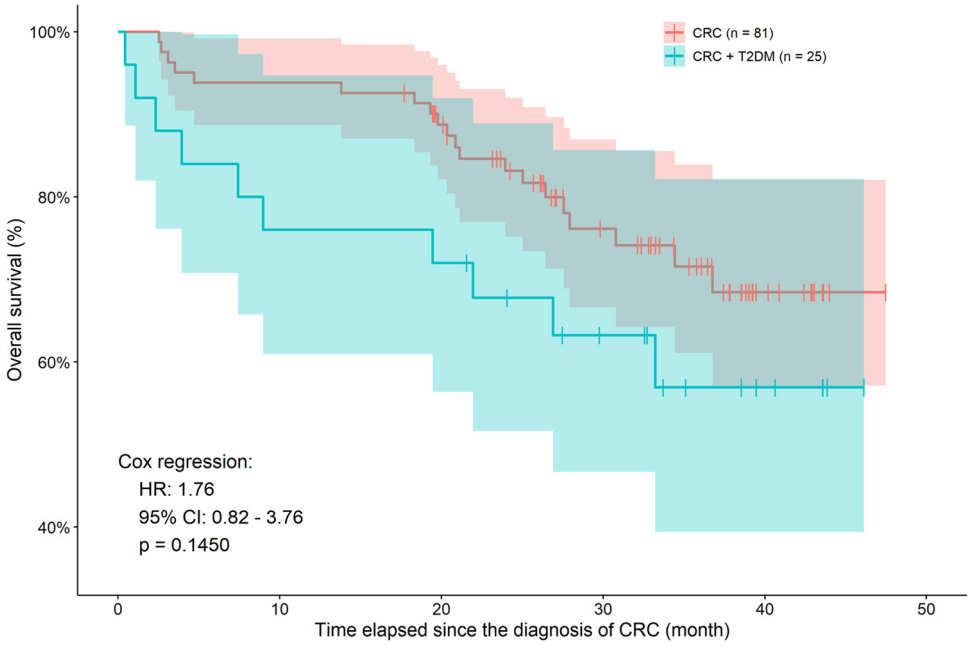

Figure 3: Naïve Kaplan–Meier curves of CRC patients with and without T2DM. It should be noted that although the two curves do not appear to be similar, neither Cox regression (p = 0.1450), nor log-rank test (p = 0.0908) could justify a statistical difference.

any antidiabetics (p = 0.2620), the presence of any diabetic complications (p = 0.2860), nor the preoperative level of HbA_{1C} (p = 0.5370) affected patient survival.

To analyze the effect of paraneoplastic thrombocytosis-related parameter changes in time, Bayesian joint models were used. First, three univariate joint models (a single parameter is analyzed within the longitudinal submodel) were constructed: the survival effect of the changes of platelet count, interleukin-6, or thrombopoietin over time was included in the longitudinal submodel, and T2DM was included in the survival submodel. An additional multivariate joint model was also constructed, where all three paraneoplastic thrombocytosis parameters were included within the longitudinal submodel, and no change was applied to the survival submodel. Interpretation of the clinical significance of parameters on patient survival was assessed as described in Section 2.

Based on the result of the univariate joint models, after the surgical removal of the primary tumor, higher platelet count and plasma interleukin-6 level is a sign of poorer survival (Figure 4). Thrombopoietin and diabetes did not affect the survival of patients in any of the univariate models. Multivariate joint model results suggested that interleukin-6 had the strongest effect on patient survival, whereas the platelet count was marginal; thrombopoietin and diabetes had no clinically relevant effect, similar to those observed in univariate models (Figures 4 and 5).

4 Discussion

In CRC, the occurrence of thrombocytosis, either prior or after the primary tumor removal surgery, is associated with shorter survival of patients [1,2,30–33]. The background of thrombocytosis may vary due to several factors, including the bleeding (reactive thrombocytosis) or metabolic changes (paraneoplastic thrombocytosis) of the tumor. In the latter, the tumor produces cytokines, such as interleukin-6, in higher quantities, and those higher cytokine concentrations stimulate hepatic thrombopoietin production, which ultimately results in the overproduction of platelets [4,34]. A platelet count of $>400 \times 10^9$/L or $>450 \times 10^9$/L is the most common definition of thrombocytosis [1,3,35], but several earlier studies reported other cut-off values [1], indicating that the definition of thrombocytosis may not be perfect. In an earlier study [20], we have reported that a personalized, relative platelet measure can predict disease outcome significantly better than “traditional” thrombocytosis, which further strengthened that the definition of thrombocytosis needs to be revised. The biochemical detection of paraneoplastic thrombocytosis in CRC is a novel research area [36]. In the current study, we observed higher interleukin-6 and thrombopoietin levels of CRC patients, compared to those of control subjects, supporting the presence of paraneoplastic thrombocytosis, which was further strengthened by

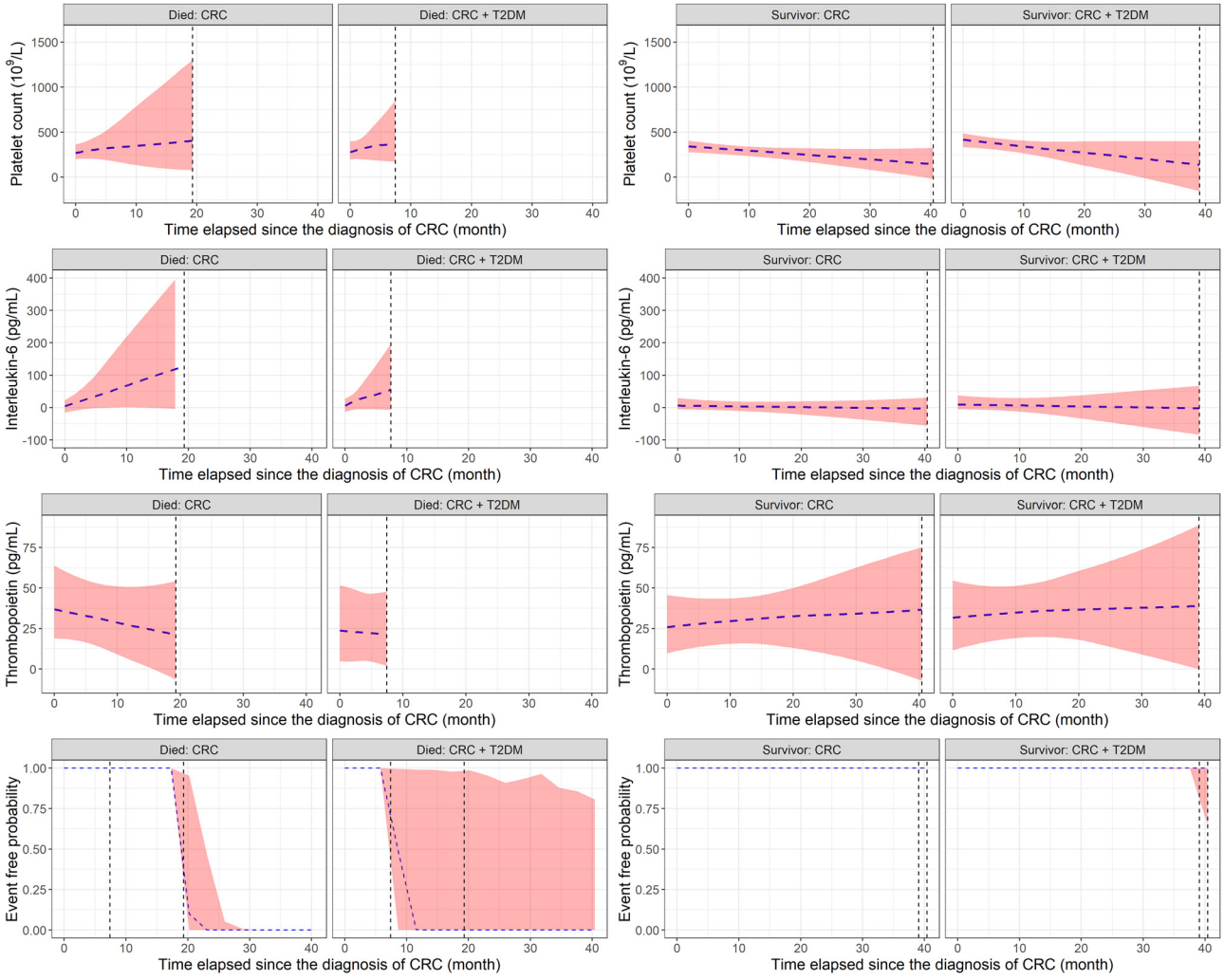

Figure 4: Longitudinal and survival predictions of the Bayesian joint models. No difference was found between CRC patients with or without T2DM. Increasing platelet count and plasma interleukin-6 level were associated with a higher risk of shorter survival times (left), whereas no change or a slow decline in these parameters is a good prognostic sign (right). Thrombopoietin levels had no clinically relevant effect neither in univariate nor in multivariate survival models.

the result of correlation and survival analysis, in line with earlier findings [1,2,31–33,36–38].

There is a strong relationship between CRC and various cytokines, including interleukin-6, which can play significant roles both in the development and progression of the disease [39–41]. Specifically, interleukin-6 is known to for its significant role in tumor proliferation, migration, induction of microsatellite instability, and angiogenesis [42,43]. It has been reported that interleukin-6 is produced by cancer-associated mesenchymal stem cells [44]. When combined with glycoprotein 130 (gp130, synonyms: IL6ST or CD130) interleukin-6 can regulate disease progression through the Shp2-Ras-ERK, JAK1/2-STAT3, and PI3K-Akt-mTOR pathways, and its higher serum levels were associated with larger tumor size, presence of metastases, and worse overall- and disease-free survival [40,41,45,46]. Due to its strong connection with CRC, interleukin-6 has been proposed as a good prognostic marker of CRC [47–49]. In the current study, we could also confirm the prognostic role of interleukin-6 over patient survival. The result that preoperative interleukin-6 did not affected survival was possibly due to the heterogeneity of study population, which was somewhat confirmed by the results obtained from the multivariate Cox models, as the predictive effect of interleukin-6 on survival increased. As a novel result, we found that constantly higher interleukin-6 level could have been observed in those patients, who died within the first 3 years after CRC diagnosis.

Research on CRC and thrombopoietin is limited. Earlier studies have identified that cancer cells can induce

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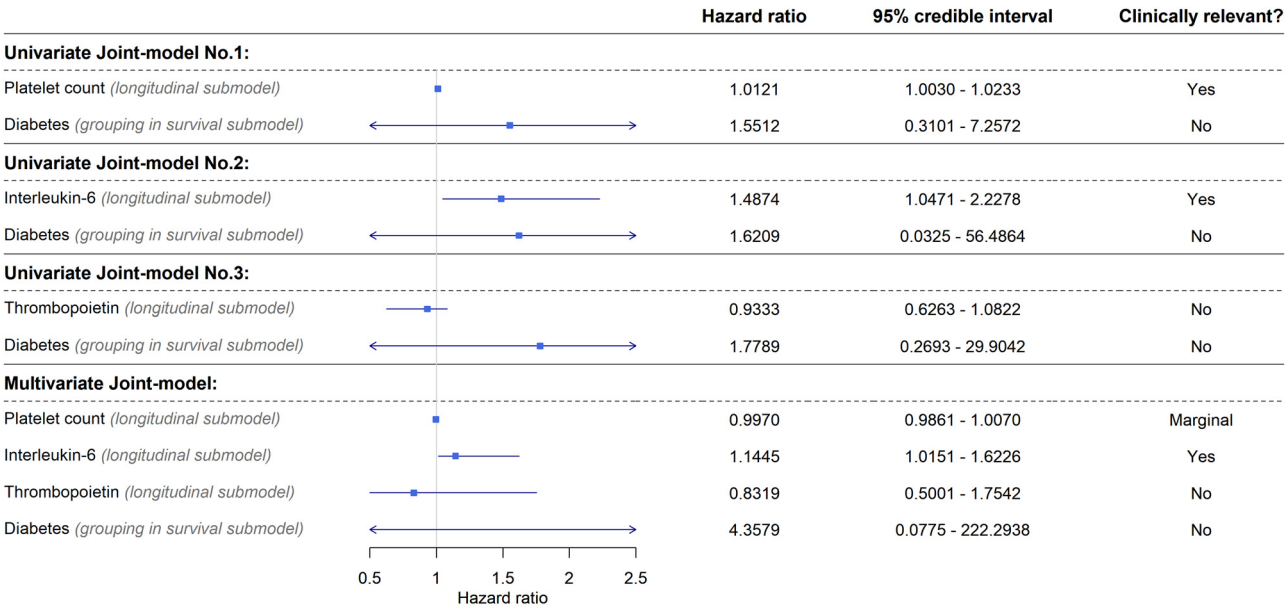

Figure 5: Results of the Bayesian joint survival models. Platelet count and/or plasma interleukin-6 level increasing over time is a poor prognostic factor, whereas diabetes and plasma thrombopoietin changes did not affect patient survival (Note: credible interval is the Bayesian equivalent of the frequentists' CI.).

circulating thrombopoietin production [50]; furthermore, higher thrombopoietin level is associated with gastrointestinal cancers, and a possible relationship between more advanced clinical stages and thrombopoietin has been also suggested [51,52]. Similar to the observations above, we found that the thrombopoietin level of CRC patients is higher than those of healthy subjects; however, the effect of thrombopoietin levels over survival was negligible, and no change with the course of the disease could have been identified in either of our longitudinal models.

T2DM, similarly to (paraneoplastic) thrombocytosis, has a known negative effect on CRC [15,53]. Several potential mechanisms link the two diseases together [19], including the increased plasma glucose levels (hyperglycemia), the presence of insulin resistance and hyperinsulinemia, increased insulin-like growth factor-1 levels, increased oxidative stress, higher cytokine concentrations, and increased platelet activation [9,10,13,19,53]. CRC is known to have an increased incidence in T2DM patients compared to those of within the healthy population [9,10]; a 1.3-fold increased risk of CRC has been reported [54,55]. T2DM has been described to have a negative effect on overall-, cancer-specific-, disease-free-, and recurrence-free survival of CRC patients [56,57]. In contrast to earlier findings, we found that T2DM did not affect patient survival in the current study statistically, but it has to be noted, that the percentage of patients who died was higher in the *CRC + T2DM* group (24 vs 40%), and similarly, the survival curves on Figure 3 also suggested a tendency toward significant difference. The effect of T2DM on patient survival was further investigated through the presence of diabetic complications, the preoperative HbA_{1C} level, and the duration of T2DM. Despite the fact that the duration of diabetes was basically twice as long in patients of the *T2DM* group, a greater number of diabetic complications were present, and a more significant proportion of patients required (intensive) insulin therapy, no significant effect could be justified for any of the above-mentioned parameters. It has to be mentioned though that the sample size of *CRC + T2DM* patients was low, but we could not even prove tendentious differences similar to that of observed in Figure 3.

The relationship between CRC-related paraneoplastic thrombocytosis and T2DM through biochemical measurements has not been investigated previously. It has to be mentioned though, that various platelet abnormalities and increased interleukin-6 and thrombopoietin production are known in T2DM [16–18,58–60]. Due to the above-mentioned effects and the high risk of cardiovascular events, the usage of platelet aggregation inhibition therapy is very common in T2DM [61,62]. Based on the available literature, our prestudy hypothesis was that CRC patients who also suffer from T2DM would probably have higher interleukin-6 and thrombopoietin levels than those who are not affected by T2DM. In contrast to our hypothesis, no differences could have been justified in any of the parameters related to paraneoplastic thrombocytosis between the *CRC* and *CRC + T2DM* groups. Furthermore, platelet aggregation inhibition also did not have any effect on the parameters of paraneoplastic thrombocytosis even though that the therapy

was more common within the *CRC + T2DM* group. Similarly, the diabetes-related parameters also did not affect the paraneoplastic thrombocytosis-related parameters. The fact that plasma thrombopoietin level of non-CRC T2DM patients was more similar to those of CRC patients than those of control subjects was most likely related to the already known fact that thrombopoietin levels are higher in T2DM [58], but no previous data are available on how similar these values of CRC and T2DM patients should be. The presented data suggest that T2DM does not increase the effect of CRC-related paraneoplastic thrombocytosis, in most probability, and the disease-worsening effect of T2DM, which has been described in earlier publications [11], must be carried out through other factors. To identify those factors, further investigations are needed.

4.1 Limitations

Limitations of the current study were the small sample size and the heterogeneity of CRC population – the latter may be also compensated with a larger sample size. The proportion of T2DM patients within the tumor cohort corresponded to the healthy Hungarian population (approximately every fourth person, over 60 years of age [8]). Due to patients' decision, a large number of potential subjects did not agree to be included in the study. Despite the low number of cases, it is important to emphasize that the presence of paraneoplastic thrombocytosis could have been already detected at such a low number of cases, showing its significance in CRC. The low number of T2DM in those of CRC patients allowed us only to pinpoint tendentious differences in several parameters.

5 Conclusion

To summarize the results of the current study, our data suggested that although some metabolic changes do occur to platelet counts and to the interleukin-6 and/or thrombopoietin synthesis in T2DM and in paraneoplastic thrombocytosis-affected CRC patients, no combined effect have been observed. Based on the current study, there is no significant relationship between the two conditions with high probability and the known disease-worsening effect of diabetes on CRC survival is presumably independent of paraneoplastic thrombocytosis.

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Author contributions: ZH, MH, and AS built the study design; MH, GH, AF, and ZH were involved in the collection of samples; ZH and ZL analyzed the data; GH, ZH, MH, MD, and ZL interpreted data; ZH and GH prepared the draft of the manuscript; all authors were involved in editing and reviewing; and AS received funding and supervised the study. All authors have read and agreed to the published version of the manuscript.

Conflict of interest: The authors declare no conflicts of interest. The funding bodies have no role in the design of the study; collection, analysis, and interpretation of data; or in writing the manuscript.

Data availability statement: The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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ORIGINAL ARTICLE

Observational Study

High plasma CD40 ligand level is associated with more advanced stages and worse prognosis in colorectal cancer

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Abstract

BACKGROUND

Colorectal cancer (CRC) is often associated with elevated platelet count (> 400 × 10^9/L), known as thrombocytosis. The role of CD40 ligand (CD40L), a member of the tumor necrosis factor family, is controversial in CRC. Circulating CD40L is higher in CRC, but its relationship with disease staging and local and distant metastasis is not clear. Although most of the circulating CD40L is produced by platelets, no previous study investigated its relationship with CRC-related thrombocytosis.

AIM

To investigate the role of CD40L to predict the outcome of CRC and its relation to thrombocytosis.

METHODS

A total of 106 CRC patients and 50 age and sex-matched control subjects were enrolled for the study. Anamnestic data including comorbidities and histopathological data were collected. Laboratory measurements were performed at the time of CRC diagnosis and 1.5 mo and at least 6 mo after the surgical removal of the tumor. Plasma CD40L and thrombopoietin were measured *via* enzyme-linked immunosorbent assay, while plasma interleukin-6 was measured *via* electrochemiluminescence immunoassay. Patient follow-ups were terminated on January 31, 2021.

RESULTS
Plasma CD40L of CRC patients was tendentiously higher, while platelet count (P = 0.0479), interleukin-6 (P = 0.0002), and thrombopoietin (P = 0.0024) levels were significantly higher as opposed to the control subjects. Twelve of the 106 CRC patients (11.3%) had thrombocytosis. Significantly higher CD40L was found in the presence of distant metastases (P = 0.0055) and/or thrombocytosis (P = 0.0294). A connection was found between CD40L and platelet count (P = 0.0045), interleukin-6 (P = 0.0130), and thrombocytosis (P = 0.0155). CD40L was constant with the course of CRC, and all baseline differences persisted throughout the whole study. Both pre- and postoperative elevated platelet count, CD40L, and interleukin-6 level were associated with poor overall and disease-specific survival of patients. The negative effect of CD40L and interleukin-6 on patient survival remained even after the stratification by thrombocytosis.

CONCLUSION
CD40L levels of CRC patients do not change with the course of the disease. The CD40L level is strongly correlated with platelet count, interleukin-6, thrombocytosis, and the presence of distant metastases.

Key Words: CD40 ligand; Colorectal neoplasms; Interleukin-6; Thrombocytosis; Thrombopoietin

Core Tip: This observational study investigated whether plasma CD40 ligand (CD40L) is related to colorectal cancer (CRC)-associated thrombocytosis and disease severity. Baseline CD40L was significantly higher in patients with distant metastasis and thrombocytosis. An association between CD40L, platelet count, and the interleukin-6 level was found. CD40L was constant with the course of the disease, and all its baseline differences persisted throughout the study. Both pre- and postoperative high CD40L levels negatively affected overall, disease-specific, and thrombocytosis-eliminated survival. A possible connection between elevated CD40L levels and increased general inflammation caused by CRC was also suggested.

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INTRODUCTION

According to the GLOBOCAN 2018 data, colorectal cancer (CRC) is the third most common cancer type with over 1.8 million new cases and causing more than 860000 deaths annually[1]. Elevated platelet count (thrombocytosis) has been described previously as a poor prognostic sign in CRC[2]. Thrombocytosis may arise in CRC due to the following: (1) Secondary thrombocytosis caused by the bleeding of the tumor; and (2) paraneoplastic thrombocytosis defined as a metabolic change caused by the tumor itself[2,3]. In the latter, the elevated platelet count can be attributed to the overproduction of cytokines by the tumor, which induces hepatic thrombopoietin production and ultimately increases bone marrow activity[3,4].

CD40 ligand (CD40L, synonym: CD154, gp39, or T-B activating molecule) is a type I transmembrane glycoprotein belonging to the tumor necrosis factor family of cytokines[5,6]. CD40L activates CD40, a transmembrane protein receptor found on antigen-presenting cells[6]. The majority of circulating CD40L is assumed to originate from platelets. A membrane-bound CD40L form is also known[6]. CD40L has been described to have antitumor effects *via* the inhibition of tumor cell proliferation and pro-apoptotic features through the activation of apoptotic pathways[6-9]. It has been found previously that CRC patients have significantly higher soluble CD40L levels than those of healthy control subjects. Its possible use as a promising biomarker in CRC was proposed[10-12]. The connection between CD40L levels and lymph node involvement or distant metastasis is controversial. One study reported increased CD40L levels in patients with worse disease conditions, while others have found the opposite. Furthermore, neoadjuvant chemoradiotherapy has decreased CD40L levels[10-12].

The role of CD40L in the course of CRC and its relationship with CRC-related thrombocytosis has not been investigated previously. Therefore, a prospective observational study was carried out. Paraneo-

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plastic thrombocytosis was investigated through the measurement of plasma interleukin-6 and thrombopoietin levels. In addition to the latter, the further aim of this study was to try to clarify the discrepancy between plasma CD40L levels and higher tumor stages. The effect of plasma CD40L on survival was also analyzed.

MATERIALS AND METHODS

The study was conducted in concordance with the World Medical Association's Declaration of Helsinki. The study was approved by the Committee of Science and Research Ethics, Hungarian Medical Research Council (ETT TUKEB 8951-3/2015/EKU) and by the institutional ethical committees of Semmelweis University (SE TUKEB 21-12/1994, approval date of latest modification: February 10, 2017) and Szent Imre University Teaching Hospital (SZIK IKEB 5/2017). Handling of patient data was in accordance with the General Data Protection Regulation issued by the European Union.

Patients and study design

A prospective, real-life observational cohort study was carried out, and a total of 106 CRC patients were enrolled for the study between 2017 and 2019. Fifty age and sex-matched control subjects were selected from a pool of 166 volunteers. Prior to any study-specific procedures, study subjects signed written informed consents. CRC patients attended the Department of Internal Medicine and Hematology, Semmelweis University, Budapest and the Department of General Surgery, Szent Imre University Teaching Hospital, Budapest. Control subjects attended the Metabolic Outpatient Clinic of the Department of Internal Medicine and Hematology, Semmelweis University, Budapest. Study exclusion criteria included age < 18 years, an Eastern Cooperative Oncology Group performance status > 2, previous malignancy, and systemic autoimmune, inflammatory bowel, inadequately controlled thyroid, hematologic, chronic kidney, or any mental diseases. The usage of erythropoiesis-stimulating agents or recent blood transfusion was also prohibited. In addition to the above, control subjects with any metabolic disease, except type 2 diabetes mellitus, which was present in several of the CRC patients, were excluded from the study.

Clinicopathological and laboratory data measurements

Body weight, height, and anamnestic data including comorbidities and recent medications were collected. Fasting blood samples were drawn: (1) At the time of CRC diagnosis; (2) at least 6 wk after tumor removal surgery; and (3) at least 6 mo after tumor removal surgery. Several chemotherapeutic agents are known to affect platelet count[13,14]. Therefore, the third measurements were timed so that patients had not received any oncological treatment for at least 6 wk prior to blood sampling. Follow-ups of patients were terminated on January 31, 2021. Routine laboratory measurements were performed at the central laboratories of Semmelweis University and Szent Imre University Teaching Hospital. Complete blood count, liver enzyme, plasma glucose, and creatinine levels were determined. The Chronic Kidney Disease-Epidemiology Collaboration equations were used to calculate the estimated glomerular filtration rate[15].

In addition to routine laboratory measurements, plasma CD40L, interleukin-6, and thrombopoietin levels were measured using the CD40L Human Enzyme-linked Immunosorbent Assay kit (ELISA kit, abcam®, Catalog Number ab99991, Cambridge, MA, United States), ELECSYS® Interleukin-6 electrochemiluminescence immunoassay (ECLIA, Roche Diagnostics GmbH, Mannheim, Germany), and the Human Thrombopoietin Quantikine® ELISA kits (catalog number: DTP00B, R&D Systems, Minneapolis, MN, United States), respectively. As per the manufacturer's description, thrombopoietin level was obtained from platelet-poor plasma.

The tumor-staging was given by histopathological examination of surgical specimens and imaging studies; the American Joint Committee on Cancer grouping was used[16]. The side of CRC was described[17] as right-sided if the tumor was originating from the cecum, ascending colon, and proximal two-thirds of the transverse colon, while left-sided was described if the tumor originated from the distal one-third of the transverse colon, descending colon, sigmoid colon, and rectum. Chemotherapy was recorded as adjuvant if no distant metastasis by imaging was detected and as first-line, second-line, *etc* in metastatic CRC. The usage of biological agents was recorded as a dummy variable.

Statistical analysis

Statistical analysis was performed with R version 4.0.4[18]. Matching of control subjects was done *via* propensity score matching (R-package Matching[19]). Wilcoxon-Mann-Whitney *U*-test, Fisher's exact test, Kruskal-Wallis test with *P* value corrected pairwise Wilcoxon-Mann-Whitney *U*-tests as post-hoc, and Spearman's rank correlation were used. To detect the changes of CD40L in time, linear mixed-effects models were used (R-package nlme)[20]. Overall and CRC-related disease-specific survival of patients were analyzed with Cox regression and cause-specific competing risk survival models (R-package survival)[21], respectively. $P < 0.05$ was considered as statistically significant, and *P* values were corrected with the Holm method[22] for multiple comparisons problem. Results were expressed as

mean ± SD and as the number of observations (percentage) for continuous and count data, respectively.

RESULTS

Baseline measurements

A total of 106 CRC patients and 50 age and sex-matched voluntary control subjects were enrolled for the study. Baseline laboratory measurements and anamnestic data were summarized in Table 1, and histopathological data of CRC patients were summarized in Table 2. The two cohorts were well balanced as no significant difference was detected in either of the anamnestic data. On the contrary, most of the parameters of complete blood count within the CRC group were out of normal range (P < 0.05), and 12 of the 106 CRC patients (11.3%) showed signs of thrombocytosis (platelet count > 400 × 10^9/L). The plasma interleukin-6 (P = 0.0002) and thrombopoietin (P = 0.0024) levels of CRC patients were significantly higher than those of the control subjects. CD40L did not differ between the two cohorts, but it should be highlighted that CD40L of control subjects was tendentiously lower (crude P value: 0.2946; Figure 1).

To test whether other factors, such as age, sex, body mass index, histopathological data, or the presence of comorbidities, affect plasma CD40L levels, further subgrouping within the individual cohorts and correlation analysis was performed. CD40L level of control subjects was affected by the presence of diabetes (without diabetes: 240.41 ± 207.37 pg/mL; with diabetes: 110.09 ± 112.06 pg/mL; P = 0.0313), while no further parameters had any effect on the CD40L level of control subjects. In contrast, the presence of diabetes had no effect on CD40L levels of CRC patients (P = 0.7377). The presence of regional lymph node metastasis alone was not associated with a higher CD40L level (P = 0.7165), but the CD40L level was significantly higher in the presence of distant metastasis (M0: 228.27 ± 293.30 pg/mL; M1: 395.11 ± 322.00 pg/mL; P = 0.0055; Figure 2A) and with thrombocytosis (without thrombocytosis: 248.15 ± 299.20 pg/mL; with thrombocytosis: 475.77 ± 323.43 pg/mL; P = 0.0294).

Furthermore, a negative correlation was found between CD40L and mean corpuscular volume (Spearman's ρ = -0.36, P = 0.0048), marginal association with hematocrit (Spearman's ρ = -0.28, P = 0.0898), mean corpuscular hemoglobin (Spearman's ρ = -0.29, P = 0.0801), and red blood cell distribution width (Spearman's ρ = +0.29, P = 0.0805). Higher platelet count was associated with more advanced stages of CRC (P = 0.0079; Figure 2B), similar to those of CD40L levels. Right-sided tumors (left sided: 300.97 ± 114.81 × 10^9/L; right sided 350.90 ± 141.27 × 10^9/L; P = 0.0121) and the presence of distant metastasis (M0: 289.16 ± 107.69 × 10^9/L; M1: 385.72 ± 140.28 × 10^9/L; P = 0.0006) were also associated with increased platelet count. Moreover, higher interleukin-6 levels were observed in patients with a higher stage range (P = 0.0025; Figure 2C), with the presence of positive regional lymph nodes (N0: 7.03 ± 7.53 pg/mL; N1+: 15.82 ± 24.67 pg/mL; P = 0.0400) and with distant metastasis (M0: 7.03 ± 8.66 pg/mL; M1: 21.88 ± 29.33 pg/mL; P = 0.0005). Plasma thrombopoietin levels were basically equal in all stages except in Stage II, where decreased thrombopoietin levels were observed compared to the other stages (P = 0.0210; Figure 2D).

To further assess the effect of thrombocytosis on CD40L, general linear models were created. Thrombopoietin did not have any effect on CD40L. Higher platelet count or the presence of thrombocytosis and higher plasma interleukin-6 levels were strongly correlated with higher CD40L levels (Table 3). It should be emphasized that both the individual and combined effect of these parameters only slightly explained the increase in CD40L. The explanatory power of the models, based on adjusted R^2, was at a maximum of 8.1%.

Changes in CD40L with the course of the disease

CRC patients were recalled for follow-ups, and 61 of the original 106 patients (call-back rate 57.4%) had at least one repeated measurement of CD40L and other laboratory parameters. Distant metastasis developed in an additional 14 CRC patients following the surgical removal of the primary tumor. Thirty CRC patients had all three sets of measurements (Figure 3). The mean durations between baseline and 6 wk after surgery and between baseline and > 6 mo after surgery were 2.07 ± 1.76 mo and 10.38 ± 3.73 mo, respectively. To determine whether CD40L or the parameters related to thrombocytosis change with respect to the course of CRC, linear mixed-effects models were constructed. A total of 197 measurements were used, where not only the paired but all the baseline and further repeated measurements from all the 106 study participants were used. Based on these estimations, a significant 1.5%-2.7% average decrease in platelet count can be expected per month (P < 0.0001; Figure 3B), while no significant changes were observed in the plasma CD40L (P = 0.6813; Figure 3A), interleukin-6 (P = 0.4497), and thrombopoietin (P = 0.2867) levels of CRC patients.

The effect of regional metastatic lymph node or distant metastasis and thrombocytosis on plasma CD40L levels within the course of the disease was also assessed. After the surgical resection of the primary tumor, the CD40L level of CRC patients with distant metastasis (P = 0.6964) or thrombocytosis (P = 0.7829) did not change over time. The same increased level could be observed throughout the observation time (M1: P = 0.0326; thrombocytosis: P = 0.0008), as described at the baseline. The strong association between CD40L level and platelet count (P = 0.0002) and interleukin-6 level (P = 0.0012),

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Table 1 Baseline laboratory results and anamnestic data of study subjects

Parameter	CRC patients (*n* = 106)	Controls (*n* = 50)	*P* value
Age (yr)	68.55 ± 8.64	63.91 ± 10.12	*P* = 0.2068
Sex [Male:Female, *n* (%)]	71 (67.0):35 (33.0)	35 (70.0):15 (30.0)	*P* = 1.0000
Body mass index (kg/m^2)	27.37 ± 4.03	29.26 ± 5.07	*P* = 0.5995
White blood cell count (10^9/L)	8.76 ± 4.56	7.41 ± 1.99	*P* = 0.7308
Neutrophil count (10^9/L)	6.05 ± 3.60	4.45 ± 1.64	*P* = 0.0205
Eosinophil count (10^9/L)	0.28 ± 0.86	0.20 ± 0.15	*P* = 1.0000
Basophil count (10^9/L)	0.06 ± 0.05	0.06 ± 0.03	*P* = 1.0000
Monocyte count (10^9/L)	0.65 ± 0.48	0.48 ± 0.12	*P* = 0.2532
Lymphocyte count (10^9/L)	1.76 ± 1.07	2.22 ± 0.72	*P* = 0.0002
Red blood cell count (10^{12}/L)	4.48 ± 0.57	4.93 ± 0.51	*P* = 0.0002
Hemoglobin (g/L)	123.67 ± 21.37	147.04 ± 12.70	*P* < 0.0001
Hematocrit (L/L)	0.38 ± 0.06	0.44 ± 0.04	*P* < 0.0001
Mean corpuscular volume (fL)	84.69 ± 8.29	89.22 ± 4.06	*P* = 0.0856
Mean corpuscular hemoglobin (pg)	27.30 ± 3.50	29.93 ± 1.77	*P* = 0.0001
Mean corpuscular hemoglobin concentration (g/L)	322.94 ± 17.84	335.38 ± 9.44	*P* < 0.0001
Red blood cell distribution width (%)	14.85 ± 3.70	13.13 ± 0.82	*P* = 0.1911
Platelet count (10^9/L)	315.58 ± 124.55	259.96 ± 73.98	*P* = 0.0479
Aspartate transaminase (U/L)	25.95 ± 20.22	26.52 ± 6.94	*P* = 0.0155
Alanine transaminase (U/L)	22.14 ± 12.74	28.62 ± 12.32	*P* = 0.0047
Gamma-glutamyl transferase (U/L)	75.07 ± 130.37	37.84 ± 31.74	*P* = 1.0000
Plasma glucose (mmol/L)	5.71 ± 1.23	5.84 ± 1.94	*P* = 1.0000
Creatinine (μmol/L)	78.26 ± 20.06	74.82 ± 15.28	*P* = 1.0000
Estimated glomerular filtration rate [mL/(min × 1.73 m^2)]	81.39 ± 17.00	87.21 ± 12.42	*P* = 0.7308
Interleukin-6 (pg/mL)	13.79 ± 28.41	3.23 ± 1.69	*P* = 0.0005
CD40 ligand (pg/mL)	273.92 ± 309.03	191.84 ± 191.82	*P* = 1.0000
Thrombopoietin (pg/mL)	43.59 ± 30.76	26.41 ± 24.15	*P* = 0.0029
Known comorbidities, *n* (%)			
Type 2 diabetes mellitus	25 (23.6)	16 (32.0)	*P* = 1.0000
Hypertension	68 (64.2)	26 (52.0)	*P* = 0.8157
Major cardiovascular event(s) prior CRC	21 (19.8)	6 (12.0)	*P* = 1.0000
Platelet aggregation inhibition, *n* (%)	23 (21.7)	18 (36.0)	*P* = 0.5517

CRC: Colorectal cancer.

observed at baseline measurements, also persisted throughout the whole time of the study.

Survival analysis

Overall and CRC-related disease-specific survival of patients was calculated. Patient follow-ups were terminated on January 31, 2021. Thirty of the 106 patients (28.3%) died during the study. The three different causes of death were postoperative complications, infection, and CRC-related in 4 cases, 1 case, and 25 cases, respectively. Both pre- and postoperative data had been analyzed; 106 and 61 cases were used for the calculations, respectively. Patients with higher preoperative plasma CD40L level [hazard ratio (HR): 1.001, 95% confidence interval (CI): 1.000-1.002, *P* = 0.0159], plasma interleukin-6 level (HR: 1.020, 95%CI: 1.010-1.030, *P* = 0.0001), and platelet count (HR: 1.003, 95%CI: 1.001-1.005, *P* = 0.0052) had significantly shorter overall survival, while preoperative plasma thrombopoietin level (*P* = 0.5550) did not affect the overall survival of patients in univariate models. In a multivariate setting, interleukin-6

Table 2 Clinico-histopathological features of colorectal cancer patients

Parameter	Number of observations
AJCC staging[16], *n* (%)	
Stage I	27 (25.5)
Stage II	26 (24.5)
Stage III	24 (22.6)
Stage IV	29 (27.4)
Regional lymph node metastasis, *n* (%)	
N0	57 (53.8)
N1+	49 (46.2)
Development of distant metastasis after the tumor removal surgery, *n* (%)	14 (13.2)
Side of CRC, *n* (%)	
Left-sided	75 (70.8)
Right-sided	31 (29.2)
Chemotherapy, *n* (%)	
None	51 (48.1)
Adjuvant	21 (19.8)
First-line	11 (10.4)
Second-line	13 (12.3)
Third or later-line	10 (9.4)
Usage of biological therapy, *n* (%)	22 (20.8)

AJCC: American Joint Committee on Cancer; CRC: Colorectal cancer.

Table 3 Results of general linear models investigating the effect of thrombocytosis on CD40 ligand

Parameter	Individual effect *P* value	Multiple effect *P* value	Multiple effect *P* value
Interleukin-6 (pg/mL)	0.0130	0.1720	0.0454
Thrombopoietin (pg/mL)	0.1620	0.2393	0.1785
Platelet count (10^9/L)	0.0045	0.0043	-
Presence of thrombocytosis	0.0155	-	0.0138

had the most prominent effect on overall survival (HR: 1.024, 95%CI: 1.010-1.039, $P = 0.0012$), while CD40L (95%CI: 0.9995-1.0020) and platelet count (95%CI: 0.9996-1.0040) had a marginal effect. Thrombopoietin did not affect the overall survival of patients. The same was observed for preoperative (Figure 4) and postoperative (Figure 5) disease-specific survival.

Using stratified survival models we could assume different preoperative baseline hazards for patients with or without thrombocytosis (platelet count > 400 × 10^9/L). In a univariate model, higher preoperative plasma CD40L level indicated poor disease-specific prognosis of CRC patients (HR: 1.001, 95%CI: 1.000-1.002, $P = 0.0332$). However, only a marginal effect was found in multivariate models (HR: 1.001, 95%CI: 0.9998-1.002, $P = 0.1196$). Neither platelet count (univariate: $P = 0.3310$; multivariate: $P = 0.6237$), nor thrombopoietin (univariate: $P = 0.9440$; multivariate: $P = 0.5387$) level affected patient survival if stratification by thrombocytosis had been applied. The strong effect of the inflammatory cytokine, interleukin-6 on survival could be demonstrated even by the elimination of the effect of thrombocytosis (univariate: $P = 0.0016$; multivariate: $P = 0.0103$).

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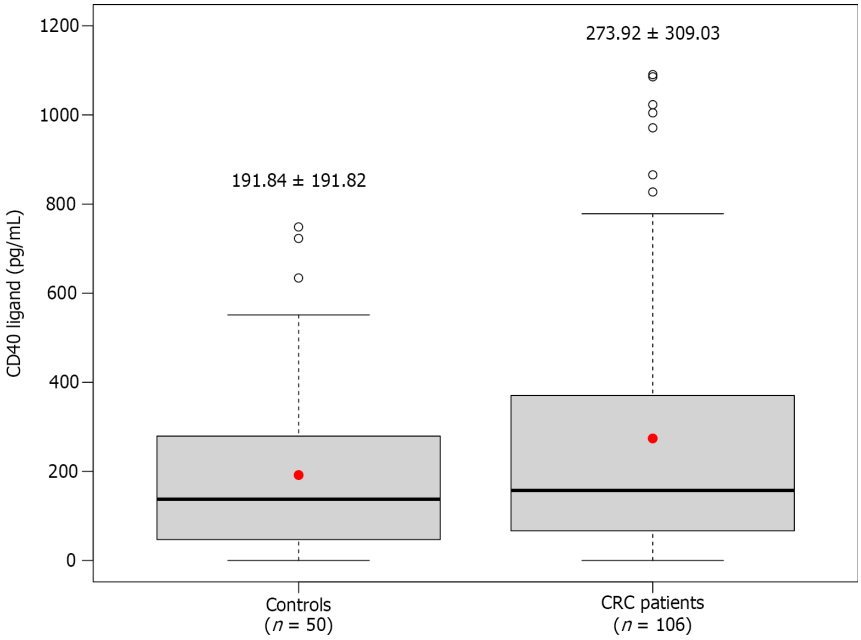

Figure 1 Baseline plasma CD40 ligand level of control subjects and colorectal cancer patients. Crude *P* value: *P* = 0.2946; the red dot and thick line represent mean and median values, respectively. CRC: Colorectal cancer.

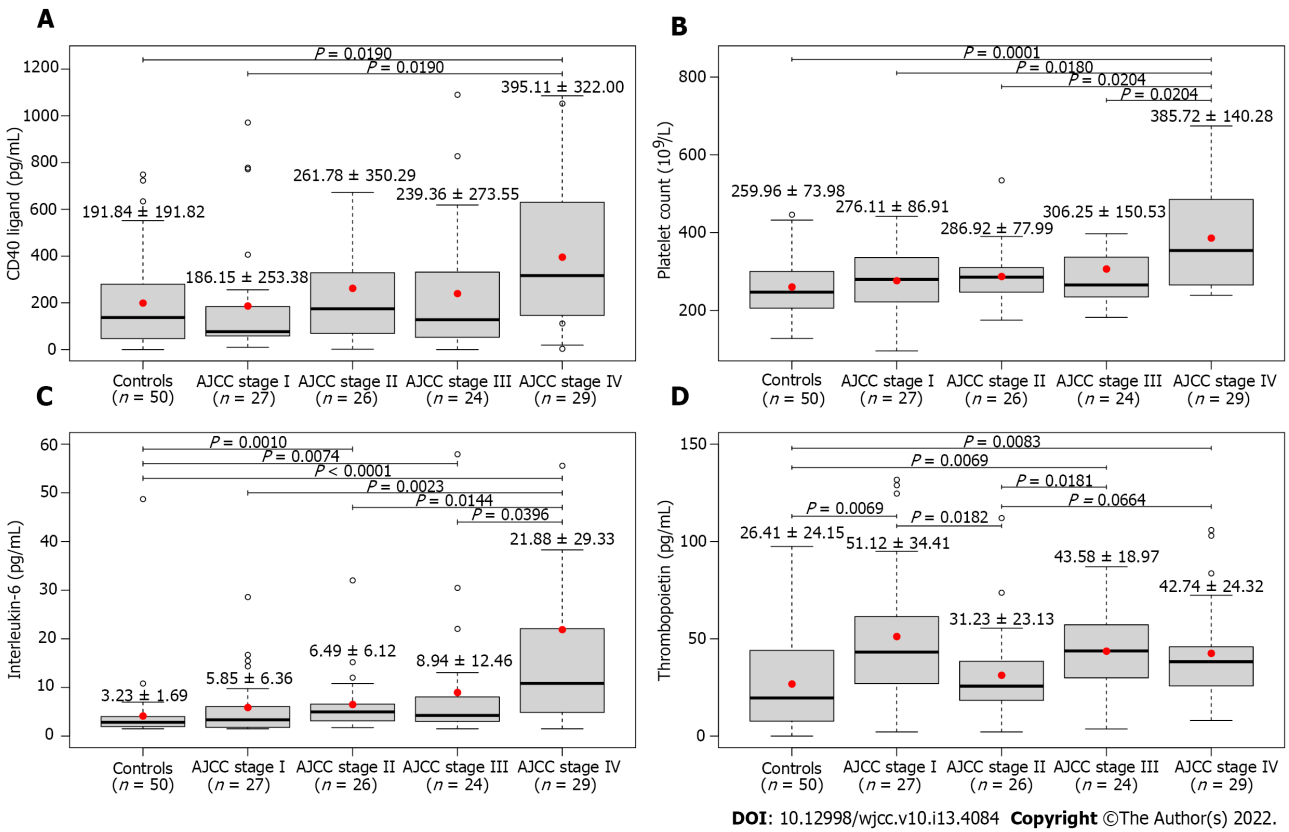

Figure 2 Baseline plasma CD40 ligand, interleukin-6, and thrombopoietin level and platelet count of study participants (mean ± SD). A: Baseline plasma CD40 ligand; B: Platelet count; C: Interleukin-6 level; D: Thrombopoietin level. CD40 ligand level (Kruskal-Wallis test: *P* = 0.0275) and platelet count (Kruskal-Wallis test: *P* = 0.0004) was the highest in Stage IV colorectal cancer (CRC) patients. Interleukin-6 (Kruskal-Wallis test: *P* < 0.0001) and thrombopoietin (Kruskal-Wallis test: *P* = 0.0002) levels of the CRC patient groups, except those in Stage II in the latter, were significantly higher than those of healthy control subjects. The red dot and thick line represent mean and median values, respectively. AJCC: American Joint Committee on Cancer.

DISCUSSION

The role of CD40L in neoplastic diseases is controversial[6]. Cellular model studies have revealed that it can significantly contribute to the immunological activity against cancer, while other studies have reported the complete opposite, *i.e.* that CD40L contributes to the progression and growth of the tumor [23,24]. The most promising results and antitumor effects have been observed in melanomas and hematological malignancies[6], including enhanced protection of dendritic cells against apoptosis-inducing factors of tumor cells, enhanced maturation and antigen production of B cells, increased T cell-dependent immune response and the CD40L activation-dependent apoptosis of cancer cells[6]. Moreover, CD40- and CD40L-based drugs have been developed recently, and active clinical trials are currently investigating their efficacy[25].

The CRC cell lines HCT116, Colo320, and Caco-2 have been shown to be positive for CD40, while SW480, HT29, Colo741, and LS174T do not express CD40[7,9,24]. The treatment of CD40-positive cell lines with interferon-γ can further increase the expression of CD40[7]. Analysis of resected tumor specimens has shown that approximately every third CRC is moderately or strongly CD40-postive[24]. While in another study CD40- and CD40L-positivity has been observed in 79% and 56% of CRC patients, respectively[8]. *Ex vivo* treatment of CD40-positive CRC cell lines with recombinant soluble human CD40L can inhibit tumor growth, induce apoptosis[7], inhibit CRC cell proliferation, stall CRC cells at the G0/G1 state, influence cell adhesion and metastasis, and increase aryl hydrocarbon receptor expression[8]. While the T cell membrane bound CD40L can induce enhanced apoptosis of *in vitro* CRC cells with CD40-positivity[24], less signaling strength has been observed in the case of the soluble form of CD40L. Soluble CD40L can induce apoptosis only following specific pharmacological interventions [24,26].

Previous clinical studies revealed that high CD40 expression and higher soluble CD40L concentration are associated with CRC[9,10], and these elevations are the most prominent with the presence of lymph node metastasis[7,10,27], venous invasion[11], and higher TNM stages[7,10,27]. *In vitro* stimulation of $CD3^+$, $CD4^+$, and $CD8^+$ T cells of CRC patients resulted in a significantly increased, approximately four-times higher, CD40L expression compared to those of healthy control subjects[28].

In contrast to the results above, Tada *et al*[11] and Lima *et al*[12] observed lower soluble CD40L levels within those CRC patients with worse clinicopathological features. To our knowledge, no previous study investigated CD40L levels with the course of the disease, and only partial data are available from the study of Tada *et al*[11]. In that study, rectal cancer patients received neoadjuvant chemoradiotherapy prior to the surgical removal of the tumor, and CD40L was measured before and after the neoadjuvant treatment. They found that the post-treatment CD40L level of patients with a high response rate to the treatment was significantly lower, while no change was observed in those patients with low response rates[11]. Results of the current study confirmed the observations of those former studies[7,10,27] where circulating CD40L level was tendentiously higher in CRC patients than those of control patients. We also observed the highest measurements in Stage IV cancer and found that the CD40L level of CRC patients is basically constant with the course of the disease. The initial differences in CD40L levels between those patients with or without distant metastasis or thrombocytosis were observable throughout the whole course of the disease. The latter observation showed, that the CD40L level was the highest in those patients with more advanced disease. This should be the cause behind higher pre- and postoperative CD40L levels associated with shorter survival of patients, with high probability. Similar to our findings, the highest soluble CD40L levels have been observed in patients with distant metastases in squamous cancer or adenocarcinoma of the lung[29], in nasopharyngeal carcinoma[30], and in gastric cancer[31].

Approximately 95% of the soluble form of CD40L is thought to be derived from platelets[32,33]. Soluble CD40L level is strongly correlated with platelet count[34]. The highest levels can be measured in reactive thrombocytosis and essential thrombocythemia, while the lowest values can be measured in patients with low platelet count[34]. Thrombocytosis is associated with several cancers[2,3], and the platelet count is higher in patients with metastasis[2]. CD40L positively correlates with platelet count in patients with a high response rate to neoadjuvant chemoradiotherapy[11]. An assumption was made by Huang *et al*[27] that in cancer patients soluble CD40L is most probably derived from activated platelets than from T cells. However, this question was never further investigated.

Our data showed that CD40L level is positively correlated with several markers of (paraneoplastic) thrombocytosis, in particular with platelet count and interleukin-6. This strong connection persisted throughout the whole observation period. It has to be mentioned though that the stratification used in our survival models should have fully eliminated the significant effect of CD40L on CRC survival. However, we could not demonstrate this expected effect, which was observed, *e.g.*, in the case of platelet count. This, together with the weaker explanatory powers observed in our linear models, suggests that the increase in CD40L levels is possibly not only influenced by (paraneoplastic) thrombocytosis alone. Increased CD40L production is known in various diseases characterized by general inflammation, like atherosclerosis, diabetes, or inflammatory bowel disease[35-38]. CRC can also be described as a disease known for its general inflammation[39], high interleukin-6 level[39], and inadequate T cell activation [27]. Furthermore, increased inflammation is also associated with metastasis[40,41]. The strong correlation between CD40L, interleukin-6, and metastases hints that the answer may be sought in the

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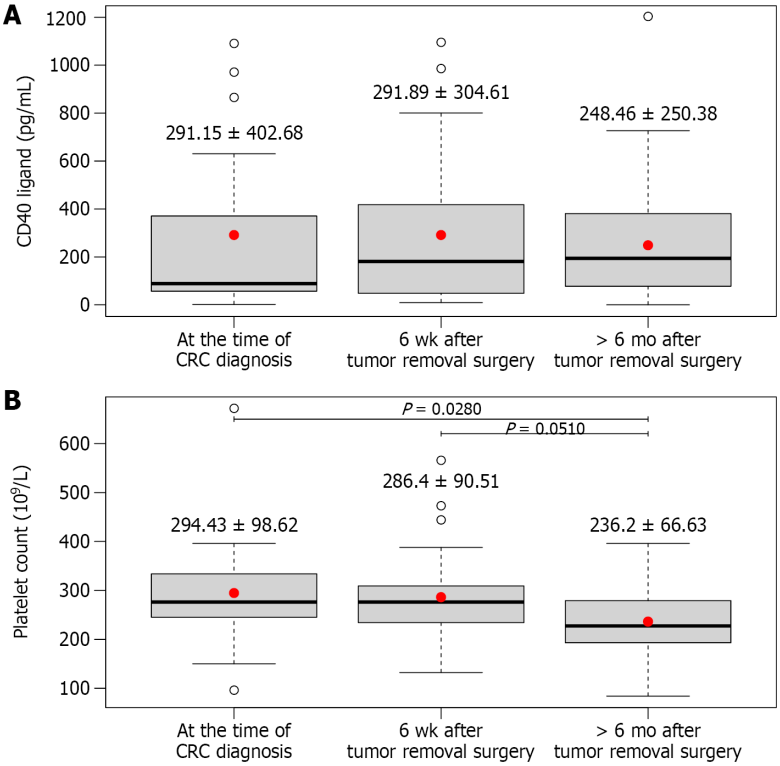

Figure 3 Change in plasma CD40 ligand level and platelet count with the course of colorectal cancer within those patients who had all three laboratory measurements (mean ± SD). A: CD40 ligand level; B: Platelet count. *n* = 30. For the platelet count, a significant decrease could be observed ($P < 0.0001$), while the CD40 ligand level of patients did not change with the course of the disease ($P = 0.6813$). The red dot and thick line represent mean and median values, respectively. CRC: Colorectal cancer.

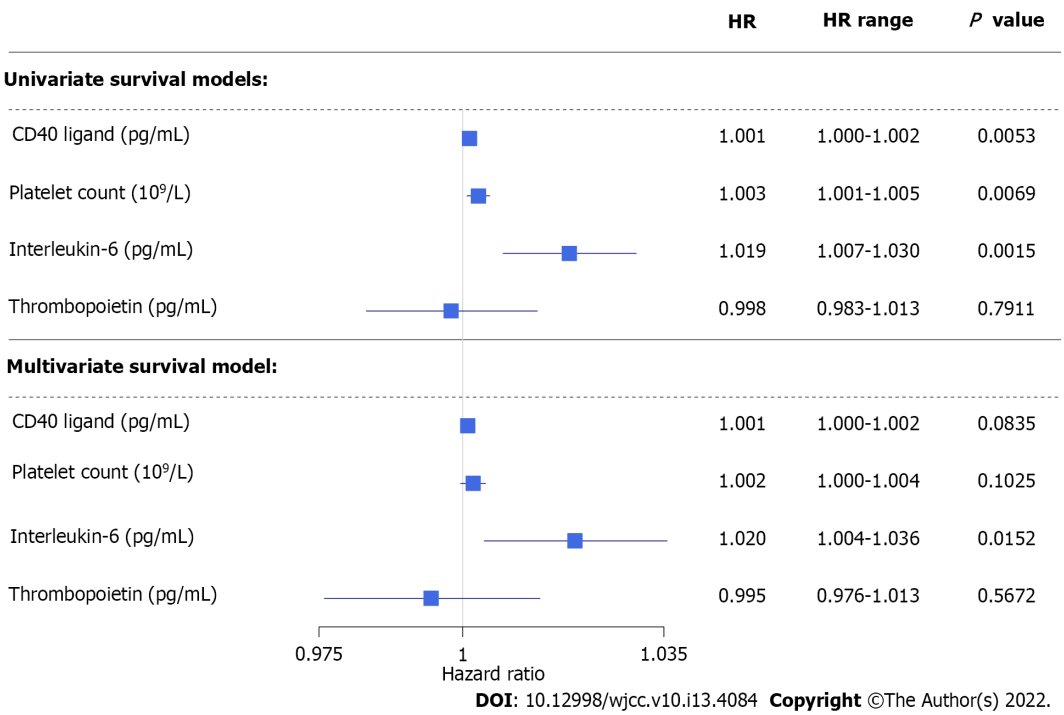

Figure 4 Results of univariate and multivariate analysis on disease-specific survival of colorectal cancer patients concerning preoperative laboratory results. HR: Hazard ratio.

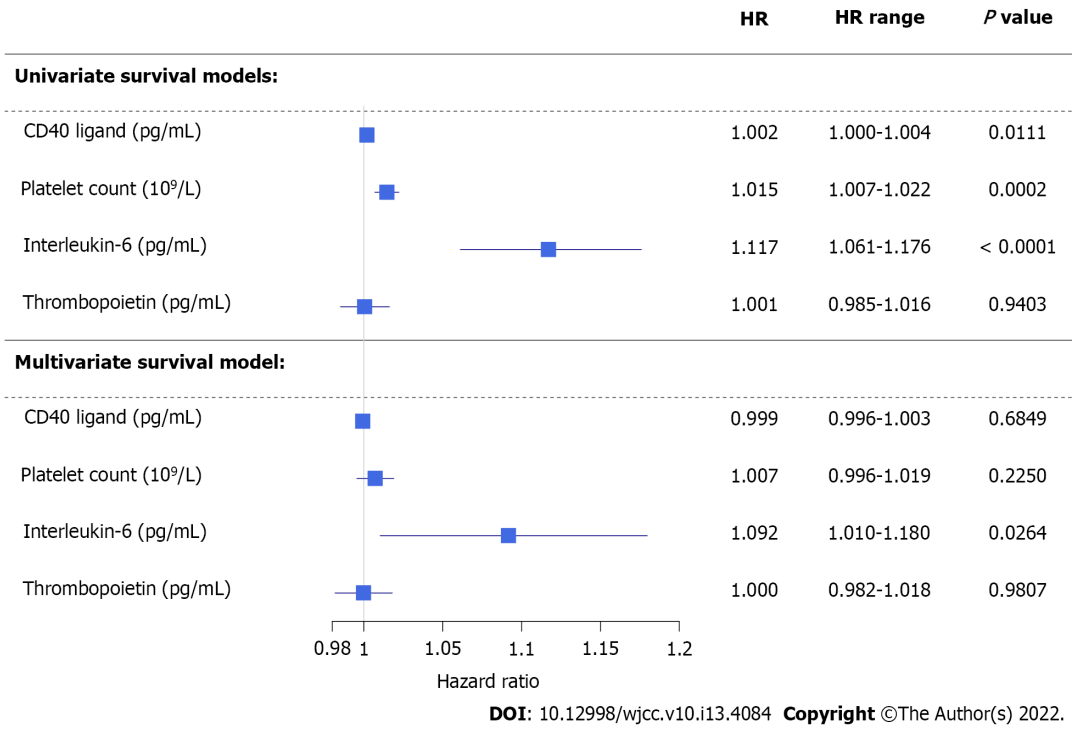

Figure 5 Results of univariate and multivariate analysis on disease-specific survival of colorectal cancer patients concerning postoperative laboratory results. HR: Hazard ratio.

increased inflammation caused by the tumor or its metastases. To clarify this question, further investigations are needed.

Limitations

Limitations of the current study were the relatively small sample size and the 57.4% follow-up rate, which did not allow us further analysis, *e.g.*, subgroup analysis or stratifications in survival models of postoperative measurements. Heterogeneity of the study population also introduced some bias.

CONCLUSION

To summarize the results of the current study, our data suggested that, in line with some previous publications[7,10,27], the plasma CD40L level is significantly higher in CRC, and the highest levels could be observed in Stage IV cancer. CRC patients with thrombocytosis had significantly higher CD40L levels, and CD40L was strongly correlated with some of the parameters of paraneoplastic thrombocytosis. The CD40L level of patients did not changed during the disease. Results from our stratified survival models and their strong association with high interleukin-6 levels and distant metastases suggest that CD40L is not only dependent on platelet count/thrombocytosis. We hypothesize that the general inflammation caused by the tumor may also play a role in the CD40L elevation of CRC patients, with high probability. To clarify this hypothesis, further investigations are needed.

ARTICLE HIGHLIGHTS

Research background

The role of CD40 ligand (CD40L) is controversial in colorectal cancer (CRC). Higher circulating CD40L levels of CRC patients are known, but their relationship with disease staging and local and distant metastasis is not clear.

Research motivation

To our knowledge, no previous study investigated the relationship between CD40L and CRC-related thrombocytosis. Furthermore, no study was conducted to observe if CD40L changes with the course of CRC.

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Research objectives
To analyze the clinical characteristics and laboratory results of 106 CRC patients and evaluate CD40L, interleukin-6, thrombopoietin level, and platelet count changes with the course of the disease; and to evaluate their effect on patient survival.

Research methods
CD40L and thrombopoietin were measured *via* enzyme-linked immunosorbent assay and interleukin-6 *via* electrochemiluminescence immunoassay. Measurements were conducted at the time of CRC diagnosis, at least 6 wk after primary tumor removal surgery, and at least 6 mo after primary tumor removal surgery.

Research results
CD40L of CRC patients was significantly higher in the presence of distant metastasis and/or thrombocytosis. CD40L was constant with the course of CRC, and all baseline differences persisted throughout the whole study. Both pre- and postoperative elevated CD40L were associated with poor overall and disease-specific survival of patients. The negative effect of CD40L on patient survival remained even after the stratification by thrombocytosis.

Research conclusions
CD40L level of CRC patients does not change with the course of the disease. The CD40L level is strongly correlated with platelet count, interleukin-6, thrombocytosis, and the presence of distant metastases. The effect of CD40L on patient survival cannot be fully eliminated *via* stratification by thrombocytosis. This suggests that the circulating amount of platelets is not the only factor behind its elevation.

Research perspectives
High plasma CD40L levels of CRC patients are with high probability not only dependent on circulating platelet count. General inflammation caused by the tumor could also contribute to CD40L elevation; therefore, further studies are required to clarify this question.

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FOOTNOTES

Author contributions: Herold Z, Herold M, and Somogyi A built the study design; Herold M, Herczeg G, Fodor A, and Herold Z were involved in the collection of samples; Herold Z analyzed the data; Herold Z, Herold M, Herczeg G, Szasz AM, and Dank M interpreted data; Herold Z prepared the draft of the manuscript; all authors were involved in editing and reviewing; Somogyi A and Herold Z received funding; Somogyi A supervised the study; all authors have read and agreed to the published version of the manuscript.

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