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Longitudinal Analysis of Soluble Vitamin D, Homocysteine, PD-L1, and PD-1 in Colorectal Cancer

PhD thesis

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List of Abbreviations

Adj-D	Patients without metastasis with vitamin D supplementation
Adj-ND	Patients without metastasis and no vitamin D supplementation
AJCC	American Joint Committee on Cancer
ALT	Alanine transaminase
APC	Adenomatous polyposis coli
AST	Aspartate transaminase
BRAF	v-Raf murine sarcoma viral oncogene homolog B gene
CCC	Cholangiocellular carcinoma
CD	Cluster of differentiation (immunophenotyping)
CRC	Colorectal cancer
CT	Computed tomography
ECOG	Eastern Cooperative Oncology Group
eGFR	Estimated glomerular filtration rate
ELISA	Enzyme-linked immunosorbent assay
FDG-PET/CT	Fluorodeoxyglucose positron emission tomography/computed tomography
GGT	Gamma-glutamyl transferase
sCRP	High-sensitivity C-reactive protein
KRAS	Kirsten rat sarcoma virus gene
LDH	Lactate dehydrogenase
mCRC	Metastatic colorectal cancer
Met-D	Patients with metastasis with vitamin D supplementation
Met-ND	Patients with metastasis and no vitamin D supplementation
MRI	Magnetic resonance imaging
NAD	Nicotinamide adenine dinucleotide
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NRAS	Neuroblastoma RAS viral oncogene homolog gene
PD-1	Programmed cell death protein 1
PD-L1	Programmed death-ligand 1
PIK3CA	Phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha

SAH	S-adenosylhomocysteine
SD	Standard deviation
TGF-β	Transforming growth factor beta
TNF	Tumor necrosis factor
TNM	TNM classification of malignant tumors
TP53	Tumor protein P53
WBC	White blood cell count

1. Introduction

Colorectal cancer (CRC) is the third most commonly diagnosed cancer type with almost 1.9 million new CRC cases being discovered worldwide annually, and CRC is responsible for 916,000 deaths, being the second leading cause of cancer deaths, according to the GLOBOCAN 2020 data [1, 2]. In Hungary, 11,126 new CRC cases were registered in 2019, of which over 56% and nearly 44% were male and female, respectively [3]. In line with international trends, the disease develops more often in elderly persons and the number of newly registered cases slowly rising every year (**Figure 1**) [1-3]. Unhealthy dietary patterns, smoking, obesity and sedentary lifestyle are risk factors associated with CRC [4, 5], moreover, family accumulation also plays a significant role: the 3-5% lifetime risk of CRC can double if the disease occurred in a first-degree relative [6].

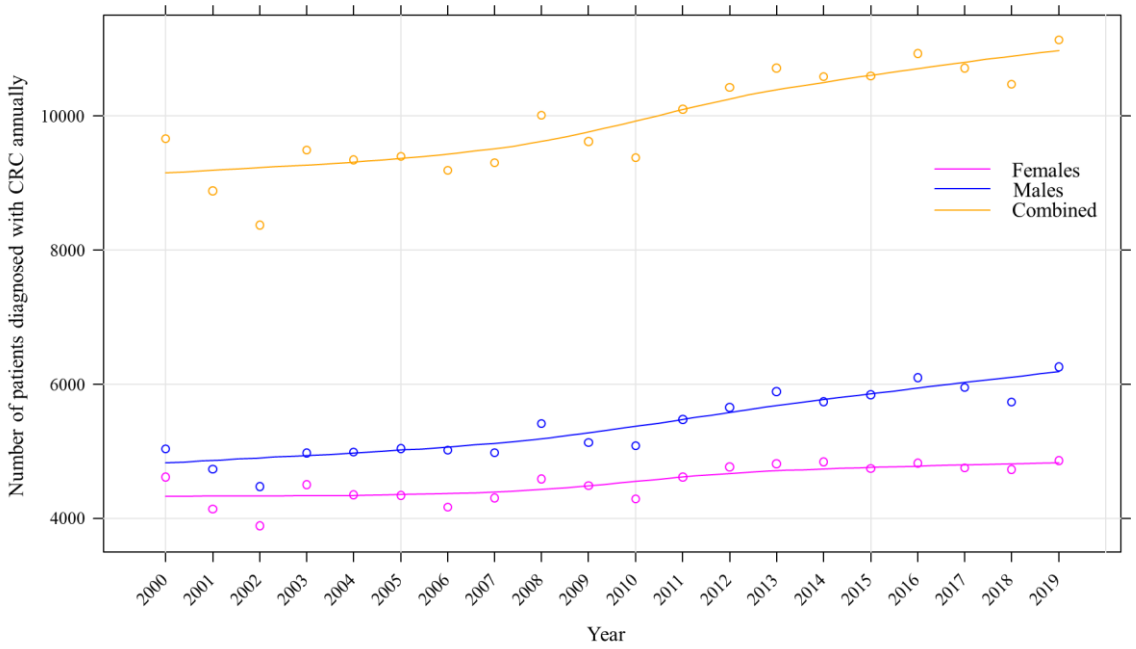

Figure 1: The incidence of CRC in Hungary between 2000 and 2019. Incidence data was obtained from: [3].

The development of CRC is a long process, it is estimated to take about 10-15 years [7]. The classical model of CRC formation (adenoma-carcinoma sequence) states that the mutation of stem cells in the Lieberkühn crypts initiates the grow of an early adenoma (< 1 cm, tubular or tubulovillous), which transforms into a late adenoma (> 1 cm, predominantly villous structure) and then into carcinoma [7]. During the time of tumor

development, mutations accumulate continuously. Common mutations occur in the APC/β-catenin, KRAS/BRAF, TGF-β, PIK3CA, and TP53 pathways, furthermore, epigenetic factors also play a significant role, of which hypermethylation is the most common [7, 8]. Moreover, CRC can be characterized by tumor-infiltrating lymphocytes, resistance to immunotherapeutic treatments, and genetic alterations leading to escaping immune surveillance [9].

The disease is asymptomatic for a long time, but with the progression and growth of the tumor bleeding and bloody stools – caused by the erosion of blood vessels around the tumor – are usually the first symptoms to attract the attention of patients. Weight loss, changes in stool habits, abdominal pain, occult bleeding and anemia of unknown cause can also be alarming symptoms. Colonoscopy followed by imaging (CT, MRI, FDG-PET/CT) is required for the diagnosis and staging of CRC [8]. Screening programs and mandatory examinations before surgeries, *e.g.*, for inguinal hernias and/or hemorrhoids, can increase the number of earlier incidental cases [10, 11].

Colorectal cancers staging is based on local invasion depth (T stage), lymph node involvement (N stage), and presence of distant metastases (M stage) [12]. The most used staging systems for CRC are the Dukes staging system [13], the modified Astler-Coller system [14], and the TNM system of the American Joint Committee on Cancer (AJCC) [15]. The 8th edition of the AJCC TNM system is the latest and most complex. In brief, stage 0 CRC is called *in situ* cancer (intraepithelial cancer or invasion of the lamina propria), stage I and II CRC have no lymph node involvement, stage III CRC has lymph node involvement, and stage IV CRC (or metastatic CRC, mCRC) is characterized by the presence of one or more distant metastases. Almost every second CRC patient will develop metastases at some point of the disease [16].

Based on the presence of metastases, the treatment of the disease can be divided into two major schemes [8]. Surgical or complete *en bloc* endoscopic resection is the mainstay curative treatment for patients with non-metastatic CRC, which may be followed by chemotherapy. According to latest guidelines, chemotherapy is needed in stage II and high-risk cases, *e.g.*, in poorly differentiated tumors, CRC with vascular, lymphatic or perineural tumor invasion, and in stage III CRC. In advanced stages, neoadjuvant chemotherapy or chemoradiotherapy can be used to reduce the size of the tumor, with which the chance of complete resection is significantly higher. Regular colonoscopic

and/or imaging follow-up of patients after tumor removal / chemotherapy is key in the early detection of tumor recurrence [17]. Nonetheless, there are some recent data – published at the ESMO 2022 meeting – suggesting that maybe there is no benefit of close follow up by imaging [18, 19]. In mCRC, the first treatment is systemic therapy, followed by surgical or ablative removal of the tumor and/or metastases if feasible, which can be followed by (radio)chemotherapy. Usually, a cytotoxic doublet with a biological agent (bevacizumab or anti-EGFR monoclonal antibody) is administered as the first-line and second-line treatments, regorafenib or trifluridine/tipiracil are administered as third-line or above [20]. Beside imaging, tumor progression can be measured using various biomarkers, including carcinoembryonic antigen (CEA), carbohydrate antigen 19-9 (CA19-9), and cancer antigens 72-4 (CA72-4) and 125 (CA125) [21].

1.1. Vitamin D and Its Role in Colorectal Cancer

Vitamin D is a pleotropic steroid hormone with known biological activity. Its natural form is dependent on ultraviolet irradiation and produced in the skin. It is transported first to the liver then to the kidneys, where first 25-hydroxyvitamin D then 1α,25-dihydroxyvitamin D is produced, respectively. Its main function is within the regulation of bone metabolism. In healthy cells, 1α,25-dihydroxyvitamin D binds to a specific vitamin D receptor in a Ca^{2+} dependent manner, however, in cancer cells the expression of these specific vitamin D receptors is downregulated, and cancer cells display limited response to vitamin D *in vitro* [22-25]. Vitamin D receptor deficient mice display hyper-proliferation and elevated levels of c-myc, which can be normalized by the administration of vitamin D from external source [26].

Low circulating levels of vitamin D are characteristic for CRC [27, 28], and associations were found between lower vitamin D levels and the increased risk of colorectal cancer [25]. In the last decades, a large number of observational and randomized clinical trials have investigated the effect of vitamin D on CRC and associations have been proposed between the circulating levels of vitamin D metabolites, CRC incidence, and patient survival [29-32]. Furthermore, in vitro studies have suggested various anticancer actions of vitamin D, including, but not limited to, through the inhibition of cyclin-dependent kinases anti-proliferative, due to gene regulatory changes pro-differentiating, pro-apoptotic, antimitotic, angiogenesis inhibitory, immune modulatory, tumorigenesis

inhibitory, through the inhibition of Wnt/β-catenin or TNF/NF-κB signaling anti-inflammatory, and regulatory effects on microRNAs and immune cells [25, 29, 33-37].

It has been previously reported that, after primary tumor removal, the high prevalence of vitamin D deficiency among CRC patients somewhat normalizes and the largest and fastest increase have been observed in those who have been on adjuvant vitamin D supplementation, as expected [38-40]. CRC survival is also affected by vitamin D levels: several studies compared pre-cancerous, preoperative and postoperative vitamin D levels of CRC patients, and significantly prolonged survival was associated with higher serum vitamin D concentrations [25].

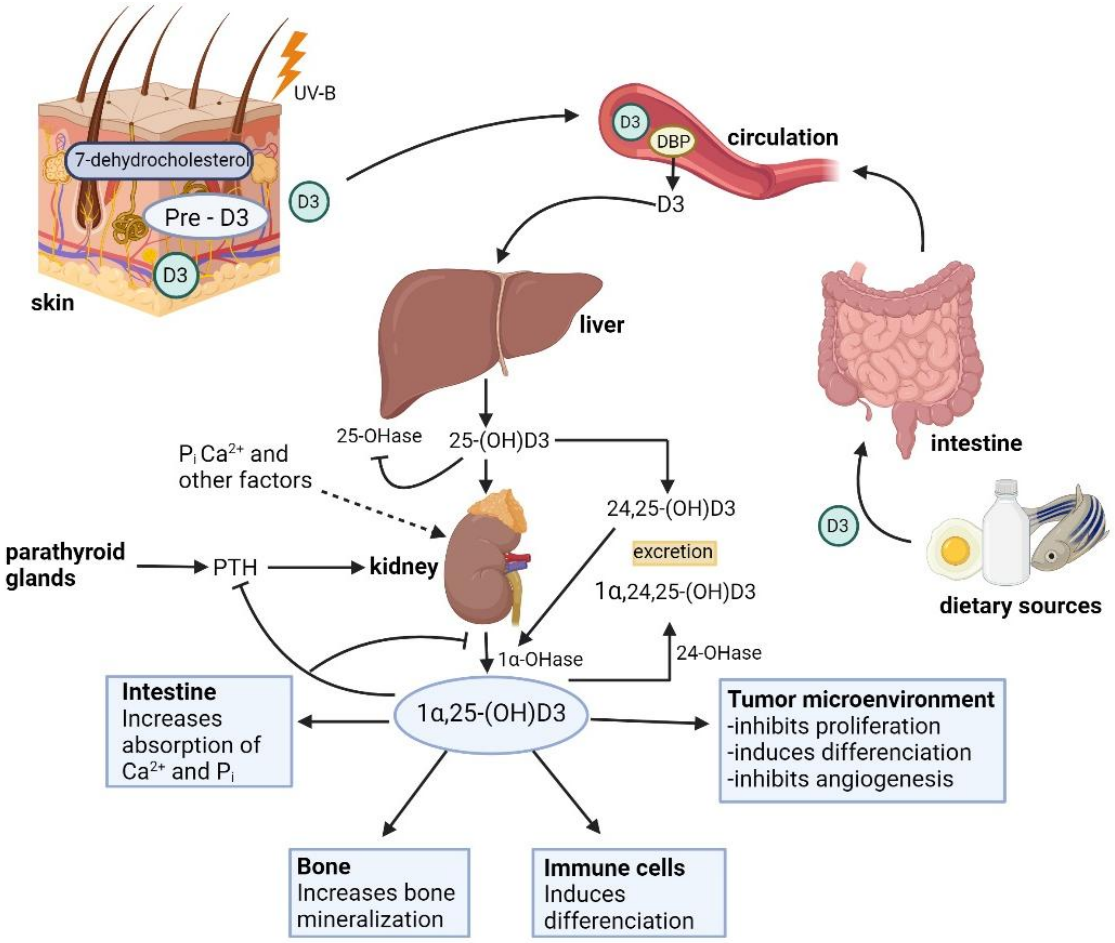

Figure 2: Role of vitamin D in human body. Based on Deeb, K., Trump, D. & Johnson, C. Vitamin D signalling pathways in cancer: potential for anticancer therapeutics. *Nat Rev Cancer* **7**, 684–700 (2007). https://doi.org/10.1038/nrc2196. The figure was created with BioRender.com.

1.2. Homocysteine and Its Role in Colorectal Cancer

Homocysteine is an essential, non-protein-forming, sulfur-containing amino acid produced by various cells throughout the human body by demethylation of methionine. Homocysteine metabolism is a vitamin B_{12}-dependent process [41]. In healthy individuals, its circulating levels are low (5–15 μmol/L), which is somewhat lower in women [42, 43]. Homocysteine is inversely proportional to the amount of hydroxyvitamin D in the blood [43].

Several diseases have been associated with pathological levels of homocysteine. Numerous studies found that high homocysteine level has major role in the development and progression of cardiovascular diseases, moreover, hyperhomocysteinemia is known to cause endothelial dysfunction. Homocysteine can affect bone mineral density and remodeling. Furthermore, elevated homocysteine levels were found in patients with Alzheimer's disease, stroke, Parkinson's disease, depression, migraine, end stage renal disease, insulin resistance, type 2 diabetes mellitus, abdominal aortic aneurysm, and in various cancers [44].

High homocysteine levels are also characteristic for CRC [45], however, in contrast to vitamin D, the relationship between homocysteine and CRC is a less investigated area. Higher homocysteine level had been found to be a risk factor for the increased incidence of CRC [41, 46, 47] and the following relations have been identified in the meta-analysis Shiao *et al*. [48]. Along with the elevated homocysteine levels, the increased risk for CRC has been also associated with decreased vitamin B_6 and B_{12} levels, TT and CT polymorphisms of the methyltetrahydrofolate reductase gene, smoking, regular alcohol intake, increased dietary B_{12} intake, and decreased dietary methionine, fiber, folate, and vitamin B_6 intake [48]. Ni *et al*. [49] reported a slow but constant decreasing level of homocysteine in CRC patients without tumor recurrence within the first year after primary tumor removal surgery, while an increase has been found in those with relapse.

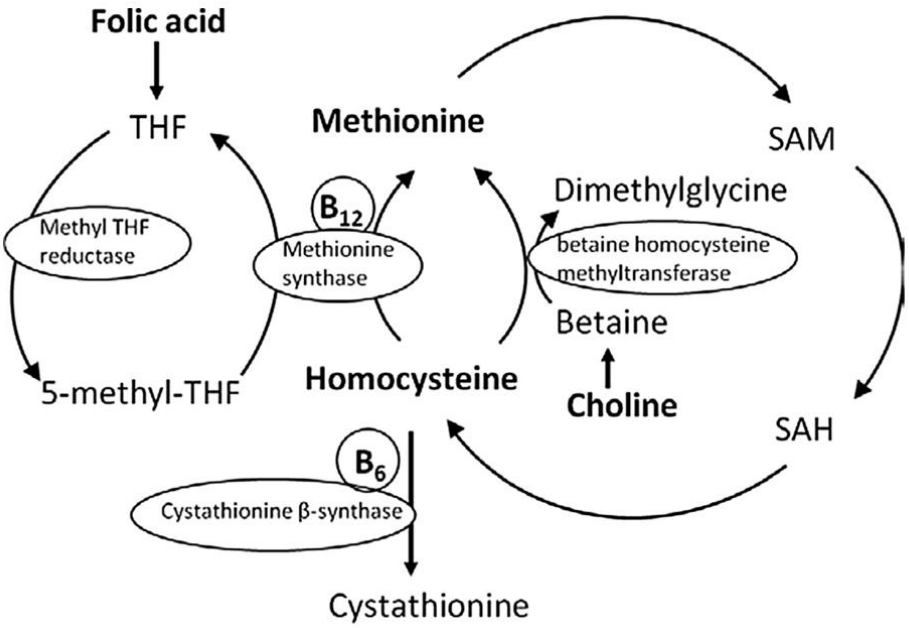

Figure 3: Role of homocysteine in human body. Based on www.etk.pte.hu/public/Klinikai_es_gyakorlati_dietetika.pdf .

1.3. Soluble PD-1 and PD-L1, and Their Role in Colorectal Cancer

Programmed death-ligand 1 (PD-L1, synonyms: CD274 and B7-H1) is an immune checkpoint molecule expressed by tumor cells that can interact with programmed cell death protein 1 (PD-1, synonym: CD279), a receptor of T cells [9, 50]. The activation of the PD-1/PD-L1 pathways can lead to an immunosuppressive, anti-apoptotic microenvironment, which helps the tumor to evade anti-tumor immunity [9, 50, 51]. Besides the originally described membrane-bound forms, soluble variants of PD-L1 and PD-1 have been also found [52]. The soluble form of PD-1 arises due to alternative splicing (lacking exon 3), while the soluble form of PD-L1 is cleaved by a metalloproteinase from the cell surface of dendritic cells [53]. The exact role of soluble PD-1/PD-L1 forms is unknown, but they are mainly involved in various malignant and inflammatory diseases [54, 55]. To our current knowledge, they are involved in immune response regulation and tumor immune escape; soluble PD-1 blocks the PD-1–PD-L1 interaction and activates $CD8^+$ T cells, while soluble PD-L1 binds to PD-1 with higher affinity than the membrane-bound forms, ultimately inhibiting the T cell response [56, 57]. Soluble PD-1/PD-L1 is independent of the amount expressed on membranes and has no association with sex, age, or histopathological type [58, 59]. The strong association

between patient survival and soluble PD-L1 levels in other malignant diseases [58-62] has led to the recommendation that the soluble PD-L1 level can also be used as a possible prognostic marker in CRC [63]. The usefulness of soluble PD-1 is controversial [58-62], and to date, only the presence of increased tissue PD-1 and/or PD-L1 is of clinical significance [56].

Studies investigating the soluble PD-1/PD-L1 forms have found that both protein is negatively associated with cancer survival [58-62, 64, 65]. Elevated soluble PD-L1 is characteristic for melanoma [66], cholangiocellular (CCC) [64], gastric [67], hepatocellular [68, 69], urothelial [61], renal [62, 70], ovarian [71], and lung cancers [58, 60, 66, 72], while elevated PD-1 was observed in gastric, lung, and bladder cancers [73]. In CRC, the circulating level of both proteins has been reported to be significantly lower compared to that of healthy controls [74]. Moreover, higher circulating PD-L1 level has been associated with a higher degree of tumor differentiation [75], $CD3^+$ and $CD8^+$ T cell counts are negatively [76], while neutrophil-to-lymphocyte ratio is positively correlated with PD-L1 and PD-1 [77]. All studies investigating soluble PD-L1 in CRC suggests that it is a good prognostic marker [59, 63, 76, 78], even for early-stage CRC [63], while soluble PD-1 seems to be independent from CRC survival [59]. PD-L1 increases significantly after neoadjuvant chemoradiotherapy [59], in progressive disease [78], but decrease after hepatic metastasectomy, however, recurrence and/or progression following hepatectomy can reintroduce the increase in PD-L1 levels [76].

2. Objectives

Despite the large and increasing number of studies investigating soluble vitamin D, homocysteine, PD-1, and PD-L1 in CRC, very little is known about their longitudinal relationship in CRC, and further data is needed about their everyday clinical application.

Therefore, the research objectives were as follows:

1.) Is there a correlation between serum vitamin D and homocysteine levels measured during colon cancer treatment and disease-specific survival?
    a. Or maybe in other clinicopathological parameters?
2.) Is there a correlation between measured parameters and possible comorbidities?
3.) Performing a longitudinal study, in which the longitudinal changes of the mentioned parameters were also monitored.
4.) Assessment of soluble PD-1 and PD-L1 levels in metastatic colon cancer.
5.) What is the prognostic value of sPD-1 and sPD-L1 in colon tumors, and is there a correlation with other routine laboratory parameters?
6.) Is there a difference between survival and other parameters of the groups with low and high baseline sPD-1 and sPD-L1 levels?

3. Methods

The study was conducted in accordance with the World Medical Association's Declaration of Helsinki; handling of patient data was in accordance with the General Data Protection Regulation issued by the European Union. The study was approved by the Regional and Institutional Committee of Science and Research Ethics, Semmelweis University (SE TUKEB 133/2015). Study subjects signed written informed consent forms prior to any study specific procedures.

3.1. Patients and Study Design

A longitudinal observational cohort study was conducted with the inclusion of eighty-six CRC patients who attended at the Division of Oncology, Department of Internal Medicine and Oncology, Semmelweis University, Budapest, between 2017 and 2018. Diagnosis of CRC was given by histopathological examination of colonoscopic biopsy or surgical specimens. Inclusion criteria of the study required at least one vitamin D and homocysteine measurement, performed at the Central Laboratory of Semmelweis University. Exclusion criteria included the following: age < 18 years, histopathological diagnosis of other tumor forms than adenocarcinoma, any previous malignancies, known hematologic, inflammatory bowel, systemic autoimmune, mental and/or inadequately controlled thyroid diseases, the usage of systemic corticosteroids 90 days prior to the baseline visit date, erythropoiesis-stimulating agents, and patients with an Eastern Cooperative Oncology Group (ECOG) performance status > 2.

Of the eighty-six patients, a total of 433 visits (5 visits/patient on average) had been processed. The first visit was performed after the primary tumor removal surgery, if technically possible in those of with metastasis, and prior the first adjuvant or metastatic chemotherapy administration in those patients without and with metastasis, respectively. Patients attended at the second and third visits one and two months after the baseline measurement, respectively. Later measurements were performed within 2–3 months intervals.

Thirty-seven of the eighty-six patients, who had mCRC were selected for further analysis. In addition to the original laboratory parameters, the soluble PD-1 and PD-L1 level of these patients were also measured prior to any metastatic setting treatments. None of the

study participants received immune checkpoint inhibitor therapy. By extending the longitudinal data with additional visits for every 4–6 weeks, if feasible, a total of 506 visits were recorded for the 37 study participants. The whole study process is shown in **Figure 4.**

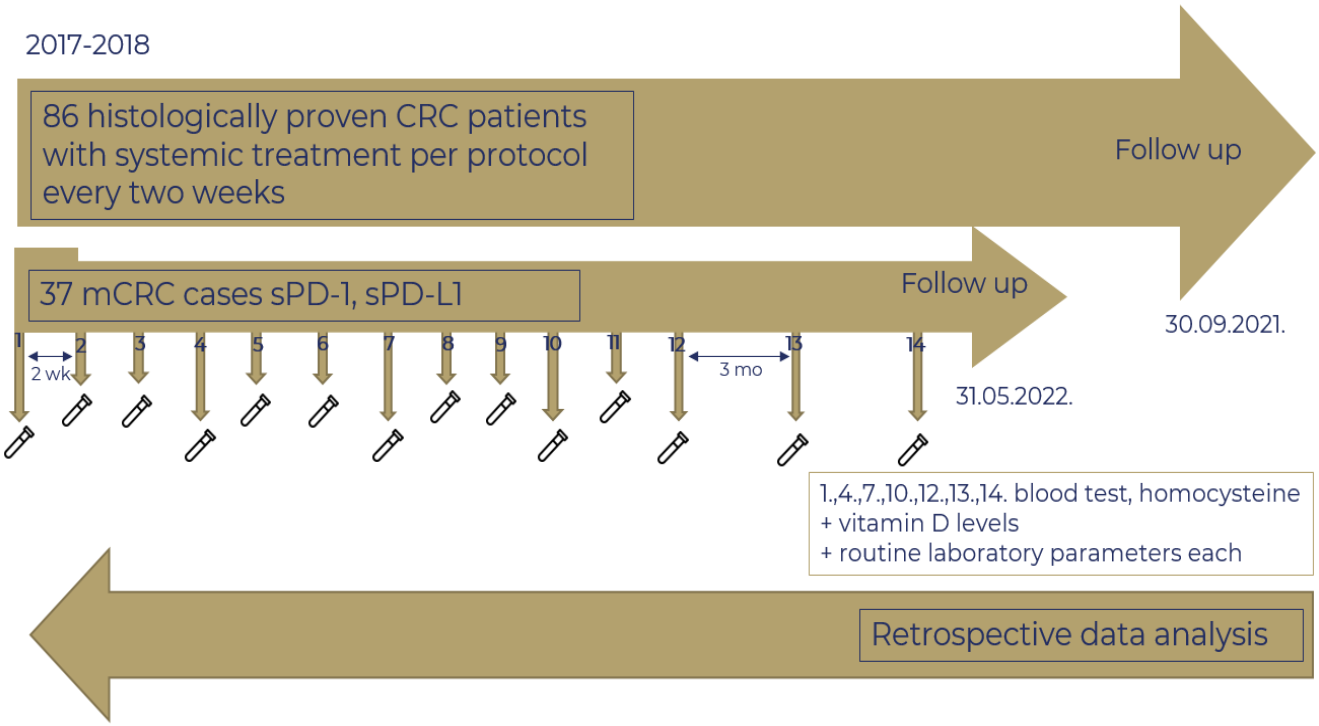

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Figure 4: Study design. 86 histologically proven CRC patients were enrolled to the study. They received standard chemotherapy regimens every two weeks. Blood samples were collected at clinical visits, vitamin D, homocysteine and standard laboratory parameters were measured. 37 metastatic patients were selected for further analysis of sPD1 and sPD-L1.

3.2. Clinicopathological and Laboratory Data Measurements

Anamnestic data, including recent medications and comorbidities, were collected at the time of the baseline measurements, while laboratory measurements from fasting blood samples were recorded during every visit. Complete blood count, aspartate and alanine transaminase, gamma-glutamyl transferase, lactate dehydrogenase, creatinine, high-

sensitivity C-reactive protein (hsCRP), plasma glucose, cholesterols and triglycerides, and CRC-related tumor markers were measured at the Central Laboratory of Semmelweis University. Estimated glomerular filtration rate (eGFR) was calculated using the Chronic Kidney Disease-Epidemiology Collaboration equations [79]:

$$eGFR_{women + \text{creatinine} < 62} = 144 * \left(\frac{creatinine}{61.6}\right)^{-0.329} * 0.993^{\text{age}}$$

$$eGFR_{women + \text{creatinin} \geq 62} = 144 * \left(\frac{creatinine}{61.6}\right)^{-1.209} * 0.993^{\text{age}}$$

$$eGFR_{men + creatinin < 80} = 141 * \left(\frac{creatinine}{79.2}\right)^{-0.411} * 0.993^{\text{age}}$$

$$eGFR_{men + creatinin \geq 80} = 141 * \left(\frac{creatinine}{79.2}\right)^{-1.209} * 0.993^{\text{age}}$$

Equation 1: Calculation of estimated glomerular filtration rate (eGFR) using the Chronic Kidney Disease-Epidemiology Collaboration equations [79].

CRC staging was given by histopathological examination of surgical specimens and imaging studies; the 8th edition of the American Joint Committee on Cancer grouping was used [15]. 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 [80, 81]. At the time of inclusion to the study, with the exception of KRAS and NRAS pathway analysis, molecular profiling of the tumors was performed only on an as-needed basis as directed by respective guidelines. Tumor burden was assessed according to medical imaging (CT, MRI or FDG-PET/CT), where tumor spread and size were measured by a radiology specialists, analysis was based on total diameter of the biggest measured tumor sizes.

Chemotherapy was grouped as adjuvant if no metastasis and as first-line, second-line etc. if metastasis was present. In brief, a cytotoxic doublet with a biological agent (bevacizumab or anti-EGFR monoclonal antibody) was administered as the first-line and second-line treatment. Regorafenib or trifluridine/tipiracil were administered mostly as third-line or above. Selection of chemotherapy protocol(s) was based on national and international guidelines [17, 20]. Vitamin D supplementation was prescribed for patients on platinum-based chemotherapies and supplementation was also recommended for the

remaining patients. Vitamin D supplementation (3000 IU/day) was recorded based on digital prescription records and paper-based patient records.

The cause of death was recorded as two separate events if patients died due to CRC-related or non-cancer related reasons. Disease-specific (DSS), overall (OS) and progression free (PFS) survival of patients was defined as the length of time from the date of study inclusion until cancer-related death, death occurring from any cause, and disease progression or death, respectively. Follow-up of all 78 CRC patients was terminated on 30 September 2021, while the subset of the 37 mCRC patients were followed-up until 31 May 2022. Patients alive at these times were right-censored. The RECIST guideline v1.1 was used to evaluate response to treatment [82].

3.3. Measurement of Serum Vitamin D

Serum vitamin D was measured using the DiaSorin LIAISON® 25OH Vitamin D TOTAL chemiluminescent immunoassay kit (DiaSorin S.p.A., Saluggia, Italy) at the Central Laboratory of Semmelweis University. To avoid degradation, vitamin D samples were taken and stored in light-protected collection tubes until measurement. As per the instructions given by the manufacturer, samples were first incubated for 10 minutes, during which vitamin D bonded to the specific antibody on the solid phase. During a second 10-minute incubation a tracer (an isoluminol derivative) was added to the solution, which was followed by washing. To start the chemiluminescent reaction, a starter reagent was added to the samples, and the vitamin D concentrations were determined using the Diasorin LIAISON® (DiaSorin S.p.A., Saluggia, Italy) automated chemiluminescence analyzer. The light signal measured in relative light units is inversely proportional to the level of the analyte.

3.4. Measurement of Serum Homocysteine

Serum homocysteine was measured using the Roche cobas® HCYS Homocysteine Enzymatic assay kit (Roche Diagnostics GmbH, Mannheim, Germany) at the Central Laboratory of Semmelweis University. The principle of the assay is as follows. Instead of assessing a co-substrate or homocysteine conversion products, the co-substrate conversion product of homocysteine is measured. In brief, oxidized homocysteine of the sample was first reduced to free homocysteine, which was then transformed into

S-adenosylhomocysteine (SAH), catalyzed by a homocysteine-S-methyltransferase. In the next step, SAH was hydrolyzed into adenosine and homocysteine. The resulting homocysteine was cycled back into the homocysteine conversion reaction in order to amplify the detection signal. The formed adenosine is used to generate the detection signal: it is hydrolyzed into inosine and ammonia, of which the latter was used to form NAD^+ from NADH. The concentration of homocysteine in the sample is directly proportional to the amount of NADH converted to NAD^+. The measurement was performed on a Roche cobas® automated analyzer (Roche Diagnostics GmbH, Mannheim, Germany).

3.5. Measurement of Plasma PD-1

Plasma PD-1 level was measured at the time of the baseline visit using the Invitrogen™ PD-1 Human ELISA kit (ThermoFisher Scientific, catalog number BMS2214, Waltham, MA, USA) at the Laboratory of the Division of Oncology, Department of Internal and Medicine and Oncology, Semmelweis University. In line with the manufacturer's description, 100 µL of plasma sample in 2x dilution with 50 µL of 100x diluted Biotin-Conjugate anti-human PD-1 monoclonal antibody was placed on the 96-well microplate. After a 120-minute incubation at room temperature, microwell stripes were buffer washed and 100 µL of 200x diluted streptavidin-HRP was added to the samples, followed by another 60-minute incubation and buffer washing. This was followed by a 30-minute incubation with 100 µL of 3,3′,5,5′-tetramethylbenzidine dihydrochloride substrate. After adding 100 µL of 0.1% 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 PD-L1 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, multiplied by the dilution factor, was used for evaluating the data.

3.6. Measurement of Plasma PD-L1

Plasma PD-L1 levels were measured at the time of the baseline visit using the Invitrogen™ PD-L1 Human ELISA kit (ThermoFisher Scientific, catalog number BMS2327, Waltham, MA, USA) at the Laboratory of the Division of Oncology, Department of Internal and Medicine and Oncology, Semmelweis University. In line with the manufacturer's description, 100 µL of plasma sample in 2x dilution was placed on the 96-well microplate. After a 120-minute incubation at room temperature, microwell stripes were buffer washed and 100 µL of 100x diluted Biotin-Conjugate anti-human PD-L1 monoclonal antibody was added to the samples, followed by another 60-minute incubation and buffer washing. This was followed by a 30-minute incubation with 100 µL of 200x diluted streptavidin-HRP, another wash, and finally a 30-minute incubation with 100 µL of 3,3′,5,5′-tetramethylbenzidine dihydrochloride substrate. After adding 100 µL of 0.1% 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 PD-L1 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, multiplied by the dilution factor, was used for evaluating the data.

3.7. Statistical Analysis

Statistical analysis was performed with R version 4.2.2 (R Core Team, R Foundation for Statistical Computing, 2022, Vienna, Austria). Group comparisons were performed using Welch's test, the Wilcoxon–Mann–Whitney U-test, Fisher's exact test, ANOVA with Tukey's HSD tests as a post hoc, and Kruskal–Wallis test with *p*-value corrected pairwise Wilcoxon–Mann–Whitney U-tests as post hoc. Spearman rank correlation was used to test associations between the investigated parameters. PD-1 and PD-L1 cut-off values were determined using a receiver operating curve (ROC) analysis (R-package pROC, version 1.18.0). To detect the changes of various parameters in time, natural cubic spline adjusted random intercept linear mixed-effect models were used (R-package "nlme", developed by Pinheiro, Bates DebRoy, Sarkar and the R Core Team, version 3.1-160). "Simple" and cause-specific Cox regression survival models were used for PFS and DSS,

respectively (R package “survival”, developed by Therneau and Grambsch, version 3.4-0). Moreover, investigations on longitudinal survival data were completed using competing risk Cox regression models with time-dependent covariate(s). All models analyzed were tested for multicollinearity and proportionality using generalized variance-inflation factors and by proportional hazards tests, respectively [83, 84]. $p < 0.05$ was considered as statistically significant and *p*-values were corrected with the Holm [85] or the false discovery rate [86] methods for multiple comparisons problem. Results were expressed as mean ± standard deviation, the number of observations (percentage), and as hazard ratio (HR) with 95% confidence interval (95% CI) for continuous, count, and survival data, respectively. Naïve Kaplan–Meier survival curves were drawn with the R-package “survminer” (developed by Kassambara, Kosinski and Biecek, version 0.4.9).

4. Results

4.1. Measurement of Vitamin D and Homocysteine in Colorectal Cancer

A total of 86 CRC patients were included in the study. The patients were divided into four cohorts based on the presence of metastases and whether they were on vitamin D supplementation during our observation time or not. Nineteen, twenty-five, ten, and thirty-two patients were assigned to the 'adjuvant without vitamin D supplementation' (Adj-ND), 'adjuvant with vitamin D supplementation' (Adj-D), 'metastatic without vitamin D supplementation' (Met-ND), and to the 'metastatic with vitamin D supplementation' (Met-D) groups, respectively. The anamnestic data of study subjects are summarized in **Table 1**.

4.1.1. Baseline Patient Characteristics and Measurements

The comparisons between the groups were performed in order to test whether the later-treated and untreated groups differed and for any confounding parameters. Only marginal differences were found both in vitamin D (Kruskal–Wallis: $p = 0.0749$, **Figure 5A**) and in homocysteine (Kruskal–Wallis: $p = 0.0535$, **Figure 5B**) levels. It was tested whether other factors, such as sex, staging, location of the tumor, and the various comorbidities, such as type 2 diabetes or hypertension, affected the parameters outlined above. Vitamin D was not affected by any of these grouping factors, while homocysteine was significantly higher if the tumor was located on the right side of the colon (left-sided: 12.48 ± 3.94 ng/mL, right-sided: 15.34 ± 5.65, $p = 0.0174$).

The comparison of laboratory results between the four cohorts revealed only the differences related to the presence of metastasis. The pathological values of high-sensitivity C-reactive protein (Kruskal–Wallis test, $p = 0.0025$), lactate dehydrogenase ($p = 0.0004$), CEA ($p < 0.0001$), and CA 19-9 ($p = 0.0080$) were found in the study subjects with metastasis, compared to those CRC patients without metastasis (Table 2). Prior to any vitamin D supplementation, no differences could have been justified between the groups Adj-ND vs. Adj-D and Met-ND vs. Met-D.

The correlation analysis between laboratory results, vitamin D, and homocysteine revealed only previously found relationships [87-92], such as higher vitamin D levels, which are associated with lower but still within the normal range platelet counts

Table 1. Demographic and clinical characteristics of study participants. The significant differences found in staging ($p < 0.0001$), lineage of chemotherapy ($p < 0.0001$), and the occurrence of death/progression ($p < 0.0001$) was related only to the presence of metastases. Continuous and count data are presented as mean ± standard deviation and number of observations (percentage), respectively.

Parameter	**Adj-ND (*n* = 19)**	**Adj-D (*n* = 25)**	**Met-ND (*n* = 10)**	**Met-D (*n* = 32)**
Age (years)	64.87 ± 10.37	62.58 ± 8.62	65.59 ± 7.16	59.49 ± 11.96
Sex (Male : Female)	12 : 7 (63.2% : 36.8%)	15 : 10 (60.0% : 40.0%)	7 : 3 (70.0% : 30.0%)	20 : 12 (62.5% : 37.5%)
Location of the tumor [80, 81]				
- Right-sided	8 (42.1%)	12 (48.0%)	1 (10.0%)	8 (25.0%)
- Left-sided [1]	11 (57.9%)	13 (52.0%)	9 (90.0%)	24 (75.0%)
Staging [15] [2]				
- Stage I	0 (0.0%)	2 (8.0%)	0 (0.0%)	0 (0.0%)
- Stage II	8 (42.1%)	10 (40.0%)	0 (0.0%)	0 (0.0%)
- Stage III	11 (57.9%)	13 (52.0%)	0 (0.0%)	0 (0.0%)
- Stage IV	0 (0.0%)	0 (0.0%)	10 (100.0%)	32 (100.0%)
Distant metastasis developed later with the course of the disease	3 (15.8%)	4 (16.0%)	–	–
Disease progression [3]	3 (15.8%)	4 (16.0%)	9 (90.0%)	28 (87.5%)

Table 1 (cont.)

Parameter	Adj-ND (*n* = 19)	Adj-D (*n* = 25)	Met-ND (*n* = 10)	Met-D (*n* = 32)
CRC-related death [4]	1 (5.3%)	1 (4.0%)	9 (90.0%)	25 (78.1%)
Chemotherapy				
- Adjuvant	17 (89.5%)	22 (88.0%)	0 (0.0%)	0 (0.0%)
- Metastatic				
o First line	2 (10.5%)	3 (12.0%)	4 (40.0%)	12 (37.5%)
o Second line	0 (0.0%)	0 (0.0%)	1 (10.0%)	10 (31.25%)
o Third line or above	0 (0.0%)	0 (0.0%)	5 (50.0%)	10 (31.25%)
Medical history				
- Type 2 diabetes mellitus	6 (31.6%)	6 (24.0%)	2 (20.0%)	5 (15.6%)
- Hypertension	12 (63.2%)	16 (64.0%)	9 (90.0%)	18 (56.3%)
- Cardiovascular diseases	3 (15.8%)	6 (24.0%)	3 (30.0%)	4 (12.5%)
- Cardiovascular event(s)	2 (10.5%)	3 (12.0%)	0 (0.0%)	4 (12.5%)
- Thyroid diseases [5]	2 (10.5%)	3 (12.0%)	3 (30.0%)	4 (12.5%)

Adj-ND: patients without metastasis and no vitamin D supplementation; Adj-D: patients without metastasis with vitamin D supplementation; Met-ND: patients with metastasis and no vitamin D supplementation; Met-D: patients with metastasis with vitamin D supplementation. [1] Three, four, three, and twelve were located in the rectum in each group, respectively. [2] Staging given at the time of inclusion to the study. [3]

Any progression that occurred between the inclusion date into the study and the latest observation date. [4] Deaths related to other diseases than CRC were treated as separate events. [5] Euthyroid status of patients was a required criteria to be included in the study.

Table 2. Baseline laboratory measurements of study participants. Besides a few parameters affected by the presence of metastases, laboratory parameter measurement data suggested that the study population was homogenous at the time of inclusion. Continuous and count data are presented as mean ± standard deviation and number of observations (percentage), respectively.

Parameter	**Adj-ND (*n* = 19)**	**Adj-D (*n* = 25)**	**Met-ND (*n* = 10)**	**Met-D (*n* = 32)**	**Signif.**
White blood cell count (10^9/L)	7.51 ± 3.17	6.80 ± 1.70	8.15 ± 2.45	7.73 ± 3.22	-
Red blood cell count (10^{12}/L)	4.52 ± 0.48	4.55 ± 0.47	4.35 ± 0.37	4.61 ± 0.52	-
Hemoglobin (g/L)	124.95 ± 18.32	126.04 ± 15.27	127.00 ± 19.09	130.38 ± 18.17	-
Hematocrit (L/L)	0.38 ± 0.05	0.39 ± 0.04	0.38 ± 0.04	0.39 ± 0.05	-
Platelet count (10^9/L)	231.26 ± 60.30	269.96 ± 86.74	332.70 ± 130.46	294.25 ± 132.94	-
Aspartate transaminase (U/L)	22.11 ± 4.70	24.58 ± 8.60	32.90 ± 19.54	50.00 ± 73.38	-
Alanine transaminase (U/L)	21.00 ± 9.46	23.12 ± 13.26	27.30 ± 20.47	38.50 ± 32.42	-
Gamma-glutamyl transferase (U/L)	34.50 ± 17.84	30.42 ± 12.83	155.50 ± 261.67	137.53 ± 163.50	-
Lactate dehydrogenase (U/L)	182.68 ± 28.79 *	178.50 ± 38.61 *	357.00 ± 291.13 *	587.44 ± 1183.24 *	$p = 0.0004$
Total cholesterol (mmol/L)	1.24	5.50 ± 1.28	6.35 ± 2.24	5.59 ± 1.45	-

Table 2 (cont.)

Parameter	Adj-ND (*n* = 19)	Adj-D (*n* = 25)	Met-ND (*n* = 10)	Met-D (*n* = 32)	Signif.
High-density lipoprotein (mmol/L)	1.54 ±0.51	1.45 ± 0.33	1.37 ± 0.34	1.37 ± 0.44	-
Low-density lipoprotein (mmol/L)	3.05 ± 0.80	3.39 ± 0.93	3.92 ± 1.43	3.54 ± 1.03	-
Triglyceride (mmol/L)	1.39 ± 0.99	1.64 ± 0.82	1.63 ± 0.89	1.58 ± 0.52	-
Creatinine (µmol/L)	79.00 ± 18.95	69.04 ± 14.06	68.80 ± 21.66	64.69 ± 12.67	-
Estimated glomerular filtration rate $\left(\frac{\text{mL}}{\text{min}\cdot 1.73\text{m}^2}\right)$	82.74 ± 18.38	90.71 ± 14.03	89.52 ± 21.13	97.48 ± 13.10	-
High-sensitivity C-reactive protein (mg/L)	3.93 ± 3.70 *	3.57 ± 3.50 *	26.90 ± 35.16 *	21.80 ± 43.92 *	$p = 0.0025$
Total protein (g/L)	75.58 ± 3.87	76.40 ± 4.55	73.98 ± 3.98	72.98 ± 5.35	-
Albumin (g/L)	44.24 ± 2.46	44.30 ± 3.13	42.53 ± 4.68	42.94 ± 3.48	-
Carcinoembryonic antigen (ng/mL)	2.33 ± 1.27 *	2.01 ± 1.48 *	117.21 ± 162.72 *	367.81 ± 1496.30 *	$p < 0.0001$
Carbohydrate antigen 19-9 (U/mL)	6.53 ± 11.39 *	7.46 ± 12.98 *	451.60 ± 813.46 *	925.56 ± 3656.01 *	$p = 0.0080$

Adj-ND: patients without metastasis and no vitamin D supplementation; Adj-D: patients without metastasis with vitamin D supplementation; Met-ND: patients with metastasis and no vitamin D supplementation; Met-D: patients with metastasis with vitamin D supplementation. * Differences in high-sensitivity C-reactive protein (Kruskal–Wallis test, $p = 0.0025$), lactate dehydrogenase ($p = 0.0004$), carcinoembryonic antigen ($p < 0.0001$), and in carbohydrate antigen 19-9 ($p = 0.0080$) were found to be significant only between patients with and those without metastasis, while all measured parameters were the same between the non-supplemented and vitamin D supplemented groups.

(Spearman's rho: −0.25, p = 0.0213), total cholesterol (Spearman's rho: −0.25, p = 0.0182), and low-density lipoprotein levels (Spearman's rho: −0.27, p = 0.0130). Higher homocysteine levels were associated with lower eGFR values (Spearman's rho: −0.38, p = 0.0003) and higher serum creatinine levels (Spearman's rho: +0.34, p = 0.0013). A marginal association was found between homocysteine and white blood cell counts (Spearman's rho: +0.21, p = 0.0533). Furthermore, significantly lower homocysteine level could have been observed with higher red blood cell counts (Spearman's rho: −0.35, p = 0.0223) within the patients without metastasis, compared to those with metastasis (p = 0.3311).

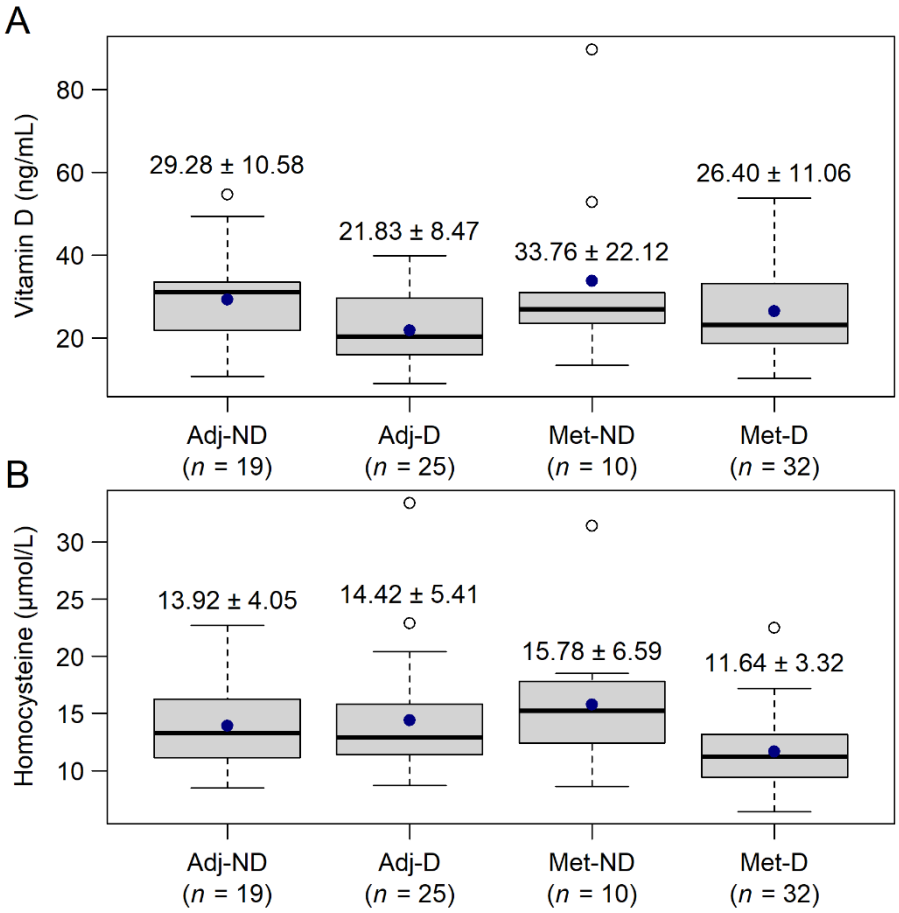

Figure 5. Baseline vitamin D (**A**) and homocysteine (**B**) level of study participants, prior to vitamin D supplementation (mean ± SD). Adj-ND: patients without metastasis and no vitamin D supplementation; Adj-D: patients without metastasis with vitamin D supplementation; Met-ND: patients with metastasis and no vitamin D supplementation; Met-D: patients with metastasis with vitamin D supplementation. The hollow black circles, blue dot, and the thick line represent outliers (> 1.5 times the interquartile range above the upper quartile), the mean and median value, respectively.

4.1.2. Longitudinal Analysis of Vitamin D and Homocysteine Levels

In order to determine whether the vitamin D and homocysteine levels changes differently in the four cohorts, with respect to the course of CRC, we chose the following approach. Linear mixed-effect models were constructed, where all of the baseline and further repeated measurements from all of the 86 study participants were used. A total of 417 and 421 vitamin D and homocysteine measurements were collected, respectively. The model prediction intervals, where an insufficient number of observations were available, were omitted as the accuracy of these model estimates would be insufficient with missing or a low number of samples. It was found that the vitamin D level of the Adj-ND patients fluctuated around an approximately constant value ($p = 0.2640$), while a constant increase was observed in those patients of the Adj-D cohort ($p = 0.0261$). A constant decrease was found for the Met-ND, while a subdued increase was found for the Met-D cohorts respectively (**Figure 6A**). In the case of homocysteine, a swoosh shape (sharp decline with slow recovery) was predicted for all of the study groups. Except for the deviation within the baseline value of the Met-D cohort ($p = 0.0195$), the only other difference between the cohorts was that the second half of the group averages resembled a saturation curve in those with vitamin D supplementation, while a steady increase in homocysteine level was observed in the non-supplemented patients (**Figure 6B**). It has to be noted, however, that although these pattern differences were assumed to be clinically different, the curves were not statistically different ($p = 0.3003$).

The effect of the various clinicopathological and laboratory parameters on the changes in the vitamin D and homocysteine levels were also investigated. The effect of staging was basically the same as belonging to any of the with or without metastasis groups (vitamin D: $p = 0.3363$, homocysteine: $p = 0.6276$). Right-sided tumors were associated with constantly higher homocysteine ($p = 0.0041$) but with similar vitamin D ($p = 0.6777$) levels (**Figure 9**). Those patients within the Adj-ND and Adj-D groups, in whom

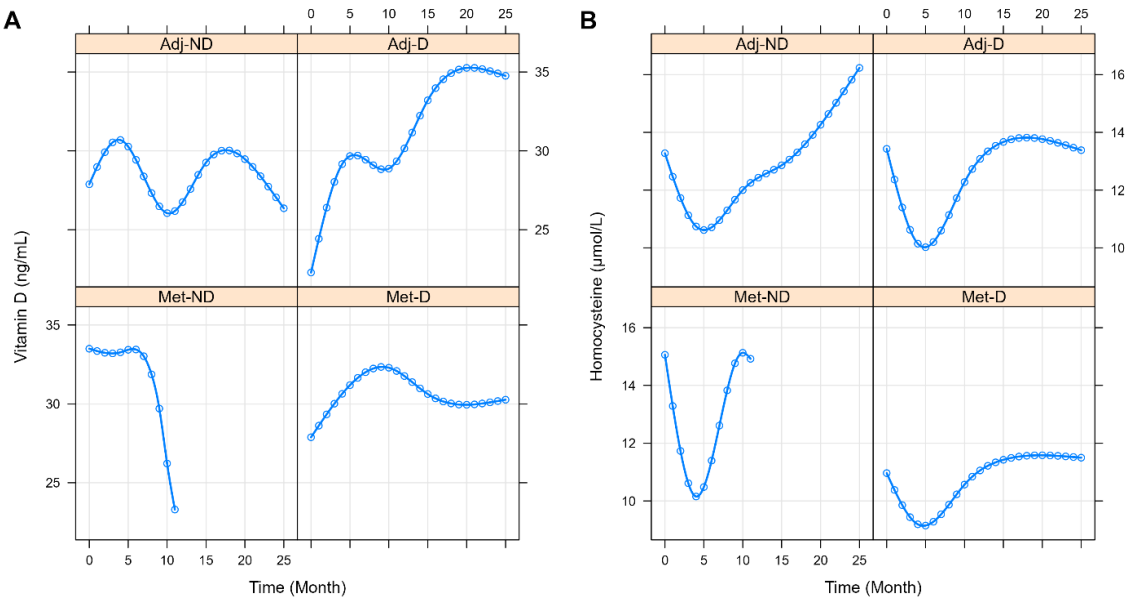

Figure 6. The predicted mean longitudinal changes in vitamin D (**A**) and homocysteine (**B**) level of the four study groups containing patients with (Adj) and without metastasis (Met) and with (D) and without (ND) vitamin D supplementation.

metachronous metastasis developed, after an initial similarity, tendentiously lower vitamin D (p = 0.1030, **Figure 8A**) levels occurred, compared to those who were metastasis-free until the end of our observations. Similarly, the vitamin D levels decreased after an initial similarity in those patients who had progressive disease (p = 0.0731) or who had died (p = 0.0267, **Figure 9A**). In contrast, the homocysteine levels were not affected statistically, neither by metachronous metastasis (p = 0.6813, **Figure 9B**), disease progression (p = 0.5225), nor by death (p = 0.2153, **Figure 10B**). Hypertension (p = 0.0585, **Figure 10A**) and other cardiovascular diseases (p = 0.0672, **Figure 11A**) caused only tendentiously higher vitamin D levels, while any previous cardiovascular event(s), type 2 diabetes mellitus, or thyroid diseases (in an euthyroid state) did not affect the change in the two parameters. The sex of patients marginally affected the change in vitamin D levels (p = 0.0878) and a marginally constant difference between the homocysteine levels of the two sexes was observed throughout the study (p = 0.0803). For both cases, the clinically advantageous effect was observable in women (**Figure 12**).

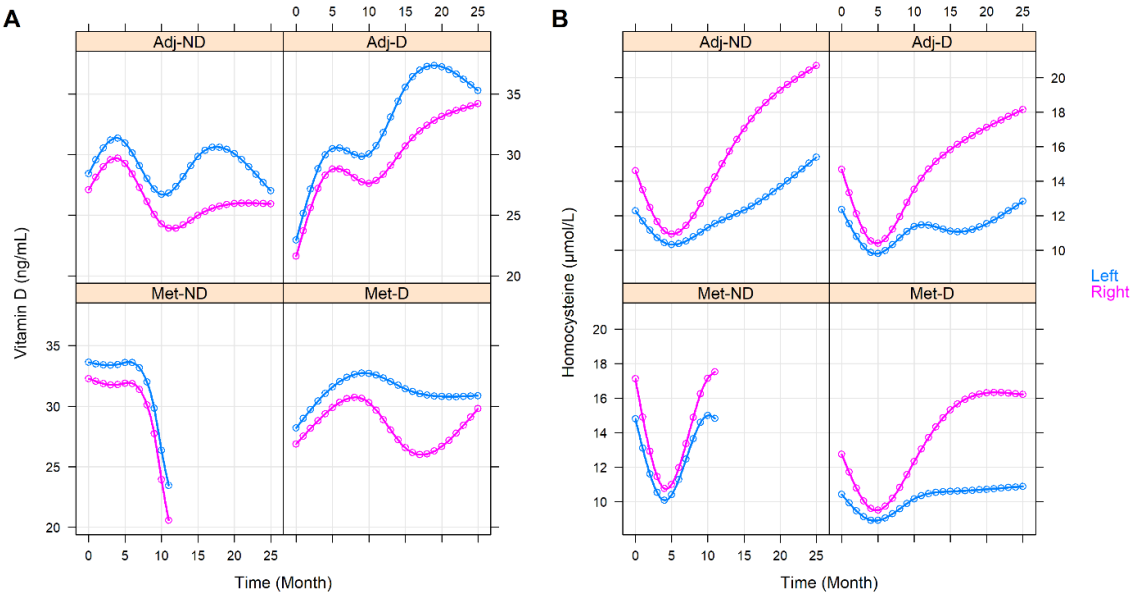

Figure 7. Average longitudinal changes in vitamin D (**A**) and homocysteine (**B**) level of the four study groups containing patients with (Adj) and without metastasis (Met) and with (D) and without (ND) vitamin D supplementation; and stratified by the sidedness of the tumor.

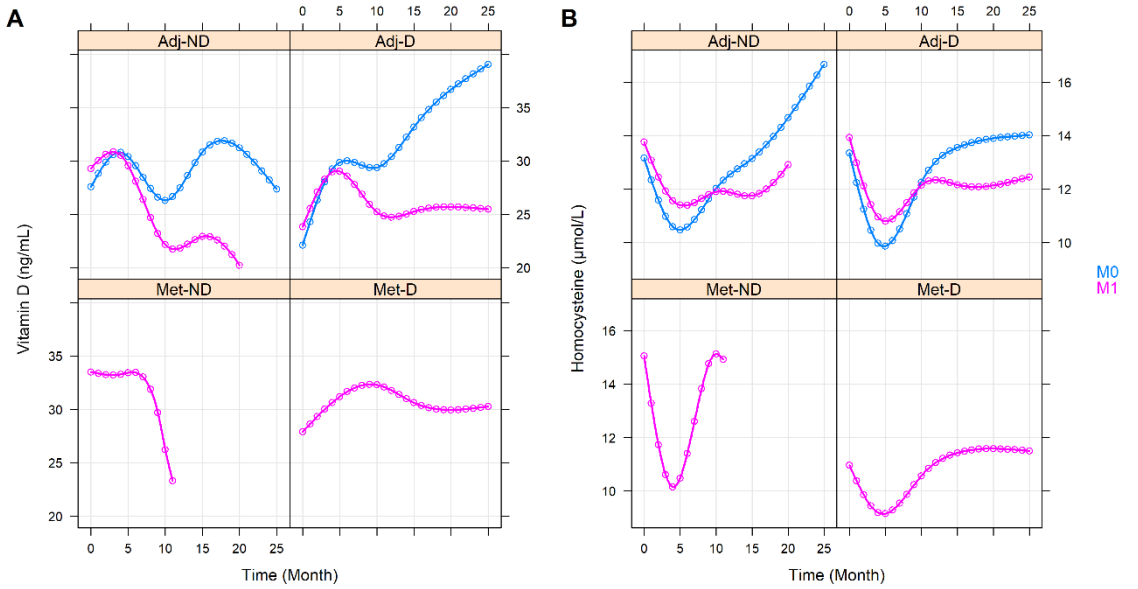

Figure 8. Average longitudinal changes in vitamin D (**A**) and homocysteine (**B**) level of the four study groups containing patients with (Adj) and without metastasis (Met) and with (D) and without (ND) vitamin D supplementation; and stratified by metachronous metastases. M0: without metachronous metastasis; M1: with metachronous metastasis.

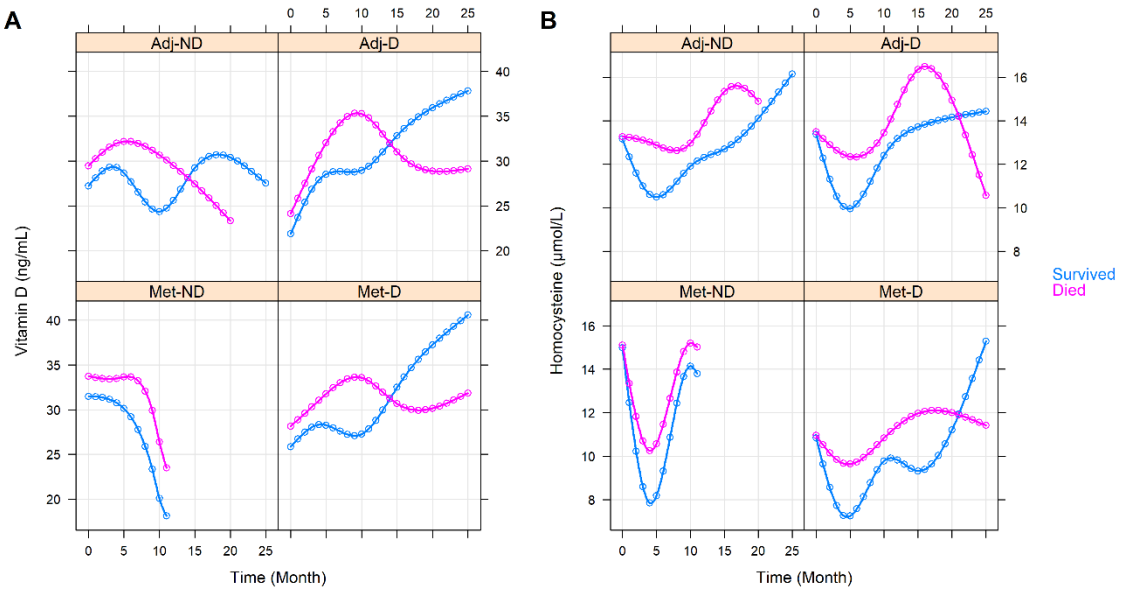

Figure 9. Average longitudinal changes in vitamin D (**A**) and homocysteine (**B**) level of the four study groups containing patients with (Adj) and without metastasis (Met) and with (D) and without (ND) vitamin D supplementation; and stratified by death.

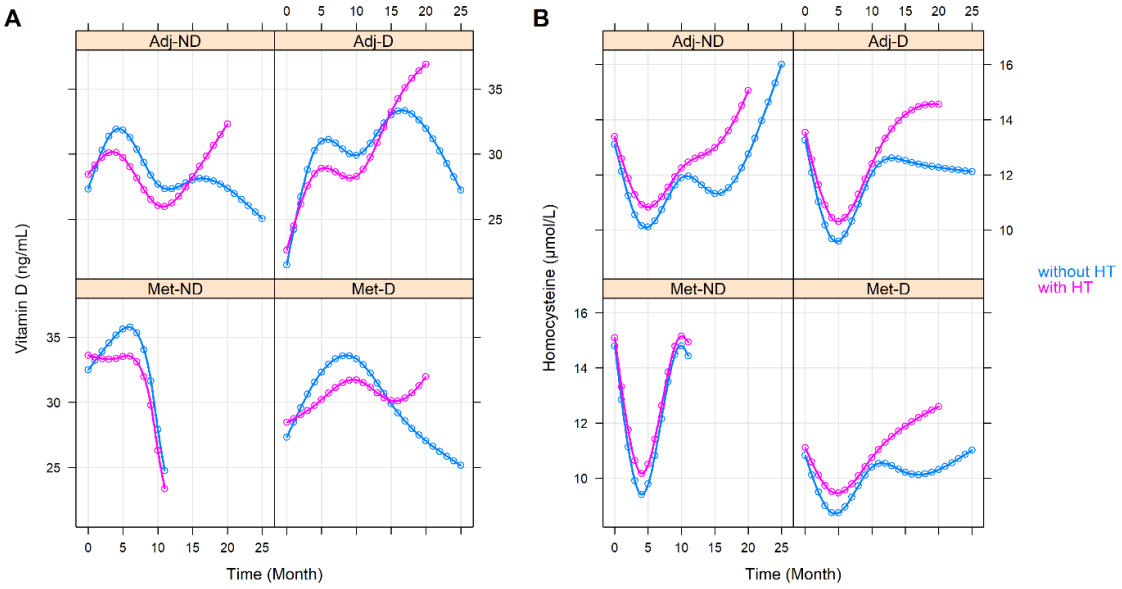

Figure 10. Average longitudinal changes in vitamin D (**A**) and homocysteine (**B**) level of the four study groups containing patients with (Adj) and without metastasis (Met) and with (D) and without (ND) vitamin D supplementation; and stratified by the presence of hypertension.

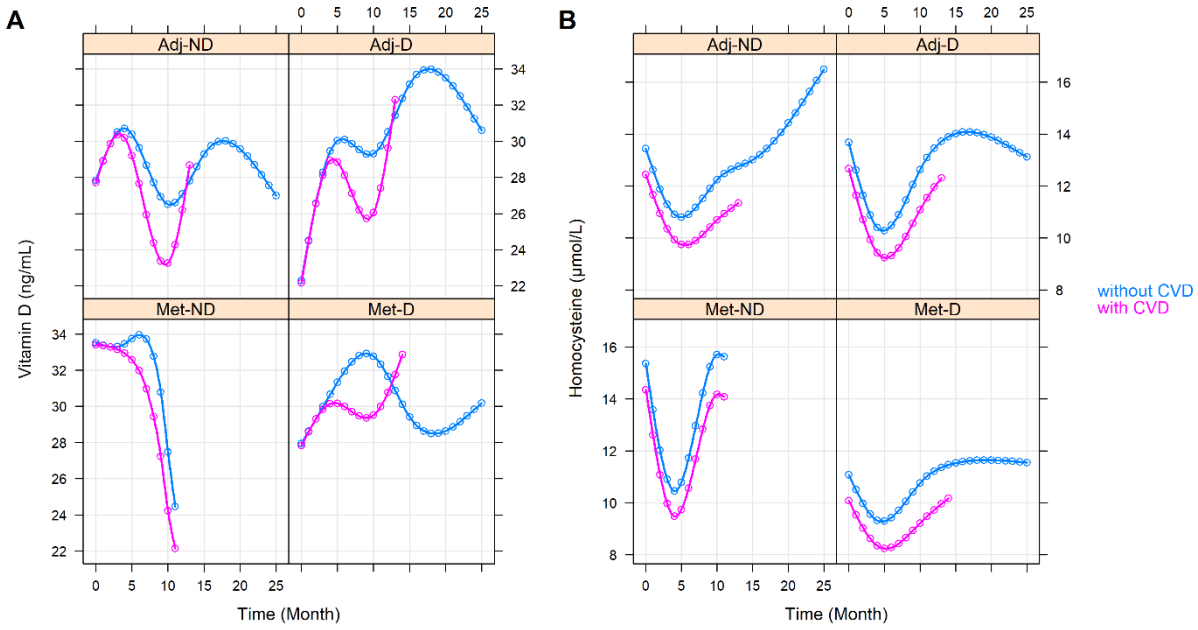

Figure 11. Average longitudinal changes in vitamin D (**A**) and homocysteine (**B**) level of the four study groups containing patients with (Adj) and without metastasis (Met) and with (D) and without (ND) vitamin D supplementation; and stratified by the presence of cardiovascular diseases other than hypertension.

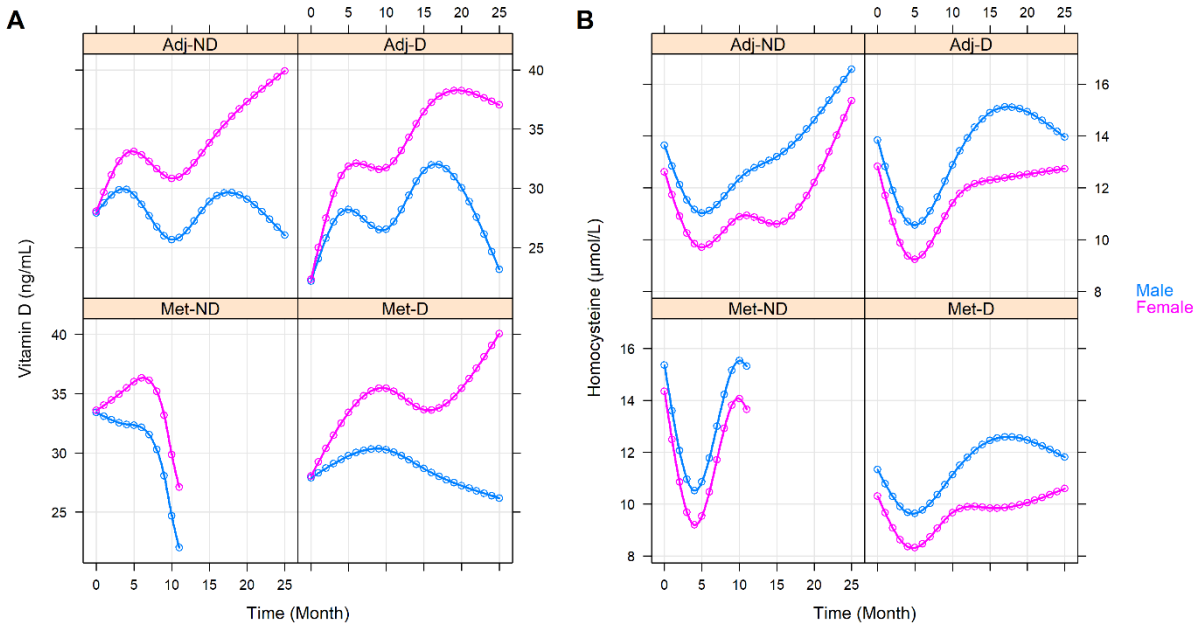

Figure 12. Average longitudinal changes in vitamin D (**A**) and homocysteine (**B**) level of the four study groups containing patients with (Adj) and without metastasis (Met) and with (D) and without (ND) vitamin D supplementation; and stratified by sex.

Despite previous literature data on a healthy population [43] and the seemingly proportionate change in opposite directions in the current study (**Figure 6**), no effect of vitamin D over homocysteine ($p = 0.5197$), and vice versa ($p = 0.6625$), could have been justified in the first months of daily 3000 IU vitamin D supplementation; even if only the first periods of our observation (vitamin D: $p = 0.1301$, homocysteine: $p = 0.2641$) were studied. By examining the longitudinal relationships between the study and other laboratory parameters, we could define the following two different types of trends. In the first, the changes in the laboratory parameters over time did not affect the changes in the vitamin D and homocysteine levels, but a constant difference related to pathological values could have been observed throughout the study. In contrast, in the other case, where an interaction was found between the two variables, the change in one of the two parameters had a significant effect on the change in the other. The first type of the two trends was observed for the serum total cholesterol ($p < 0.0001$), low density-lipoprotein cholesterol ($p < 0.0001$), and for triglycerides ($p < 0.0001$); in all cases, constant high levels were associated with lower vitamin D levels. Higher homocysteine level was associated with constantly higher white blood cell counts ($p = 0.0092$), platelet counts ($p = 0.0092$), serum aspartate aminotransferase ($p = 0.0021$), lactate dehydrogenase ($p = 0.0009$), total cholesterol ($p = 0.0146$), low-density lipoprotein cholesterol ($p = 0.0441$), and creatinine ($p < 0.0001$) levels, and with constantly lower eGFR rates ($p < 0.0001$). Interaction was found between the change in vitamin D levels and the change in serum albumin ($p = 0.0158$): Over time, as albumin levels normalized, the speed of vitamin D saturation slowed down. Normalized levels of serum total protein were more likely to be associated with constant homocysteine levels at the end of the observation period ($p = 0.0072$). In addition to investigating the univariate effects of the laboratory parameters, their combined multivariate effect was also analyzed, where the first type of influencing effects made the interaction terms statistically not significant. No effect could have been justified for the remaining laboratory parameters, which are listed in **Table 2**.

4.1.3. Survival Analysis

The overall and progression-free survival of patients was investigated. In the former, the following two endpoint events had been defined: (1) death related to CRC and (2) death related to natural causes (e.g., old age) or other, non-cancerous diseases. The first event

occurred in 36 (41.9%) and the second in 4 (4.7%) cases, while the follow-up of the survivors was terminated on 30 September 2021. Two and three patients in the non-metastatic groups, and 34 and 1 within the metastatic groups died due to CRC-related or other causes, respectively. Disease progression occurred in 3, 4, 9, and 28 cases of the Adj-ND, Adj-D, Met-ND, and Met-D cohorts, respectively. Non-adjusted, naïve overall, and progression-free Kaplan–Meier survival curves of the four study cohorts are shown in **Figure 13**.

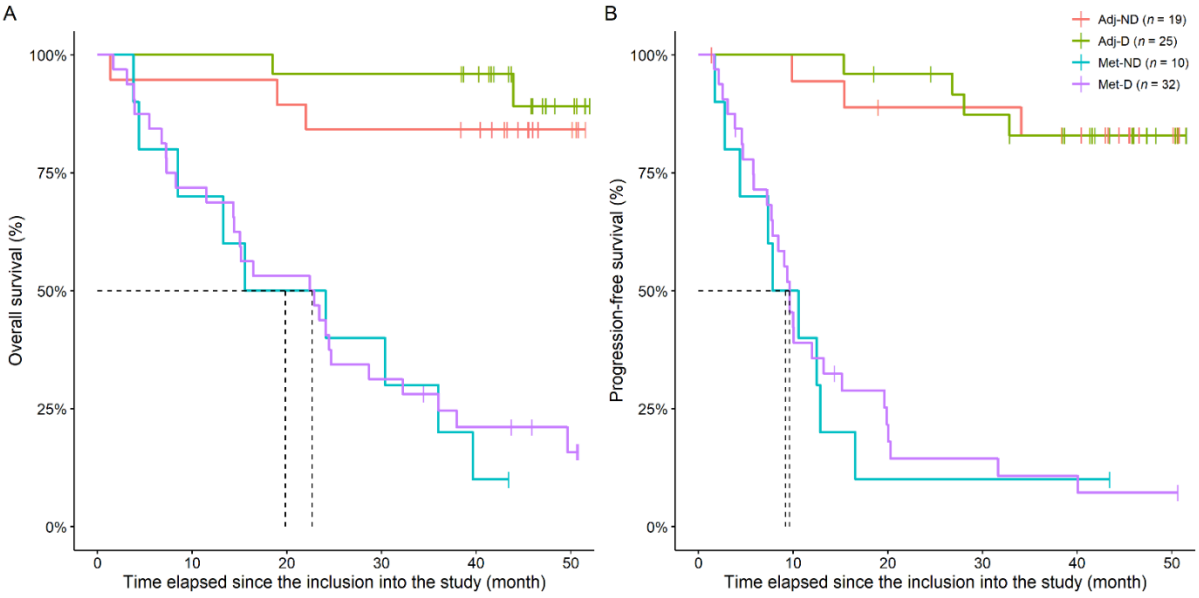

Figure 13. Naïve overall (**A**) and progression-free (**B**) Kaplan–Meier survival curves of the four study groups containing patients with (Adj) and without metastasis (Met) and with (D) and without (ND) vitamin D supplementation.

Being in any of the metastatic groups, as expected, significantly worsened patients' life expectancy. Increasing levels of serum vitamin D had a positive effect on the overall survival of the patients without metastasis (HR: 0.9130, 95%CI: 0.8511 – 0.9794, p = 0.0111), however, the survival-reducing effect of metastases practically cancelled out this positive effect (p = 0.0107, adjusted HR of vitamin D of the Met-ND group: 1.0034, 95%CI: 0.8698 – 1.1567), even with daily 3000 IU vitamin D supplementation (p = 0.0224, adjusted HR of vitamin D of the Met-D group: 0.9979, 95%CI: 0.8622 – 1.1567). No association was found between disease progression and vitamin D changes (HR: 0.9868, 95%CI: 0.9482 – 1.0270, p = 0.5150), while the increase in homocysteine level was a negative effector of both the overall (HR: 1.0940, 95%CI: 1.0250 – 1.1680, p =

0.0067) and the progression-free survival (HR: 1.0845, 95%CI: 1.0221 – 1.1510, p = 0.0073).

Similar to the independent effects, if both of the parameters were included in an extended model, the increase in serum vitamin D (HR: 0.9173, 95%CI: 0.8567 – 0.9822, p = 0.0133) and homocysteine (HR: 1.0890, 95%CI: 1.0140 – 1.1690, p = 0.0193) level had the same positive and negative effect on the overall survival, respectively. As above, the positive effect of vitamin D was cancelled out by the presence of metastases (Met-ND p = 0.0124, Met-D p = 0.0420). However, the progression-free survival was only significantly affected by the presence of metastases (Met-ND: p = 0.0006, Met-D: p = 0.0162), while neither homocysteine (p = 0.7757) nor vitamin D (p = 0.5707) had any effect on disease progression if both of the parameters were included in the same model.

4.2. Measurement of PD-1 and PD-L1 in Metastatic Colorectal Cancer

4.2.1. Baseline Measurements

A total of thirty-seven mCRC patients were included in this part of the study. The average pre-treatment PD-L1 and PD-1 level of the patients was 16.01 ± 1.09 pg/mL and 27.73 ± 1.20 pg/mL, respectively. RAS, microsatellite, and BRAF molecular profiling of patients were performed in 32, 12, and 8 of the 37 cases, respectively. Thirteen RAS mutant, one microsatellite instable, and one BRAF mutant patient were diagnosed. The clinicopathological data of the study participants are summarized in **Table 3**.

Table 3. Baseline anamnestic data of study participants (*n* = 37). Continuous and count data are presented as mean ± standard deviation and number of observations (percentage), respectively.

Clinicopathological Characteristics	**Average ± SD or No. of Obs.**
Age (years)	60.95 ± 10.99
Male : female ratio	24 : 13 (64.9% : 35.1%)
AJCC staging [15] [1]	
- Stage II	2 (5.4%)
- Stage III	4 (10.8%)
- Stage IV	31 (83.8%)
Distant metastases:	
- Synchronous : metachronous	31 : 6 (83.8% : 16.2%)
Location:	
- Liver (single : multiple)	4 : 22 (10.8% : 59.5%)
- Lung	13 (35.1%)
- Gynecological	2 (5.4%)
- Intraabdominal	24 (64.9%)
- Advanced local invasion	13 (35.1%)
- Other	12 (32.4%)

Table 3 (cont.)

Clinicopathological Characteristics	Average ± SD or No. of Obs.
Patients with multiple metastatic sites	25 (67.6%)
Diameter of largest liver/lung metastasis (mm)	51.67 ± 44.66
Regional lymph node metastasis	33 (89.2%)
Primary tumor resection [2]	26 (70.3%)
RAS (wild : mutant) [3]	19 : 13 (51.4% : 35.1%)
Location of the tumor [80, 81]	
- Left-sided	28 (75.7%)
- Right-sided	9 (24.3%)
Final lineage of chemotherapy	
- First line	14 (37.8%)
- Second line	10 (27.0%)
- Third line or above	13 (35.1%)
Medical history	
- Diabetes mellitus	6 (27.0%)
- Hypertension	24 (64.9%)
- Cardiovascular disease(s), except events and hypertension	5 (13.5%)
- Major cardiovascular event(s) prior to CRC	4 (10.8%)
- Thyroid disease (in euthyroid state)	4 (10.8%)

[1] At the time of primary tumor removal surgery. [2] Prior to the first metastatic chemotherapy session. [3] RAS analysis results were not available for 5 patients. AJCC: American Joint Committee on Cancer; RAS: rat sarcoma virus gene; SD: standard deviation.

Significantly lower PD-L1 levels were found in those patients with metachronous metastases (metachronous: 9.96 ± 3.17 pg/mL; synchronous: 17.23 ± 11.43 pg/mL; p = 0.0412; **Figure 14A**), and in those who did not show any signs of disease progression (without progression: 10.30 ± 1.58 pg/mL; with progression: 16.70 ± 11.32 pg/mL; p = 0.0443; **Figure 14B**). No connections were found between PD-L1 levels and sex,

sidedness of the tumor, chemotherapy, staging, or co-morbidities. The plasma PD-1 levels did not differ in any of the abovementioned groupings. Correlation analyses revealed positive associations between PD-L1 and hsCRP (Spearman's ρ: +0.60; p = 0.0011), aspartate transaminase (AST, Spearman's ρ: +0.48; p = 0.0253), alanine transaminase (ALT, Spearman's ρ: +0.45; p = 0.0386), and gamma-glutamyl transferase (GGT, Spearman's ρ: +0.52; p = 0.0103). Marginally positive associations were found between PD-L1 and white blood cell (WBC, Spearman's ρ: +0.35), monocyte (Spearman's ρ: +0.35), and platelet (Spearman's ρ: +0.43; p = 0.0556) counts. No associations were found between PD-1 and any of the laboratory parameters.

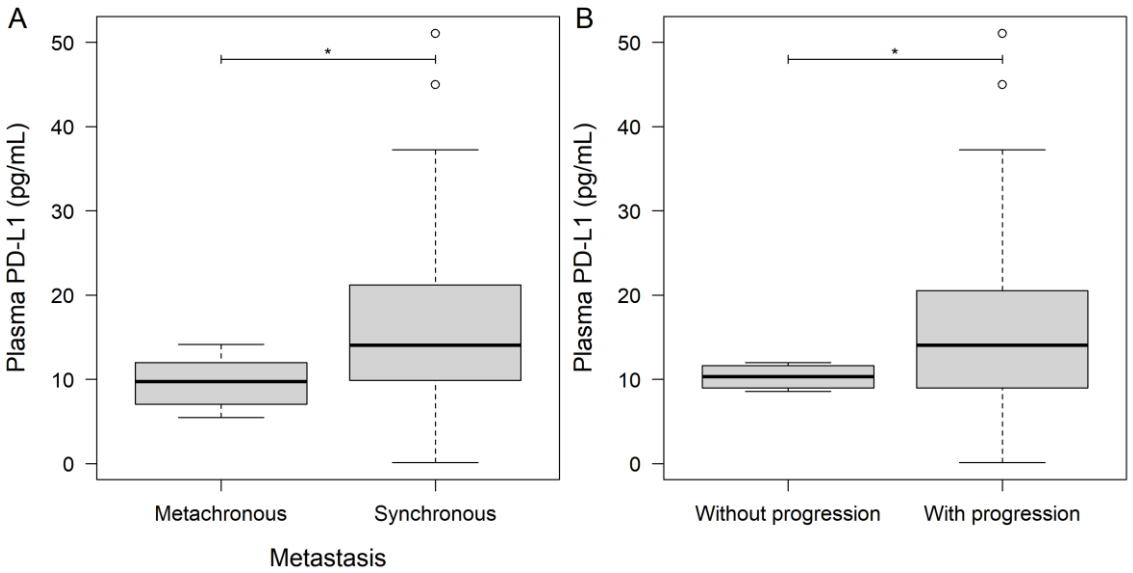

Figure 14. Pre-treatment measurements of plasma programmed death-ligand 1 (PD-L1) of metastatic colorectal cancer patients with (**A**) synchronous (n = 31) and metachronous (n = 6) metastases, and (**B**) who had disease progression (n = 33) or not (n = 4) during our observation period. Thick lines and hollow circles represent median and outliers, respectively. * $p < 0.05$.

Thirty-two (86.5%) and thirty-three (89.2%) mCRC-related death and progression events were observed, respectively. Additionally, one further but non-mCRC-related death was registered. In univariate models, patients with higher baseline plasma PD-L1 levels had significantly shorter DSS (HR: 1.0396; 95% CI: 1.0073 – 1.0730; p = 0.0160) and PFS (HR: 1.0498; 95% CI: 1.0130 – 1.0880; p = 0.0074). In contrast, higher baseline plasma PD-1 levels were only associated with a marginal increase in decreased DSS (HR: 1.0269; 95% CI: 0.9987 – 1.0559; p = 0.0617) and no difference was observed for PFS (HR:

1.0226; 95% CI: 0.9914 – 1.0550; $p = 0.1580$). The same results were obtained if the models were stratified by synchronous and metachronous metastases (PD-L1 DSS: $p = 0.0257$; PD-L1 PFS: $p = 0.0141$; PD-1 DSS: $p = 0.1680$; PD-1 PFS: $p = 0.2690$).

Similar results were found in a multivariate setting. The following parameters were investigated in relation to patient survival: age, sex, tumor sidedness, final lineage of chemotherapy, the presence of type 2 diabetes and/or hypertension, platelet count, and the plasma level of PD-1 or PD-L1. PD-L1 levels marginally and significantly affected DSS and PFS, respectively, while no effect of PD-1 on patient survival was found. In addition to PD-1 and PD-L1, patient survival was most affected by sidedness, type 2 diabetes, and platelet count (**Table 4**). The same results were obtained if the models were adjusted for synchronous and metachronous metastases as well.

Table 4. The *p*-values obtained for survival models investigating the multivariate effect of PD-1 and PD-L1. The 4 individual multivariate models investigated DSS and PFS, and either PD-L1 or PD-1 were included as predictor

Parameter	**Disease-specific survival**		**Progression-free survival**	
	Model 1	**Model 2**	**Model 3**	**Model 4**
Age (years)	0.3825	0.3284	0.8433	0.6069
PD-1 (pg/mL)	0.2499	–	0.1652	–
PD-L1 (pg/mL)	–	0.0932	–	0.0215
Sex (male vs. female)	0.3173	0.1289	0.1953	0.0598
Sidedness (left-sided vs. right-sided)	0.1544	0.3149	0.3324	0.4389
Lineage of chemotherapy				
- First-line vs. second-line	0.5425	0.6244	0.4332	0.3344
- First-line vs. third-line or above	0.4964	0.4270	0.3320	0.3635
Type 2 diabetes mellitus	0.1435	0.0601	0.7066	0.2831
Hypertension	0.4842	0.5690	0.4708	0.6070
Platelet count (10^9/L)	0.0210	0.0954	0.0074	0.0699

PD-1: plasma programmed cell death protein 1; PD-L1: programmed death-ligand 1.

4.2.2. Investigating the Association Between Plasma PD-1, PD-L1 Level and Tumor Burden

To investigate the effect of tumor burden on the plasma levels of PD-1 and PD-L1, the two parameters were first compared based on the presence of various metastasis sites and between RAS wild and mutant cases. As presented above, the timing of the metastasis occurrence was significantly associated with plasma PD-L1 levels (p = 0.0412; **Figure 14A**), but not with PD-1 (p = 0.4569). The presence of hepatic metastases was associated with significantly higher plasma PD-L1 levels (p = 0.0499; **Figure 15A**) and with marginally higher PD-1 levels (p = 0.0618; **Figure 15B**). In contrast, if a patient had lung metastases, both PD-L1 (**Figure 15C**) and PD-1 (**Figure 15D**) were lower, but a significant difference was only observed in the case of PD-L1 (p = 0.0209). No difference was found for peritoneal metastases (PD-1: p = 0.4985; PD-L1: p = 0.1100) and other metastasis locations (PD-1: p = 0.3282; PD-L1: p = 0.5953), for patients with carcinosis (PD-1: p = 0.3329; PD-L1: p = 0.5580), or for patients with advanced local invasion (PD-1: p = 0.4778; PD-L1: p = 0.3973). Patients with multiple metastases affecting ≥ 2 other organs had marginally higher plasma PD-1 levels (metastasis at one site: 22.89 ± 9.44 pg/mL; metastases at ≥ two locations: 30.06 ± 12.55 pg/mL; p = 0.0630), but the same PD-L1 (p = 0.5602). Moreover, neither RAS mutations (PD-1: p = 0.7255; PD-L1: p = 0.7689) nor inoperable primary tumors (PD-1: p = 0.2289; PD-L1: p = 0.9723) affected the PD-1/PD-L1 levels of patients. A strong association was found between plasma PD-L1 levels and the diameter of the largest hepatic/lung metastases (Spearman's ρ: +0.51; explanatory power of the model (adjusted R^2): 24.68%; p = 0.0059; **Figure 16**).

The effect of tumor burden on patient survival was analyzed using both univariate and multivariate survival models. Stratified univariate survival models revealed that PD-L1 had a strong effect on DSS and PFS as well, regardless of the location of metastases, RAS mutations, or whether the tumor was operable/inoperable. In contrast, PD-1 had basically no effect on PFS, but if the model was adjusted for the presence of hepatic metastases, the presence of carcinosis peritonei, or whether the tumor was operable/inoperable, higher PD-1 levels indicated inferior survival outcomes of mCRC patients (**Table 5**).

Multivariate survival analyses showed that higher PD-L1 levels were associated with shorter survival times of patients, while no such effect could be justified for plasma PD-1 levels. The strongest effect on survival was found for peritoneal metastases and for

metastasis locations other than the peritoneum, liver, lung, or ovarium. DSS was also significantly affected if the tumor was inoperable with HRs between two and three compared to those patients who underwent primary tumor removal surgery (**Table 5**).

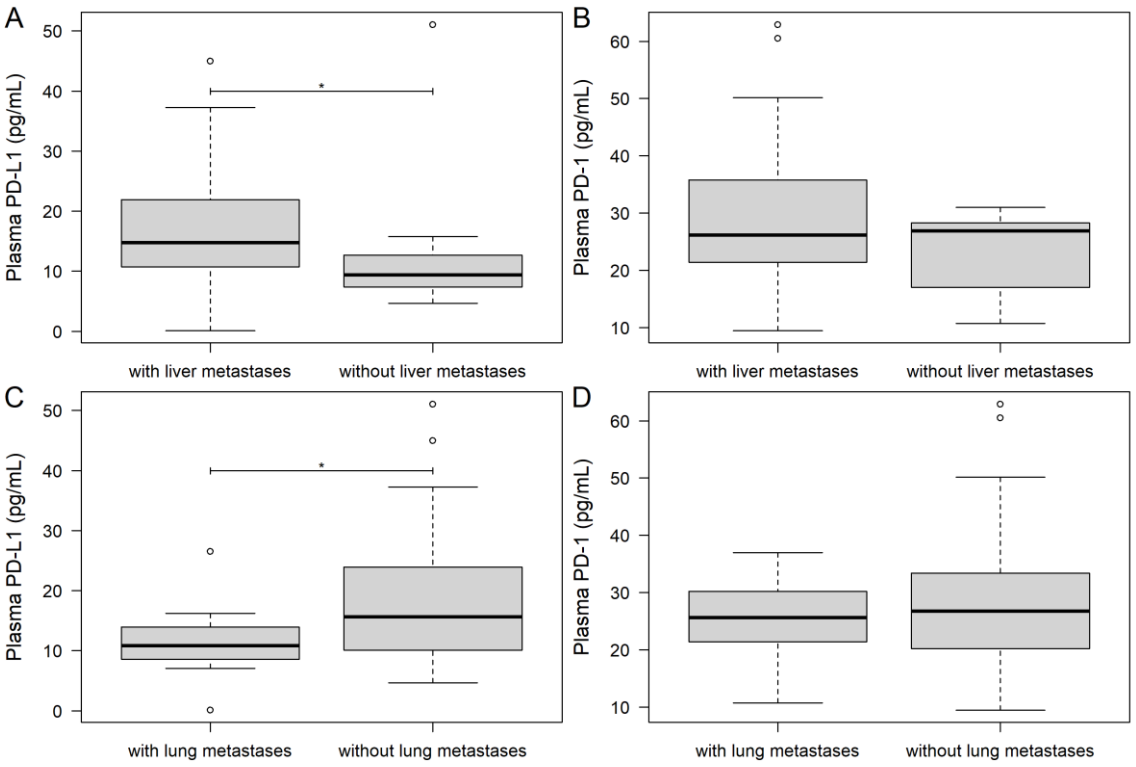

Figure 15. Baseline PD-1 and PD-L1 measurements of study participants grouped by the presence of hepatic (**A**,**B**) and lung (**C**,**D**) metastases. Thick lines and hollow circles represent medians and outliers, respectively. PD-1: plasma programmed cell death protein 1; PD-L1: programmed death-ligand 1. * $p < 0.05$. With hepatic metastases: $n = 26$; without hepatic metastases: $n = 11$; with lung metastases: $n = 13$; without lung metastases: $n = 24$.

Table 5. The *p*-values obtained for survival models investigating the effect of tumor burden on PD-1 and PD-L1.

Parameter	**Disease-specific survival**		**Progression-free survival**	
Stratified univariate model *p*-values:				
	PD-1	PD-L1	PD-1	PD-L1
Location of metastases:				
Liver (none vs. present)	0.0331	0.0211	0.3240	0.0253
Lung (none vs. present)	0.0651	0.0461	0.1710	0.0063
Other location (none vs. present)	0.4353	0.0076	0.8480	0.0539
Advanced local invasion	0.1280	0.0096	0.4040	0.0329
Multiple metastatic sites (no vs. yes)	0.3431	0.0011	0.7450	0.0020
Primary tumor resection (no vs. yes)	0.0224	0.0932	0.0625	0.0041
RAS (wild vs. mutant)	0.2800	0.0803	0.5350	0.0050
Multivariate model *p*-values:				
PD-1 (pg/mL)	0.1731	–	0.4018	–
PD-L1 (pg/mL)	–	0.0987	–	0.0058
Location of metastases:				
Liver (none vs. present)	0.9128	0.4816	0.2072	0.7377
Lung (none vs. present)	0.1594	0.2623	0.9651	0.7952
Other location (none vs. present)	0.0146	0.0050	0.0312	0.0085
Advanced local invasion	0.6798	0.8232	0.6379	0.3933
Primary tumor resection (no vs. yes)	0.0101	0.0488	0.1605	0.1825
RAS (wild vs. mutant)	0.9388	0.6880	0.3124	0.3753

PD-1: plasma programmed cell death protein 1; PD-L1: programmed death-ligand 1. RAS: rat sarcoma virus gene.

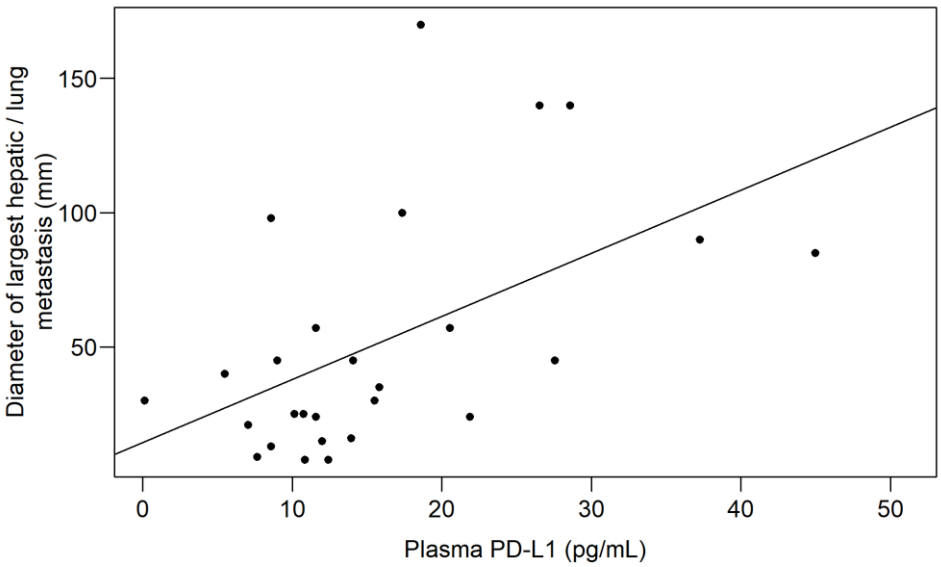

Figure 16. A strong association was found between plasma programmed death-ligand 1 (PD-L1) levels and the diameter of the largest hepatic/lung metastases (Spearman's ρ: +0.51, $p = 0.0059$).

4.2.3. Comparison of Low and High PD-1/PD-L1 Subgroups

Based on the differences in PD-1 and PD-L1 levels between the different subgroups detailed above, we hypothesized that the study population might be divided into high and low plasma level groups. Therefore, we performed ROC analyses to obtain optimal cut-off values. Although most of the ROC curves had lower area-under-the-curve values (< 75%), most of the models predicted cut-off points within the same range (**Figure 17**). The values of 26 pg/mL and 13 pg/mL became the cut-off values for PD-1 and PD-L1, respectively. Both values coincided with both the median of the obtained cut off values and roughly with the median values of the measured PD-1 and PD-L1 levels. A total of 19, 18, 18, and 19 patients were assigned to the PD-L1 < 13 pg/mL, PD-L1 > 13 pg/mL, PD-1 < 26 pg/mL, and PD-1 > 26 pg/mL groups, respectively.

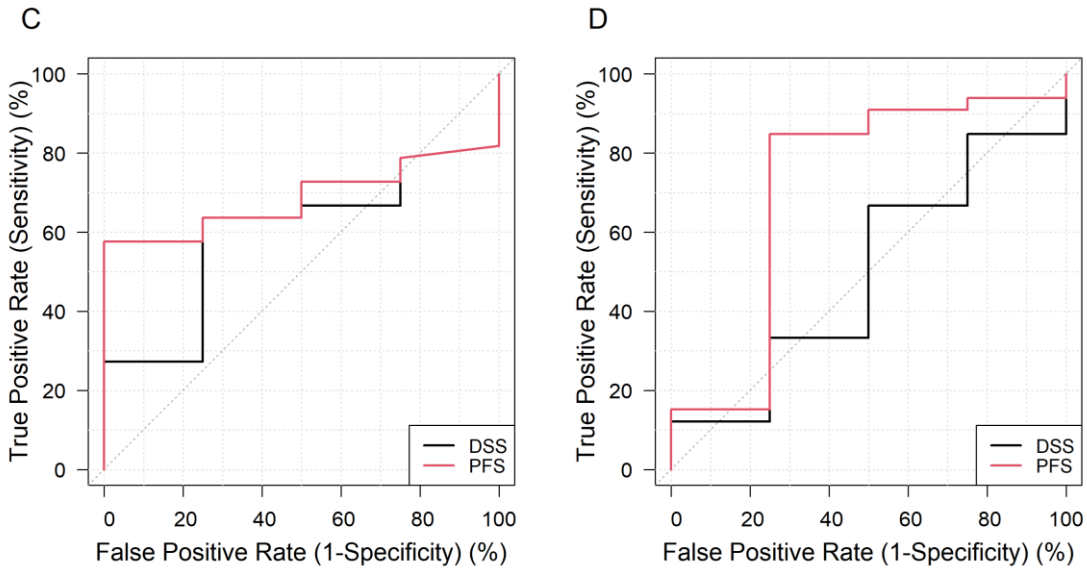

Figure 17. ROC curves of PD-1 and PD-L1.

4.2.3.1. Baseline Measurements

Both laboratory and clinicopathological features of the groups were compared. Clinically worse values were characteristic for the patients with higher PD-L1 levels. Namely, the hsCRP levels were significantly higher (p = 0.0478), the WBC, monocyte count, platelet count, AST, ALT, GGT, lactate dehydrogenase (LDH), vitamin D, homocysteine and CEA levels were clinically higher, and the albumin levels were clinically lower (**Table 6**). Lower PD-1 levels were associated with higher vitamin D, and lower homocysteine, hematocrit, hemoglobin, and plasma glucose levels (**Table 7**). There was no difference in the clinicopathological parameters for either the PD-L1 or PD-1 subgroups.

A total of 15 (78.9%), 17 (94.4%), 15 (83.3%), and 17 (89.5%) mCRC-related death events and 15 (78.9%), 18 (100%), 15 (83.3%), and 18 (94.7.0%) progression events occurred within the low and high PD-L1, and low and high PD-1 groups, respectively. Shorter DSS times were approximately two times more likely to occur in the PD-L1 > 13 ng/mL group (HR: 1.9830; 95% CI: 1.0120 – 3.8850; p = 0.0462; **Figure 18A**); furthermore, belonging to the PD-L1 > 13 pg/mL group was associated with a 2.40-fold probability of shorter PFS times (HR: 2.4027; 95% CI: 1.2080 – 4.7800; p = 0.0125; **Figure 18B**). No difference could be demonstrated between the PD-1 groups, either for DSS (p = 0.8330, **Figure 18C**) or for PFS (p = 0.7300; **Figure 18D**). No additional information could be obtained via multivariate survival models beyond those described in **Table 4**.

Table 6. Comparison of age and laboratory measurements of study participants with plasma PD-L1 levels under or over 13 pg/mL. Continuous data are presented as mean ± standard deviation using Wilcoxon–Mann–Whitney U-tests.

Parameter	**< 13 pg/mL (*n* = 19)**	**> 13 pg/mL (*n* = 18)**	**Crude *p*-value**	**Adjusted *p*-value**
Age (years)	60.47 ± 12.70	61.45 ± 9.20		0.0000
PD-1 (pg/mL)	25.12 ± 6.99	30.49 ± 15.40		0.6388
PD-L1 (pg/mL)	8.77 ± 3.04	23.65 ± 10.97		–
White blood cell count (10^9/L)	6.85 ± 196	9.24 ± 3.65	0.0322	0.1329
Lymphocyte count (10^9/L)	1.52 ± 0.51	1.81 ± 0.59		0.4380
Monocyte count (10^9/L)	0.57 ± 0.15	0.77 ± 0.30	0.0432	0.1383
Red blood cell count (10^{12}/L)	4.66 ± 0.44	4.67 ± 0.59		0.4380
Hemoglobin (g/L)	134.79 ± 18.72	124.00 ± 17.89		0.3151
Hematocrit (L/L)	0.40 ± 0.05	0.37 ± 0.05	0.0887	0.2027
Mean corpuscular volume (fL)	86.43 ± 6.48	83.98 ± 6.46		0.4380
Mean corpuscular hemoglobin (pg)	28.93 ± 2.83	27.84 ± 2.99		0.4380
Mean corpuscular hemoglobin concentration (g/L)	334.17 ± 10.49	330.86 ± 14.07		0.5216
Red blood cell distribution width (%)	14.91 ± 2.54	16.01 ± 2.82		0.4737
Platelet count (10^9/L)	257.05 ± 76.71	368.39 ± 163.97	0.0217	0.1257
Aspartate transaminase (U/L)	26.37 ± 10.28	65.50 ± 93.05	0.0225	0.1257
Alanine transaminase (U/L)	26.79 ± 17.16	44.94 ± 34.98	0.0635	0.1694
Gamma-glutamyl transferase (U/L)	72.79 ± 82.78	225.83 ± 243.04	0.0143	0.1257

Table 6 (cont.)

Parameter	< 13 pg/mL (*n* = 19)	> 13 pg/mL (*n* = 18)	Crude *p*-value	Adjusted *p*-value
Alkaline phosphatase (U/L)	110.68 ± 34.22	256.73 ± 245.55		0.4380
Lactate dehydrogenase (U/L)	219.37 ± 66.61	943.22 ± 1517.84	0.0374	0.1329
Plasma glucose (mmol/L)	5.30 ± 0.82	5.21 ± 0.95		0.6717
Creatinine (μmol/L)	68.26 ± 12.50	64.28 ± 18.12		0.4483
Estimated glomerular filtration rate $\left(\frac{\text{mL}}{\text{min}\cdot 1.73\text{m}^2}\right)$	94.01 ± 13.76	96.32 ± 17.59		0.4880
Total cholesterol (mmol/L)	5.32 ± 1.07	5.99 ± 2.05		0.4882
High-density lipoprotein cholesterol (mmol/L)	1.42 ± 0.35	1.23 ± 0.31		0.3151
Low-density lipoprotein cholesterol (mmol/L)	3.25 ± 0.76	3.90 ± 1.43	0.0780	0.1919
Triglycerides (mmol/L)	1.62 ± 0.77	1.56 ± 0.46		1.0000
Total protein (g/L)	73.28 ± 4.21	72.88 ± 6.21		0.9075
Albumin (g/L)	44.69 ± 2.75	40.83 ± 4.16	0.0138	0.1257
High-sensitivity C-reactive protein (mg/L)	7.01 ± 8.56	42.89 ± 57.19	0.0015	0.0478
Thyroid stimulating hormone (mU/L)	1.18 ± 0.87	1.88 ± 2.25		0.4882
Vitamin D (ng/mL)	25.84 ± 8.03	29.36 ± 18.79		0.4499
Homocysteine (μmol/L)	12.02 ± 3.27	14.12 ± 5.65		0.8304
Carcinoembryonic antigen (ng/mL)	458.10 ± 1916.54	220.39 ± 425.38	0.0373	0.1329
Carbohydrate antigen 19-9 (U/mL)	266.59 ± 682.01	1602.17 ± 4813.00		0.4882

PD-1: plasma programmed cell death protein 1; PD-L1: programmed death-ligand 1.

Table 7. Comparison of age and laboratory measurements for study participants with plasma PD-1 levels under or over 26 pg/mL. Continuous data are presented as mean ± standard deviation using Wilcoxon–Mann–Whitney U-test.

Parameter	**< 26 pg/mL (*n* = 18)**	**> 26 pg/mL (*n* = 19)**	**Crude *p*-value**	**Adjusted *p*-value**
Age (years)	59.88 ± 11.72	61.96 ± 10.48		0.8812
PD-1 (pg/mL)	19.31 ± 5.21	35.71 ± 11.13		–
PD-L1 (pg/mL)	16.39 ± 11.54	15.64 ± 10.51		0.8812
White blood cell count (10^9/L)	8.16 ± 3.80	7.87 ± 2.39		0.8812
Lymphocyte count (10^9/L)	1.60 ± 0.53	1.72 ± 0.59		0.8812
Monocyte count (10^9/L)	0.72 ± 0.26	0.61 ± 0.23		0.5203
Red blood cell count (10^{12}/L)	4.41 ± 0.58	4.72 ± 0.42		0.4799
Hemoglobin (g/L)	124.61 ± 19.69	134.21 ± 17.29		0.5203
Hematocrit (L/L)	0.37 ± 0.05	0.40 ± 0.04		0.5203
Mean corpuscular volume (fL)	84.76 ± 6.54	85.68 ± 6.61		0.8793
Mean corpuscular hemoglobin (pg)	28.29 ± 2.89	28.50 ± 3.03		0.8812
Mean corpuscular hemoglobin concentration (g/L)	333.27 ± 12.84	331.88 ± 12.08		0.8812
Red blood cell distribution width (%)	15.41 ± 3.00	15.48 ± 2.63		0.8812
Platelet count (10^9/L)	342.50 ± 176.18	281.58 ± 80.13		0.8793
Aspartate transaminase (U/L)	59.72 ± 93.02	31.84 ± 22.31	0.0463	0.4242
Alanine transaminase (U/L)	43.00 ± 31.76	28.63 ± 23.68		0.5203
Gamma-glutamyl transferase (U/L)	144.83 ± 149.40	149.53 ± 231.46		0.8793

Table 7 (cont.)

Parameter	< 26 pg/mL (*n* = 18)	> 26 pg/mL (*n* = 19)	Crude *p*-value	Adjusted *p*-value
Lactate dehydrogenase (U/L)	724.50 ± 1415.71	426.58 ± 714.76		0.8793
Alkaline phosphatase (U/L)	200.12 ± 215.17	150.12 ± 132.75		0.8793
Plasma glucose (mmol/L)	4.97 ± 0.79	5.55 ± 0.86		0.4799
Creatinine (μmol/L)	66.83 ± 17.47	65.84 ± 13.63		0.8812
Estimated glomerular filtration rate $\left(\frac{\text{mL}}{\text{min}\cdot 1.73\text{m}^2}\right)$	96.10 ± 17.15	94.21 ± 14.31		0.8793
Total cholesterol (mmol/L)	5.38 ± 1.23	5.90 ± 1.94		0.8735
High-density lipoprotein cholesterol (mmol/L)	1.29 ± 0.35	1.37 ± 0.34		0.8793
Low-density lipoprotein cholesterol (mmol/L)	3.46 ± 1.05	3.68 ± 1.28		0.8793
Triglycerides (mmol/L)	1.47 ± 0.58	1.71 ± 0.66		0.8735
Total protein (g/L)	71.53 ± 6.07	74.57 ± 3.83	0.0499	0.4242
Albumin (g/L)	41.44 ± 3.68	44.12 ± 3.88	0.0417	0.4242
High-sensitivity C-reactive protein (mg/L)	33.49 ± 58.18	15.92 ± 21.63		0.8793
Thyroid stimulating hormone (mU/L)	1.83 ± 2.27	1.23 ± 0.85		0.8793
Vitamin D (ng/mL)	28.69 ± 18.62	26.47 ± 8.63		0.8798
Homocysteine (μmol/L)	11.92 ± 5.68	14.10 ± 3.20	0.0098	0.1760
Carcinoembryonic antigen (ng/mL)	555.18 ± 1961.43	140.93 ± 389.14		0.8812
Carbohydrate antigen 19-9 (U/mL)	1440.29 ± 4841.00	419.94 ± 768.91		0.9757

PD-1: plasma programmed cell death protein 1; PD-L1: programmed death-ligand 1.

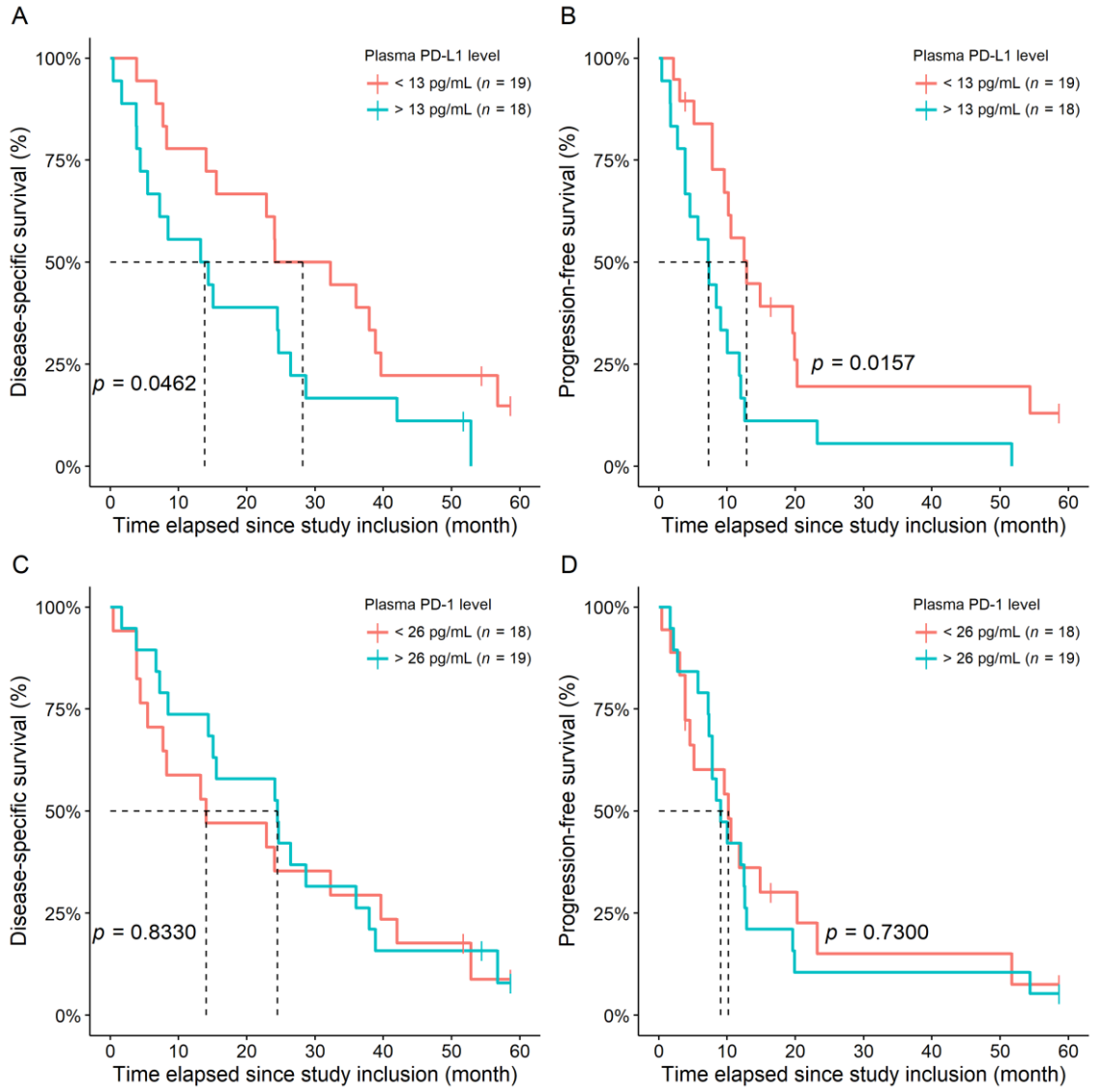

Figure 18. Disease-specific (**A**,**C**) and progression-free (**B**,**D**) survival differences between the high and low PD-L1 (**A**,**B**) and the high and low PD-1 (**C**,**D**) groups. Statistical analysis was performed using competing risk Cox regression models. Significantly shorter survival times were found between the low– and high PD-L1 groups. While no difference was found between the survival of the PD-1 groups. PD-1: plasma programmed cell death protein 1; PD-L1: programmed death-ligand 1.

4.2.3.2. Longitudinal Analysis

A total of 506 visits were recorded for the 37 study participants: on average, 13.68 ± 7.94 visits per patient. To determine the changes in laboratory parameters with the course of mCRC, linear mixed-effects models were created. The fixed effect of the model was

either the PD-L1 or the PD-1 low/high group, while the random effects were the patients' IDs. Measurements were not available for every visit in the case of LDH (367 of the 506 measurements were available, 72.52%) and high-density lipoprotein (HDL) cholesterol (236 of the 506 measurements were available, 46.64%). The LDH and HDL cholesterol values in the data set were missing at random. The model predictions for these two parameters had to be cut at an earlier observation time due to a lower number of data points at later visits.

First, the two PD-L1 groups were compared. Patients within the PD-L1 > 13 ng/mL group had consistently higher WBC (p = 0.0267; **Figure 19A**), monocyte (p = 0.0206; **Figure 19B**), lymphocyte (p = 0.0317; **Figure 19C**), and platelet (p = 0.0021; **Figure 19D**) counts. The mean corpuscular hemoglobin levels (p = 0.0374; **Figure 19E**), mean corpuscular hemoglobin concentration (p = 0.0355; **Figure 19F**), mean corpuscular volume (p = 0.0707; **Figure 19G**), albumin levels (p = 0.0181; **Figure 19L**), and HDL cholesterol levels (p = 0.0593; **Figure 19N**) were consistently lower in the PD-L1 > 13 ng/mL group throughout the observation time. Furthermore, the red blood cell distribution width (p = 0.0022; **Figure 19H**), hsCRP levels (p = 0.0132; **Figure 19K**), and LDH levels (p = 0.0123; **Figure 19M**) were significantly higher. Except for a short increase with a peak level around the second year of our observation, the hemoglobin (p = 0.0569; **Figure 19I**) and hematocrit (p = 0.0711; **Figure 19J**) values were lower in those patients with a higher baseline plasma PD-L1 level. Despite the apparent difference graphically represented (**Figure 20A**), vitamin D level of patients did not differ statistically between the two PD-L1 cohorts (p = 0.2163), while homocysteine was marginally lower in the PD- L1 < 13 ng/mL group (p = 0.0979; **Figure 20B**). In general, the direction of all longitudinal changes was towards the clinically worse state (**Figures 19** and **20**).

Second, the same comparisons were performed between the two PD-1 groups as well. In contrast to the PD-L1 groups, where an elevated platelet count was more common with higher PD-L1 levels, patients of the PD-1 > 26 pg/mL group had significantly lower platelet counts during the study compared to those patients with a lower baseline plasma PD-1 level (p = 0.0061; **Figure 21**). Vitamin D and homocysteine did not differ between the PD-1 groups (**Figures 20C** and **20D**). No further differences were found between the two PD-1 groups.

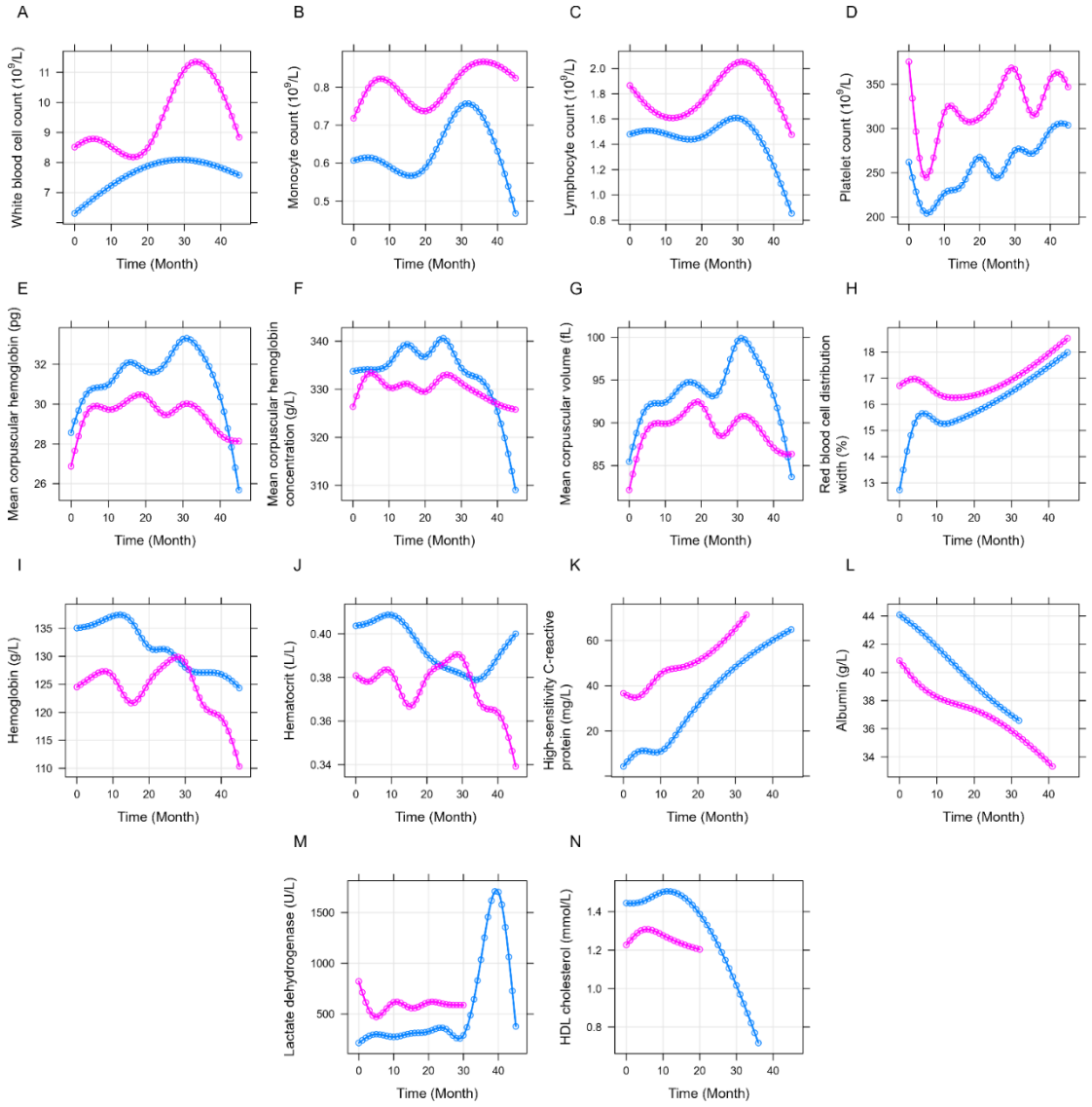

Figure 19. Longitudinal changes in the various laboratory measurements throughout the study within the PD-L1 < 13 ng/mL (blue) and PD-L1 > 13 ng/mL (magenta) groups. Significantly higher (**A**) white blood cell, (**B**) monocyte, (**C**) lymphocyte, and (**D**) platelet counts, as well as higher (**H**) red blood cell distribution width, (**K**) high-sensitivity C-reactive protein levels, and (**M**) lactate dehydrogenase levels were found in the PD-L1 > 13 ng/mL group. Higher (**E**) mean corpuscular hemoglobin levels, (**F**) mean corpuscular hemoglobin concentration, (**G**) mean corpuscular volume, (**I**) hemoglobin levels, (**J**) hematocrit levels (**L**) serum albumin levels, and (**N**) high-density lipoprotein (HDL) cholesterol levels were characteristic for the PD-L1 < 13 ng/mL group. PD-L1: programmed death-ligand.

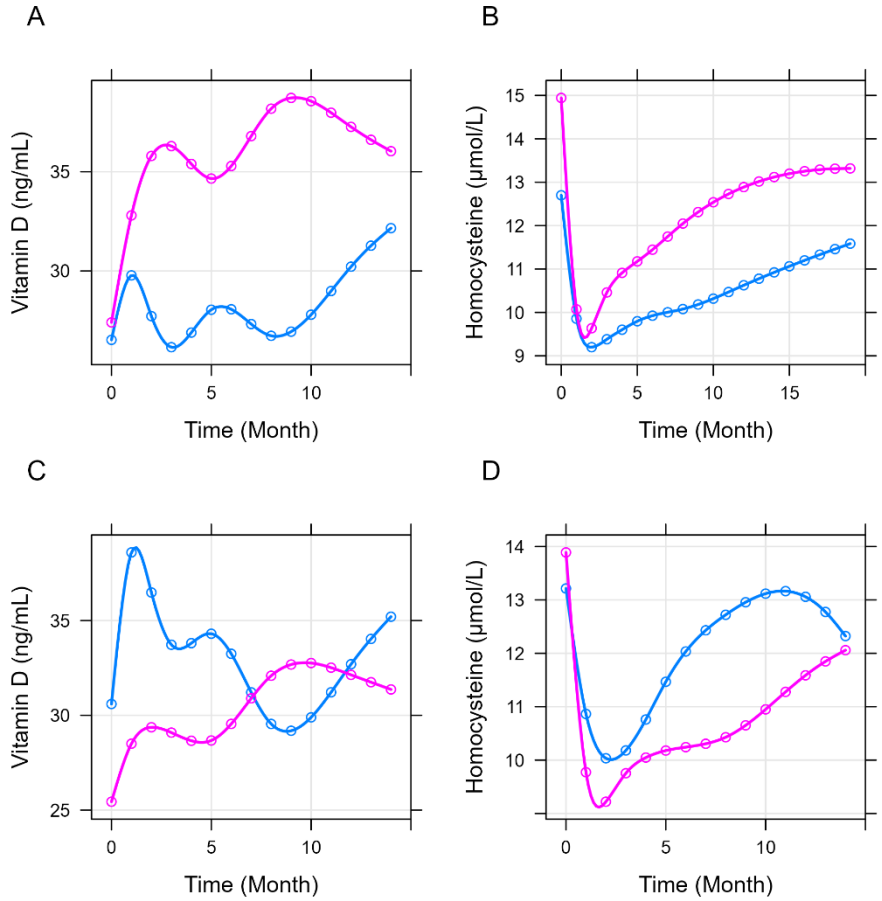

Figure 20. Longitudinal changes in vitamin D (**A,C**) and homocysteine (**B,D**) levels throughout the study within low and high PD-L1 (**A,B**) and low and high PD-1 (**C,D**) groups. Blue and magenta line represent the low and high groups, respectively. PD-1: plasma programmed cell death protein 1; PD-L1: programmed death-ligand.

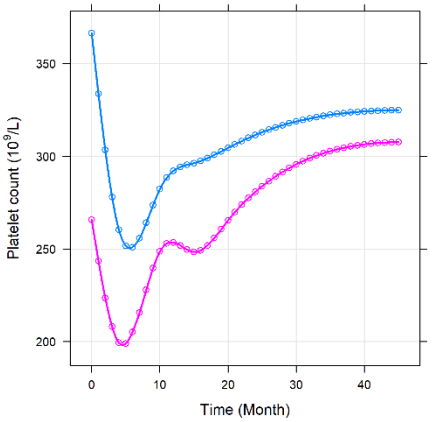

Figure 21. Longitudinal changes in platelet counts throughout the study within the PD-1 < 26 ng/mL (blue) and PD-1 > 26 ng/mL (magenta) groups. PD-1: plasma programmed cell death protein 1.

5. Discussion

5.1. The Investigation of Vitamin D in Colorectal Cancer

Our knowledge about vitamin D has changed significantly in the last two decades. The latest research has highlighted that vitamin D is acting more similar to a hormone rather than a “simple” vitamin [93]. Vitamin D deficiency develops in most chronic diseases, including various forms of cancers [94]. Although vitamin D supplementation seems to have beneficial effects in various diseases and several observational studies proposed association between cancer mortality and low circulating vitamin D levels, due to the lack of large-scale clinical trials on vitamin D supplementation, insufficient evidence exists on this presumed positive effect of vitamin D over patient survival [32, 95]. It has been suggested that supplementation may have a dose-dependent effect on reducing mortality/disease progression [96]. A randomized clinical trial reported marginally better progression-free survival if metastatic CRC patients were treated with higher levels of the active ingredient [97]. In the current study the positive effect of vitamin D supplementation on circulating vitamin D level was basically detectable only in those patients without any metastasis: a sustained increase, and the positive effect of increasing vitamin D levels on patient survival was confirmed. In contrast, despite an initial increase, a similar lasting effect could not be achieved in metastatic patients and circulating vitamin D levels returned to almost the same level as the baseline, simultaneously cancelling out the positive effect on patient survival. It should be mentioned that, based on the available documentation, this trend has been accompanied by a continuous supplementation of daily 3000 IU vitamin D. Taking into account the results of previous studies [96, 97] and in light of our results in those patients with metastatic CRC detailed above, we hypothesize that vitamin D supplementation should be regularly supervised with continuous monitoring through the laboratory measurement of vitamin D until a sufficient saturation response is achieved. E.g., the dosage titration of daily vitamin D should be increased until the serum levels are constantly within the middle of normal range. Our hypothesis is that the tumor burden, accompanied by metastatic CRC, at first is counterweighted via the introduction of vitamin D supplementation, but afterwards the negative effect of the advanced disease slowly completely suppresses the positive effects of vitamin D. The setting of the current study was insufficient to answer this hypothesis,

therefore, a prospective study is suggested to properly investigate this effect of metastases over vitamin D supplementation.

Lately, with the possibility of analyzing longitudinal data much more easily, the knowledge about serum level changes in various biomarkers has significantly increased, which includes vitamin D metabolites as well. In CRC, increasing levels of circulating vitamin D metabolites have been found in patients whose primary tumor was surgically removed [39], which was accompanied by the decrease in serum high-sensitivity C-reactive protein levels, but no relationship between the two parameters could be justified via mixed effect modeling, similarly to our results. Another study [38] reported increasing hydroxyvitamin D levels of non-metastatic CRC patients, which, similarly to the results of [39], was preceded by a brief decrease right after tumor resection. The study also reported [38] differences between the hydroxyvitamin D levels of males and females, patients with and without vitamin D supplementation, and between those patients who received chemotherapy and those who did not. Furthermore, increasing serum levels were also associated with better quality of life questionnaire results. The difference between the sexes could have been justified in our study as well. In addition to all of the above, patients, who eventually died as a result of their tumorous disease, had progressive disease, or those who developed a metastasis at a later stage of the disease showed a similar trend in the first interval of our observation to that of survivors, while a decrease was more pronounced in later times. Right-sided tumors were associated with only tendentiously lower levels of serum vitamin D and no difference was found in the lower disease stages (stage II vs. III).

Investigating the effect of various comorbidities on the change in vitamin D was an additional objective of our study. Interestingly, a higher increase in serum levels of vitamin D was more likely to occur in those patients with comorbidities. This observation is presumably due to the fact that comorbid patients are usually under continuous medical supervision with well-established disease treatment prior to CRC as well, which, apart from known disease-related deviations, can also cause their general better condition, compared to those patients without any comorbidities. CRC is known to have a strong relationship with various comorbidities, such as hypertension [98], other cardiovascular diseases including major events [99], and type 2 diabetes mellitus [100]. The observation that comorbid patients are seemingly more protected suggests that the assessment of

comorbidities at the time of tumor diagnosis and their appropriate subsequent follow-up may have a positive effect on the outcome of CRC, with high probability. Therefore, the early detection of new, or the balancing of existing comorbidities, in cancer patients is highly recommended, in addition to the routine oncology care.

The relationship between vitamin D metabolites and cholesterols is controversial. While one study [101] has reported a strong positive correlation between vitamin D and HDL cholesterol, another study [102] has found a significant positive correlation between vitamin D and triglycerides, and total and low-density lipoprotein cholesterol only. In a recent publication [103], a strong connection between various vitamin D metabolites and serum lipid parameters in CRC has also been justified, suggesting that lower vitamin D levels are more likely to be associated with high lipids. Our results extend these findings, suggesting that low vitamin D levels are inherent in high lipid levels. Another observation from our longitudinal analysis was that there was a very strong connection between albumin and vitamin D levels, which is known for renal diseases [104, 105], and in the case of CRC, with the most probability it is connected to disease progression [106].

5.2. The Investigation of Homocysteine in Colorectal Cancer

Another major focus of our study was to further elucidate the role of homocysteine in CRC. Homocysteine related molecular pathways are suggested to have a significant role in the development of CRC [41] and its higher serum level has been found as a risk factor for increased incidence of CRC [41, 46, 47] and diseases progression [107]. Moreover, a study [49] investigated its change with the course of the disease and it has been found that the homocysteine level reduced from baseline levels in CRC patients without tumor recurrence, while homocysteine levels returned or even exceeded the baseline levels in those patients with tumor recurrence. In contrast to the above, our results revealed a swoosh-shaped trend in the change in the homocysteine level of CRC patients, regardless of any clinicopathological features. The different trends in the change in homocysteine levels between our results and those from the study of Ni *et al*. [49] are possibly related to the distinct patient selection, as follows: 1.) Hungarian patients with European ancestry vs. Chinese patients with Asian ancestry, 2.) less rectal cancer cases were included in the present study (25.6% vs. 57.6%), 3.) patients younger than 50 years of age were less pronounced (10.5% vs. 29.7%), 4.) half of our study subjects were composed of

metastatic disease (48.8% vs. less than 40.7%, patients with Dukes' C or D were not assigned to different cohorts), and 5.) the observation period lasted longer in our study (25 vs. 12 months). In addition, higher homocysteine levels were poor prognostic signs for both the overall and the progression-free patient survival.

Analysis of the effect of clinicopathological and laboratory parameters on the change in homocysteine level revealed the previously known relationships that hyper-homocysteinemia is associated with elevated serum lipids [88, 108], white blood cell [91, 109] and platelet counts [110, 111], liver enzymes [112, 113], reduced renal function [90, 114, 115], cardiovascular diseases [116], type 2 diabetes mellitus [117, 118], and male sex [40]. To our knowledge, the observations that serum homocysteine level is higher in the right-sided colorectal tumors and the normalization of serum total protein level entails a constant homocysteine level, were never described previously. Right-sided tumors are known for their increased disease severity [80, 81], therefore, the observation above fits into the knowledge so far; additionally, the latter may be also related to lighter disease severity/lower tumor burden, with high probability.

5.3. Investigating the Combined Effect of Vitamin D and Homocysteine in Colorectal Cancer

Our knowledge about the relationship between homocysteine and vitamin D is very limited. In health, the relationship between the two is inversely proportional [40], lower homocysteine level is associated with higher vitamin D concentrations. Furthermore, a study [119] investigating the supplementation of healthy subjects with a combination therapy of vitamins (500 µg folic acid, 500 µg vitamin B_{12}, 50 mg vitamin B_6, 1200 IU vitamin D, and 456 mg calcium) found that after a 1-year supplementation period, homocysteine level reduced by approximately one-third from the baseline. In other cancerous diseases than CRC, it was found that gastric lymphoma patients requiring total gastrectomy have higher homocysteine and lower vitamin D level than those who only required partial gastrectomy or were not operated on at all [120]. A study of breast cancer patients [121] reported that the homocysteine level of all of the patients was over the median of the normal range (5 to 15 µmol/L) and almost every second patient had vitamin D deficiency. Another study of breast cancer patients [122] reported significantly lower vitamin D, but the same homocysteine levels, compared to those of healthy subjects. In

the case of CRC, it has to be highlighted that we were the first to investigate the relationship between serum homocysteine and vitamin D and their combined effect on CRC. It was found that neither of the two affected each other's change with the course of the disease, and a positive and negative effect on patient survival was found in a multivariate setting for vitamin D and homocysteine, respectively.

5.4. The Investigation of PD-1 and PD-L1 in Colorectal Cancer

Both PD-1 and PD-L1 have membrane-bound and soluble forms [52]. The latter ones arise due to alternative splicing and cleavage by metalloproteinases [53]. The overexpression of PD-L1 and CD80/CD86 on tumor cells, which can respectively bind to PD-1 and cytotoxic T lymphocyte antigen 4 (CTLA4), results in the inhibition of T cell activation. Furthermore, recent research has also described a significant interaction between the PD-1/PD-L1 axis and the EGFR pathway [123]. These mechanisms ultimately help the tumor to escape anti-tumor immunity [9, 50, 51, 123-126]. Therefore, monoclonal antibodies have been developed to antagonize this inhibitory signaling, and in the last decade, several randomized clinical trials have investigated the efficacy and safety of immune checkpoint inhibitors, including anti-PD-1 and anti-PD-L1 drugs [124-129]. The most immunological response is expected in those (metastatic) CRC patients who have tumors with deficient mismatch-repair and/or high levels of microsatellite instability [124, 130, 131]. Most studies have reported promising results: a significantly improved overall survival and PFS, better response to treatment, and a higher occurrence of partial and complete responses have been found in those patients for whom almost no responses were observed previously [125, 127].

Although the literature about PD-1/PD-L1 and cancer is extremely broad, however, the majority of studies have investigated the membrane-bound forms only. Studies investigating the soluble forms in various cancers have reported that both proteins might be used as independent prognostic factors for patient survival [58-62]. Significantly higher soluble PD-L1 is known to occur in CCC patients with progressive disease, compared to those with stable disease [64]. Moreover, CCC patients with higher baseline soluble PD-L1 levels had shorter survival times [64, 65]. Similar findings could have been observed for melanoma [66], gastric [67], hepatocellular [68, 69], urothelial [61], renal [62, 70], ovarian [71], and lung cancers [58, 60, 66, 72]: baseline soluble PD-L1

measurements can serve as a good prognostic marker for patient survival and increasing levels are associated with progressive disease. Soluble PD-1 may serve as a good prognostic factor in gastric, lung, and bladder cancers [73].

Even less studies have investigated soluble PD-1/PD-L1 in CRC. Compared to control subjects, both protein levels were significantly lower in CRC patients, but with a large SD [74], and significant alterations can be found in various colitis forms as well [132]. Higher circulating PD-L1 levels have been found to be associated with a higher degree of tumor differentiation [75]. $CD3^+$ and $CD8^+$ T cell counts are negatively correlated with PD-L1 and PD-1 [76], and a positive association between PD-L1 and the neutrophil-to-lymphocyte ratio has been also reported [77]. Furthermore, basically all studies investigating soluble PD-L1 in CRC have reported it as a good prognostic marker [59, 63, 76, 78], even for early-stage CRC [63], while soluble PD-1 seems to be independent from CRC survival to date [59]. In our study, we further strengthened these observations. The baseline plasma PD-L1 levels of mCRC patients were found to be good prognostic markers both for DSS and PFS: higher plasma PD-L1 levels were significantly associated with shorter survival. However, no such strong relations could be justified in the case of plasma PD-1 levels. The latter observation was supplemented by the fact that PD-1 could be also confirmed as a weaker, but significant, effector of patient survival if the survival models were adjusted for tumor burden. To our knowledge, we are the first to describe this relationship between PD-1 and tumor burden in the case of CRC.

Studying PD-1/PD-L1 during the course of the disease is a less-documented area. To date, three studies have investigated the longitudinal changes of soluble PD-L1 and/or PD-1 in CRC: no changes in PD-1 levels were observed between the measurements before and after neoadjuvant chemoradiotherapy, while PD-L1 increased significantly [59]. In another study [78], PD-L1 elevation had been described for progressive disease, but not for stable disease/partial response to the treatment. Furthermore, the resection of colorectal liver metastases can also reduce PD-L1 levels, while recurrence and/or progression following hepatectomy reintroduces the increase in PD-L1 levels [76]. Although our study also contained a longitudinal analysis of various laboratory parameters, the fact that the additional blood samples were taken only at baseline prevented us from analyzing PD-1/PD-L1 changes during the course of the disease. Instead, we could only sub-group our study population into low and high PD-L1 and PD-

1 groups based on their baseline measurement values, and the following novel results were found: High PD-L1 levels predicted consistently higher WBC, monocyte, lymphocyte, and platelet counts, red blood cell distribution width, hsCRP levels, homocysteine levels, and LDH levels throughout our observation period. Mean corpuscular hemoglobin levels, mean corpuscular hemoglobin concentration, mean corpuscular volume, serum albumin levels, HDL cholesterol levels, hemoglobin levels, and hematocrit levels were consistently lower in patients with a higher baseline PD-L1 level. No association with vitamin D was found. Higher PD-1 levels showed a strong connection only with lower platelet counts.

As shown above, soluble PD-L1 is strongly associated with progressive disease and tumor burden, both in CRC [59, 63, 75, 76, 78, 133] and in other malignant diseases [58, 60-62, 64, 65, 68-73, 134]. Most studies have found higher serum levels if metastases were present [135-140], and our results, such as the finding that patients with hepatic metastases had higher plasma PD-1/PD-L1 levels, are in line with previous literature, but the observation that patients with metastases in the lung had lower plasma PD-L1 levels has not been described anywhere so far. A previous animal study has shown that bispecific antibodies against gp52 and CD3 can inhibit lung metastasis growth [141]. Furthermore, Kleef *et al*. [142] presented a case report previously, where a low-dose immune checkpoint blockade treatment (nivolumab and ipilimumab) with concurrent hyperthermia resulted in major remission of the patient's pulmonary metastases. Although the soluble forms were not investigated in a similar setting, controversial expression results have been found in other cancers as well. While no difference in the different metastatic sites of non-small cell lung carcinomas has been found [143], a lower PD-L1 expression has been described in skin, liver, and bone metastases of triple-negative breast cancer; however, the same expression levels have been found for lung, soft tissue, and lymph node metastases [144]. Similarly, the lung and lymph-node metastases of renal cell carcinoma express PD-L1 and PD-1 in larger quantities [145]. Therefore, further examination of these observations is needed.

We hypothesize that our longitudinal observations between laboratory parameters and plasma PD-L1 are related to disease progression and to the higher tumor burden as well, with high probability. It is known that numerous laboratory results change for the clinically worse as the disease progresses [146-149]. Ninety percent of the study

populations showed signs of progressive disease throughout our observation, and the direction of longitudinal change in the parameters detailed in the Results section was towards the clinically worse conditions; *e.g.*, it is known that increasing platelet count [150] or decreasing serum albumin [151] levels are poor prognostic signs and are related to an increased tumor burden. The observation that plasma PD-1 was associated with lower platelet counts needs further analysis. To our knowledge, no previous study investigated the potential mechanism linking PD-1/PD-L1, metastases, and other laboratory parameters together, if any. Furthermore, longitudinal changes of various laboratory parameters in relation to high and low PD-1/PD-L1 groups have not been investigated before, but some limited single-time findings are available. In primary and secondary brain tumors, a negative correlation has been described between soluble PD-L1 and hsCRP, neutrophil counts, and other systemic inflammation markers such as $CD3^+$ and $CD8^+$ T cell counts [152]. Platelets have been identified as a possible source of soluble PD-L1 in various tumors [153, 154], and platelet-originated PD-L1 was positively correlated with hsCRP, LDH and, as expected, platelet counts [153]. Platelets have a significant role in CRC as well; they are known to be involved in metastasis formation, and a platelet-inducing mechanism of the tumor itself, known as paraneoplastic thrombocytosis, is also known [154]. Metastases, progression, and increased tumor burden can also affect the extent of paraneoplastic thrombocytosis, ultimately increasing the platelet count in those conditions [155, 156]. The similarity between the present results, the known effects of paraneoplastic thrombocytosis, and the observation that to a certain extent soluble PD-L1 might originate from platelets [153, 154] suggests the hypothesis that there might be a connection between these seemingly different mechanisms, which may be due to a more advanced tumor disease/more severe metastatic disease. Compared to healthy cells, it is known that the tumor/metastasis cells express various proteins and cell markers in a different pattern, which is also associated with disease stage and progression status [157]. To answer the question of whether a direct relationship between tumor cells, metastases, platelets, and PD-1/PD-L1 really exists, more mechanistic studies are needed.

6. Conclusions

The aim of our study was to better understand the relationship between vitamin D, homocysteine, soluble PD-1 and PD-L1, and colorectal cancer. The following conclusions can be draw from the results:

- Vitamin D supplementation has the most benefits in CRC patients without metastases.

- The presence of metastases can cancel out the positive effects of vitamin D / vitamin D supplementation, therefore, it was suggested that in metastatic CRC, the daily dose of vitamin D supplementation should be increased until an appropriate titration level is reached.
    - Patients, who eventually died, had progressive disease, or developed metachronous metastasis showed a similar trend initially to that of survivors, but a significant decrease in the vitamin D level occurred thereafter.
    - The effectiveness of the treatment should be monitored by the regular measurement of vitamin D metabolites.

- Homocysteine level changes have a swoosh-shaped trend in CRC, regardless of any clinicopathological features.

- Serum homocysteine level is higher in the right-sided colorectal tumors.

- The normalization of serum total protein level entails a constant homocysteine level.

- In contrast to previous, single-timepoint based literature data [40], serum vitamin D and homocysteine levels do not change proportionally opposite to each other, no significant longitudinal correlation could have been justified between the two parameters.

- Elevated baseline plasma PD-1/PD-L1 levels may predict not just a poorer survival, but also clinically worse levels of laboratory parameters for mCRC patients. The differences related to the baseline plasma PD-1/PD-L1 levels persisted throughout the course of the disease.

- A strong relationship was found between plasma PD-1/PD-L1 levels and a higher metastatic tumor burden.

- No association between PD1/PD-L1 levels and vitamin D and homocysteine can be justified.

- The strong associations found between vitamin D, homocysteine, PD-1 and PD-L1 and the comorbidities, serum lipids, and the other laboratory parameters refer to the need for a more complex oncology that is more dependent on interdisciplinary solutions.

7. Summary

Background: Vitamin D, homocysteine, programmed cell death protein 1 (PD-1), and programmed death-ligand 1 (PD-L1) are known to play a role in the pathophysiology of colorectal cancer (CRC). However, little is known about their combined and longitudinal effect in CRC.

Methods: The serum vitamin D and the homocysteine level of 86 CRC patients was measured in a longitudinal observational study. Plasma PD-1 and PD-L1 levels were measured in a subset of the original cohort, in 37 metastatic CRC (mCRC) patients.

Results: The 86 CRC patients were enrolled into four cohorts based on the presence of metastases (Adj vs. Met) and vitamin D supplementation (ND vs. D). Vitamin D was constant (Adj-ND), increased significantly (Adj-D, $p = 0.0261$), decreased (Met-ND), or returned close to the baseline after an initial increase (Met-D). Its longitudinal increase positively affected the overall survival in non-metastatic CRC, however, this effect was cancelled out in those with metastasis ($p = 0.0107$). The longitudinal change in homocysteine had a swoosh-shaped trend and it negatively affected both the overall (HR: 1.0940, $p = 0.0067$) and the progression-free survival (HR: 1.0845, $p = 0.0073$). No statistically justifiable connection was found between the two target variables.

Disease progression ($p = 0.0443$) and baseline high-sensitivity C-reactive protein ($p = 0.0011$), aspartate transaminase ($p = 0.0253$), alanine transaminase ($p = 0.0386$), and gamma-glutamyl transferase ($p = 0.0103$) were associated with higher PD-L1 levels. Disease-specific survival and progression-free survival were significantly shorter in patients with high PD-L1.

Based on the baseline PD-1/PD-L1 levels, low and high PD-1/PD-L1 groups were created. Constant, pathological levels of complete blood count values, high-sensitivity C-reactive protein, serum albumin, high-density lipoprotein cholesterol, and lactate dehydrogenase were characteristic for patients with high baseline PD-L1. High PD-L1 levels were significantly associated with increased tumor burden.

Conclusions: Abnormal levels of laboratory parameters and intensified tumor burden can be expected if elevated baseline plasma PD-1/PD-L1 levels are found. A measurement-based titration of vitamin D supplementation, and a more complex oncology that is more dependent on interdisciplinary solutions is recommended for CRC.

8. References

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9. Bibliography of the Candidate's Publications

9.1. Publications Related to the Dissertation

Dank M, **Mühl D**, Herold M, Hornyák L, Szász AM, Herold Z. (2022) Does Elevated Pre-Treatment Plasma PD-L1 Level Indicate an Increased Tumor Burden and Worse Prognosis in Metastatic Colorectal Cancer? *J Clin Med*, 17: 4815. **IF: 3.9**, SJR: Q1 (Medicine – miscellaneous)

Mühl D, Herold M, Herold Z, Hornyák L, Szász AM, Dank M. (2022) Longitudinal Analysis of 1α,25-dihidroxyvitamin D_3 and Homocysteine Changes in Colorectal Cancer. *Cancers (Basel)*, 14:658. **IF: 5.2**, SJR: Q1 (Oncology).

9.2. Publications Not Related to the Dissertation

Szentmártoni Gy, **Mühl D**, Csanda R, Szász AM, Herold Z, Dank M. (2024) Predictive Value and Therapeutic Significance of Somatic BRCA Mutation in Solid Tumors. *Biomedicines*, 12: 593. IF: **4.7 (2022)**, SJR: Q1 (Medicine – miscellaneous).

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