Dissertation

THE ROLE OF MELATONIN AND GLYCINE IN COLORECTAL CANCER LIVER METASTASES TREATMENT

submitted by

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Declaration

I hereby declare that this thesis is my own original work and that I have fully acknowledged by name all of those individuals and organisations that have contributed to the research for this thesis. Due acknowledgement has been made in the text to all other material used. Throughout this thesis and in all related publications I followed the “Guidelines of the Medical University of Graz on Good Scientific Practice”.

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Disclosures

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Abbreviations and Definitions

Akt	Protein kinase B
APC	Adenomatous polyposis coli
CEA	Carcinoembryonic antigen
CEUS	Contrast-enhanced ultrasonography
CRC	Colorectal cancer
CRLM	Colorectal cancer liver metastasis
CT	Computed tomography
CTx	Chemotherapy
EGFR	Epidermal growth factor receptor
ESMO	European Society for Medical Oncology
FLR	Future liver remnant
HCC	Hepatocellular carcinoma
HIF-1α	Hypoxia induced factor 1 α
IHC	Immunohistochemistry
IL	Interleukin
iNOS	Nitric oxide synthase
KRAS	Kirsten rat sarcoma viral oncogene
MAPK	Mitogen-activated protein kinase
LVEF	Left ventricle ejection fraction
MMR	Mismatch repair
MRI	Magnetic resonance imaging
MSI	Microsatellite instability
MVD	Microvascular density
NCCN	National Comprehensive Cancer Network
NF-κβ	Nuclear factor κβ
PET	Positron emission tomography
PIK3	Phosphoinositide 3 kinase
PVE	Portal vein embolization
RAS	Rat sarcoma viral oncogene
US	Ultrasonography
VEGFR	Vascular endothelial growth factor receptor

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Abstract in German

Hintergrund: Trotz multimodalen Behandlungsstrategien bleiben klinische Ergebnisse von Patienten mit kolorektalem Karzinom im fortgeschrittenen Stadium (mit Lebermetastasen) hinter den Erwartungen zurück. Die Wirksamkeit neoadjuvanter/adjuvanter Chemotherapie ist hauptsächlich aufgrund von Chemoresistenz, Toxizität und Nebenwirkungen begrenzt. Es konnte gezeigt werden, dass Melatonin und Glyzin krebsbekämpfende und organschützende Wirkungen besitzen. Ziel dieser Studie war es den positiven Effekt von Melatonin- und Glyzinsupplementierung während der Behandlung von kolorektalen Lebermetastasen (CRLM) in einem Modell mit Ratten zu zeigen.

Materialien und Methoden: Die Studie wurde an 180 männlichen Wistar-Ratten mit induziertem CRLM (indem Adenokarzinom-Rattendickdarm-Zelllinie CC531 Zellen eingesetzt wurden) durchgeführt, die mit Melatonin ± Glyzin ± FOLFOX Chemotherapie für 14 Tage behandelt wurden. Es wurden verschiedene Blutparameter, Computertomographie (Tumorvolumen im Zeitablauf), Herzultraschall (linksventrikuläre Ejektionsfraktion im Zeitablauf), Anti-Ki67- und Anti-CD31-Expressionen im Tumorgewebe ausgewertet und zwischen den Gruppen verglichen.

Ergebnisse: Melatonin- und Glyzin-Supplementierung allein reduzierte das Tumorvolumen um 63.2 % ($p = 0.002$) bzw. um 43 % ($p = 0.044$) im Zeitablauf, während in der Kontrollgruppe das Tumorvolumen um 8.7 % zunahm. Die Behandlung mit Melatonin, Glyzin und deren Kombination hat die mikrovaskuläre Dichte im Tumorgewebe wesentlich reduziert ($p < 0.001$, $p = 0.018$ und $p = 0.003$), während der Proliferationsindex im Tumor nur nach der Supplementierung mit Melatonin und Glyzin allein ($p = 0.005$ und $p = 0.044$) im Vergleich zu Kontrollen wesentlich reduziert wurde. Nach FOLFOX Therapie hat die linksventrikuläre Ejektionsfraktion in der Kontrollgruppe um 9.5 % abgenommen, während die Supplementierung mit Melatonin und Glyzin allein und mit ihrer Kombination die Herzfunktion deutlich verbesserte (jeweils $p < 0.001$, $p = 0.013$ und $p = 0.023$).

Schlussfolgerungen: Die Wirkung von Melatonin alleine und in Kombination mit Glyzin wurde in einem Rattenmodell mit CRLM untersucht. Die Ergebnisse dieser Studie weisen auf proliferationshemmende Eigenschaften von Melatonin und Glyzin als Nahrungsergänzungsmittel während Therapie von CRLM hin. Neben den antiangiogenetischen Faktoren konnte gezeigt werden, dass Melatonin immunmodulatorische Effekte ausübt und damit möglicherweise die weitere Tumorprogression beeinflusst. Außerdem konnte gezeigt werden, dass Melatonin und Glyzin sich positiv auf die kardiotoxischen Effekte der FOLFOX-Chemotherapie auswirken.

Abstract in English

Background: Despite multidisciplinary approach, treatment outcomes of patients with advanced stage colorectal cancer are relatively low. Neoadjuvant/adjuvant chemotherapy efficacy is mainly limited due to chemoresistance, toxicity and negative side effects. Recently, melatonin and glycine have been shown to possess anticancer and organoprotective activities. We aimed to investigate the effects of dietary melatonin and glycine supplementation for colorectal cancer liver metastases (CRLM) treatment in a rat model.

Materials and Methods: This study was conducted on 180 male Wistar rats with induced CRLM (by implanting rat colon adenocarcinoma cell line CC531 cells) treated with melatonin ± glycine ± FOLFOX chemotherapy for 14 days. Various blood parameters, computed tomography scan (tumour volume over time), heart ultrasound (left ventricular ejection fraction over time), anti-Ki67, and anti-CD31 expression in tumour tissue were analysed and compared between groups.

Results: Melatonin and glycine supplementation alone significantly reduced the tumour volume by 63.2 % ($p = 0.002$) and 43 % ($p = 0.044$) over time, respectively, while in the control group tumour volume increased by 8.7 %. Treatment with melatonin, glycine, and their combination significantly reduced microvascular density in tumour tissue ($p < 0.001$, $p = 0.018$, and $p = 0.003$, respectively), while tumour proliferation index was significantly reduced only after supplementation with melatonin and glycine alone ($p = 0.005$ and $p = 0.044$, respectively) compared to controls. The leukocyte count at the end of experiment was significantly increased ($p = 0.012$) in the melatonin supplemented group and this was associated with a higher count of lymphocytes ($p < 0.001$) compared with control group. After FOLFOX application left ventricular ejection fraction significantly decreased by 9.5 % in the control group, while supplementation with melatonin and glycine alone and their combination significantly rescued heart function ($p < 0.001$, $p = 0.013$ and $p = 0.023$, respectively).

Conclusions: In this study potential inhibitory effect of melatonin and glycine supplementation on CRLM growth was demonstrated, most likely due to inhibition of angiogenesis in cancer tissue with significant cancer cell proliferation reduction. Additionally, melatonin was found to exert immunomodulatory effects possibly influencing further tumour progression. Moreover, this study demonstrated beneficial properties of melatonin and glycine for protection against toxic effects on heart function of FOLFOX chemotherapy.

1. Introduction

The liver is the most prevalent location for colorectal cancer (CRC) metastases, accounting for 50 – 60 % of all CRC cases. About 30 % of individuals with CRC develop liver metastases (CRLM), which can occur together with primary CRC diagnosis (a synchronous metastatic disease in one-third of cases) or later after completion of the initial curative treatment (metachronous metastases in two-thirds of patients) (1). Previously, the majority of CRLM were defined as non-resectable. However, recent advancements in CRLM therapy have enabled some patients to be downstaged, leading to a growth in the number of patients cured or living with controlled disease for an extended period of time (2). The initial step in treating CRLM patients is to determine if they have a resectable disease (evaluation of primary tumour and metastases) or one that will be resectable following systemic therapy (3). Due to the fact that care depends on the evaluation of advanced clinical, radiological, and biomarker data, first-line treatment should be defined by a multi-disciplinary team involving surgeons, oncologists, radiologists, and pathologists (2-6). Regardless of the lack of evidence in this extremely heterogeneous group of patients, maximising resection rate of CRLM by utilizing various different treatment strategies including neoadjuvant chemotherapy (CTx), optimization of liver remnant through portal vein embolization or associating liver partition and portal vein ligation for staged hepatectomy (ALLPS), and combining surgical resection with various ablation techniques, remains the major goal that offers the best chance of long-term disease-free and overall survival (7). Prior to patient management, it is critical to evaluate the number, size, and localization of CRLM, as well as their major biliary and vascular relationships. Additionally, preoperative response to neoadjuvant CTx and liver volumetric analysis must be taken into account (3). For those patients initially defined as non-resectable, treatment options, such as systemic CTx, including biological therapy, intraarterial infusion or bead delivered CTx, tumour ablation, and radiotherapy improve survival and may eventually result in the disease becoming resectable (7). Notwithstanding the wide variety of available treatment strategies, survival rates of CRLM patients remain relatively poor.

Currently developing fields in CRLM treatment include research of new biomarkers, the investigation of new systemic drugs and their combinations that target particular pathways of oncogenesis, and the development of full radical surgical methods such as liver transplantation (LTx).

1.1. Overview: Epidemiology, Diagnostic Modalities, Biomarkers, Treatment Strategies, and Surveillance of CRLM

1.1.1. Epidemiology

CRC is the third most often diagnosed malignancy, accounting 10.2 % of the total cases, and the second leading reason of death caused by cancer in the world for both sexes combined (8). Men have a greater incidence and death rate than women, although having a comparable lifetime risk of CRC diagnosis (4.6 vs 4.2 due to women's longer life expectancy (9). The incidence of CRC varies significantly by country, with over two-thirds of all cases and approximately 60 % of fatalities occurring in nations with a high or very high human development index (10). Globally, the incidence between 2005 and 2015 increased by 37 % from 1.2 million to 1.7 million cases per year. The majority of this growth may be attributed to an older and expanding population (11). As well, lifestyle-related factors, such as greater intake of sugar and red or processed meat, less consumption of fibre and physical activity, which result in an increased amount of overweight people are involved (12). In 2018, it was anticipated that almost 2 million new CRC cases and 900,000 deaths will be diagnosed globally (8).

When CRC is detected early, the 5-year survival rate surpasses 90 %. In contrast, metastases significantly lower it to less than 10 % (13). Depending on the stage of primary CRC, many patients will develop synchronous or metachronous CRLM, and it represents the major cause of death in CRC (14, 15). According to the population-based studies, the incidence of CRLM is slightly lower than the 50 % often presented in the publications. For example, in the large study, including more than 1 million inhabitants from two administrative areas in Burgundy (France), 14.5 % of patients had synchronous and the same number of patients (14.5 %) had metachronous CRLM (16). Comparable results were obtained in other studies in different populations: Western Europe (France, Italy, the Netherlands, Poland, Spain, and the UK) (17), Sweden (18), and Australia (19). Meanwhile in Germany, for 24.7 % CRC patients developed hepatic metastases, whereas synchronous metastases accounted for 17.7 % and metachronous metastases for 7.1% of them (20). Data of French study suggested that the liver represents 75.7 % of all synchronous metastases with higher incidence in males than in females (15.9 vs. 12.8 %) (16). Furthermore, the age at primary diagnosis had a significant impact on the incidence rate of synchronous CRLM: 19.8 % before the age of 55 years, 16.7 % between 55 and 64, 16 % between 65 and 74, and 11.7 % in patients 75 and older. Primary tumour location was similar in all groups. However, a recent population-based study indicated that CRLM were less frequently discovered in right-sided CRC (22.1 vs. 28.4 %), but with greater extense than in left-sided cancer (18). They also confirmed that CRLM occurs more

often in the younger patients than in the older ones, but no difference comparing the sex ratio was found.

1.1.2. Diagnostic Modalities

During the last few decades, the clinical results of patients with CRLM have substantially enhanced (21). This could be explained not only by new more effective treatment strategies but also by more advanced imaging methods and better accessibility of them. All of this led clinicians to diagnose more precisely and in the earlier stage the extension of disease, which has fundamental importance for achieving disease control.

Surgical resection plays an essential role in treating patients with CRLM (15). To succeed, appropriate preoperative imaging should be carried out to establish hepatic and extrahepatic disease extension. Currently, imaging techniques can estimate future liver remnant volume and therefore the risk of insufficient liver function and subsequent mortality after major or extended hepatectomy could be reduced. Those aspects are crucial during the discussion about patient-personalised optimal management strategy in a multidisciplinary group.

According to the guidelines for the treatment of patients with metastatic CRC disease presented by European Society for Medical Oncology (ESMO), imaging should always include an abdominal/pelvic and thoracic computed tomography (CT) scan. In the event of uncertainty, additional methods such as contrast-enhanced ultrasonography (CEUS), magnetic resonance imaging (MRI) or CT and positron emission tomography (PET)/CT scan depending on the localisation of the metastases might improve diagnosis. Ultrasonography (US) may assist to better characterise metastases in liver, MRI in liver, peritoneal cavity or pelvis and PET/CT extrahepatic disease (6).

The main objectives of imaging in this circumstance are to locate all metastases, establish the feasibility for local resection, rule out the existence of extrahepatic disease, and assess the likelihood of adjuvant CTx and radical surgery (22). Further, the most important diagnostic imaging modalities mentioned above will be discussed.

1.1.2.1. CEUS

The transabdominal US is a cheap, quick and non-invasive way to assess patients who may have CRLM (23). However, the sensitivity of the US to identify CRLM has previously been shown to be poor and varied, ranging from 50 – 76 %. Mostly due to the limited contrast between the liver metastases and parenchyma (22). In general, isoechogenic CRLM are hard to identify because their acoustic impedance is comparable to that of the surrounding liver parenchyma, and hyperechogenic metastases are difficult to distinguish from benign lesions, such as haemangiomas (24). Based on guidelines published in 2013, those limitations could

be avoided by using the application of US contrast agents (25). Previous studies have shown that the CEUS techniques significantly improve sensitivity and specificity in detecting liver metastases, up to 91 and 98 %, respectively (26-29). However, diagnostic characteristics vary in a wide range (Table 1). This disparity could be explained by different study design and performance technique. Studies, which have included selected patients with established liver metastases, reported greater accuracy than studies with unselected patients. Nevertheless, unlike other contrast-enhanced imaging methods, such as CT and MRI, CEUS allows for the real-time and continuous visualization of the parenchymal microvasculature and the enhancement patterns of metastases throughout all vascular phases (24). Therefore, it seems to be a fast and relatively reliable method for assessing liver involvement by primary malignancies and for further disease surveillance. However, for surgery planning, CEUS does not provide enough information; therefore, other imaging methods, such as CT or MRI, should be used.

Table 1. The characteristics of different diagnostic modalities most often used in the detection of CRLM.

Methods	**Sensitivity (%)**	**Specificity (%)**
CEUS	64 – 91	84.4 – 98
CT	60 – 92.8	92.9 – 96.5
MRI	64.8 – 97.1	89.5 – 98
PET/CT	94.2 – 97.9	92.8 – 99

Data summarized from references (23, 26-31). CEUS: contrast-enhanced ultrasonography; CT: computed tomography; MRI: magnetic resonance imaging; PET: positron emission tomography.

1.1.2.2. CT

CT remains the backbone of imaging modalities, which covers most of the clinical situations for patients with CRC, since it can detect both intra and extrahepatic disease extent extension (23). It is the best option for initial staging. Significant developments in CT, including spiral and multi-section scanning methods, have been implemented throughout the years (32). Previous studies have shown a high sensitivity and specificity (up to 92.8 and 96.5 %, respectively) of multi-detector CT in the detection of CRLM, whereas single or few slice CT has a significantly lower sensitivity of only about 49–59 % (30, 31, 33). Due to short acquisition time CT provides the opportunity to evaluate an entire liver in specific vascular phases by optimizing contrast administration and scan protocols, depending on the available technology (34). In patients diagnosed with CRC, initial CT scan including both arterial and portal venous phases is

advised, and CT images with slice thickness < 3 mm is optimal (35). When the slice thickness is reduced to 1 mm, there is no additional improvement in lesion detection, but there is a significant increase in image noise, resulting in picture quality reduction (36). High-resolution scans using the highest intensity approach in combination with volumetric three-dimensional rendering may describe precise characteristics of CRLM. Vascular reconstruction enables invaluable information about the anatomy of arterial and venous systems, eliminating the necessity for conventional angiography before surgical resection (37). In a survey, including 66 Dutch hospitals, CT was found to be as primary preferred imaging method for the assessment of patients with synchronous CRLM in 52 hospitals (78.8 %) and US in only 12 hospitals (18.2 %). While the second choice was US in 34 hospitals (51.5 %) and CT in 11 hospitals (16.7 %) (38). Other modalities, such as MRI, PET and PET/CT were not frequently utilized as first or second option.

1.1.2.3. MRI

The MRI technology creates pictures with great spatial resolution and tissue contrast by combining powerful magnetic fields and radiofrequency pulses (22). Over the last decade, the sensitivity of MRI in CRLM has increased mostly due to the following improvements: diffusion-weighted MRI sequences and the hepatospecific phase using gadoxetic acid. The best sensitivity is achieved by combining both MRI techniques (39). In a large meta-analysis of prospective trials of CRC patients who have not previously received therapy, MRI was selected as a first-line tool for assessing the intrahepatic involvement (30). With its high overall diagnostic parameters, it accurately depicts lesions < 10 mm (30, 40). It should be considered when planning liver resection because a significant proportion of liver lesions in patients with CRC are of small size. The presented incidence of CRLM ≤ 10 mm is about 30 – 50% in the literature (41). Furthermore, MRI seems to be the most suitable imaging method for preoperative evaluation of CRLM patients after the neoadjuvant CTx (41, 42).

1.1.2.4. PET/CT

PET using the glucose analogue ^{18}F-fluorodeoxyglucose (FDG-PET) has become a complementary tool in the diagnosis of intra- and extrahepatic lesions for CRC patients (43). It differentiates malignant cells by detecting their higher glucose metabolism. Integrated FDG-PET/CT techniques can provide not only functional but also morphological information in a single scan session. Although previous studies showed excellent diagnostic parameters (30) and found the prognostic value of FDG-PET/CT (44), the guidelines do not recommend to use it for all patients (6, 35). Due to limited availability and high cost, FDG-PET and PET/CT should only be utilized in selected cases whose diagnosis is unclear after traditional diagnostic

approach (e.g., when carcinoembryonic antigen (CEA) level increase and CT fails to determine the location of disease) (7, 23). Additionally, it should also be used in individuals who are unable to have an MRI due to absolute contraindications (45).

1.1.3. Biomarkers

Historically, traditional prognostic factors, such as primary CRC characteristics (tumour localization and stage of cancer according to the TNM classification), CRLM features (size of largest CRLM, number of lesions, differentiation grade, and resection margin status), and other variables including CEA, existence of extrahepatic extension, and time until CRLM occurred, have been utilized as biomarkers to predict key oncologic outcomes (progression-free, recurrence-free, or overall survival) and assist in selection for individualised CRLM therapy (7, 46). The ability to integrate numerous treatment modalities, including surgery and other locoregional therapies, CTx, and targeted and biological therapies, has resulted in the development of new prognostic and predictive biomarkers during the last few years (47). Recently, it was recommended that in addition to the standard prognostic criteria described above, patient selection for surgical resection should incorporate molecular profiling, since surgery and systemic therapy could be customized to individual biology of CRLM to enhance results (48).

A deeper knowledge about the molecular pathways underlying the development and CRLM has allowed identification of mutations in BRAF, KRAS, PIK3CA, APC, and P53 genes and the presence of microsatellite instability (MSI) as important prognostic molecular biomarkers (46). Information on the mutational status of CRLM should be included in multidisciplinary debate to assist in determining the best treatment strategy, kind of CTx, and also optimal time and technique of surgical resection (49). Further, clinical significance of these mentioned biomarkers will be discussed.

1.1.3.1. BRAF

BRAF, a RAF gene kinase and immediate downstream effector of the rat sarcoma viral oncogene (RAS) shows mutations in nearly 10 % of CRLM. It has recognized as a crucial prognostic biomarker and therapeutic target for metastatic CRC in recent years (50, 51). Around 90 % of these BRAF mutations in V600 are missense mutations, and roughly 90 % of V600 mutations are glutamic acid substitutions (V600E). V600 is required for BRAF to stay inactive in the absence of a RAS activation signal (51). Numerous studies have revealed that the presence of a BRAF mutation is linked with considerably lower recurrence-free and overall survival in patients having CRLM resection, with some research showing that BRAF mutations may be more predictive for poor outcomes than RAS mutations (46).

Previously, Tran *et al.* found that patients with BRAF mutant CRLM had a median overall survival of 10.4 months, while 34.7 months with BRAF wild-type lesions ($p < 0.001$) (52). More recently, much better overall survival was presented in a multicenter analysis of 1497 patients: 40 and 81 months, respectively ($p < 0.001$) (53). In a study by Margonis *et al.*, BRAF V600E status has been linked with both a worse overall survival (HR = 2.76; 95 % CI: 1.74 to 4.37; $p < 0.001$) and disease-free survival (HR = 2.04, 95 % CI: 1.30 to 3.20; $p = 0.002$), and it seems to be the strongest prognostic determinant in their cohort (54). A large meta-analysis, including 5192 CRLM patients, confirmed that BRAF mutations impair both overall survival (OR = 1.98; 95 % CI: 1.61 to 2.43) and recurrence-free survival (OR = 1.49; 95 % CI: 1.01 to 2.21) following liver surgery (55). Moreover, risks of both hepatic (OR = 0.42; 95 % CI: 0.18 to 0.98) and extrahepatic recurrences (OR = 0.53; 95 % CI: 0.33 to 0.83) were significantly higher in BRAF mutant patients (55). Although individuals with BRAF mutation have a worse prognosis, the mutation does not exclude long-term survival. Better results among this group of patients are related with node-negative initial lesions, CEA < 200 µg/L, and clinical risk score < 4. Thus, based on existing data, BRAF mutant individuals with resectable CRLM should be proposed, but with careful selection, for hepatic surgery due to the high risk of disease recurrence (53).
Current guidelines urge the examination of BRAF status in all CRLM cases (recommendation level I, grade B), considering its potential as a prognostic biomarker and a predictive marker for CTx treatment options (6, 46).

1.1.3.2. RAS

Mutations in RAS subfamily genes have recently acquired popularity as CRLM molecular biomarkers. KRAS (Kirsten RAS) plays an important regulatory role in the cell signal transduction pathways, such as PI3K-Akt and RAS-RAF-MAPK (mitogen-activated protein kinase) signalling pathways, which have a role in the growth of cells. These mutations are more frequent than BRAF mutations: approximately 30 – 50 % of patients with metastatic CRC bear mutations in KRAS (46, 51, 56). In general, KRAS operates as a molecular “power” switch in the pathway of epidermal growth factor receptor (EGFR), a mitogenic pathway, and predicts responsiveness to anti-EGFR therapy in its wild-type form (57). In addition, KRAS mutation has been implicated in the development of extrahepatic disease, an unfavourable response to CTx, a positive resection margin, and poor overall and recurrence-free survivals following surgery regardless of the CTx scheme used, as well as a decreased survival following reoperation for recurrence (4).
A recent meta-analysis including 1809 individuals established that KRAS mutation was an independent unfavourable prognostic factor linked with substantially lower overall survival (HR = 2.24; 95 % CI: 1.76 to 2.85) and recurrence-free survival (HR = 1.89; 95 % CI: 1.54 to 2.32)

after resection of CRLM (56). KRAS-mutant patients have encountered a higher incidence (60.3 vs. 40.8 %; p = 0.002), higher number of micrometastases (2 vs. 0; p < 0.001), more involved resection margins (21.5 vs. 9.2 %; p = 0.007) and narrower margin widths (2.0 vs. 4.3 mm; p = 0.002) than KRAS wild-type patients (58).

Surgical resection plays an essential role in the treatment of CRLM patients with wild-type KRAS mutation and resectable disease. In such circumstances, R0 resection with a clear margin width of 1 to 4 mm is considered to be adequate; In contrast, results show that broader resections, including anatomic hepatic resections, may confer a survival advantage in CRLM patients with KRAS mutation (49). However, due to the inferior prognosis, some authors proposed that aggressive surgical approach for KRAS mutant patients with several risk factors (node-positive primary tumour, hepatic metastases > 3 cm, and > 7 cycles of cycles of preoperative CTx) would not be beneficial and therefore other modalities and further systemic therapy should be pursued (4, 59).

According to the guidelines, evaluation of RAS mutation status for all CRLM patients is recommended, since it is required prior to therapy with the EGFR-targeted monoclonal antibodies cetuximab and panitumumab (recommendation level I, grade A) (6).

1.1.3.3. PIK3CA

PIK3CA is a proto-oncogene that encodes a catalytic subunit of class IA phosphoinositide 3-kinase (PI3K), phosphorylating protein kinase B (Akt) and activation of the PI3K/Akt/mTOR signalling pathway and contributes to the development, proliferation, progression and survival of numerous solid tumours (48, 60). Mutations in PIK3CA have been found in 7 – 28 % of patients with CRLM (46). Currently, the association between PIK3CA mutational status and prognosis of CRLM patients is still debatable.

In a systematic review, PIK3CA gene mutation had an effect on long-term outcomes (overall survival and recurrence-free survival) (49). A meta-analysis showed consistent trends towards shorter progression-free survival (HR = 1.91; 95 % CI: 0.78 to 4.68; p = 0.16), shorter overall survival (HR = 1.43; 95 % CI: 1.02 to 2.00; p = 0.04) and lower objective response rate (RD = −23 %; 95 % CI: −35 to −10 %; p < 0.001) among patients with PIK3CA mutations when compared to PIK3CA wild-type (61). Recently, Wang *et al.* found that PIK3CA mutation correlates with first-line CTx resistance: patients who were resistant to first-line CTx had an increased incidence of PIK3CA mutation than those who were not (14.49 vs. 5.15 %, p = 0.003) (60). In another study, patients with CTx refractory CLRM treated with radioembolization and mutations in this pathway had significantly decreased cumulative incidence of local progression compared to patients with wild type PI3K pathway genes (33 vs. 76 % and 55 vs. 92 %) at 6 and 12 months, respectively (62). Moreover, it was a significant indicator of longer

time to local progression in both univariate (HR = 0.31; 95 % CI: 0.11 to 0.90; p = 0.031) and multivariate (HR = 0.27; 95 % CI: 0.09 to 0.77; p = 0.015) analysis (62).
Currently, clinical implication of PIK3CA remains unclear and further research is required to completely understand the mechanism involved in downstream effects of this mutation in CRLM.

1.1.3.4. APC

The formation of polyps, which are induced by hereditary or somatic loss-of-function mutations in the tumour suppressor gene called adenomatous polyposis coli (APC), is one of the first phases in CRC formation (63). These mutations are very prevalent in CRLM patients, occurring at a rate of 42 – 73 % (46). While the effect of single APC mutations on CRLM patients is unknown, a growing number of studies demonstrates that APC may alter tumour biology via its cooperative interaction with other mutations such as PIK3CA.
Based on previous findings, combined mutations of APC and PIK3CA predict poor response to preoperative CTx and inferior survival in patients with CRLM. Recurrence-free survival (3.1 vs. 20 %; p < 0.001) and overall survival (44 vs. 84 %; p < 0.001) after hepatectomy were worse in patients with double mutation, while absence of double mutation was an independent predictor of main pathologic response (OR = 2.91; 95 % CI: 1.47 to 5.86; p = 0.002) (64). However, a comparable research on patients with resected CRLM was unable to verify the independent effect of concomitant APC and PIK3CA mutations on survival (63). Further studies are warranted to fully determine the mechanism of downstream effects of co-occurring APC and PIK3CA mutations in CRLM.

1.1.3.5. TP53

The TP53 gene encodes a critical transcription factor involved in tumourigenesis. After activation, as a consequence of various cellular triggers, including DNA damage, TP53 modifies the regulatory pathways of a variety of target genes resulting in cell cycle arrest, DNA repair, apoptosis and angiogenesis (48). TP53 deficiency is prevalent and has been documented in 50 – 66 % of CRLM patients (46). Currently, the link between TP53 mutations and survival is inconsistent in the literature. Several studies suggest no effect of TP53 status on overall and disease-free survivals (49). However, a recent study of 490 patients reported that TP53 mutation was an independent negative prognostic factor related to significantly reduced rates of overall survival (HR = 2.21; 95 % CI: 1.49 to 3.28; p < 0.001) and recurrence-free survival (HR = 1.40; 95 % CI: 1.11 to 1.78; p = 0.005) after resection of CRLM (65). Moreover, patients with coexisting mutations in RAS and TP53 genes had poorer outcomes,

including overall and recurrence-free survival, compared with patients with mutations in one or none of these genes (65, 66).

1.1.3.6. MSI

Epigenetic inactivation or germline defect of DNA mismatch repair (MMR) genes, that encoded four proteins including MLH1, MSH2, MSH6, and PMS2, are responsible for accumulation of errors on the microsatellite site. Deficiency of MMR genes lead to truncation, non-function or loss of a protein that causes microsatellite instability (MSI) of DNA that increases the likelihood of mutation accumulation and tumourigenesis (67). MSI-positive tumours account for only 4 – 8 % of CLRM cases, and data on the prognostic and predictive significance of MSI phenotype in the metastatic CRC are limited (6).

Previous data have not suggested a role for MSI in stratifying good versus poor prognosis in CRLM patients (68). However, in a more recent study, tumours with high levels of MSI have shown significantly poorer survival compared to microsatellite stable tumours (22.1 vs. 11.1 months; p = 0.017) (52). Interestingly, MSI is more prevalent in cancer stage II (20 %) rather than III (12 %) and even stage IV (4 %), offering that these kinds of tumours have a lower potential to spread (63).

In general, MSI testing for patients with CRLM should be considered because it assists physicians in genetic counselling, and it has high predictive value for the treatment with immune checkpoint inhibitors (recommendation level II, grade B) (6).

1.1.4. Treatment Strategies

Surgical resection is the preferred therapeutic strategy for CRC metastases limited to the liver, whereas radiofrequency or microwave ablation procedures are considered for patients who are unsuitable for surgery (45, 69). Metastatic spread to the liver is common during the course of CRC, for which the overall survival without treatment is about 9 months. However, if a patient is suitable for surgical liver resection, 5-year survival can be expected somewhere in between 25 % and 40 % (70).

CRLM patients may be categorized into three categories based on their primary resectability status: 1) the hepatic lesions are distinctly resectable, 2) although the hepatic lesions are unresectable, they may be converted to resection after initial CTx, and 3) the hepatic lesions are unresectable, and most likely conversion will not be reached even after CTx (69).

1.1.4.1. Surgery

While over a half of all CRC patients will be diagnosed with liver metastases throughout the course of their disease, less than quarter of CRLM patients are classified as resectable at the

time of initial diagnosis. There are five conditions used as criteria indicative for CRLM resectability: 1) the patient is physically fit for surgery, 2) the primary tumour has been controlled or can be controlled, 3) R0 resection can be achieved, 4) there is no evidence for extrahepatic lesions, or they can be controlled, and 5) the sufficient function of the future liver remnant (FLR) can be preserved (71). Much effort has been directed toward predicting liver functional reserve preoperatively. In patients who receive short-duration preoperative CTx, it has been suggested that an FLR of a minimum of 20 to 30 % is sufficient to be subjected to hepatic resection (72). However, those receiving long-duration (> 12 weeks) preoperative CTx should have a FLR volume of at least 30 %, and in patients with hepatic cirrhosis or fibrosis, the FLR volume is suggested to be at least 40 to 50 % of total liver volume (72). Several different approaches are being utilized to maximize the resectability status of CRLM patients, including downsizing CTx, portal vein embolization (PVE), Associating Liver Partition and Portal vein Ligation for Staged hepatectomy (ALPPS), and ablation techniques (radiofrequency, microwave or electroporation) (7).

Surgical resection for CRLM typically involves either anatomical resection or parenchymal-sparing hepatectomy, which is utilized increasingly. Recent data demonstrate that parenchymal-sparing hepatectomy has a comparable safety and efficacy profile compared to traditional anatomical resection and do not compromise oncologic outcomes. However, this technique facilitates preservation of hepatic parenchyma, thereby increasing the potential for salvage repeat hepatectomy for those patients who experience intrahepatic recurrence of CRLM (73). When synchronous CRLM is present, the surgical approach should be determined by the liver tumour burden. For patients with multiple bilobar CRLM, recent data of the LiverMetSurvey registry of 7360 patients recommended the liver-first strategy as the benchmark since it has been shown better short-term outcomes and higher survival rates than other treatment approaches (74).

For patients who are considered to have unresectable disease, treatment methods, such as systemic CTx, intra-arterial infusion or bead delivered CTx, targeted biological agents, tumour ablation, and radiotherapy, lead to increased survival and may allow for the resection of initially unresectable patients (7). Partial palliative surgeries for CRLM are not indicated.

1.1.4.2. Neoadjuvant CTx

There is currently a scarcity of data on the role of neoadjuvant CTx in the therapy of resectable CRLM patients, with majority of results presented from retrospective studies with high variability in CTx protocols (75). The primary challenge is to identify those who can benefit significantly from neoadjuvant CTx, since neither the National Comprehensive Cancer Network (NCCN) nor the European Society for Medical Oncology (ESMO) consensus guidelines in the

management of resectable CRLM have given a clear answer to this question up to now (76). Previous retrospective multi-institutional analysis concluded that CTx administered after but not before resection of synchronous CRLM was related to increased recurrence-free and overall survival (77). This was also proven for metachronous CRLM (78). Zhu *et al.* retrospectively analysed 466 patients with resectable CRLM and found that neoadjuvant CTx before surgical resection increase neither mortality nor complications. Among all resectable patients, only those with at least two risk factors (primary tumour at stage T4, ≥ 4 liver metastases, the largest liver metastasis ≥ 5 cm in diameter, and a serum CEA level ≥ 5 ng/mL), received survival benefit from neoadjuvant CTx (79). In 2013, long-term overall survival analysis (EORTC 40983 randomised-controlled, phase 3, trial) found no difference in overall survival with the addition of perioperative CTx with 6 cycles of FOLFOX (folinic acid, fluorouracil, and oxaliplatin) compared to surgery alone for patients with resectable CRLM (80). The largest meta-analysis including 6254 patients from 17 cohorts and one randomised control trial (mentioned above) presented relatively high level of evidence of a significant advantage of neoadjuvant CTx in high-risk CRLM patients survival; although compiled data were heterogenic (81). A recent multicentre study (LM02-trial) of anti-EGFR-targeted therapy with panitumumab in combination with FOLFIRI (folinic acid, fluorouracil, and irinotecan) as preoperative therapy for operable CRLM in RAS wild-type patients showed in a radiological objective response rate in 65.7 % of patients, while progression-free survival and overall survival are still monitored (82).

In summary, there is still no validated evidence to consider CTx administered prior to surgery as standard treatment approach of initially resectable CRLM patients. More protective trials are necessary. Furthermore, the efficiency of neoadjuvant CTx should be studied while considering tumour biology based on pathological features and molecular biomarkers.

1.1.4.3. Adjuvant CTx

There is no solid evidence to support the use of adjuvant CTx in CRLM patients with adequate oncological and technical (surgical) criteria who have not received perioperative CTx (recommendation level II, grade C). However, those patients with negative prognostic factors may have advantage from adjuvant CTx (recommendation level III, grade B) (6). The trials of modern postoperative adjuvant CTx suggest that an oxaliplatin-based CTx, CAPOX (capecitabine with oxaliplatin) or FOLFOX for 6 months following surgery of CRLM improves the overall results, unless patients failed an adjuvant oxaliplatin-based therapy for stage II or III cancer within 12 months (recommendation level IV, grade B) (6, 83).

Two randomized control trials revealed that median disease-free survival significantly increased in response to fluorouracil and folinic acid after curative surgery of CRLM compared

to surgery alone (17.6 vs. 24.4 and 8.4 vs. 17.4 months, respectively). However, there was no benefit on overall survival (46.4 vs. 62.1 months and 5-year overall survival: 66.1 vs. 66.8 %, respectively) (84, 85). The additional use of irinotecan (FOLFIRI scheme) in the adjuvant setting following complete resection of CRLM does not significantly improve patient disease-free survival than therapy with fluorouracil and folinic acid (86).

Anti-EGFR-targeted treatment with cetuximab in combination with FOLFIRI or FOLFOX has been demonstrated to substantially enhance response rate, prolong progression-free survival and facilitate radical resections of liver metastases in RAS wild-type patients with primary unresectable metastatic CRC (87, 88). In another study, anti-VEGFR-targeted (vascular endothelial growth factor receptor) treatment with bevacizumab plus FOLFOXIRI (fluorouracil, folinic acid, oxaliplatin, and irinotecan) was related to increased rates of response and resection, as well as a slower progression when compared with bevacizumab-FOLFOX in patients with initially unresectable CRLM (89). Since data from these recent randomized clinical trials with biological therapeutics showed substantial improvements in outcomes of patients with KRAS wild-type unresectable CRLM, further research into implication for adapting multimodal therapies in the adjuvant setting of CRLM is needed.

1.1.4.4. Conversion Therapy

About 15 – 30 % of patients diagnosed with initially nonoperable CRLM, may be converted to resectable after CTx (4). Several studies reported even higher rates of conversion, up to 12 – 39 %, when there is excellent effect of CTx (90). The conversion therapy includes various combinations of CTx regimens (irinotecan- or oxaliplatin-based), with or without the use of biological therapy targeting VEGFR or EGFR (90). Currently, there is a lack of randomised controlled trials aimed to analyse conversion CTx as potential treatment approach for patients with initially unresectable CRLM, making it difficult to provide any clinical recommendations regarding the optimal regimens (6).

Other modalities, including tumour ablations (electroporation, microwave or radiofrequency), chemo- and radioembolization, and intra-arterial CTx, may be useful to reach disease conversion (from unresectable to resectable).

1.1.4.5. LTx

For highly selected patients with malignant liver tumours like hepatocellular carcinoma (HCC), LTx is often the part of a standard treatment (91). LTx in individuals with unresectable liver-only CRC metastases has been shown to be associated with a 5-year overall survival rate of 56 %, compared with 9 % in patients receiving standard palliative CTx. This was demonstrated in a recent LTx pilot study, Secondary CAncer (SECA) study, including 21 CRLM patients (92,

93). Maximal tumour diameter > 5.5 cm and level of CEA before LTx > 80 µg/L before LTx, as well as CTx response failure, and short interval (< 2 years) from initial resection to LTx were all negative elements associated with decreased survival (93). Recently, the SECA-II study including 15 CRLM patients presented improved 5-year overall survival (83 %) which is similar to other indications for LTx (94).

Although, in highly selected CRLM patients LTx is a surgical method with an acceptable long-term overall survival, the scarcity of donor grafts requires that criteria for LTx of non-resectable CRLM patients need to be further developed and refined to ensure long post-transplant survival.

1.1.5. Surveillance

Despite advances in CRLM therapy, up to 70 % of patients still experience disease recurrence which is most common in the first three years following surgery (95). Surveillance practice vary widely (3 vs. 5 years), and it remains under debate with special focus on irradiation (multiple CT scans) and healthcare expenses (7). Some authors suggest, that low-risk CRLM patients may not benefit from the extra two years of surveillance, whereas high-risk patients should be observed at least 5 years. This highlights the role of a personalised, long-term follow-up plan following CRLM therapy with the goal of curing the disease (95).

Currently, NCCN recommends to physically inspect patients with metastatic CRC and check CEA levels in serum every 3 – 6 months for 2 years, later every 6 months for following 3 years (5 years in total). One year after colon resection, colonoscopy should be performed, except in the case of colonoscopy was not completely performed before resection (e.g. bowel obstruction) when it is recommended in 3 – 6 months (recommendation level II, grade A). CT imaging is recommended every 3 – 6 months for the first 2 years, later at intervals of 6 – 12 months for following 3 years (5 years in total) (96).

1.2. Melatonin and Cancer

Melatonin (N-acetyl-5-methoxytryptamine) is a ubiquitous amphipathic indolamine molecule found in all species, from bacteria to humans. It was established in 1958 at Yale University by Aaron Lerner, a North American dermatologist (97). In human beings, the pineal gland is the central place responsible for synthesis and secretion of melatonin, from N-acetyl serotonin, under the control of the retinohypothalamic tract in response to darkness. Recently it was found that some extrapineal tissues, including the gastrointestinal tract, spleen, heart, thymus, muscle, and others, also enable production of melatonin (98). This endogenous hormone exhibits lipophilic and hydrophilic features enabling receptor-independent diffusion through cell membranes. Melatonin stimulates a broad range of cellular mechanisms via both receptor-

dependent and independent signalling pathways (99). It affects a variety of physiological processes, including the circadian rhythm, the sleep–wake cycle, neuroprotection, gonadal activity, redox homeostasis, and immunoregulation. Abnormal production of melatonin results in significant health problems (e.g. premature aging, development and progression of cancer, adipose tissue formation, cardiovascular diseases, and initiation of type 2 diabetes) (100). Melatonin seems to be safe at even high doses (up to 100 mg/kg per day) since no serious adverse effects have been previously documented (98).

Recently, potential effect of melatonin in cancer development and progression has gained attention. Significant number of epidemiological and experimental researches have shown that melatonin has the potential to suppress several types of cancer (colorectal, breast, prostate, ovarian, liver, kidney, hematologic, and etc.) *in vitro* and *in vivo* conditions. Melatonin is involved in multiple anticancer processes, including activation of apoptosis, inhibition of cell proliferation and angiogenesis resulting in decreased tumour development and metastasis (98, 101, 102). In addition, melatonin supplementation was related to a reduction in the adverse effects of CTx and radiotherapy, a decrease in drug resistance in cancer treatment, and an enhancement of the therapeutic benefits of traditional anticancer therapies (102). Several clinical studies demonstrated that supplementation with melatonin is an effective adjunctive therapy method (98).

Melatonin targets delivery of nutrients and oxygen to cancer cells through a number of pathways. The regulation of glucose transporters, in addition to the regulation of glucose uptake, is a novel mechanism by which melatonin might inhibit tumour growth (103). At the transcriptional level, hypoxia induced factor 1 α (HIF-1α), as well as genes under its modulation, such as VEGF and endothelin-1, that functions as the major angiogenesis growth factors and induces progression of cancer, are the key targets of melatonin (101). Moreover, melatonin reduces activation of FoxO-1, nuclear factor κβ (NF-κβ) and Ca^{2+} /calmodulin-dependent protein kinase II α (CaMKIIα) resulting in apoptosis promotion through caspase-dependent apoptotic pathway, thereby limiting development and further progression of cancer cells (101, 104). Moreover, melatonin has anti-invasive/anti-metastatic properties involving various pathways, including inhibition of p38 MAPK and repression of epithelial–mesenchymal transition (105). By reducing aromatase expression and associated enzymes, melatonin reduces its activity, resulting in a decrease in estrogen synthesis. Due to the close association between the level of estrogen and cancer growth, inhibiting estrogen production is critical for estrogen-dependent cancer treatment (106). Finally, the capability of melatonin to transfer glucose oxidation from the cytosol to the mitochondria reflects how cancer cells that have

developed resistance to standard CTx drugs may be resensitized to the same therapy when melatonin is delivered (107).

Taken together, melatonin can be involved in the treatment of cancer with other anticancer therapeutics. In contrast to other anticancer drugs, melatonin has been shown to be safe and nontoxic, with a low risk of adverse events. However, deeper knowledge about the mechanisms of its multiple modes of effect is needed to ensure its clinical use.

1.3. Glycine and Cancer

Glycine is a common amino acid composed of one carbon atom, an amino group, and a carboxyl group (108). H. Braconnot, a French chemist, was the first to identify glycine molecule from protein acid hydrolysates (i.e., gelatine) in 1820. Glycine was determined to be as sugary as glucose, hence its name was taken from the Greek word "glykys", which means sweet (109, 110). The daily human diet consists approximately 2 g of glycine. In clinical studies, a daily dose of up to 90 g of glycine has been demonstrated to be safe without serious adverse events (111, 112). This high dose of glycine raises the level of glycine in serum to more than 900 μM. Normally, the level of glycine in serum is about 300 μM (112).

Glycine is frequently thought to be physiologically neutral, and it has even been employed as an isonitrogenous control in amino acid supplementation researches (108). However, it is engaged in a multiple of metabolic and pathophysiological processes critical to the development and survival of proliferating cells, including malignant cells (101, 113). Previously, *in vivo* studies found that dietary glycine inhibited tumour development by roughly 65 % in cases of induced hepatic tumours in rats and subcutaneous melanoma in mice (114, 115). It was suggested that the action of glycine in these models was related to the suppression of endothelial cell proliferation, thereby inhibiting angiogenesis and tumour progression (116). In another study, treatment with dietary glycine has led to reduced growth of breast adenocarcinoma cells *in vivo* through reduction of inducible nitric oxide synthase (iNOS) expression (117). Nitric oxide generally stabilizes HIF-1α and promotes secretion of VEGF from endothelial cells and macrophages, which then induces angiogenesis (118). It was suggested that glycine blunts VEGF-induced elevations in intracellular calcium concentration, a crucial signal for endothelial cell proliferation and migration (116, 119). These findings support the concept that glycine triggers its receptor, allowing chloride influx and hyperpolarization of the endothelial cell membrane, decreasing the opening time of voltage-dependent calcium channels. *In vivo* experiments, glycine blocks tumour growth and angiogenesis most likely via this mechanism (116).

More recently, *in vivo* studies approved, that dietary glycine reduces angiogenic signalling of endothelial cells and development by about 40 % in a model of CRLM and preserves the heart

function during therapy with FOLFOX (120-122). Additionally, glycine protects skeletal muscle against cancer-induced atrophy and function loss, lowers oxidative and inflammatory stress, and inhibits the expression of genes linked with muscle protein breakdown occurring during cancer cachexia (123). In a rodent model, this molecule preserves the liver against CTx-induced damage, most likely through inhibiting the activation of Kupffer cells and improving intrahepatic microperfusion (112), since the synthesis of tumour necrosis factor α (TNF-α) by Kupffer cells and stimulation of NF-κβ are blocked by glycine (114).

Currently, the anticancer effects of glycine represent an attractive area for combination therapy. However, the optimal dose of glycine and administration route are unknown, limiting its usage in clinical practice.

1.4. Aims and Objectives

We aimed to assess the anti-cancer effects, such as anti-proliferative and anti-angiogenic, of both glycine and melatonin for CRLM treatment in experimental animal model. Furthermore, a possible negative influence of FOLFOX CTx on heart function and potential protective effects of melatonin alone and in combination with glycine.

The primary objective of this dissertation was to assess the effects of dietary melatonin and glycine supplementation with and without FOLFOX CTx on CRLM growth in experimental rat model. The secondary objectives were: 1) to assess tumour proliferation index and microvascular density (MVD) based on histological analyses; 2) in addition, to analyse the effects of different treatments on body weight, food intake and various blood parameters overtime; 3) furthermore, demonstrate the negative effect of FOLFOX CTx on heart ejection fraction and potential protective effects of melatonin and glycine alone and in their combination.

2. Materials and Methods

The description of some parts of Material and Methods section may be similar to those published in Kvietkauskas *et al*., 2021 (101).

2.1. Experimental Design and Groups

The experimental model and animal groups are fully described in Figure 1. A total of 180 male Wistar rats (six-week-old; weight 200 – 250 g) were used in this experimental study. The rats were randomly allocated to either sham groups (n = 10/group) or experimental groups (CRLM) with or without CTx (n = 15/group or 20/group, respectively; Table 2). After an acclimatization period, standard diet was changed to 5 % glycine-enriched diet, containing 15 % casein and 5 % glycine, for half of the animals in each group, while the others received a control diet, containing 20 % casein and 0 % glycine. These special diets were prepared as hard pellets without any visual difference and purchased from Altromin International (Lage, Germany). CRLM was induced in experimental groups, while sham surgery (both procedures are described below in the paragraph “CRLM Induction and Sham Surgery”) was performed in sham groups six days after diet switch mentioned above. Immediately after intervention, rats received 1.5 mL of milk (3.5 % fat) containing either melatonin (100 mg/kg of rat body weight) or the corresponding amount of microcrystalline cellulose (control treatment) daily via gavage. Both, melatonin and microcrystalline cellulose, were obtained from Sigma-Aldrich (St. Louis, MO, USA) as powder. Seven days after CRLM induction or sham procedure, first abdominal microCT scan and heart US were performed in order to evaluate tumour size and heart function (both procedures are described below in the paragraphs “MicroCT Scanning Procedure and Analysis” and “Heart US Examination and Analysis”, respectively). Right after this, CTx based on the FOLFOX (oxaliplatin, folinic acid and 5-fluoruracil) regimen was administered under a short duration of 2 % isoflurane anaesthesia. Firstly, 85 mg/m² oxaliplatin and 200 mg/m² folinic acid were administered intraperitoneally (i.p.). Five hours later, 1000 mg/m² 5-fluorouracil was administered via another i.p. injection. On the morning of the following day, 200 mg/m² folinic acid and 1000 mg/m² 5-fluorouracil were administered via i.p. injection. For animals in sham and experimental groups without CTx, CTx drugs were replaced with an equivalent amount of 0.9 % sodium chloride solution (control treatment). Detailed CTx protocol is presented in Figure 2. Later, after another four days of treatment (food diet and further daily gavage) and observation, a second abdominal microCT scan and heart US were performed followed by animal sacrification via terminal blood collection and tissue sampling for further investigation. Food intake and body weight were all monitored on a regular basis during the research period.

Table 2. Study groups.

Surgery	Sham				CRLM							
CTx	None				None				FOLFOX			
Daily gavage	Cellulose		Melatonin		Cellulose		Melatonin		Cellulose		Melatonin	
Diet	Casein	Glycine	Casein	Glycine	Casein	Glycine	Casein	Glycine	Casein	Glycine	Casein	Glycine
n =	10	10	10	10	15	15	15	15	20	20	20	20

CRLM: colorectal liver metastases; CTx: chemotherapy.

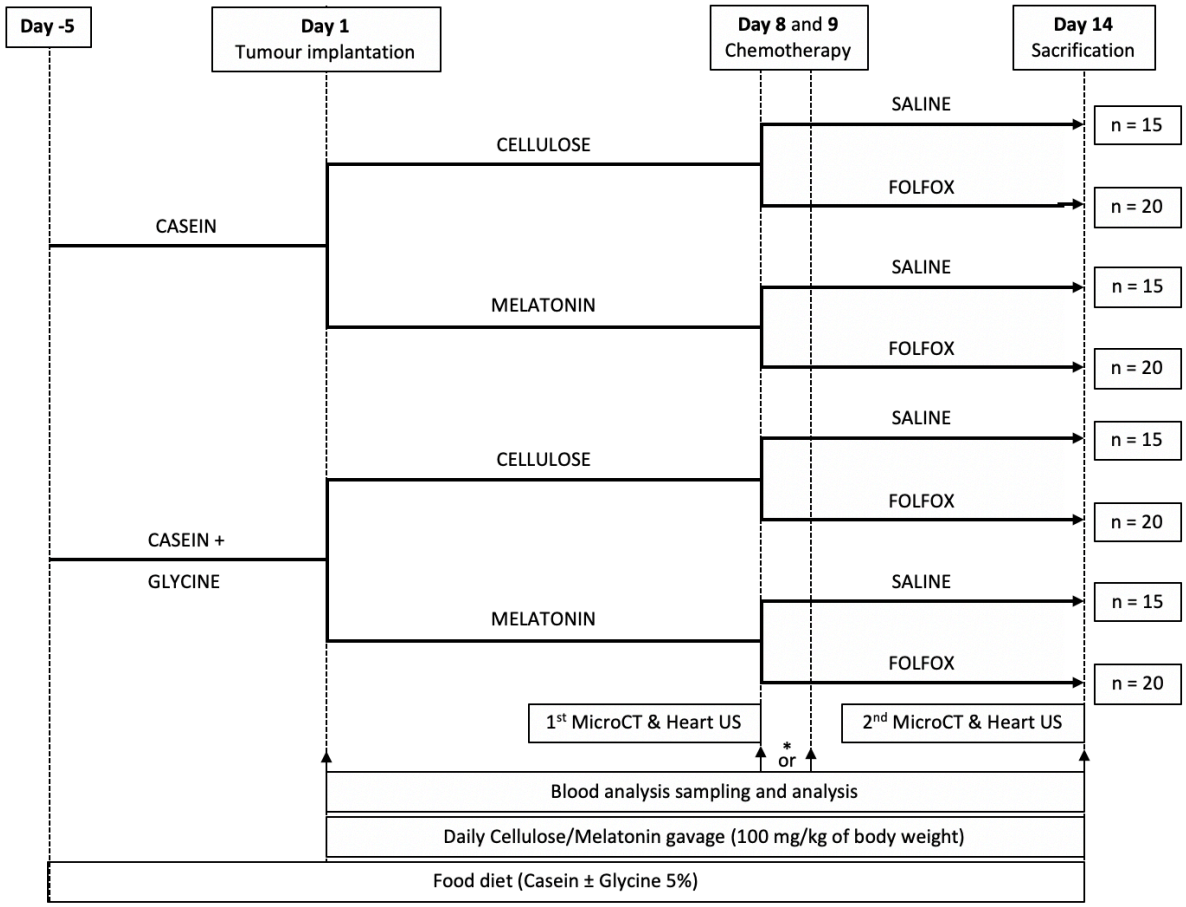

Figure 1. Experimental design. **In half of the animals, blood samples were collected before first CTx injection (on experimental day 8) while in another half after last CTx injection (on experimental day 9). Sham groups are not presented in this figure. CT: computed tomography; US: ultrasound.*

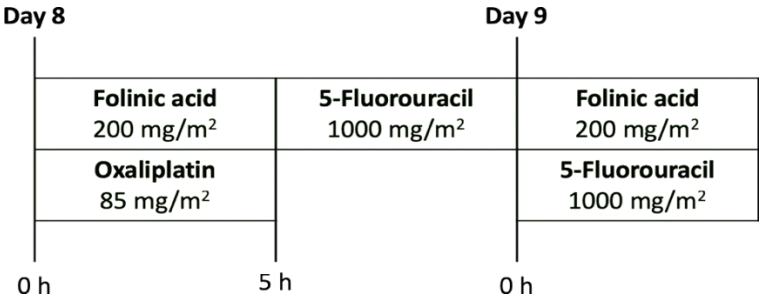

Figure 2. Adapted protocol of FOLFOX CTx for administration via i.p. injection.

2.2. Animal Care

Wistar rats were acquired from Janvier Labs (Le Genest-Saint-Isle, France) and allowed to acclimatize at least six days until the first intervention (food diet switch). Rats were housed in groups up to four per cage under a temperature-regulated environment (22 ± 1 °C) with 12/12 h light/dark cycle and unlimited, *ad libitum,* access to fresh water and chow (Figure 3). This study was conducted according to the 3Rs and the guidelines issued by the Federation of European Laboratory Animal Science Associations (FELASA). The approval by Austrian Federal Ministry of Education, Science and Research was obtained for this study (BMWF-66.010/0154-V/3b/2019, 17 September 2019).

Figure 3. Rats housing conditions and environment.

2.3. CC-531 Cell Line Maintenance

The rat colon adenocarcinoma cell line CC-531 (Cell Lines Service, Eppelheim, Germany) was used for CRLM induction. Cells were cultured at 37 °C under a 5 % CO_2 atmosphere. RPMI 1640 growth medium (GE Healthcare Life Sciences, Logan, UT, USA) supplemented with 10 % heat inactivated foetal bovine serum, 2 % L-glutamine, 1 % penicillin–streptomycin, and 25 mM HEPES buffer was used. The medium was renewed every other day, and cells were passaged once they grew to approximately 95 % confluence. On the day of CRLM induction, a suspension of 5 × 10^7 CC-531 cells/mL of phosphate-buffered saline (PBS) was prepared and stored at room temperature until use.

2.4. CRLM Induction and Sham Surgery

CRLM was induced and sham surgery was performed under general anaesthesia by 2 % 2 L/min isoflurane inhalation and single intramuscular injection of fentanyl (5 µg/kg). During

intervention, rats were kept in a supine position on an automatically regulated heating pad to maintain normothermia (Figure 4A). The surgical site was shaved using a clipper blade and skin was disinfected before incision. A small horizontal laparotomy (about 1 cm) was performed approximately 0.5 cm below the edge of the right ribs in order to expose the median liver lobe (Figure 4B). Then, the edge of liver lobe was gently lifted and 100 µL of the previously prepared cell suspension (containing 5×10^6 CC-531 cells) were injected directly under the liver capsule using a 26 G needle. Appearance of a whitish protrusion in the injection site was the sign for successful inoculation (Figure 4C). To stop bleeding and prevent potential backflow/leakage of cancer cells in the abdomen, gentle compression with a sterile swab was applied. Laparotomy was closed by a single nod double-layer (muscular and subcutaneous). Additionally, a small layer of tissue adhesive was applied for skin closure (Figure 4D). Immediately after wound closure, a mixture of 4 mg/kg carprofen and 0.02 mg/kg buprenorphine was injected subcutaneously to maintain adequate analgesia during the early postoperative period. Moreover, for the following five days, drinking water was supplemented with 2.5 mg/100 mL tramadol. After opioid withdrawal, rats were observed regularly to make sure there were no signs of chronic pain.

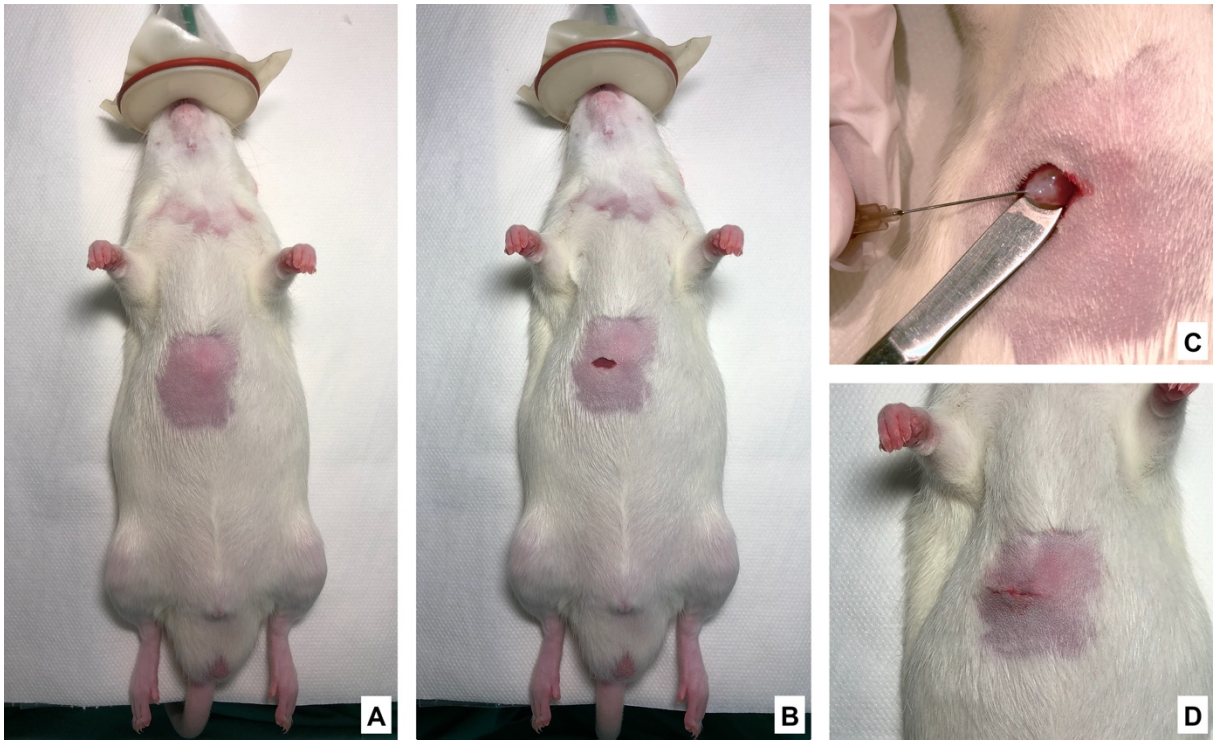

Figure 4. CRLM induction and sham surgery. *(A) Prepared surgical site; (B) Small laparotomy in the right upper quadrant; (C) Subcapsular injection of tumour cells into liver; (D) Closed incision. Reproduced from reference (101).*

Sham surgery (control) was carried out identically as described above, except the suspension of cancer cells was replaced with 100 µL sterile PBS.

2.5. MicroCT Scanning Procedure and Analysis

The day prior to the first microCT scan, 800 µL contrast medium ExiTron Nano 12,000 (Viscover, nanoPET Pharma GmbH, Berlin, Germany) was injected in a tail vein under 2 % isoflurane anaesthesia to better visualize hypoattenuating lesions (CRLM). The time of the contrast agent delivery was selected based on our previous research (101, 121). This single injection of the contrast medium was sufficient to ensure good tumour visibility for the entire duration of the study. The first abdominal microCT scan was performed seven days after CRLM induction on experimental day 8 and repeated at the end of the study on experimental day 14. A Siemens Inveon microCT scanner (Siemens Medical Solutions, Ann Arbor, MI, USA) was used to obtain images with the following settings: 0.5 mm filter at 70 kV X-ray voltage, 500 µA anode current, and 800 ms exposure time. The scanning procedure was performed under 1.5 – 2 % isoflurane anaesthesia with the rats in a prone position (Figure 5). The whole scanning procedure took approximately 23 min. Raw data were reconstructed using a modified Feldkamp filtered back-projection in Siemens Inveon Acquisition Workplace software version 2.1.272 (Siemens Medical Solutions, Ann Arbor, MI, USA). The reconstructed datasets were converted to DICOM format using Siemens Inveon Research Workplace software version 4.2 (Siemens Medical Solutions, Ann Arbor, MI, USA) for further analysis described below.

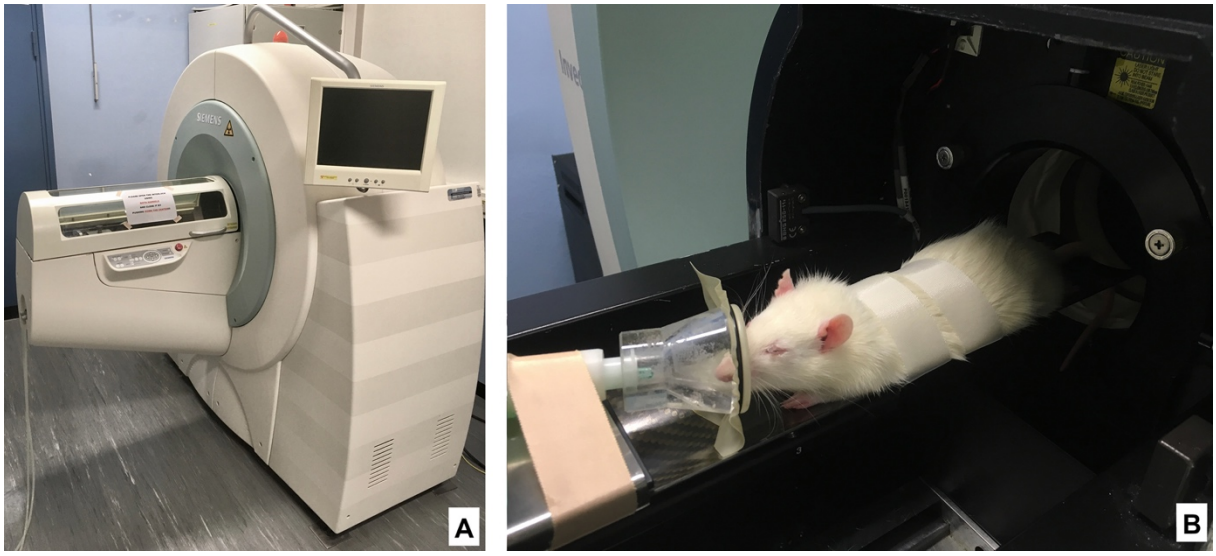

Figure 5. MicroCT scanning procedure. *(A) MicroCT scanner; (B) Procedure was performed under inhalation anaesthesia while rat was in a prone position.*

Mimics Research software version 21.0.0.406 (Materialise NV, Leuven, Belgium) was used to analyse microCT images. The volume (mm^3) of the contrast-enhanced liver and unenhanced

tumour were measured by creating a mask with predefined upper and lower threshold ranges (-650 HU and -247 HU for the liver and -806 HU and -609 HU for the tumour, respectively). The mask was then split into separate masks (liver and tumour) by manually marking each of the regions in at least 12 representative views in the transverse plane, and a 3D model was created using the "Calculate Part" function (Figure 6). Change in tumour volume was expressed in % and calculated as tumour volume (mm^3) on experimental day 14 x 100 % / tumour volume (mm^3) on experimental day 8.

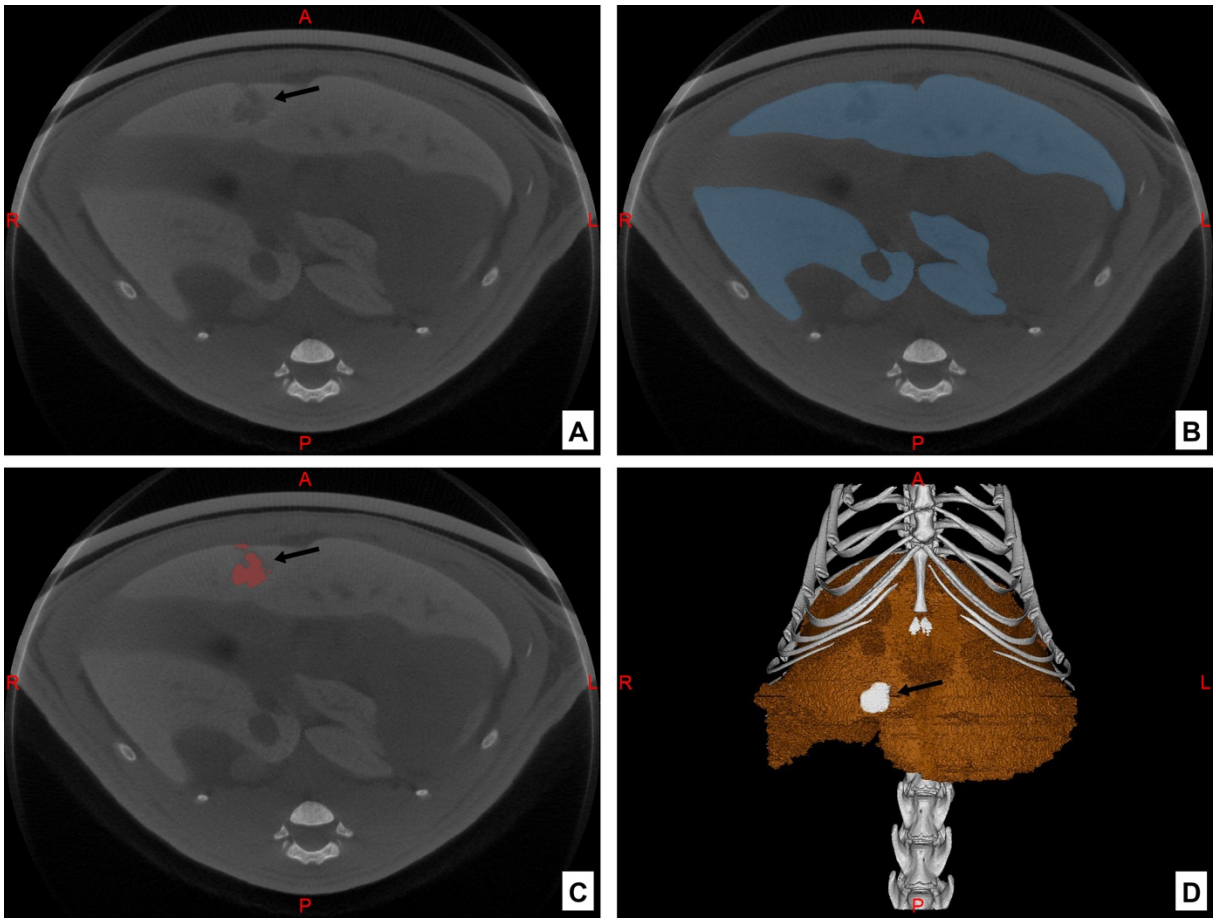

Figure 6. MicroCT analysis. *(A) Prepared surgical site; (B) Liver region including tumour was manually marked (blue) in one representative CT image to create the mask; (C) The marked tumour region (red); (D) 3D model with liver (brown) and tumour (white). Orientation: A – anterior; P – posterior; R – right; L – left.*

2.6. Heart US Examination and Analysis

Heart US was performed using a Vevo 3100 Imaging System (FUJIFILM VisualSonics, Toronto, Canada) together with a MX250 (13-24 MHz, axial resolution: 75 µm) ultra-high frequency linear array transducer, specifically designed for small animals. Immediately after microCT scan, rats were transferred and secured in a supine position on the heated platform

(Vevo Imaging Station, FUJIFILM VisualSonics, Toronto, Canada). Anaesthesia was maintained throughout the US examination via inhalation of 1 – 2 % isoflurane in 100 % oxygen to obtain constant and comparable heart rates (350 – 450 bpm). Before US examination all hair was removed from the ventral thorax using a shaver and depilatory cream to prevent hair-based artefacts (Figure 7). After depilation, pre-warmed US gel was applied on the chest of the rat. The left ventricle (LV) was imaged using B-Mode and M-Mode from parasternal long axis (PLAX) and parasternal short axis (PSAX) view. B-Mode and M-Mode images of LV in PLAX view were taken in its maximum dimension from apex to base. In PSAX view, B-Mode images were acquired at the level of mitral valve (base), papillary muscles and apex, while M-Mode images only at the level of papillary muscles. At least 5 consecutive cardiac cycles were sampled for further analysis.

US data analysis was performed in a blinded fashion by one examiner. Percentage changes in LV ejection fraction (LVEF) were calculated and recorded using Vevo® LAB desktop software version 32.0 (FUJIFILM VisualSonics, Toronto, Canada). Measurement of LV dimensions in systole and diastole was performed on PSAX view images and LVEF was calculated using the cardiac package. Change in LVEF (%) was expressed as LVEF (%) after CTx (at experimental day 14) x 100 % / LVEF (%) before CTx (at experimental day 8).

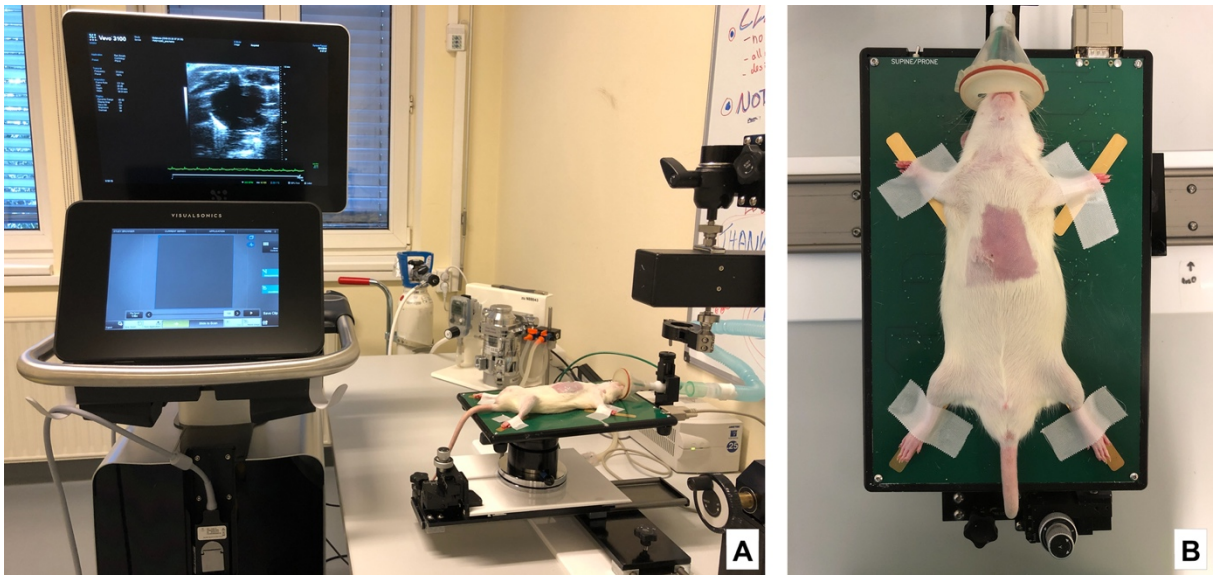

Figure 7. Heart US examination. *(A) US machine; (B) Examination was performed under inhalation anaesthesia while rat was in a supine position on the warmed platform.*

2.7. Blood Samples and Analysis

At experimental day 1 (before CRLM induction) and 8 (before CTx) or 9 (after CTx), blood samples were collected from the subclavian vein under 2 % isoflurane inhalation anaesthesia.

After the last abdominal microCT scan and heart US examination (on experimental day 14), all rats were euthanized by intramuscular injection of a xylazine (20 mg/kg) and ketamine (100 mg/kg) mixture, immediately followed by terminal blood collection from the inferior vena cava. Various blood parameters were analysed including standard blood test and biochemical analyses of levels of albumin, blood urea nitrogen, creatine kinase, aspartate and alanine aminotransferase, high-density lipoprotein, lactate dehydrogenase, total bilirubin, total cholesterol, triglyceride and total protein.

A V-Sight Analyzer (A. Menarini Pharma GmbH, Vienna, Austria) was used to measure complete blood count immediately (up to maximum 4 h) after collection, while biochemical blood analyses were performed later from prepared serum samples stored at −80 °C with SPOTCHEM Analyser (A. Menarini Pharma GmbH, Vienna, Austria) and respective SPOTCHEM reagent strips (A. Menarini Pharma GmbH, Vienna, Austria) according to the manufacturer's instructions.

Terminal blood was centrifuged at 1970xg at 4 °C for 10 min, plasma and serum samples were collected and subsequently stored at −80 °C. Determination of glycine levels in serum at the end of the experiment (on experimental day 14) was performed in the routine hospital laboratory.

2.8. Immunohistochemical Staining and Analysis

Tissue samples including liver and tumour were collected after sacrification (Figure 8), fixed in 4 % formalin and prepared for immunohistochemistry (IHC) according to the standard laboratory procedure. Paraffin-embedded tissue was thin sectioned at 3 µm. For staining with anti-Ki67 antibodies (Thermo Fisher Scientific, Waltham, MA, USA; dilution 1:200, rabbit IgG Clone SP6) was performed to evaluate the tumour proliferation index and with anti-CD31 antibodies (Abcam, Cambridge, UK; dilution 1:2000) to evaluate MVD in the tumour. The UltraVision LP Detection System HRP Polymer (Thermo Fisher Scientific, Waltham, MA, USA) in combination with DAB Chromogen (Dako, Via Real Carpinteria, CA, USA) and counterstained with haematoxylin was used. For positive controls, rat intestinal and heart tissue was used for Ki67 and CD31, respectively, while for negative controls, primary antibodies were omitted.

The slides were scanned and analysed using the QuPath software version 0.2.0-m5 (Belfast, Northern Ireland) (124) by a single examiner. For Ki67 analysis, the number of positive cells was automatically counted and expressed as the percentage of stained cells of total nuclei. Single endothelial cells or a cluster of cells positive for CD31 were considered as a vessel, and tumour microvascular density (MVD) was expressed as number of vessels / (analysed tumour area (mm^2) x 10^{-4}).

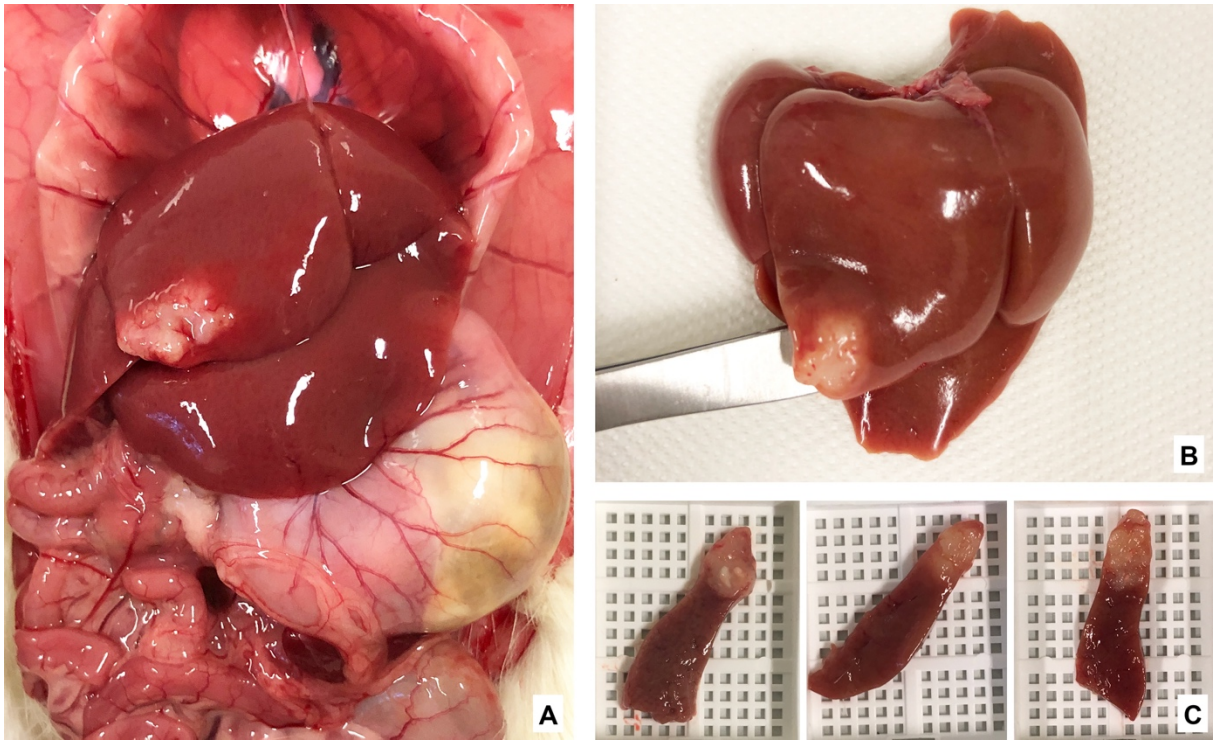

Figure 8. Collection of tissue samples (liver including tumour) for IHC. *(A) General view of abdominal cavity; (B) Removed livers with tumour (CRLM) in the median lobe; (C) Thick slices of liver and tumour prepared for fixation in 4 % formalin. Reproduced from reference (101).*

2.9. Statistical Analyses

Statistical analyses were performed using SPSS software version 23.0 (IBM Corp., Armonk, NY, USA). The data are presented as median and quartiles (Q1; Q3). Non-parametric tests, including Mann-Whitney U test (for independent samples) and Wilcoxon signed-rank test (for paired samples), were used to analyse statistical difference between groups and over time, respectively. A p value < 0.05 was considered as statistically significant.

3. Results

Some parts of the results are reported in Kvietkauskas *et al*., 2021 (101).

3.1. Final Sample Size

One hundred sixty-four (91.1 %) rats underwent the procedure as planned: one (0.6 %) animal dropped out prematurely because of problems with anaesthesia during CRLM induction procedure, two (1.1 %) animals related to contrast medium injection, one (0.6 %) was prematurely euthanized during the first 24 h after tumour implantation, and 12 (6.7 %) were prematurely euthanized due to poor general condition after CTx.

3.2. General Data

The mean body weight of rats not receiving CTx (sham and CRLM) increased about 1.46-fold from 231 (216; 255.5) g at the beginning of the study to 338 (315.5; 352.5) g at the end ($p < 0.001$), while application of FOLFOX CTx drastically reduced this increase to about 1.13-fold ($p < 0.001$) from 232.3 (209.6; 261.3) g to 261.1 (235.7; 285.4) g. Although the average daily food intake was similar in groups without CTx (sham and CRLM), melatonin supplementation significantly reduced the body weight gain compared to the control (133.8 (128.4; 137.3) vs. 153.5 % (144.3; 157.8), $p < 0.001$) and glycine (139.2 % (135.1; 145.8), $p = 0.03$) in sham groups, and compared to the control (131.7 (128.7; 143.1) vs. 146.6 % (144.7; 156.1), $p < 0.001$) and melatonin + glycine (155.6% (142.4; 162.1), $p = 0.002$) in the CRLM groups without CTx (Figure 9). Moreover, glycine supplementation resulted in a reduction in body weight gain in the sham cohort ($p = 0.01$), but not in CRLM without CTx (145.3 % (135.5; 148.9), $p = 0.055$) when compared to controls. FOLFOX CTx significantly reduced body weight gain ($p < 0.001$) during the study period being 109.7 % (104.2; 121.3) in control, 112 % (107.6; 116.5) in melatonin, 108.6 % (100.5; 118.4) in glycine, and 106.1 % (97.7; 112.2) in melatonin + glycine group. This reduced body weight gain in CTx groups was associated with about 4-fold decreased average food intake per day (22.1 ± 2.2 vs. 5.4 ± 0.7 g, $p < 0.001$). The presence of the tumour itself did not affect body weight gain, except for the CRLM combined treatment group without CTx, where it was increased (131.5 (124.9; 134.1) vs. 155.6 % (142.4; 162.1), $p < 0.001$).

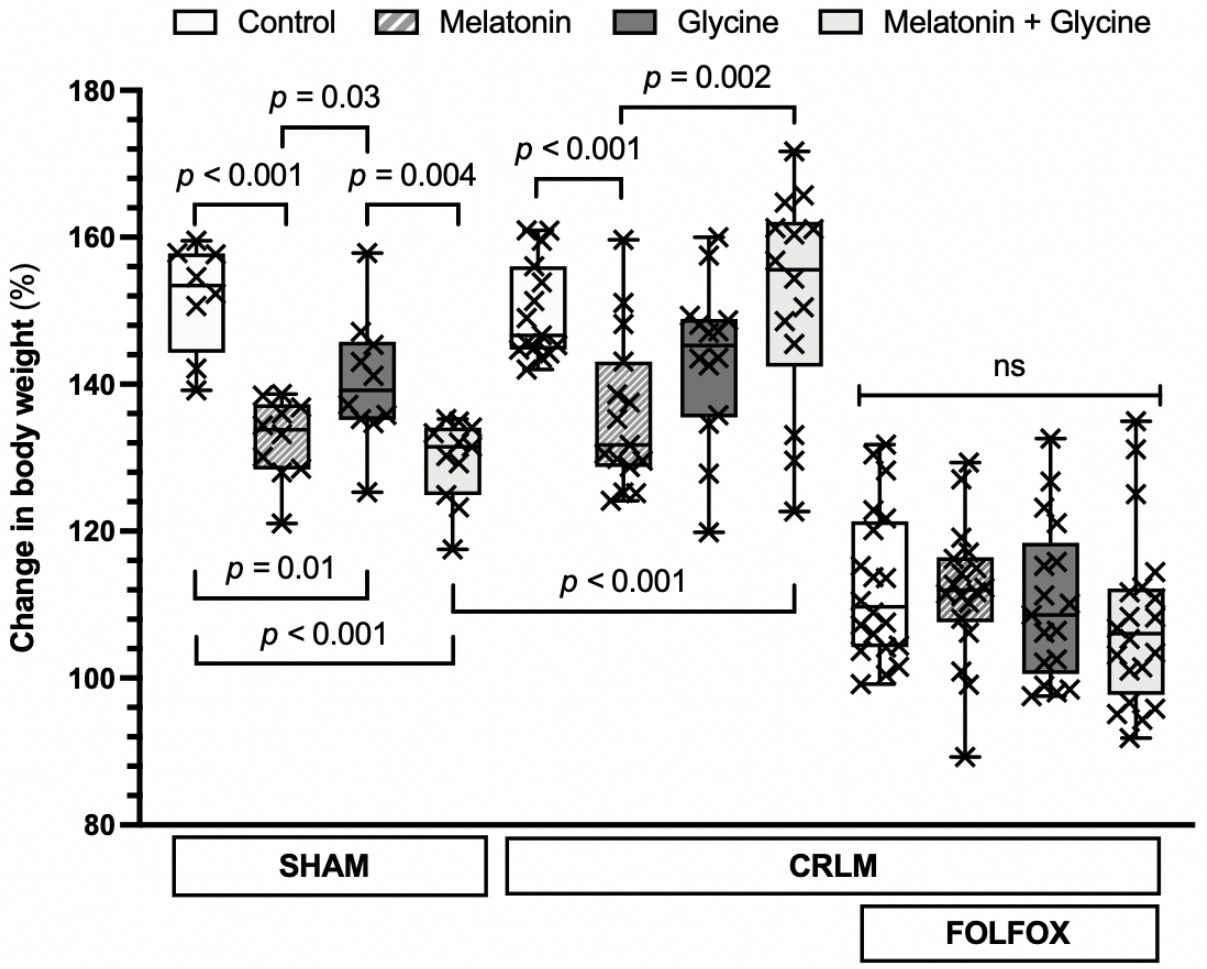

Figure 9. Change in body weight at the end of experiment in study groups. *Change in body weight was expressed as ratio of body weight (g) at the end and beginning of the study x 100 % in study groups. Data presented as median and interquartile range. Each cross mark represents individual case. CRLM; colorectal cancer liver metastasis.*

3.3. Blood Test

Supplementation with melatonin alone and in combination with glycine was associated with an increased leukocyte count at the end of experiment (p = 0.022 and p = 0.002, respectively) in sham groups (Figure 10 and Table 3). CRLM without CTx rats treated with melatonin alone had a significantly higher number of leukocytes compared to the control (p = 0.012) and glycine (p = 0.041) groups, while supplementation with melatonin and glycine did not reach significance (p = 0.111). This increase in leukocytes was associated with an increased number of lymphocytes in all groups (Figure 11 and Table 3). FOLFOX CTx significantly induced severe leukopenia in all rats independently from treatment (p < 0.001). The presence of the tumour itself did not affect leukocyte and lymphocyte counts. Only CRLM without CTx rats supplemented with melatonin had significantly higher numbers of lymphocytes compared to

the corresponding sham group (p = 0.041), while the combined supplementation of melatonin and glycine did not reach significance (p = 0.082).

In general, a glycine-enriched diet increased the median serum glycine concentration by 8.1-fold (186.6 (160; 212.8) vs. 1506.6 (1339.2; 1676.2) μmol/L, p < 0.001) at the end of experiment as compared to the control, casein diet, in groups without CTx (sham and CRLM) (Figure 12 and Table 3). However, FOLFOX CTx significantly reduced this increase to only about 3.7-fold in respective groups (200.1 (158.3; 287) vs. 737.2 (537.3; 976.5) μmol/L, p < 0.001) due to decreased food intake. The presence of the tumour itself and supplementation with melatonin did not impact on glycine levels.

No other clinically significant results were found during data analysis of blood results. FOLFOX CTx did not induce hepatotoxicity measured by levels of liver enzymes.

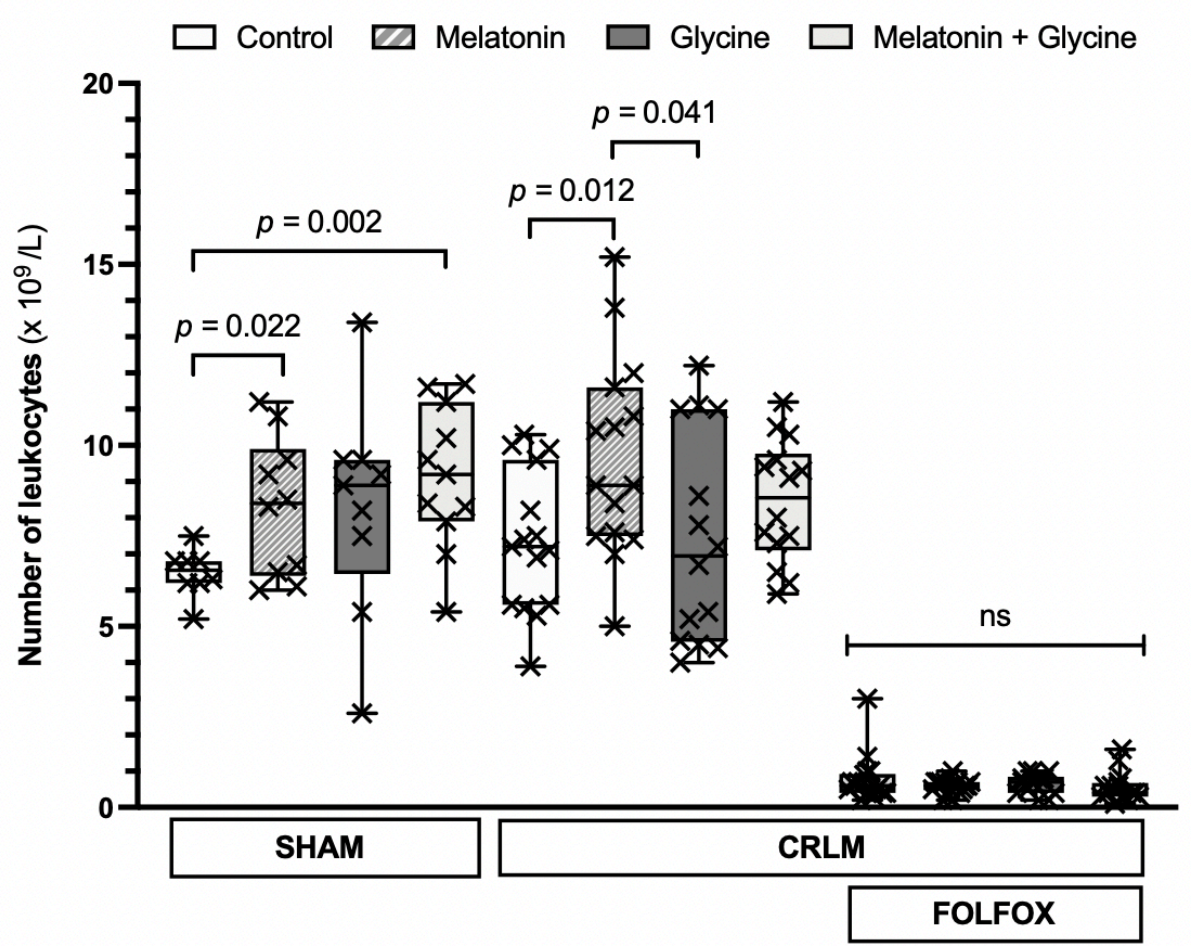

Figure 10. Change in number of leukocytes at the end of experiment in study groups. *Data presented as median and interquartile range. Each cross mark represents one individual case. CRLM; colorectal cancer liver metastasis.*

Table 3. Detailed data about leukocytes and lymphocytes count and glycine concentration in serum at the end of the study in study groups.

	SHAM				CRLM							
					without FOLFOX CTx				with FOLFOX CTx			
	Control	Melatonin	Glycine	Melatonin + Glycine	Control	Melatonin	Glycine	Melatonin + Glycine	Control	Melatonin	Glycine	Melatonin + Glycine
Leukocytes (x 10^9/L)	6.5 (6.2; 6.8)	8.4 (6.4; 9.9)	8.9 (6.5; 9.6)	9.2 (7.9; 11.2)	7.2 (5.6; 9.6)	8.9 (7.5; 11.6)	7 (4.6; 11)	8.6 (7.1; 9.8)	0.6 (0.4; 0.9)	0.6 (0.5; 0.7)	0.7 (0.4; 0.9)	0.4 (0.3; 0.7)
Lymphocytes (x 10^9/L)	3.5 (3.2; 3.8)	5.2 (4; 5.4)	5.1 (3.7; 6.9)	6.4 (5.1; 6.8)	4.1 (3.3; 5)	5.5 (5.1; 7.2)	3.4 (2.9; 6.2)	5.3 (4.5; 5.9)	0.1 (0; 0.7)	0 (0; 0.4)	0 (0; 0.3)	0.1 (0; 0.5)
Glycine (µmol/L)	161.1 (146; 247.2)	201 (171.8; 225.1)	1480.4 (1307.1; 1675.6)	1553.9 (1336.8; 1634.9)	171.1 (167; 210.7)	188.8 (162.6; 221.7)	1455.5 (1058.2; 1721.1)	1548.3 (1345.6; 1803.5)	232.6 (178.4; 315.1)	186.4 (147.7; 282.4)	830.6 (730.2; 1000)	580.9 (381.5; 740.1)

CRLM: colorectal liver metastases; CTx: chemotherapy.

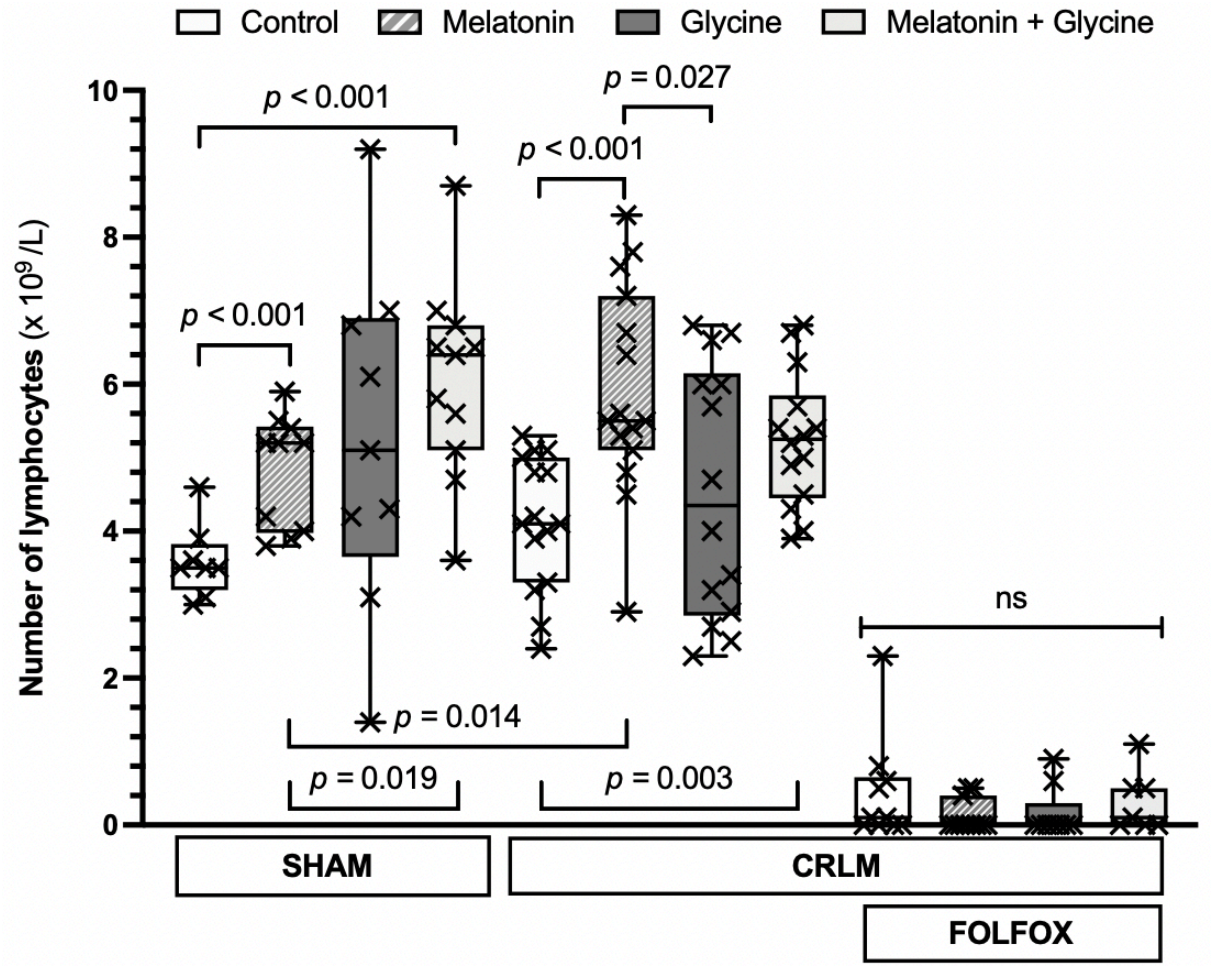

Figure 11. Change in number of lymphocytes at the end of experiment in study groups. *Data presented as median and interquartile range. Each cross mark represents one individual case. CRLM; colorectal cancer liver metastasis.*

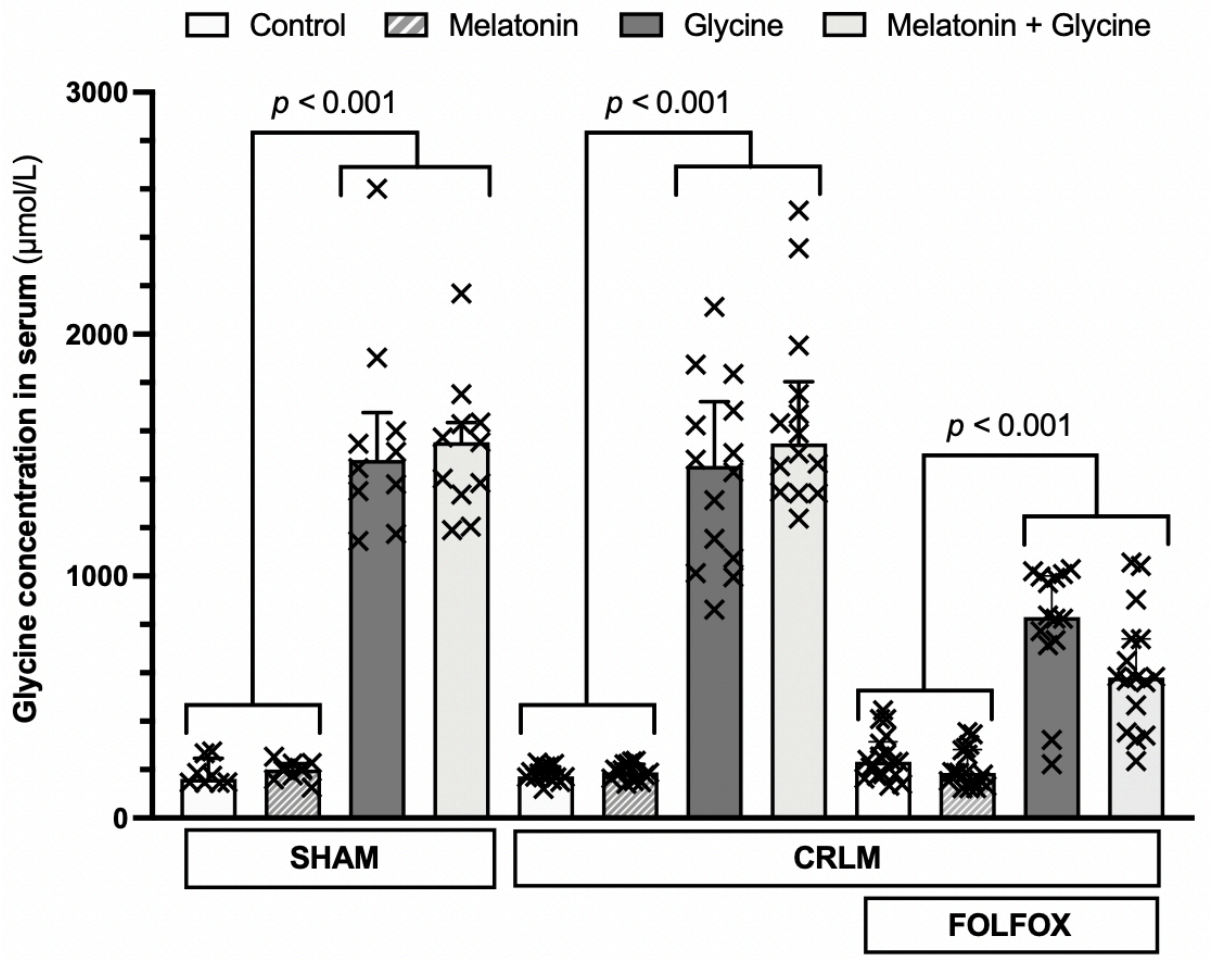

Figure 12. Change in glycine concentration in serum at the end of experiment in study groups. *Data presented as median and interquartile range. Each cross mark represents one individual case. CRLM; colorectal cancer liver metastasis.*

3.4. Tumour Volume

Tumour volume increased (tumour size at experimental day 8 vs. day 14) by 8.7 % (−17.5; 40.9) in the control group, while it decreased by 63.2 % (−3.1; 71.1) in melatonin, 43 % (−12.6; 70.1) in glycine, and 47.7 % (−116.9; 60.6) at combined supplementation with melatonin and glycine. Melatonin and glycine supplementation alone significantly reduced the tumour volume compared to the respective control group (p = 0.002 and p = 0.044, respectively). However, combined supplementation was found to be non-significant (Figure 13 and Table 4).

In CRLM groups receiving FOLFOX CTx, tumour volume increased independently from treatment (Figure 13 and Table 4). Change in tumour volume was found to be 143.1 % (100.5; 213.5) in control group, 141.1 % (112.3; 203.1) in melatonin, 159.6 % (119.7; 188.5) in glycine, and 147.8 % (31.5; 203.7) in melatonin + glycine group (p = 0.898).

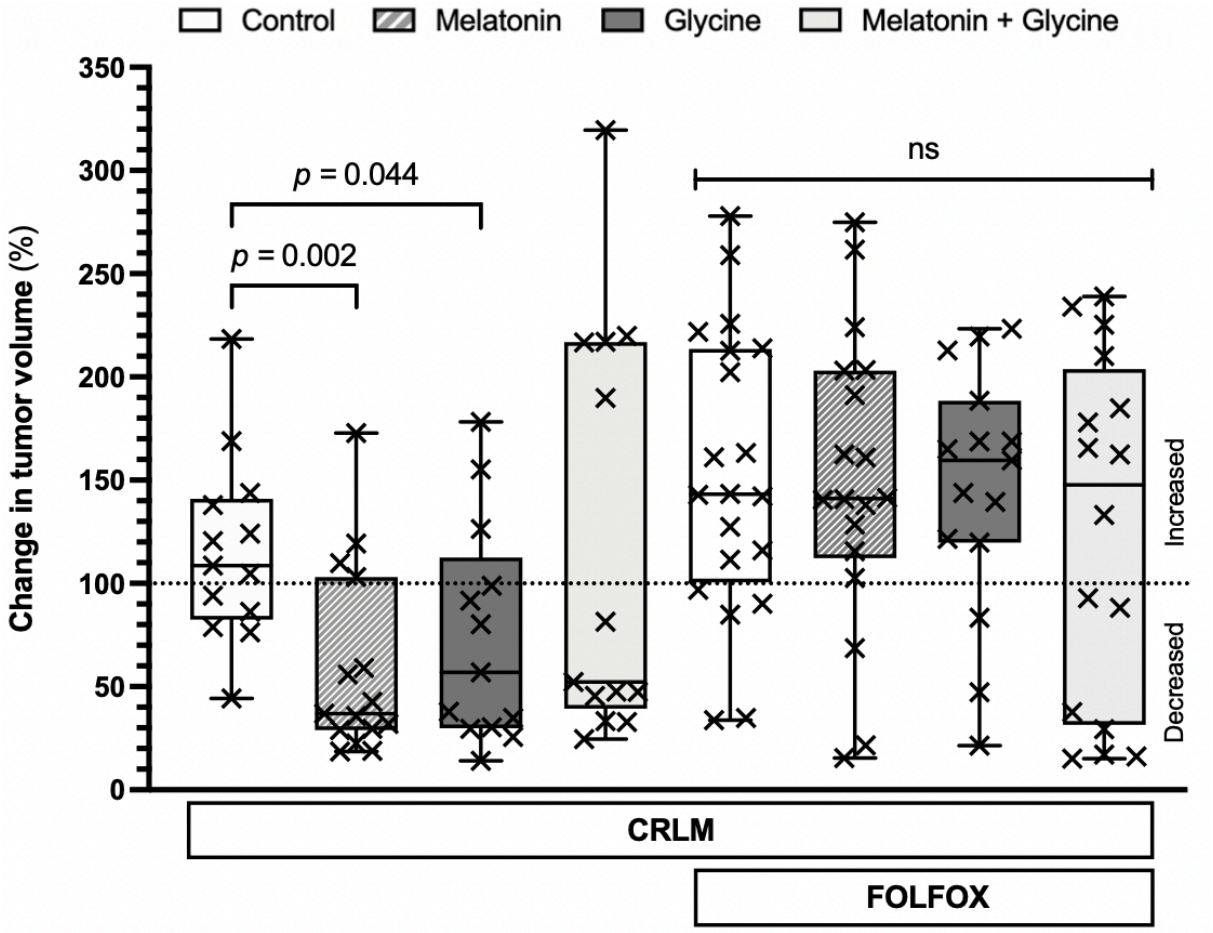

Figure 13. Change in tumour volume in study groups. *Change in tumour volume was expressed as tumour volume (mm^3) on day 14 / tumour volume (mm^3) on day 8 x 100 %. Data presented as median and interquartile range. Each cross mark represents one individual case. CRLM; colorectal cancer liver metastasis.*

3.5. Tumour MVD and Proliferation Index

Supplementation with melatonin, glycine, and their combination in CLRM without CTx revealed significantly lower MVD in tumour tissue compared to representative controls ($p < 0.001$, $p = 0.018$, and $p = 0.003$, respectively) (Table 4 and Figure 14). No difference between these three treatments ($p = 0.099$) could be found. This reduction was statistically insignificant in CRLM groups with FOLFOX CTx.

In CRLM without FOLFOX CTx groups, supplementation with melatonin and glycine significantly reduced tumour proliferation index compared to controls ($p = 0.005$ and $p = 0.044$, respectively), while their combination did not reduce it significantly (Table 4 and Figure 15). There was no difference when comparing the different supplementation regimens ($p = 0.535$). In this model, treatment with FOLFOX CTx was associated with higher tumour proliferation index independently from supplementation.

Table 4. Tumour characteristics in study groups.

	CRLM							
	without FOLFOX CTx				with FOLFOX CTx			
	Control	Melatonin	Glycine	Melatonin + Glycine	Control	Melatonin	Glycine	Melatonin + Glycine
Change in tumour volume (%)	108.7 (82.5; 140.9)	36.8 (28.9; 103.1)	56.9 (29.9; 112.6)	52.3 (39.4; 216.9)	143.1 (100.5; 213.5)	141.1 (112.3; 203.1)	159.6 (119.7; 188.5)	147.8 (31.5; 203.7)
Tumour MVD (number of vessels/area (mm^2) x 10^{-4})	3.2 (2.5; 4.7)	1.8 (1.2; 2.6)	2.1 (1.8; 3)	1.1 (0.7; 2.8)	3.3 (2.4; 3.9)	2.4 (2; 3.3)	2.8 (2.2; 3.4)	2.5 (1.9; 3.6)
Tumour proliferation index (%)	12.2 (10.7; 17.5)	7.5 (6.4; 11.3)	8.3 (7.2; 14.4)	10.1 (7.4; 13.4)	15.6 (9.4; 22.4)	14.4 (12; 18.2)	18.3 (12.2; 23.8)	15.9 (8.1; 23.6)

Change in tumour volume was expressed as tumour volume (mm^3) on day 14 / tumour volume (mm^3) on day 8 x 100 %. Data presented as median and quartiles (Q1; Q3). CRLM: colorectal liver metastases; CTx: chemotherapy; MVD: microvascular density.

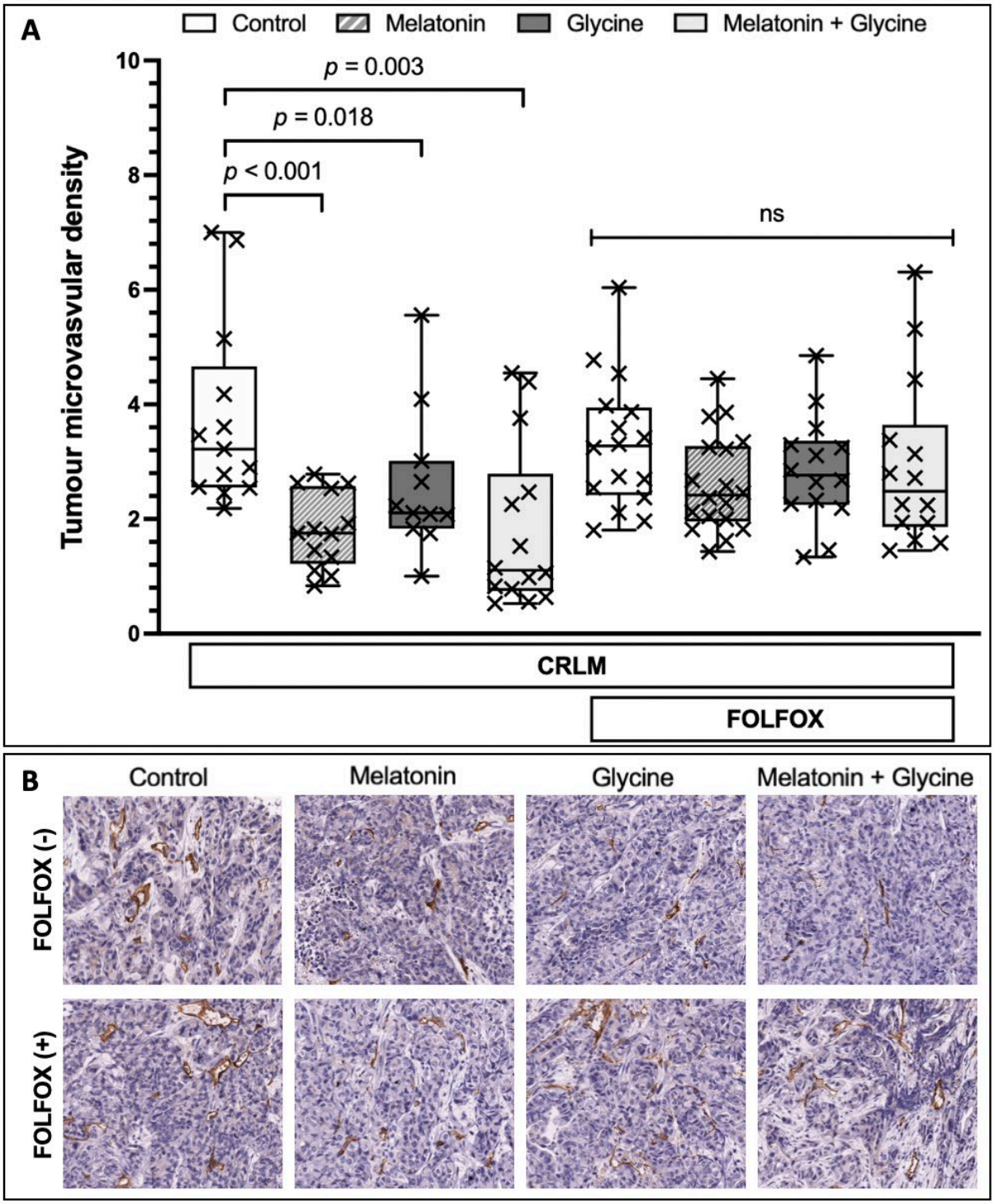

Figure 14. Tumour MVD in study groups. *Tumour MVD (A) was expressed as number of vessels and tumour area (mm^2) ratio x 10^{-4} based on IHC images of CD31 (B). Data presented as median and interquartile range. Each cross mark represents one individual case. CRLM: colorectal liver metastases.*

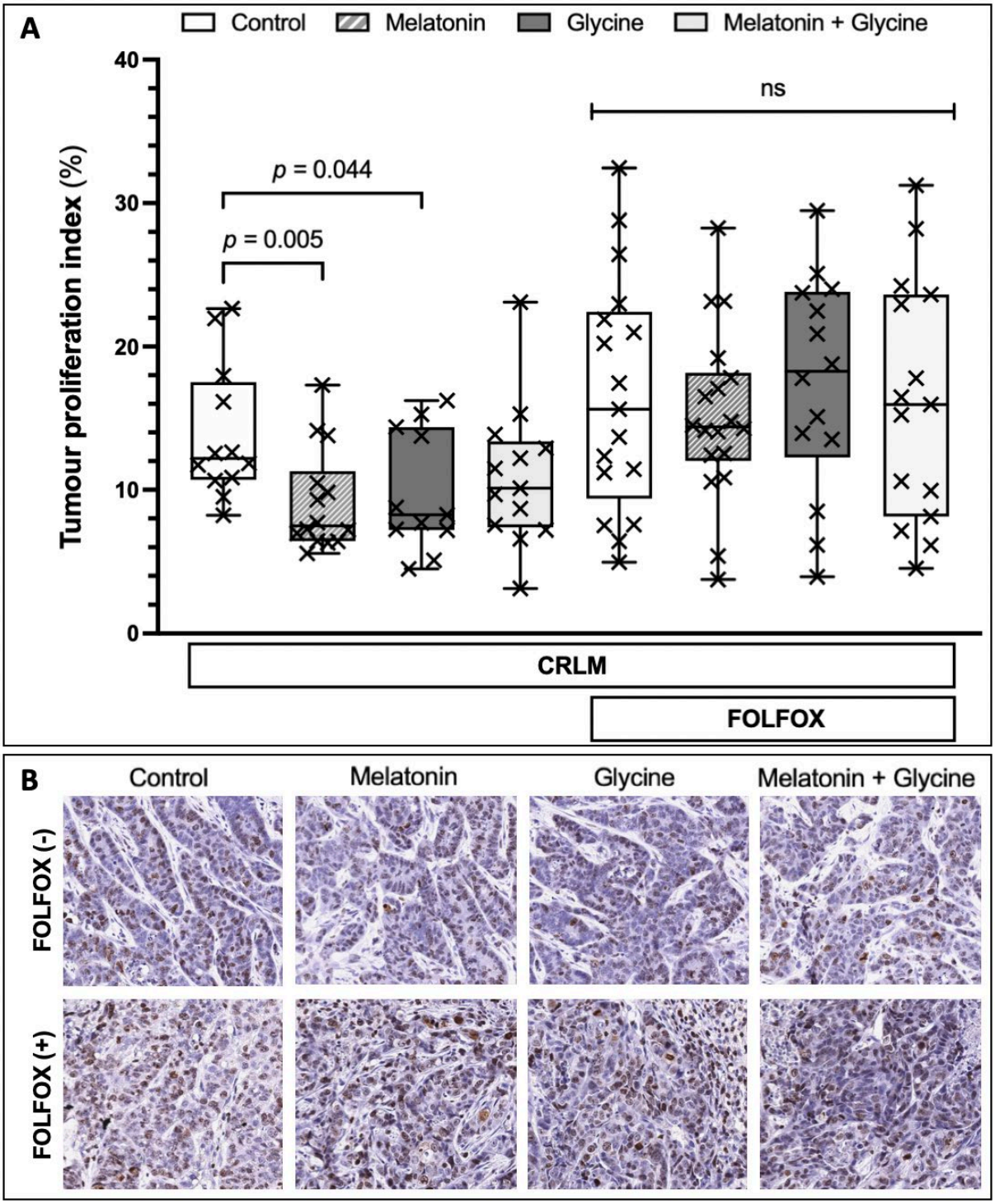

Figure 15. Tumour proliferation index in study groups. *Tumour proliferation index in study groups (A) based on IHC images of Ki67 (B). Data presented as median and interquartile range. Each cross mark represents one individual case. CRLM: colorectal liver metastases.*

3.6. CRLM without CTx: Melatonin + Glycine (Subgroup Analysis)

Additional subgroup analyses of CRLM without FOLFOX CTx combined supplementation group (melatonin + glycine) were performed between those animals with increased (n = 5) and decreased tumour volumes (n = 8) (Figure 13). These two subgroups had more than 4-fold difference in the median change in tumour volume (216.8 (47.6; 219.9) vs. 46.4 % (32.9; 51.1), $p < 0.001$); although tumour volume at day 8 (92.5 (58.5; 213.3) vs. 55.4 (30.2; 216.5) mm^3, $p = 0.699$) and glycine concentration in serum at the end of the study (1506.6 (1344.5; 1793) vs. 1527.4 (1369.8; 2183.4) µmol/L, $p = 0.539$) were similar. A significant increase in number of platelets before tumour implantation (1088 (900; 1208) vs. 625.5 (258; 942.8) × 10^9/L, $p = 0.019$) was found in the cases of tumour enlargement. Moreover, animals from this subgroup had a higher tumour MVD (3.8 (2.4; 4.5) vs. 0.8 (0.6; 1.1) number of vessels/area (mm^2) x 10^{-4}, $p < 0.001$) and proliferation index (14.6 (13.1; 21.2) vs. 8.7 % (6.9; 10.8), $p < 0.001$).

3.7. Heart Function

Baseline values of the median LVEF measured before FOLFOX CTx (at experimental day 8) in study groups were non-significantly different and varied between 81.5 % and 84.8 % (Table 5). There was no difference in change of LVEF (at experimental day 8 vs. day 14) in study groups without CTx (sham and CRLM) (Figure 15 and Table 5). It was 102.4 % (95.1; 105.1) in sham control, 98.6 % (94.2; 106.7) in sham melatonin, 99.8 % (94.3; 101.7) in sham glycine, and 101.1 % (95.9; 104.6) in melatonin + glycine group. The presence of the tumour itself and different supplementation did not affect LVEF. Change in LVEF was 102.4 % (94.4; 105.4), 101.9 % (94.4; 103.9), 97.2 % (94.4; 102.3) and 99.2 % (96.3; 102.9) in respective CRLM groups without CTx.

After FOLFOX CTx application, LVEF significantly decreased by 9.5 % (5.6; 14.9) in the control group, by only 3.8 % (0.8; 9) in melatonin, 0.8 % (-3.6; 5.1) in glycine, and 1.1 % (-1.1; 10.2) in the combined treatment with melatonin and glycine group (Figure 15). Melatonin and glycine supplementation alone and their combination significantly prevented from the decrease of LVEF induced by FOLFOX CTx ($p < 0.001$, $p = 0.013$, and $p = 0.023$, respectively). There was no statistical difference between these three treatments ($p = 0.168$).

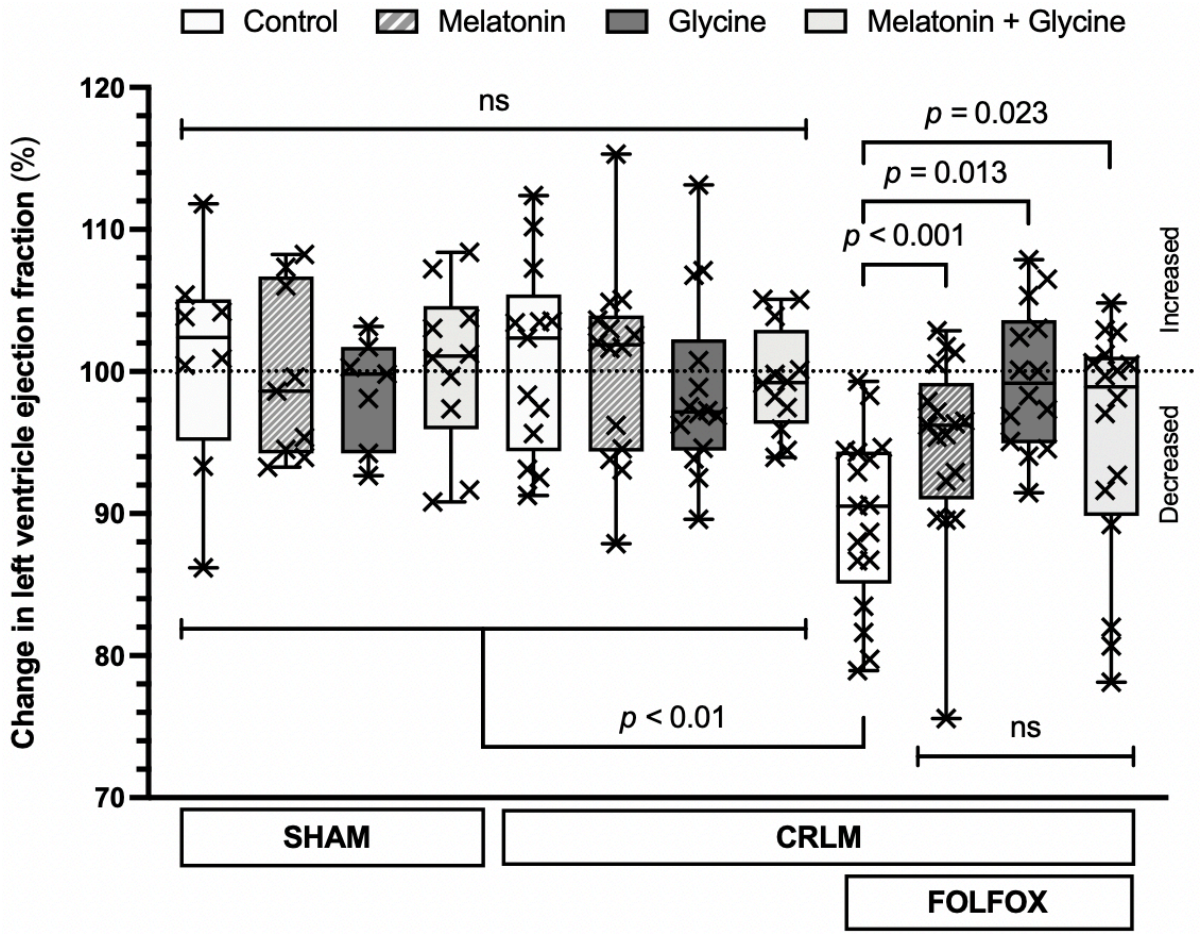

Figure 16. Change in LVEF in study groups. *Change in LVEF was expressed as LVEF (%) on day 14 / LVEF (%) on day 8 x 100 %. Data presented as median and interquartile range. Each cross mark represents one individual case. CRLM; colorectal cancer liver metastasis.*

Table 5. Heart function in study groups.

	SHAM				CRLM							
					without FOLFOX CTx				with FOLFOX CTx			
	Control	Melatonin	Glycine	Melatonin + Glycine	Control	Melatonin	Glycine	Melatonin + Glycine	Control	Melatonin	Glycine	Melatonin + Glycine
LVEF at experimental day 8 (%)	82.2 (75.8; 86.5)	82.8 (80; 84.7)	83 (81.9; 84.6)	84.7 (78.4; 87.9)	83.3 (80.2; 85.5)	82.9 (79.5; 89.1)	81.8 (79.4; 85.1)	81.5 (77.1; 86.3)	83.7 (81.2; 87.4)	84.3 (77.9; 87)	83.6 (81; 85.8)	84.8 (79.6; 87.7)
LVEF at experimental day 14 (%)	83.4 (79.5; 84.7)	84.2 (77.1; 87.6)	83 (79.1; 85.8)	83.9 (80.5; 85.7)	81.7 (79.2; 85.1)	81.8 (79.7; 88.3)	82.2 (77.7; 87.6)	82.5 (79.5; 85.7)	75.7 (69.6; 82.3)	79.5 (75; 82.5)	81.7 (80.2; 86.2)	79.3 (70.7; 84.2)
Change in LVEF (%)	102.4 (95.1; 105.1)	98.6 (94.2; 106.7)	99.8 (94.3; 101.7)	101.1 (95.9; 104.6)	102.4 (94.4; 105.4)	101.9 (94.4; 103.9)	97.2 (94.4; 102.3)	99.2 (96.3; 102.9)	90.5 (85.1; 94.4)	96.2 (91; 99.2)	99.2 (94.9; 103.6)	98.9 (89.8; 101)

Change in LVEF was expressed as LVEF (%) on day 14 / LVEF (%) on day 8 x 100 %. Data presented as median and quartiles (Q1; Q3).

CRLM: colorectal liver metastases; CTx: chemotherapy; LVEF: left ventricle ejection fraction.

4. Discussion

The idea that melatonin and glycine given separately have antineoplastic potential is not new and has been previously proven in different types of cancer, including CRC (98, 121). However, the combined treatment with these two natural and nontoxic substances, and treatment with melatonin alone was not yet analysed in the model of CRLM. Hence, in this study, antiangiogenic and antiproliferative effects, of melatonin alone and in combination with glycine were evaluated in an experimental rat model of CRLM. Moreover, the effect of melatonin and glycine supplementation on the effectiveness of a standard regimen of CTx for CRLM treatment, FOLFOX, was assessed, and negative influence of FOLFOX CTx on heart function and potential protective effects of melatonin alone and when combined with glycine was demonstrated for the first time.

4.1. The Effect of Melatonin on CRC

4.2.1. Literature Review

There is sufficient evidence that the use of melatonin may be beneficial in preventing the formation and progression of cancer. Several cohort studies have previously indicated that working at night may have a higher risk of developing cancer, including CRC (125, 126). Night-shift workers experience the disturbance of the circadian rhythm by prolonged exposure to residential light, leading to disruption of the diurnal production of melatonin (127). Since melatonin secretion increases soon after the onset of darkness, this finding supports the idea that ambient light inhibits melatonin generation, hence promoting cancer. In terms of reducing the incidence of cancer development, one of the mechanisms may be the potency of melatonin to mitigate severe DNA injury caused by unstable oxygen and nitrogen-containing reactants, either free radicals or related metabolites (128). Moreover, the process of DNA repair can be accelerated by melatonin, but the efficiency of this system is less than perfect and the residual injured DNA may eventually mutate and proceed to oncogenesis (128). The molecular junk created by toxic reactants, over a life-time, may be a major cause of cancer in the elderly Melatonin increases the ability for DNA repair, most likely via altering genes involved in DNA damage response pathways (129).

Once tumours are formed, melatonin exerts its anticancer properties in a variety of distinct ways, such as apoptosis, proliferation, angiogenesis, autophagy, endoplasmic reticulum stress, and oxidative stress (130). In many preclinical studies of cancer development, melatonin significantly inhibited cancerogenesis despite intentional exposure to multiple carcinogenic substances (127). Despite these encouraging results, melatonin is not often used in cancer therapy in humans.

4.2.1.1. *In Vitro* Studies

A significant amount of *in vitro* studies with different cell lines have demonstrated that melatonin has indisputable anticancer effects on CRC. Early experiments of Winczyk and Pawlikowski *et al.* with Colon 38 cells suggested that melatonin receptors are not required for melatonin to exert its oncostatic effect, and hence that alternative pathways, such as nuclear signaling and receptor-independent processes, may be implicated as well (131-133). Melatonin activates cell death programs (apoptosis) through histone deacetylase 4 (HDAC4) nuclear import mediated by CaMKIIα inactivation (134), the type 1 sodium/calcium exchanger and type 1 inositol trisphosphate (IP3) receptor (135), and via superoxide-mediated stress of endoplasmic reticulum by inhibiting cellular prion protein (PrP^C) expression (136). Melatonin inhibits migration by downregulating Rho-associated protein kinase expression through interference with p38/MAPK in RKO human rectal cancer cells (137, 138). Moreover, it is able to supress angiogenesis by inhibiting HIF-1α stabilization under the lack of oxygen (139), by down-regulation of endothelin-1 expression and production in CRC cells through the inactivation of FoxO-1 and NF-κB (140).

4.2.1.2. *In Vivo* Studies

However, only a limited number of these *in vitro* findings were approved *in vivo* (131, 133, 141). Additionally, *in vivo* experiments suggested that anticarcinogenic effects of melatonin are associated with elevated numbers of CD8+ lymphocytes (Fas-positive T cells) (142) and an initial response to pericryptal colonic stroma alterations, specifically cell clusters of CD68+ and CD133+ (143). In a murine model, melatonin administration reduced the progression of colitis-associated colon carcinogenesis by modulating cell-death mechanisms through Beclin-1 expression, light chain (LC)3B-II/LC3B-I ratio and p62 (144).

4.2.1.3. Clinical Studies

Currently, there are several clinical studies evaluating the effects of melatonin on CRC. The first was published by Lissoni *et al.* in 1987 (145). In this study, 19 patients diagnosed with advanced solid tumours, including CRC (four patients), unresponsive to conventional treatment, were treated with melatonin administered via intramuscular injection (20 mg per day) for two months, followed by further therapy with lower melatonin doses (10 to 18 mg per day) given orally or intramuscularly in case of disease remission or at least stabilization, or an improved score of performance status. Treatment with melatonin improved not only the overall performance status but also the quality of life in 6 of 10 (60 %) patients with a performance status, evaluated by using Karnofsky's scale, higher than 20 before beginning the treatment. This early preliminary study suggested that melatonin may be of some value in treating cancer

patients in whom standard anticancer therapies have failed, especially by improvements in their performance status score and quality of life (145). However, in the following study published by Barni *et al.* in 1990, the findings revealed a lack of anticancer properties for melatonin in patients with metastatic CRC not responding to standard anticancer therapy, such as 5-FU (146). This study included only 14 patients and melatonin was administered in a similar manner, intramuscular injection (20 mg per day) for 2 months followed by oral intake (10 mg per day). A significant enhancement in performance status was found in only 36 % (5 out of 14) of patients (146).

In general, it seems that adequate number of results, *in vitro* and *in vivo*, supports the involvement of melatonin in the formation of CRC and further disease progression by various different pathways. However, there is lack of clinical data from randomised controlled trials, and the optimal administration route and dosage for CRC therapy remains the topic of debates and further studies.

4.2.2. Our Evidence

In our CRLM model, the significant reduction of tumour growth, by 63 %, was observed in a short period of time after supplementation with melatonin. This could be attributed to its immunomodulatory capabilities, while treatment with melatonin significantly increased the count of leukocytes, specifically lymphocytes, and there is evidence supporting the idea that white blood cells are critical in the host's immune response to malignancy (101). Perforin and granzymes are the effector molecules used by cytotoxic lymphocytes (cytotoxic T cells and natural killer cells) to eliminate neoplastic cells during cancer immunosurveillance and immunotherapy, due to their ability to overcome several mutations involved in the process of apoptosis, such as p53 deletion/mutation, up- and down-regulation of Bcl-2 family members, and caspase suppression. Hence, in this case, the initiation of apoptosis is not necessary for cytotoxic lymphocytes-mediated target cell death (147).

In addition, data of this study suggested antiangiogenic properties for melatonin, since melatonin significantly reduced tumour vascularization resulting in reduction of tumour growth. The molecular mechanism remains unclear. However, melatonin has previously documented ability to affect HIF-1α and several genes it regulates, including VEGF, the most significant growth factor involved in cancer neovascularisation leading to its progression (101, 148-150). Moreover, melatonin also inhibits angiogenesis by reducing endothelin-1, important CRC survival factor, production and release in tumour cells by supressing FoxO-1 and NF-κβ activation, affecting formation and progression of CRC (101, 140, 151).

More extensive studies of VEGF and endothelin-1 expression in cancer tissue would assist in confirming our findings that melatonin inhibits CRLM development by acting as a natural antiangiogenic molecule.

4.3. The Synergistic Effect of Melatonin and Anticancer Drugs in CRC Treatment

4.3.1. Literature Review

Currently, data on the synergistic effects of melatonin in synergy with other CTx agents on CRC lacks confirmation *in vivo,* while the majority of studies were conducted *in vitro.*

4.3.1.1. *In Vitro* Studies

Nevertheless, it seems that standard treatment supplementation with melatonin improved the individual cytotoxic effects of different anticancer therapeutics, such as doxorubicin, irinotecan, cisplatin, 5-FU, and oxaliplatin on various types of CRC cells, as well as those resistant to drugs (13, 152-161). The most important pathways limiting proliferation of cancer cells and tumour-mediated formation of new blood vessels include (98): 1) downregulation of PrP^C (13, 157); 2) apoptosis induction through sequential regulation of cytochrome c/caspase, matrix metallopeptidase 9 (MMP9)/ cyclooxygenase 2 (COX-2), and p300/ NF-κB signalling pathways (161); 3) inhibition of PI3K/ Akt and NF-κB/ iNOS signalling pathways (153).

4.3.1.2. *In Vivo* Studies

To date, there are only few studies evaluating the synergistic potential of melatonin in combination with other anticancer agents in CRC treatment *in vivo* (162, 163). Previously, Melen-Mucha *et al.* studied the effect of melatonin and somatostatin analogue, octreotide, on cancer cell proliferation and cell death via apoptosis in the model of induced Colon 38 cancer in mice (162). In this experimental study, both substances were administered by direct subcutaneous injection for six days at a daily dose of 10 μg/animal. Interestingly, regardless of the fact that both melatonin and octreotide applied individually exert antiproliferative and proapoptotic effects and decrease the ratio of proliferation and apoptosis on the examined tumours, the joint treatment with these therapeutics did not show additive properties (162). Authors assumed that melatonin and octreotide may act through the common intracellular mechanism, for instance using the same pool of G proteins. More recently, Bakalova *et al.* demonstrated the potential anticancer features of melatonin in synergy with SN38/EF24, active irinotecan form, in a model of CRC-grafted mice (163). In this study, Colon 26 cells were inoculated subcutaneously in one hind-paw to trigger a development of colon cancer. Seven days post tumour induction, mice underwent treatment with SN38/EF24 ± melatonin (10 mg/kg of body weight) via daily subcutaneous injection under the tumour for 22 days. This treatment

combination diminished tumour volume three times by up-regulating oncogenic and down-regulating onco-suppressive reactive oxygen species in tumour tissue (163). However, the low number of animals per group (only 3 animals/group) limits the explanatory power of this study.

4.3.1.3. Clinical Studies

Primary synergistic anticancer effect of melatonin and interleukin-2 (IL-2) has been presented in a study of 35 individuals with advanced tumours, including CRC (14 patients), gastric cancer, HCC and pancreas adenocarcinoma (164). Seven days before IL-2 application (6 days/week), melatonin was administered for four consecutive weeks at 50 mg/day, corresponding to a single cycle of immunotherapy. The overall rate of response to treatment was 23 % (8 out of 35) suggesting this supplementation as a novel promising treatment modality for advanced gastrointestinal malignancies (164). Later a similar study demonstrated that cancer patients tolerate well the therapy composed of a small dose of IL-2 plus 40 mg/day of melatonin, preventing surgery-induced lymphocytopenia, since a reduction of lymphocyte count greater than 30 % occurred in 80 % (8 out of 10) control patients, while only in 10 % (1 out of 10) using IL-2 and melatonin (165). In 2002, the results of two large clinical trials were presented by Lissoni (166). In an initial clinical study, the effect of melatonin was demonstrated in a large cohort consisting of 1,440 patients with highly advanced tumours (279 patients with CRC), who were randomly admitted to a group of supportive care alone or supportive care + melatonin. In another study, the impact of melatonin on the effectiveness and cytotoxicity of CTx in a group of 200 patients with CTx-resistant metastatic tumours (51 patients with CRC), who were randomized to receive CTx (5-FU and FA or raltitrexed) alone or CTx plus melatonin (166). In both these studies, melatonin was administered orally at a dose of 20 mg/day. Cachexia, asthenia, thrombocytopenia, and leukopenia were considerably less common in melatonin-treated patients than in supportive care-only patients. Moreover, melatonin-supplemented patients had significantly higher disease control and 1-year survival rates. Furthermore, melatonin diminished CTx-induced several adverse effects (stomatitis, cardiotoxicity, neurotoxicity, asthenia and thrombocytopenia) (166). More recently, Cerea *et al.* looked at the effect of melatonin supplementation on irinotecan therapeutic efficacy in advanced (metastatic) CRC patients who had progressed after at least one CTx line including 5-FU (167). A total of 30 individuals, after randomisation, received oral melatonin at a daily dose of 20 mg for 9 consecutive weeks. The control of disease was more often obtained with the combined therapy of irinotecan and melatonin than with irinotecan alone, 85.7% vs. 43.8%, respectively (167). More recently, a randomized study was conducted to establish the impact of a concomitant melatonin (20 mg/day) administration on effectiveness and cytotoxicity of several CTx combinations in 370 metastatic cancer patients (168). In this study, one third of the patients

had CRC (122 individuals) and were treated with oxaliplatin + 5-FU, or weekly irinotecan or 5-FU and FA. Patients who received CTx with melatonin had a significantly higher tumour regression rate than those who received CTx alone. Moreover, the higher 2-year survival rate was achieved in patients supplemented with melatonin (168).

In summary, these clinical investigations suggest that the pineal hormone melatonin may be effectively used in oncology to provide supportive treatment for patients with incurable advanced CRC and to minimize CTx-induced damage. Moreover, current evidence suggests that melatonin should be applied in therapeutic dosages rather than its physiological ones, which do not provide enough protection for cells against CTx toxicity (146).

4.3.2. Our Evidence

In our model, CTx alone and combined treatment with melatonin did not reduce tumour volume. Controversially, an unexpected tumour volume increase after CTx administration was observed. This could be related to severe general condition and immunosuppression after CTx. The average body weight was significantly reduced after FOLFOX administration and this reduction was not influenced by treatment with melatonin. After FOLFOX CTx, the average food and water intake per day was drastically reduced independent from treatment. Moreover, CTx induced severe leukopenia in all animals leading to immunosuppression and initial tumour progression. The FOLFOX administration protocol and drug doses were adopted from previous experiments from our colleagues (120, 121), except that we used a different but genetically similar strain of rats (Wistar instead of WAG/Rij) which was probably more sensitive to CTx. Furthermore, only one cycle of FOLFOX and short observation period post CTx are probably not enough to induce and observe tumour cell death. A higher number of FOLFOX CTx cycles was not feasible due to its adverse effects mentioned above (animals suffered from severe leukopenia, substantial bodyweight loss, and poor overall health). Moreover, there may be inadequate melatonin uptake caused by malabsorption associated with intestinal mucositis after therapy with FOLFOX, which was reported previously (121).

4.4. Organoprotective and Other Properties of Melatonin

Although chemotherapeutic agents increased survival of oncological patients, they have serious side effects including neuropathy, haematological and organ toxicity, especially cardiological, as well as skin necrosis, gastrointestinal symptoms (nausea and vomiting), and others. The formation of free oxygen radicals has a role in the negative effects of these medicines, at least to a certain extent. The usage of melatonin as a supplementary substance may provide significant protection and prevent against the side effects of multiple CTx agents (169, 170). Interesting results were found after we analysed heart function. After FOLFOX

application LVEF significantly decreased by about 10 % in the control group whereas therapy with melatonin significantly preserved from the decrease of heart function. This corresponds to literature data. A recent meta-analysis found that administering melatonin to adults as a heart-protective drug had a beneficial impact on LVEF by ameliorating cardiac function (171). Melatonin is a potent anti-inflammatory and antioxidant agent with a broad spectrum of therapeutic features contributing to this cardioprotective effect. We failed to demonstrate other protective properties of melatonin against haematological toxicity and hepatotoxicity of CTx, although we analysed various blood tests including liver enzymes. However, these were secondary objectives and this CRLM model was mainly designed to analyse antiangiogenic effects of melatonin and glycine. Only one cycle of FOLFOX is probably not enough to induce significant liver damage and short observation period post CTx is not enough to observe haematoprotective effects. A higher number of FOLFOX CTx cycles was not feasible due to its adverse effects mentioned before.

Furthermore, in this study we found that melatonin supplementation at large dose resulted in a modest reduction in body weight growth overtime without influencing the amount of daily food intake. This observation is consistent with the previously published evidence indicating the ability of melatonin to control metabolism and adipogenesis in mammalian vertebrates, including humans (172-174). Several different hypotheses have been proposed to explain this effect, including indirect pathways by alteration of gonadal steroid hormones, glucocorticoid and adipocytokines secretion, as well as by activation of the sympathetic nervous system acting directly on fat depots, controlling the storage and mobilisation of adipose tissue (101).

4.5. The Effect of Glycine on CRC

4.5.1. Literature Review

A growing number of evidence supports the hypothesis that glycine inhibits the growth of cancer cells, most likely through antiangiogenic properties (116). However, currently this was demonstrated only in a limited number of *in vitro* and *in vivo* experiments concerning CRC.

A glycine receptor-mediated counteractive action on endothelial cells after stimulation with both VEGF-conditioned and CRC-conditioned culture media was shown *in vitro*, in the study by Bruns *et al.* in 2016 (122). VEGF and conditioned media enhanced cancer cell growth, migration, and capillary development by 267 %, while glycine completely neutralized this effect and strychnine, an antagonist of glycine, completely blunted glycine's effect. Moreover, in the same study, they demonstrated that oral intake of glycine has a direct impact on tumour angiogenesis in a rodent model of CRC (122). After CRC induction in subcutaneous tissue of rats' right flank, tumour proliferation and vessel formation were analysed between those rats

who received a glycine-enriched diet (5 % glycine) and those who received a control diet without glycine for two consecutive weeks. Tumour size and weight, as well as vessel density reduced by 35 % ($p = 0.02$), 34 % ($p = 0.03$), and 55 % ($p = 0.04$) in glycine-supplemented animals (122).

More recently, Maneikyte *et al.* performed *in vitro* experiments with the rat colon adenocarcinoma cell line CC-531 and found that glycine had no impact on their viability at any tested levels ranging from 0.1 to 5 mM (121). Furthermore, they demonstrated that 5 % glycine food diet has no effect on tumour proliferation in CRLM rat model; however, glycine significantly suppressed tumour growth to about 40 % of controls with a 60 % reduced tumour vascularization (121).

Although current available data is very limited, it seems that glycine blunts VEGF-stimulated angiogenesis by inhibiting proliferation, transformation and migration of endothelial cells through glycine receptor-dependent mechanisms.

4.2.2. Our Evidence

This study confirmed the results of previously published experiments performed by our colleagues (121), in which dietary glycine reduces vascularization and thus significantly reduces tumour growth in a similar CRLM rat model, except we used different but genetically similar strain of rats (Wistar instead of WAG/Rij). We demonstrated that treatment with glycine alone significantly diminished tumour size by 43% in a short period of time, and this reduction was related to a decreased tumour vascularization and proliferation of cancer cells. This study brings additional evidence and encourages further investigation of glycine as a potential anticancer agent with antiangiogenic features.

4.6. The Synergistic Effect of Glycine and Anticancer Drugs in CRC Treatment

Currently, there is single study which had evaluated the synergistic effect of glycine with other anticancer drugs in CRC treatment. In 2019, Maneikyte *et al.* presented the antitumorigenic effect of dietary glycine combined with CTx, the one cycle of FOLFOX, *in vivo* model of CRLM (121). The results of this study demonstrated that glycine-enriched diet (5 % glycine) substantially decreases the growth of CRLM. First, they performed *in vitro* experiments with rat colon adenocarcinoma, CC-531 cells, and found that glycine had no influence on the viability of CC-531 cells at any concentration tested (0.1 to 5 mM), while both 5-FU and oxaliplatin were cytotoxic to CC-531. Combined treatment with glycine and 5-FU and/or oxaliplatin did not show additional effects on cell viability (121). Later, CRLM were induced by direct CC-531 cell injection under the liver capsule of WAG/Rij rats and treated with 5 % dietary glycine with or without CTx. Although treatment with glycine alone had no influence on tumour

cell proliferation, single cycle of FOLFOX reduced cell proliferation without reaching significance (121). It seems that glycine works synergistically with FOLFOX through distinct mechanisms and that does not counteract anticancer properties of CTx.
Clinical research is warranted to establish the clinically relevant value of glycine since it possesses promising antiangiogenic properties and is nontoxic in contrast to the standard CTx drugs used nowadays.

4.7. Organoprotective Properties of Glycine

The hepatotoxicity of neoadjuvant CTx for CRLM contributes to increased rates of perioperative morbidity and mortality. In 2011, Mikalauskas *et al.* established for the first time that glycine protects the liver against CTx-induced damage (112). It dramatically reduced transaminases up to 50 % of control levels after CTx. Additionally, phagocytosis of latex beads was diminished by about half, and adhesion of leukocytes in central and midzonal subacinar zones reduced to 60 – 80 % after glycine therapy. The activation of Kupffer cells and enhanced intrahepatic microperfusion are the most likely mechanisms (112). We were not able to induce significant liver injury, measured by liver enzymes analysed in blood serum, in our model of CRLM and FOLFOX CTx though the general condition of animals was significantly worse after CTx. Therefore, hepatoprotective effects of glycine were not investigated.
More recently, it was demonstrated, that dietary glycine prevents the heart function from FOLFOX-induced damage during CRLM therapy (120). In this study, FOLFOX CTx significantly decreased LVEF by 10 %, and glycine-enriched diet (5 % glycine) significantly preserved from this decrease of LVEF, as well as reduced cardiac fibrosis and cardiomyocyte apoptosis in a rat model. We were able to replicate these protective properties of glycine against cardiotoxicity of CTx. In our study, after FOLFOX application LVEF significantly decreased by about 10 % in control group while treatment with glycine significantly preserved from the decrease of heart function. Further investigation is warranted to explore underlying mechanisms.

4.8. Combined Treatment with Melatonin and Glycine for CRC

To our knowledge, the combination of melatonin and glycine was not yet investigated in any model. In our study, we observed that tumour volume declined, in association with reduced tumour vascularization and cell proliferation, in most rats when treated with melatonin and glycine in combination. However, in few rats an unanticipated significant higher tumour vascularization and cell proliferation was found resulting in significant tumour volume increase. As a possible cause of this unexpected observation, we were able to exclude several performance biases associated with the tumour induction procedure, including cell batch, since

information about all important steps was recorded in case report forms. Furthermore, tumour volume at the first microCT and serum levels of glycine at the end of the study were comparable in all cases treated with combination of melatonin and glycine. We could speculate that this discrepancy might be attributed to the effects on TNF-α of both these molecules. Glycine has been previously demonstrated the ability to diminish TNF-α, and thus resulting in reduction of proliferation of the cells (114, 115, 175). Conversely, melatonin is able to reduce the expression of TNF-α (176) which obviously might counteract the anticancer effects of glycine in small tumours used in our model (101). However, this does not explain tumour growth discrepancy between animals in this group.

Interesting results were found after we analysed different fractions of blood cells between animals treated with combination of melatonin and glycine. We observed that the number of platelets prior to tumour implantation procedure was significantly higher in the rats whose tumour volume increased compared to those for whom tumour volume decreased. In recent meta-analysis, thrombocytosis has been linked to the development and progression of CRC (177). Platelets seem to have a role in tumour growth through angiogenesis. Furthermore, number of platelets is associated with serum VEGF levels in oncological patients (178). We believe that, especially in this model, with angiogenesis playing an important role immediately after implantation of tumour cells, higher numbers of platelets might potentially release more proangiogenic factors and significantly more promote further tumour progression (101).

Moreover, we could not exclude biological variability of experimental animals and drug interaction as a possible explanation for this unexpected observation, since currently data concerning the combination of melatonin and glycine as supplementation in a cancer treatment or other setting are deficient, making further research mandatory.

4.9. Future Perspectives

Despite the encouraging results of preclinical studies, the use of melatonin and glycine in cancer treatment in clinical practice is currently limited. The effects of melatonin and glycine alone and in combination with other anticancer agents have been investigated in *in vitro* experiments, only in limited number of *in vivo* models, and in several clinical trials, exceptionally with advanced cancer patients. The preliminary results are promising; however, the best route of administration and dosage of both melatonin and glycine for CRC treatment is still unknown, and bioavailability of these molecules requires further research to truly clarify potential variations among individuals. Furthermore, more fundamental research with respect to determining the signalling mechanisms modulated by these substances in tumours needs to be pursued. Organoprotective properties are promising, and they could be used as supplements for standard CTx, especially in patients with additional comorbidities. Overall,

melatonin’s and glycine's low toxicity, multimodal activity, and remarkable efficacy encourage their usage in order to prevent and treat cancer. Clinical trials are warranted and should not only include advanced cancer patients.

4.10. Conclusions

This study demonstrated inhibitory properties of both melatonin and glycine alone, administered as food supplements, in the case of CRLM growth by acting as potential angiogenesis inhibitors with significant cancer cell proliferation reduction. Moreover, melatonin was found to exert immunomodulatory effect possibly influencing further tumour progression. Despite the fact that dietary melatonin and glycine given separately exert anticancer properties, the combination therapy of these natural and nontoxic molecules had no additional effects. Moreover, this study demonstrated beneficial properties of treatment with melatonin and glycine for protection against toxic effects on heart function of FOLFOX CTx. Further investigations are warranted to deepen the knowledge about underlying molecular mechanisms. Extensive literature review revealed that there is sufficient evidence that both melatonin and glycine are involved in carcinogenesis, development, and progression of cancer. As a result, more clinical studies are required to incorporate these molecules as novel therapeutic agents for CRLM therapy.

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