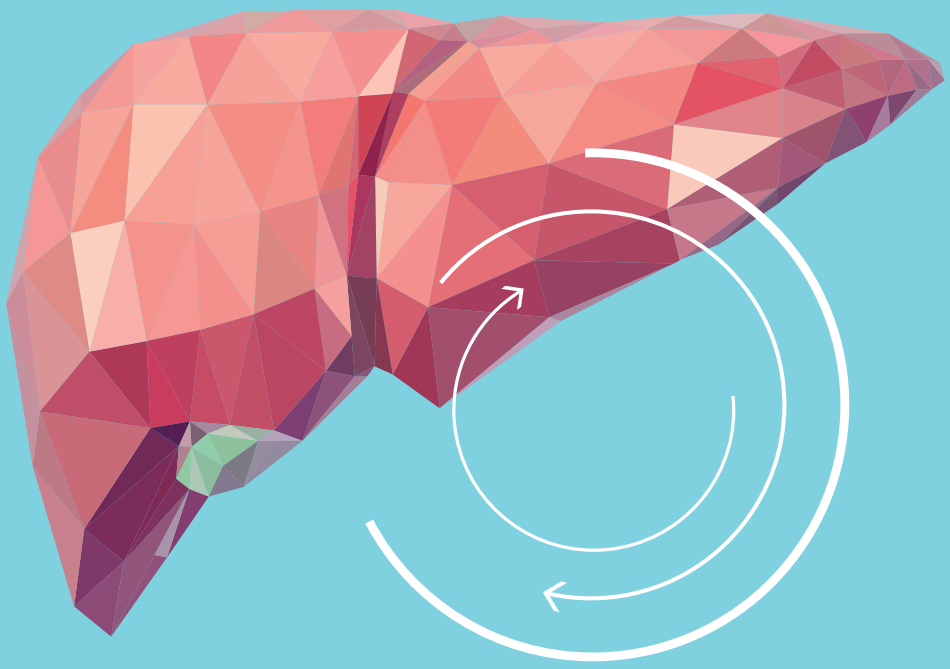

EXPLORING NEW WAYS TO IMPROVE TREATMENT OUTCOME OF COLORECTAL LIVER METASTASES

Sander A. Huisman

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Exploring New Ways to Improve Treatment Outcome of Colorectal Liver Metastases

Verkennen van nieuwe manieren om resultaten van behandeling van colorectale levermetastasen te verbeteren

Proefschrift

ter verkrijging van de graad van doctor aan de
Erasmus Universiteit Rotterdam
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Our lives are not in the lap of the gods, but in the lap of our clocks

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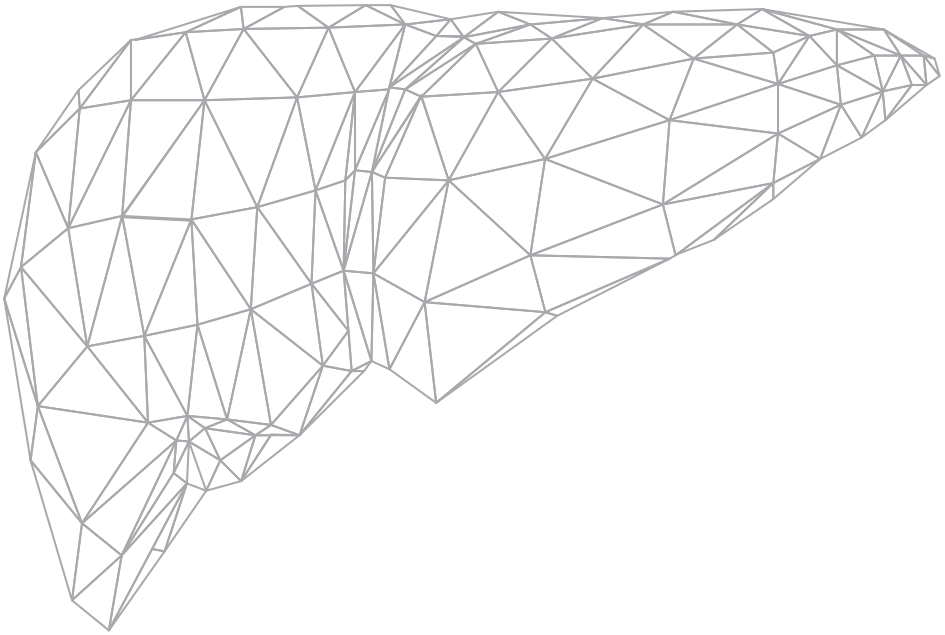
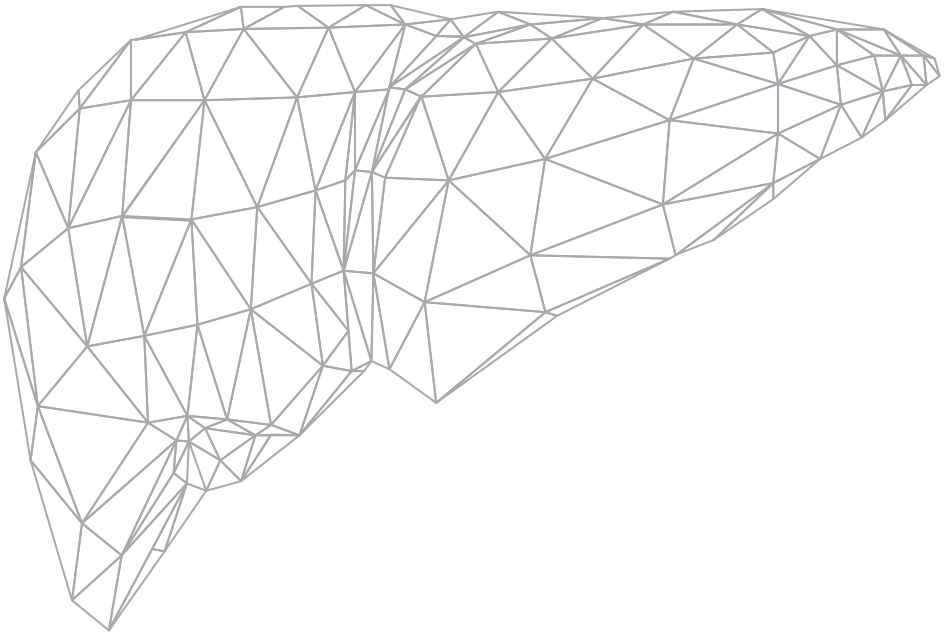


PART ONE

INTRODUCTION

Chapter 1 **Introduction and outline of this thesis**



CHAPTER 1

INTRODUCTION AND OUTLINE OF THIS THESIS

Exploring new ways to improve treatment outcome of colorectal liver metastases by studying the behavior of the biological clock in cancer cells and the effects of dietary restriction.

Dysregulation of the circadian clock in cancer cells

The biological functions of many organisms display a rhythm with a periodicity of approximately 24-hours, called the circadian rhythm. These rhythms are controlled by circadian clocks (1). Circadian clocks are present in almost all life forms on earth, ranging from single cellular organisms like bacteria and yeast to multicellular organisms like plants, animals and humans. In a simple way, the mammalian circadian clock is composed of three components: a master clock (an internal oscillator located in the neurons of the suprachiasmatic nuclei, SCN), an input (keeping the clock in phase with environmental cues, the light-dark cycle), and an output (coupling the circadian clock to different biological processes) (2, 3). Circadian (Latin for "about one day") rhythms generated by these clocks have a period of approximately 24-hours, and it is necessary that they are synchronized to the environment to meet the exact 24-hour light/dark cycle. The synchronization of clocks between body time and solar time is called entrainment of the clock and is adjusted every day by light (2). The SCN regulates peripheral clocks trough both the autonomic nervous system and neuroendocrine systems. Circadian clocks in the SCN neurons as well as in the peripheral clocks make use of the same set of clock genes (4).

The circadian clock regulates about 10-15% of the transcriptome and influences diverse functions as the rest-activity period, physiological processes, and cellular proliferation. Hence, the circadian timing system plays an important role in the development of cancer. Disruption of circadian rhythmicity, either genetic or functional may contribute to genomic instability and accelerate cellular proliferation, two conditions that favor carcinogenesis (5). Recent studies indicate that clock genes influence susceptibility to chemotherapeutic agents (6-8). Treatment with chemotherapy usually damages both malignant and healthy cells, leading to toxicity and adverse side-effects which is a major limitation to their use. Not only susceptibility to the efficacy of chemotherapeutic drugs, but also the adverse side-effects may vary over a 24-hours period (7, 9). Chronotherapy takes advantage of the fact that there are asynchronies in circadian rhythm between normal and cancer tissue, by administering chemotherapy when minimal side effects and maximal antitumor effect is anticipated (10). Although the clinical relevance of chronotherapy has been demonstrated in randomized multicenter trials, chronomodulated therapy is not yet part of daily clinical practice (11-13).

Dietary restriction induced stress resistance

Dietary restriction (DR) is a non-invasive intervention that prolongs lifespan in a variety of model organisms including worms, yeast, fruit flies, mice and rats (14-19). DR can be achieved by different regimens including caloric restriction (CR) and fasting. CR is defined as a reduction in food intake without causing malnutrition, while fasting is abstinence of food, with *ad libitum* access to water. The fact that DR increases lifespan indicates that it slows down the aging process. In 1956, Harman proposed the free radical theory of aging, which is based on the recognition that metabolic use of oxygen is a major source of free radicals (20). DR lowers steady state levels of oxidative stress, decreases mitochondrial electron and proton leak, increases bio-energetic efficiency, and attenuates damage resulting from intracellular oxidative stress (21-25). A full understanding of the underlying mechanistic basis of DR is far from complete. However, fasting turned out to be a powerful means to increase resistance against acute stressors such as oxidative stress. It has been previously shown that preoperative fasting and CR protect against acute oxidative damage including ischemia-reperfusion injury in both liver and kidney (26-29), surgical trauma (30), heat shock (31), and paraquat toxicity (32). Irinotecan is one of the chemotherapeutic agents which has been reported to cause high levels of oxidative stress, which will lead to disturbance of normal cellular functions in healthy cells and may contribute to severe dose-limiting toxicities (33, 34). Rats that were caloric restricted for 35% during 3 weeks were protected against a lethal dose of the hepatotoxic compound thioacetamide. CR rats showed 70% survival compared with 10% in *ad libitum* fed rats (35, 36). Bleomycin, a cytotoxic drug that is associated with DNA damage and oxygen radical production was injected into rats kept on a four week 40% CR diet and *ad libitum* control rats. The DNA mutant frequency was higher in *ad libitum* fed- compared to CR rats, indicating that CR reduced mutagenesis to bleomycin (37). Importantly, it has been shown that the beneficial effects of CR can be induced rapidly by a short-term fasting period. The benefit of fasting above CR is that fasting can be induced more rapidly, is non-invasive, and a cost-free method of protecting against multiple forms of stress. Three days of fasting and 2-4 weeks of 30% CR both led to a similar protection against oxidative damage induced by renal ischemic injury (26). A short-term fasting regimen provided complete protection to mice from a high dose of the chemotherapeutic agent etoposide. All *ad libitum* fed animals died within 5 days after administration of 110 mg/kg etoposide, whereas all fasted animals survived without showing visible signs of toxicity (38). Taken together, these findings demonstrate that CR and fasting increase stress resistance and protect against the adverse effects of toxic agents.

In this thesis we studied two new strategies to improve the chemotherapeutic treatment of colorectal liver metastases. We investigated the behavior of the biological clock

in a colorectal cancer model in mice, and the potential benefits of a restrictive diet on irinotecan induced side effects. The colorectal model was chosen as this type of cancer is one of the main causes of cancer related mortality in western countries.

Aims and outline of the thesis

Chemotherapeutic agents are part of the multimodality approach for treatment in patients with CRLM. The associated toxicity is a major limitation to their use, and leads to early discontinuation of treatment. Therefore, the aim of the studies described in this thesis was to describe two experimental subjects to ameliorate chemotherapeutic treatment in the multi-modality approach of colorectal liver metastases: the impact on the circadian clock and dietary restriction.

Chapter 2 gives a systematic review of the literature on the function of the clock gene machinery and circadian gene expression in relation to frequently seen abdominal cancer types. In **chapter 3** we compared the 24-hour expression levels of important circadian clock genes in liver and kidney of healthy control mice with those of mice bearing C26 colorectal tumor metastases in the liver. We also investigated clock periodicity in kidney and liver tissue in healthy and C26 tumor bearing mice. In **chapter 4** we set out to translate this to a clinical setting and therefore we studied clock gene expression levels in colorectal liver metastases in patients. We compared clock gene expression levels of the liver to those of colorectal liver metastases and primary tumors, and related these expression levels to clinicopathological factors. In **chapter 5** we explored the value of dietary restriction and the benefits on chemotherapy associated toxicity. We studied the effects of 3-days fasting prior to irinotecan treatment on toxicity and anti-tumor activity in a FabplCre;Apc15lox/+ mouse model, which spontaneously develops intestinal tumors. In **chapter 6** we explored the effects of fasting on irinotecan induced toxicity and pharmacokinetics in plasma, liver, and tumor. In **chapter 7** we tried to unravel the mechanisms of protection induced by fasting and investigated the transcriptional responses to fasting and irinotecan treatment in both tumor and healthy liver tissue using array analyses. In **chapter 8** the results of the studies performed in this theses are summarized and discussed.

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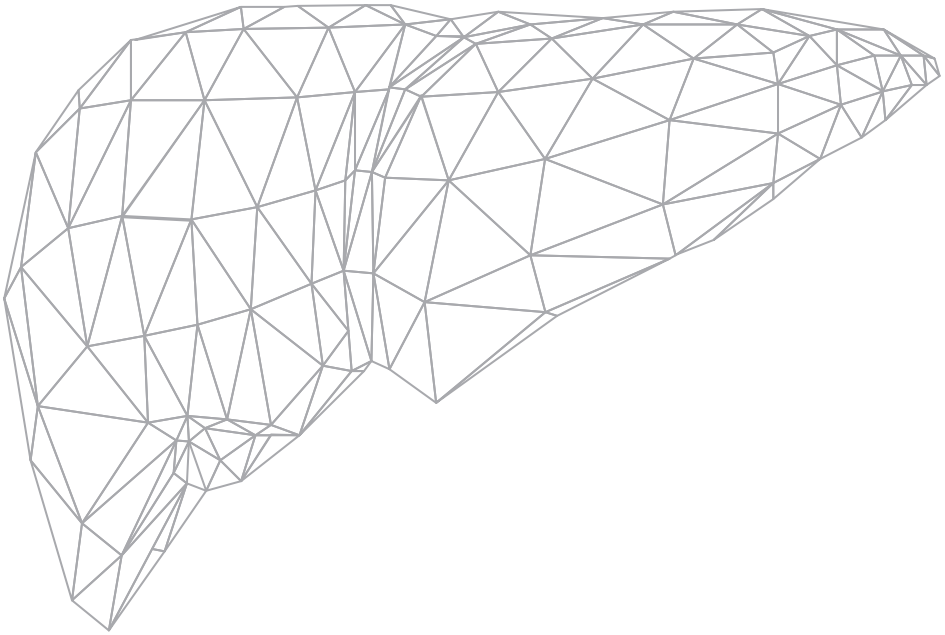


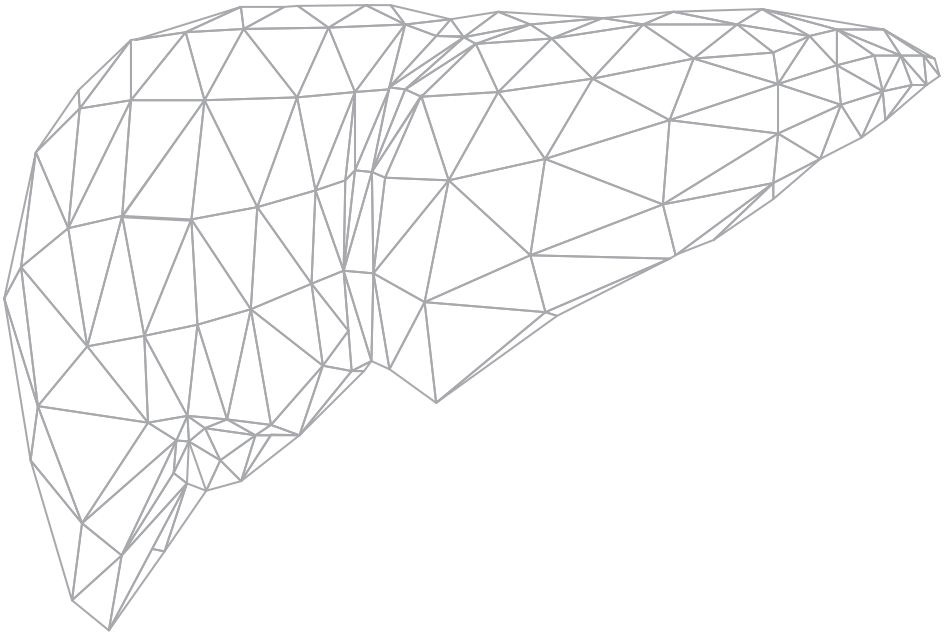
PART TWO

DISRUPTION OF THE CIRCADIAN CLOCK AND CANCER

Chapter 2 **Circadian gene expression in human abdominal cancer: A systematic review**
Submitted

Chapter 3 **Colorectal liver metastases with a disrupted circadian rhythm phase shift the peripheral clock in liver and kidney**
International Journal of Cancer, 2015, Mar 1;136(5):1024-32

Chapter 4 **Disruption of clock gene expression in human colorectal liver metastases**
Submitted



CHAPTER 2

CIRCADIAN GENE EXPRESSION IN HUMAN ABDOMINAL CANCER: A SYSTEMATIC REVIEW

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Ali R Ahmadi*
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Jan NM IJzermans
Gijsbertus TJ van der Horst
Ron WF de Bruin

Submitted

*Both authors contributed equally to this study.

Abstract

Circadian clocks impose near (circa) 24-hour (dies) rhythmicity on important cellular and physiological functions. The mammalian circadian system is composed of a light-entrainable central clock in the suprachiasmatic nucleus (SCN) in the hypothalamic region, which in turn synchronizes peripheral clocks, present in virtually all other cells and tissues. Circadian rhythms are generated by a cell-autonomous molecular oscillator composed of a set of clock genes that act in positive and negative transcription-translation feedback loops. Numerous studies have investigated the role of the circadian machinery in relation to different types of malignancies. There is mounting evidence that circadian genes may play a pivotal role in oncogenesis and prognosis of the disease. In this review, we evaluate the expression levels of circadian genes in human abdominal cancerous tissue in relation to disease development, prognosis and treatment. Comprehensive searches were carried out in multiple databases until February 2016 to search for articles regarding expression levels of circadian genes in human tissues. Of the 3571 studies retrieved, 27 articles fell within the scope of our review. Based on our findings, we conclude that the expression levels of the circadian genes are disturbed in malignant tissue in comparison to normal tissue. The genes *PER1*, *PER2*, and *PER3* seem to be consistently down regulated in different types of abdominal cancers. The other clock genes show discrepancies in expression levels in different studies. These aberrant expression levels between normal and cancerous tissue may serve as biomarkers for prognosis and may facilitate chronomodulated cancer therapy.

Introduction

The circadian clock is an internal time keeping system that imposes temporal organization to an organisms biological functions and is present in nearly all organisms (1). Circadian rhythms have a periodicity of approximately 24-hours (circa diem: about one day) and allow an organism to anticipate daily recurring changes in the environment, notably the light-dark cycle. In mammals, the circadian system is composed of a master clock in the suprachiasmatic nuclei (SCN), located in the hypothalamus, and peripheral clocks in virtually all other cells and tissues. To keep pace with the day-night cycle, the SCN clock is daily synchronized by light. In turn, the SCN synchronizes the peripheral clocks through humoral and neuronal stimuli (2). Circadian rhythms are generated by a cell autonomous molecular oscillator, involving transcription-translation feedback loops composed of a set of interplaying clock genes and proteins. The maintenance and generation of rhythms is similar in the central and the peripheral clocks, although, the resulting output pathways can be different and more tissue specific.

The positive branch of the clock machinery consists of the *CLOCK* (Circadian Locomotor Output Cycles Kaput) and *BMAL1* (Brain-Muscle Arnt-Like protein 1) genes. CLOCK and BMAL1 are transcription factors that heterodimerise and induce the expression of core circadian genes by binding to E-box elements in their promotors. Core circadian genes are defined as genes whose protein products are necessary for the generation and regulation of circadian rhythms within individual cells throughout the organism (3). There are two families of core circadian genes, the period (*PER1*, *PER2* and *PER3*) and cryptochrome (*CRY1* and *CRY2*) families, that encode proteins which translocate back to the nucleus to repress their own transcription by acting on the CLOCK/BMAL1 complex. The core mechanism is more complex than a single autoregulatory loop. Several interconnected feedback loops operate in concert (4, 5). *BMAL1* is regulated by two orphan nuclear receptors: retinoic acid related orphan receptor-α (RORα) and REV-ERBα. These proteins are transcription factors that bind to the *BMAL1* promotor at *REV-ERBα* and *RORα* response elements. RORα activates BMAL1 transcription (6), instead of REV-ERBα, which inhibits it (7). The interacting positive and negative feedback loops of the circadian timing system take approximately 24-hours to complete a cycle and are governed by post-translational modification like phosphorylation and ubiquitination. Casein kinase 1 e.g. plays an important role in phosphorylation by affecting the stability of clock proteins (**Fig1**) (8). The molecular core oscillator is coupled to circadian output processes through a series of clock-controlled genes. Some of these genes play an essential role in cell-cycle control. CLOCK-BMAL1 directly regulates cell-cycle genes such as *WEE1* (G2-M transition), *TIM* and *TIPIN* (DNA replication), and *SERPINE* (protease inhibition).

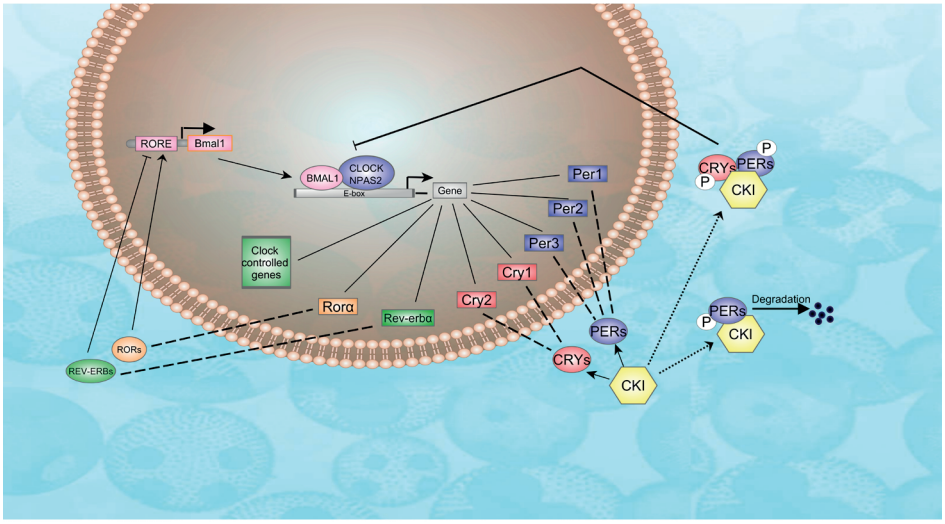


Fig.1 Molecular mechanism of the circadian clock.
The core mammalian clock is comprised of the heterodimeric activators CLOCK and BMAL1 that activate transcription of E-box genes, including the repressors *PERIOD* (*PER*) and *CRYPTOCHROME* (*CRY*). An interlocked regulatory loop directs alternating activation and repression of *BMAL1* expression by the nuclear receptors RORα and REV-ERBα, respectively, via binding at the ROR enhancer elements (ROREs) in the *BMAL1* promoter.

The circadian clock controls physiology, metabolism, and behavior through clock-controlled genes, which may encompass up to 10-15% of a tissues transcriptome (9-13). There is building evidence that disturbances in the expression of these genes may lead to disturbances in physiology and behavior which in turn may lead to pathology (14). From epidemiological and experimental studies it is known that rotating shift-workers face a higher risk of developing cancer (15-18). Altered expression of circadian clock genes could have important effects on the transactivation of downstream targets that control the cell cycle and on the ability of cells to undergo apoptosis. This may lead to genomic instability and accelerated cellular proliferation which promotes carcinogenesis (19). Desynchronizing this rhythmicity seems to be implicated in several pathologic conditions, including tumorigenesis and progression of cancer (20-23). During the last years, human studies have described involvement of the circadian clock in the development and progression of specific cancer types. In this systematic review we provide an overview of the role of the clock gene machinery and circadian gene expression in relation to human abdominal cancer.

Methods

All aspects of the Cochrane Handbook for Interventional Systematic Reviews were followed and the manuscript was written according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) statement (24).

Literature search strategy

Comprehensive searches were carried out in Embase, Medline OvidSP, CENTRAL (the Cochrane Library 2013, issue 10), Web-of-Science, PubMed Publisher and Google Scholar. The search was performed for articles published up to February 2016 using terms specific for each search-engine, provided in the supplemental data.

Inclusion and exclusion criteria

Studies which reported on the expression of circadian genes in specimens of human tissue by real-time quantitative polymerase chain reaction, immunohistochemistry (IHC), microarray analysis, or by RNA sequencing were included. Only studies that reported expression of circadian genes in abdominal cancers were included. Other cancer types were not included. Animal studies, *in vivo*, *in vitro* and pharmacological experimental studies, letters, conference abstracts and editorials were excluded. Articles not written in English were also excluded.

Literature screening

All titles obtained by the search were screened for relevance and all those that were irrelevant were identified and discarded. Articles were considered relevant if the expression of one or multiple circadian genes were studied in human tissues, according to our inclusion criteria. Subsequently studies were evaluated for inclusion by two independent researchers (SAH, ARA) for relevance to the subject by screening the abstracts of the remaining studies. If necessary, the full texts were obtained and screened for relevance. In case of discrepancy, a third author was consulted (RWFdB) to reach consensus.

Results

The systematic search in multiple databases retrieved a total of 3571 articles. Of these, 27 fell within the scope of the review. Two additional references were added that were not included in the search. The up and down regulation of the core circadian genes as described in the reviewed studies is shown in **table 1.** The PRISMA flow diagram for systematic reviews is presented in **table 2**.

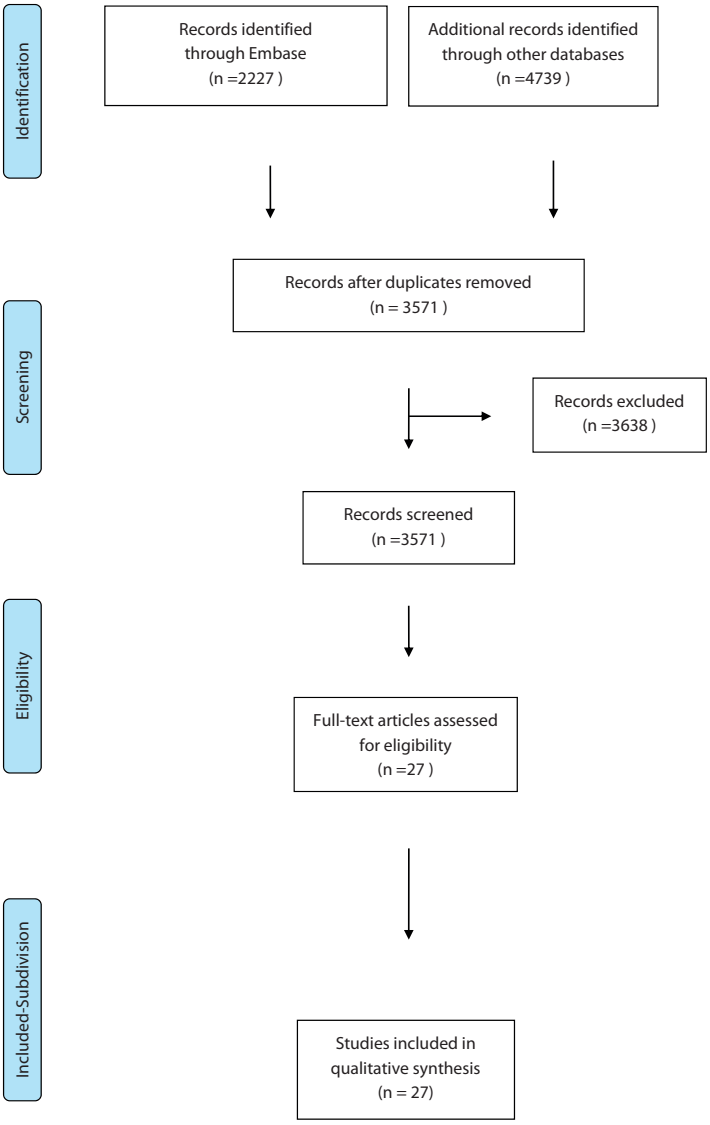
Table 1. Expression of core circadian genes in human abdominal cancer

	Genes												
Author	**CLOCK**	**BMAL1**	**BMAL2**	**PER1**	**PER2**	**PER3**	**CRY1**	**CRY2**	**TIM**	**TIPIN**	**CSNK1**	**SERPINE1**	**WEE1**
Colorectal Cancer													
Mazzocolli et al. 2011		↓		↓	↓	↓	**NS**	↓	↑	**NS**	**NS**	**NS**	
Mazzocolli et al. 2012			↑									↑	
Mazzocolli et al. 2016	**NS**	↓		↓	↓	↓	**NS**	↓	↑	**NS**	**NS**		
Mazzocolli et al. 2016							↓	↓					
Fang et al. 2015								↑					
Yu et al. 2013							↑						
Wang et al. 2011					↓								
Zeman et al. 2008					↓								
Herichova et al. 2008					↓								
Wang et al. 2012						↓							
Wang et al. 2013		↑											
Karantanos 2013	↑	↑		↓		↓							
Mostafaie et al. 2009	↑			↓									
Oshima et al. 2011	↑	↑		↓		↓							
Hepatocellular Carcinoma													
Lin et al. 2008				↓	↓	↓		↓	↓				
Kogiso et al. 2012													↑
Lv et al.													↑
Lin et al. 2015											↓		

Table 1 (continued)

Author		Genes											
		CLOCK	**BMAL1**	**BMAL2**	**PER1**	**PER2**	**PER3**	**CRY1**	**CRY2**	**TIM**	**TIPIN**	**CSNK1**	**SERPINE1**	**WEE1**
	Pancreatic Cancer													
Relles et al.		↓	↓		↓	↓	↓	↓	↓	↓	↓	↓		
	Renal Cancer													
Mazzocolli et al.						↓				↓	↓	↓		

Table 2. PRISMA flow-chart of the systematic literature search.



Gastrointestinal tumors

Colorectal cancer

Colorectal cancer (CRC) is the third most common cancer in men and the second most common in women. Mortality of CRC is 8.5% of the total mortality caused by all forms of cancer (25). Mounting evidence suggests that deregulation of circadian genes play a crucial role in tumorigenesis and prognosis of patients with CRC.

Mazzoccoli et al. studied the expression levels of core clock genes in tumor tissue and healthy mucosa of patients with CRC (26). Expression levels of *BMAL1*, *PER1*, *PER2*, *PER3*, and *CRY2* were lower in tumor tissue compared to adjacent healthy mucosa. The expression levels of *TIM* were higher in tumor tissue. No significant differences were observed for the expression levels of *TIPIN*, *CSNK1*, *CRY1* and *CLOCK*. A significant association was found between low *CRY1* expression levels in the tumor, age, and female sex, whereas high *CRY1* expression levels in tumor mucosa were associated with cancer location in the distal colon. Authors conclude that abnormal expression levels of core clock genes in CRC tissue may be related to the process of tumorigenesis and exert an influence on host/tumor interactions. In a recent study, the same authors studied expression levels of specifically *CRY1* and *CRY2* in CRC tissue and adjacent normal tissue of 50 patients by qRT-PCR. The mRNA levels were significantly lower in CRC tissue. Moreover, low *CRY1* mRNA levels correlated with patients of 62-74 years of age. Also, lower *CRY1* mRNA levels were found in female patients. Survival was better in CRC patients expressing lower *CRY1* and *CRY2* levels in tumor tissue (27). In a study where *CRY2* expression was evaluated in relation to chemoresistance, authors found that *CRY2* overexpression was correlated with poor patient survival. They also found in these patients with stage III primary rectal cancer, treated with neoadjuvant chemotherapy that *CRY2* was overexpressed in chemoresistant CRC patients (28).

Several studies have been conducted to assess the ability of deregulated circadian genes as potential biomarkers for clinical characteristics and prognosis. Mazzoccoli et al. found up-regulation of *BMAL2* and *SERPINE1* in 50 CRC specimens (29). A statistically significant association was found between high *BMAL2* mRNA levels and vascular invasion, and between high *SERPINE1* mRNA levels and microsatellite instability. Sorting the subjects into quartile groups, a statistically significant association was found between high *BMAL2* expression and lymph node involvement, between high *SERPINE1* expression and grading, and between high *SERPINE1* expression and micro satellite instability (MSI) status.

Yu et al. demonstrated that *CRY1* over expression correlates with tumor progression and poor prognosis in patients with CRC (30). Clinical pathological analysis showed that high *CRY1* expression was significantly correlated with lymph node metastasis and the tumor/node/metastasis (TNM) stage. Furthermore, high *CRY1* expression corresponded with poor overall survival in CRC patients. These results suggest that *CRY1* is important in the development and progression of CRC.

PER2 expression levels in CRC have been determined in 38 human specimens (31). Using IHC most colorectal cells in non-cancerous tissues were homogeneously stained. In contrast, in the cancerous tissue, a heterogeneous pattern was found with a significant portion of cancer cells displaying negative or weak PER2 staining. In over 60% of the cases, the staining for PER2 was stronger in healthy cells than in the cancerous cells.

Furthermore, decreased PER2 levels correlated with younger patients with age no more than 50, a higher histological grade, a more advanced TNM stage, and a higher Ki-67 score. qRT-PCR for *PER2* showed similar results. Similar deregulated expression of *PER2* was found in another study (32).

Wang et al. aimed to determine whether *PER3* expression was associated with CRC incidence number and progression (33). As compared to normal tissue, a 2.8-fold decrease in *PER3* mRNA levels was observed in cancerous tissue. In addition, altered *PER3* expression was related to multiple clinicopathological factors, including tumor location, differentiation, and stage. Furthermore, mortality rates were higher in patients in which *PER3* was down-regulated in tumor tissue.

An analysis of CLOCK protein and gene expression was performed in 30 cases of CRC (34). Higher levels of CLOCK expression was observed in CRC mucosa tissue compared with non-cancerous healthy mucosa. CLOCK expression was significantly higher in poorly differentiated, or late-stage, Dukes' grade tumors and in 64.3% of tumor cases with lymph node metastasis.

Karantanos et al. evaluated the expression levels of clock genes in 42 CRC patients and sought to correlate them with prognosis (35). In cancerous tissue, *CLOCK*, and *BMAL1* expression levels were higher, while *PER1*, and *PER3* expression levels were lower compared to matched healthy tissue. No difference was observed in the expression levels of *PER2*. Except for the differential clock gene expression levels between tissues, no correlations were found with clinicopathological factors or prognosis of the patients. Several other studies investigated the role of circadian genes and CRC and their relationship to prognosis. They hypothesized that core clock genes are functionally involved in the process of carcinogenesis, and that the relevance of circadian gene expression to determine prognosis should be considered in relation to tumor staging (36-41).

In summary, in CRC *PER1*, *PER2*, *PER3*, and *CRY2* are down-regulated, and this down regulation is associated with poor survival, high tumor staging and development of metastasis. *CLOCK* and *CRY1* over expression is correlated with poor differentiation, high grading, and lymph node metastasis.

Hepatocellular carcinoma

Hepatocellular carcinoma (HCC) mainly occurs in Asia and Africa. It is the fifth most common cancer in men (554.000 cases, 7.5% of the total) and the ninth in women (228.000 cases, 3.4%). Hepatic cancer is the second cause of cancer related deaths worldwide. With an overall ratio of mortality to incidence of 0.95, the prognosis is poor (42).

Several studies evaluated the role of circadian genes in HCC. Expression levels of 9 circadian genes were measured in 46 HCC tissues and paired non-cancerous tissues of the liver (43). Decreased expression levels were shown for *PER1*, *PER2*, *PER3*, *CRY2* and *TIM*. Lin et al. state that the decreased expression of circadian genes may result in dis-

turbances of cell cycle, and is correlated with bigger tumor size. These disturbances may disrupt the control of the central pacemaker and benefit selective survival of cancerous cells and promote carcinogenesis.

The expression of WEE1 kinase, and its correlations to clinicopathological factors has been investigated in 26 HCC patients (44). *WEE1* is a key regulator of mitosis, and its transcription is directly driven by the circadian clock. Reduction of *WEE1* could result in less mitosis control and therefore faster cell cycling. Using IHC, non-cancerous tissues and cirrhotic tissues did not show any expression of WEE1. WEE1 expression was detected in 30% of the HCC samples, and was correlated with higher levels of albumin, and prothrombin time. Furthermore, the prognosis was better, and the duration until recurrence longer in WEE1-postive HCC patients. It is thought that WEE1 kinase may suppress the hyper cell-cycle in HCC at the G2/M phase. WEE1 up-regulation was also found in 42 HCC specimens compared with 20 cirrhosis, and 23 normal liver tissues (45). In this study however, up-regulated WEE1 expression was correlated with worse tumor differentiation and pathological grade.

One of the main functions of casein kinase 1 epsilon (CK1ε) is to regulate circadian rhythm by phosphorylation and degradation of *PER* genes (46). A recent study used IHC to examine CK1ε expression in 230 HCC specimens by tissue microarray. It showed that low cytoplasmic CK1ε expression was significantly correlated with a decrease in 5-year survival. Furthermore, low CK1ε expression correlated with high tumor vascular invasion potential (47).

In summary, down-regulated expression of clock genes *PER1*, *PER2*, *PER3*, *CRY2* and *TIM* has been found in HCC and is correlated with bigger tumor size and selective survival of cancerous cells which may promote carcinogenesis. Two studies found up-regulated expression of *WEE1* in HCC specimens, but clinicopathological factors were shown to be differentially distributed and its significance remains uncertain. Low *CK1ε* expression was correlated with a decreased survival rate.

Pancreatic cancer

Pancreatic cancer has a relatively low incidence rate of 337.872 cases worldwide (2.4% of total cancer incidence). However, pancreatic cancer is an aggressive form with a high mortality rate of 4.0% of all cancer-related deaths worldwide (42).

Evidence that circadian genes might play a significant role in pancreatic ductal adenocarcinoma (PDA) was published by Pogue-Geile et al. in 2006 (48). In tumor tissues of PDA patients, expression levels of circadian (controlled) genes were lower, including *BMAL1*, *CLOCK*, *PER1*, *PER2*, *PER3*, *CRY1*, *CRY2*, *TIPIN*, *TIM*, and *CKIε*. Comparing PDA tumors with adjacent normal pancreatic tissue, or benign pancreas specimens like intraductal papillary mucinous neoplasms (IPMNs), adenomas, cystadenomas, or epithelial cysts; circadian genes were also significantly lower expressed for *PER1*, *PER2*, *PER3*, *CRY2*, *TIPIN*,

and *CKIε*. A significant association between low expression levels in tumors and reduced survival was found for almost all genes. Higher gene expression levels in benign tissues and matched adjacent tissues may be indicative of their role during the process of tumorigenesis. The potential of using circadian genes as predictive markers for the outcomes and survival and distinguishing PDA from benign pancreas must be studied in larger populations to validate and demonstrate their eventual clinical relevance (49).

In summary, core circadian genes and their downstream effectors were down-regulated in PDA which may indicate that the peripheral clock is disrupted and that circadian genes in healthy tissue act as tumor suppressors.

Renal cancer

Renal cancer has an incidence of 337.860 cases (2.4% of total cancer incidence) with a mortality of 143.369 cases (1.7% of total cancer related mortality) worldwide (42). Renal cancer is associated with an alteration in pathways regulated by the von Hippel-Lindau protein and hypoxia inducible factor-α. Hypoxia-inducible transcription factors are transcriptional regulators of genes controlling mammalian oxygen homeostasis, energy metabolism, neovascularization, internal pH, cell survival, and migration and are considered powerful promoters of tumor growth. Tight interrelationships have been evidenced between hypoxia response pathways and circadian pathways (50). Severe deregulation of genes involved in the circadian clock circuitry and response to hypoxia has been found in patients affected by renal cancer, influencing the process of carcinogenesis, as well as disease progression and outcome.

The clock gene machinery was evaluated in primary renal carcinoma and matched adjacent non-cancerous tissues in a cohort of 11 consecutive patients (51). A significant down-regulation was found for *PER2*, *TIM* and *TIPIN*. The only up-regulated gene was *SERPINE1*. A statistically significant correlation was found between mRNA levels of *PER2* and *CSNKIE*, *TIPIN*, and *SERPINE1*. Other significant correlations were found between *TIMELESS*, *TIPIN*, and *CSNKIE*. Authors concluded that the circadian clock circuitry is deregulated and the altered expression of clock genes might be involved in disease onset and progression.

In summary, kidney cancer is associated with alteration in the pathways regulated by von Hippel-Lindau protein and hypoxia inducible factor-α, which plays an important role in interaction with the circadian pathway. A single study shows the dysregulation of several key circadian genes in kidney cancer. More research is needed to confirm these first findings and determine their clinical significance.

Discussion

Circadian disruptions may lead to pathology, such as cardiovascular diseases, metabolic disease and behavioral changes. The link between circadian disturbance (e.g. by shiftwork and jetlag), and the association with cancer has been demonstrated both experimentally (52) and in humans (16, 53). A meta-analysis by Wang et al. estimated that the risk increase in breast cancer is 3% for every five year of rotating shift work and 13% for every 500 night shifts (54). However whether this is caused by disruption of the circadian clock or by stress or other factors related to an altered lifestyle is difficult to determine and remains controversial (55). Nonetheless mounting evidence suggests the pivotal role of the circadian clock in the development and prognosis of cancer (20). We have reviewed the expression of circadian genes in abdominal cancers and their association with clinicopathological factors. Many studies showed altered expression of circadian genes in different abdominal cancer types, however it is difficult to distinguish whether the altered expression leads to oncogenesis or whether the process of oncogenesis leads subsequently to altered clock function. Down-regulation of clock genes with tumor suppressor functions as *PER1*, *PER2*, and *PER3* may enhance oncogenesis and up-regulation of *TIM* and *CLOCK* are correlated with higher tumor staging, malignant tumors and overall poorer survival. These alterations in clock gene expression have a direct effect on fundamental biological rhythms, the cell cycle, apoptosis and the DNA damage response (56). These disturbances in gene expression can be attributable to methylation or hypermethylation of the promotor sites or by structural changes. Aberrant expression of these genes or their downstream targets may prevent the cells from apoptosis and may even promote cell division by losing cell cycle regulators. The expression of these genes or the proteins they produce are being explored as biomarkers in clinical use for early detection of the disease, tumor staging, tumor aggressiveness and the potential of metastasis or as a predictor of survival. In general the majority of the studies show similar expression in cancer in terms of down- or up-regulation and the relationship with clinicopathological factors. However, whereas some studies show elevated expression of specific clock genes, others demonstrate decreased expression of the same specific clock genes with different clinicopathological factors. Thus there seems to be a discrepancy, which may be explained by a difference in pathways where these genes are up- or down-regulated.

To our knowledge this is the first review describing the aberrant expression of circadian genes in human abdominal cancers. This study further strengthens that circadian genes play a significant role in carcinogenesis. A major drawback in human studies is that all studied samples are surgical resection specimens taken at only one time point, and not all resection specimens are collected at the same time.

As discussed above the aberrant expression of clock genes in cancer tissue may be used to develop novel therapies or be used as biomarkers to assess prognosis of patients. As a result of our increased understanding of the circadian system, the term chronotherapy was born (57-59). Chronotherapy consists in the administration of medication according to biological rhythms and has already proven to be effective. The cellular circadian clocks are coordinated by endogenous physiological rhythms, so that they tick in synchrony in the host tissues that can be damaged by anticancer agents (60). As a result, circadian timing can modify the tolerability of anticancer medications in experimental models and in cancer patients and may improve drug tolerability. Conversely, host clocks are disrupted whenever anticancer drugs can be administered at their most toxic time. On the other hand, circadian disruption accelerates experimental and clinical cancer processes. The clinical relevance of the chronotherapy principal has been demonstrated in randomized, multicenter trials (60-62). Chronotherapeutic schedules have been used to document the safety and activity of oxaliplatin against metastatic CRC and have formed the basis for a new approach of management of the disease. Several trials investigating the role of chronotherapy have been deployed and used successfully against metastatic colon cancer for example (59, 61, 63-66). Kloth et al. investigated the chronopharmacology of the orally administrated drug sunitinib, and found that there is variation in pharmacokinetics of sunitinib dependent on the time of administration. They showed that administration in the afternoon or evening resulted in higher plasma concentrations (67). Chronotherapy can be a powerful tool to add to our consisting arsenal of cancer treatment options. Options to apply chronotherapy on different forms of human cancer have been investigated for years now (58, 68). Investigating the circadian machinery may also be useful to elucidate some general mechanisms in the development and progress of cancer. New technologies such as recent advances in DNA sequencing have the potential to shed light on mutations and epigenetic changes in molecular clocks and on how human cancer develops (69). Many circadian regulated genes seem to be down regulated in cancerous tissue of different types of abdominal cancer. This down regulation is most consistently seen in core clock genes like *PER1*, and *PER2*. Therefore, it is suggested that these genes could serve as biomarkers to determine the patients' prognosis in several abdominal cancer types. However more research is needed in human cancer specimens to confirm previous findings and fully understand the role of the circadian clock in development, treatment, and prognosis of human cancer. Time will tell.

Supplemental Data

Literature search

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(('circadian rhythm'/de AND genetics/exp) OR 'transcription factor CLOCK'/de OR 'transcription factor ARNTL'/de OR 'molecular clock'/de OR 'PER1 protein'/de OR 'PER2 protein'/de OR 'cryptochrome 1'/de OR 'cryptochrome 2'/de OR 'protein BMAL1'/de OR 'casein kinase I'/de OR chronotherapy/de OR 'circadian rhythm signaling protein'/de OR (((circadian OR clock OR rhythm*) NEAR/6 (genetic* OR gene OR expression*)) OR per1 OR per2 OR per3 OR cry1 OR cry2 OR ARNTLOR ((per OR cryptochrome OR cry) NEXT/1 (1 OR 2 OR 3)) OR bmal1 OR 'bmal 1' OR wee1 OR 'wee 1' OR 'Rev Erb' OR 'casein kinase I' OR CKI OR chronotherap*):ab,ti) AND ('human tissue, cells or cell components'/exp OR ((human/de OR (human* OR patient*):ab,ti AND ('cell, tissue or organ culture'/exp OR (tissue* OR culture* OR cell*):ab,ti)))) AND (neoplasm/exp OR ((tumo* OR cancer* OR carcinom* OR metasta* OR malign* OR carcinogen* OR neoplas*)):ab,ti)

Medline (OvidSP)

("circadian rhythm"/ge OR "circadian clocks"/ge OR exp "Circadian Rhythm Signaling Peptides and Proteins"/ OR exp "casein kinase I"/ OR exp chronotherapy/ OR (((circadian OR clock OR rhythm*) ADJ6 (genetic* OR gene OR expression*)) OR per1 OR per2 OR per3 OR cry1 OR cry2OR ARNTL OR ((per OR cryptochrome OR cry) ADJ (1 OR 2 OR 3)) OR bmal1 OR "bmal 1" OR wee1 OR "wee 1" OR "Rev Erb" OR "casein kinase I" OR CKI OR chronotherap*).ab,ti.) AND (((human/ OR (human* OR patient*).ab,ti. AND (exp "Cells, Cultured"/ OR (tissue* OR culture* OR cell*).ab,ti.)))) AND (exp neoplasms/ OR ((tumo* OR cancer* OR carcinom* OR metasta* OR malign* OR carcinogen* OR neoplas*)).ab,ti.)

Cochrane

((((circadian OR clock OR rhythm*) NEAR/6 (genetic* OR gene OR expression*)) OR per1 OR per2 OR per3 OR cry1 OR cry2 OR ARNTL OR ((per OR cryptochrome OR cry) NEXT/1 (1 OR 2 OR 3)) OR bmal1 OR 'bmal 1' OR wee1 OR 'wee 1' OR 'Rev Erb' OR 'casein kinase I' OR CKI OR chronotherap*):ab,ti) AND ((((human* OR patient*):ab,ti AND ((tissue* OR culture* OR cell*):ab,ti)))) AND (((tumo* OR cancer* OR carcinom* OR metasta* OR malign* OR carcinogen* OR neoplas*)):ab,ti)

Web-of-science

TS=(((((circadian OR clock OR rhythm*) NEAR/6 (genetic* OR gene OR expression*)) OR per1 OR per2 OR per3 OR cry1 OR cry2 OR per-1 OR per-2 OR per-3 OR cry-1 OR cry-2 OR cryptochrome-1 OR cryptochrome-2 OR ARNTL OR bmal1 OR "bmal 1" OR wee1 OR

"wee 1" OR "Rev Erb" OR "casein kinase I" OR CKI OR chronotherap*)) AND ((((human* OR patient*) AND ((tissue* OR culture* OR cell*)))))) AND (((tumo* OR cancer* OR carcinom* OR metasta* OR malign* OR carcinogen* OR neoplas*))))

Scopus

TITLE-ABS-KEY(((((circadian OR clock OR rhythm*) W/6 (genetic* OR gene OR expression*)) OR per1 OR per2 OR per3 OR cry1 OR cry2 OR per-1 OR per-2 OR per-3 OR cry-1 OR cry-2 OR cryptochrome-1 OR cryptochrome-2 OR ARNTL OR bmal1 OR "bmal 1" OR wee1 OR "wee 1" OR "Rev Erb" OR "casein kinase I" OR CKI OR chronotherap*)) AND ((((human* OR patient*) AND ((tissue* OR culture* OR cell*)))))) AND (((tumo* OR cancer* OR carcinom* OR metasta* OR malign* OR carcinogen* OR neoplas*))))

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((((circadian[tiab] OR clock[tiab] OR rhythm*[tiab]) AND (genetic*[tiab] OR gene[tiab] OR expression*[tiab])) OR per1[tiab] OR per2[tiab] OR per3[tiab] OR cry1[tiab] OR cry2[tiab] OR ARNTL [tiab] OR per-1[tiab] OR per-2[tiab] OR per-3[tiab] OR cry-1[tiab] OR cry-2[tiab] OR cryptochrome-1[tiab] OR cryptochrome-2[tiab] OR bmal1[tiab] OR bmal-1[tiab] OR wee1[tiab] OR wee-1[tiab] OR Rev-Erb[tiab] OR casein-kinase-I[tiab] OR CKI[tiab] OR chronotherap*[tiab])) AND ((((human*[tiab] OR patient*[tiab]) AND ((tissue*[tiab] OR culture*[tiab] OR cell*[tiab])))))) AND (((tumor*[tiab] OR tumors*[tiab] OR tumour*[tiab] OR cancer*[tiab] OR carcinom*[tiab] OR metasta*[tiab] OR malign*[tiab] OR carcinogen*[tiab] OR neoplas*[tiab])))) AND publisher[sb]

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per1|per2|per3|cry1|cry2|ARNTL|"per1|2|3"|"cry1|2"|"cryptochrome1|2"|bmal1|"bmal 1"|wee1|"wee 1"|"Rev Erb"|"casein kinase I"|CKI ~human tissue|culture|celltumor|tumour|cancer|carcinoma|metastasis|malignancy|neoplasm|neoplasms

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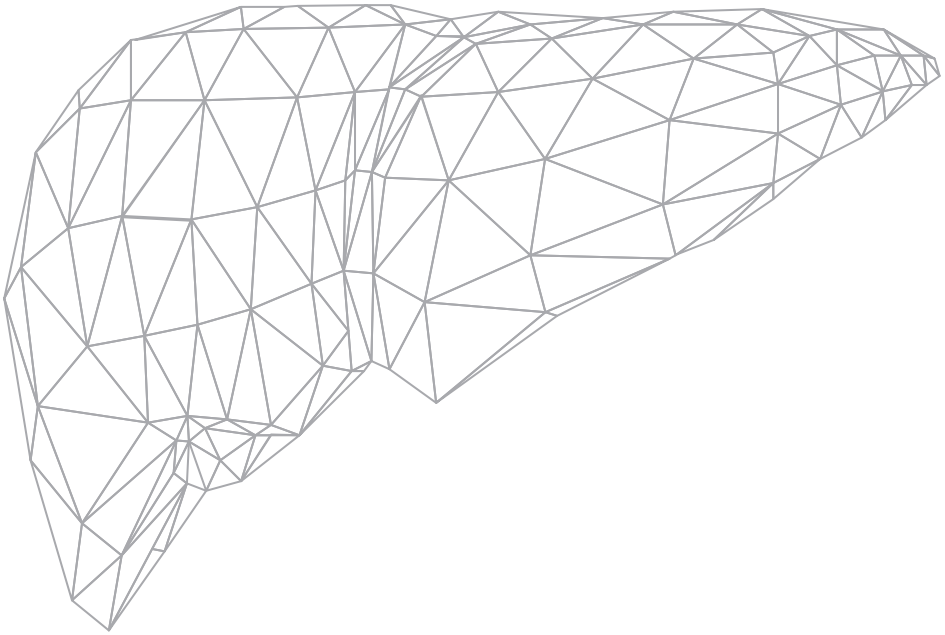

CHAPTER 3

COLORECTAL LIVER METASTASES WITH A DISRUPTED CIRCADIAN RHYTHM PHASE SHIFT THE PERIPHERAL CLOCK IN LIVER AND KIDNEY

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Abstract

Circadian clock genes regulate 10-15% of the transcriptome, and might function as tumor suppressor genes. Relatively little is known about the circadian clock in tumors and its effect on surrounding healthy tissues. We therefore compared the 24-hour expression levels of key circadian clock genes in liver and kidney of healthy control mice with those of mice bearing C26 colorectal tumor metastases in the liver. Metastases were induced by injection of C26 colorectal carcinoma cells into the spleen. Subsequently, tumor, liver and kidney tissue was collected around the clock to compare circadian rhythmicity. Expression levels of 5 clock genes (*Rev-Erbα*, *Per1*, *Per2*, *Bmal1*, *Cry1*) and 3 clock controlled genes (*Dbp*, *p21* and *Wee1*) were determined by qRT-PCR. Liver and kidney tissue of healthy control mice showed normal 24-hour oscillations of all clock and clock-controlled genes, consistent with normal circadian rhythmicity. In colorectal liver metastases, however, 24-hour oscillations were completely absent for all clock and clock-controlled genes except *Cry1*. Liver and kidney tissue of tumor-bearing mice showed a shift in clock periodicity relative to control mice. In the liver we observed a phase advance, whereas in the kidney the phase was delayed. These data show that hepatic metastases of C26 colon carcinoma with a disrupted circadian rhythm phase shift liver and kidney tissue clocks, which strongly suggests a systemic effect on peripheral clocks. The possibility that tumors may modify peripheral clocks to escape from ordinary circadian rhythms and in this way contribute to fatigue and sleep disorders in cancer patients is discussed.

Introduction

The circadian timing system plays an essential role in the development of cancer. Cancer cells carry a similar machinery of increased proliferation rate, reduced apoptotic sensitivity and escaping cell cycle control. These parameters are controlled by the circadian clock (1-4).

Circadian rhythms are generated by a molecular oscillator composed of a set of clock genes that act in a cell-autonomous way and are present in all cell types. These clocks are coordinated by a master clock in the neurons of the suprachiasmatic nuclei (SCN), which is located in the anterior hypothalamus (5). Peripheral clocks are regulated by the SCN through both the autonomic nervous system and neuroendocrine systems. Clocks in the SCN neurons and peripheral cells make use of the same set of clock genes (6). The positive branch of the mammalian clock machinery consists of CLOCK (Circadian Locomotor Output Cycles Kaput) and BMAL1 (Brain-Muscle Arnt-Like protein 1). CLOCK and BMAL1 are transcription factors that heterodimerize and activate transcription of the *Cryptochrome* (*Cry1* and *Cry2*) and *Period* (*Per1* and *Per2*) genes by binding to E-box elements in their promoters. After being synthesized in the cytoplasm, CRY and PER proteins heterodimerize and enter the nucleus where they inhibit CLOCK-BMAL1-mediated transcription and accordingly close the negative feedback loop (7). The core mechanism is more complex than a single auto regulatory loop. Several interconnected feedback loops operate in concert. *Bmal1* is regulated by two orphan nuclear receptors: RORα and REV-ERBα. These proteins are transcription factors that bind to the *Bmal1* promoter at REV-ERBα and RORα response elements. RORα activates *Bmal1* transcription (8) whereas REV-ERBα, inhibits it (9).

This molecular core oscillator is coupled to circadian output processes through a series of clock-controlled genes (CCGs). Some of these CCGs play an essential role in cell-cycle control. CLOCK-*BMAL1* directly regulates cell-cycle genes such as *Wee1* (G2-M transition), *c-Myc* (G0-G1 transition), and *Cyclin D1* (G1-S transition) (10).

The circadian timing system plays an important role in the development of cancer. Genetic or functional disruption of the molecular circadian clock may contribute to genomic instability and accelerate cellular proliferation, two conditions that favor carcinogenesis (11). The role of circadian dysfunction in tumor development has been well documented during the last decades. One of the first discoveries of circadian disruption in rodents was seen in mammary tumors (12-14). Subsequently, Fu et al. showed that mice deficient in the *mPer2* gene are cancer prone after γ-radiation (10, 15). Other animal studies were performed comparing disabled circadian clocks with healthy controls which showed a higher incidence of spontaneously developing tumors (2, 16). Furthermore, mice with SCN ablation or exposure to experimental chronic jetlag showed significantly accelerated tumor growth (17). In SCN-lesioned mice, transplanted tumors grow twice

as fast as in sham-lesioned animals (18). In chemically induced colorectal tumors using azoxymethane (AOM) and dextran sodium sulfate (DSS), daily expression levels of clock genes remained mostly coordinated but damped in amplitude. Especially, the rhythm of *Bmal1* was completely abrogated in colorectal tumors. In the same study a temporal shift of clock gene rhythms in the liver of tumor-bearing mice was observed (19).

Down-regulation of *Per2* expression increases the proliferation of cancer cells *in vitro*, and stimulates tumor growth *in vivo*, whereas *Per2* over-expression inhibits cell growth both *in vitro* and *in vivo* (20, 21). The same effects have been observed for *Per1* (22). In addition, *Bmal1* and *Cry* mutant mice show accelerated spontaneous or radiation induced tumor development (15). Epidemiological findings of relevance includes several studies showing that shift workers, flight attendants and pilots are more likely to develop malignancies because of their disrupted circadian cycles (23-26). These findings suggest that there is a close connection between the circadian organization and development of different types of cancer.

The prognosis of almost 60 percent of patients with colorectal cancer (CRC) is worsened by the development of liver metastases (27, 28). Disruption of circadian genes in CRC has been reported. The functioning of the circadian clock in Colorectal Liver Metastases (CRLM) remains unclear, and the effect of CRLM on peripheral clocks is not well established. In this study we therefore investigated the 24-hour expression levels of key circadian clock genes in C26 derived colorectal liver metastases, adjacent liver tissue and kidney tissue, and compared them with clock gene expression in healthy control mice.

Materials and methods

Cell culture

The murine colon carcinoma cell line C26 (kindly provided by Dr. R. Schiffelers, Utrecht University, the Netherlands) was cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Sigma Aldrich, St. Louis, MO, USA), supplemented with 10% fetal calf serum (Lonza, Verviers, Belgium), penicillin (100 units/ml) and streptomycin (100 units/ml) (Invitrogen, Auckland, NZ) at 37 degrees Celsius in a 5% carbon dioxide environment. Cells were harvested by brief trypsinization (0.05% trypsin in 0.02% ethylenediamine tetra-acetic acid (EDTA)). Cell suspensions were prepared to a final concentration of 5.0×10^3 cells/ml.

Animals

Male BALB/c mice of 6-8 weeks weighing approximately 25 grams were obtained from Charles River, Maastricht, the Netherlands. Upon arrival, animals were housed at random in individually ventilated cages (n= 3-4 animals/cage) in a licensed biomedical facility

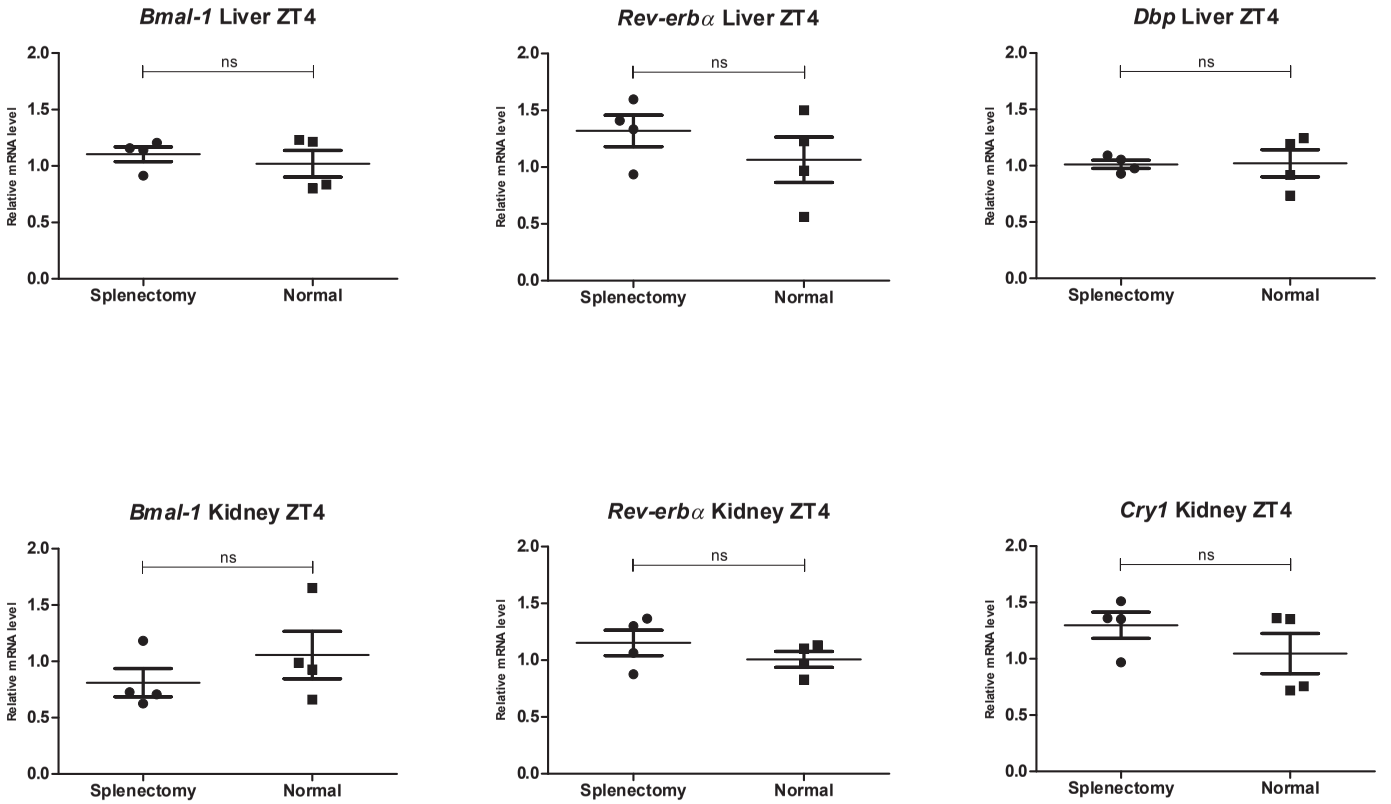

Fig 1. Effect of splenectomy on circadian rhythm. Splenectomized mice (N=4) and control mice (N=4) were sacrificed at ZT4 three weeks after splenectomy. ZT4 was chosen as time point where most robust phase differences were detected between tumor-free liver and healthy control liver (Fig.4) and kidneys from mice bearing colorectal liver metastases and healthy control kidneys (Fig.5).

at Erasmus University Medical Center, Rotterdam, the Netherlands. Standard laboratory conditions were maintained, i.e. temperature ~22°C, humidity ~50%, and a 12 hr light/12 hr dark cycle. All mice had free access to water and food (Special Diet Services, Witham, United Kingdom). Animals were allowed to acclimatize for one week before the start of the experiments. The experimental protocol was approved by the Animal Experiments Committee under the Dutch National Experiments on Animals Act, and complied with the 1986 directive 86/609/EC of the Council of Europe.

Intrahepatic tumor growth

Mice were anaesthetized with isoflurane inhalation (2%) and placed on a heating pad to maintain their body temperature. A left lateral incision was made to expose the spleen. Next, 5.0×10^4 C26 colorectal carcinoma cells (resuspended in PBS in a volume of 100 µl) were injected into the spleen, allowing transport to the liver through the portal vein where they develop into intrahepatic micro-metastases. After 10 minutes, the spleen was removed to prevent intrasplenic tumor growth. In control animals, the spleen was not removed. An additional experiment comparing splenectomized mice with control animals revealed no differences in clock gene expression (**Fig1**). There were no differences in handling and tissue sampling between the groups.

Experimental setup

To compare the performance of the circadian clock in different tissues, mRNA expression levels of clock genes were determined at six time points (08.00h, 12.00h, 16.00h, 20.00h, 0.00h, 04.00h, corresponding to Zeitgeber Time ZT0, ZT4, ZT8, ZT12, ZT16 and ZT20, in which ZT0 represents "lights on"). Three weeks after tumor inoculation, macroscopically visible tumor noduli were present, and the 24-hour time point experiment was started. At each time point, four mice were sacrificed by exsanguination under anesthesia with isoflurane inhalation. Liver and kidneys were removed, and the hepatic tumor load was scored on a semi-quantitative macroscopic tumor load scale ranging from 0-3 (0: no macroscopic tumor, 1: 1-30% of liver surface covered by tumor nodules, 2: 31-70% of liver surface covered by tumor nodules, 3: 71-100% of liver surface covered by tumor nodules). Once tumor-free liver tissue and tumor tissue were identified, small pieces of both were immediately snap frozen in liquid nitrogen until analysis. We used tumor tissue from the viable tumor border where no necrotic cells were present. At the ZT12, ZT16 and ZT20 time points, animals were sacrificed in dark using red light to prevent influence on the circadian clock. To study the circadian rhythm of healthy liver and kidney, age matched mice without tumor inoculation were used.

Real-time quantitative RT-PCR (qRT-PCR)

RNA was isolated from all obtained tissues using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. To avoid genomic DNA contamination, RNA was purified by a DNAse treatment (RQ1 RNase-Free DNase; Promega, Madison, WI, USA). RNA was then reverse transcribed into cDNA using random primers (Invitrogen) and Superscript II RT (Invitrogen). Gene expression was analyzed by real-time quantitative PCR using an Applied Biosystems 7700 PCR machine (Foster City, CA, USA). RT-PCR was performed for 5 core clock transcripts: *Bmal1*, *Rev-Erba*, *Per2*, *Per1*, *Cry1* and 3 clock-controlled genes: *Dbp*, *p21* and *Wee1* (for primer sequences see **table 1**). Each sample was tested in triplicate.

Normalization of RT-PCR data using multiple internal control genes

Because different tissue types were used, a stringent method was applied to identify the most stable internal control genes. We selected four RNA samples from different liver tissues, and four tumor samples from different animals taken at different time points. Primers were designed for 12 commonly used housekeeping genes: *Ywhaz*, *Actb*, *Tbp*,

Table 1: Primer sequences of clock genes and housekeeping genes.

Clock gene	Forward primer	Reverse primer
Per1	CTGGGGACCAGGTCATTAAGT	CACACACGCCATCACATCAA
Per2	CCATCCACAAGAAGATCCTAC	GCTCCACGGGTTGATGAAGC
Rev-erba	AGCTCAACTCCCTGGCACTTAC	CTTCTCGGAATGCATGTTGTTC
Dbp	ACCGTGGAGGTGCTAATGAC	CCTCTTGGCTGCTTCATTGTT
Bmal1	GCAGTGCCACTGACTACCAAGA	TCCTGGACATTGCATTGCAT
p21	GAGCAAAGTGTGCCGTTGTC	GGTTGGAGACTGGGAGAGG
Cry1	CAGACTCTCGTCAGCAAGATG	CAAACGTGTAAGTGCCTCAGT
Wee1	GCTGGAGAAAATTGGTTCTGGA	CTCAAAGCATTCTGCTCATCAAC
Housekeeping gene	**Forward primer**	**Reverse primer**
Ywhaz	ACGGAGCTGCGTGACATCTG	GCAACCTCGGCCAAGTAACG
Actb	GACCCAGATCATGTTTGAGACC	GATGGGCACAGTGTGGGTGAC
Tbp	CCTGCCACACCAGCTTCTGA	AAGTGCAATGGTCTTTAGGT
Rpl13a	GGTGGTGGTCGTACGCTGTG	TCATCCGCTTCCGGAGAAAG
18s	GATCCCTGAGAAGTTCCAGCACA	TGTCTGCTTTCCTCAACACCACA
CypA	CATGGCAAATGCTGGACCAA	CCAAAACGCTCCATGGCTTC
Gapdh	CATCCTGCACCACCAACTGC	ACGCCACAGCTTTCCAGAGG
Hprt	AAGCAGTACAGCCCCAAAATGG	CCAACAAAGTCTGGCCTGTATCC
Ubc	AAGGTCAAACAGGAAGACAGACGTA	CATCACACCCAAGAACAAGCACA
SdhA	TACGCACCTGTTGCCAAGGA	GGCCACAGCCTCTTCCTTCA
B2m	TCACTGACCGGCCTGTATGC	GAGGCGGGTGGAACTGTGTT
Hmbs	GTGCCATTGTCCTGGCTGTG	TGCATTCCTCTGGGTGCAAA

Rpl13a, *18S rRNA*, *CypA*, *Gapdh*, *Hprt*, *Ubc*, *SdhA*, *B2M* and *Hmbs* (for primer sequences see **table 1**). The 8 RNA samples were tested using a gene-stability measure developed by Vandensompele et al (29). This measure relies on the principle that the expression ratio of two ideal internal control genes is identical in all samples. All samples were tested in triplicate. The most stable housekeeping genes were identified with the use of a Microsoft Excel-based Visual Basic Application, geNorm (v3.5) (provided by Dr. J. Vandesompele, Ghent University Hospital, Belgium) that automatically calculates the gene-stability measure "M" for all control genes. The most stable housekeeping genes proved to be TATA-binding proteins (TBP), YWHAZ and beta-actin (ACTB). For accurate averaging of the control genes, the geometric mean was used.

Statistical analyses

For each set of parameters means and standard errors of the mean were computed. To detect circadian oscillation, CircWave v1.4 was used. CircWave software is using the cosinor analyses in addition with a linear harmonic regression fit (30). A rhythm was present when the null hypothesis was rejected by $p < 0.05$. Multiple-way analyses of variance (ANOVA) was used to test three variables 1) an overall difference between tissues, 2) a possible overall difference among time points and 3) the interaction between the two. We report a significance level for the interaction to show a possible significant difference between measured expression through time. All standard statistical tests were performed using SPSS 21 for Windows software (Statistical Package for Social Sciences, Chicago, IL), and $p < 0.05$ was considered to be significant.

Results

Hepatic tumor load

Three weeks after intrasplenic injection of 5.0×10^4 tumor cells (n=24 mice), animals were taken from the experiment at 4 hour intervals (n=4 per time point) and hepatic tumor load was assessed using a macroscopic tumor load score as described. The murine colon carcinoma cell line C26 forms multiple tumors in the liver after intrasplenic injection (31). Four mice had a tumor load 1, 6 mice a tumor load 2 and 14 mice a tumor load 3 (**Fig2**). We did not observe major differences in hepatic tumor load between different time points ($p>0.05$). In all animals multiple tumors were observed which allowed adequate sampling of tumor tissue. No tumors were found in kidney tissue.

Gene expression of core clock genes

To compare circadian gene expression in liver tissue and colorectal liver metastases, we analyzed mRNA expression levels over a time course of 24 hours in samples of liver and

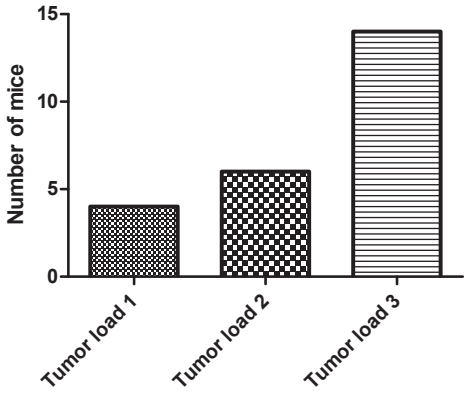

Figure 2. Tumor load. Three weeks after the induction of hepatic metastases, there were 4 mice with tumor load 1, 6 mice with tumor load 2 and 14 mice with tumor load 3 (n=24). On average mice had the same tumor load per time point (2.42 ± 0.16 per time point; p>0.05).

tumor tissue by qRT-PCR. In tumor-free liver tissue from tumor bearing mice the cosinor analysis showed circadian rhythms on the mRNA level of all core clock genes, except for *Per1* (**Table 2A**). As expected, the expression profile of *Bmal1* shows an opposite phase as compared to the other (E-box promoter containing) core clock genes, particularly *Rev-Erbα* and *Per2*. Since BMAL1 is highly expressed during the dark phase of the day, it will together with CLOCK induce the transcription of the other core clock genes. REV-ERBα represses the transcription of *Bmal1*, after ZT4, when *Rev-Erbα* is highly expressed.

In colorectal liver metastases, the cosinor analysis showed the absence of circadian rhythms of all core clock genes, except *Cry1* ($p<0.01$; **Table 2A**). The ANOVA analysis revealed significant differences in expression between liver metastases and tumor-free liver for *Bmal1* ($p<0.001$), *Rev-Erbα* ($p<0.001$) and *Cry1* ($p<0.05$). No circadian rhythm was observed for *Per2* using the cosinor analysis and, ANOVA showed no significant difference between tumor-free liver and liver metastases ($p=0.2$). Moreover, there was no statistical difference in *Per1* expression (**Fig3**).

In conclusion, circadian rhythms were detected in tumor-free liver tissue from tumor bearing mice for *Bmal1*, *Rev-Erbα*, *Cry1* and *Per2*. Colorectal liver metastases showed no circadian rhythms for *Bmal1*, *Rev-Erbα*, *Per2* and *Per1*.

Gene expression of clock-controlled genes

Next, we measured the expression of the clock-controlled genes *Dbp*, *Wee1* and *p21* to determine whether the clock machinery provides output in colorectal liver metastases and tumor-free liver (**Fig4**). Using cosinor analysis, *Dbp*, *Wee1* and *p21* were shown to be rhythmically expressed in tumor-free liver (**Table 2B**).

Table 2: Significance levels and circadian oscillation parameters

Circadian rhythmicity analysis was performed with CircWave cosinor analysis. Subset analysis of core clock genes (A) and clock-controlled genes (B) with significance of cosinor fitting for tumor free liver compared to liver metastases. (C) Subset analysis of tumor free liver compared to control liver and (D) subset analysis of gene expression in kidneys from mice bearing liver metastases and control kidney. Acrophase is ZT when maximum values are most likely reached and mesor is the weighted average value.

	Gene	**CircWave oscillation**		**Acrophase (ZT)**		**Mesor**		**Amplitude**		
A		**Tumor free liver**	**Liver metastases**	**Tumor free liver**	**Liver metastases**	**Tumor free liver**	**Liver metastases**	**Tumor free liver**	**Liver metastases**	
	Bmal1	P<0.001	n.s.	19.6	-	1.8	-	2.4	-	
	Rev-erba	P<0.001	n.s.	3.4	-	1.9	-	4.2	-	
	Cry1	P<0.001	P<0.01	15.9	13.8	5.3	1.8	12.0	3.1	
	Per2	P<0.001	n.s.	10.7	-	2.6	-	6.7	-	
	Per1	n.s	n.s.	-	-	-	-	-	-	
B	*Dbp*	P<0.001	n.s.	6.1	-	2.1	-	3.0	-	
	Wee1	P<0.001	P<0.05	10.8	12.1	2.1	1.4	2.9	0.4	
	P21	P<0.05	n.s.	18.3	-	1.1	-	0.5	-	
C		**Tumor free liver**	**Control liver**	**Tumor free liver**	**Control liver**	**Tumor free liver**	**Control liver**	**Tumor free liver**	**Control liver**	**Phase-shift**
	Bmal1	P<0.001	P<0.001	19.6	21.1	1.8	1.9	2.4	3.0	1.5
	Rev-erba	P<0.001	P<0.001	3.4	6.1	1.9	2.3	4.2	3.3	2.7
	Dbp	P<0.001	P<0.001	5.1	7.9	2.1	1.3	3.0	3.0	2.8
	Per2	P<0.001	P<0.001	11.7	12.2	2.6	2.7	6.7	7.1	0.5
	Cry1	P<0.001	P<0.001	15.9	17.1	5.3	4.9	12.0	5.1	1.2
	Wee1	P<0.001	P<0.001	10.8	10.9	2.1	1.9	2.9	3.8	0.1
	P21	P<0.05	P<0.001	18.3	18.1	1.1	1.0	0.5	0.9	-0.2
	Per1	n.s.	P<0.001	-	11.2	-	2.3	-	8.2	-

Table 2 (continued)

	Gene	CircWave oscillation		Acrophase (ZT)		Mesor		Amplitude		
D		**Tumor free kidney**	**Control kidney**	**Tumor free kidney**	**Control kidney**	**Tumor free kidney**	**Control kidney**	**Tumor free kidney**	**Control kidney**	
	Bmal1	P<0.001	P<0.001	0.6	20.4	2.4	1.2	3.3	2.2	-4.2
	Rev-erbα	P<0.001	P<0.001	8.3	3.6	1.8	2.8	2.3	5.3	-4.7
	Per2	P<0.001	P<0.001	16.4	11.6	2.6	2.1	2.2	3.8	-4.8
	Cry1	P<0.001	P<0.001	18.4	15.5	2.4	2.5	1.9	3.3	-2.9
	Dbp	P<0.001	P<0.001	10.8	8.0	1.8	1.2	3.6	2.5	-2.8
	Per1	P<0.05	P<0.001	11.5	10.2	2.3	3.4	0.8	3.3	-1.3
	Wee1	P<0.01	P<0.001	14.1	12.9	3.3	2.9	1.8	3.5	-1.2
	P21	P<0.01	P<0.01	23.1	23.0	2.1	2.2	2.9	2.9	-0.1

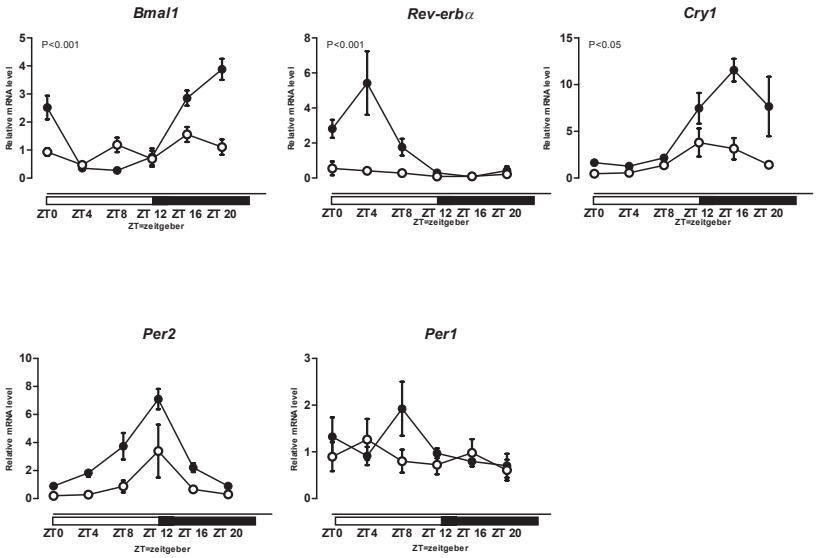

Figure 3. Circadian mRNA expression of core clock genes in colorectal liver metastases (○) and tumor-free liver (●). The relative mRNA expression of each gene of interest was normalized to *Tbp*, *Ywhaz* and *Beta-actin*. Each point represents the mean of 4 mice (±SEM). The sampling circadian times (ZTs) are relative to the time of light onset, ZT0. White rectangles indicate daily light span (ZT0 – ZT12) and black rectangles indicate nightly dark span (ZT12 – ZT20). P value indicates significance between liver metastases and tumor-free liver using multiway ANOVA analysis.

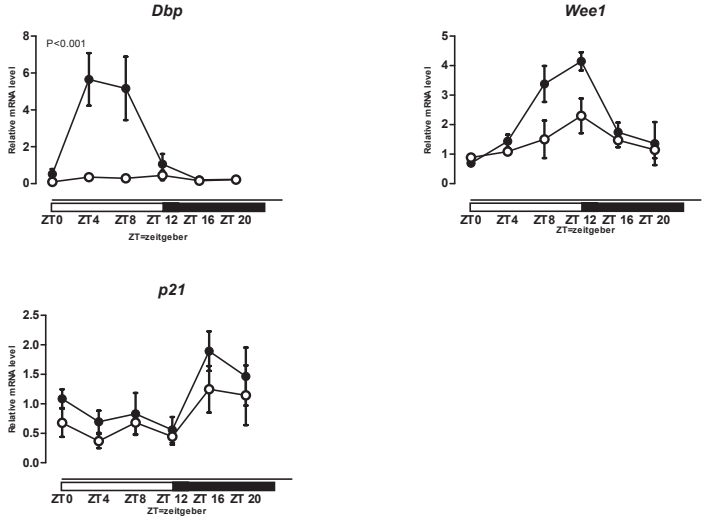

Figure 4. Circadian mRNA expression of clock-controlled genes in colorectal liver metastases (○) and tumor-free liver (●). The relative mRNA expression of each gene of interest was normalized to *Tbp*, *Ywhaz* and *Beta-actin*. Each point represent the mean of 4 mice (±SEM). The sampling circadian times (ZTs) are relative to the time of light onset, ZT0. White rectangles indicate daily light span (ZT0 – ZT12) and black rectangles indicate nightly dark span (ZT12 – ZT20). P value indicates significance between liver metastases and tumor-free liver using multiway ANOVA analysis.

In liver metastases, *Dbp* mRNA levels are strongly reduced (almost zero) and not oscillating (**Table 2B**). ANOVA analysis revealed significant differences between liver metastases and tumor-free liver for *Dbp* ($p<0.001$) but not for *Wee1* and *p21*. In contrast to *Dbp*, *Wee1* and *p21* showed small amplitude oscillations. However, using cosinor analyses tumor tissue showed only a circadian rhythm for *Wee1* ($p<0.05$; **Table 2B**). The mRNA expression levels of clock-controlled genes are explicitly lower in liver metastases compared to tumor-free liver. In summary, in line with the rhythmic clock gene expression in tumor-free liver, circadian rhythms have been observed for *Dbp*, *Wee1* and *p21* gene expression while, surprisingly, in the absence of an active core oscillator *Wee1* remains rhythmically expressed in liver metastases.

Clock gene expression in liver tissue of tumor bearing mice and healthy controls

To determine the effect of liver tumors on the circadian rhythm of the liver, we compared tumor-free liver of tumor bearing mice with the liver of healthy control mice for clock gene expression patterns. In the liver of healthy controls, circadian rhythms were observed for all core clock genes and clock-controlled genes that were tested (**Table 2C**). Interestingly, in tumor bearing liver a phase advance was found for *Rev-Erba* and *Dbp* (approximately 2.75 h), *Bmal1* (1.5 h), *Cry1* (1.2 h), and *Per2* (0.5 h) while clock-controlled *Wee1* and *p21* showed similar phase as in healthy control livers (**Fig5, Table 2C**). Since there is no circadian rhythm observed in tumor bearing liver tissue for *Per1*, it is not possible to compare this with the rhythm found in healthy control mice. Cosinor analysis showed circadian rhythms for all genes in healthy control livers (**Table 2C**).

Clock gene expression in the kidney

To determine whether the presence of tumor in the liver affects clocks in peripheral tissues (systemic) other than the liver (local), we measured circadian rhythmicity in kidneys of mice with liver metastases as well as in healthy controls. Again, circadian rhythmicity was detected for all clock- and clock-controlled genes (**Fig6, Table 2D**). Cosinor analysis showed a circadian rhythm in the kidneys of liver-tumor-bearing-mice as well in the kidneys of healthy controls (**Table 2D**). Phase delays of circadian oscillation were observed for all measured genes ranging from -0.1 h for *p21* expression to -4.8 h for *Per2* expression (**Table 2D**). Interestingly, *Per1*, *Wee1* and *p21* showed less robust differences between tumor bearing mice and healthy controls (**Fig6; Table 2D**). Thus, in the kidney we observed a phase delay whereas in the liver we found a phase advance in tumor bearing mice.

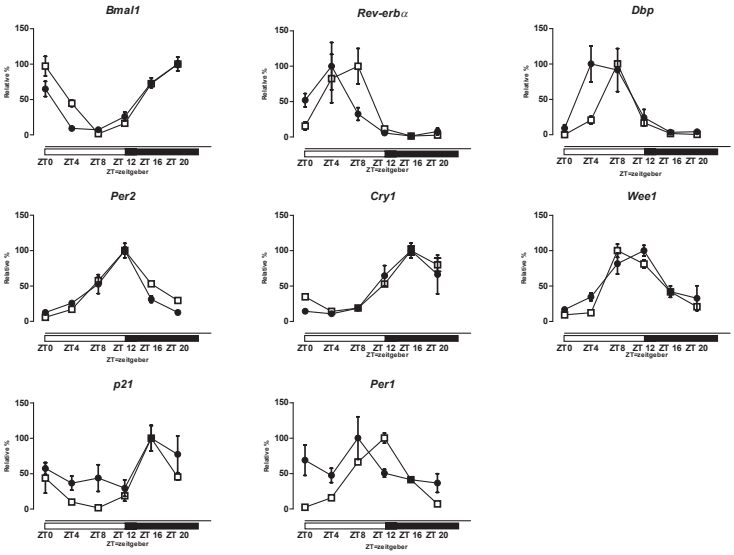

Figure 5. Clock gene expression in healthy and tumor bearing liver. Graphs represent percentages of relative mRNA expression where the highest value is set to 100% to compare results between healthy control liver (□) and tumor-free liver (●). Each point represent the mean of 4 mice (±SEM). The sampling circadian times (ZTs) are relative to the time of light onset, ZT0. White rectangles indicate daily light span (ZT0 – ZT12) and black rectangles indicate nightly dark span (ZT12 – ZT20).

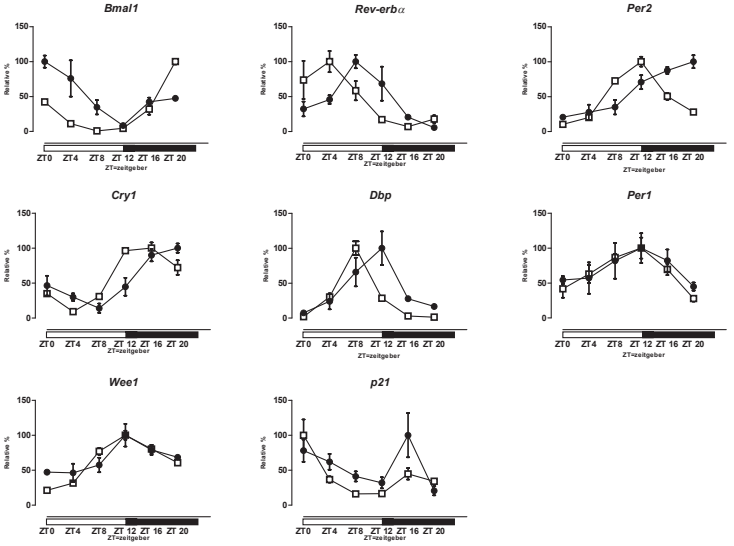

Figure 6. Clock gene expression in healthy kidney and in kidney from mice bearing liver metastases. Graphs represent percentages of relative mRNA expression where the highest value is set to 100% to compare results between healthy control kidney (□) and kidneys from mice bearing colorectal liver metastases (●). Each point represent the mean of 4 mice (±SEM). The sampling circadian times (ZTs) are relative to the time of light onset, ZT0. White rectangles indicate daily light span (ZT0 – ZT12) and black rectangles indicate nightly dark span (ZT12 – ZT20).

Discussion

The experiments presented here show that the circadian clock in C26 colorectal liver metastases is disrupted. We also observed a phase shift of the clock in healthy areas of the liver and in kidney tissue of tumor bearing animals. The circadian alterations are most pronounced in the tumor tissue obtained from our C26 liver metastatic tumor model. Core clock genes like *Bmal1*, *Rev-Erbα* and *Cry1* show a significant reduction in expression, absence of circadian rhythm, and reduction in circadian output, as demonstrated by a significant depression of *Dbp* gene expression in colorectal liver metastases. Other clock-controlled genes that regulate the cell cycle were deregulated as well. The expression of *Wee1*, a key regulator of mitosis, was severely repressed in the tumor compared to tumor-free liver. *Wee1* transcription is directly driven by the circadian timing system, as shown by low *Wee1* expression in *Clock*-mutant mice(4). Reduction of *Wee1* could result in less mitosis control and therefore faster cell cycling. For the CDK inhibitor *p21*, there were no statistical differences in mRNA expression levels.

To our knowledge, this is the first study that reveals changes in daily circadian rhythmicity in colorectal liver metastases, and shows that the presence of these metastases induces a circadian phase shift. In the liver a phase advance was observed whereas in the kidney a phase delay was found, suggesting that this is a systemic effect. In healthy control animals, the transcription of clock genes *Bmal1*, *Rev-Erbα*, *Per1*, *Per2* and *Cry1* varied largely as a function of circadian time in the liver. Peak times and amplitudes are consistent with those presented in previous reports (9, 10, 32). The expression of BMAL1, the transcription factor that heterodimerizes with CLOCK to drive the expression of genes, was completely disrupted in liver metastases, but showed normal 24- hour gene expression in tumor-free liver tissue. The perturbation in the tumor is in accordance with previous findings where *Bmal1* was also disrupted in a C26 colon carcinoma (33). The low expression of *Bmal1* in the tumor indicates that the core mechanism of the clock is less active. This is supported by the fact that other core clock genes as well as clock controlled genes show a lower expression in the tumor. In osteosarcoma bearing mice, circadian amplitudes were also dampened in the tumor compared to liver tissue (32).

While *Per2* did not oscillate in the tumor according to the cosinor analyses, there was a significant difference in expression level in time. This could be explained by the fact that *Per2* expression is not only controlled by the circadian clock, but also food driven in the liver. In the liver of $Cry1^{-/-}/Cry2^{-/-}$ mice under restricted (i.e. daytime) feeding conditions, *Per2* mRNA expression levels increase immediately after food intake (34). Postprandial rise in body temperature may activate *Per2* transcription via Heat Shock Factor (HSF) binding to *cis*-acting elements in the *Per2* promoter (35). The time of *Per2* induction between ZT 8 and 12 coincides with feeding activity, which underscores the food induced, rather than circadian induced *Per2* activity.

We found a phase advance in tumor-free liver tissue compared to healthy control livers for *Bmal1*, *Rev-Erbα*, *Cry1* and *Dbp* mRNA rhythms. Remarkably, in the kidneys from these tumor-bearing mice we found a phase delay for *Bmal1*, *Rev-Erbα*, *Per2*, *Cry1* and *Dbp*. This phase shift can be caused by different processes regulated systemically via the SCN or locally by the tumor itself. Tumor cells secrete a plethora of mediators such as TGF-β that is able to modulate the circadian clockwork by activating the ALK-SMAD3-DEC1 pathway. Activation of this pathway induces *Dec1* expression and resets the peripheral clock *in vivo*. Injection of TGF-β was shown to induce a phase shift in kidney and adrenal gland tissue. In the same study, it was shown that the phase-shifting effect of TGF-β was absent in *Dec1*$^{-/-}$ mice. Variation in TGF-β levels in peripheral tissues can provide phase shifts in different directions, both advanced and delayed (36). TGF-β could activate different pathways to entrain peripheral clocks. This might be a manner of the tumor to manipulate surrounding cells and escape from the natural tumor suppressive effect of the circadian clock. Alternatively, the phase shifts could be caused by other cytokines produced by host cells secondary to tumor invasion, that signal to the central nervous system via the SCN. For example, elevated levels of transforming growth factor-alpha (TGF-α) and IL-6 were correlated with altered behavior and dampened 24-hour rest-activity patterns in patients with metastatic colorectal cancer (37). In an animal model, micromolar infusion of TGF-α into the hypothalamic subparaventricular zone blocks signaling from the SCN. In this way hypothalamic signaling for the circadian regulation of i.e. sleep, motor activity and body temperature was impaired (38).

Cancer-related fatigue is one of the most prevalent symptoms experienced by cancer patients and is reported by as many as 40% of patients at time of diagnosis. It is not only highly prevalent, but also affects the patient's quality of life and daily activities in a negative way (39). There is a great lack in understanding cancer-related fatigue. Disruption of circadian rhythms in cancer patients has been documented mainly via salivary cortisol and locomotor activity (40). In a recent trial, patients received first-line chemotherapy for metastatic colorectal cancer, to identify toxic symptoms associated with circadian disruption. Patients with an impaired circadian function, showed a higher incidence of clinically relevant fatigue and weight loss (41). It may well be that the phase shifts observed in our study also occur in cancer patients, and provide a mechanistic basis to understand cancer-related fatigue.

In this experimental study we show a disrupted circadian clock in colorectal liver metastases, while liver and kidney still show circadian output. These results could support the hypothesis that the host circadian timing system is a target for tumor-related substances, also used in the field of chronotherapy. One of the first experimental studies in this field of research was performed by treating mice with 5-FU at different times of the day, showing that circadian timing of chemotherapy administration influences both toxicity and anti-tumor activity in mice (42). In more recent studies it has been shown

that chronotherapy in combination with highly toxic hepatic arterial infusion is feasible, well tolerated and resulted in a doubling of secondary surgical resection rate in patients with colorectal liver metastases (43, 44).

In a recent study by Sotak and coworkers, azoxymethane (AOM)/dextran sodium sulfate (DSS) induced colorectal tumors showed significantly reduced circadian mRNA expression levels compared with healthy colon tissue. Subsequent analysis of the liver, where AOM is primarily metabolized, revealed a temporal phase delay for *Bmal1*, *Rev-Erba*, *Dbp* and *Wee1* mRNA rhythms in AOM treated mice (19). Whether this phase shift is the result of the AOM treatment or induced by the colon tumor itself could not be determined. We show that the presence of C26 colon carcinoma metastases in the liver induces a phase advance in the adjacent liver tissue. These discrepancies in phase shifts could be explained by major differences in experimental models. Sotak and coworkers used a different mouse strain, older mice and induced cancer chemically by AOM treatment.

In summary, the present study shows for the first time that the core clock machinery is severely disrupted in a murine colorectal liver metastases model, and results in reduced expression levels of output genes such as *Dbp*. We also show that the presence of a tumor in the liver induces a phase shift in liver and kidney tissue clocks, strongly suggesting that this is a systemic effect. How these perturbations are involved in tumor progression and cancer related fatigue remain intriguing questions to be answered.

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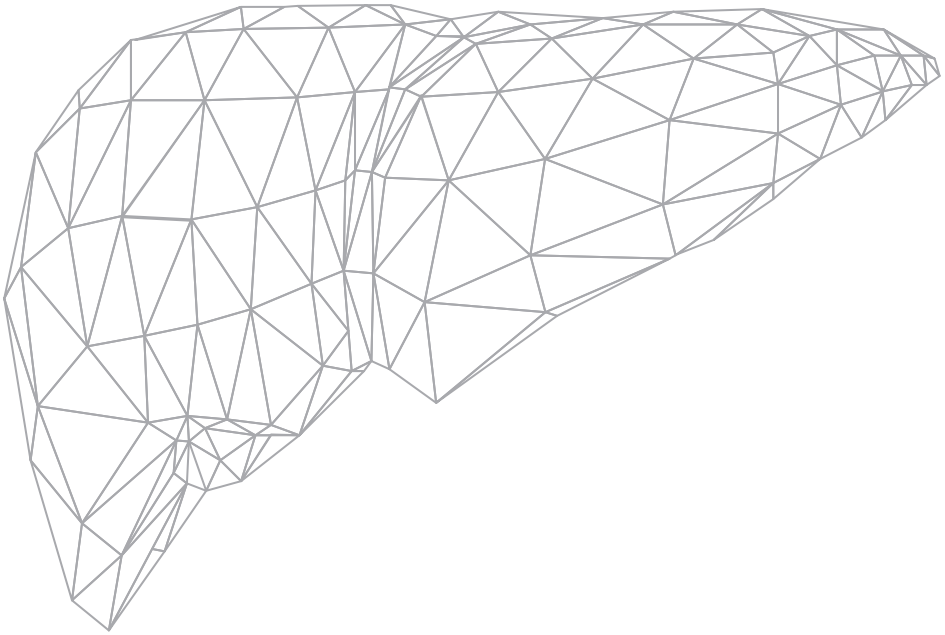

CHAPTER 4

DISRUPTION OF CLOCK GENE EXPRESSION IN HUMAN COLORECTAL LIVER METASTASES

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Submitted

Abstract

The circadian timing system controls about 10-15% of the transcriptome, and is important in the regulation of a wide variety of biological processes including metabolic and proliferative functions. Disruption of the circadian clock could have significant effect on human health, and has an important role in the development of cancer. Here, we compared the expression levels of core clock genes in primary colorectal cancer (CRC), colorectal liver metastases (CRLM), and liver tissue within the same patient. Surgical specimens of 15 untreated patients with primary CRC and metachronous CRLM were studied. Quantitative real-time polymerase chain reaction (qRT-PCR) was used to measure the expression of 10 clock genes: *CLOCK*, *BMAL1*, *PER1*, *PER2*, *PER3*, *CRY1*, *CRY2*, *CSNK1E*, Timeless (*TIM*), and timeless interacting-protein (*TIPIN*). Expression levels of 7 core clock genes were down-regulated in CRLM: *CLOCK* ($p=0.006$), *BMAL1* ($p=0.003$), *PER1* ($p=0.003$), *PER2* ($p=0.002$), *PER3* ($p<0.001$), *CRY1* ($p=0.002$), and *CRY2* ($p<0.001$). In CRC, 5 genes were down-regulated: *BMAL1* ($p=0.02$), *PER1* ($p=0.004$), *PER2* ($p=0.008$), *PER3* ($p<0.001$), and *CRY2* ($p<0.001$). *CSNK1E* was up-regulated in CRC ($p=0.02$). Related to clinicopathological factors, a significant correlation was found between low expression of *CRY1* and female gender, and low *PER3* expression and the number of CRLM. Our data demonstrate that the core clock is disrupted in CRLM- and CRC tissue from the same patient. This disruption may be linked to altered cell-cycle dynamics and carcinogenesis.

Introduction

The circadian timing system controls important biological processes, including metabolic and proliferative functions (1-3). The rhythmic behavior of these processes takes approximately 24 hours and is called circadian rhythm (rhythms of approximately one day). The circadian clock consists of a master oscillator which is located in the neurons of the suprachiasmatic nuclei (SCN) in the anterior hypothalamus of the brain (4-6). The master circadian oscillator coordinates peripheral circadian clocks through both the autonomic nervous system and neuroendocrine systems in most cells of the body (7). Peripheral circadian oscillators all consist of the same set of clock genes, but regulate their expression in a tissue specific way (8).

The human molecular clock system involves a set of core clock genes that act in transcription-translation feedback loops. The primary feedback loop consists of CLOCK (Circadian Locomotor Output Cycles Kaput) and BMAL1 (Brain-Muscle Arnt-Like protein 1) which heterodimerize and subsequently activate transcription of the *Cryptochrome* (*CRY1* and *CRY2*) and *Period* (*PER1*, *PER2*, and *PER3*) genes by binding to E-box elements in their promoters. The PER and CRY proteins translocate to the cytoplasm where PER proteins are phosphorylated by CKIε. Phosphorylated PER proteins are unstable and are degraded by ubiquitination. CRY proteins promote the formation of PER/CRY complexes and re-enter the nucleus, where they inhibit the transcription of their own genes by blocking CLOCK/BMAL1. This molecular core oscillator is coupled to circadian output processes through a series of clock-controlled genes (CCGs), which together regulate about 10-15% of the transcriptome (9, 10).

Perturbations in the function of circadian clock genes may have significant effects on human health, and may cause sleep disorders, depression, and gastrointestinal and cardiovascular diseases. Furthermore, the circadian timing system plays an important role in the development of cancer. Epidemiological studies have demonstrated that circadian disruption in shift-workers increases the risk of various epithelial cancers (11-14). An important part of the cell cycle is regulated by the circadian clock. CLOCK/BMAL1 directly regulates cell-cycle genes that control cell proliferation, DNA damage, and apoptosis. These CCGs include *Wee-1*, *c-Myc*, and *Cyclin D1*. Disruption of the circadian timing system may lead to a deregulated cell cycle which favors carcinogenesis (15).

It has been demonstrated that inhibition of *Per1* caused reduced apoptosis in HCT116 colon cancer cells, while overexpression of *Per1* leads to DNA damage-induced apoptosis (16). Inactivation of *Per2* caused deregulation of *Bmal1* expression which contributed to a high incidence of tumor formation. In addition, mice deficient in *Per2* showed an increase in tumor formation after γ-radiation (17). Recently we showed that the core clock machinery is severely disrupted in murine colorectal liver metastases (CRLM) and that the presence of tumor in the liver induces a phase-shift in liver and kidney tissue clocks (18).

In humans, CRLM worsen the prognosis of almost 60% of patients with colorectal cancer (19). In animal models the core clock machinery is disrupted in several types of cancer. The functioning of the circadian clock in patients with CRLM has remained unclear. A better understanding of how tumors affect the circadian clock may help elucidate the role of the clock in cancer patients. We therefore investigated the expression levels of core clock genes in human CRLM tissue, adjacent liver tissue, and the primary colorectal tumor. Furthermore, we related the expression levels to clinicopathological factors in these patients.

Material and Methods

Patients

Surgical resection specimens of the primary colorectal tumor, liver metastases, and adjacent normal liver tissue were obtained from 15 CRLM (male: 8, female: 7) patients who did not receive neo-adjuvant chemotherapy treatment. The patients underwent surgery at the Erasmus MC Cancer Institute, Erasmus University Medical Center, Rotterdam, The Netherlands between January 2005 and January 2012. Clinical data including tumor characteristics of these patients are shown in **Table 1**. All operations started between 8:00 AM and 11:00 AM and mean time patients were in the operation room was 3.30h. All tissues were collected between 09:00 AM and 13:30 PM and immediately frozen into liquid nitrogen and stored at -80^{o}C until further analysis. Informed consent was obtained from all patients and the study was approved by the Ethics Committee at our institution.

Sampling procedure

All surgical resection specimens from primary colorectal tumor, liver metastases, and adjacent normal liver tissue were first macroscopically, than microscopically identified by an experienced pathologist. Frozen resection specimens were retrieved from the archives of the pathology department and at least 1 cm in diameter of viable tumor tissue was included using a frozen tissue slicer (Leica CM1850 UV, Leica Biosystems). There was no admixture of stromal tissue and no necrosis was identified in the included samples. Tumor characteristics are shown in **Table 1**.

Fresh frozen tissue, RNA extraction, and cDNA synthesis

RNA was isolated from all tissues by phenol extraction using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The amount of extracted RNA was measured by Nanodrop Spectrophotometry (Nanodrop Technology, Wilmington, DE, USA). To avoid genomic DNA contamination, RNA was purified by DNAse treatment (RQ1 RNase-Free DNase; Promega, Madison, WI, USA). RNA was then

Table 1. Characteristics of clinicopathological factors from 15 patients evaluated for circadian rhythm and outcome

	N	(%)	Male/Female
Age (years), mean (±SD)	67.5 ± 9.8		M 66.9 ± 10.2
			F 68.3 ± 10.0
Sex			
Male (M)/ Female (F)			8/7
Number of metastases			
1	7	(46.7)	M4/F3
2	3	(20.0)	M2/F1
3	2	(13.3)	M1/F1
4	1	(6.7)	M0/F1
5	1	(6.7)	M0/F1
6	1	(6.7)	M1/F0
Diameter of largest metastasis (cm)			
1.20-2.20	5	(33.3)	M2/F3
2.40-4.40	7	(46.7)	M4/F3
5.00-9.00	3	(20.0)	M2/F1
CRLM in number of segments			
1	4	(26.7)	M3/F1
2	6	(40.0)	M3/F3
3	1	(6.7)	M0/F1
4	4	(26.7)	M2/F2
Primary tumor location			
Ascending colon	2	(13.3)	M1/F1
Transverse colon	-	-	-
Descending colon	5	(33.3)	M4/F1
Ascending + descending colon	1	(6.7)	M0/F1
Rectum	7	(46.7)	M3/F4
Histological type CRC			
Moderately differentiated adenocarcinoma	13	(86.7)	M7/F6
Poorly differentiated adenocarcinoma	2	(13.3)	M1/F1
Depth of tumor invasion CRC			
T1	-	-	-
T2	4	(26.7)	M2/F2
T3	9	(60.0)	M5/F4
T4	1	(6.7)	M1/F0
Missing	1	(6.7)	M0/F1
Lymph node metastasis CRC			
N0	8	(53.3)	M5/F3
N1-N2	7	(46.7)	M3/F4

reverse transcribed into cDNA using random primers (Invitrogen) and Superscript II RT (Invitrogen). cDNA samples were stored at -20°C until further analysis.

Quantitative Real-Time Reverse Transcriptase Polymerase Chain Reaction (qRT-PCR)

Gene expression was analyzed by real-time quantitative PCR to assess differential expression of clock genes in CRC tissue, CRLM, and adjacent liver tissue using an Applied Biosystems 7700 PCR machine (Foster City, CA, USA). RT-PCR was performed using SYBR Green-based Quantitect Primer Assay (Qiagen, Venlo, The Netherlands) for 10 clock transcripts; *CLOCK* (QT00054481), *BMAL1* (QT00011844), *PER1* (QT00069265), *PER2* (QT00011207), *PER3* (QT00097713), *CRY1* (QT00025067), *CRY2* (QT00094920), *TIM* (QT00019789), *TIPIN* (QT00054334), and *CSNK1E* (QT02323916). PCR reactions were carried out in a total volume of 25 μL using the Quantifast SYBR Green PCR kit (Qiagen, Venlo, The Netherlands). Each sample was tested in triplicate according to the following PCR protocol: 10 min at 50°C, 5 min at 95°C, followed by 40 cycles at 95°C for 10 seconds, and at 60°C for 30 seconds. ΔCt values of the genes of interest were calculated as described by the method of Pfaffl et al. using the glutaraldehyde-3-phosphate dehydrogenase (*GAPDH*; QT00079247) as a housekeeping gene (20). *GAPDH* is a commonly accepted marker for normalization of qPCR data obtained from human tissues. Triplicate values of GAPDH showed low standard deviations, and a one-way ANOVA analysis between GAPDH values of all tissues showed no significant differences ($p>0.05$). ΔCt values were normalized to the average ΔCt of the normal liver tissue. The fold change was calculated using the Pfaffl equation, $2^{-\Delta\Delta Ct}$. Results are expressed as median with the interquartile range (IQR). The IQR is the difference between the upper and lower quartiles ($IQR= Q_3 - Q_1$).

Statistical analysis

Gene expression levels of CRLM, and CRC were compared with those of adjacent liver tissue and calculated using the Pfaffl equation, $2^{-\Delta\Delta Ct}$. To assess the statistical significance of the up- or down-regulation of genes the Wilcoxon signed-rank rest was used. Correlation analyses between gene expression levels and all nine clinical pathological factors was performed using Spearman correlation. All analyses were corrected for multiple testing by the Bonferroni method. All statistical tests were two-sided and performed using SPSS 21 for Windows software (Statistical Package for Social Sciences, Chicago, IL). $p < 0.05$ was considered to be significant, unless otherwise mentioned.

Results

Clock gene mRNA expression in CRLM compared to adjacent liver

To compare clock gene expression in CRLM with adjacent normal liver tissue, we analyzed mRNA expression levels of 10 clock genes (*CLOCK*, *BMAL1*, *PER1*, *PER2*, *PER3*, *CRY1*, *CRY2*, *CSNK1E*, *TIM*, *TIPIN*). Liver metastasis and liver tissue were used from 15 patients. Relative mRNA expression levels of clock genes in the liver and CRLM are presented in **Figure 1**. Tumor samples were normalized to the average ΔCt of the liver tissue, and 7 genes were subsequently observed to be down-regulated in CRLM: *CLOCK* (median=0.46, Q1-Q3=0.22-0.75, p=0.006), *BMAL1* (median=0.21, Q1-Q3=0.14-0.36, p=0.003), *PER1* (median=0.14, Q1-Q3=0.05-0.52, p=0.003), *PER2* (median=0.63, Q1-Q3=0.25-0.86, p=0.002), *PER3* (median=0.14, Q1-Q3=0.04-0.37, p<0.001), *CRY1* (median=0.50, Q1-Q3=0.32-0.98, p=0.002), and *CRY2* (median=0.31, Q1-Q3=0.09-0.61, p<0.001). The expression levels of TIM and TIPIN showed no significant difference (median=0.81, Q1-Q3=0.73-1.04, p=0.54, and median=1.05, Q1-Q3=0.62-1.48, p=0.74, respectively).

Clock gene mRNA expression in the primary colon tumor compared to liver tissue

To determine whether clock gene expression was also impaired in the primary tumor, we measured mRNA expression levels of colorectal tumors of the same patients. Five of 10 genes were down-regulated namely *BMAL1* (median=0.37, Q1-Q3=0.16-0.53, p=0.02), *PER1* (median=0.13, Q1-Q3=0.06-0.6, p=0.004), *PER2* (median=0.33, Q1-Q3=0.17-0.80, p=0.008), *PER3* (median=0.09, Q1-Q3=0.04-0.22, p<0.001), *CRY2* (median=0.11, Q1-Q3=0.07-0.20, p<0.001). Again, *CSNK1E* was up-regulated (median=1.35, Q1-Q3=1.11-2.22, p= 0.02). The expression of four genes did not show significant differences: *CLOCK* (median=0.73, Q1-Q3=0.29-1.16, p=0.43), *CRY1* (median=0.51, Q1-Q3=0.47-1.30, p=0.23), *TIM* (median=1.04, Q1-Q3=0.78-1.23, p=0.52), *TIPIN* (median=1.11, Q1-Q3=0.53-1.43, p=0.70) (**Fig1**).

Relation between clock gene mRNA expression levels and clinicopathological factors

Patients clinical and pathological features were retrieved using the electronical patient database (**Table 1**), and associations between mRNA expression levels and clinicopathological factors were determined. A statistically significant correlation was found between CRLM mRNA levels of *PER3* and the number of metastases. Lower *PER3* mRNA levels were found with an increasing number of metastases (r= 0.645, p=0.009) (**Fig2A**). Another significant correlation was found between CRLM *CRY1* mRNA levels and patient gender. Lower *CRY1* mRNA levels were found in female patients compared to male patients (r=0.700, p=0.005) (**Fig2B**). There were no other significant correlations found between mRNA expression levels and clinicopathological factors.

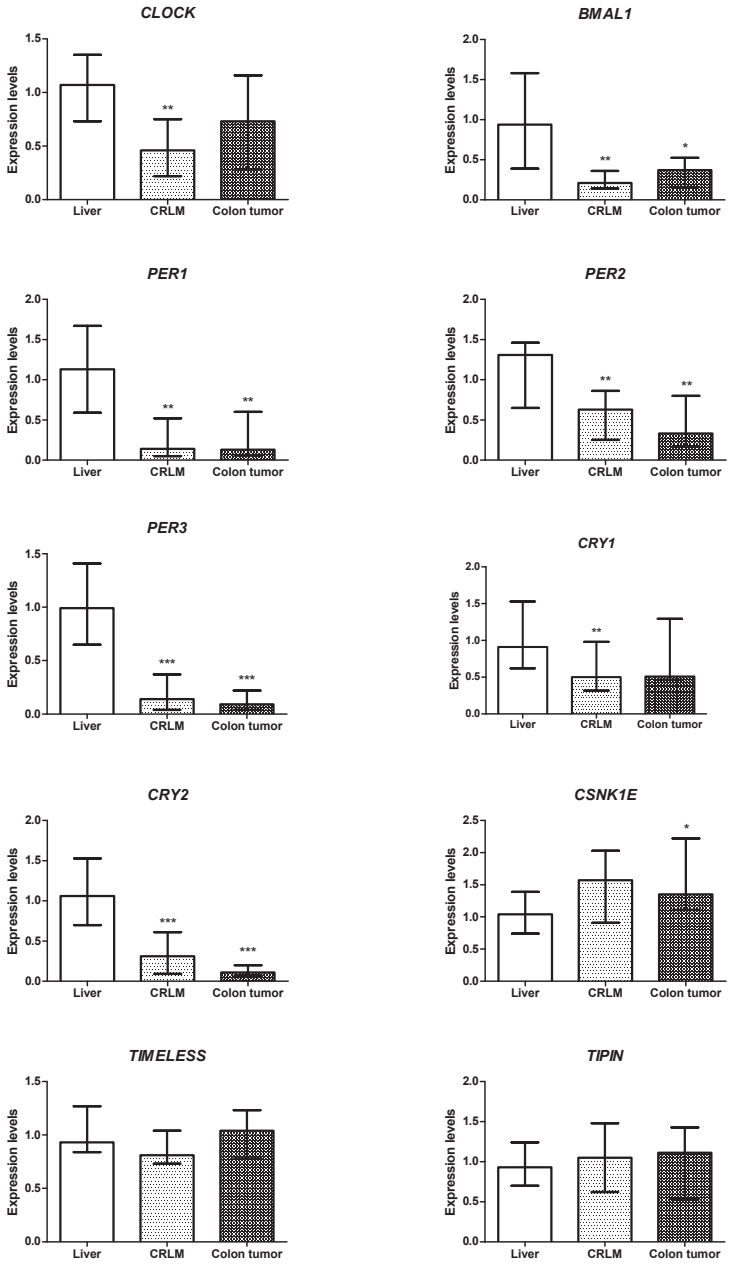

Figure 1. mRNA expression levels of clock- and clock-controlled genes in liver, colorectal liver metastases (CRLM), and colon tumor. The relative mRNA expression of each gene of interest was normalized to glutaraldehyde-3-phosphate dehydrogenase (*GAPDH*). ΔCt values were normalized to the average ΔCt of the normal liver tissue. For each gene, boxes show the median with the interquartile range (IQR= Q3-Q1). Asterisks indicate significance of the difference in expression of each gene in liver as compared to CRLM and CRC as assessed by the Wilcoxon signed-rank test (*p<0.05, **p<0.01, ***p<0.001).

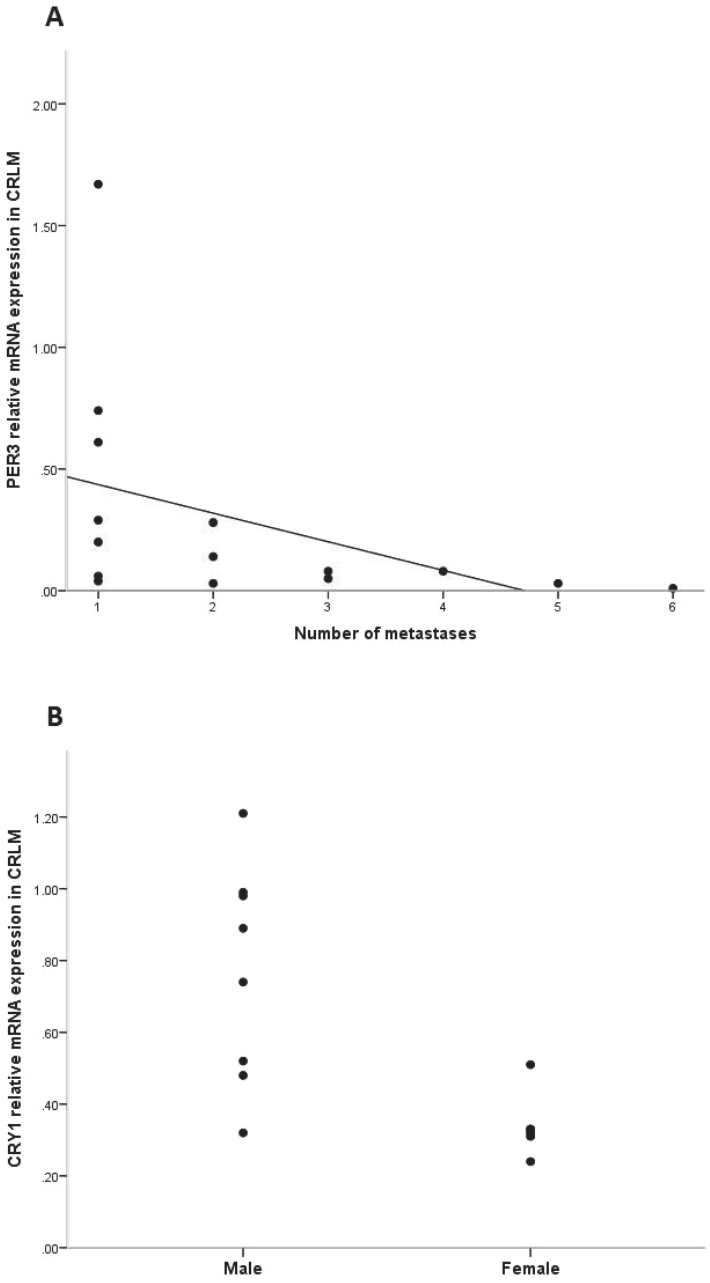

Figure 2. Correlation between clock gene mRNA expression levels, CRLM, and gender. (A) The correlation between *PER3* mRNA expression levels in CRLM and the number of metastases, evaluated by Spearman test (r= 0.645, p=0.009). **(B)** The correlation between *CRY1* mRNA expression levels in CRLM and patient gender evaluated by Spearman test (r=0.700, p=0.005).

Discussion

In the current study, we examined the expression levels of clock genes in colorectal liver metastases (CRLM), the primary colorectal tumor, and liver tissue in surgical resection specimens of CRC patients. We also studied possible relations between gene expression levels and clinical and pathological factors of these patients. We used quantitative real-time polymerase chain reaction (qRT-PCR) to identify the expression levels of *CLOCK*, *BMAL1*, *PER1*, *PER2*, *PER3*, *CRY1*, *CRY2*, *CSKN1E*, *TIM*, and *TIPIN*. We observed a down-regulation of core clock mRNA levels in both liver metastases and colorectal cancer. The genes encoding CLOCK and BMAL1, the two core clock proteins that heterodimerize and drive transcription of clock (controlled) genes, were both significantly down-regulated in CRLM. *BMAL1* expression was also lowered in colorectal tumors. In line with the lower expression levels of *CLOCK* and *BMAL1*, genes activated by the CLOCK/BMAL1 complex, such as *PER1*, *PER2*, *PER3*, *CRY1*, and *CRY2* all show a significant reduction in expression compared to normal liver tissue. The only gene that was significantly up-regulated in the primary tumor was *CSKN1E*. We observed no differences in the expression levels of *TIM*, and *TIPIN*.

To our knowledge this is the first study describing severe down-regulation of clock genes in human CRLM. Our findings are in line with previous studies describing circadian disruption in other malignancies. In more than 95% of breast cancer tissue from 55 women, expression levels of *PER1*, *PER2*, and *PER3* were severely disrupted in comparison with adjacent non-cancerous tissue (21). Pancreatic cancer has a low incidence rate, but is very aggressive with high mortality rates. Especially *PER1* and downstream effectors of the circadian clock are lower expressed in pancreatic cancer which further suggests they may act as tumor suppressor genes in healthy tissue (22). Our data are supported by a study in human primary colorectal cancer. A down regulation in expression of *BMAL1*, *PER1*, *PER2*, *PER3*, and *CRY2* was found. Furthermore, differential expression of clock genes was associated with differences in survival (23). In a study of 202 untreated CRC patients, *PER1* and *PER3* expression levels were significantly lower compared to normal tissue. In contrast, the expression of *CLOCK* and *CKIε* was significantly higher in cancer tissue. *PER2* was shown to be differentially expressed related to survival, with a better survival corresponding with a high *PER2* expression (24).

In this study, the only gene that was significantly up-regulated in CRC and showed a trend towards increased expression in CRLM was *CSNK1E*. The *CSNK1E* gene encodes the CKIε protein, whose main function is to regulate circadian rhythm by phosphorylation and degradation of Period genes (25). We showed that *PER1*, *PER2*, and *PER3* gene expression levels were all lower in cancer tissue than in liver tissue, while *CSNK1E* gene expression was higher in cancer tissue. The decreased expression levels of both transcription activator-, (BMAL1) and transcription inhibitor genes (CRYs and PERs) suggest

that the clock in the primary tumor and CRLM may be dampened and/or less robust. Up-regulation of *CSNK1E* may lead to enhanced phosphorylation of the PER2 protein which is known to destabilize the PER protein and target it for ubiquitination and subsequent proteosomal degradation. Furthermore, *CKIε* plays an essential role in the early development of CRC. *CKIε* is involved in cell proliferation by stabilizing β-catenin and mimicking the effect of WNT-signaling. Subsequently, this will lead to increased levels of β-catenin in the nucleus to control transcription and maintain tumorigenesis (26, 27). Knocking down *CSNK1E* in a human sarcoma cell-line led to growth inhibition of cells, and *CSNK1E* was found to be up-regulated in ten different human cancer tissues compared to normal tissue (28).

In contrast to others, we found no significant difference in *TIMELESS* (*TIM*) and timeless-interacting protein (*TIPIN*) mRNA expression levels. These genes interact with components of the DNA replication system to regulate DNA replication processes under normal and stress conditions and are essential in regulating different phases of the cell-cycle (29). Down-regulation of *TIM* increased doxorubicin toxicity in HCT116 cancer cells, and it is suggested that *TIM* inhibition could be used to enhance cytotoxic effectiveness of chemotherapeutic drugs (30). Down-regulation of *TIM* and *TIPIN* was found in kidney cancer patients compared to normal kidney tissue (31).

We related the expression levels of clock genes to clinical and pathological factors. Low expression of *PER3* was correlated with a higher number of metastases. In another study, low *PER1* expression was correlated with the development of CRLM in CRC patients (24). We also found a significant correlation between gender and the expression of *CRY1* in CRLM. The lowest levels of *CRY1* mRNA expression were found in female patients. This correlation was also found in a study where differential expression levels of core clock genes were determined in tumor specimens of CRC patients (23). The fact that female patients show lower *CRY1* expression levels could be related to a difference in metabolic pathways and xenobiotic detoxification between genders. In the Chronotherapy Group Trial, including a schedule of chronomodulated delivery of chemotherapy, female patients were shown to have shorter survival and greater toxicity when treated with 5-fluorouracil and leucovorin (32).

The mRNA expression levels of CRLM of all core clock genes in this study show differential expression compared to liver tissue. These results support the hypothesis of the apparent coupling between the circadian rest-activity cycle and the time-dependent toxicity of drugs, which may be exploited in the field of chronotherapy. The basis of chronotherapy relies on the principle of administering chemotherapy at times when toxicity is expected to be lowest (33, 34). A phase III study in CRC patients has shown better tolerability and anti-tumor activity compared with conventional therapy when chemotherapy was administered according to least toxic dosing times (32). In a phase II study, patients with unresectable CRLM were treated with chronotherapy and highly

toxic hepatic arterial infusion which resulted in a doubling of secondary surgical resection rates (35).

Albeit our study shows evidence of a disrupted timing system in CRLM and CRC in patients, we studied a relatively small cohort of patients. By expanding the number of patients, more correlations might be found between mRNA expression levels and clinicopathological factors. The mRNA expression levels of CRC are normalized to the levels of adjacent liver, but not colon tissue. Based on experiments with rats, it is expected that the circadian timing system in the colon is in phase with that in the liver (36). Furthermore, we were only able to study gene expression at the timepoint at which the resection specimen was obtained. Since patients are operated on at different times of the day, and surgical resections are not procedures with a fixed time frame, this is a limitation inherent to a clinical study. To further elucidate this issue, we are currently investigating the impact of clock gene expression levels in cancer cells in vitro by knocking down and overexpressing clock genes in various tumor cell lines, followed by systematic phenotyping of cancer properties of the cell (i.e. proliferation rate, cell migration and invasion properties, and drug sensitivity).

In summary, the present study shows that there are differences in clock gene expression in CRLM- and CRC tissue compared to liver in patients without neo-adjuvant chemotherapy treatment. The differential expression might be related to carcinogenesis, tumor burden and survival, and supports the application of chronomodulated chemotherapy.

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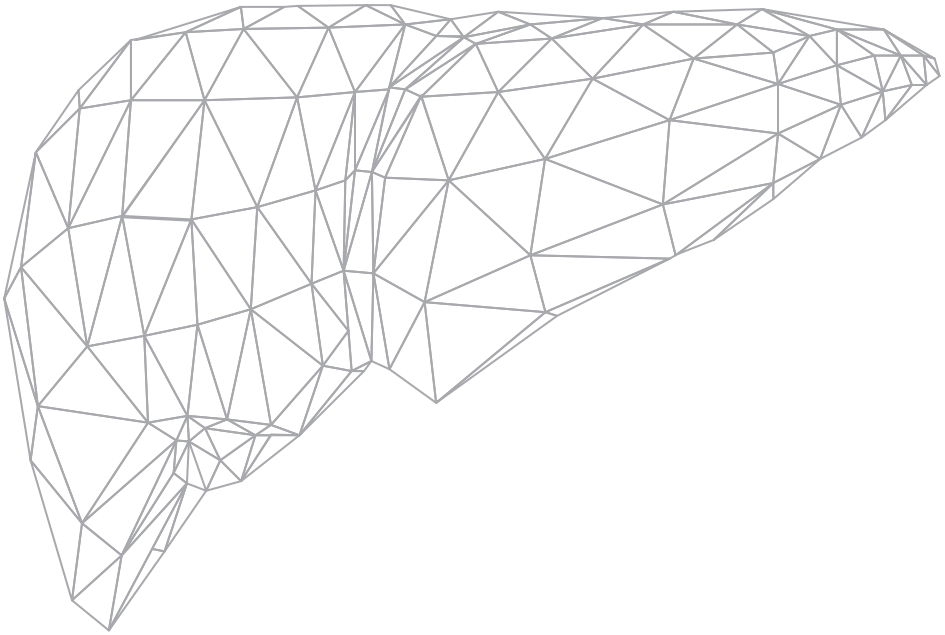


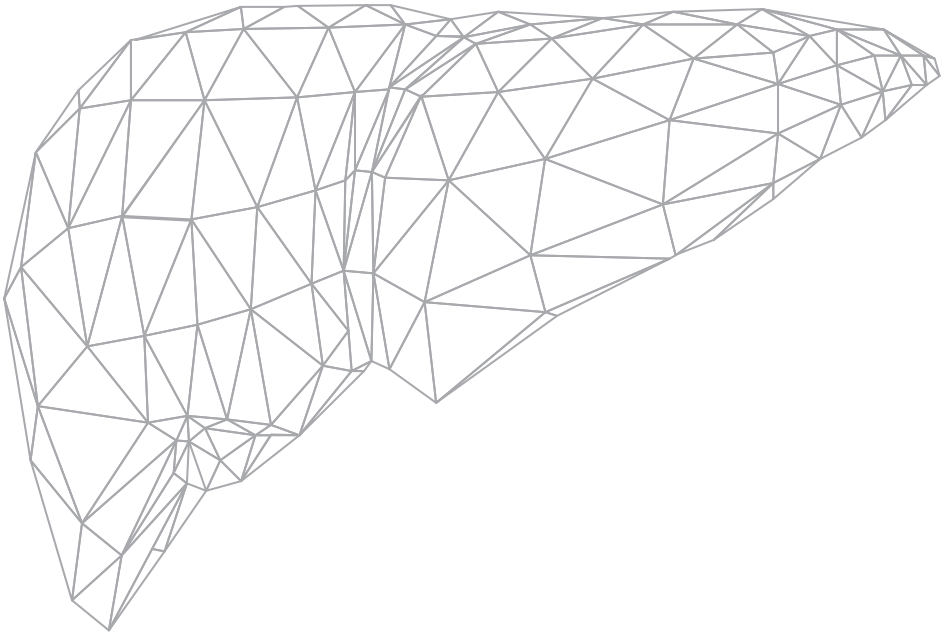
PART THREE

DIETARY RESTRICTION AND PROTECTION AGAINST CHEMOTHERAPY INDUCED TOXICITY

Chapter 5 **Fasting protects against the side effects of irinotecan but preserves its anti-tumor effect in Apc15lox mutant mice**
Cell Cycle, 2015;14(14):2333-9

Chapter 6 **Fasting protects against the side-effects of irinotecan treatment but does not abrogate anti-tumor activity in mice**
British Journal of Pharmacology. 2016, Mar; 173(5): 804-14

Chapter 7 **Transcriptomic analysis of the response to irinotecan in fasted tumor bearing mice**
In preparation



CHAPTER 5

FASTING PROTECTS AGAINST THE SIDE EFFECTS OF IRINOTECAN BUT PRESERVES ITS ANTI-TUMOR EFFECT IN APC15LOX MUTANT MICE

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Cell Cycle, 2015;14(14):2333-9

Abstract

Irinotecan is a widely used topoisomerase-I-inhibitor with a very narrow therapeutic window because of its severe toxicity. In the current study we have examined the effects of fasting prior to irinotecan treatment on toxicity and anti-tumor activity. Fabpl*Cre*;Apc$^{15lox/+}$ mice, which spontaneously develop intestinal tumors, of 27 weeks of age were randomized into 3-day fasted and *ad libitum* fed groups, followed by treatment with a flat-fixed high dose of irinotecan or vehicle. Side-effects were recorded until 11 days after the start of the experiment. Tumor size, and markers for cell-cycle activity, proliferation, angiogenesis, and senescence were measured. Fasted mice were protected against the side-effects of irinotecan treatment. *Ad libitum* fed mice developed visible signs of discomfort including weight loss, lower activity, ruffled coat, hunched-back posture, diarrhea, and leukopenia. Irinotecan reduced tumor size in fasted and *ad libitum* fed groups similarly compared to untreated controls (2.4 ± 0.67 mm and 2.4 ± 0.82 mm *versus* 3.0 ± 1.05 mm and 2.8 ± 1.08 mm respectively, $p<0.001$). Immunohistochemical analysis showed reduced proliferation, a reduced number of vascular endothelial cells, and increased levels of senescence in tumors of both irinotecan treated groups. In conclusion, 3 days of fasting protects against the toxic side-effects of irinotecan in a clinically relevant mouse model of spontaneously developing colorectal cancer without affecting its anti-tumor activity. These results support fasting as a powerful way to improve treatment of colorectal carcinoma patients.

Introduction

Irinotecan is extensively used in first and second line treatment for unresectable and metastatic disease of colorectal cancer (1). In 1996, approval was obtained in the United States for the second-line treatment of patients with metastatic colorectal carcinoma. Today, irinotecan is approved as a single agent as well as in combination with other drugs (i.e. oxaliplatin, and fluorouracil) for treatment of colorectal cancer (2, 3). It is a pro-drug of the topoisomerase I inhibitor SN-38, and is metabolized by CYP3A into inactive metabolites (4). Irinotecan is typically known for its narrow therapeutic window, which explains its unpredictable toxicities, including severe myelosuppression, massive diarrhea, and in some cases even death as a complication of other side-effects (5, 6). These side-effects may lead to dose reductions and early discontinuation of treatment, and limit the anti-tumor activity of this chemotherapeutic agent (7).

A potentially protective intervention against these severe side-effects is dietary restriction (DR), i.e. a reduction of caloric intake before drug delivery. We previously showed that 3-days of fasting up-regulates cytoprotective and antioxidant enzymes, and induces protection against oxidative stress (8, 9).

While much information is obtained from studies that revealed beneficial effects of fasting and DR using transplantable tumor models, there is a need to perform these studies in more clinically relevant animal models (10-12). Fabpl*Cre*;Apc$^{15lox/+}$ mice express an adenomatous polyposis coli (*APC*) mutant allele, *Apc*^{15lox}, based on *loxP* sites flanking exon 15, and a Cre-mediated knockout by deletion of this exon (13). These mice are genetically predisposed to develop macroscopic adenomas in the distal small and large intestine emerging at approximately 3 months of age. Humans with germ-line inactivating mutations of this gene are also predisposed to develop many adenomatous polyps in the colon and rectum, a hereditary cancer syndrome called familial adenomatous polyposis (FAP). In addition, 80% of the sporadic colorectal tumors are initiated by a germ-line mutation in *APC* (14, 15).

To study the role of APC in prevention and treatment of colorectal cancer, many inactivating mutant alleles of the mouse *Apc* gene were investigated. Morbidity and mortality rates of most of these models were high at a rather young age (16, 17). An advantage of the conditional *Apc*-mutant mouse model discussed here is the slow onset of tumor development, mimicking the growth of a spontaneously developing tumor in a more mature mouse model. Furthermore, this model accounts for neoplasia in the small intestine as well as in the large intestine, which makes it a relevant model to study colorectal cancer.

In this study we examined the effects of fasting before administration of a high dose irinotecan on the occurrence of side-effects, and number and size of tumors in this conditional *Apc*-mutant mouse model for colorectal carcinoma. In addition, we performed

immunohistochemistry on tumor tissue to compare the anti-tumor activity of irinotecan in fasted and *ad libitum* fed animals.

Materials and methods

Animals

FabplCre;Apc15lox/+ mice (n=36) with a C57Bl/6 background were generated, bred, and maintained under pathogen-free conditions at a licensed biomedical animal facility, Erasmus University Medical Center, Rotterdam, the Netherlands, as previously reported (13). During 27 weeks they were housed in individually ventilated cages (n=3-4 animals per cage) where standard laboratory conditions were maintained, i.e. temperature ~22ºC, humidity ~50%, and a 12 h light/12 h dark cycle. During this period, there were no visible signs of disease in the studied animals. All mice had free access to water and food (Special Diet Services, Witham, UK) unless mentioned otherwise. Previously we have shown that after 27 weeks of age, intestinal tumors were macroscopically present. Mice were divided into four groups; 1. Ad libitum group (n=9; males (m) n=4, females (f) n=5), 2. Fasted group (n=9; m, n=4, f, n=5), 3. Ad libitum group with irinotecan treatment (n=10; m, n=5, f, n=5), and 4. Fasted group with irinotecan treatment (n=8; m, n=4, f, n=4). The experimental protocol was approved by the Animal Experiments Committee under the Dutch National Experiments on Animals Act, and complied with the 1986 directive 86/609/EC of the Council of Europe.

Fasting

The two *ad libitum* fed groups were allowed unrestricted access to food. Before the start of the experiment, all mice were transferred to a clean cage and the 2 fasted groups were withheld food for 3 days starting at 4:00 PM and were fed *ad libitum* again 3 days later at 10:00 AM. All animals were given continuous access to water. No mortality occurred during fasting.

Chemotherapy

Irinotecan, HCl-trihydrate 20 mg/mL (Hospira, Benelux) was diluted in sodium chloride 0.9% (Braun, Melsungen, Germany) to a final volume of 200 µL per injection, and was given intraperitoneally. The optimal cumulative drug dose (400 mg/kg) was determined in a pilot experiment and defined as the concentration that induces severe toxicity without causing mortality (data not shown). All animals received the same amount of irinotecan, called a flat-fixed dose, based on the average weight of *ad libitum* fed male mice, which was 25 gram. All animals in the *ad libitum* fed group as well as in the fasted group received 167 µL irinotecan supplemented with 33 µL sodium chloride 0.9% per injection.

Experimental setup

All mice in the irinotecan treated groups received a cumulative flat-fixed dose of 400 mg/kg irinotecan. On days 1,3 and 5 after fasting, mice received 133 mg/kg irinotecan . The control groups received vehicle (sodium chloride 0.9%). Following the first irinotecan injection mice were weighed and scored daily for adverse side effects by a mouse wellbeing-score protocol adapted from 'the guidelines for welfare of animals in experimental neoplasia research' (1988: United Kingdom Co-ordinating Committee on Cancer Research, UKCCCR). Assessing wellbeing by one researcher took 20 minutes per cage with 4 mice. Side-effects were scored independently by two experienced researchers. Mouse cages were removed from racks and placed on a bench to facilitate improved visualization of mice, but cages were not opened at any point during the scoring process, except for the determination of the stool consistency at the end of the process. Mouse activity level was scored according to the amount each mouse moved in its cage. A score of 2 indicates an animal that moved around the cage normally. A score of 1 indicates an animal that was moving slowly with an altered gait. A score of 0 indicated an animal that was not moving and was taking no more than 5 steps. The appearance of the coat was scored according to the smoothness. A score of 2 indicated a healthy, smooth uninterrupted coat. A score of 1 indicated a slightly fluffy coat. A score of 0 indicated a severe fluffy coat with evident parts of visible skin. Posture was scored according to a hunched appearance. A score of 2 indicated a normal body posture. A score of 1 indicated a moderately hunched posture. A score of 0 indicated a severely hunched posture. Severity of diarrhea was assessed according to the stool consistency score (0: normal, 1: loose stool, 2: loose/some diarrhea, 3: diarrhea, 4: severe watery diarrhea) (34). Before every stool consistency measurement clean white tissues were placed at the bottom of the cage to allow determination of the consistency of the stool. Results are expressed as mean ± SD. Leukocyte numbers were determined on day 8 after the first irinotecan injection with a Z series Coulter Counter (Beckman Coulter, Woerden, The Netherlands). Ten days after the first irinotecan injection mice were sacrificed by exsanguination.

Determination of tumor burden

Directly after sacrificing the animals, the entire gastrointestinal tract was removed for dissection but the stomach, duodenum and cecum were omitted from the analysis because of their low tumor incidence. The intestinal tract was opened along the cephalocaudal axis, flushed with phosphate-buffered saline (PBS), cut into five segments of approximately equal lengths, and spread out flat on filter paper. These preparations were fixed overnight at 4°C in 10% phosphate-buffered formalin and thereafter stored in 70% EtOH until further analysis. Tumor enumeration was performed using a dissecting microscope. Diameter of each tumor was determined using an ocular micrometer that allows precise measurements with a resolution of 0.1 mm. The smallest detected

tumor was 0.8 mm. All tumors were scored by two experienced researchers blinded to the treatment.

Immunohistochemistry

After determination of tumor size, 4 tumors per intestinal tract were embedded in paraffin, sectioned at 5µm, and stained with the following antibodies: 1. polyclonal antibody against pHiston H3 (Ser10; 9701S, dilution 1:200, Cell Signaling, Danvers, MA). 2. monoclonal antibody against Cyclin D1 (VP-RM03, dilution 1:500, Bio-Connect, Huissen, The Netherlands). 3. Monoclonal antibody against Ki-67 (D3B5; 12202S, dilution 1:500, Cell Signaling, Danvers, MA). 4. Monoclonal antibody against CD146 (EPR3208; ab75769, dilution 1:200, Abcam, Cambridge, UK) 5. Monoclonal antibody against p21 (Waf1/Cip1; 12D1; 2947S, dilution 1:500, Cell Signaling, Danvers, MA). All primary antibodies were visualized with a polyclonal horseradish peroxidase-Y-conjugated secondary antibody (goat-anti-rabbit IgG/HRP, P0448, dilution 1:500, DAKO, Denmark). Slides were scored using the number of positive cells for pHiston H3, CD146, and p21. For Cyclin D1, and ki-67 the percentage of positive cells was used. Two independent observers blinded to the treatment scored the slides at a magnification of 200-400x. Five microscopic fields per tumor were measured. Results are expressed as mean ± SD.

Statistical analyses

Categorical data are presented as number (percentage) and continuous variables as mean ± SD. Means between two groups were compared using the t-test for parametric data. One-way analysis of variance (ANOVA) in combination with the Bonferroni multiple comparison test was used to assess whether fasting significantly altered irinotecan induced toxicity in treated mice compared to the other groups. Two-way ANOVA was used to assess if fasting or irinotecan treatment significantly influenced expression of immunohistochemical markers. All standard statistical tests were performed using SPSS version 21 for Windows software (Statistical Package for Social Sciences, Chicago, IL), and $p < 0.05$ was considered to be significant.

Results

Adverse side-effects in irinotecan treated Apc-mutant mice

During the first 3 days of the experiment, animals in the fasting group were withheld from food, and had *ad libitum* access to water. Bodyweight was presented as a relative percentage of the starting weight. Weight loss was 22.5 ± 3.0% during fasting relative to the animals starting weight in both fasting groups. The *ad libitum* fed groups gained 2.3 ± 1.9% body weight during this period. Directly after the first irinotecan injection, fasted

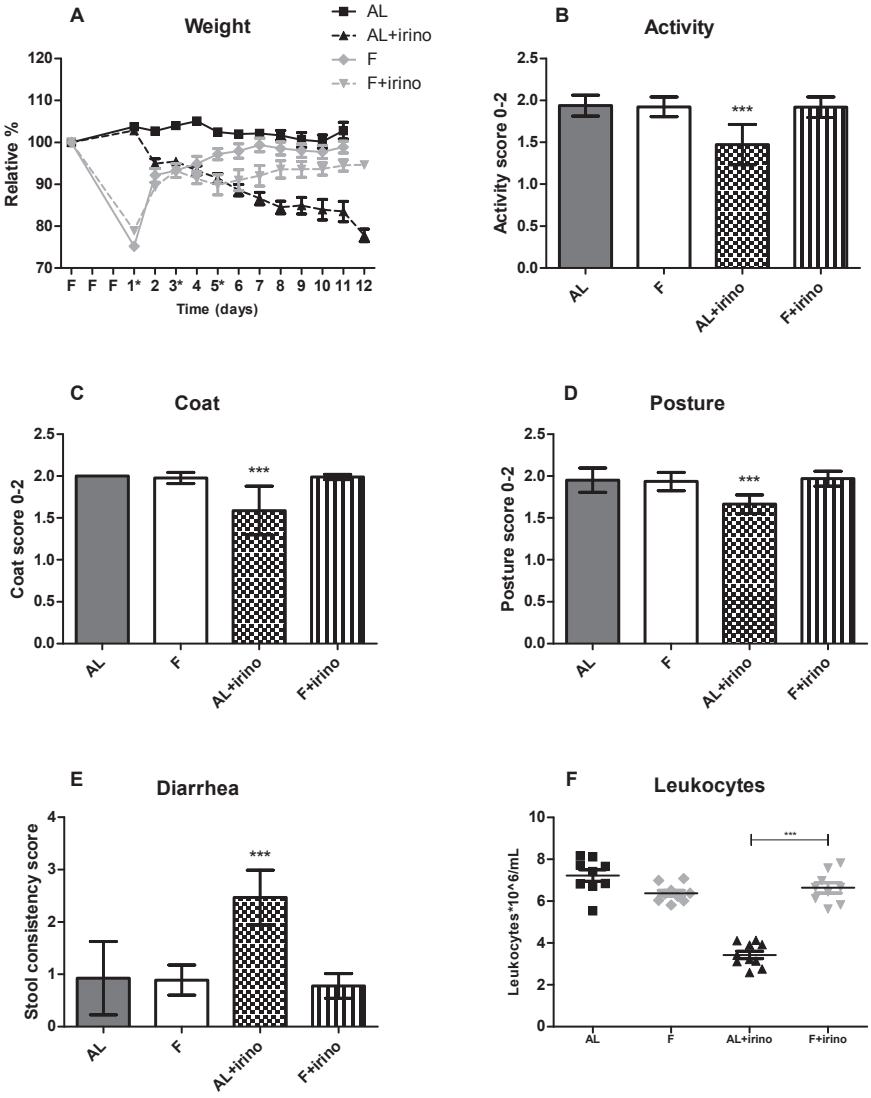

Figure 1. Protection of fasting against side-effects of irinotecan in *Apc*-mutant mice. Side-effects were monitored from the start of the fasting regimen until 11 days after the first irinotecan injection (*). Graphs represent a total of 36 mice: 1. Ad libitum group (n=9; males (m) n=4, females (f) n=5), 2. Fasted group (n=9; m, n=4, f, n=5), 3. Ad libitum group with irinotecan treatment (n=10; m, n=5, f, n=5), and 4. Fasted group with irinotecan treatment (n=8; m, n=4, f, n=4). (**A**) Effects of fasting and irinotecan treatment on body weight. Fasted (F) mice lost weight during the fasting regimen, but gained weight during irinotecan treatment. In contrast, a*d libitum* (AL) fed animals lost weight during irinotecan administration. (**B-E**) Effects of fasting and irinotecan treatment on activity, coat, posture, and stool. ***: indicates significant difference ($p<0.001$) between *ad libitum* fed animals treated with irinotecan compared to each of the other groups. (**F**) Effect of fasting and irinotecan treatment on bone marrow toxicity. Number of leukocytes on day 8 after the first irinotecan injection was significantly lower in *ad libitum* fed animals compared to fasted animals in the irinotecan treated groups. ***: $p<0.001$.

mice were fed *ad libitum* again. In the *ad libitum* fed group, mice showed weight loss following the first irinotecan injection, and at the end of the experiment they had lost 22.1 ± 3.7% of their starting weight. In contrast, the fasted mice gained weight following the first injection and at the end of the experiment their weight had returned to baseline levels (94.8 ± 3.5% of their starting weight) (**Fig1A**). From day 3 after the first irinotecan injection, mice in the *ad libitum* fed group were less active ($p<0.001$) and showed a ruffled coat compared with fasted mice treated with irinotecan and untreated controls ($p<0.001$). Furthermore, *ad libitum* fed mice treated with irinotecan showed a hunched-back posture ($p<0.001$) and suffered from diarrhea from day 3 after the first injection, whereas this was not observed in fasted mice treated with irinotecan and untreated controls ($p<0.001$) (**Fig1B-E**). Thus, mice in the fasted group treated with irinotecan did not show signs of toxicity and showed no significant differences in side-effects compared with both untreated control groups. Eight days after the first irinotecan injection the number of leukocytes was determined to measure bone marrow toxicity. Among the mice receiving irinotecan, the *ad libitum* fed group showed a significant reduction in leukocyte numbers compared to fasted animals ($3.4 \pm 0.6*10^6$/mL vs. $6.6 \pm 0.7*10^6$/mL, $p<0.001$) (**Fig1F**). There was no significant difference between the control groups.

Number and size of intestinal tumors

At the end of the experiment the entire gastrointestinal tract from each mouse was removed, and the small and large intestine were opened for tumor enumeration. The average

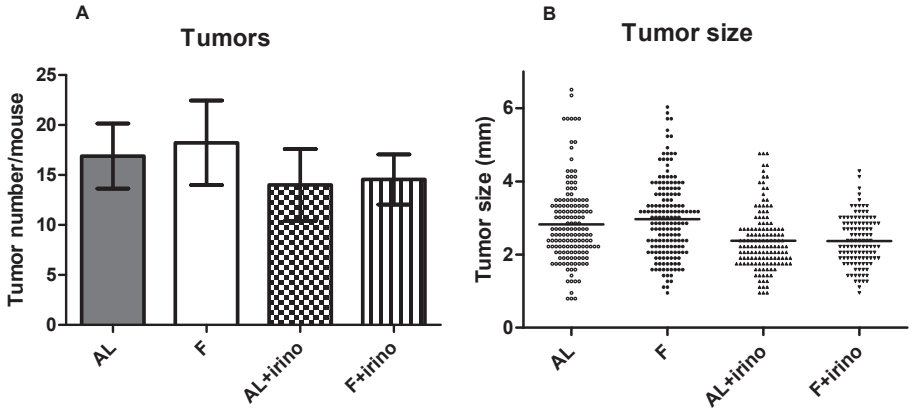

Figure 2. Number and size of intestinal tumors. Tumors from the gastrointestinal tract were counted and measured under a dissection microscope. (**A**) Number of intestinal tumors per mouse. In the irinotecan treated groups, *ad libitum* (AL) fed and fasted (F) mice had respectively 14.0 ± 3.6 and 14.6 ± 2.5 tumors per intestine. In control groups, *ad libitum* (AL) fed and fasted (F) mice had respectively 16.4 ± 3.1 and 17.3 ± 3.3 tumors per intestine. (**B**) Size of all intestinal tumors. Tumor size in irinotecan treated mice was 2.4 ± 0.82 mm in fasted, and 2.4 ± 0.67 mm in *ad libitum* fed and fasted mice respectively. In control groups this was 2.8 ± 1.08 mm and 3.0 ± 1.05 mm ($p<0.001$).

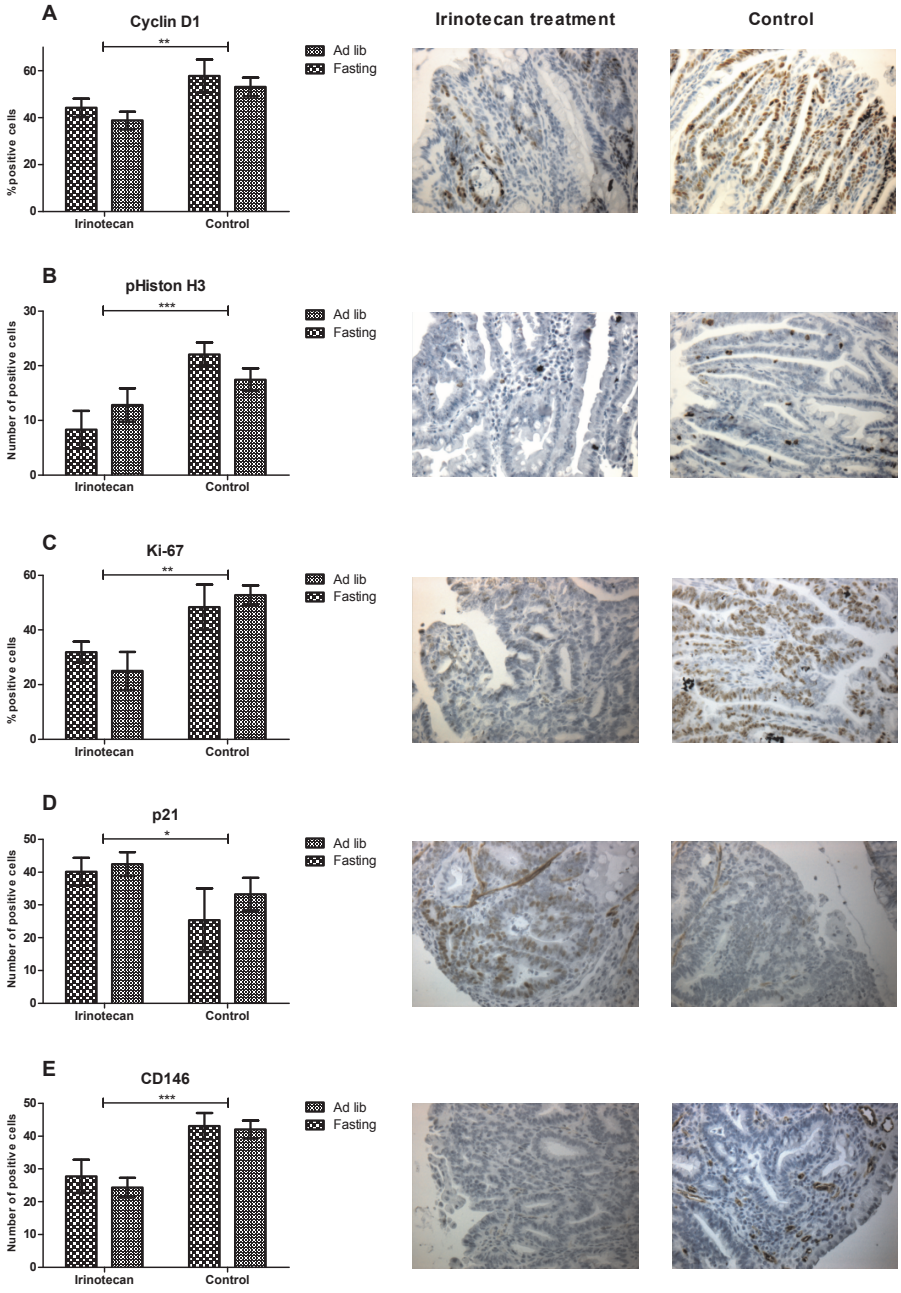

Figure 3. Anti-tumor effect of irinotecan. (**A**-**C**) Irinotecan treatment significantly inhibited tumor cell proliferation as shown by differential expression of Cyclin D1, pHiston H3, and Ki-67. (**D**) This corresponded with an up-regulation of the CDKI p21. (**E**) CD146, a marker for endothelial cells, was significantly down-regulated in irinotecan treated animals. Representative photomicrographs of irinotecan treated (middle panels) and control (right panels) tumor specimens (magnification 200x). *:p<0.05, **:p<0.01, ***:p<0.001.

number of tumors was 17% lower in the irinotecan-treated groups compared to controls. There were 16.4 ± 3.1 and 17.3 ± 3.3 tumors per mouse in the *ad libitum* and fasted group without irinotecan treatment, whereas in the *ad libitum* and fasted group with irinotecan treatment mice had 14.0 ± 3.6 and 14.6 ± 2.5 intestinal tumors, respectively (**Fig2A**). Interestingly, the size of the tumors showed a significant difference between irinotecan treated and control groups. Tumor size of a*d libitum* fed and fasted control groups was 2.8 ± 1.08 mm (range: 0.8 mm – 6.5 mm) and 3.0 ± 1.05 mm (range: 1.0 mm – 6.0 mm) respectively, while tumor size in irinotecan treated groups was 2.4 ± 0.82 mm (range: 1.0 mm – 4.8 mm) and 2.4 ± 0.67 mm (range: 1.0 mm – 4.3 mm) ($p<0.001$) (**Fig2B**).

Antitumor effect of irinotecan

We examined whether fasting changed the effects of irinotecan on the cell cycle, cellular proliferation, induction of senescence, and angiogenesis by immunohistochemistry on four randomly chosen tumors per intestinal tract. Cyclin D1, a marker that indicates the G1/S-phase of the cell cycle and that is often seen overexpressed in tumors, showed a significantly lower expression in irinotecan treated animals compared to controls ($p<0.01$) (**Fig3A**). This corresponded with a significantly lower expression of the mitosis marker pHiston H3 in irinotecan treated animals ($p<0.001$) (**Fig3B**). Ki-67, the proliferation marker that is present in all active phases of the cell cycle was also significantly lower expressed in both irinotecan treated groups ($p<0.01$) (**Fig3C**). Expression of the cyclin dependent kinase inhibitor (CDKI) p21, indicating senescence and S/G2-phase, was significantly up-regulated in both irinotecan treated groups ($p<0.05$) (**Fig3D**). CD146, present on vascular endothelial cells and a marker for angiogenesis, showed significantly lower expression in both treated groups ($p<0.01$) (**Fig3E**). There were no significant differences between irinotecan treated *ad libitum* and fasted animals. Collectively these data show that irinotecan treatment significantly inhibited tumor cell proliferation, and has a comparable anti-tumor effect in both *ad libitum* fed-, and fasted mice.

Discussion

The experiments presented here show that 3-days of fasting prior to treatment with a high dose of the chemotherapeutic agent irinotecan protects against its side-effects in an *Apc*-mutant mouse model that spontaneously develops intestinal tumors. We observed a similar reduction in tumor size in fasted and *ad libitum* fed irinotecan treated groups compared to controls. Furthermore, we found a significant decrease in cell cycle activity, proliferation, and angiogenesis, while senescence was increased in both fasted and *ad libitum* fed irinotecan treated groups, indicating that fasting does not affect the anti-tumor activity of irinotecan.

To our knowledge, this is the first study that shows beneficial effects of fasting on chemotherapy toxicity in a mouse model that spontaneously develops colorectal cancer. In support of these observations, others have shown that long term dietary-restricted rodents are more resistant against a variety of toxic agents (18-21). We showed that beneficial effects seen after long-term dietary restriction, can also be induced rapidly using a 3-day fasting regimen (8). Others have observed the beneficial effects of short-term fasting on chemotherapy treatment as well. Mice injected with neuroblastoma cells and subsequently fasted for 48 hours were protected against side-effects of etoposide, while anti-tumor activity was preserved (11). In previous studies, tumor-cells or tissues were placed subcutaneously, whereas in the current study we used an *Apc*-mutant mouse model that mimics spontaneous intestinal tumor formation (13). Spontaneously developing tumor models are needed to better develop strategies to control malignant cell growth, including chemotherapy treatment. In this regard, the *Apc*$^{Min/+}$ mouse model is one of the most studied models of intestinal tumorigenesis in which tumor growth is suppressed by different anticancer agents (22, 23).

In the present study we used fasting as a pre-conditioning method to protect against the side-effects of irinotecan. Previously, we reported that 3-days of fasting increases stress-resistance, including upregulation of the Nrf2 pathway, which is involved in the protection against oxidative stress (8, 24). In contrast to the up-regulation of protective pathways by normal cells, it is thought that cancer cells are unable to obtain a protected state by dietary restriction. This phenomenon is called differential stress sensitization (DSS) and is based on the fact that cancer cells have acquired a number of mutations that progressively decrease their ability to adapt to diet restriction (25).

A key finding in this study is that 3-day fasted *Apc*-mutant mice do not show any signs of toxicity in response to a very high dose irinotecan while the anti-tumor response is still present. Using immunohistochemistry, we found no significant differences between *ad libitum* fed- and fasted animals in both the irinotecan treated groups as well as the control groups, indicating that the effects of irinotecan are unimpaired in fasted animals. Phosphorylation of Histone H3 at serine 10 takes place during mitosis of the cell (26). The reduced expression of pHiston H3 in tumors of irinotecan treated mice indicates that mitosis is suppressed. Cyclin D1 regulates the progression of cells into the proliferative stage (27). Both fasted and *ad libitum* fed irinotecan treated animals showed a similarly reduced expression of cyclin D1. Ki-67, a widely used proliferation marker, confirmed the anti-proliferative effect of irinotecan in all treated animals.

Due to irinotecan induced DNA damage, the p53/p21 pathway is activated and subsequently arrests cells from division and causes cellular senescence (28, 29). We evaluated the expression of p21 as a senescence marker, and showed that irinotecan treated tumors express significantly higher levels of p21 compared to controls. The expression of CD146, a marker present on vascular endothelial cells, was significantly reduced in

tumors from irinotecan treated mice. The effects of irinotecan on vascular endothelial cells remain largely unknown. Irinotecan decreases hypoxia inducible factor 1α accumulation in tumors, which may inhibit tumor angiogenesis (30). These findings support our observation that irinotecan reduces CD146 expression and thus angiogenesis.

We investigated both male and female Fabpl*Cre*;Apc$^{15lox/+}$ mice. The number of male and female mice was evenly distributed between the different groups, and we observed no differences in side-effects, number and size of tumors, and anti-tumor effect of irinotecan between genders. The only difference was seen in bodyweight; at the start of the experiment, at 27 weeks of age, male mice were on average 30% heavier. Therefore, bodyweight is presented as a relative percentage of the starting weight. Several studies have compared gender differences in the *Apc*$^{Min/+}$ model. The small molecule compound SHetA2 significantly reduced incidence and size of intestinal polyps, both in males and females (22). In contrast, in a dietary study using the retinoid-x-receptor agonist bexarotene, a greater reduction of intestinal tumors was found in male mice compared to female mice (31). The observation that we did not find differences between genders could be attributed to the fact that we administered a flat-fixed dose to all mice, based on the bodyweight of male mice. Thus, female mice received a higher concentration irinotecan relative to their bodyweight, but are known to be less susceptible to the toxicity induced by irinotecan (32).

Based on existing evidence from *in vitro* and *in vivo* experiments, fasting has a great potential to be implemented in clinical cancer patients. Although the introduction of fasting remains challenging, a study with self-reported patients showed a decrease in side-effects and an increase in subjective well-being after chemotherapy treatment (33). Therefore, randomized controlled clinical trials are needed to validate the beneficial effects of fasting in a clinical setting.

In conclusion, fasting significantly prevented the occurrence of side-effects of irinotecan in a genetic mouse model that spontaneously develops intestinal neoplasms through an inactivating mutation of the *Apc* gene. Irinotecan significantly reduced tumor size and proliferation in fasted and *ad libitum* fed animals, indicating that fasting does not abrogate the anti-tumor response. These results support fasting before irinotecan treatment as a powerful way to improve treatment for colorectal carcinoma patients.

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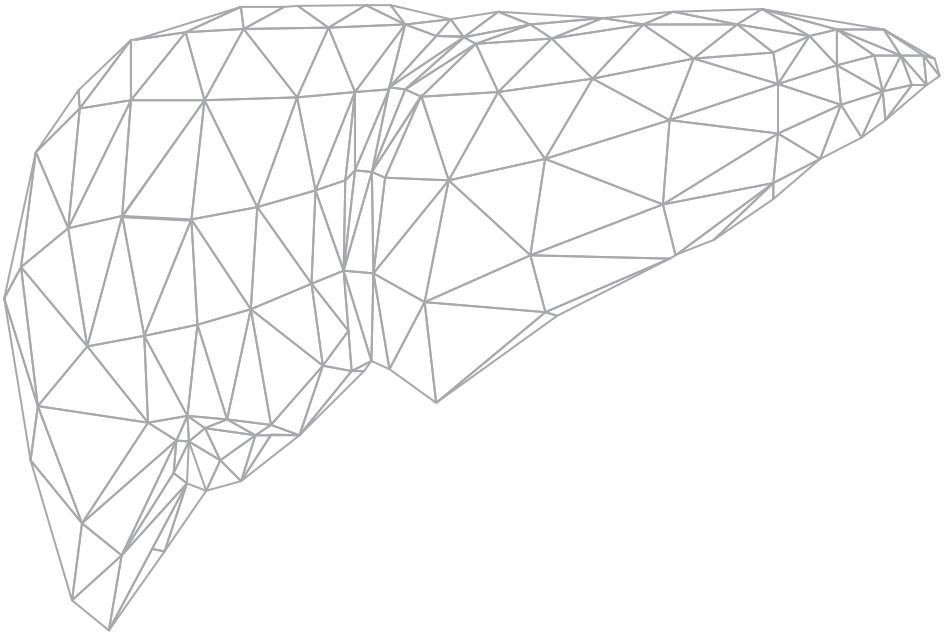

CHAPTER 6

FASTING PROTECTS AGAINST THE SIDE-EFFECTS OF IRINOTECAN TREATMENT BUT DOES NOT ABROGATE ANTI-TUMOR ACTIVITY IN MICE

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Abstract

The main limitation to the use of irinotecan in the treatment of colorectal cancer is the severity of side-effects, including neutropenia and diarrhea. Here, we explored the effects of 3 days of fasting on irinotecan induced toxicities, plasma, liver and tumor pharmacokinetics, and anti-tumor activity in mice. Male Balb/c mice received C26 colon carcinoma cells subcutaneously. They were randomized 1:1 into *ad libitum* fed and fasted groups after which they were treated with irinotecan. Weight and adverse side-effects were recorded daily. At the end of the experiment, tumors were resected, weighed, and concentrations of irinotecan and its active metabolite SN-38 were determined. Fasting prevented side-effects induced by irinotecan as shown by absence of diarrhea and visible signs of discomfort. *Ad libitum* fed animals developed leukopenia compared to untreated controls whereas fasted mice did not ($3.2\pm0.1*10^6$ leukocytes/mL *versus* $6.5\pm0.2*10^6$/mL, $p<0.001$). Irinotecan significantly suppressed tumor growth in both the fasted and *ad libitum* fed groups compared to untreated controls (1,263±54 mg and 1,278±36 mg in fasted and *ad libitum* fed animals *versus* 2,195±113 mg in untreated controls, $p<0.001$). SN-38 AUC values were significantly lower in fasted mice in both plasma and liver, but not in tumor tissue ($p=0.02$, $p=0.003$, and $p=0.27$, respectively). Our data demonstrate that fasting protects against irinotecan induced side-effects without interfering with its anti-tumor efficacy. Fasting induces a lower systemic exposure to SN-38 which may explain the absence of adverse side-effects, while tumor AUC values remain unchanged. These data offer important new opportunities to improve irinotecan treatment in patients.

Introduction

Colorectal cancer is the second most prevalent cancer, with more than 1,2 million new cancer cases and over 600,000 deaths per year (1). About 15% to 20% of patients at initial presentation have liver metastases, and an additional 45% is diagnosed with metastases in follow-up after resection of the primary tumor (2). A potent anticancer agent in first and second line treatment for unresectable and metastatic disease is irinotecan (CPT-11), today's most widely used topoisomerase-I-inhibitor. Irinotecan can produce severe and unpredictable hematologic, intestinal and systemic toxicities, including deep myelosuppression, massive diarrhea, vomiting, fatigue and in some cases even death as a complication of the other side-effects (3-6). It is generally accepted that this toxicity is mediated by the active metabolite of irinotecan, SN-38 (7). Importantly, irinotecan is one of the chemotherapeutic drugs which has been reported to generate high levels of oxidative stress (8), which will lead to failure of normal cellular functions and may contribute to the discussed toxicities (9).

Fasting is a powerful means to increase resistance against acute stressors such as oxidative stress. We have previously shown that preoperative fasting and dietary restriction protect against acute oxidative damage induced by ischemia-reperfusion injury in both kidney and liver (10-13). In addition, preoperative dietary restriction reduces hepatic tumor load and the hepatic expression of the endothelial cell specific adhesion molecule, E-selectin (14). It has been suggested that fasting protects normal cells by rearrangement of energy into maintenance pathways instead of reproduction and growth (15, 16). In this way, dietary restriction could protect against toxic side-effects of anti-cancer drugs, but how this affects pharmacokinetics and anti-tumor activity is still largely unknown.

Therefore, in this study we examined the effects of fasting prior to administration of a high dose of the chemotherapeutic agent irinotecan on the occurrence of side-effects and anti-tumor activity in C26 colon carcinoma bearing mice. In addition, to elucidate the mechanism involved, we have examined the pharmacokinetics of irinotecan in 3-day fasted mice in plasma, liver, and tumor.

Materials and methods

Animals

Male BALB/c mice of 6-8 weeks old --weighing approximately 25 grams-- were obtained from Charles River, Maastricht, the Netherlands. Upon arrival, animals were housed at random in individually ventilated cages (n= 3-4 animals per cage) in a licensed biomedical facility at Erasmus University Medical Center, Rotterdam, the Netherlands. Standard

laboratory conditions were maintained, i.e. temperature ~22°C, humidity ~50%, and a 12 h light/12 h dark cycle. All mice had free access to water and food (Special Diet Services, Witham, UK) unless mentioned otherwise. Animals were allowed to acclimatize for one week before the start of the experiments. The experimental protocol was approved by the Animal Experiments Committee under the Dutch National Experiments on Animals Act, and complied with the 1986 directive 86/609/EC of the Council of Europe. For *in vivo* experiments the ARRIVE guidelines were used (17).

Fasting protocol

Mice in the *ad libitum* fed groups were allowed unrestricted access to food. Before the start of the experiment, all mice were transferred to a clean cage and mice in the fasting groups were withheld food for 3 days starting at 4:00 PM. All animals were given continuous access to water.

C26 colon carcinoma cells

The murine colon carcinoma cell line C26 was kindly provided by dr. R. Schiffelers (Utrecht Medical Centre, The Netherlands). The C26 cell line originally derived from the BALB/c mouse and was cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Sigma Aldrich, St. Louis, MO), supplemented with 10% fetal calf serum (Lonza, Verviers, Belgium), penicillin (100 units/ml) and streptomycin (100 units/ml) (Invitrogen, Auckland, NZ) at 37 degrees Celsius in a 5% carbon dioxide environment (18). Cells were harvested by brief trypsinization (0.05% trypsin in 0.02% ethylenediamine tetra-acetic acid (EDTA)). For subcutaneous injection, cells were harvested and after centrifugation, single-cell suspensions were prepared in phosphate buffered saline (PBS) to a final concentration of 5.0×10^5 cells/100 µL. Cell viability was determined by trypan blue staining, and was always ≥90%.

Chemotherapy

Irinotecan, HCl-trihydrate 20 mg/mL (Hospira, Benelux) was used for *in vivo* experiments. Irinotecan was diluted in sodium chloride 0.9% (Braun, Melsungen, Germany) to a final volume of 200 µL per injection, and was given intraperitoneally. Optimal drug doses were determined in a pilot experiment (**Fig.1**). All drug and molecular target nomenclature follows the standard of 'Concise guide to pharmacology' (19).

Effect of fasting on irinotecan induced side-effects

A total of 24 mice were anaesthetized with isoflurane inhalation (2%). Body temperature was maintained by placing the mice on heating pads. A lateral incision on the right flank was made to implant a tumor cube, derived from mice carrying C26 tumors subcutaneously. All mice received 0.05 mg/kg buprenorphine (Temgesic, Schering Plough, Houten,

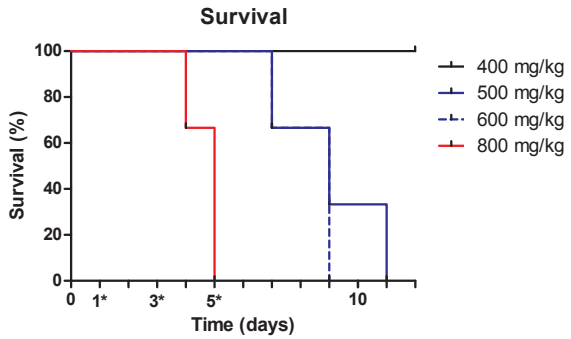

Figure 1. Survival of ad libitum fed mice treated with different doses of irinotecan. *Ad libitum* fed mice (n=6 per group) were treated on 3 intermittent days with a cumulative dose of 400 mg/kg, 500 mg/kg, 600 mg/kg, or 800 mg/kg irinotecan (*). Using 500-, 600-, and 800 mg/kg irinotecan, all animals had to be sacrificed because of severe discomfort induced by irinotecan within 4, 8, and 10 days respectively. With a concentration of 400 mg/kg all mice survived and recovered from the side-effects of irinotecan.

The Netherlands) via subcutaneous injection before the implantation of tumors. Tumor cubes measured approximately 15 mm^3, and were implanted at least 4 mm away from the incision site. Mice were randomly divided into four groups (n=6/group); group 1: *ad libitum* fed group receiving vehicle treatment, group 2: fasting group receiving vehicle treatment, group 3: *ad libitum* fed group receiving irinotecan, group 4: fasting group receiving irinotecan treatment. After 3 days of fasting as well as in the *ad libitum* fed group, mice received a cumulative weight-adjusted dose of 400 mg/kg irinotecan administered on days 1,3 and 5 relative to fasting. The control groups received vehicle treatment (sodium chloride 0.9%). From the first irinotecan injection mice were weighed and inspected daily for adverse side effects by a mouse wellbeing-score protocol adapted from 'the guidelines for welfare of animals in experimental neoplasia research' (1988: United Kingdom Co-ordinating Committee on Cancer Research, UKCCCR). Assessing wellbeing by one researcher took approximately 10 minutes per cage with 4 mice. Side-effects were scored independently by two experienced researchers. Mouse cages were removed from racks and placed on a bench to facilitate visualization of the mice, but cages were not opened at any point during the scoring process, except for the determination of the stool consistency at the end of the process. Mouse activity level was scored according to the amount each mouse moved in its cage. A score of 2 indicates that an animal moved around the cage normally. A score of 1 indicates that an animal was moving slowly or less frequently and with an altered gait. A score of 0 indicated that an animal was not moving and was taking no more than 5 steps. The appearance of the coat was scored according to the smoothness. A score of 2 indicated a healthy, smooth uninterrupted coat. A score of 1 indicated a slightly fluffy coat. A score of 0 indicated a severe fluffy coat with evident parts of visible skin. Severity of diarrhea

was assessed according to the stool consistency score (0: normal, 1: loose stool, 2: loose/some diarrhea, 3: diarrhea, 4: severe watery diarrhea) (20). Before every stool consistency measurement clean white tissues were placed at the bottom of the cage to allow determination of the consistency of the stool. Posture was scored as follows; A score of 2 indicated a normal body posture. A score of 1 indicated a moderately hunched posture. A score of 0 indicated a severely hunched posture. Results are expressed as mean ± SEM. Leukocyte numbers were determined on day 8 after the first irinotecan injection with a Z series Coulter Counter (Beckman Coulter, Woerden, The Netherlands). Ten days after the first irinotecan injection, mice were sacrificed by exsanguination. Tumors were resected, and weighed.

Effects of fasting on irinotecan pharmacokinetics

A total of 54 mice were anaesthetized with isoflurane inhalation (2%). The right lateral flank was shaved for precise injection. 5.0 x 10^5 C26 cells were injected in a volume of 100 µL suspension, using a 21G needle. Growth kinetics following C26 tumor cube implantation are similar to growth after injection of C26 cells (**Fig2**). Tumors were allowed to grow for 14 days before start of the experiment. The mice were randomly divided into three groups (n=18/group). Two groups were fasted for 3 days and one group was fed *ad libitum*. After the fasting period, mice were fed *ad libitum* again. The *ad libitum* fed group, and one group of fasted animals, were treated with a single weight-adjusted dose of 100 mg/kg (±2.5 mg, and ±2.0 mg respectively) of irinotecan intraperitoneally. The other fasted group received a flat-fixed dose. This dose contained the same concentration as in *ad libitum* fed mice (2.5 mg), which were approximately 20% heavier compared to the fasted mice. The flat-fixed dosed group was included to provide an alternative dosing

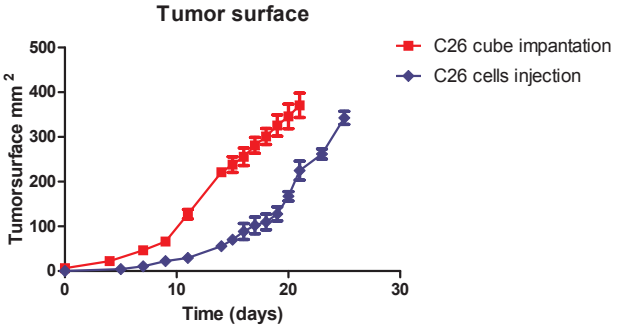

Figure 2. Tumor surface of ad libitum fed mice implanted with tumor cubes and injected with cells. *Ad libitum* fed mice (n=6 per group) were implanted with C26 tumor cubes from approximately 15mm^3 or injected with 5.0 x 10^5 C26 tumor cells. At T=0 tumor cubes were implanted and tumor cells were injected. On day 5 after injection, tumors were macroscopically visible in this group and showed similar growth kinetics as the tumor cubes at times of implantation. At the day of harvesting the tumors (day 21 and day 25 respectively), both groups had a similar tumor surface ($p>0.05$).

to correct for bodyweight loss in fasted animals, especially during the first irinotecan injection. Mice were sacrificed by exsanguination and 100 µL plasma, 100 µg liver and 100 µg tumor were collected at 1,4,8,12, 24 and 48h after irinotecan injection. Each time point included three mice per group.

Tissue pretreatment

Tumor and liver samples were obtained and kept frozen at -70ºC until used. Tissues were diluted in human plasma (1:4 w/v) into a 2-mL Eppendorf tube. Hereafter a 5-mm stainless steel bead (Qiagen, Venlo, The Netherlands) was added and the samples were homogenized with a Tissuelyser (Qiagen, Venlo, The Netherlands) and processed for 4 minutes at 40 Hz. Beads were removed and homogenized samples were stored at -70ºC until analyses. Homogenized tissue samples were further processed as plasma samples.

Determination of irinotecan, SN-38, and SN-38G concentrations

Total irinotecan and total SN-38 were quantified using a validated method involving reversed-phase high-performance liquid chromatography with fluorescence detection as described before (21-23). All frozen samples were thawed at 4°C and were homogenized by vortex-mixing. A 250-aliquot of plasma was mixed for 10 min with 500 µL 5% perchloric acid- methanol (1:1, v/v) in 1.5-mL polypropylene tubes (Eppendorf, Hamburg, Germany). After centrifugation for 5 min at 18,000**g*, 250 µL of the clear supernatant was mixed with 250 µL phosphate buffer. Hereafter, 100 µL was injected into the HPLC system. Chromatographic analysis was performed using an Agilent® HPLC system 1100 series (Agilent Technologies, Santa Clara, CA). Chemstation software was used for data monitoring and analysis. Separation of the compounds was achieved using a Inertsil ODS 80A (4.6 mm x 150 mm, 5 µm particle size) analytical column. The analytical column was maintained at a temperature of 50°C. A gradient at a flow-rate of 1.00 mL/min was achieved with mobile phase A, composed of 0.1 M ammonium acetate containing 0.01 M tetra-butyl-ammonium sulphate and mobile phase B composed of methanol. A linear gradient was used, with 67-50% mobile phase A, from 0 to 25 min, followed by 50-10% mobile phase A for 5 min. This was succeed by a linear gradient back to 67% mobile phase A from 30 to 31 min which was held for 2 min to re-equilibrate. The column eluent was monitored fluorimetrically at an excitation wavelength of 375 nm and an emission wavelength of 460 nm for detection of irinotecan and an excitation wavelength of 380 nm and an emission wavelength of 532 nm for the detection of SN-38. Calibration curves were computed using the ratio of the peak height of irinotecan or SN-38 versus the nominal concentration, with a lower limit of detection of 5.00 ng/mL for irinotecan and 0.500 ng/mL for SN-38. Uridine diphosphate glucuronosyltransferase 1A (UGT1A) is capable of inactivating SN-38 to its glucuronide form, SN-38G. SN-38G in plasma samples was quantified by the amount of SN-38 released following treatment for 3 h at 37°C with

100U of E. coli ß-glucuronidase. Concentrations from 48 hour samples were below the detection limit and therefore we decided to include 0-24h time points.

Real-time quantitative RT-PCR (qRT-PCR)

RNA was isolated from liver and tumor tissues collected at 1, 8 and 12h after irinotecan injection from the *ad libitum* fed and the fasted group (flat-fixed dosed) using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. To avoid genomic DNA contamination, RNA was purified by a DNAse treatment (RQ1 RNase-Free DNase; Promega, Madison, WI, USA). RNA was then reverse transcribed into cDNA using random primers (Invitrogen) and Superscript II RT (Invitrogen). Gene expression was analyzed by real-time quantitative PCR using an Applied Biosystems 7700 PCR machine (Foster City, CA, USA). RT-PCR was performed for carboxylesterase 2 (CES2) forward, 5-ggccatgtgtctgcaaaatc-3 and reverse, 5-caccatcacaggcaggttag-3. Glutaraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a housekeeping gene. Each sample was tested in triplicate. ΔCt values were normalized to the average ΔCt of the *ad libitum* fed group. The fold change was calculated using the Pfaffl equation, 2-ΔΔCt. Results are expressed as mean with the standard error of the mean (SEM).

Statistical analyses

For analyzing the pharmacokinetic (PK) data Phoenix WinNonlin version 6.1 (Certara USA Inc, St. Louis, MO) software was used. All standard statistical tests were performed using SPSS version 21 for Windows software (Statistical Package for Social Sciences, Chicago, IL), and $p < 0.05$ was considered to be significant. For each set of parameters means and standard errors of the mean were computed. One-way analyses of variance (ANOVA) was used to assess whether fasting significantly altered side-effects. Bonferroni correction was applied to correct for multiple testing. To show statistics between pharmacokinetic differences, the highest, intermediate, and lowest area under the plasma concentration time curve (AUC) value for each group was calculated. The students t-test was used to determine statistical differences.

Results

To study the effect of fasting on irinotecan toxicity, we first treated *ad libitum* fed mice with different doses of irinotecan. Using cumulative doses of 500,600 and 800 mg/kg all mice died within 11 days from the first injection (**Fig1**). Using a cumulative dose of 400 mg/kg mice experienced serious adverse effects like diarrhea, hair loss, and a ruffled coat but survived and recovered in the 15-day observation period. Therefore, we decided to use the cumulative dose of 400 mg/kg in our first experiment.

Fasting protects against the adverse side-effects of irinotecan and does not abrogate anti-tumor activity

To examine the effects of fasting before irinotecan administration on the occurrence of irinotecan-induced side-effects and anti-tumor effect in C26 colon carcinoma bearing mice, we compared 3 day-fasted with *ad libitum* fed mice. In the *ad libitum* fed group, mice showed weight loss from the first irinotecan injection, while the fasted mice gained weight during the treatment and observation period (**Fig3A**). From day 4 after the first irinotecan injection, other side-effects were observed in the *ad libitum* fed group. They were less active ($p=0.003$), had a ruffled coat ($p<0.001$), suffered more from diarrhea ($p<0.001$) and showed a hunched-back posture ($p<0.001$) (**Fig3B-E**). The fasted mice showed no visible side-effects. On day 8 after the first irinotecan injection the fasted group had a significantly higher blood leukocyte count than the *ad libitum* group ($6.5 \pm 0.2*10^6$/mL vs. $3.2 \pm 0.1*10^6$/mL, $p<0.001$) (**Fig3F**). Survival during the observation period was 100% in the chemotherapy treated groups. At the end of the experiment all tumors were resected and weighed. Irinotecan treated tumors were significantly smaller both in fasted and *ad libitum* fed groups compared to untreated controls ($1,263 \pm 54$ mg and $1,278 \pm 36$ mg vs. $2,195 \pm 113$ mg, $p < 0.001$). Fasting alone had no significant effect on tumor growth (**Fig4**).

Taken together, these results show that 3 days of fasting prior to treatment with a high dose of irinotecan prevents against side-effects but does not abrogate its anti-tumor effects.

Fasting reduces the systemic exposure to SN-38

To elucidate the mechanism of fasting induced resistance against its side-effects, we have examined the pharmacokinetics of total irinotecan and its active metabolite SN-38 in plasma, liver, and tumor tissue of 3 day fasted and *ad libitum* fed mice. Furthermore, SN-38G concentrations were measured in plasma to indicate activity of uridine diphosphate glucuronosyltransferase 1A (UGT1A). Area under the plasma concentration time curve (AUC) data were computed, normalized, and set to 100% for *ad libitum* fed groups (**Fig5**). To express differences between AUC curves more clearly, and to perform statistics on these curves, figures were computed where the highest, intermediate and lowest AUC values were plotted (**Fig6A-G**).

AUC values of the active metabolite SN-38 were significantly lower in plasma and liver in fasted animals compared to *ad libitum* fed animals while in the tumor SN-38 AUC values did not differ between fasted and *ad libitum* fed animals.

Plasma AUC values of irinotecan were increased with 35% and 47% in the weight-adjusted dosed fasting group and flat-dosed fasting group respectively compared with *ad libitum* fed controls ($AUC_{0\text{-}inf}$ 56.1 vs. 41.7 and $AUC_{0\text{-}inf}$ 61.4 vs. 41.7 µg*h/mL, **Fig6A**).

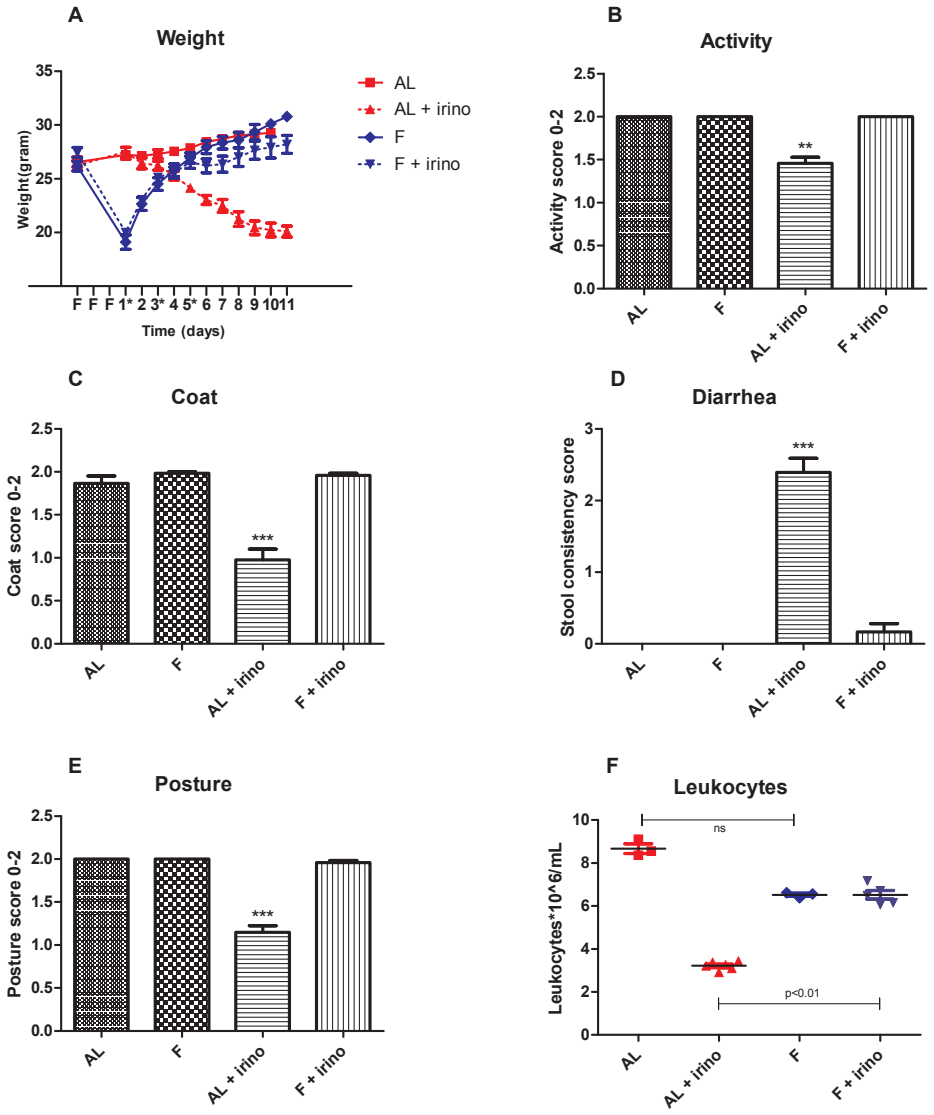

Figure 3. Protective effects of fasting against irinotecan toxicity. A total of 24 mice, 6 mice per group, were observed for adverse side-effects until ten days after the first irinotecan injection (*). (**A**) Effect of fasting on body weight. Fasted (F) mice lost about 20% of their body weight during 3-days fasting. During irinotecan treatment ad libitum (AL) fed mice showed 20% weight loss, while fasted mice gained weight and reached their starting weight at day 5. (**B** to **E**) Effects of fasting on activity (p=0.003) (B), coat (p<0.001) (C), diarrhea(p<0.001) (D), and the posture of the mice (p<0.001) (E). (**F**) Effect of fasting on leukocyte numbers. On day 8 after the first irinotecan injection, treated ad libitum (AL) fed mice showed leukopenia compared to fasted (F) mice (6.5 ± 0.2*10^6/mL vs. 3.2 ± 0.1*10^6/mL, p<0.001).

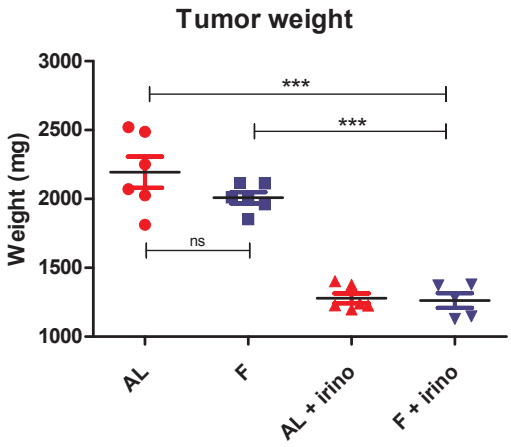

Figure 4. Effect of irinotecan treatment on tumor growth in fasted and ad libitum fed groups. At the end of the experiment irinotecan treated tumors were significantly smaller in both the fasted (F) and *ad libitum* (AL) fed groups compared to untreated controls (1,263 ± 54 mg and 1,278 ± 36 mg vs. 2,195 ± 113 mg, $p < 0.001$). Fasting alone had no significant effect on tumor weight.

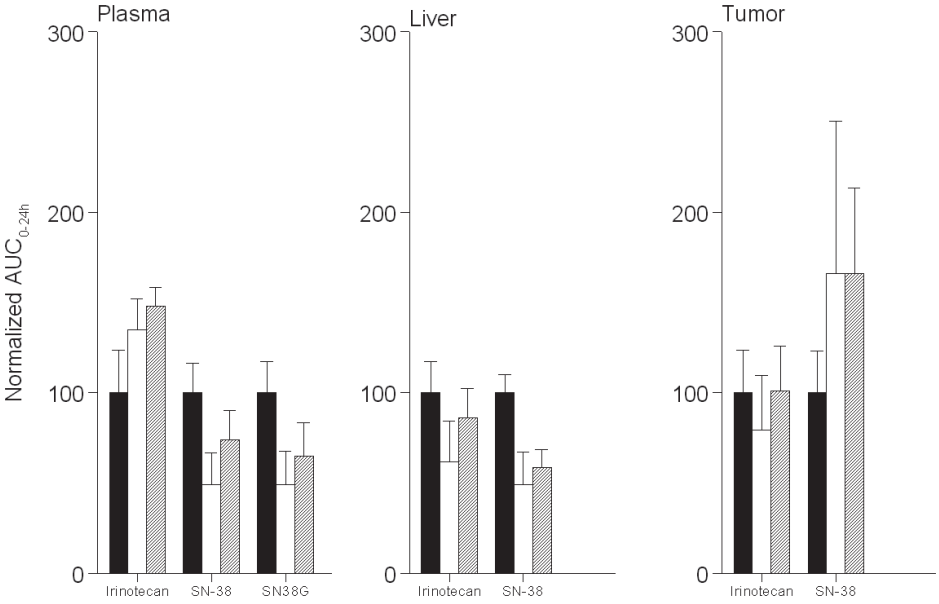

Figure 5. Pharmacokinetics of irinotecan and SN-38 in plasma, tumor and liver. Area under the plasma concentration time curve (AUC) data was normalized, and set to 100% for *ad libitum* fed groups (black rectangles). Fasted weight-adjusted dosed (white rectangles) and fasted flat-fixed dosed (striped rectangles) animals show a trend towards higher plasma irinotecan AUC values, while irinotecan AUC values in the liver and tumor are lower in fasted animals. SN-38 AUC values are lower in both fasted groups in plasma and liver, and SN-38G AUC values are lower in plasma. SN-38 AUC values in tumor tissue show large variation.

SN-38 AUC values were reduced by 51% and 26% in the weight-adjusted dosed fasting group, and the flat-dosed fasting group respectively compared with *ad libitum* fed controls ($AUC_{0\text{-}24}$ 0.56 vs.1.15 µg*h/mL, p=0.02 and $AUC_{0\text{-}24}$ 0.85 vs. 1.15 µg*h/mL, **Fig6B**). SN-38G AUC values were reduced by 51% and 35% in the weight-adjusted dosed fasting group, and the flat-dosed fasting group respectively compared with *ad libitum* fed controls ($AUC_{0\text{-}24}$ 8.3 vs.16.9 µg*h/mL, p=0.01 and $AUC_{0\text{-}24}$ 11.0 vs. 16.9 µg*h/mL, p=0.04, **Fig6C**).

In the liver, a reduction of 39% and 14% in irinotecan AUC values was observed in the weight-adjusted dosed fasting group, and the flat-dosed fasting group respectively compared with *ad libitum* fed controls ($AUC_{0\text{-}inf}$ 354 vs. 575 ng*h/mg and $AUC_{0\text{-}inf}$ 495 vs. 575 ng*h/mg, **Fig6D**). SN-38 AUC values showed a 47% and 41% reduction in the

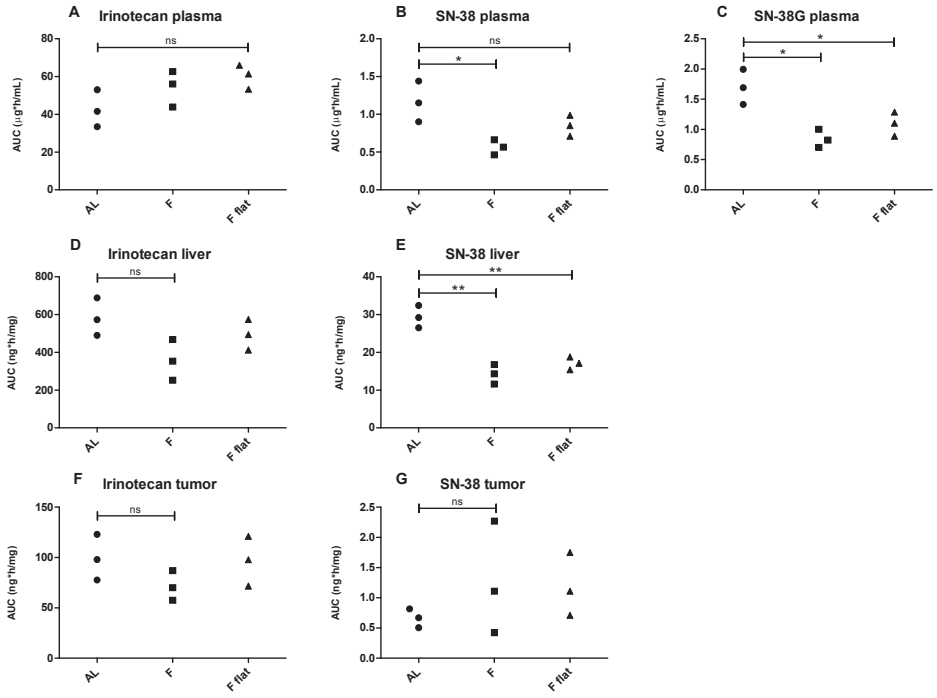

Figure 6. Statistical validation of pharmacokinetic differences. Graphs represent the highest, intermediate, and lowest area under the plasma concentration time curve (AUC) value for each group. No significant differences were found for irinotecan AUC values between *ad libitum* (AL) fed, weight-adjusted dosed fasted (F), and flat-fixed dosed fasted (F flat) groups in plasma (**A**+**B**), liver (**D**+**E**), and tumor (**F**+**G**). SN-38 AUC values in plasma were significantly lower in weight-adjusted dosed fasted animals (p=0.02), and SN-38G AUC values were significantly lower in weight-adjusted dosed fasted animals and fasted flat-fixed dosed animals (p=0.01, and p=0.04 respectively, **C**). SN-38 AUC values in the liver were significantly lower in both fasted groups (p=0.003 and p=0.003). No significant differences were seen in SN-38 AUC values for tumor tissues (p=0.27).

weight-adjusted dosed fasting group, and the flat-dosed fasting group respectively compared with *ad libitum* fed controls (AUC_{0-24} 15.5 vs. 29.2 ng*h/mg, p=0.003 and AUC_{0-24} 17.1 vs. 29.2 ng*h/mg, p=0.003, **Fig6E**).

In the tumor, a reduction of 21% in irinotecan AUC values was observed in the weight-adjusted dosed fasting group. In the flat-dosed fasting group no difference was found compared with *ad libitum* fed controls (AUC_{0-inf} 77.9 vs. 98.4 ng*h/mg, **Fig6F**). Interestingly, SN-38 AUC values showed an increase of 166% in both fasted groups, compared with *ad libitum* fed controls (AUC_{0-24} 1.11 and 1.11 vs. 0.669 ng*h/mg, **Fig6G**). Although this increase is not significantly different, it implies that the intratumoral SN-38 concentrations do not decrease after fasting.

These data demonstrate that irinotecan AUC values are not changed in fasted animals, while SN-38(G) AUC values are significantly lower in plasma and liver tissue from fasted animals, indicating that 3 days of fasting prior to irinotecan administration induces an important change in its metabolism, and lowers the systemic exposure to toxic SN-38.

CES2 activity in liver and tumor

Conversion of irinotecan to SN-38 primarily occurs in the liver by CES enzymes. Fasting is known to affect liver function and size. Therefore, irinotecan metabolism by CES may be changed during fasting. We measured CES2 activity by mRNA expression at 1,8, and 12 hour(s) after irinotecan injection in liver and tumor tissue from *ad libitum* fed and fasted animals. There are no significant differences in CES2 expression levels between *ad libitum* fed and fasted groups in liver and tumor tissue (**Fig7**).

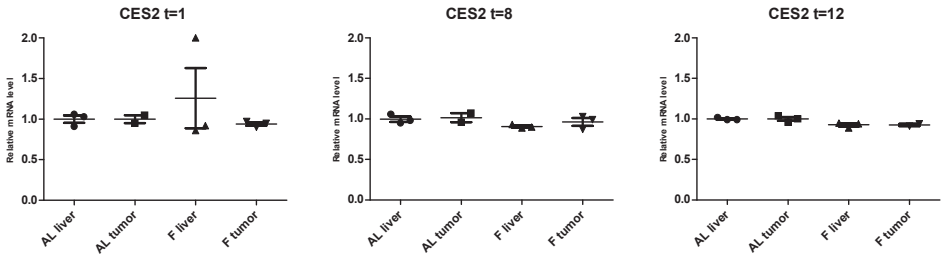

Figure 7. CES2 mRNA expression in liver and tumor tissue from ad libitum fed and fasted mice. CES2 expression at 1, 8, and 12 hour(s) after irinotecan injection in liver and tumor tissue from *ad libitum* (AL) fed and fasted (F) animals did not reveal any significant differences.

Discussion

The experiments presented in the current study show that 3 days of fasting prior to treatment with a high dose of irinotecan prevents the occurrence of drug related side-effects while anti-tumor efficacy is not affected. To elucidate the mechanisms behind

the fasting-induced protection to irinotecan induced side-effects we studied its pharmacokinetics in fasted and fed mice. We found no differences in irinotecan AUC values between fasted and *ad libitum* fed animals and demonstrated that the AUC of the active and toxic metabolite of irinotecan, SN-38, is lower in plasma and liver tissue in fasted animals. Furthermore, SN-38G AUC values are significantly lower in plasma from fasted animals. The intratumoral SN-38 AUC values in fasted mice remained unchanged. These data indicate that fasting induces important changes in irinotecan metabolism, and lowers the systemic exposure to irinotecan and SN-38.

A remarkable finding in this study is that 3-day fasted BALB/c mice do not show any signs of toxicity in response to a high dose of irinotecan. In contrast, animals regained the weight that was lost during the fasting period, approximately 20%, during high dose irinotecan treatment, while control animals lost approximately 20% of their bodyweight during both irinotecan exposure and the subsequent observation period. It is currently thought that fasting induces a hormetic response. This is a common biological phenomenon in which exposure to a low intensity stressor induces a general adaptive response that has beneficial effects, including protection against different types of stress (24, 25). We observed relevant differences in irinotecan pharmacokinetics between *ad libitum* fed and fasted mice. In plasma from fasted mice, there is a trend towards higher AUC values of irinotecan compared with *ad libitum* fed mice, while SN-38 AUC values in plasma from fasted mice are lower compared with *ad libitum* fed groups. In the liver, there is a trend towards lower AUC values of irinotecan in fasted mice, and AUC values of SN-38 are significantly lower in fasted groups compared to *ad libitum* groups. SN-38 is approximately 100-1,000-fold more cytotoxic than the pro-drug (7). The conversion of irinotecan to SN-38 occurs primarily in the liver by carboxylesterase (CES) enzymes, but in patients this conversion is rather inefficient (only 2-5%) when irinotecan is injected intravenously (26, 27). Therefore it is thought that intratumoral *CES* expression is responsible for the anti-tumor effect in patients (28). We found reduced levels of SN-38 in liver and plasma of fasted mice, but *CES* expression is not altered in these mice. Tumor cells have the same if not higher levels of SN-38, but *CES* expression is not higher in tumor cells of fasted mice. This may indicate that tumor cells of fasted mice are taking up more SN-38 rather than metabolism by CES is higher in fasted mice. UGT1A is capable to inactivate SN-38 to its glucuronide form SN-38G, leading to excretion from the circulation (29). We showed significantly lower SN-38G AUC values in plasma from fasted mice, indicating that the lower amount of SN-38 in plasma is not due to induced UGT1A activity.

In an attempt to explain the differential effects of fasting on the systemic level versus tumor tissue, the term 'differential stress resistance' was coined (16, 30). Drug metabolism can be largely divided into 3 phases: phase-I (redox and hydrolysis), phase-II (conjugation), and phase-III (transport). Diet and nutrient signaling pathways have important effects on phase-I enzymes (31). These enzymes consist primarily of cytochrome P450

(CYP) superfamily proteins (32) and phase II proteins including enzymes regulated by Keap1-Nrf2 (33). During fasting, significant modulation of these pathways occurs in healthy tissue, but not in tumor tissue. We have previously shown that 3 days of fasting up-regulates canonical stress resistance pathways such as Nrf2, 'xenobiotic metabolism' and effector genes such as heme-oxygenase I, superoxide dismutase, and glutathione-S-transferase (10, 34). These data may partly explain the concept of differential stress resistance, which predicts that these pathways are differentially regulated by fasting in healthy-, versus tumor tissue. How this affects pharmacokinetics and anti-tumor efficacy has remained unknown. We hypothesize that during fasting cells in the liver (among others) up regulate stress resistance pathways, which decreases conversion of irinotecan whereas in the tumor these pathways are unaffected. This supposition is strengthened by the fact that SN-38 AUC values remained unchanged in tumors of fasted mice.

Fasting down regulates Insulin-Like Growth Factor-1 (IGF-1) levels (10), which has a plethora of downstream effects that are likely linked to the protective effect induced by fasting. Many intrinsic factors may influence the PK of irinotecan (7). IGF-1 has been related to cetuximab + irinotecan treatment outcome in human (35). However, to the best of our knowledge, IGF-1 has never been related to the PK of irinotecan in vivo. Potential interactions between IGF-1 and irinotecan PK can, based on this study, not be determined.

Previously, protective effects against toxic agents have also been observed when animals were subjected to long term dietary restriction. Mice subjected to 5-8 months 40% dietary restriction were significantly more resistant against paraquat toxicity than *ad libitum* fed mice (36). Rats allowed to eat 65% of their normal intake for 3 weeks showed increased resistance to thioacetamide due to increased liver tissue repair mechanisms (37). Rats that were 40% calorically restricted for 4 weeks showed increased resistance against bleomycin induced DNA damage (38).

We showed that the beneficial effect of dietary restriction on acute stress resistance can be induced rapidly; 3 days of fasting led to protection against oxidative damage induced by renal ischemic injury similar in magnitude to 2-4 weeks of 30% dietary restriction (DR) (10). On a global level we found an overlapping transcriptional response, indicating that short-term fasting and long-term DR induce potentially overlapping mechanisms. Furthermore, it has been shown that an *ad libitum* diet deficient in protein or amino acids protects mice in a surgical ischemia-reperfusion injury model (39). More recently, a study in dogs with lymphoma that were treated with doxorubicin showed that 24 hours of fasting the night before administration resulted in significantly lower incidence of vomiting (40).

Implementation of dietary restriction and fasting in the clinic is still laborious. We have shown that preoperative dietary restriction and fasting are feasible and safe in surgical patients (11, 41). Furthermore, women at risk for breast cancer showed reduced

inflammation and oxidative stress during a 2 days/week fasting diet (42). Asthma patients maintained for 8 weeks on an alternate day fasting diet exhibited less clinical symptoms, pulmonary functions were improved, and oxidative stress and inflammation declined during the diet (43). Based on the existing evidence from animal and human studies, there is a great potential translating these results to clinical cancer patients. In a self-reported study of patients with a variety of malignancies, a voluntarily fasting regimen prior to (48-140 hours) and/or following (5-56 hours) chemotherapy resulted in a decrease of common side-effects and increased subjective well-being (44). These data suggest that fasting is safe, feasible, and might offer important new opportunities to improve chemotherapy treatment in cancer patients. However, development and completion of randomized controlled clinical trials is essential in unleashing its clinical potential.

It is known that ectopic and orthotopic tumor environments could respond differently on a variety of treatments. However, in a recent study using FabplCre;Apc15lox/+ mice, which spontaneously develop intestinal tumors, fasting also reduced tumor growth similarly as in ad libitum fed mice and protected against the side effects of irinotecan (45). However, the results from this orthotopic tumor model not directly prove that differential alteration of PK is the primary mechanism, and needs further investigation. We show that fasting protects against side-effects of one cycle of irinotecan treatment. It remains unclear if cancer cells are sensitized or not by multiple cycles of fasting/ irinotecan treatment in this experimental model. However, a recent study suggested that multiple cycles of fasting induces differential stress sensitization in a range of tumors (15).

In summary, the present study shows that short-term fasting protects against adverse side-effects of irinotecan in C26 colorectal carcinoma bearing mice without interfering with anti-tumor activity. The reduction of side effects may be the result of the lower systemic exposure to SN-38 in fasted compared to ad libitum fed mice. When confirmed in clinical trials, fasting before irinotecan treatment may offer important new opportunities to improve this treatment for colorectal carcinoma patients.

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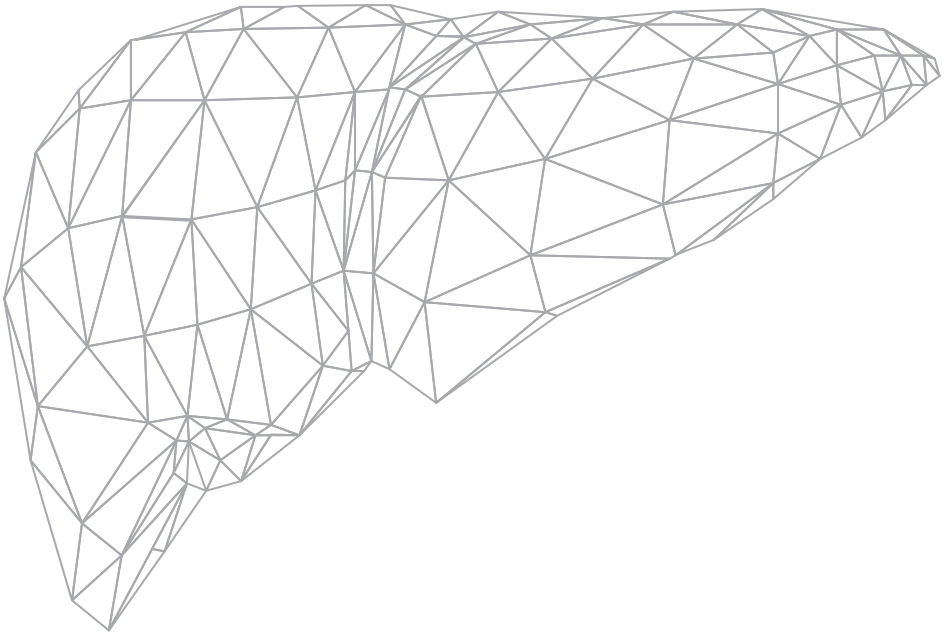

CHAPTER 7

TRANSCRIPTOMIC ANALYSIS OF THE RESPONSE TO IRINOTECAN IN FASTED TUMOR BEARING MICE

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Abstract

Irinotecan is an effective and frequently used chemotherapeutical agent, however its use is accompanied by severe toxicity. Previously, we reported that 3 days of fasting protects against side effects of irinotecan while preserving its antitumor activity. The mechanisms behind the protective effects of fasting have yet to be elucidated. Here, we investigated the transcriptional responses to fasting and irinotecan treatment in both tumor and healthy liver tissue.

Male BALB/c mice (n=36) were subcutaneously injected with murine colon carcinoma cell line C26. After 12 days, two groups were fasted for 3 days and two groups were allowed food ad libitum (AL). One group of each received irinotecan intraperitoneally, the other groups vehicle. Twelve hours after injection mice were sacrificed, and blood, tumor and liver samples were harvested. Blood samples were analyzed for organ function and leukocyte numbers, collected tissues were used for microarray analyses.

The AL fed irinotecan group had significantly altered liver and kidney function and decreased leukocyte numbers. These effects were abated in the fasted animals. At gene expression level, fasting led to a differential and ameliorated stress response, with fewer toxicity pathways, in liver upon irinotecan treatment compared to AL fed mice. In contrast, such diet-induced adaptation after irinotecan exposure was absent in tumor tissue, which showed a signature of increased sensitivity in the fasted group.

In conclusion, fasting reduces toxicity of irinotecan by inducing a protective stress response in healthy liver tissue, while it preserves or even increases the anti-tumor activity of irinotecan on a transcriptional level. These data give insight in the mechanisms involved in the protective effects of fasting on the side effects of irinotecan, and may pave the way to develop dietary restriction mimetics which may induce similar effects without the disadvantages of fasting.

Introduction

The occurrence of cancer is rising continuously. Colorectal cancer (CRC) is the second most diagnosed cancer in women and the third most diagnosed in men. Estimated new colorectal cancer cases account for 1.4 million cases and 693.900 deaths worldwide occurring in 2012 (1). At initial presentation, 15-20% of patients already have liver metastases and another 45% is diagnosed with liver metastases in the follow-up after resection of the primary tumor (2). Irinotecan is a pro-drug of the topoisomerase-I inhibitor SN-38, and is applied in first and second line chemotherapy treatment for colorectal carcinoma (3, 4). Irinotecan can induce severe and unpredictable side effects including deep myelosuppression, diarrhea, and in some cases even death as a complication of side-effects (5).

Dietary restriction (DR) is a method to trigger highly conserved survival mechanisms that enhance protection of organisms against stressors and diseases (6). In previous research we showed that 3 days of fasting induces a protection against oxidative stress (7, 8). Recently, we showed that 3-day fasting prior to a toxic dose of irinotecan significantly prevented the occurrence of side effects in an *Apc*-mutant mouse model. Furthermore, tumor size and proliferation were reduced equally in *ad libitum* fed and fasted animals, indicating that the anti-tumor effect of the drug was unimpaired by the dietary preconditioning (9). One of the theories on how this effect is initiated is called differential stress resistance. It has been suggested that fasting protects normal cells by shifting from growth to maintenance. The tumor cells are unable to make this shift and remain driven towards growth because of mutations and alterations (10, 11). It remains largely unknown what the mechanism behind this beneficial effect is.

Mechanistic insight and clarification of the underlying genes and pathways should facilitate translational research into the clinic since it may reveal alternative approaches, e.g. specialized forms of diet restriction, or dietary restriction mimetics which may induce similar effects without the disadvantages of fasting.

Therefore, in this study we investigated the transcriptional responses to fasting and irinotecan exposure in tumor and in healthy liver tissue.

Materials and methods

Animals

Male BALB/c mice of 6-8 weeks old, weighing approximately 25 grams, were obtained from Charles River, Maastricht, the Netherlands. Upon arrival, animals were housed at random in individually ventilated cages (n=4 animals per cage) in a licensed biomedical facility at Erasmus University Medical Center, Rotterdam, the Netherlands. Standard

laboratory conditions were maintained, i.e. temperature ~22°C, humidity ~50%, and a 12 h light/12 h dark cycle. All mice had free access to water and food (Special Diet Services, Witham, UK) unless mentioned otherwise. Animals were allowed to acclimatize for one week before the start of the experiments. The experimental protocol was approved by the Animal Experiments Committee under the Dutch National Experiments on Animals Act, and complied with the 1986 directive 86/609/EC of the Council of Europe.

C26 colon carcinoma cells

The murine colon carcinoma cell line C26 originally derived from the BALB/c mouse and was cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Sigma Aldrich, St. Louis, MO), supplemented with 10% fetal calf serum (Lonza, Verviers, Belgium), penicillin (100 units/ml) and streptomycin (100 units/ml) (Invitrogen, Auckland, NZ) at 37 degrees Celsius in a 5% carbon dioxide environment. Cells were harvested by brief trypsinization (0.05% trypsin in 0.02% ethylenediamine tetra-acetic acid (EDTA)). For subcutaneous injection, cells were harvested and after centrifugation, single-cell suspensions were prepared in phosphate buffered saline (PBS) to a final concentration of 2.5×10^5 cells/100 µL. Cell viability was determined by trypan blue staining, and was always ≥90%.

Experimental setup

Mice (n=36) were anaesthetized (isoflurane inhalation, 5% isoflurane inhalation initially and then 2% isoflurane with a 1:1 air:oxygen mixture for maintenance of anesthesia) (**Fig1**). Both flanks were shaved for precise injection. 2.5×10^5 C26 cells were injected on both sides in a volume of 100 µL, using a 21G needle. Tumors were allowed to grow for 12 days before start of the experiment. Mice were weighed and tumors were measured daily with digital calipers. The mice were randomly divided into four groups (n=6/group). Two groups were fasted for 3 days and two groups were fed *ad libitum* (AL). After the fasting period, mice were fed *AL* again. One *AL* fed group, and one group of fasted animals, were treated with a single weight-adjusted dose of 133 mg/kg (±3.3 mg, and ±2.7 mg respectively) of irinotecan intraperitoneally. The control groups received

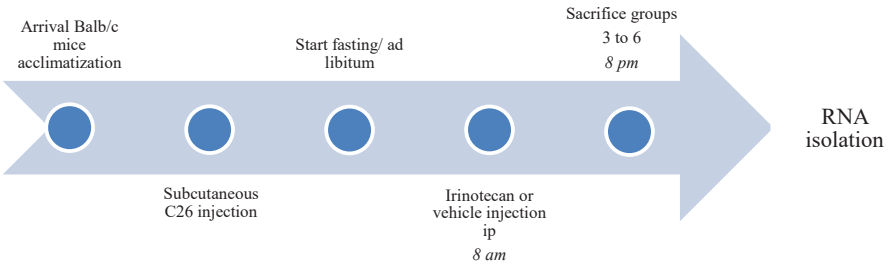

Figure 1. Schematic overview of the experimental set-up, including a timeline from arrival of the mice until sacrifice and subsequent RNA isolation for further analyses. IP = intraperitoneal

vehicle treatment (sodium chloride 0.9%). Two hours after injection, the fasted groups received a weighed amount of AL food. Mice were sacrificed 12 hours after administration of irinotecan by exsanguination.

Fasting protocol

Mice in the AL fed groups were allowed unrestricted access to food, and amount of food eaten per cage was measured daily. Before the start of the fasting period, all mice were transferred to a clean cage and mice in the fasting groups were withheld food for 3 days starting at 4:00 PM on a Friday until 10:00 AM on a Monday. All animals were given continuous access to water.

Chemotherapy

Irinotecan, HCl-trihydrate 20 mg/mL (Hospira, Benelux) was used for *in vivo* experiments. Irinotecan was diluted in sodium chloride 0.9% (Braun, Melsungen, Germany) to a final volume of 200 µL per injection, and was given intraperitoneally.

Serum measurements

Mice were killed by exanguination with cardiac puncture under anesthesia (isoflurane inhalation, 5% isoflurane inhalation initially and then 2% isoflurane with a 1:1 air:oxygen mixture for maintenance of anesthesia). After cardiac puncture, ± 900 µL of blood per mouse was transferred directly into 1 mL tubes (MiniCollect, Greiner Bio-one), containing EDTA. Samples were directly centrifuged (3,500 rpm; 10 min) after which the serum was transferred to a separate tube. Serum aspartate transaminase (AST), alanine transaminase (ALT), lactate dehydrogenase (LDH), urea, and creatinine levels were analyzed at the Central Clinical Chemical Laboratory of the Erasmus University Medical Center. Fifty µL of blood was used to measure the number of leukocytes with a Z series Coulter Counter (Beckman Coulter, Woerden, The Netherlands).

Tissue sampling

Liver and tumors were collected and weighed. The median liver lobe was isolated for array analysis and directly stored in RNA*later*® Solution (Life Technologies Europe BV, Bleijswijk, the Netherlands) and stored at 4°C until further analysis. Parts of viable tumor border were identified and also directly stored in RNA*later*® until further analysis.

RNA isolation

Tumor and liver samples obtained were kept at 4°C in the RNA*later*® Solution until further analyses. RNA isolation took place between 24 hours and 96 hours after sample collection. Total RNA was extracted via the QIAzol lysis Reagent and miRNAeasy Mini Kits (QIAGEN, Hilden, Germany), according to Qiagen protocol. Concentrated buffers

RPE and RWT (QIAGEN) for washing of membrane-bound RNA and purification were added mechanically by using the QIAcube (QIAGEN, Hilden, Germany) via the miRNeasy program. Subsequently, isolated RNA was stored at -80°C. RNA concentrations were measured using the Nanodrop (Thermo Fisher Scientific™, Breda, the Netherlands) and RNA quality was assessed using the 2100 Bio-Analyzer (Agilent Technologies, Amstelveen, the Netherlands), according to manufacturer's instructions. The RNA quality was quantitatively expressed as the RNA Integrity Number (RIN, range 0-10). Out of 6 tumor and 6 liver samples per group, the 4 samples with the highest RIN were used for microarray analyses. RIN-values of the tumor samples ranged between 7.8 and 10, the RIN-values of the liver samples ranged between 7.6 and 8.6.

Array analysis

Microarray hybridization was done at the Microarray Department of the University of Amsterdam (the Netherlands) to Affymetrix HT MG-430 PM Array Plates, according to the Affymetrix protocols. For each group, 4 biological replicates were used. The output of the hybridization contained raw mean expression data put into .CEL files. Subsequent quality control and normalization were done using the pipeline at the www.array-analysis.org website (Maastricht University, the Netherlands) (12). Normalization was performed via the Robust Multichip Average (RMA) algorithm, and the output of the normalization consisted of 45141 probes (13). Both raw and normalized microarray data and their MIAME compliant metadata were deposited at the Gene Expression Omnibus (GEO) database, with number GSE72484 (www.ncbi.nlm.nih.gov/geo).

Statistical analyses

For each set of parameters means and standard errors of the mean were computed. All standard statistical tests were performed using SPSS version 21 for Windows software (Statistical Package for Social Sciences, Chicago, IL) and GraphPad Prism (GraphPad Software Inc., version 5.01) . A $p<0.05$ was considered to be significant. Microarray analyses were performed using the free software package R (R foundation). Gene expression profiles were compared using the Linear Models for Microarray Data (limma) method with correction for multiple testing using the false discovery rate (FDR) according to Benjamini and Hochberg (14). Fold changes were expressed as the geometric mean per diet group against the corresponding AL fed control group, and cutoff values for a significant difference were put at FDR <5%. Functional annotation and analyses were performed using the Ingenuity software (http://www.ingenuity.com/products/ipa). Inhibition or activation prediction of the upstream transcription regulators (upstream analysis) was predicted with Ingenuity software by calculating statistical z-scores based on the observed gene expression changes in our dataset. Via z-scores, the chance of significant prediction based on random data is reduced (http://ingenuity.force.com/ipa/

articles/Feature_Description/Upstream-Regulator-Analysis). Cutoff values for a significant activation or inhibition were put at a z-score of ≥2 or ≤-2, respectively.

Results

Fasting induced chemoprotective phenotype

The direct systemic effects of chemotherapy and the effects of fasting were analyzed via markers of liver function, kidney function and leukocyte numbers at 12 hours after injection of irinotecan. Compared to AL fed mice injected with vehicle, a significant increase

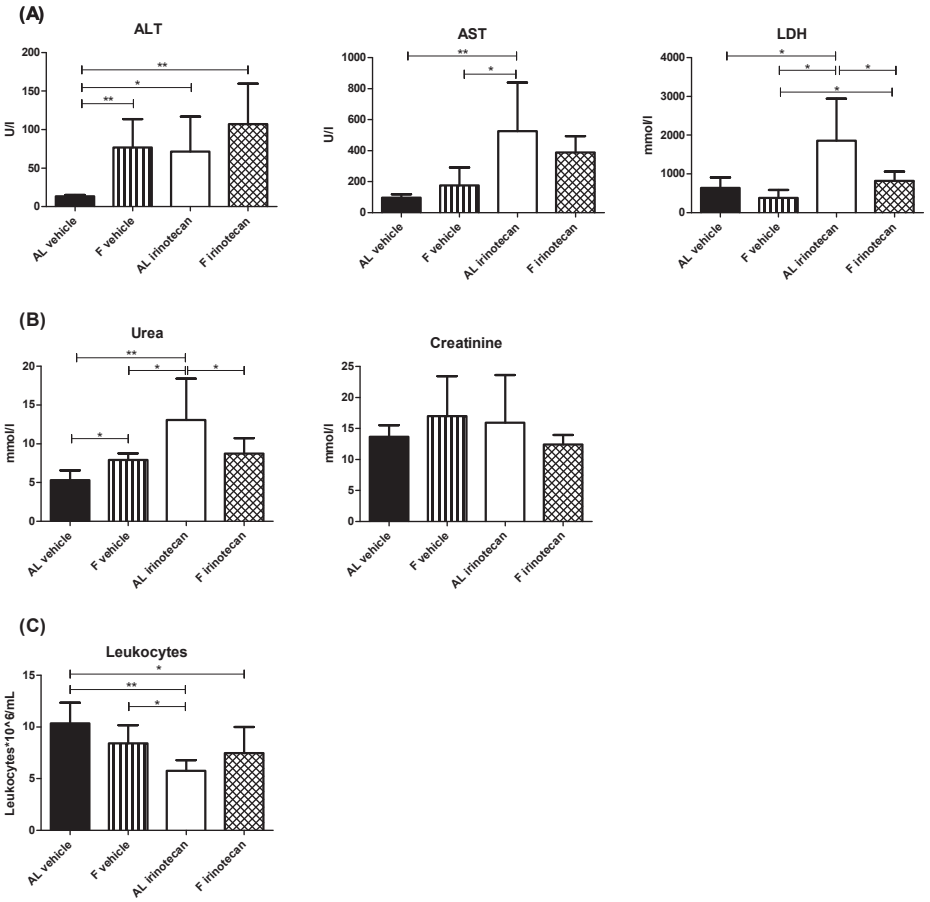

Figure 2. (A) Liver function of the different experimental groups, measured via serum AST, ALT and LDH levels. **(B)** Kidney function of the experimental groups via serum urea and creatinine levels. **(C)** Leukocyte numbers of all the experimental groups. All values were determined 12 hours after administration of either vehicle or irinotecan. AST = aspartate transaminase, ALT = alanine transaminase, LDH = lactate dehydrogenase. *= P<0.05, **=P<0.01.

in serum levels of ALT (P<0.05), AST (P<0.01) and LDH (P<0.05) was observed in AL fed irinotecan treated mice (**Fig2A**). The fasted vehicle group had significantly higher serum ALT levels than the AL vehicle group. The fasted irinotecan group had significantly lower levels of serum LDH than the AL irinotecan group (P<0.05).

Serum urea levels were significantly lower in the AL vehicle group than in fasted vehicle (P<0.05), the AL irinotecan (P<0.01) and fasted irinotecan (P<0.01) group (**Fig2B**). The fasted irinotecan group had significantly lower serum urea levels than did the AL irinotecan group (P<0.05). Serum creatinine did not significantly change in any of the 4 groups (**Fig2B**).

Both the AL irinotecan (P<0.01) and fasting irinotecan (P<0.05) group had a significantly lower number of leukocytes than the AL vehicle group (**Fig2C**). The AL irinotecan group also had significantly lower leukocyte numbers than the fasted vehicle group (P<0.05). In contrast the fasted vehicle and fasted irinotecan group had comparable leukocyte numbers.

Liver array analyses

Principal component analysis

To investigate variability between and among our experimental groups, we performed an unbiased principal component analysis (PCA) of liver and tumor samples including all probe sets in the microarray. In the PCA plot of the liver samples, 50.77% of the variance was explained by principal components (PC) 1 and 16.81% by PC2 (**Fig3**). A pattern of distinct clustering of the 4 individual groups was seen, with the smallest intragroup variability in the fasting vehicle group. The fasting vehicle group was most different from the AL vehicle group, mainly on the PC1, while the difference with the AL irinotecan group was for the most part on PC2, indicating distinct gene expression patterns among these groups. The fasting vehicle group was closely related to the fasting irinotecan group, and showed the smallest difference observed between groups.

Gene expression profiles

To compare gene expression profiles between the different experimental groups, the numbers of differentially expressed probe sets (DEPS) were calculated. In the liver, 2667 DEPS were found between the AL vehicle and AL irinotecan group, with 1144 DEPS down-regulated and 1523 up-regulated (**Fig4A**). The number of DEPS between fasting vehicle and fasting irinotecan was 754, a 3.5 fold lower number than found in the AL vehicle and AL irinotecan comparison. Of these 754 genes, 459 DEPS were down-regulated and 295 were up-regulated. To analyze common expression patterns between the AL and fasting groups, the overlapping DEPS were visualized using a Venn diagram and a scatterplot. The Venn diagram showed that 329 DEPS overlapped between the AL irinotecan and

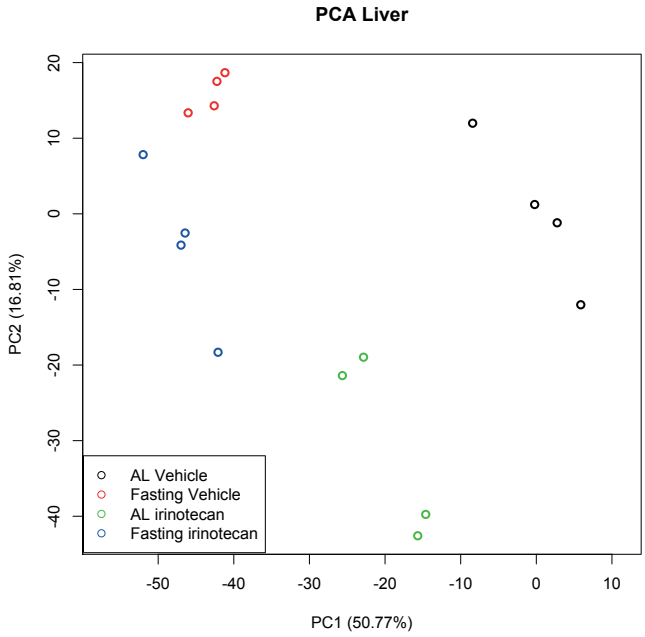

Figure 3. Unbiased principal component analyses (PCA) of liver samples, based on all probe sets in the microarray. Principal component (PC) 1 is depicted on the x-axis and PC2 is depicted on the y-axis, including the percentage of variance explained by each PC. Each symbol represents one sample of one mouse. Samples of the same group are shown in the same color. AL = ad libitum.

fasting irinotecan groups, corresponding to 44% of the DEPS found after fasting, and only 14% of the DEPS after AL vehicle vs. AL irinotecan (**Fig4B**). Directionality and expression intensity of the unique and overlapping genes was visualized in a scatterplot. This scatterplot showed that 83% of all DEPS had a similar directionality in both comparisons, but fold change expressions of the AL irinotecan groups (red symbols) were on average higher than the fasting irinotecan groups (green symbols). Of the overlapping DEPS (black symbols) the vast majority had similar directionality patterns (**Fig4C**). Collectively, these results point towards a dampened response in de fasted group pertaining the significantly regulated genes in the AL group and more pronounced expression changes for a different subset of genes (green symbols).

Pathway analyses

To explore the pathways regulated by irinotecan, the liver of AL fed mice (2667 DEPS) was analyzed. A total of 19 pathways were regulated, defined as pathways with a significant p-value of <0.05 and a z-score of ≥1 or ≤-1 (**Table 1A**). The activated pathways were mainly involved in induction of cytokine signaling, cellular immune response and tyrosine/kinase system signaling. The inhibited pathways were mainly part of the

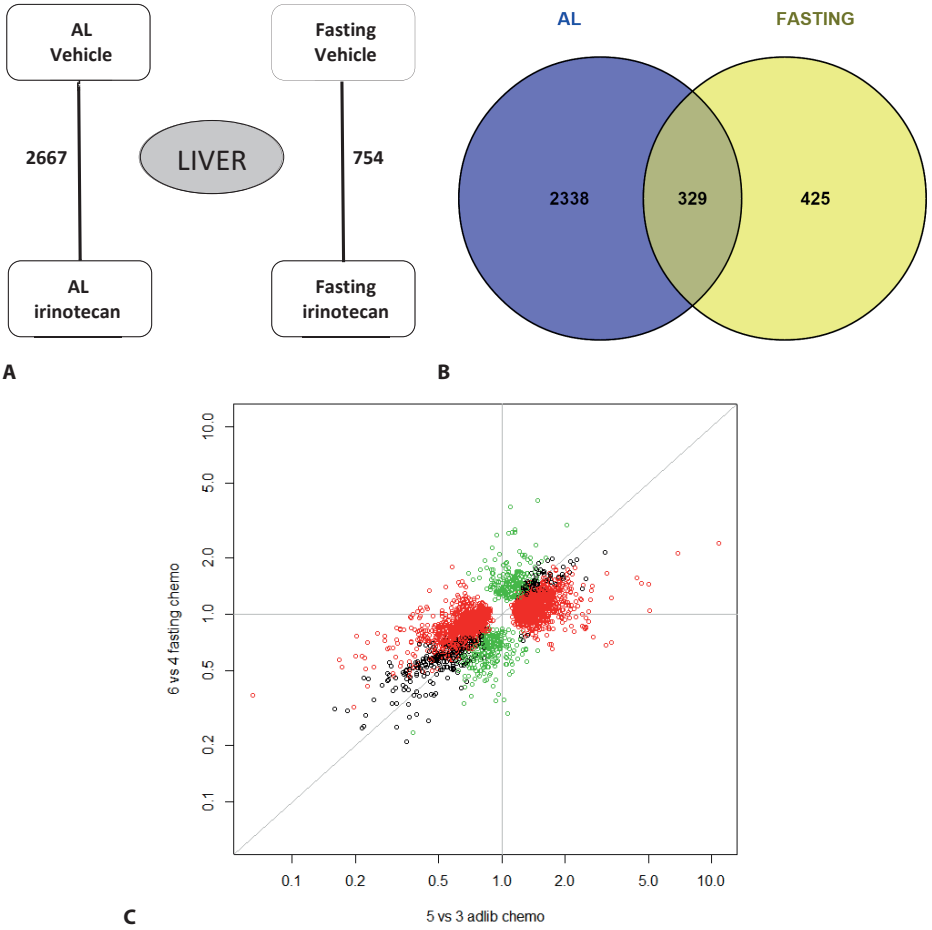

Figure 4. (A) Number of significantly differentially expressed probe sets (DEPS) in the liver in the ad libitum (AL) and fasted groups. **(B)** Venn diagram of overlapping DEPS between the DEPS after the AL groups and the fasting groups, including the unique DEPS of both comparisons. **(C)** Scatterplot of the DEPS found in the liver with false discovery rate of 5%. On the x-axis are the fold changes found in the comparison of AL vehicle vs. AL irinotecan, on the y-axis the fold changes of fasting vehicle vs. fasting irinotecan. Red symbols = AL groups; green symbols = fasting groups; black symbols = overlapping DEPS. Symbols in the upper-right quadrant are up-regulated in both groups; symbols in the lower-left quadrant are down-regulated in both groups; symbols in the upper-left quadrant resemble DEPS down-regulated in the AL groups and up-regulated in the fasting groups; the lower-right quadrant resemble DEPS that are up-regulated in the AL groups and down-regulated in the fasting groups.

cAMP system, cardiovascular signaling and inhibition of cellular growth, proliferation, development, and cellular stress and injury. To examine the changes on pathway level due to fasting, the differentially expressed pathways found in the AL fed groups were examined in the fasting groups. Of the 19 pathways regulated in the AL groups, 5 were regulated in the opposite direction in the fasting groups (**Table 1A**). These pathways

Table 1 Overview of the top overrepresented canonical pathways in liver following each comparison ranked by their –log P-value

(A) AL vehicle – AL irinotecan					
Canonical Pathway	**Pathway classification**	**P-value**	**Genes Ratio**	**Z-score**	**Z-score FV - FI**
AMPK Signaling	Intracellular and second messenger signaling; cellular growth and proliferation and development	2.90E-02	19/134 (14.2%)	-1.000	N/A
Calcium-induced T Lymphocyte Apoptosis	Apoptosis; cellular immune response	3.79E-03	13/64 (20.3%)	-1.732	-0.816
CXCR4 Signaling	Cytokine signaling; cellular immune response	6.91E-05	29/152 (19.1%)	+1.000	-0.832
Dopamine-DARPP32 Feedback in cAMP Signaling	Intracellular and second messenger signaling; neurotransmitter and other nervous system signaling	1.02E-03	27/161 (16.8%)	-1.279	-2.121
EIF2 Signaling	Intracellular and second messenger signaling; cellular stress and injury; cellular growth and proliferation and development	7.43E-03	27/185 (14.6%)	+2.138	N/A
fMLP Signaling in Neutrophils	Cytokine signaling; cellular immune response	1.89E-04	22/108 (20.4%)	+1.342	-0.707
Growth Hormone Signaling	Growth factor signaling; cellular growth and proliferation and development	4.10E-02	11/69 (15.9%)	-1.265	0.000
IL-1 Signaling	Cytokine signaling	6.33E-03	16/91 (17.6%)	+1.604	+0.378
IL-8 Signaling	Cytokines signaling; cellular immune response	3.04E-05	34/183 (18.6%)	+1.219	-1.000
Integrin Signaling	Cell cycle regulation; intracellular and second messenger signaling; cellular growth and proliferation and development	2.21E-02	27/202 (13.4%)	+1.225	-0.905
LPS/IL-1 Mediated Inhibition of RXR Function	Nuclear Receptor Signaling	6.39E-03	31/218 (14.2%)	+3.051	N/A
LPS-stimulated MAPK Signaling	Apoptosis	2.73E-02	12/73 (16.4%)	+1.155	+0.707
Nitric Oxide Signaling in the Cardiovascular System	Cardiovascular Signaling	6.36E-03	17/99 (17.2%)	-1.000	-0.333
P70S6 Signaling	Cellular stress and injury; cellular growth and proliferation and development	4.17E-05	25/119 (21.0%)	-1.043	-0.707
Remodeling of Epithelial Adherens Junctions	Cellular growth and proliferation and development	3.74E-02	11/68 (16.2%)	+1.414	N/A
RhoA Signaling	Intracellular and second messenger signaling	1.17E-02	19/122 (15.6%)	+2.668	N/A

Table 1 (continued)

Canonical Pathway	Pathway classification	P-value	Genes Ratio	Z-score	Z-score FV - FI
Role of BRCA1 in DNA Damage Response	Cellular stress and injury	2.50E-02	11/64 (17.2%)	+1.134	N/A
Signaling by Rho Family GTPases	Intracellular and second messenger signaling	5.69E-03	33/234 (14.1%)	+2.985	-1.265
Sphingosine-1- phosphate Signaling	Organismal growth and development	1.62E-02	17/109 (15.6%)	+1.000	0.000
(B) Fasting vehicle – Fasting irinotecan					
Canonical Pathway	**Pathway classification**	**P-value**	**Genes Ratio**	**Z-score**	**Z-score ALV-ALI**
Androgen Signaling	Nuclear receptor signaling	2.82E-03	9/111 (8.1%)	-1.134	+0.302
Aryl Hydrocarbon Receptor Signaling	Cell cycle regulation; apoptosis; xenobiotic metabolism; nuclear receptor signaling	3.34E-02	8/140 (5.7%)	-2.121	N/A
Cardiac Hypertrophy Signaling	Cardiovascular signaling	2.64E-03	14/223 (6.3%)	-1.387	+0.186
Chemokine Signaling	Cytokine Signaling; Organismal Growth and Development	1.14E-02	6/71 (8.5%)	-1.633	-0.302
Cholecystokinin/Gastrin-mediated Signaling	Neurotransmitters and other nervous system signalling	4.50E-07	14/101 (13.9%)	-1.069	0.447
Dopamine-DARPP32 Feedback in cAMP Signaling	Intracellular and second messenger signaling; neurotransmitter and other nervous system signaling	2.82E-02	9/161 (5.6%)	-2.121	-1.279
Glioblastoma Multiforme Signaling	Cancer	1.61E-02	9/146 (6.2%)	-1.000	+0.209
GNRH Signaling	Neurotransmitters and other nervous system signalling	1.70E-04	12/129 (9.3%)	-1.732	+0.853
IL-8 Signaling	Cytokines signaling; cellular immune response	3.17E-05	16/183 (8.7%)	-1.000	+1.219
Leukocyte Extravasation Signaling	Cellular Immune response	3.91E-02	10/198 (5.1%)	-1.000	N/A
NRF-mediated Oxidative Stress Response	Cellular stress and injury	1.07E-03	13/180 (7.2%)	+1.414	N/A
P2Y Purigenic Receptor Signaling Pathway	Cardiovascular signaling	4.49E-03	9/119 (7.6%)	-1.000	-0.894
PTEN signaling	Apoptosis; cancer	4.24E-03	9/118 (7.6%)	+1.000	N/A
Renin-Angiotensin Signaling	Cardiovascular signaling; growth factor signaling	2.49E-03	9/109 (8.3%)	-1.000	+0.471
RhoGDI Signaling	Intracellular and second messenger signaling	6.62E-03	11/173 (6.4%)	+2.333	-0.471
Signaling by Rho Family GTPases	Intracellular and second messenger signaling	1.54E-03	15/234 (6.4%)	-1.265	+2.985

Table 1 (continued)

Canonical Pathway	Pathway classification	P-value	Genes Ratio	Z-score	Z-score ALV-ALI
Sperm Motiliy	Organismal growth and development	1.48E-02	8/120 (6.7%)	-1.134	-0.243
Tec Kinase Signaling	Intracellular and second messenger signaling	3.37E-03	11/158 (7.0%)	-1.000	N/A
Thrombin Signaling	Cardiovascular signalling	6.05E-04	14/191 (7.3%)	-1.069	+0.192
Toll-like Receptor Signaling	Apoptosis; humoral/cellular immune response	4.68E-02	5/74 (6.8%)	+1.342	N/A

All canonical pathways with a z-score of ≤-1.000 or ≥+1.000 are listed. Pathways with a significant z-score of ≤-2.000 or ≥+2.000 are depicted in bold. Genes ratio= the number and percentage of genes differentially expressed in ratio to the total number of genes involved in the pathway.

involved inhibition of cytokine signaling and cellular immune responses, and inhibition of the intracellular and second messenger signaling as well as cell cycle regulation and cellular growth and proliferation. Six pathways showed the same directionality, yet with lower activity scores than in the AL groups. These pathways were also mainly involved in cytokine signaling and cellular stress and injury. Eight pathways were not regulated in the fasting groups, mainly cellular growth and proliferation signaling pathways as well as the growth hormone inhibition.

Secondly, the isolated effect of fasting on irinotecan treatment was examined by analyzing the pathways regulated by the 754 DEPS found after fasting. This analysis revealed 20 pathways, of which 16 were down-regulated and 4 were up-regulated (**Table 1B**). Most of the down-regulated pathways were connected to cellular immune response, cellular stress and injury, apoptosis and cardiovascular signaling pathways. Of these 16 pathways, 12 were either not activated or were inhibited in the AL liver comparison. The 4 up-regulated pathways were involved in pathogen-influenced signaling and cellular stress and injury. These up-regulated pathways were not differentially regulated in the comparison between AL vehicle and AL irinotecan (**Table 1B**).

Irinotecan-related gene expression

We have previously shown that SN-38 levels are lower in the livers of fasted mice. To identify the direct effect of irinotecan on liver tissue, target proteins of the metabolite SN-38 were highlighted and explored. The main target of SN-38 is topoisomerase-I (*TOP1*), and irinotecan directly interacts via inhibition of this gene (15). In AL fed irinotecan treated animals, *TOP1* showed a fold change of 1.373, while in the fasted group no differences were seen. Other molecules involved in the metabolism of irinotecan and colorectal carcinoma (CRC) are nuclear factor-kappa beta (*NF-κβ*) (16), mammalian target of rapamycine (*mTOR*) (17) and mouse double minute 2 homolog (*MDM2*) (18). *NF-κβ* was up-regulated (1.069) in the AL fed irinotecan group and inhibited in fasted animals

(-1.293). Expression of *mTOR* was lower in the AL fed irinotecan treated group (-1.749), while it was slightly higher in fasted mice treated with irinotecan (1.116). Dihydropyrimidine dehydrogenase (*DPYD*) and methylenetetrahydrafolate (*MTHFD1*) deficiencies are both involved in drug clearance and toxicity (19, 20). Both genes were down-regulated in the AL irinotecan group compared to the AL vehicle group (*DPYD*: -2.455, *MTHF*D1: 1.742). Both were not differentially expressed in the fasted liver groups.

Tumor transcriptome analysis

Principal component analysis

With a total of 59.49% of the variance explained by PC1 and 10.88% by PC2, the PCA plot of the tumor samples of the 4 experimental groups showed high heterogeneity among the groups with large intragroup variability (**Fig5**), also compared to the liver PCA plot (**Fig3**). The individual groups were not clearly separated as clusters, and showed only minor differences between groups. The tumor samples of the fasting vehicle group

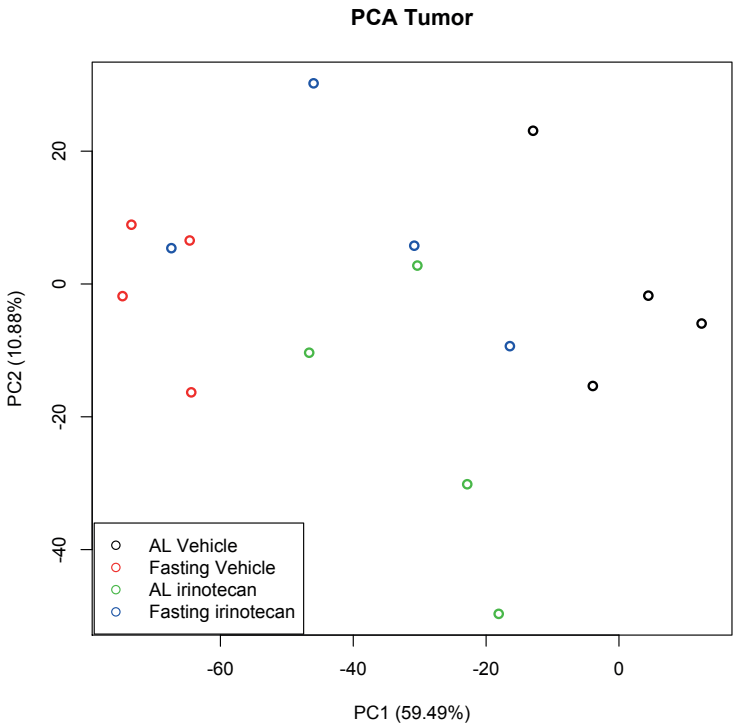

Figure 5. Unbiased principal component analyses (PCA) of tumor samples, based on all probe sets in the microarray. Principal component (PC) 1 is depicted on the x-axis and PC2 is depicted on the y-axis, including the percentage of variance explained by each PC. Each symbol represent one sample of one mouse. Samples of the same group are shown in the same color. AL = ad libitum.

showed the smallest intragroup variability. A shift on the PC1 axis could be detected between the AL vehicle and fasting vehicle groups, while no shift on the PC2 axis was obvious. The irinotecan-treated groups showed high overlap within groups and no clear clustering of groups.

Gene expression profiles

Based on the heterogeneous PCA plot, a small number of differentially regulated genes between the AL groups was suspected. Indeed, comparison of AL vehicle and AL irinotecan showed 610 DEPS, of which 321 were down-regulated and 289 up-regulated (**Fig6A**). The number of regulated DEPS between fasting vehicle and fasting irinotecan was 3093. Comparing overlapping genes, we found 138 genes in common between the AL vehicle vs. AL irinotecan and the fasting vehicle vs. fasting irinotecan comparison, corresponding to 22,6% of the DEPS in the AL groups and only 4,5% of the fasting groups (**Fig6B**). All DEPS considered, only 17% had the same directionality in both comparisons, while the other DEPS had opposite directionalities (**Fig6C**).

Pathway analyses

To identify pathways in common between liver and tumor tissue treated with irinotecan, the pathways found regulated in the liver were examined in the tumor (**Table 2**). Firstly, of the pathways regulated in the AL vehicle vs. AL irinotecan groups, only one pathway was regulated in the tumor vehicle vs. tumor irinotecan groups. This downregulated pathway was the *Nitric Oxide Signaling in the Cardiovascular System*, a pathway involved in cardiovascular signaling, which was more downregulated in tumor tissue based on the z-score. Of the 19 pathways regulated in the liver in the AL groups, 10 were also found in the fasting vehicle vs. fasting irinotecan DEPS. Eight out of 10 showed the same directionality, yet showed on average less activation. These were involved in cytokine signaling and cellular immune responses as well as cellular growth and proliferation. The apoptosis pathway was unaffected and was still strongly activated. Also, 2 pathways were oppositely regulated, involved in cellular growth inhibition and activation of a cellular stress and injury pathway.

Effect of irinotecan on gene expression in tumor tissue

The main target of metabolized irinotecan, *TOP1* had a fold change of 1.689, in the tumor AL vehicle vs. AL irinotecan groups. In the fasting groups, the fold change was 1.772. Analysis of tumor-related effects was performed with tumor-specific genes. The gene tumor protein 53 (*Tp53*) was higher regulated in the AL irinotecan than in the AL vehicle group (1.310), while it was lower in the fasting irinotecan than in the fasting vehicle group (-1.815). Tumor suppressor phosphatase and tensin homolog (*Pten*) was not differentially expressed due to irinotecan treatment in the AL group, while it was higher

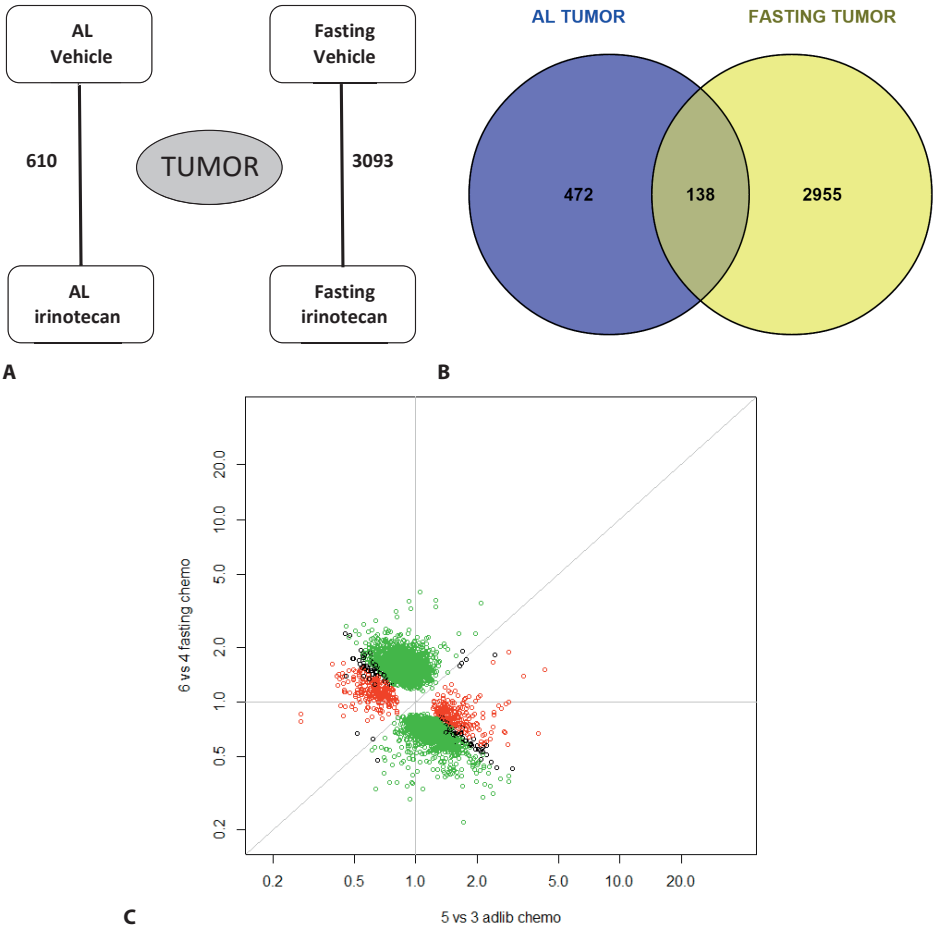

Figure 6. (A) Number of significantly differentially expressed probe sets (DEPS) in the tumor in ad libitum (AL) and fasted groups. **(B)** Venn diagram of overlapping DEPS between the DEPS after the AL tumor groups and the fasting tumor groups, including the unique DEPS of both comparisons. **(C)** Scatterplot of the DEPS found in the tumor with false discovery rate of 5%. On the x-axis are the fold changes found in the comparison of AL vehicle vs. AL irinotecan, on the y-axis the fold changes of fasting vehicle vs. fasting irinotecan. Red symbols = AL groups; green symbols = fasting groups; black symbols = overlapping DEPS. Symbols in the upper-right quadrant are up-regulated in both groups; symbols in the lower-left quadrant are down-regulated in both groups; symbols in the upper-left quadrant resemble DEPS down-regulated in the AL groups and up-regulated in the fasting groups; the lower-right quadrant resemble DEPS that are up-regulated in the AL groups and down-regulated in the fasting groups.

in the fasting irinotecan group than in the fasting vehicle group. Irinotecan is known to induce apoptosis via activation of the cysteine-aspartic acid protease (caspase) group, amongst which caspase 3 (*Casp3*), caspase 9 (*Casp9*) and cyclin-dependent kinase inhibitor 1A (*CDKN1A*, also known as *p21*). Using the upstream analysis, the caspase group showed no differences between the AL vehicle and AL irinotecan groups. Following the

Table 2. Overview of the top overrepresented canonical pathways in tumor following each comparison ranked by their –log P-value

TUMOR

(A) AL vehicle – AL irinotecan			
Canonical Pathway	**P-value**	**Genes Ratio**	**Z-score**
Glioma Signaling	1.39E-03	8/95 (8.4%)	-2.121
Neuropathic Pain Signaling in Dorsal Horn Neurons	1.93E-03	8/100 (8.0%)	-2.121
CREB Signaling in Neurons	5.60E-03	10/171 (5.8%)	-2.333
PTEN Signaling	1.77E-02	7/118 (5.9%)	+2.449
Nitric Oxide Signaling in the Cardiovascular System	2.48E-02	6/99 (6.1%)	-2.000
Thrombin Signaling	2.99E-02	9/191 (4.7%)	-1.000
eNOS Signaling	4.12E-02	7/141 (5.0%)	-1.342
(A) Fasting vehicle – Fasting irinotecan			
Canonical Pathway	**P-value**	**Genes Ratio**	**Z-score**
CD40 Signaling	9.76E-04	14/65 (21.5%)	+2.138
TNFR2 Signaling	9.45E-03	7/29 (24.1%)	+2.646
ATM Signaling	9.30E-05	15/59 (25.4%)	-1.604
Angiopoietin Signaling	9.22E-03	12/66 (18.2%)	-1.265
Colorectal Cancer Metastasis Signaling	8.96E-04	35/236 (14.8%)	+1.826
4-IBB Signaling in T-lymphocytes	8.31E-04	9/31 (29.0%)	+1.414
B Cell Activating Factor Signaling	8.23E-05	12/40 (30.0%)	+2.111
UVC-induced MAPK Signaling	7.85E-03	9/42 (21.4%)	-1.000
IL-6 Signaling	7.45E-04	21/116 (18.1%)	+1.528
PDGF Signaling	6.65E-04	16/77 (20.8%)	+1.000
Estrogen-dependent Breast Cancer Signaling	5.92E-04	14/62 (22.6%)	+1.069
Signaling by Rho Family GTPases	5.23E-03	32/234 (13.7%)	+2.887
Androgen Signaling	5.19E-05	23/111 (20.7%)	+1.667
ILK Signaling	5.07E-04	30/186 (16.1%)	+1.512
Aryl Hydrocarbon Receptor Signaling	4.62E-05	27/140 (19.3%)	+2.200
P2Y Purigenice Receptor Signaling pathway	4.62E-05	27/140 (19.3%)	+2.200
Ephrin B Signaling	4.43E-02	11/73 (15.1%)	+1.633
Prolactin Signaling	4.43E-02	11/73 (15.1%)	+1.000
EGF Signaling	3.51E-03	27/183 (14.8%)	+1.633
IL-8 Signaling	3.51E-03	27/183 (14.8%)	+1.633
Ga12/13 Signaling	3.27E-04	22/117 (18.8%)	+1.091
iNOS Signaling	3.06E-02	8/44 (18.2%)	+1.890
Acute Phase Response Signaling	3.02E-02	22/169 (13.0%)	+1.091
Rac Signaling	2.88E-03	18/104 (17.3%)	+1.213
PPARa/RXRa Activation	2.70E-06	35/179 (19.6%)	-1.569
TGF-B Signaling	2.57E-03	16/87 (18.4%)	-1.941
Death Receptor Signaling	2.35E-02	14/92 (15.2%)	+1.069

Table 2 (continued)

Canonical Pathway	P-value	Genes Ratio	Z-score
NF-kB Signaling	2.32E-05	32/173 (18.5%)	+1.768
Pancreatic Adenocarcinoma Signaling	2.32E-04	23/122 (18.9%)	+1.528
RhoA Signaling	2.32E-04	23/122 (18.9%)	+1.528
PPAR Signaling	2.31E-03	17/94 (18.1%)	-1.213
Toll-like Receptor Signaling	2.20E-02	12/74 (16.2%)	+1.134
AMPK Signaling	2.12E-03	22/134 (16.4%)	+1.213
April Mediated Signaling	2.12E-03	22/134 (16.4%)	+1.213
TNFR1 Signaling	2.12E-02	9/49 (18.4%)	+1.000
FcyRIIB Signaling in B-lymphocytes	2.07E-02	8/41 (19.5%)	-1.414
WNT/Ca+ pathway	1.88E-02	10/56 (17.9%)	+2.333
Inhibition of Angiogenesis by TSP1	1.71E-03	9/34 (26.5%)	-1.134
Wnt/B-catenin Signaling	1.70E-02	23/169 (13.6%)	+1.414
Acute Myeloid Leukemia Signaling	1.58E-04	23/119 (19.3%)	+1.091
Remodeling of Epithelial Adherens Junctions	1.17E-02	12/68 (17.6%)	+1.134
PKCθ Signaling in T Lymphocytes	1.10E-02	18/118 (15.3%)	+1.414

All canonical pathways with a z-score of ≤-1.000 or ≥+1.000 are listed. Pathways with a significant z-score of ≤-2.000 or ≥+2.000 are depicted in bold. Genes ratio= the number and percentage of genes differentially expressed in ratio to the total number of genes involved in the pathway.

comparison in the fasting groups, the caspase group was more activated in the fasted animals treated with irinotecan than in the fasting vehicle group (z-score 1.387). Specific targets of the caspase group that were up-regulated in the fasting irinotecan group, were *CCND3*, *CDKN1A* and *CDKN1B*, *CDK4* and *CKD6*, and *APP*.

Discussion

Fasting is known to reduce the adverse side effects of chemotherapy treatment, while it preserves or even enhances its anti-tumor activity. To unravel the mechanism behind this remarkable effect, we performed transcriptome analysis on liver and tumor tissue of fasted and fed mice carrying subcutaneous colorectal carcinoma. We found that 3-days of fasting prior to irinotecan treatment reduces the side effects of irinotecan via down-regulation of the gene expression profiles in the liver and activation of a cellular stress response, thereby possibly decreasing the accessibility of irinotecan in extra-tumor tissue. Specific sets of genes involved in liver damage and toxicity were decreased due to fasting, indicating that metabolism from irinotecan to SN-38 is decreased and that SN-38 is not able to exert its toxic actions in the liver. These effects were not present in tumor tissue, where the anti-tumor activity of irinotecan was not compromised by fasting. Even

so, expression analyses hinted towards increased sensibility for the chemotherapy in the tumor. These results increase our knowledge about the mechanistic processes underlying dietary restriction (DR), making it a possible to facilitate translational research into the clinical setting, and may help us to develop DR mimetics that induce similar effects without the disadvantages of fasting.

The topoisomerase-I inhibitor irinotecan is a frequently used chemotherapeutic agent and recognized as first and second line treatment for colorectal carcinoma, which contributes to increased survival rates and the chance of a potential curative approach in patients with colorectal liver metastases (3, 4). The use of irinotecan is limited by the frequent occurrence of severe side effects, including hepatotoxicity (5). In a recent publication, we showed that fasting is a strong protector against side effects induced by irinotecan in a mouse model, while leaving its anti-tumor activity unaffected (9, 21). In the present study, we examined the mechanistic actions of irinotecan in both healthy liver and colorectal tumor tissue, and confirmed above-mentioned findings on gene expression level. Irinotecan resulted in increased liver and kidney injury, and bone marrow depression, as indicated by increased AST, LDH and urea levels, and a significant leukocyte depletion 12 hours after one high dose of irinotecan. These changes were not seen in fasted mice, demonstrating that, in this study, irinotecan induced toxicity in AL fed animals was reduced by fasting. Indeed, over 3 fold fewer gene expression changes occurred in healthy liver tissue when mice were fasted prior to injection of irinotecan. The gene profiles between the AL and fasting comparisons overlapped only partially as evidenced by the genes and pathways in common. In the AL irinotecan treated mice, the majority of pathways indicated activation of chemokine and cytokine signaling as well as DNA damage response, indicating the induction of cell damage and inflammation. Both failed to appear after 3 days of fasting or were of lower magnitude. Also, fasting mostly inhibited pathways, and down-graded most actions involved in irinotecan treatment. More interestingly, fasting induced an additional independent response, mediated by stress pathways such as *Nrf2* and Aryl Hydrocarbon Receptor, which are known to induce differential stress sensitization (DSS) (11, 22). Especially activation of *Nrf2* plays a key role in induction of DSS, and has come up in previous gene expression analyses in fasting and DR mouse models (23, 24). A possible underlying mechanism of DSS is the inability of irinotecan to enter the healthy liver cells via activation of these pathways. An extra indication for this decreased permeability of the cells is the failure to activate genes involved in the irinotecan metabolism, such as topoisomerase-I (*TOP1*). Several studies showed the correlation between the *TOP1* expression levels and the sensitivity to SN-38, the active metabolite of irinotecan (25-27). Further down the cascade of irinotecan is *NF-κβ*, a nuclear factor initiated by *Nrf2* and involved in the effects of the drug. An increase in *NF-κβ* may lead to an extra suppression of cell growth and an increase in apoptosis in addition to that induced by irinotecan (16). This effect stopped out in the

AL groups, while even an inhibition of *NF-κβ* occurred in the fasted groups, including family member v-rel avian reticuloendotheliosis viral oncogene homolog A (*RELA*) (28).

Side effects of chemotherapy are difficult to measure at the transcriptional level, since efficacy and toxicity are closely correlated (29). Nevertheless, the list of genes possibly involved in toxicity of specific chemotherapeutic agents continues to lengthen. Genes that have been examined recurrently are dihydropyrimidine-dehydrogenase (*DPYD*) and methylenetetrahydrofolate dehydrogenase (*MTHFD1*) (30). Partial deficiencies of *DPYD* occur in 3-5% of the cancer patients, and are related to decreased drug clearance and prolonged exposure to toxicity (30, 31). The role of *MTHFD1* deficiencies remains controversial with studies showing decreased toxicity, while others document no effect or even worsening of side effects of different chemotherapeutic agents (30). Both genes, normally highly expressed in the liver, were down-regulated in the AL irinotecan treated mice while these changes were nullified by fasting. Other genes associated with side effects of different chemotherapeutic agents, such as *TYMS*, *GSTM1* and *CDA*, were not regulated in any of the treated groups (20, 30). However, expression levels of these genes in the liver are usually low. Taken together, our data suggest that fasting is able to decrease the metabolization of irinotecan to SN-38 in healthy liver tissue, thereby preventing the drug to exert its toxic effects.

On the contrary, the protective effect of fasting as seen in healthy tissue was absent in tumor tissue preconditioned by 3 days of fasting. The majority of differentially regulated pathways found in the liver of fasted irinotecan treated mice, were either oppositely regulated in fasted tumor tissue or to a lesser extent. Interestingly, the analysis of the AL tumor groups revealed less regulation due to irinotecan than in the mice that were fasted. Although the phenotypical results as well as the presence of *TOP1* expression in tumor indicate that irinotecan is metabolized and active. The higher number of regulated genes as well as detailed analyses demonstrate an additional effect in tumor due to fasting. This effect might be dual, with a role for increased induction of chemotherapy-mediated apoptosis as well as sensitization to the actions of irinotecan. For example, the higher expression of genes such as *NF-κβ (16)*, *JUN (32)* and *MYC (33)* as well as genes involved in the caspase cascade (34), *i.e.* caspase 9, *p21* and *p27*, points towards an increase in chemotherapy-mediated apoptosis, and even hint towards higher sensitivity to chemotherapeutical agents (35, 36). Also, fasting might increase sensitivity to irinotecan by modulating the expression of tumor-specific genes. Several p53 knockout-models showed an increase in sensitivity to different chemotherapeutic agents (37, 38). Further research is needed to elucidate the role of these findings in the DSS.

Since pharmacokinetic studies showed that the half-life of the active metabolite SN-38 is approximately 12 hours (21), we choose to explore the transcriptional changes in the therapeutic window in which the effects of both irinotecan and fasting would

be maximal. Analyses on multiple time points and with different dosages would likely improve our understanding of the results obtained in our study.

In conclusion, we showed that 3-days of fasting results in protection against the side effects of irinotecan treatment in healthy liver tissue, possibly via decreased metabolization from irinotecan to SN-38 in the liver, together with the activation of a protective stress response in healthy, but not tumor tissue. Although fasting leads to an increase in differentially regulated pathways in tumor tissue following irinotecan treatment, it does not abrogate its antitumor efficacy but possibly increases its sensitivity to irinotecan. These data give insight in the mechanisms involved in the protective effects of fasting on the side effects of irinotecan, and may pave the way to develop dietary restriction mimetics which may induce similar effects without the disadvantages of fasting.

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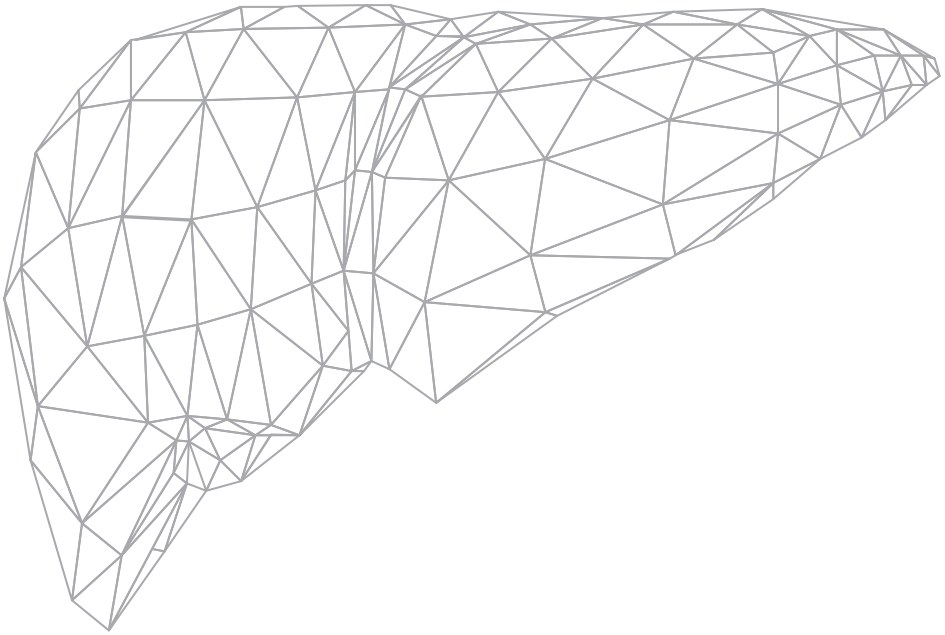
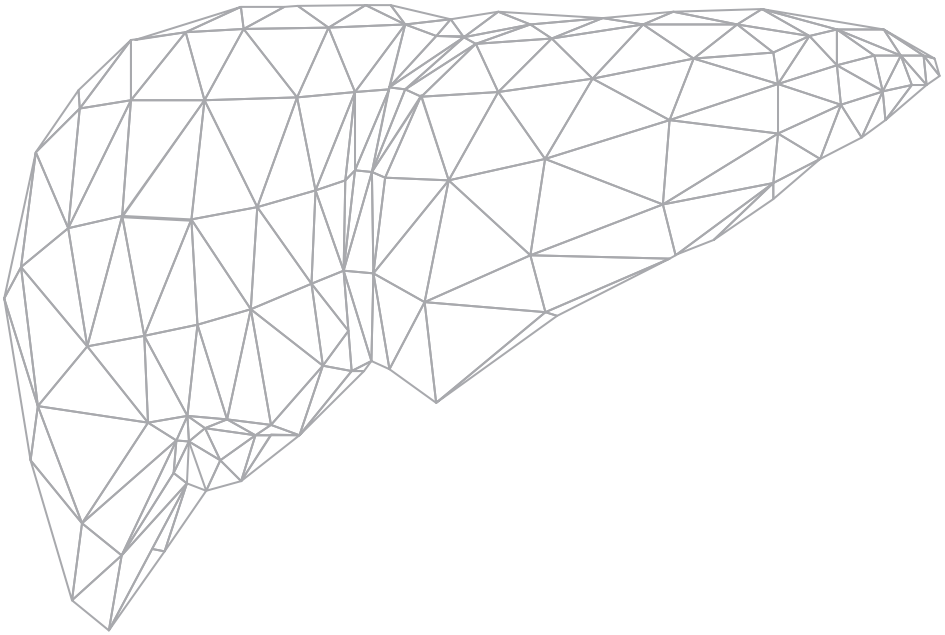


PART FOUR

SUMMARY AND DISCUSSION

Chapter 8 **Summary, discussion, and future perspectives**



CHAPTER 8

SUMMARY, DISCUSSION, AND FUTURE PERSPECTIVES

Summary and discussion

According to the latest numbers colorectal cancer remains the third most common cancer diagnosis in men and the second in women worldwide (1). In approximately 20% of patients distant metastases are present at the time of diagnosis (2). The liver is by far the most common site for metastases, and approximately 50% of patients with early-stage disease will develop colorectal liver metastases (CRLM) (3, 4). Over the past 30 years, the treatment of CRLM has undergone major changes. Patients who were considered to be terminal and incurable, started to show promising improvements using the modern multi-modality approach. At the time of introduction of systemic treatment, 5-fluoruracil based regimens were standard of care in CRLM and already improved the overall survival from 6 to 10 months. Subsequently, the development of chemotherapeutic agents like irinotecan improved this overall survival to a median up to 24 months. However, the associated toxicity of chemotherapeutic agents is a major limitation to their use. There are several severe adverse side-effects seen during irinotecan treatment which may lead to early discontinuation of treatment. There is an urgent need for further optimization of chemotherapeutic treatment to diminish the adverse side-effects, to tolerate higher doses and subsequently improve its efficacy. The overall aim of this thesis was to examine the role of the circadian clock and of fasting in improving chemotherapeutic treatment in CRLM.

In **chapter 2** we reviewed the literature on the role of the circadian clock machinery and circadian gene expression levels in relation to human abdominal cancer types. We concluded that the expression levels of multiple circadian genes are disturbed in malignant tissue in comparison to healthy tissues. Circadian output genes like *PER1*, *PER2*, and *PER3* are consistently downregulated in tumors of different types of cancer. Because of the aberrant expression of multiple circadian, and circadian regulated genes in cancer, novel therapies were conducted to exploit these aberrations as means to improve chemotherapeutic treatment of patients. An important therapy in this field is chronotherapy (5, 6). Chronotherapy consists of administrating chemotherapeutical agents according to the biological rhythm. The perfect time of administration would be when the tumor tissue is most active and the surrounding healthy tissues are not. It is often seen that tumors show a disrupted or altered circadian function in contrast to the circadian entrainment of healthy tissues. In this case, improved efficacy is also seen when chemotherapeutic agents are given near their respective times of best tolerability. In a recent clinical study it has been shown that chronotherapeutic administration of irinotecan, oxaliplatin, and 5-fluorouracil via highly toxic hepatic arterial infusion in heavily pretreated patients is feasible, well tolerated and resulted in a doubling of secondary resection rate in patients with otherwise unresectable colorectal liver metastases (7, 8). We conclude that many

circadian (regulated) genes are down regulated in tumor tissue of different types of abdominal cancer, and it is therefore suggested that these genes could help to improve chemotherapy treatment.

In **chapter 3** we compared the 24-hour expression levels of key circadian clock genes in liver and kidney of healthy control mice with those of mice bearing C26 colorectal tumor metastases in the liver. Metastases in the liver were induced in these mice by injection of C26 colorectal carcinoma cells into the spleen. To prevent intrasplenic tumor growth, the spleen was removed after 10 minutes of injection. Tumors were grown for three weeks, and subsequently tumor, liver, and kidney tissue was collected around the clock to compare circadian rhythmicity. qRT-PCR expression levels of important clock genes revealed normal 24-hour oscillations of all measured clock- and clock-controlled genes in liver and kidney tissue. However, in colorectal liver metastases 24-hour oscillations were absent for all clock- and clock-controlled genes except for *Cry1*. In addition, we observed a phase shift of the circadian clock in healthy liver and in kidney tissue of tumor bearing animals. In the liver a phase advance was observed whereas in the kidney a phase delay was found, suggesting that this is a systemic effect. We conclude that the core clock machinery is severely disrupted in murine colorectal liver metastases which results in altered expression levels of output genes such as *Dbp*. It would be of interest to investigate how this phase shift between healthy liver and kidney tissue can be caused. It is known that tumor cells secrete a plethora of mediators such as TGF-β that is able to modulate the circadian clockwork by activating the ALK-SMAD3-DEC1 pathway. Variation in TGF-β levels in peripheral tissues can induce phase shifts in peripheral clocks (9).

Next, we were interested to determine whether these clock gene expression levels were also disrupted in human colorectal liver metastases. In **chapter 4** we studied surgical resection specimens of 15 patients with primary colorectal carcinoma (CRC) and metachronous CRLM. We compared the expression levels of 10 clock genes in primary CRC, CRLM, and liver tissue within the same patient. Clock genes that were studied included *CLOCK*, *BMAL1*, *PER1*, *PER2*, *PER3*, *CRY1*, *CRY2*, *CSNK1E*, *Timeless* (*TIM*), and *timeless interacting-protein* (*TIPIN*). Seven of the clock genes showed a down-regulation of mRNA levels in CRLM, while in CRC five clock genes were down-regulated. One clock gene was upregulated in in CRC, namely *CSNK1E*. This gene encodes the CKIε protein whose main function is to regulate circadian rhythmicity by phosphorylation and degradation of Period genes (10). Upregulation of *CSNK1E* expression may lead to enhanced phosphorylation of the PER2 protein which is known to destabilize the PER2 protein and target it for ubiquitination and subsequent proteosomal degradation. Furthermore, CKIε plays an essential role in the early development of CRC (11-13). We also studied possible relations between gene expression levels and clinical and pathological factors of the included

patients. We found a significant correlation between gender and the and the expression of *CRY1* in CRLM. Lowest levels of *CRY1* mRNA expression were found in female patients. We also found that low expression of *PER3* correlates with a high number of metastases. In other studies, circadian gene expression levels were also correlated with clinicopathological factors. Mazzoccoli et al. showed a significant association between low *CRY1* expression levels in tumor tissue and a higher tumor grade, advanced age, and female sex. However, survival was better in CRC patients expressing lower *CRY1* and *CRY2* levels in tumor tissue (14, 15). These results are in line with another study which showed that high *CRY1* expression in the tumor corresponded with poor overall survival in CRC patients (16).

In **chapter 5** we started to examine the potential beneficial effects of dietary restriction (DR) on chemotherapy induced adverse side effects. There is emerging evidence that DR, if well timed and titrated may improve a patients resistance to stress. Thus, we speculated that a dietary intervention may protect cancer patients against drug toxicities. Reduction of food intake of approximately 30% (calorie restriction, CR) during life is a powerful means to increase resistance to multiple stressors, reduce inflammation, and increase lifespan in a variety of model organisms (17-22). We have previously extended these observations and showed that short-term CR and periods of fasting also improve acute stress resistance and protect against acute oxidative damage in the kidney and liver (23-26). This protection was associated with increased baseline levels of cytoprotective and anti-oxidant genes, and robust reduction of inflammation. In the present study we have examined the effects of fasting prior to irinotecan treatment on toxicity and anti-tumor activity in FabplCre;Apc15lox/+ mice, which spontaneously develop intestinal tumors. We showed that 3-days of fasting protects against irinotecan induced side-effects, and we observed a similar reduction of tumor size in fasted and *ad libitum* fed treated groups compared to controls. In addition, we found a significant decrease in cell cycle activity, proliferation, and angiogenesis, while senescence was increased in both fasted and ad libitum fed irinotecan treated groups, indicating that fasting does not affect the anti-tumor activity of irinotecan. Previous research from our lab showed that 3-days of fasting increases stress-resistance, including upregulation of the Nrf2 pathway, which is involved in the protection against oxidative stress (23, 27). In contrast to the up-regulation of protective pathways by normal cells, it is thought that cancer cells are unable to obtain a protected state by dietary restriction. This phenomenon is called differential stress sensitization (DSS) and is based on the fact that cancer cells have acquired a number of mutations that progressively decrease their ability to adapt to dietary restriction (28, 29).

To extend and verify our findings and include a more mechanistic approach, we designed the experiments as shown in **chapter 6**. Again, we examined the effects of fasting prior to a high dose of irinotecan on the occurrence of side-effects and anti-tumor activity. In this model we made use of an ectopic model, C26 colon carcinoma cells were grown as subcutaneous tumors in the flank of BALB/c mice. In addition, to elucidate the mechanisms behind the fasting-induced protection to irinotecan induced side-effects we studied its pharmacokinetics in fasted and fed mice in plasma, liver, and tumor. These experiments showed again that 3 days of fasting prior to treatment with irinotecan prevents the occurrence of drug-related side-effects while anti-tumor efficacy was not affected. As in the previous study, we showed that fasted BALB/c animals did not show any signs of toxicity at all in response to high dose irinotecan. We demonstrated that levels of the active and toxic metabolite of irinotecan, SN-38, were lower in plasma and liver tissue of fasted mice. In contrast, intratumoral SN-38 values in fasted mice compared to *ad libitum* fed mice remained unchanged. These data indicate that fasting induces important changes in irinotecan metabolism, and lowers the systemic exposure to SN-38.

Evidence from *in vitro* and *in vivo* research suggests that fasting has a great potential to be implemented in clinical cancer patients (28-34). Nonetheless, introduction of fasting to the clinic remains challenging. A better understanding of different pathways and genes involved in the theory of fasting is necessary, and will help us to extend ideas translating this method to cancer patients.

Therefore, in **chapter 7** we investigated transcriptional responses using microarray analysis after fasting and irinotecan treatment in CRLM and liver tissue in mice. BALB/c mice were subcutaneously injected with C26 colorectal carcinoma cells. After 12 days of tumor growth, two groups were fasted for 3 days and two groups were fed *ad libitum*. One group of each received irinotecan treatment, the other groups received vehicle. 12 hours after injection mice were sacrificed and blood, liver, and tumor tissue were collected. Microarray analyses on tumor and liver tissue were performed. We found that 3 days of fasting prior to irinotecan treatment reduces the side-effects of irinotecan via down-regulation of the gene expression profiles in the liver and activation of a cellular stress response. Several specific sets of genes involved in liver damage and toxicity were decreased due to fasting. However, the effects of irinotecan were preserved in the fasted tumor group, with a hint towards increased sensitivity for irinotecan. These findings support the differential stress resistance theory, stating that cancer cells are unable to adapt to a protected state by dietary restriction (29). We conclude that these results increase our knowledge about the mechanistic processes underlying DR, and may pave the way to find a DR mimetic. We believe that short-term fasting can improve both the quality of life as well as the sensitivity to chemotherapy in colorectal cancer patients.

Future perspectives

The studies described in this thesis showed that circadian regulated genes are significantly down-regulated compared to non-cancerous tissues in abdominal cancer types in animal experiments as well as in human studies. It is suggested that circadian genes could serve as biomarkers for development, treatment, and prognosis of human cancer patients. In mice bearing C26 colorectal tumor metastases in the liver, we showed that there was an absence of circadian rhythm in CRLM. Furthermore, we observed a phase shift of the circadian clock in healthy liver and in kidney tissue of tumor bearing animals. It would be of interest for future research to investigate how this phase shift between healthy liver and kidney tissue can be caused. It is suggested that the tumor can affect the circadian rhythm of surrounding healthy tissue by excreting e.g. TGF-β. Future experiments need to focus on how the tumor can affect circadian rhythms in distant organs like kidney tissue. In cancer patients, this would also be an interesting research question. In our study with mice bearing C26 colorectal tumors, we concluded that oscillations were absent in tumor tissue. However, it could also be that all cancer cells are ticking in a different phase. More research on single cell level could elucidate this topic.

In general, circadian clock research in human tissues suffers from serious drawbacks that may contribute to the failure to successfully understand human circadian rhythms in tumor tissue. For instance, in the clinical setting we are dependent on the time of collection of tissues, and we usually only have one time point to measure the expression of circadian genes. In an ideal situation we would like to have several time points over the day to obtain a 24-hour circadian rhythm, and future research needs to focus on a way to display circadian rhythm in patients without the need for surgical resection specimen. Chronotherapy consists of the administration of drugs at a specific moment of the day in order to minimize the side-effects and cause damage predominantly to the malignant cells (35). Over the last years it has been shown that treatment at a specific circadian time might improve drug tolerance, the activity of anti-cancer drugs, and inhibit cancer progression (36, 37). More randomized controlled trials should be conducted to investigate the added value of chronomodulated therapy for cancer patients.

The data described in this thesis demonstrate that a short term fasting regimen of 3 days protects against irinotecan induced side-effects, while a similar reduction of tumor size was found in fasted and *ad libitum* fed treated groups. Irinotecan was used in these studies, because it is known for its severe toxicity also in non-cancerous cells. However, it remains unknown if fasting also protects against the adverse effects of other anticancer drugs. Therefore, further research is needed.

Translation of the results of fasting prior to chemotherapy from animal studies to the clinical setting poses a challenge. We showed that 3 days of fasting protected against the side-effects of irinotecan. Mice, however, have a metabolic rate that is approximately 7 times higher than that of humans (38). This suggests that the fasting period in humans should be prolonged by 7 times to obtain the same results. A fasting period of 3 weeks seems impossible. DR of 3 days 30% restriction and 1 day fasting pre-operatively has been shown to be feasible and safe in humans undergoing surgery (39). A fasting regimen is found to be more difficult and controversial. However, in a case reported study of 10 patients who voluntarily fasted prior to different chemotherapy regimens, less side-effects were reported, and subjective wellbeing improved (31). Recently, we started a pilot study to investigate the feasibility, pharmacokinetics, and effects of a short-term dietary restriction regimen given before second line irinotecan treatment in CRLM patients. More clinical trials are required to explore the possible beneficial effects of dietary interventions. We should keep in mind that cancer patients are often malnourished and already lost a substantial amount of weight before the start of chemotherapy. Restricting these patients from a balanced diet may cause morbidity and mortality. To overcome the problem of undernutrition in cancer patients, pharmacological mimetics that induce the beneficial effects of fasting without the disadvantages of fasting should be developed. Our microarray analysis data may be used to identify more differences in gene expression patterns and may reveal additional pathways that are involved in the beneficial effects of fasting, which may aid in finding a DR mimetic.

In the final part of this thesis we attempted to unravel some of the underlying mechanisms of the beneficial effects of fasting, and therefore we investigated transcriptional responses using microarray technology of fasting and irinotecan treatment in CRLM and liver tissue in mice. We found that fasting reduced toxicity of irinotecan and it induced a protective stress response in healthy liver tissue. Furthermore, fasting preserved the anti-tumor activity on a transcriptional level. It is thought that the up-regulation of protective pathways by normal cells e.g. the Nrf2 pathway, is involved in the protection against oxidative stress (23, 27). However, Nrf2 knock-out mice still exhibited the protective effects after DR, therefore more research need to be conducted on the role of Nrf2 in the protective effects of DR (40). Raffaghello et al. introduced the phenomenon that cancer cells are unable to obtain a protected state by DR, which he coined differential stress sensitization. It is based on the fact that cancer cells have acquired a number of mutations that decrease their opportunities to adapt to DR (28, 29). Although some of the mechanisms by which DR induces its protective effects are now known, further studies are warranted to fully understand the complex molecular response to dietary restriction.

In conclusion, the use of differential circadian gene expression between cancerous tissue and host tissue, and the beneficial effects of fasting prior to chemotherapy are promising strategies to optimize chemotherapeutic treatment in CRLM patients. Future research is needed to fully clarify the role of the circadian clock and dietary interventions in cancer patients.

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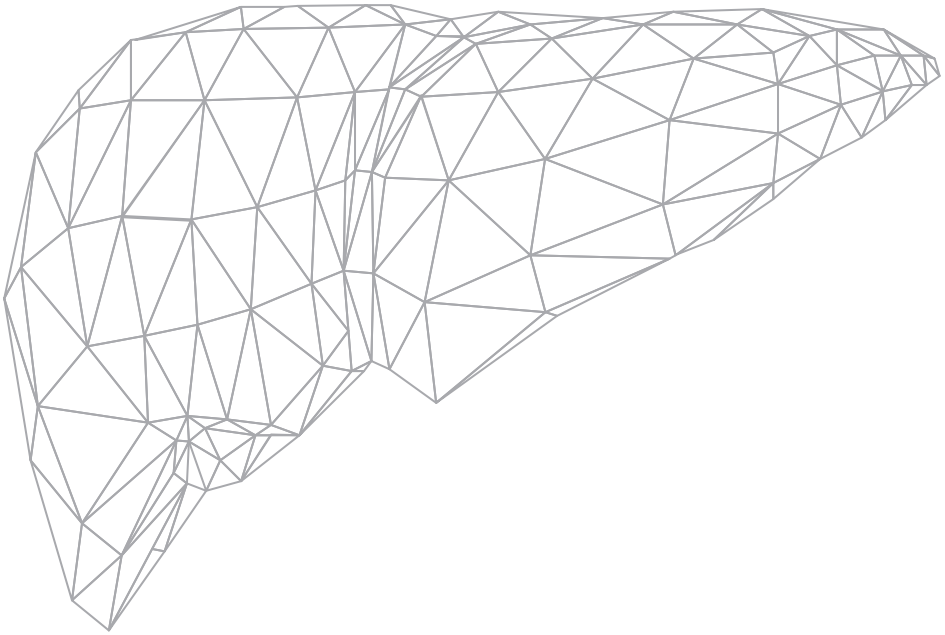

CHAPTER 9

NEDERLANDSE SAMENVATTING

Colorectaal carcinoom (CRC) is volgens recente cijfers de op twee na meest voorkomende kankersoort bij mannen en de op één na meest voorkomende soort bij vrouwen wereldwijd (1). Bij ongeveer 20% van de patiënten zijn reeds metastasen op afstand aanwezig tijdens het stellen van de diagnose (2). De lever is veruit de meest voorkomende plaats voor metastasen, en ongeveer 50% van de patiënten met CRC zal colorectale levermetastasen (CRLM) ontwikkelen (3, 4). In de afgelopen 30 jaar heeft de behandeling van CRLM grote veranderingen ondergaan. Patiënten die in het verleden ongeneeslijk ziek werden verklaard, zijn nu beter te behandelen met de multimodaliteit aanpak. Deze aanpak bestaat uit het combineren van verschillende chemotherapeutische middelen om zo voorheen niet operabele tumoren te verkleinen en alsnog chirurgisch te verwijderen. Op het moment dat chemotherapie werd geïntroduceerd voor CRLM was de behandeling gebaseerd op toediening van 5-fluorouracil en was de totale overleving 6 tot 10 maanden. De ontwikkeling van chemotherapeutische middelen zoals irinotecan in combinatie met de multimodaliteit aanpak verbeterde de algemene overleving tot maximaal 24 maanden. De extreme toxiciteit van chemotherapeutische middelen is een belangrijke beperking voor het gebruik. Er zijn verschillende ernstige bijwerkingen van irinotecan die vaak leiden tot het vroegtijdig beëindigen van de behandeling. Zodoende is er een dringende behoefte aan verdere optimalisatie van de chemotherapeutische behandeling om bijwerkingen te verminderen en om hogere doseringen te tolereren. In dit proefschrift is de rol van de circadiane klok en van vasten onderzocht met als uitgangspunt de chemotherapeutische behandeling van CRLM te verbeteren.

Hoofdstuk 2 biedt de uitkomsten van een literatuurstudie naar de rol van de circadiane klok in abdominale kankersoorten. mRNA expressieniveaus van meerdere circadiaan gereguleerde genen waren verstoord in tumor weefsel in vergelijking met gezond weefsel. Circadiane genen als *PER1*, *PER2* en *PER3* vertoonden consequent minder expressie in tumoren van verschillende soorten kanker. Er zijn nieuwe behandel schema's ontwikkeld om de afwijkende expressie van meerdere circadiane en circadiaan gereguleerde genen bij kanker te benutten. Chronotherapie is hier een voorbeeld van en bestaat uit het toedienen van chemotherapeutische middelen op basis van het biologische ritme (5, 6). Het perfecte tijdstip van toediening zou zijn wanneer de tumor het meest actief is en het omliggende gezonde weefsel het minst actief. In een recent klinisch onderzoek is aangetoond dat chronotherapeutische toediening van irinotecan, oxaliplatin en 5-fluorouracil via hepatische arteriële infusie bij zwaar voorbehandelde patiënten mogelijk is. De behandeling bleek goed te verdragen en leidde tot een verdubbeling van secundaire resectie bij patiënten met voorheen inoperabele CRLM (7, 8). We concludeerden dat veel circadiane (gereguleerde) genen verlaagd tot expressie komen in tumorweefsel van verschillende soorten abdominale kanker.

In **hoofdstuk 3** zijn 24-uurs mRNA expressieniveaus van essentiële circadiane-, en circadiaan gereguleerde genen in lever en nieren van gezonde controlemuizen vergeleken met die van muizen die C26 colorectale lever metastasen hadden. Metastasen in de lever werden geïnduceerd door injectie van C26 colorectaal carcinoom cellen in de milt. Om tumorgroei in de milt te voorkomen werd deze 10 minuten na injectie verwijderd. Tumoren kregen de kans om drie weken te groeien, waarna in een 24-uurs schema tumor, lever- en nierweefsel werd geoogst om expressie van circadiane genen te vergelijken. Met behulp van qRT-PCR werd een normaal 24-uurs ritme van alle klokgenen in lever en nierweefsel gevonden. In CRLM was het 24-uurs ritme afwezig voor alle klokgenen behalve voor *Cry1*. Tevens was er een significant verminderde expressie van output genen zoals *Dbp*. Daarnaast zagen we een faseverschuiving van de circadiane klok in gezond lever en nierweefsel van tumordragende dieren. In de lever zagen we een faseversnelling terwijl we in de nier een fasevertraging zagen. Dit wijst op een systemisch effect. We concludeerden dat de circadiane klok ernstig verstoord is in CRLM. Een nuttig vervolg onderzoek zou zijn om te onderzoeken hoe de faseverschuiving tussen lever en nierweefsel wordt veroorzaakt. Het is bekend dat tumorcellen verschillende mediatoren uitscheiden zoals TGF-β. Deze zijn in staat de circadiane klok te moduleren door activering van de ALK-Smad3-DEC1 route (9).

Vervolgens hebben we onderzocht of deze verschillen in circadiaan ritme ook aanwezig waren in patiënten met CRLM. In **hoofdstuk 4** hebben we chirurgische resectie preparaten van 15 patiënten met primair CRC en CRLM onderzocht. Van 10 verschillende klokgenen is de mRNA expressie vergeleken in primair CRC, CRLM en leverweefsel van dezelfde patiënt. Klokgenen die onderzocht zijn *CLOCK*, *BMAL1*, *PER1*, *PER2*, *PER3*, *CRY1*, *CRY2*, *CSNK1E*, *TIM* en *TIPIN*. Zeven van deze klokgenen vertoonden een verminderde mRNA expressie in CRLM, terwijl in CRC vijf klokgenen verminderde mRNA expressie vertoonden. Alleen *CSNK1E* vertoonde een hogere mRNA expressie in CRC. *CSNK1E* codeert voor het eiwit CKIε en een van de belangrijkste functies is het reguleren van het circadiane ritme door fosforylering en afbraak van *PERIOD* genen (10). *CKIε* speelt een essentiële rol bij de vroege ontwikkeling van CRC (11-13). Ook is onderzocht of er relaties bestaan tussen de genexpressie niveaus van CRC en CRLM patiënten en klinische en pathologische factoren. Er bestond een significante correlatie tussen het geslacht en de expressie van *CRY1* in CRLM. Bij vrouwelijke patiënten werd een lager mRNA expressie niveau van *CRY1* gevonden. Tevens vonden we dat een lage *PER3* expressie correleerde met een groter aantal metastasen. In andere studies zijn ook correlaties aangetoond tussen circadiane genexpressie en klinische en pathologische factoren. Mazzoccoli et al. toonden een significant verband tussen lage *CRY1* expressie in tumorweefsel en een hogere tumor gradering, oudere leeftijd en het vrouwelijk geslacht. De overleving was echter beter in CRC patiënten met een lagere *CRY1* en *CRY2* expressie in tumorweefsel

(14, 15). Deze resultaten zijn ook gevonden in een studie waaruit bleek dat een hoge *CRY1* expressie in de tumor gecorreleerd was aan een slechtere overleving in CRC patienten (16).

In **hoofdstuk 5** is zijn we begonnen met het onderzoeken van de positieve effecten van calorische restrictie (DR) op de bijwerkingen van chemotherapie. Er zijn steeds meer aanwijzingen dat DR, mits goed getimed en gedoseerd, de weerstand tegen stress van patiënten kan verbeteren. Vermindering van de voedselinname van ongeveer 30% tijdens het leven is een krachtig middel om resistentie tegen meerdere stressoren te verhogen en de levensduur van verschillende organismen te verlengen (17-22). In eerder onderzoek, uitgevoerd in ons laboratorium, is aangetoond dat kortdurende dieetinterventies en vasten beschermen tegen acute oxidatieve schade in nieren en lever (23-26). De bescherming werd in verband gebracht met een verhoogde aanwezigheid van cytoprotectieve en anti-oxidant genen. In deze studie is het effect van vasten op de bijwerkingen van irinotecan in een FabplCre;Apc15lox/+ muismodel onderzocht. Dit genetisch gemodificeerde muizenmodel zorgt ervoor dat de muizen spontaan tumoren in het darmstelsel krijgen. In dit model hebben wij gevonden dat dat 3 dagen vasten beschermt tegen het ontstaan van ernstige bijwerkingen bij het gebruik van irinotecan. In zowel gevaste als *ad libitum* gevoede dieren vonden we een gelijke tumorreductie. Daarnaast was er een significante afname in celcyclusactiviteit, proliferatie en angiogenese, terwijl markers voor senescence verhoogd waren in zowel gevaste als *ad libitum* gevoede groepen. Eerder onderzoek toonde aan dat 3 dagen vasten de stressbestendigheid verhoogd, onder andere door verhoogde expressie van Nrf2, welke betrokken is bij de bescherming tegen oxidatieve stress (23, 27). In tegenstelling tot de verhoogde expressie van beschermende routes in normale cellen, wordt gedacht dat kankercellen niet in een beschermende toestand kunnen komen door dieetrestrictie. Dit verschijnsel wordt 'differential stress sensitization' (DSS) genoemd en is gebaseerd op het feit dat kankercellen een aantal mutaties ondergaan waardoor ze zich niet meer kunnen aanpassen aan dieetrestrictie (28, 29).

Om onze bevindingen verder uit te breiden en op zoek te gaan naar het mechanisme hierachter, zijn de experimenten in **hoofdstuk 6** ontworpen. Nogmaals onderzochten we het effect van vasten op de bijwerkingen en anti-tumor effect van een hoge dosis irinotecan. In deze experimenten is ook de farmacokinetiek van irinotecan onderzocht. C26 coloncarcinoomcellen werden subcutaan geïnjecteerd in de flanken van BALB/c muizen en groeiden uit tot subcutane tumoren. De resultaten bevestigden dat 3 dagen vasten vóór behandeling met irinotecan bijwerkingen voorkomt, terwijl de anti-tumoractiviteit niet werd beïnvloed. De actieve en toxische metaboliet van irinotecan, SN-38 was significant lager aanwezig in plasma en lever weefsel van muizen die gevast hadden.

Daarentegen waren de concentraties van SN-38 in de tumor van gevaste in *ad libitum* gevoede dieren niet verschillend. Deze resultaten geven aan dat vasten een verandering teweeg brengt in het metabolisme van irinotecan en zo de systemische blootstelling aan SN-38 verminderd. Resultaten uit voorgaande studies suggereren dat het toepassen van vasten in de kliniek veel potentie heeft (28-34). Dit is echter een uitdagend proces. Om dit te bereiken zal een beter begrip nodig zijn van de verschillende pathways en genen die betrokken zijn bij de inductie van bescherming door vasten.

In **hoofdstuk 7** is de transcriptionele respons na vasten van muizen die behandeld waren met irinotecan onderzocht met behulp van microarrays. BALB/c muizen werden subcutaan geïnjecteerd met C26 colorectale carcinoomcellen. Na 12 dagen tumorgroei werden twee groepen muizen gevast voor 3 dagen en twee groepen werden *ad libitum* gevoed. Vervolgens ontving één van deze groepen irinotecan behandeling, de andere groepen kregen vehiculum. Twaalf uur na injectie met chemotherapie werden de muizen opgeofferd en werd bloed, lever en tumorweefsel verzameld. Microarray analyses van tumor en leverweefsel werden uitgevoerd. Resultaten toonden dat 3 dagen vasten voorafgaand aan irinotecan behandeling de bijwerkingen vermindert via downregulatie van genexpressieprofielen in de lever en activering van een cellulaire stressrespons. Een aantal specifieke sets van genen die betrokken zijn bij schade aan de lever en toxiciteit waren afgenomen als gevolg van het vasten. Deze bevindingen ondersteunen de DSS theorie, die stelt dat kankercellen zich niet kunnen aanpassen aan een beschermde toestand door calorische restrictie (29). We concludeerden dat de resultaten bijdragen om onze kennis over de mechanistische processen die ten grondslag liggen aan DR te vergroten. Met meer onderzoek naar de gunstige effecten van DR kunnen we uiteindelijk op zoek gaan naar een mimeticum dat deze effecten kan induceren zonder te hoeven vasten.

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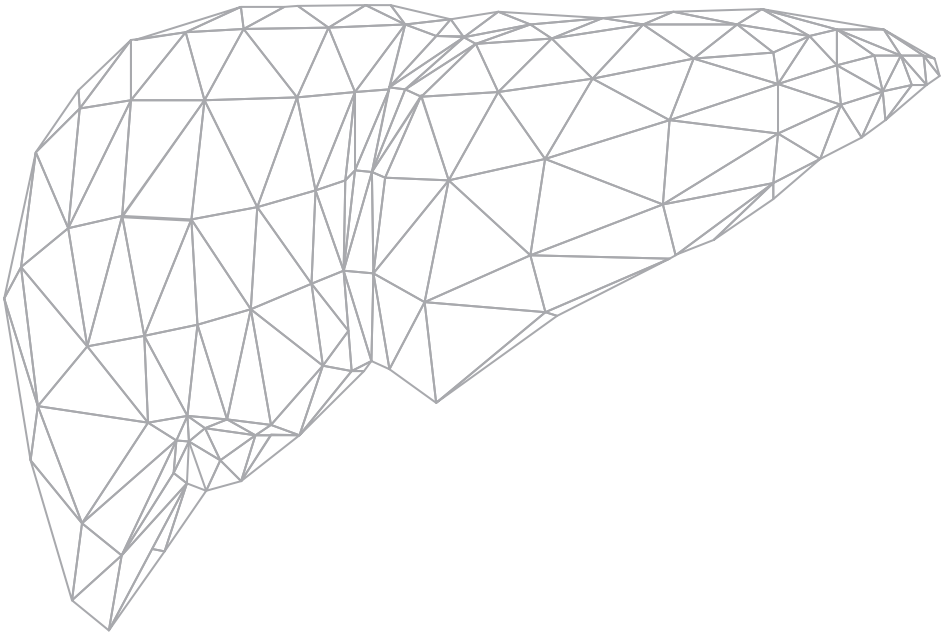

APPENDICES

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DANKWOORD

Dit proefschrift is tot stand gekomen dankzij de steun, hulp en inzet van velen. Een aantal mensen wil ik in het bijzonder noemen, omdat zij nóg meer dan anderen hebben bijgedragen aan de totstandkoming van dit proefschrift.

Mijn copromotor, dr. R.W.F. de Bruin, beste Ron, tijdens mijn co-schappen kreeg ik de tip met jou contact op te nemen voor het doen van onderzoek binnen de Heelkunde. Kijk eens wat daarvan terecht is gekomen. Jij bent het brein achter dit onderzoek. Dank voor al het geduld en de mooie tijd op het lab. Naast de basale wetenschap hebben we ook erg veel gelachen. De congressen waren legendarisch. Laten we binnenkort weer een whisky drinken samen.

Mijn promotor, professor J.N.M. IJzermans, beste Jan, ik kan me onze kennismaking nog goed herinneren. Toen bleek dat we beide op school hebben gezeten in Bergen op Zoom, was het ijs snel gebroken. Ik wil je voornamelijk bedanken voor de vrijheid die ik de afgelopen jaren van je kreeg. Tijdens overleg wist je altijd die ene vraag te stellen die mij weer extra alert en enthousiast maakte.

Professor A.H.J. Mathijssen, beste Ron, de samenwerking tussen de labs ontstond 2 jaar nadat ik begon met mijn onderzoek. Dit had vanaf het begin al zo moeten zijn. De frisse blik waarmee jij naar onderzoek kijkt is prachtig. Altijd heb je goede ideeën en ben je bereikbaar voor overleg. Ik weet zeker dat ook de klinische studie een succes zal worden! Ook bedankt voor het deelnemen aan de kleine commissie, jammer dat je er op 3 juni niet bij kunt zijn.

Professor G.T.J. van der Horst, beste Bert, je kennis over de klok is groot en heeft een belangrijke bijdrage aan mijn proefschrift geleverd. Ik heb veel van je mogen leren, dank hiervoor. Dank ook voor je laagdrempeligheid en de prettige samenwerking tussen de labs. Fijn dat je zitting wilt nemen in mijn promotie commissie.

Dr. M.J.M. Smits, beste Ron, dank voor de samenwerking en het gebruik maken van het *Apc*-model. Mooie experimenten hebben we gedaan en talloze tumoren samen geteld. Als derde Ron was je van onmiskenbare waarde.

Overige leden van de kleine en grote commissie, hartelijk dank voor het beoordelen van mijn proefschrift en het zitting nemen in de commissie.

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Beste chirurgen en arts-assistenten uit het Franciscus ziekenhuis, enorm blij ben ik dat ik mag leren opereren in dit prachtige ziekenhuis. Er zullen de komende jaren veel mooie momenten volgen. Zo zal de skireis 2017 legendarisch worden!

Mijn paranimfen, Freek en Wouter. Freek, als studiegenoot, dispuutsgenoot, clubgenoot, maar vooral als heel goede vriend wist jij de afgelopen jaren precies waar ik mee bezig was. Dank dat je ook nog mijn paranimf wilt zijn. Wouter, grote broer en vriend, Kneeke, erg trots en dankbaar ben ik dat je mijn paranimf wilt zijn.

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Tot slot, lieve Renate, wat ben ik ongelooflijk gelukkig met jou. Jouw steun en liefde hebben ervoor gezorgd dat er altijd iets leuks was om naar uit te kijken. Enorm bedankt voor het ontwerpen van de prachtige omslag, beter had het niet gekund. Het leven is prachtig met jou en we gaan er nog zó lang van genieten!!

LIST OF PUBLICATIONS

S.A. Huisman, M. Oklejewicz, F. Tamanini, A.R. Ahmadi, J.N.M. IJzermans, G.T.J van der Horst, R.W.F. de Bruin. Colorectal liver metastases with a disrupted circadian rhythm phase shift the peripheral clock in liver and kidney. Int J Cancer 2015.

S.A. Huisman, W. Bijman-Lagcher, J.N.M. IJzermans, R. Smits, R.W.F. de Bruin. Fasting protects against the side effects of irinotecan but preserves its anti-tumor effect in Apc15lox mutant mice. Cell cycle 2015.

S.A. Huisman, P. de Bruijn, I.M. Ghobadi Moghaddam-Helmantel, J.N.M. IJzermans, E.A. Wiemer, R.H.J. Mathijssen, R.W.F. de Bruin. Fasting protects against the side-effects of irinotecan treatment but does not abrogate anti-tumor activity in mice. British Journal of Pharmacology 2015.

S.A. Huisman, A.R. Ahmadi, J.N.M. IJzermans, C. Verhoef, G.T.J van der Horst, R.W.F. de Bruin. Disruption of circadian clock gene expression in human colorectal liver metastases. Submitted.

S.A. Huisman, A.R. Ahmadi, J.N.M. IJzermans, G.T.J van der Horst, R.W.F. de Bruin. Circadian gene expression in human abdominal cancer: A systematic review. Submitted.

S.A. Huisman, F. Jongbloed, M.E.T. Dollé, H. van Steeg, J.L.A. Pennings, M. Luijten, W. Rodenburg, J.N.M. IJzermans, R.W.F. de Bruin. Transcriptomic analysis of the response to irinotecan in fasted tumor bearing mice. In preparation.

CURRICULUM VITAE

Sander Arjan Huisman was born on November the 18th of 1985 in Bergen op Zoom, The Netherlands. A large part of his youth, he grew up on the island Curaçao. After graduating from high school at R.S.G 't Rijks secondary school in Bergen op Zoom in June 2004, he started medical school at the Erasmus University Rotterdam. During medical school, he worked as a student assistant and tutor teaching surgical anatomy in extra-curricular classes to students. During his study, he did an internship at the department of Surgery at the St. Elisabeth Hospital in Curaçao. In 2010 he performed research at the Department of Surgery, Laboratory of Experimental Surgery, Erasmus University Rotterdam (dr. R.W.F. de Bruin). In November 2010, he graduated from medical school, after which he started working as a PhD candidate at the Department of Surgery at the Erasmus University Medical Center in Rotterdam, under supervision of prof. dr. J.N.M. Ijzermans and dr. R.W.F. de Bruin. His studies focused on optimizing chemotherapy for colorectal liver metastases, which has resulted in this thesis. In November 2014 he started working as a surgical resident at the Department of Surgery at the Ikazia Hospital in Rotterdam (dr. P.T. den Hoed). In January 2016, he started his surgical training at the Franciscus Hospital (dr. T.M.A.L. Klem) and the Erasmus MC (dr. B.P. Wijnhoven).

PhD PORTFOLIO SUMMARY

Erasmus MC
Universitair Medisch Centrum Rotterdam

Summary of PhD training and teaching activities

Name PhD student: Sander A. Huisman
Erasmus MC Department: Surgery
Research School: Molecular Medicine

PhD period: 01-11-2010 – 01-11-2014
Promotor(s): Prof.dr. J.N.M. IJzermans
Co-promotor: Dr. R.W.F. de Bruin

1. PhD training		
	Year	**Workload (ECTS)**
General academic skills		
- Biomedical English Writing (short)	2011	2.0
- Biomedical English Writing and Communication	2013	5.7
- Laboratory animal science	2011	5.7
- Classical methods for data-analysis	2011	5.7
Research skills		
- Basic introduction course on SPSS	2011	1.0
- Basis cursus oncologie (NVvO)	2013	3.0
Presentations		
- National conferences	2011	3.0
	2012	4.0
	2013	2.0
- International conferences	2011	1.0
	2012	1.0
	2013	2.0
	2014	1.0
Seminars and workshops		
- Photoshop and Illustrator CS4 workshop	2011	0.3

2. Teaching activities		
	Year	**Workload (ECTS)**
Supervising practicals and excursions, Tutoring		
- Supervising first aid examinations (Medical students)	2012	0.2
- Supervising first aid examinations (Medical students)	2013	0.2
- Supervising first aid examinations (Medical students)	2014	0.2
Supervising Master's theses		
- Supervising enthusiastic students in the lab	2012/2014	5.0