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Coffee interferes with HCV replication by inhibition of wnt signaling pathway

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Für meine Eltern

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1. Introduction

1.1. Projektskizze/ Aim of Study

Die chronische Infektion mit Hepatitis C Virus (HCV) ist durch neue Medikamente mittlerweile gut beherrschbar. Dennoch ist die Entstehung einer Leberzirrhose und möglicherweise eines hepatozelluläres Karzinom (HCC) weiterhin eine Folge der chronischen Hepatitis C. Noch vor einigen Jahren hielt sich die Meinung, dass Kaffeekonsum schädlich für den menschlichen Körper sei. Es konnten jedoch in den letzten Jahren verschiedene positive Wirkungen von Kaffeekonsum nachgewiesen werden. Neben positiven Effekten bei Krankheiten wie Alzheimer oder Parkinson, konnte auch eine Wirkung des Kaffees auf verschiedene Viren (HBV, HIV, HSV) festgestellt werden. Bislang wurden Kaffeewirkungen auf das Hepatitis C Virus noch nicht untersucht. Die Effekte bei anderen Virusinfektionen werfen nun die Frage auf, inwieweit Kaffeekonsum auch den Verlauf einer HCV Infektion günstig beeinflussen könnte.

Zur Untersuchung der HCV Replikation stehen in unserem Labor die Replikonzelllinien HUH5-15 und Luc-Ubi-Neo/ET zur Verfügung. Es handelt sich dabei um Zelllinien, welche auf der humanen Hepatomzelllinie HUH7 basieren, die die HCV Proteine NS3 bis NS5B stabil unter der Kontrolle der HCV UTRs exprimiert. Im Fall der Linie Luc-Ubi-Neo/ET dient ein zusätzlich vorhandenes Luziferase-Reportergen zur einfachen Quantifizierung der HCV Replikation. Als primäre Messgröße verwenden wir dabei die durch real-time RT-PCR zu messende Expression der Gene für NS3 bis NS5B, die mit der HCV Replikation korrelieren. In dieser Arbeit sollen die Effekte von koffeinhaltigem und entkoffeiniertem Kaffee, Koffein und verschiedenen Kaffeeinhaltsstoffen und Abbauprodukten auf die HCV Replikation gestestet werden.

1.2. Introduction

Coffee

Coffee is the most frequently consumed legal drug all over the world. It is a beverage derived from roasted fruits of the coffee tree, known as coffee cherries. Historically, coffee originates from the region Caffa in Ethiopia and was first described in the 9th century. First reports of coffee in Europe are from the 15th-century and the first German coffeehouse was built in 16th century. The most common species of coffee beans are *Coffea arabica* („Arabica“) and *Coffea canephora* („Robusta“). After roasting and grinding, the coffee flour is brewed to coffee using different techniques, e.g. boiling coffee in hot water in Turkey or using the French press or a coffee percelerator, which is common in Europe and the United States. Coffee is consumed as a hot or cold beverage, with cream and sugar or just as black coffee. Enhanced concentration and less fatigue are the reasons for many people to consume high amounts of coffee.

Bad Coffee?

During the long tradition of coffee drinking, coffee effects were discussed controversially. Despite its well-known effects on fatigue and concentration, coffee was accused to have bad side effects on human health. Therefore, coffee intake was suspected to cause and aggravate chronic diseases. In particular, damages of the gastrointestinal tract and the cardiovascular system have been discussed to be caused by coffee consumption. In fact, it has been shown that coffee activates gastric acid secretion and thereby can promote gastro-oesophageal reflux. Nevertheless an association between coffee-consumption and dyspepsia could not be confirmed (Boekema PJ et al. 1999). Another study showed that caffeine reduces exercise-induced myocardial blood flow especially in patients with

coronary heart diseases or arteriosclerosis (Namdar M et al. 2006), and thereby raises the question whether caffeine consume is safe for patients with coronary artery diseases.

Healthy Coffee!

In the last years, many studies investigated the effects of coffee on human health and it turned out that coffee seems to have overly beneficial effects (Butt MS, Sultan MT 2011). Clinical studies revealed that coffee consumption reduces general mortality, especially mortality due to cardiovascular diseases (Lopez-Garcia E et al. 2008; Liu J et al. 2013). The reduced risk of heart diseases, especially in women, (Wu JN et al. 2009) and stroke (Lopez-Garcia E et al. 2009) might be caused by antioxidants contained in coffee. The risk to develop a metabolic syndrome or diabetes typ-2 (van Dam RM, Hu FB 2005) was found to be reduced among coffee consumers. This might also be due to anti-oxidative components in coffee, which enhance insulin sensitivity. Likewise, the risk of Alzheimer's disease (Eskelinen MH, Kivipelto M 2010) or Parkinson's disease (Costa J et al. 2010) was found reduced via the antioxidant defense by coffee. Recently it has been shown that coffee consumption decreases the risk of liver fibrosis formation (Modi AA et al. 2010). Furthermore, coffee has been shown to reduce the risk to develop colorectal (Galeone C et al. 2010), liver (Larsson SC, Wolk A 2007) and breast cancer (Lowcock EC et al. 2013).

Coffee ingredients

Coffee contains more than 2000 components (Tuomilehto J. 2013), of which many are not yet characterized. Those substances can be divided into groups, e.g. carbohydrates, lipids, proteins, acids, alkaloids, minerals and aromatic compounds. During roasting, grinding and brewing, concentrations of all these substances change.

The most commonly known substance in regular coffee is caffeine. In the human body, caffeine is metabolized in the liver into its main degradation product paraxanthine (81%) as well as theofylline (11%) and theobromine (5%) by cytochrome oxidases (Gu L et al. 1992;

Orrú M et al. 2013). As a clinical study has shown, caffeine consumption is associated with reduced liver fibrosis formation (Modi AA et al. 2010) and reduced histological activity in patients with chronic hepatitis C (Costentin CE et al. 2011). The caffeine degradation product paraxanthine has been shown to reduce intercellular collagen deposition in the cholestatic rat liver (Klemmer I et al. 2011) and to be an inhibitor of CTGF (connective tissue growth factor) expression in liver parenchymal cells (Gressner OA et al. 2009). Paraxanthine, theofylline and theobromine act as phosphodiesterase (PDE) inhibitors. The PDE-inhibitor pentoxifylline has been shown to have hepatoprotective properties and antiviral effects in HCV patients (Gutierrez-Reyes G et al. 2006). Furthermore, supplementation of HCV treatment (PEG-IFN/ribavirin) with pentoxifylline improved the sustained virological response (SVR) (Jiménez-Luévano MÁ et al. 2013). Per definition SVR is the absence of detectable HCV RNA in blood serum up to six months after therapy.

Other coffee ingredients have been shown to have positive effects on different types of cancer as well. Cafestol and kahweol are diterpens belonging to the coffee lipids and are mostly consumed with unfiltered coffee. Both have been reported to exhibit anti-carcinogenic activity in hepatic, intestinal and kidney tissue (Cavin C et al. 2002). Chlorogenic acid is part of the coffee acids. It is the ester of caffeic acid and has been reported to affect colon cancer (Kang NJ et al. 2011). Genistein belongs to the group of isoflavones, which are flavonoides belonging to the secondary plant compounds. Secondary plant compounds are nonessential plant compounds, which do not directly affect growth and development of an organism. Genistein has been shown to inhibit the wnt/beta-catenin signaling pathway (Zhang Y, Chen H 2011), which is linked to cancer development.

Hepatitis C Virus – HCV

The acute infection with HCV displays no specific symptoms and therefore remains mostly undetected and untreated. As a result, almost 70% of all acute HCV infections become chronic and about 20% of chronically infected patients develop liver cirrhosis. Chronic HCV infection currently affects about 3% of the world's population (Tang H, Grisé H 2009) and is a major cause for the development of hepatic cancer (Blonski W, Reddy KR 2008). In Germany, HCV prevalence is about 0.5%, while in high-risk areas in Africa the prevalence is up to 14%. HCV, which is a small RNA virus and belongs to the family of Flaviviridae, is transmitted parenterally. Up to now, seven major genotypes have been identified, which vary in their prevalence between the continents. In Germany, genotype 1 still is the major genotype with the highest prevalence, while the percentage of patients with genotype 3 infections is increasing (Cornberg M et al. 2011). Because of the high mutation rate of the virus and the thereby fast increasing number of subtypes, it has not been possible to develop a vaccine so far. Since the most common way of transmission is via blood and blood products, there is a higher risk of HCV infection in dialysis patients, intravenous drug users and after blood transfusion. Target cell of HCV is the hepatocyte, which is entered by the virus via cell surface receptors. So far CD81, CLDN1, OCLN (Fraquhar MJ et al. 2012) and LDL-R (Albecka A et al. 2012) have been identified as essential HCV receptors.

HCV therapy

Until very recently, treatment of acute and chronic hepatitis C infection has predominantly been performed by a combination of pegylated interferon alpha2 (PEG-IFN) with the nucleoside analogue ribavirin. IFN alpha activates the JAK-STAT-signaling pathway, which at least leads to inhibition of viral proteinsynthesis and as well cause degradation of viral mRNA (Bandurska K et al. 2013). Ribavirin is a nucleoside analogue, which has been

shown to have antiviral activity via direct and indirect mechanisms e.g. inhibition of inosine-monophosphate-dehydrogenase and immune modulation. With this combined treatment, a sustained virological response (SVR) could be achieved. Due to PEG-IFN/ribavirin therapy only 45% of genotype 1 patients achieve SVR in contrast to 80% of genotype 2 or 3 infected patients (Di Bisceglie AM, Hoofnagle JH 2002). The recent approval of the HCV NS3-4A protease inhibitors Telaprevir and Boceprevir raises hopes for an improvement of therapy (Vermehren J, Sarrazin C 2011). In fact, a combination of protease inhibitors with PEG-IFN/ribavirin enhanced SVR rates in patients infected with HCV genotype 1 (Vagu C et al. 2013). In phase III clinical studies, Daclatasvir, an NS5A inhibitor, has been shown to effectively interfere with HCV genotype 1a and especially HCV genotype 1b in patients (Herbst DA, Reddy KR 2013).

Coffee and HCV – what this work is about

In 2011 a clinical study reported that coffee consumption improved the effects of PEG-IFN/ribavirin therapy in HCV patients (Freedman ND et al. 2011). Therefore, this thesis was conducted to investigate, if coffee in fact has anti-HCV activity and to elucidate mechanisms and mediators in vitro. We decided to incubate hepatocytes and HCV replicating human cell lines with concentrations of coffee corresponding to up to 3 cups of coffee (200 mL per cup), an amount easily consumed during a day. Cell lines were also incubated with coffee ingredients and degradation products to break down effects of coffee to single substances.

2. Materials and Methods

2.1 Materials

2.1.1 Technical Equipment

Equipment	***Supplier***
Casy Cell Counter	Roche Innovatis AG, Reutlingen, D
Centrifuge 5417 R	Eppendorf, Hamburg, D
Centrifuge 5810 R	Eppendorf, Hamburg, D
CFXTM Real-Time sytem	Bio-Rad, München, D
CK40 Microscope	Olympus, Hamburg, D
Electrophoresis Unit	Bio-Rad, München, D
Eppendorf Research® Plus Pipettes	Eppendorf, Hamburg, D
Incubator HERAcell 240 CO2	Thermo Fisher, Langenselbold, D
LCachN/20X/0.40 Phc/1/FN22 UIS objective	
Light Cycler, CFX96 Real-Time System	Bio-Rad, München, D
Neubauer Improved Chamber	Marienfeld GmbH, Lauda-Königshofen, D
Pipetboy acu	Integra Biosciences GmbH, Fernwald, D
Precision balance TE1245	Sartorius, Göttingen, D
Peristaltic pump	Medorex, Nörten-Hardenberg, D
Sterilbench HERAsafe	Thermo Fisher, Langenselbold, D
Tecan® infinite M2000	Tecan Group Ltd., Männedorf, CH

Thermal Cycler Primus 96 plus	Eurofins MWG Operon, Ebersberg, D
VersaDoc 4000MP	Bio-Rad, München, D
Vortex, Reax200	Heidolph Instruments, Schwabach, D
Water bath	Lauda GmbH&Co , Lauda-Königshofen, D

2.1.2 Consumables

Consumable	***Supplier***
Cell culture bottle Cellstar®	Greiner Bio-One GmbH, Frickenhausen, D
Cell culture plates, 24-well, 96-well	Thermo Fisher, Roskilde, DK
Centrifuge tubes	Sarstedt, Nürnbrecht, D
Cuvette (single-use)	Roth, Karlsruhe, D
Mµltiguard®-Tips	Roth, Karlsruhe, D
PCR-Tubes	ABgene, Thermo Fisher, Hamburg, D
Pipets Research® variabel	Eppendorf, Hamburg, D
Pipets (single-use)	Sarstedt, Nürnbrecht, D
Pipet Tips Plastibrand®	Brand, Wertheim, D
Quali-PCR-Tube	Kisker GbR, Steinfurt, D
Tubes, 1,5ml, 2ml	Roth, Karlsruhe, D

2.1.3 Reagents and Kits

Reagent	***Supplier***
(3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-	Sigma-Aldrich, St Louis, USA

Tetrazolium bromide (MTT)	
4,5,6,7-tetrabromo-2-azabenzimidazole (TBB)	Sigma Aldrich, St Louis, USA
Bradford	Bio-Rad,München, D
BSA	Roth, Karlsruhe, D
Cafestol	Sigma Aldrich, St Louis, USA
Caffeine	Sigma Aldrich, St Louis, USA
Chlorogenic acid	Sigma Aldrich, St Louis, USA
Coffee regular/ decaffeinated (100% Arabica)	
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich, St Louis, USA
Distilled water, RNase free	Thermo Fisher Scientific, Waltham, USA
DMEM (Dulbecco´s Modified Eagle´s Medium) + 4,5 g/l Glucose, + L-glutamin, - Pyruvate	Gibco®, Invitrogen, Darmstadt, D
Fetal calf serum (FCS)	Invitrogen, Darmstadt, D
Genistein	Sigma Aldrich, St Louis, USA
IFN alpha	
L-glutamine	Invitrogen, Darmstadt, D
LiChrosolv Water	Merck, Darmstadt, D
Lipofectamine^{TM} 2000	Invitrogen GmbH, Karlsruhe, D
Luciferase Assay System	Promega, Mannheim, D
Master mix (2x)	Thermo Fisher Scientific, Waltham, USA
Nicotinic acid	Sigma Aldrich, St Louis, USA
Optimem (1X)	Gibco®, Invitrogen, Darmstadt, D
Paraxanthine	Sigma Aldrich, St Louis, USA

Percoll	GE Healthcare, Chalfont St. Giles, UK
PFA	Roth, Karlsruhe, D
Quercetin hydrate	Fisher Scientific, Schwerte, D
rDNase Kit	Macherey-Nagel, Düren, D
Sodium chloride (NaCl)	AppliChem, Darmstadt, D
Sodium dodecyl sulfate (SDS)	AppliChem, Darmstadt, D
Sodium hydrogen carbonate ($NaHCO_3$)	Roth, Karlsruhe, D
Sodium hydroxide (NaOH)	Roth, Karlsruhe, D
Theofylline	Sigma Aldrich, St Louis, USA
Theobromine	Sigma Aldrich, St Louis, USA
Triton	Roth, Karlsruhe, D
Trizol Reagent	Invitrogen, Karlsruhe, D
Trypan blue	Sigma-Aldrich, St Louis, USA
VersoTM cDNA Kit Thermo	Fisher Scientific, Waltham, USA
William's medium E (1X) + GlutaMAXTM-I	Gibco®, Invitrogen, Darmstadt, D

2.1.4 Buffers and Solutions

PBS

· 137.9 mM NaCl

· 6.5 mM Na2HPO4 x 2 H2O

· 1.5 mM KH2PO4

· 2.7 mM KCl

· pH 7.4 (NaOH)

SDS-lysis-buffer	10 g SDS
	ad to 50ml PBS
	100 µl 10 M HCl

2.1.5 Oligonucleotides

Oligonucleotides for subsequent PCR-reactions were obtained from Metabion International AG (Martinsried, Germany) (Table 1).

Table 1: Oligonucleotides and Sequences

Oligonucleotide	**Sequence 5′-3′**
5’GAPDH	5′-ACCCAGAAGACTGTGGATGG -3′
3’GAPDH	5′-TTCTAGACGGCAGGTCAGGT-3′
5’ATPsy	5’-GCCCACTTCTTACCACAAGG-3’
3’ATPsy	5’-GCGACAGCGATTTCTAGGAT-3’
5' HCV214	5′-TGCGGAACCGGTGAGTACA-3′
3' HCV214	5′-AGGTTTAGGATTCGTGCTCAT-3′
5’ Conductin	5’- AGGGAGAAATGCGTGGATAC-3’
3’ Conductin	5’-TGGAATCAATCTGCTGCTTC-3’
5’ Beta-catenin	5’-AAAGCGGCTGTTAGTCACTGG-3’
3’ Beta-catenin	5’-CGAGTCATTGCATACTGTCCAT-3’
5’ CK2alpha	5’-GAACGCTTTGTCCACCGTG -3’
3’ CK2alpha	5’-GTTGGCAGCAGCAATCACTG -3’
5’ CK2alphaprime	5′-CTTGTTCGCATTGCCAAGGTTC-3′
3’ CK2alphaprime	5′-CACTGGAAAGCACAGCATTGTC-3′
5’ LDLR	5’-GTGCTCCTCGTCTTCCTTTG-3’
3’ LDLR	5’-TAGCTGTAGCCGTCCTGGTT-3’
5’ Claudin-1	5′-CCGTTGGCATGAAGTGTATG -3′
3’ Claudin-1	5′-CCAGTCAAGAGAGCCTGACC-3′

2.1.6 Plasmids

TOPFlash plasmid (M50)	kind gift from Dr. Wege, UKE
FOPFlash (M51)	kind gift from Dr. Wege, UKE

2.1.7 Antibodies

anti-NS3	Biofront Technologies;	1:3000
2E3	Biofront Technologies;	1:3000
anti-mouse-HRP	Sigma; A4416;	1:10000
HCV-2E3	BioFront Technologies	
NS3	BioFront Technologies	
A3R3	gift of Mansun Law, The Scripps Research Institute	
E2	gift of Mansun Law, The Scripps Research Institute	
Alexa-594	Molecular Probes	
Alexa-488	Molecular Probes	

2.1.8 Software

MS Office 2003	Microsoft, Redmond, USA
GraphPad Prism 5	GraphPad Software, La Jolla, USA
Bio-Rad CFX Manager 2.0	Bio-Rad, Hercules, USA
iControl 5.0	Tecan, Crailshaim, D

2.2 Methods

2.2.1 Cell lines

Huh-7

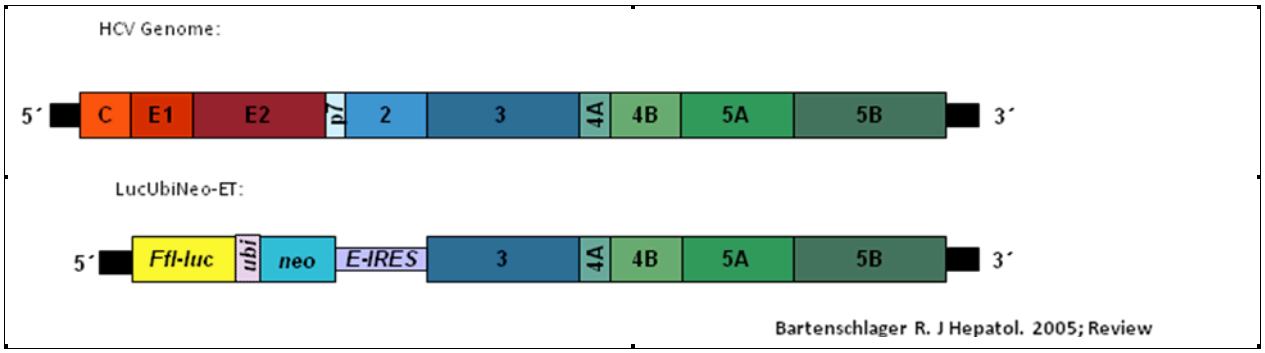
Huh-7 is a human hepatoma cell line, which was isolated from a hepatocellular carcinoma in 1982 (Nakabayashi et al., 1982). This cell line grows adherend.

Huh-5-15

Huh-5-15 is a cell line, which is stabile transfected with the HCV genome (Lohmann V et al. 1999). Its maternal cell line is Huh7. The replicon contains the non-structural proteins NS3 to NS5B.

LucUbiNeo replicons:

LucUbiNeo-ET (GT-1b; Frese M et al. 2003) and LucUbiNeo-JFH (GT-2a; Zitat) are Huh7 human hepatoma cell lines stably transfected with HCV genomes. The replicons contain the non-structural proteins NS3 to NS5B and as well the genetic information for luciferase, facilitating the quantification of viral replication via measurement of luciferase activity.



2.2.2 Isolation of primary murine hepatocytes

To isolated murine hepatocytes method of Seglen (Seglen, 1976) was used. This is a modification of the two-step collagenase perfusion method. Before perfusion the hosepipe was sterilized with 50 ml ddH_2O and 50 ml PPML. Mice were then anesthetized with KHX.

After opening the abdomen, the upper part of the vena cava superior was ligated. 10 ml PPML, followed by 25 ml PM (42 °C), containing 1 mg Liberase (Roche, Basel, CH), were used to perfuse the portal vein at a flow-rate of 1.8 ml/min. In the next step the liver was perfused with 25 ml PM. After excising the liver it was transferred to a petri-dish with 25 ml PM (42 °C), where it was homogenized with tweezers by gentle shaking. This was followed by sterile filteration through a sterile cell strainer (100 µm) into a sterile 50 ml tube. All following steps were carried out under sterile conditions inside a clean bench. In the next 15-20 minutes the hepatocytes were incubated at room temperature for sedimentation, after this step 25 ml of the supernatant were discarded. Isolated hepatocytes were gently resuspended by pipetting up and down and transferred to 25 ml of a 4 °C Percoll-gradient containing 90% Percoll (GE-Healthcare, Chalfont St. Giles, UK) and 10% 10x DPBS (Gibco®, Invitrogen, Darmstadt, D). The mixture was inverted and centrifugated at 72 g and 4 °C for 10 min. After discharging the supernatant the hepatocyte pellet was washed in 30 ml William's_E_GlutaMAXTM-I medium (Gibco®, Invitrogen, Darmstadt, D), supplemented with 10% fetal bovine serum (Invitrogen, Darmstadt, D), 1% penicillin/streptomycin (Gibco®, Invitrogen, Darmstadt) and 1% L-glutamine (Invitrogen, Darmstadt) and centrifugated at 72 g and 4 °C for 5 min. Again supernatant was discarded and the pellet was resuspended in William's medium supplemented as above. The hepatocytes were stained with trypan blue to determine cell viability. Cells were counted with a Neubauer counting cell chamber. Cells were seed in concentrations of 1,5 x 10^5 cells per well in 24-well plates and 3 x 10^4 cells in 96-well plates and incubated in a 40% O_2 and 5% CO_2 humidified atmosphere at 37 °C. William's medium was exchanged 4h after isolation.

2.2.3 Reagents' preparation

1 g of regular or decaffeinated coffee (100% Arabica) was extracted in 5 ml of LiChrosolv Water. Following centrifugation at 4000 rpm for 5 minutes, supernatants were sterile filtered (0.2 µM), aliquoted and stored at -20°C until use. Control cells were incubated with water as used for coffee preparations. Caffeine, theofylline, theobromine, paraxanthine, chlorogenic acid and genistein (all: Sigma Aldrich GmbH, Steinheim, Germany) were dissolved in aqua destillata and stored at -20°C until use. Quercetin hydrate (Acros Organics, part of Thermo Fisher Scientific, Waltham, MA, USA), 4,5,6,7-tetrabromo-2-azabenzimidazole (TBB) and cafestol (both: Sigma Aldrich GmbH, Steinheim, Germany) were dissolved in DMSO. Vehicle control (DMSO) was dissolved in cell culture medium to concentrations used on incubated cells and measured in parallel as indicated in the Figures and Figure Legends.

2.2.4 Analysis of cell viability

Cell viability was measured by using (3-4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT; Sigma Aldrich GmbH, Steinheim, Germany) according to the manufacturer's instructions. Viability of cells in immunohistochemistry was visualized by bright field microscopy.

2.2.5 Luciferase reporter assay

Luc-Ubi-Neo-ET cells were seeded in 24-well plates and allowed to adhere over night. Treatment with different substances was performed for 24 hours. After treatment the medium was discarded and cells were washed with PBS. After washing cells were incubated with 100 µl 1 x cell culture lysis-buffer (Promega, Madison, USA) at room temperature for 5 min, followed by scratching of the cells with a pipette-tip. The cell-lysates were then transferred into a 1.5 ml tube and centrifugated at 20,800 g and 4 °C for 1 min. After this

step the lysates were kept on ice. To measure luciferase-activity, 70 µl of the lysate supernatant were transferred to a white 96-well plate (Nunc A/S, Roskilde, DK) and 50 µl luciferase assay reagent (Promega, Madison, USA) were added. Luciferaseluminescence was measured photometrically with the Infinite M200 Photometer (TECAN, Crailshaim, D) according to the manufacturer's instructions. Protein concentrations were quantified via Bradford assay (Bradford 1976). Luciferase activity was normalized to the protein concentrations of the cells.

2.2.6 Beta-catenin-regulated transcription (CRT)

CRT was monitored using a luciferase reporter system consisting of Super8xTOPFlash and Super8xFOPFlash. The TOPFlash plasmid (M50) contains 8 TCF/LEF binding sites enhancing firefly luciferase expression. The control plasmid FOPFlash (M51) carries mutant TCF/LEF binding sites. 24h after transfection cells were assayed using a Reporter Assay Systems (Promega GmbH, Mannheim, Germany).

2.2.7 Detection of mRNA by real-time RT-PCR

RNA isolation was performed using Trizol Reagent (Invitrogen, Darmstadt, D) according to the manufacturer's' instructions. Cells were cultured on 24-well plates for 24 hours. After 24 hours the culture medium was discarded and cells were washed with PBS. Cells were homogenized with 500 µl Trizol for 5 min at RT after washing. Lysates were then transferred into sterile, RNase-free 1.5 ml tubes and 100 µl chloroform were added. The samples were shaken for 15 s, followed by 15 min centrifugation at 12,000 g at 4 °C to separate phases. The upper phase, containing the RNA was transferred into a new sterile, RNase-free tube after centrifugation, mixed with 250 µl isopropanol and stored at -20 °C for 20-30 min. This was followed by 10 min centrifugation at 12,000 g and 4 °C. After

discarding the supernatant 500 µl of 70% ethanol solved in DEPC-treated ddH_2O were added to the tubes, followed by 5 min centrifugation at 7,500 g and 4 °C. The RNA pellets were dried in a heat block for 10 min at 55 °C after discarding the ethanol, to ensure that no ethanol remained. Afterwards RNA pellets were resolved in 40 µl RNase-free ddH_2O by incubation at 55 °C for 10 min in a heat block. mRNA was transcribed into cDNA by reverse-transcriptase, to analyse altered gene expression. PCR was performed by using the VersoTM cDNA Kit (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. Oligonucleotides for PCR-reactions were obtained from Metabion International AG (Martinsried, Germany). Oligonucleotide pairs used for real-time PCR are summarized in section 2.1.5. CFX^{TM} Real-Time system (BIO-RAD, Munich, Germany) and reagents from ABgene® (Epsom, UK) were used to performe real-time RT-PCR. To confirm amplification specificity PCR products were subjected to a melting curve analysis. For normalization Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and Actin were used as a housekeeping-genes.

2.2.8 Infection with virus particles

Huh7.5 cells were seeded into 12-well plates at 4×10^4 cells/well 24h prior to infection. For infection experiments cells were pre-incubated with coffee for 2 hours, followed by washing. Infection was carried out for 24 hours before cells were washed and incubated with coffee for 24 hours. Infection was carried out using HCV genotype 2a strain JC1 (Pietschmann T et al. 2006) at an MOI of 0.5.

2.2.9 Immunofluorescence

Mouse monoclonal antibody HCV-2E3 against NS3 (BioFront Technologies), human monoclonal antibody A3R3 against E2 (a kind gift of Mansun Law, The Scripps Research

Institute, La Jolla, CA, USA), goat anti-mouse Alexa-594 and chicken anti-human Alexa-488-conjugated antibodies from Molecular Probes were used for staining. To visualize HCV infection, NS3 and E2 proteins were stained. The procedure included fixation (4% PFA; 20 min. at RT), permeabilization (0.1% Triton X100; 4°C for 10 min) and blocking (5% BSA; 20 min. at RT). Pictures were taken in an inverted microscope CKX41 (Olympus) with an LCachN/20X/0.40 Phc/1/FN22 UIS objective.

2.2.10 TCID50

Huh7.5 cells were seeded 24h prior to infection at $1.1x10^4$ in a 96 well plate. Supernatants from JC1 infected cells were serially diluted starting from 1:5, 6 wells per dilution were incubated. 72h post infection cells were fixed (4% of PFA; 20 min. at RT), permeabilized (0.1% Triton X100; 10 min. at 4°C) and blocked (5% BSA; 20 min. at RT). After incubation with the first antibody (anti-NS3, 2E3, Biofront Technologies; 1:3000) for 1h at RT, cells were incubated with the second antibody (anti-mouse-HRP; Sigma; A4416; 1:10000) for 1h at RT. Washing consisted of three rinses with PBS and bound peroxidase was detected. TCID50 was calculated based on as described previously (Muench H 1938).

2.2.11 Statistical analysis

Results were analyzed using Student's *t* test, if two groups were compared and the Dunnett's test if more groups were tested against a control group. If variances were inhomogeneous in the Student's *t* test, the results were analyzed using the Welsh test. All data in this study are expressed as a mean ± SEM. $P \leq 0.05$ was considered significant.

3. Results

3.1 Coffee reduces HCV replication of genotype 1b, replicon system

Coffee consumption has been shown to interfere with fibrosis formation (Modi AA et al. 2010) and to improve the effects of interferon alpha and ribavirin treatment in HCV patients (Freedman ND et al. 2011). Using the sub-genomic replicon system for genotype 1b we now investigated effects of coffee on HCV replication in vitro. HCV replication was detected by measurement of luciferase activity, which in this reporter assay is directly linked to HCV replication. Our results show that both, regular or decaffeinated coffee, were able to dose-dependently reduce HCV replication (Figure 1A). These effects were not due to cytotoxicity for the replicon cell culture system, since cell viability was not significantly altered within an incubation period of 72 hours (Figure 1B).

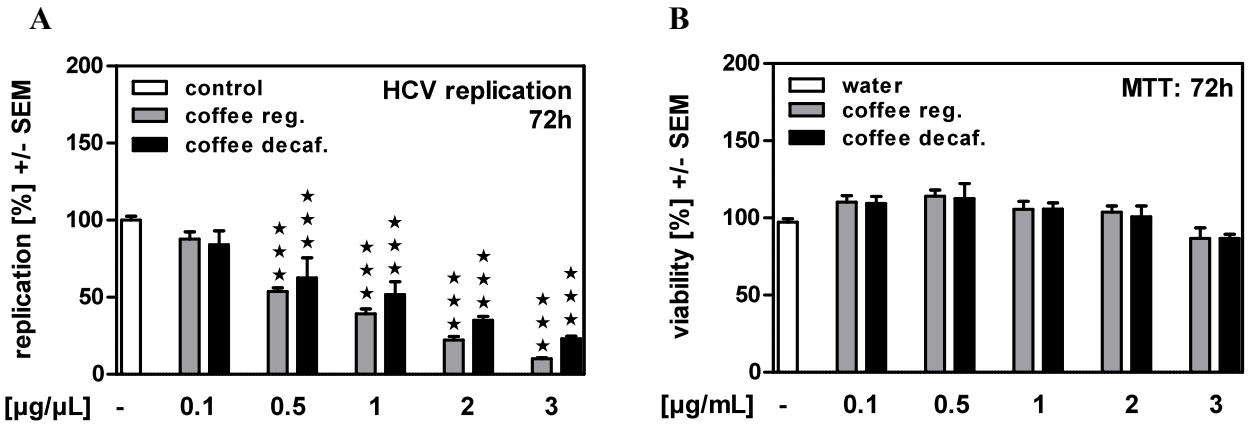


Figure 1: Coffee reduces HCV replication after 72h treatment in subgenomic replicon for genotype 1b
LucUbiNeo-ET replicon cells were incubated with regular or decaffeinated coffee at concentrations indicated for 72 hours (**A**+**B**). Viral replication was measured by luciferase reporter assay (**A**). Cell viability was measured by MTT assay (**B**). * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

Effects on HCV replication were already detectable after 24 hours of incubation. (Figure 2)

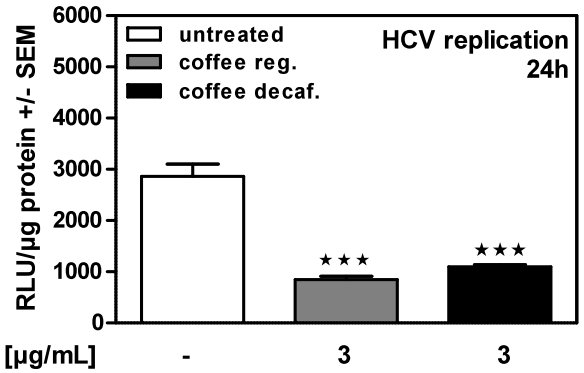


Figure 2: Coffee reduces HCV replication after 24h treatment in subgenomic replicon for genotype 1b
LucUbiNeo-ET replicon cells were incubated with regular or decaffeinated coffee at concentrations indicated for 24 hours. Viral replication was measured by luciferase reporter assay. * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

Additionally, we measured HCV replication by real time RT-PCR for HCV non-translated region (UTR) and found significant reduction of HCV RNA by coffee incubation, while there was no signal detectable in Huh7 cells, the parental cell line of the replicon system (Figure 3).

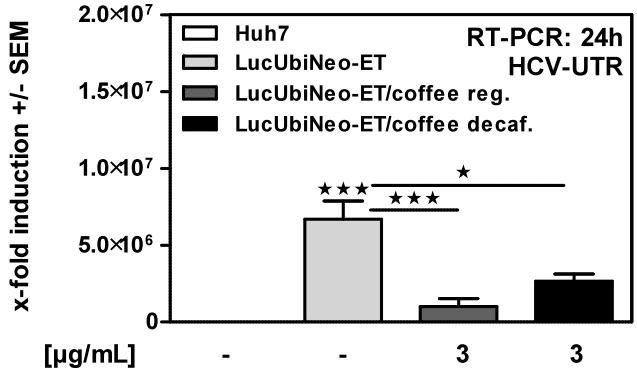


Figure 3: Coffee reduces HCV-UTR in real time RT-PCR in subgenomic replicon for genotype 1b
LucUbiNeo-ET replicon cells were incubated with regular or decaffeinated coffee at concentrations indicated for 24 hours. Viral replication was measured by real time RT-PCR. * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

3.2 Coffee reduces HCV replication of genotype 2a, subgenomic replicon system

As shown in Figure 1, 2 and 3 coffee was able to reduce HCV replication in vitro in the sub-genomic replicon system for genotype 1b. Genotype 1b is the most frequent HCV sub-type in Europe and all over the world, but genotype 2 and 3 infections are increasing. To investigate whether coffee exclusively interferes with genotype 1b replication, effects of coffee were tested in a sub-genomic replicon system for genotype 2a as well (Figure 4).

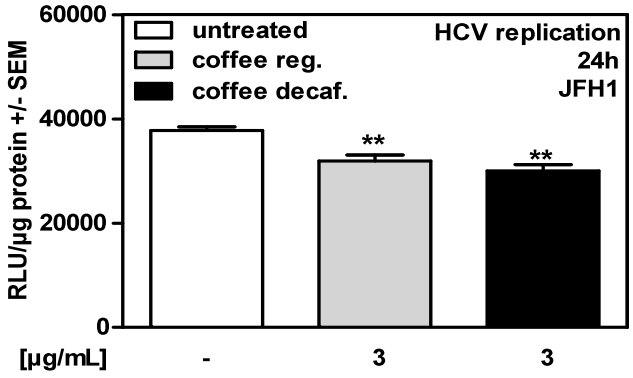


Figure 4: Coffee reduces HCV replication after 24h treatment in subgenomic replicon for genotype 2a
JFH cells were incubated with regular or decaffeinated coffee at concentrations indicated for 24 hours. Viral replication was measured by luciferase reporter assay. * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

3.3 Coffee reduces HCV replication and infectivity of genotype 2a, infectious system

The subgenomic replicon systems do not contain genetic information for the HCV proteins core, E1, E2, and NS2. To investigate effects of coffee on the whole HCV genome experiments were performed in the infectious system for genotype 2a (Pietschmann T et al. 2006). Here cells were transfected with the infectious HCV clone JC1 encoding for genotype 2a. HCV replication was detected by measuring luciferase activity and normalization to the protein content of each sample. In this system coffee was also found to interfere with HCV replication. Therefore coffee is able to affect HCV replication in infectious HCV cells as well and furthermore is not dependent on a special HCV genotype.

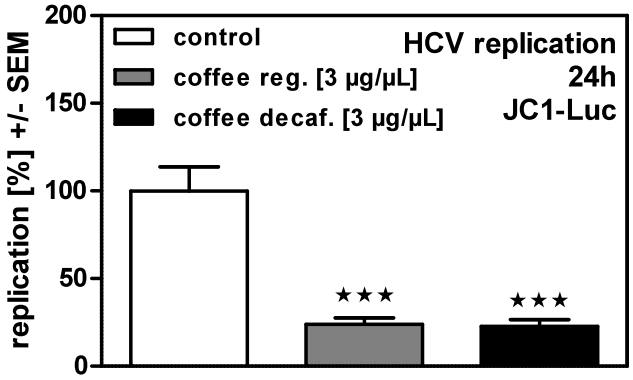


Figure 5: Coffee reduces HCV replication after 24h treatment in the infectious system
JC1 infected Huh7.5 cells were incubated with regular or decaffeinated coffee at [3 µg/µL] for 24 hours. Viral replication was measured by luciferase reporter assay. * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

Additionally, effects on HCV replication in the infectious system for genotype 2a were visualized by immuno-fluorescent staining for the HCV non-structural protein NS3 in red (Figure 6) and for the HCV structural protein E2 in green (Figure 7). Visual field through microscope is shown below in grey.

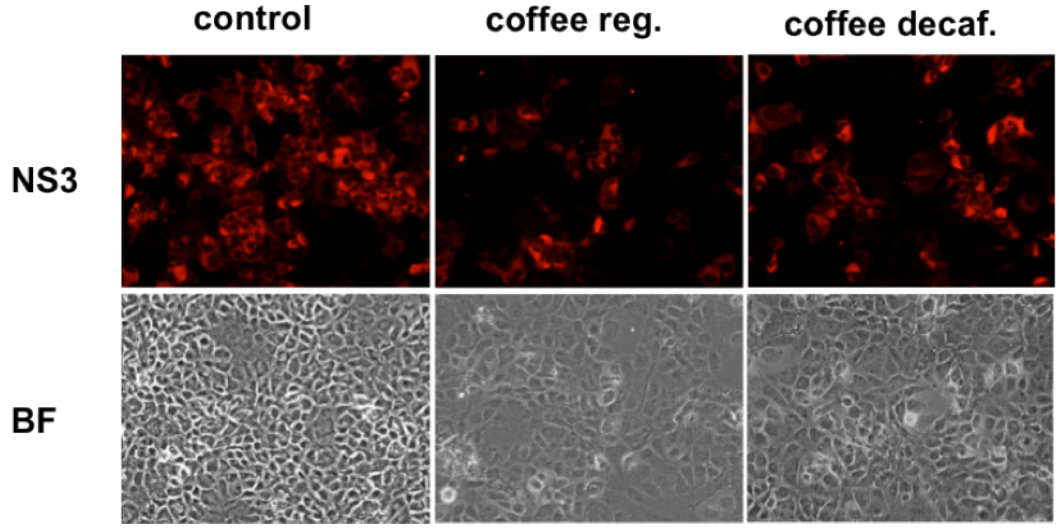


Figure 6: **Coffee reduces non-structural protein NS3 in the infectious system**
JC1 infected Huh7.5 cells were incubated with regular or decaffeinated coffee at [3 µg/µL] for 48 hours. Immuno-fluorescent staining for NS3 was performed. Representative images are shown.

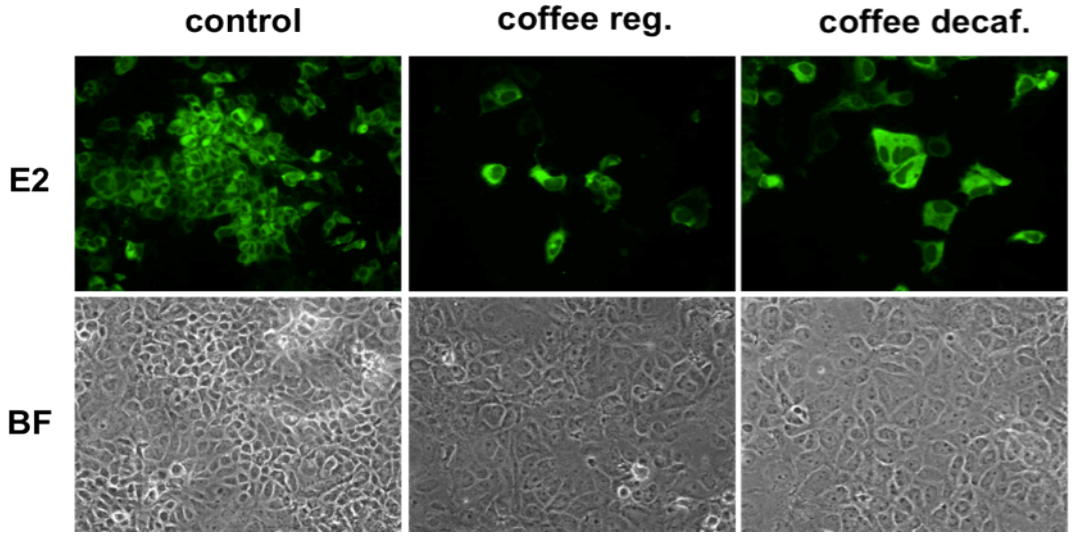


Figure 7: **Coffee reduces structural protein E2 in the infectious system**
JC1 infected Huh7.5 cells were incubated with regular or decaffeinated coffee at [3 µg/µL] for 48 hours. Immuno-fluorescent staining for E2 was performed. Representative images are shown.

Coffee is not only able to reduce expression of HCV non-structural protein NS3 and HCV structural protein E2. Moreover, coffee interfered with infectivity of infectious viral particles (Figure 8). So incubation with coffee was able to reduce cell infection with HCV. Infectivity was measured by TCID50 titration of infectious viral particles in supernatants.

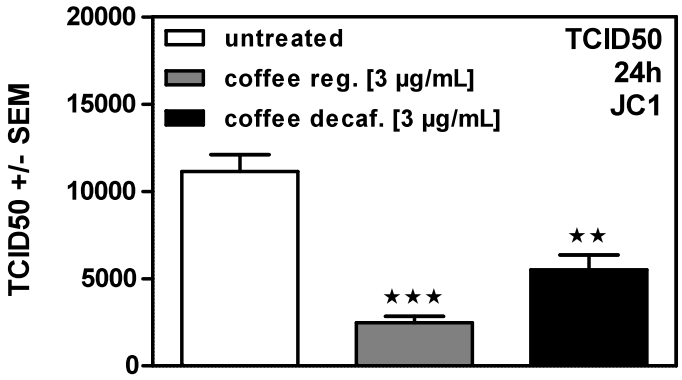


Figure 8: Coffee reduces HCV infectivity in the infectious system, genotype 2a
JC1 infected Huh7.5 cells were incubated with regular or decaffeinated coffee at [3 µg/µL] for 24 hours. Viral infectivity was measured by titration of the TCID50. * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

3.4 Coffee interferes with viral entry by HCV receptor down-regulation

To investigate effects of coffee on HCV entry, Huh 7.5 cells were pre-incubated with coffee for 2 hours before 2 hours of infection with the HCV clone JC1 encoding genotype 2a. Experiments were evaluated after an incubation period of 48 hours. Pre-incubation with coffee interfered with HCV infection, as detected by immuno-fluorescent staining for non-structural protein NS3 (Figure 9). Visual field through microscope is shown below in grey.

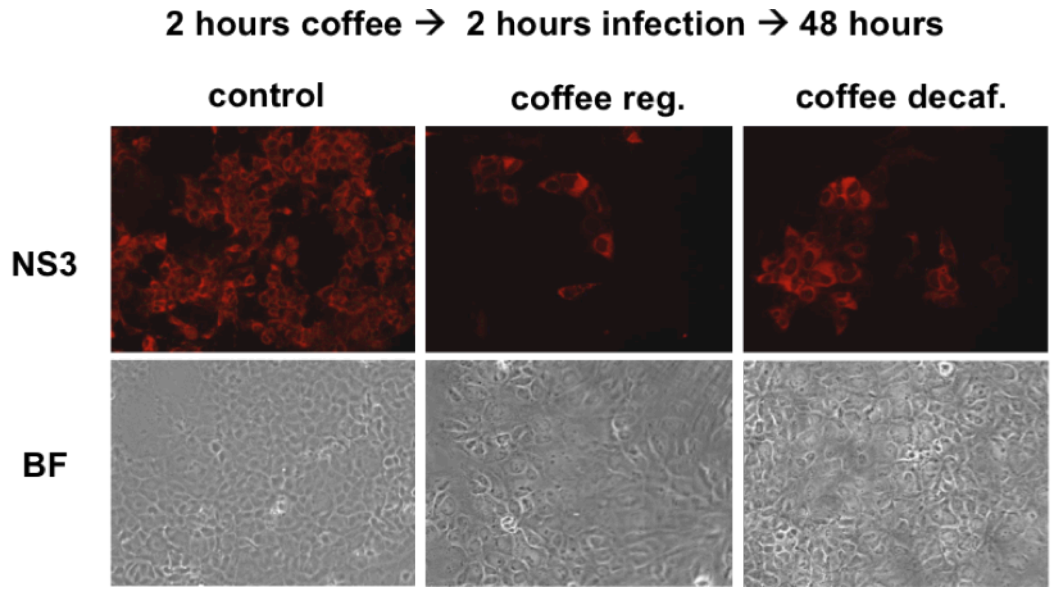


Figure 9: Coffee pre-incubation reduces non-structural protein NS3 in the infectious system
Huh7.5 cells were incubated with regular or decaffeinated coffee [3 µg/µL] for 2 hours. After pre-incubation cells were transfected with JC1 for 2 hours. After 48 hours immuno-fluorescent staining for NS3 was performed. Representative images are shown.

This observation prompted us to investigate effects of coffee on essential HCV entry receptors, of which tetraspanin CD81, scavenger receptor class B member I and tight junction proteins claudin-1 and occludin have been described (Fraquhar MJ 2012) (Albecka A et al. 2012). Human hepatocytes were incubated with regular coffe for 2 and 24 hours. Receptor expression was measured by real-time RT-PCR. Our results show that LDL-receptor was already down regulated after 2 hours of coffee incubation, while claudin-1 was down regulated after 24 hours (Figure 10).

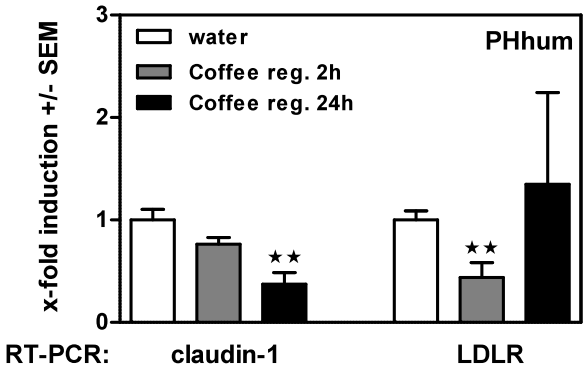


Figure 10: Coffee leeds to HCV entry receptor downregulation
Human hepatocytes were incubated with 3µg/µl regular coffee for 2 and 24 hours. Receptor expression was measured by real-time RT-PCR * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$

3.5 Caffeine interferes with replication of HCV genotype 1b, replicon system

Although decaffeinated coffee was able to reduce HCV replication effects of caffeine-containing coffee were more pronounced. This prompted us to investigate effects of caffeine on HCV replication. Our results show that caffeine decreased HCV replication dose-dependently (Figure 11A) without decreasing cell viability (Figure 11B).

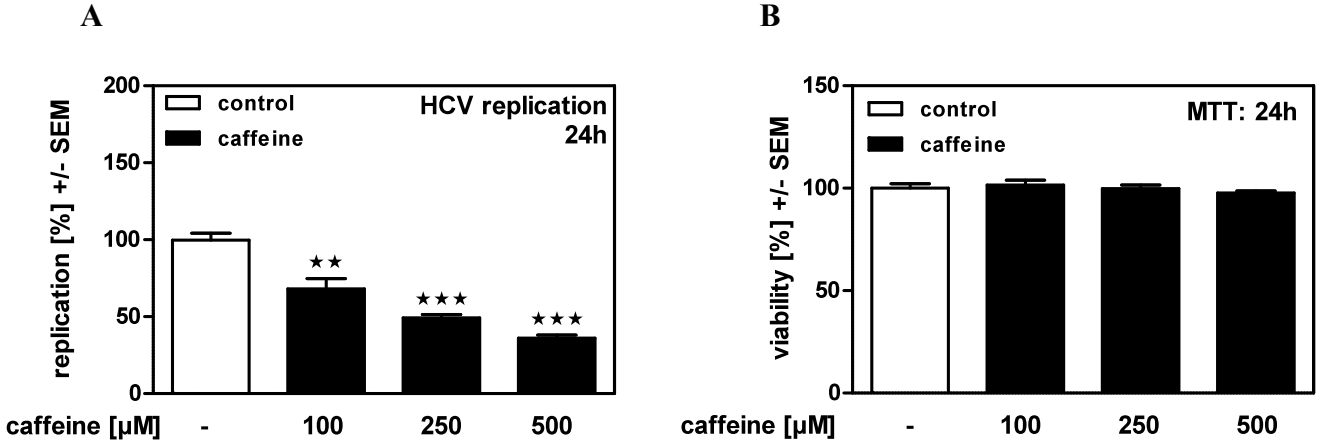


Figure 11: Caffeine reduces HCV replication in subgenomic replicon system, genotype 1b
LucUbiNeo-ET replicon cells were incubated with Caffeine at concentrations indicated for 24 hours (**A**+**B**). Viral replication was measured by luciferase reporter assay (**A**). Cell viability was measured by MTT assay (**B**). * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$

Caffeine is metabolized to about 11% theofylline, 5% theobromine and predominantly paraxanthine (81%) by cytochrome oxidases (Gu L et al. 1992). While theofylline (Figure 12A+B) and theobromine (Figure 12C+D) could not reduce HCV replication at concentrations achievable by coffee consumption, paraxanthine was able to inhibit HCV replication as efficiently as caffeine and therefore seems to be the main antiviral metabolite (Figure 12E+F).

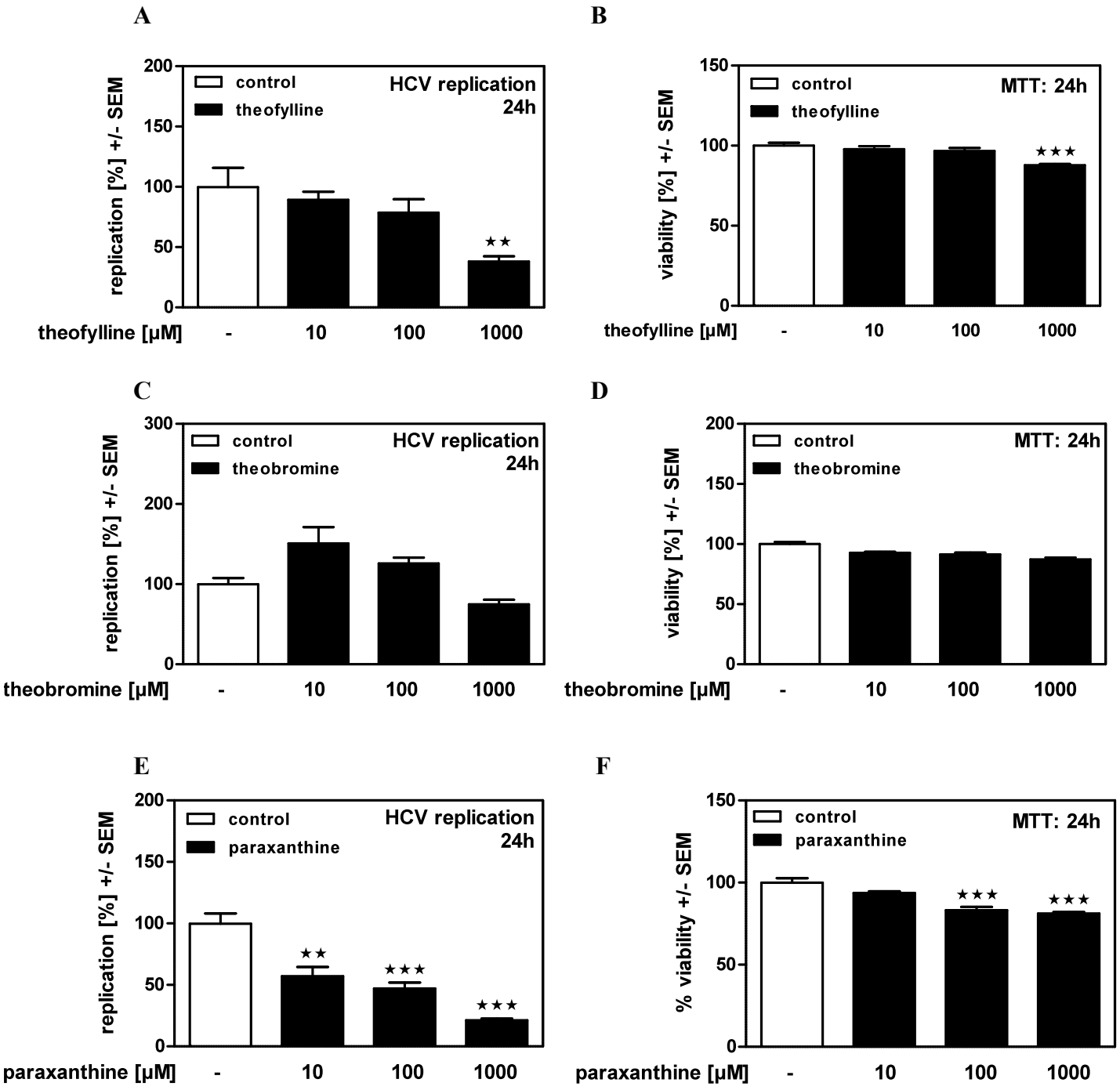


Figure 12: **Caffeine metabolites interfere with HCV replication**
LucUbiNeo-ET replicon cells were incubated with theofylline (**A**+**B**), theobromine (**C**+**D**) or paraxanthine (**E**+**F**) at concentrations indicated for 24 hours. Viral replication was measured by luciferase reporter assay (**A**+**C**+**E**). Cell viability was measured by MTT assay (**B**+**D**+**F**) * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$

3.6 Chlorogenic acid and genistein do not interfere with HCV replication

Different coffee ingredients have been reported to interact with different diseases or cancer in different organs. Chlorogenic acid (ChA) is another predominant compound in coffee, which is found at concentrations of 70 to more than 300 mg/cup (Olthof MR et al. 2001) and has been shown to affect colon cancer (Kang NJ et al. 2011). The concentration of ChA, which translates to 3 µg/µL of arabica coffee in vitro, would be about 80 µM. Our results show that incubation of replicon cells in the presence of ChA at concentrations of up to 200 µM did not interfere with HCV replication. Given that ChA is resorbed by about 30% in vivo, in vitro concentrations above 200 µM would exceed blood concentrations achievable by consumption of coffee.

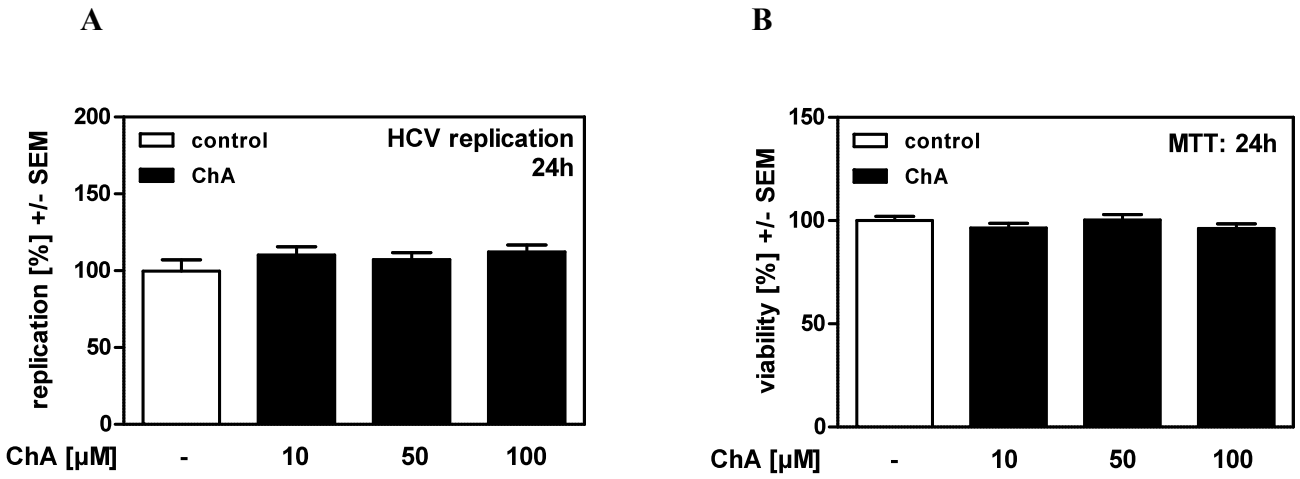


Figure 13: Chlorogenic acid did not interfere with HCV replication
LucUbiNeo-ET replicon cells were incubated with ChA at concentrations indicated for 24 hours. Viral replication was measured by luciferase reporter assay (**A**), cell viability was measured by MTT assay (**B**). * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$

Genistein is another coffee component; it has been shown to inhibit HSV-1 replication in vitro (Yura Y et al. 1993). We investigated effects of genistein on HCV replication. But we could not show effects of Genistein on HCV replication in non-toxic concentrations.

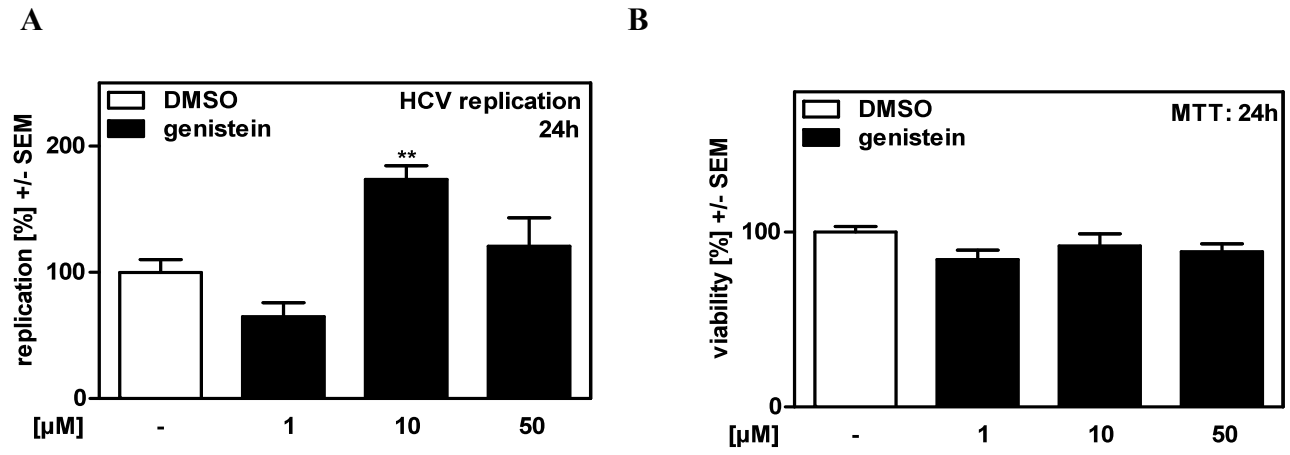


Figure 14: G**enistein did not interfere with HCV replication**
LucUbiNeo-ET replicon cells were incubated with Genistein at concentrations indicated for 24 hours. Viral replication was measured by luciferase reporter assay (**A**), cell viability was measured by MTT assay (**B**). * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$

3.7 Cafestol does not interfere with HCV replication

Coffee beans also contain lipids, mostly triglycerides and diterpenesters. The diterpenes cafestol and kahweol have been shown to exert anti-carcinogenic activity in vitro (Cavin C et al. 2002). This prompts us to investigate effects of cafestol on HCV replication. Our results show that cafestol did not interfere with HCV replication in concentrations used. Furthermore cafestol concentration of 50 µM reduced cell viability.

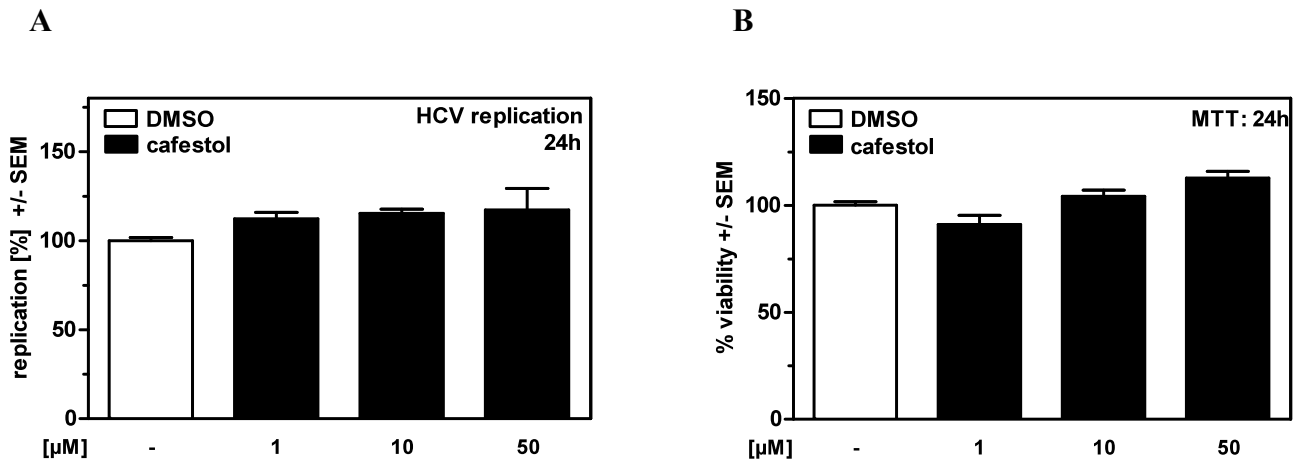


Figure 15: Cafestol did not interfere with HCV replication
LucUbiNeo-ET replicon cells were incubated with cafestol at concentrations indicated for 24 hours. Viral replication was measured by luciferase reporter assay (**A**), cell viability was messured by MTT assay (**B**). * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$

3.8 Coffee and caffeine increase interferon effects on HCV replication

It has been reported that coffee increases effects of interferon alpha and ribavirin therapy in HCV patients (Freedman ND et al. 2011). Our results confirm this finding in vitro, since in the HCV replicon system for genotype 1b regular coffee (Figure 16A) as well as caffeine (Figure 16B) significantly increased antiviral effects of interferon. Regular coffee had a more pronounced effect compared to decaffeinated coffee, which in multiple experiments did not increase interferon effects significantly (Figure 16A). Therefore, caffeine seems to play a pivotal role in increasing interferon effects.

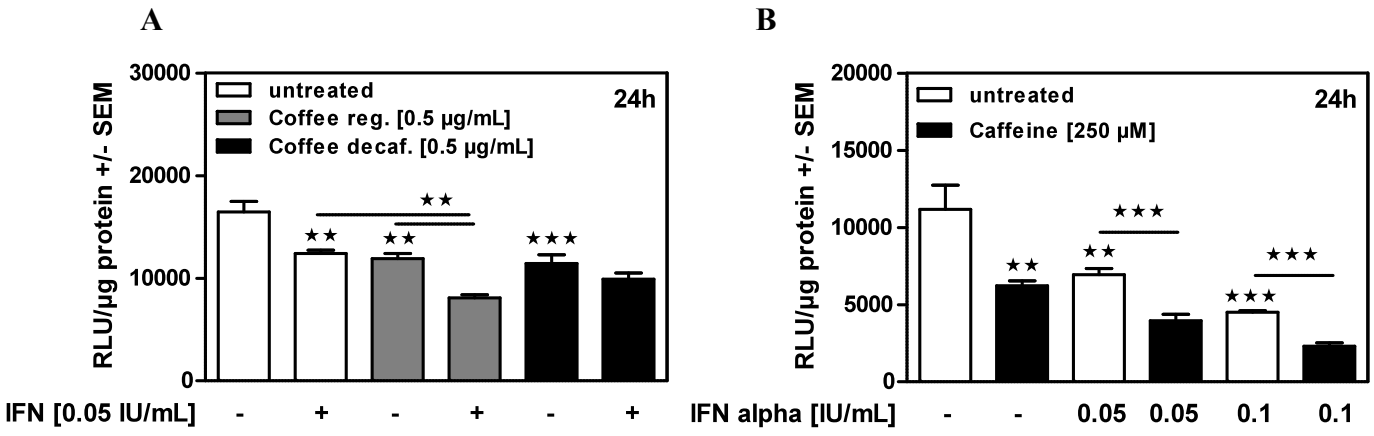


Figure 16: Coffee and caffeine increase interferon effects on HCV replication
LucUbiNeo-ET replicon cells were co-incubated with interferon and regular or decaffeinated coffee (**A**) or caffeine (**B**) at concentrations indicated for 24 hours. Viral replication was measured by luciferase reporter assay. * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$

3.9 Coffee, but not caffeine, decreases wnt signaling activity

Interferon alpha exhibit its antiviral potential by anti-proliferative effects on the viral cells and and immunsystem activating effects in the human body. But it has also been reported to inhibit the wnt signaling pathway (Thompson MD et al. 2011). IFN alpha is able to decrease wnt pathway activity factor beta-catenin through increasing beta-catenin nuclear export factor RanBP3 (Thompson MD et al. 2011). It has been described that beta-catenin

expression is elevated in patients with chronic HCV infection in dependence on the HCV non-structural protein NS5A (Park CY et al. 2009), implicating that the wnt signaling pathway might affect HCV replication. Comparing our replicon cell lines to their parental cell line Huh7 we observed significantly enhanced wnt activity measured by a reporter assay for beta-catenin driven transcription (CRT). CRT was significantly decreased in the presence of regular or decaffeinated coffee, but not in the presence of caffeine alone.

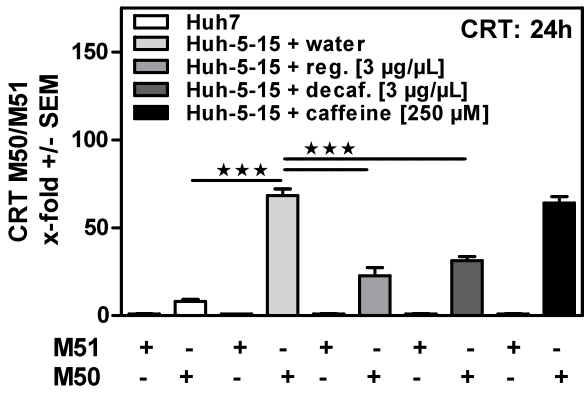


Figure 17: **Coffee, but not caffeine, decreases wnt signaling activity**
Parental cell line Huh7 and HCV replicating Huh5-15 cells were transfected with M50 and M51 plasmids for 24 hours. After transfection cells were incubated with regular or decaffeinated coffee or caffeine at concentrations indicated for another 24 hours. Viral replication was measured by CRT assay. * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$

Increased wnt signaling activity in HCV replicating cells was also indicated by enhanced expression of the wnt activation markers conductin and beta-catenin as well as the beta-catenin stabilizing kinase CK2 catalytic subunits alpha (CK2a) and alpha' (CK2ap). As observed for CRT, incubation with coffee, but not caffeine significantly reduced expression of conductin, beta-catenin and CK2 subunit alpha.

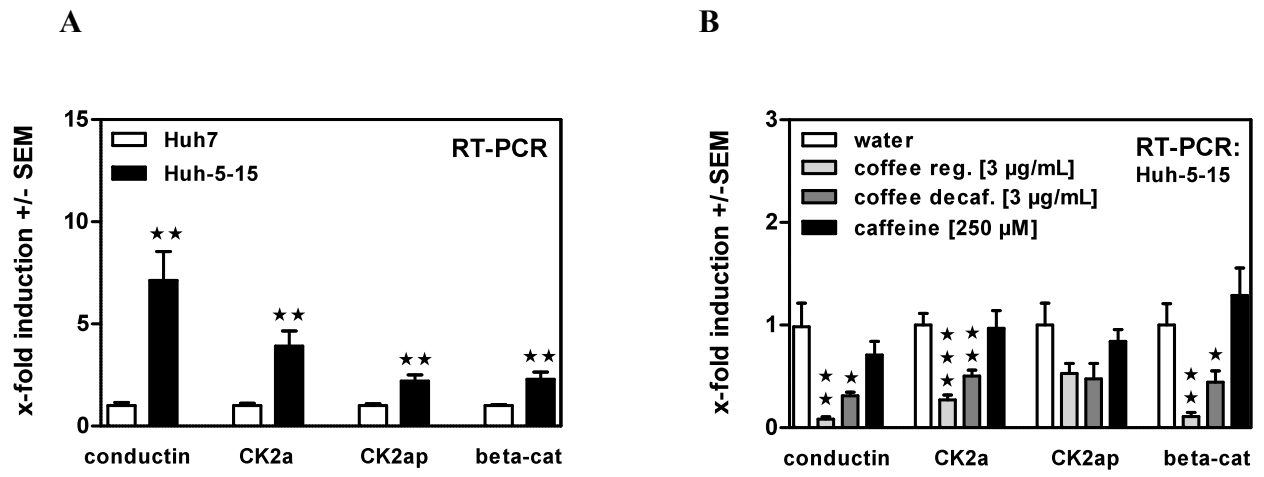


Figure 18: Coffee, but not caffeine, decreases wnt activation markers
Huh7 and Huh-5-15 cells were incubated with regular or decaffeinated coffee or caffeine at concentrations indicated for 24 hours. Different wnt activation markers were measured by real time RT-PCR. * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

Using inhibitors of beta-catenin activity (Quercetin, Figure 19) or CK2 activity (TBB, Figure 20) we found that HCV replication was significantly reduced. Our results show that wnt signaling promotes HCV replication and that coffee interferes with this mechanism.

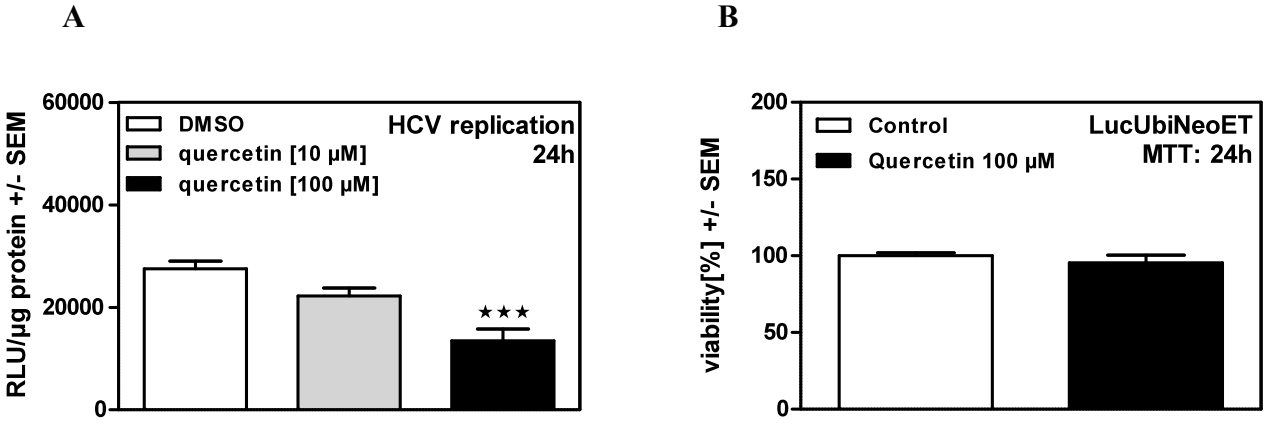


Figure 19: Quercetin reduced HCV replication in vitro
LucUbiNeo-ET replicon cells were incubated with beta-catenin activity inhibitor quercetin (A) or CK2 activity inhibitor TBB (B) at concentrations indicated for 24 hours. Viral replication was measured by luciferase reporter assay. * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$

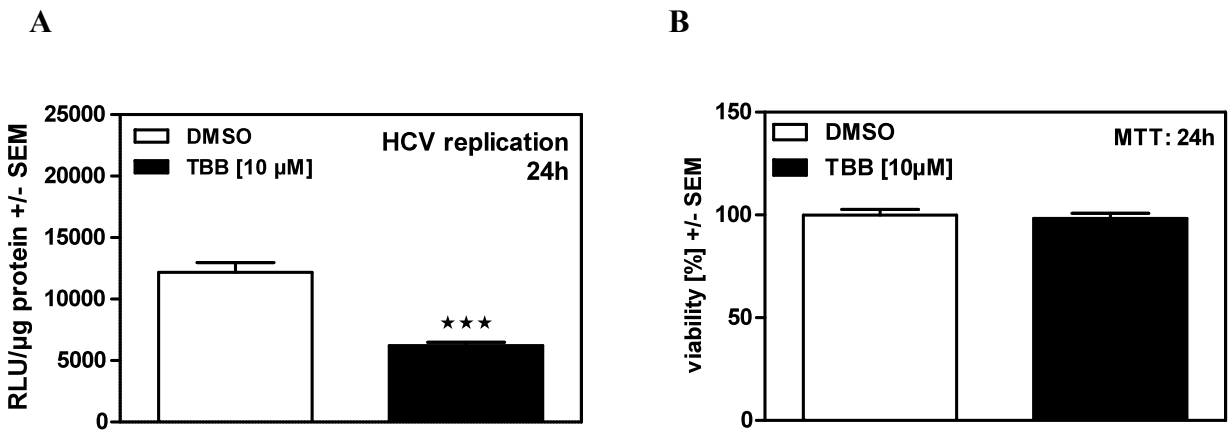


Figure 22: TBB reduced HCV replication in vitro
LucUbiNeo-ET replicon cells were incubated with beta-catenin activity inhibitor quercetin (A) or CK2 activity inhibitor TBB (B) at concentrations indicated for 24 hours. Viral replication was measured by luciferase reporter assay. * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$

4. Discussion

Coffee is the most favourite beverage in Germany. With 150 litres per person per year the Germans consume more coffee than water or beer (Deutscher Kaffeeverband e.V., www.kaffeeverband.de). Regular coffee increases levels of alertness and concentration via competitive antagonism at adenosine-receptors (Chou CC, Vickroy TW 2003), and therefore, it is the worldwide most frequently used legal psychoactive substance (Dórea JG, Da Costa THM 2005).

Coffee has been discredited for a long time. Even the children song "C-A-F-F-E-E " (Carl Gottlieb Hering, 1766-1853) warns of higher coffee consumption. But in recent years, many publications have shown that coffee consumption is associated with multiple positive effects on the human organism. Coffee contains phytoprotectants, of which coffee acids, alkaloids, melanoids, flavinoids and polyphenols exhibit anti-oxidative properties, at least in vitro (Svilaas A et al. 2004). Since free radicals seem to play a role in cardiovascular and metabolic diseases as well in tumor development (Davì G et al. 2005), this might serve as an explanation for the ability of coffee to reduce general mortality (Lopez-Garcia E et al. 2008). This is in line with findings that coffee drinking decreases the risk to develop e.g. heart diseases (Wu JN et al. 2009), stroke (Lopez-Garcia E et al. 2009), diabetes typ-2 (van Dam RM, Hu FB 2005), Alzheimer's (Eskelinen MH, Kivipelto M 2010), and Parkinson's disease (Costa J et al. 2010), or gastrointestinal cancers (Galeone C et al. 2010), (Larsson SC, Wolk A 2007) and breast cancer (Lowcock EC et al. 2013).

Coffee contains over 2000 components (Tuomilehto J. 2013), which makes it difficult to break down the mechanisms for all those described effects to single substances. The most commonly known substance in regular coffee is caffeine. But in all these clinical studies, the effects of coffee could not be clearly ascribed to the caffeine in regular coffee. Decaffeinated

coffee showed the same reductive effects as regular coffee, but not as strong as caffeine-containing coffee. Nevertheless, part of this effect could be attributed to caffeine (Utsunomiya H et al. 2008).

The idea that coffee might interfere with viral replication is not new. In 1998, Dorothy Bonn suggested that green coffee beans might contain substances able to interfere with viral infection, showing inhibition of HIV integrase (Bonn D 1998). It has also been reported that coffee extracts are able to decrease HSV type 1 and poliovirus multiplication, possibly due to interference with infectivity and virus particle formation (Utsunomiya H et al. 2008). With regard to hepatitis virus infections, regular and decaffeinated coffees have been shown to interfere with HBV replication due to the coffee ingredients chlorogenic acid and caffeic acid (Wang GF et al. 2009). This observation could be broadened, showing that HCC due to HBV infection and was less pronounced in coffee drinkers, while there was no reduction found in consummates of green tea (Inoue M et al. 2009).

Epidemiological studies now provide evidence for effects of coffee on HCV. A reduced risk for coffee drinking patients with chronic HCV infection to develop or die from HCC has been reported (Ohfuji S et al. 2006; Wakai K et al. 2007). Although a lot of evidence points to direct effects of coffee on HCV replication, this has not been investigated in vitro to define active substances and molecular mechanisms of coffee on viral replication. Our results show that concentrations of coffee, which are achievable by consumption of 1-3 cups (200 mL per cup) per day are able to significantly and dose-dependently reduce HCV replication in vitro without exerting toxic effects on the cell culture system.

We tested coffee effects on replication in the replicon system for genotyp 1b and in the infectious system for genotype 2a and could show comparable results. Coffee did not only exhibit antiviral effects on replication, but also coffee incubation decreased infectivity of viral particles. This is new for HCV, but decrease in multiplication by coffee extracts has

already been shown for HSV type 1 and poliovirus (Utsunomiya H et al. 2008). Incubation of uninfected cells with coffee prior to infection resulted in reduced infection compared to control pre-incubated cells, which could be due to a reduction in the expression of HCV entry receptors. LDL-receptor (Albecka A et al. 2012) and Claudin-1 (Fraquhar MJ 2012) are described to act as HCV entry receptors and our results show significant reduction for LDL-receptor and Claudin-1 expression on human primary hepatocytes after treatment with regular coffee. Therefore, coffee incubation seems to have several target points of antiviral activity, interfering with viral replication, decreasing infectivity of viral particles and directly protecting host cells from infection.

We performed experiments with regular and decaffeinated coffee, which both reduced HCV replication, although effects of regular coffee turned out to be stronger. This prompted us to investigate effects of caffeine in the HCV replicon system. Higher caffeine consumption is associated with less liver cirrhosis, among others caused by chronic HCV infection (Modi AA et al. 2010). Our results show that concentrations of caffeine corresponding to 3-4 cups of coffee (200 mL per cup) were sufficient to significantly impair HCV replication in vitro without having toxic effects on the cell culture system. The alkaloid caffeine and its metabolites paraxanthine, theobromine and theofylline are known to act as competitive adeonsine receptor antagonists in the brain (Fisone G et al. 2004), (Chou CC, Vickroy TW 2003). Adenosine is a purine nucleoside, which promotes sleep by inhibition of release of activating neurotransmitters. So inhibition of adenosine receptors by caffeine is primarily used to avoid fatigue. Beside its sleep promoting effect, adenosine is also known for promoting wound healing and matrix production by activation of its receptors (Macedo L 2007). Furthermore adenosine has been reported to promote fibrosis in skin and lungs as well as in the liver via excess matrix production (Cronstein BN 2011). This might imply that inhibition of adenosine receptors by caffeine could inhibit fibrosis development, which

might be another effect besides inhibition of HCV replication, why coffee is able to slow down hepatic fibrosis development in HCV infected patients (Modi AA et al. 2010). Of the three caffeine degradation products paraxanthine, theobromine and theofylline, we found inhibition of HCV replication only by the main degradation product paraxanthine. Besides the effect on adenosine receptors, all three degradation products are phosphodiesterase inhibitors as well, but just paraxanthine is responsible for the lipolytic effects of caffeine (Jiang M et al. 1998). So it seems that caffeine is able to inhibit HCV replication via its degradation product paraxanthine.

Since decaffeinated coffee is also able to interfere with HCV replication we tried to identify other antivirally active substances in coffee. Besides caffeine, coffee beans contain about 2000 other substances: Coffee beans contain about 17% of lipids, mostly triglycerides and diterpenesters. The diterpens cafestol and kahweol have been shown to exert anti-carcinogenic activity in vitro (Cavin C et al. 2002). In our experiments, we did not observe decreased HCV replication at non-toxic concentrations of cafestol in replicon cell lines. The polyphenols caffeic acid and chlorogenic acid exhibit antioxidative effects and have been shown to affect colon cancer (Kang NJ et al. 2011). Incubation of HCV replicating cells with chlorogenic acid did not reduce HCV replication. The isoflavone genistein has been shown to inhibit HSV-1 replication in vitro (Yura Y et al. 1993), but did not inhibit HCV replication in vitro. Therefore, we were not able to identify another substance contained in coffee, besides caffeine, which was able to reduce HCV replication at non-toxic concentrations. On the other hand, it might also be a combination of substances, which in a concerted action are able to inhibit HCV replication.

Concerning the mechanism by which coffee is able to interfere with viral replication, we found hints to an involvement of the wnt signaling pathway. This pathway transduces signals from the outside of the cell via surface receptors to the inside of the cell. The cascade is

started by the signal protein ligand “wnt”, which binds to the receptor “frizzled“ and leads to inhibition of GSK3beta and thereby inhibits beta-catenin degradation. Concomitantly, activation of the wnt signaling pathway leads to beta-catenin accumulation in the cytoplasm, causing stabilized beta-catenin to act as a co-activator of transcription factors and leads to changes in target gene transcription (Nusse R, Varmus HE. 1992). Recently beta-catenin activity was found to be enhanced in HCV patients by direct interaction between the non-structural HCV protein NS5A and components of the wnt signaling pathway (Park CY et al. 2009); furthermore, wnt signaling pathway activity has also been found to be enhanced by HCV core protein (Liu J et al. 2011). This met our observations that wnt signaling activity was significantly enhanced in the HCV replicon system in comparison to its parental cell line not containing the replicon. A connection from wnt pathway activity to enhanced HCV replication could be made by showing that interference with wnt signaling, either by use of the beta-catenin inhibitor quercetin or a knockdown of beta-catenin expression, resulted in a direct inhibitory effect on HCV replication.

In fact we found that both regular and decaffeinated coffee, but not caffeine, were able to interfere with wnt signaling pathway activity in replicon cells. HCV seems to up-regulate wnt pathway inducers while down-regulating wnt pathway inhibitors. This results in higher expression of wnt target genes like conductin, myc or claudin-1, implicating that HCV might force infected cells towards higher HCV expression but also towards cancer development, as recently shown for the development of colon (Jeong WJ et al. 2012) and breast (Zhang H et al. 2012) cancer. Higher wnt pathway activity has recently been described to be associated with hepatocellular carcinoma (Li ZQ et al. 2012).

Until very recently, treatment of chronic hepatitis C infection has predominantly been performed by a combination of pegylated interferon alpha2 with the nucleoside analogue ribavirin. Interferon alpha exhibits its antiviral potential by anti-proliferative effects on the

viral cells and immunsystem activating effects in the human body. But it has also been reported to inhibit the wnt signaling pathway (Thompson MD et al. 2011). Interestingly, also the response to interferon/ribavirin treatment of HCV patients was found enhanced by coffee drinking (Freedman ND et al. 2011). We were able to show that inhibition of HCV replication in vitro increased when interferon alpha was co-incubated with regular coffee or caffeine. Decaffeinated coffee did not significantly increase interferon effects on HCV replication. Therefore, this effect seems to be due to caffeine. Further research is needed to determine possible positive effects of caffeine supplementation of HCV therapy.

So there might be two different mechanisms why coffee is able to reduce HCV replication and increase IFN alpha therapy effects. While inhibition of HCV replication could be achieved by regular and decaffeinated coffee and caffeine itself, decaffeinated coffee could not increase IFN alpha effects on HCV replication. Accordingly, this is in line with our suspicion that caffeine is not the only antiviral effective substance in coffee and the coffee effects on HCV replication and HCV therapy might be due to combination of substances. This thesis is furthermore provided by the finding that other caffeine-containing beverages besides coffee, were not able to reduce liver fibrosis formation in HCV infected patients (Modi AA et al. 2010).

In this work, we could show antiviral properties of regular and decaffeinated coffee, caffeine and the caffeine degradation product paraxanthine on HCV replication in vitro. Additionally, coffee had effects on HCV entry and HCV infection in vitro, and did not show restriction to a special genotype. HCV therapy with IFN/ribavirin was shown to be improved by regular and decaffeinated coffee, but not by caffeine. Our data indicate that wnt signaling pathway inhibition significantly contributes to the antiviral effects of coffee, but not caffeine. So non-caffeine induced effects of coffee on HCV replication seems to be due to interference with wnt signaling and might improve IFN/ribavirin therapy. Our results also show that caffeine

is not the only antivirally active substance in coffee, but all other substances contained in coffee, which have been part of our experiments could not be identified to have effects on HCV replication in vitro. Further experiments need to be done to identify a combination of substances or a single substance, which is responsible for the effects of coffee on the Hepatitis C Virus.

In conclusion, coffee consumption of about 3 cups per day might interfere with HCV replication and support HCV therapy and also slow down development of liver cirrhosis and hepatocellular carcinoma (HCC).

5. Abstract

5.1 Abstract

Background & Aims: Coffee is the most frequently consumed legal drug all over the world. Coffee consumption has been shown to decrease the risk of fibrosis formation and to improve the effects of interferon treatment in HCV patients. We investigated effects of regular and decaffeinated coffee and coffee ingredients on HCV replication and infection in vitro.

Methods: To investigate HCV replication, infectious as well as replicon systems for genotypes 1b and 2a were used. Cells were incubated in the presence of regular or decaffeinated coffee, coffee ingredients, caffeine and caffeine metabolites. Effects on wnt signaling pathway activity and antiviral effects in co-incubation with interferon alpha were measured. Furthermore effects of coffee on viral entry have been tested in the infectious system.

Results: Both, regular and decaffeinated coffee, were able to reduce HCV infection and replication significantly with regular coffee having a more pronounced effect. Caffeine as well as its main metabolite paraxanthine significantly reduced HCV replication. While caffeine and its metabolite paraxanthine did not; both, regular and decaffeinated coffee, were able to reduce wnt pathway activity, which has been found markedly increased by HCV replication. Likewise, inhibition of wnt signaling by the beta-catenin inhibitor quercetin, siRNA directly against beta-catenin or inhibition of the wnt pathway inducer CK2 by its inhibitor 4,5,6,7-tetrabromo-2-azabenzimidazole (TBB) significantly interfered with HCV replication. Furthermore coffee as well as caffeine was able to increase inhibitory effects of interferon alpha on HCV replication. Nevertheless coffee pre-incubated cells show significantly less HCV infection compared with water pre-incubated cells. Furthermore

coffee incubated uninfected hepatocytes showed significant down-regulation of essential HCV entry receptors.

Conclusions: Coffee effects in HCV infected patients, which have been described by clinical studies are due effects on HCV replication level. Coffee, but not caffeine, seems to exhibit antiviral activity by inhibition of the wnt signaling pathway, which might represent a novel target for HCV therapy. Furthermore coffee increases effects of interferon alpha therapy in vitro and therefore could take place in therapy guidelines. Nevertheless coffee pre-incubation decreases essential HCV receptors and thereby decreases HCV infection and could act as a preventive substance.

5.2 Zusammenfassung

Hintergrund: Kaffee ist die am häufigsten konsumierte legale Droge der Welt. Studien belegen, dass regelmäßiger Kaffeekonsum das Risiko einer Leberfibrose signifikant senken und die Effekte der Interferon Therapie bei HCV Patienten verbessern kann. Wir haben in dieser Arbeit die Effekte von koffeinhaltigem und entkoffeiniertem Kaffee auf die HCV Replikation und die HCV Infektion in vitro getestet

Methoden: Für die Experimente wurden sowohl ein infektiöses System für Genotyp 2a, als auch Replikonzelllinien für die Genotypen 1b und 2a verwendet. Die Zellen wurden mit koffeinhaltigem und entkoffeiniertem Kaffee, Kaffeeinhaltsstoffen, Koffein und Koffeinabbauprodukten behandelt. Es wurden die Effekte auf die Aktivität des wnt signaling pathway und antivirale Effekte von der Kombinationsbehandlung mit Interferon gemessen. In weiteren Versuchen im infektiösen System wurden die Effekte von Kaffee auf den Vireneintritt in die Zelle gemessen.

Ergebnisse: Sowohl koffeinhaltiger als auch entkoffeinierter Kaffee konnten die HCV Infektion und die HCV Replikation signifikant reduzieren, wobei der koffeinhaltige Kaffee einen etwas ausgeprägteren Effekt hatte. Koffein und das Hauptabbauprodukt von Koffein, Paraxanthin konnten die HCV Replikation signifikant reduzieren. Es konnte eine deutlich erhöhte Aktivität des wnt signaling pathways in HCV replizierenden Zellen gezeigt werden. Koffeinhaltiger als auch entkoffeinierter Kaffee reduzierten die Aktivität des wnt signaling pathway, während Koffein und Paraxanthin keinen Effekt auf den wnt signaling pathway hatten. Dies deckt sich mit den Beobachtungen, dass eine Hemmung des wnt signaling pathways durch den beta-catenin Inhibitor Quercetin, eine siRNA gegen beta-catenin oder einen Inhibitor des wnt pathway Induktors CK2 4,5,6,7-tetrabromo-2-azabenzimidazole (TBB) die HCV Replikation signifikant senken konnten. Es konnte zusätzlich gezeigt werden, dass koffeinhaltiger Kaffee und Koffein in der Lage waren die Effekte von

Interferon auf die HCV Replikation zu verstärken. Eine Vorbehandlung der Zellen mit Kaffee vor der Infektion mit dem HCV Virus im infektiösen System, zeigte eine signifikant reduzierte Infektionsrate im Vergleich mit Zellen, welche mit Wasser vorbehandelt wurden. Gesunde Hepatozyten zeigten nach Behandlung mit Kaffee eine deutliche reduzierte Expression von essentiellen Rezeptoren für den HCV Eintritt in die Zelle.

Schlussfolgerungen: Die in Studien beschriebenen Kaffeewirkungen bei HCV infizierten Patienten sind auf Effekte des Kaffees auf die HCV Replikation zurückzuführen. Kaffee selbst wirkt antiviral über die Hemmung des wnt signaling pathways, für Koffein konnte dies nicht gezeigt werden. Die Hemmung des wnt signaling pathways könnte ein neues Angriffsziel von Therapien für HCV sein. Es konnte zusätzlich eine Verstärkung der Wirkung von Interferon alpha durch Kaffee gezeigt werden, eine entsprechende Empfehlung könnte in die Therapieleitlinien für HCV aufgenommen werden. Außerdem konnte eine Vorbehandlung von gesunden Zellen die Eintrittsrezeptoren für HCV vermindern und scheint somit auch einer HCV Infektionsprävention zu dienen.

6. Abbreviations

CD81:	Cluster of Differentiation 81
CLDN1:	Claudin-1
CRT:	Beta-catenin-regulated transcription
CTGF:	connective tissue growth factor
GSK3beta:	Glycogen synthase kinase 3 beta
HBV:	Hepatitis B Virus
HCC:	Hepatocellular carcinoma
HCV:	Hepatitis C Virus
HIV:	Human immunodeficiency virus
HSV:	Herpes simplex Virus
OCLN:	Occludin
PDE:	Phosphodiesterase
PEG-IFN:	pegylated interferon alpha
RT-PCR:	real-time Polymerase chain reaction
TCID50:	Tissue Culture Infection Dose 50
UTR:	un-translated region

7. References

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7.2 Internet

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"Everything will be okay in the end. If it is not okay, it is not the end"

John Lennon

9. Curriculum vitae

entfällt aus datenschutzrechtlichen Gründen

10. Eidesstattliche Versicherung

Ich versichere ausdrücklich, dass ich die Arbeit selbständig und ohne fremde Hilfe verfasst, andere als die von mir angegebenen Quellen und Hilfsmittel nicht benutzt und die aus den benutzten Werken wörtlich oder inhaltlich entnommenen Stellen einzeln nach Ausgabe (Auflage und Jahr des Erscheinens), Band und Seite des benutzten Werkes kenntlich gemacht habe.

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Ich erkläre mich einverstanden, dass meine Dissertation vom Dekanat der Medizinischen Fakultät mit einer gängigen Software zur Erkennung von Plagiaten überprüft werden kann.

Unterschrift: .....