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**Colon cancer chemopreventive potential of apple juice
and apple polyphenols in the APC^{Min/+} model**

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ABBREVIATIONS

AAPH	2,2-Azobis-(2-amidinopropane) dihydrochloride
Ab	Antibody
ABC	Avidin biotin complex
ACF	Aberrant crypt foci
Akt/PkB	Protein Kinase B
AJ	Apple juice
AP	Apple polyphenols
APC	Adenomatous polyposis coli
APS	Ammonium persulfate
BCA	Bicinchoninic acid
BSA	Bovine serum albumin
b.w.	Body weight
CAJ	Cloudy apple juice
CBP/p300	CREB binding protein
CD	concentration required to double the specific activity of NAD(P)H: quinone oxidoreductase
cDNA	Complementary deoxyribonucleic acid
Cox	Cyclooxygenase
Cpm	Counts per minute
CRC	Colorectal cancer
Cyp1A1	Cytochrome P450 1A1
DAB	3,3'-Diaminobenzidine
DMBA	7,12-Dimethylbenzo[a]anthracene
DMH	1,2-Dimethylhydrazine
DMSO	Dimethylsulfoxide
DNA	Desoxyribonucleic acid
DTT	Dithiothreitol
dNTP	2-Desoxynucleotide-5'-triphosphate
DPPH	1,1-Diphenyl-2-picrylhydrazyl
DTT	Dithiothreitol
EDTA	Ethylenediamine tetraacetic acid
EGCG	(+)-Epigallocatechingallate
EGF	Epidermal growth factor
ENU	N-ethyl-N-nitrosourea
EP	Prostaglandin E receptor
ERK	Extracellular signal-regulated kinase
FAD	Flavin adenine dinucleotide
FAP	Familial adenomatous polyposis
FCS	Fetal calf serum
g	Gram

GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GBP	GSK-3 β binding protein
GSK	Glycogen synthase kinase
h	Hour
HE	Hematoxylin-Eosin
HPLC	High performance liquid chromatography
HRP	Horseradish peroxidase
IC ₅₀	Half maximal inhibitory concentration
INDO	Indomethacin
iNOS	Inducible nitric oxide synthase
kDa	kiloDalton
kg	Kilogram
K-RAS	Kirsten-Ras (rous avian sarcoma homologue)
l	Liter
LOH	Loss of heterozygosity
LOX	Lipoxygenase
LPS	Lipopolysaccharide
MAPK	Mitogen activated protein kinase
mg	Milligram
min	Minute
ml	Milliliter
mm	Millimeter
MOM	Mouse on mouse
MOPS	3-(N-Morpholino)-propanesulphonic acid
MMP	Matrix metalloproteinases
mRNA	Messenger ribonucleic acid
NAD(P)H	β -Nicotinamide adenine dinucleotide (phosphate)
NP	Normal phase
NSAID	Non-steroidal antiinflammatory drug
NF- κ B	Nuclear factor- κ B
NO	Nitric oxide
NOS	Nitric oxide synthase
NR	Nitrate reductase
OD	Optical density
OPC	Oligomeric procyanidins
ORAC	Oxygen radical absorbance capacity
OR	Odds ratio
PAE	Polyphenolic apple extract
PARP	Poly(ADP ribose) polymerase
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PG	Prostaglandin

PI3K	Phosphatidyl-inositol 3-kinase
PKC	Protein kinase C
PLC	Phospholipase C
PPAR	Peroxisome Proliferator Activated Receptor
PMSF	Phenylmethyl sulfonyl fluoride
PVDF	Polyvinyliden fluoride
QR	NAD(P)H:quinone oxidoreductase
r	Correlation coefficient
RNA	Ribonucleic acid
RNS	Reactive Nitrogen species
ROS	Reactive oxygen species
rpm	Rotations per minute
RT	Reverse transcriptase
RTK	Receptor tyrosine kinase
RR	Relative risk
s	Second
SD	Standard deviation
SDS	Sodium dodecyl sulfate
SEM	Standard error of mean
SFN	Sulforaphane
SI	Small Intestine
SOD	Superoxide dismutase
TBE	Tris/borate/EDTA
TBS	Tris-buffered saline
TBS-T	TBS containing Tween 20
TCA	Trichloroacetic acid
TCF/LEF	T-cell factor/lymphoid enhancer-binding factor 1
TEMED	N,N,N',N'-tetramethylethylenediamine
TGF	Transforming growth factor
U	Unit
UV	Ultraviolet
V	Volt
VEGF	Vascular endothelial growth factor
vs.	versus
Wnt	Wingless int-1
Wt	Wild type
XTT	Sodium-2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide

Summary

Colorectal cancer is the second and third most leading cancer in Europe and the US, respectively. One of the main risk factors of this malignancy of the large bowel has been associated to western-style diets rich in high saturated fat and meat and low in fiber. During the last years, it has been reported that a regular uptake of fruits and vegetables may reduce the incidence of colorectal cancer. One of the most consumed fruits in western diet patterns are apples. Several epidemiological and case-control studies have indicated that usual consumption of one or more apples a day may reduce the risk for colon cancer. But so far, the possible preventive effects of apple juice have not been completely investigated.

Our previous examinations showed that polyphenolic extracts from apple juice are biologically active in terms of radical scavenging, influence on drug metabolism as well as anti-inflammatory and anti-hormonal potential *in vitro*. Moreover, we used the colorectal chemoprevention $Apc^{Min/+}$ mouse model to study the chemopreventive effect of apple juices. This strain is highly susceptible to spontaneous intestinal adenoma formation. The results showed that cloudy apple juice and polyphenolic apple juice extract had a significant impact diminishing the tumor development in the small intestine of $Apc^{Min/+}$ mice. However, the possible mechanism remained to be understood.

In the present study, the possible molecular mechanism was investigated in a second animal experiment. Animals were treated with water (as a control), cloudy apple juice (CAJ), placebo juice (apple juice without polyphenols), 0.2% B-PAE (procyanidin-rich apple extract) and a combination between cloudy apple juice and 0.2% B-PAE.

Both cloudy apple juice and B-PAE intervention significantly reduced adenoma development by 24% and 34%, respectively. These findings indicated polyphenols as the main chemopreventive compounds in this model. On the other hand, placebo juice and cloudy apple juice plus B-PAE treatments, inhibited intestinal adenomas in a non-significant manner by 7% and 17%, respectively. Moreover, placebo juice intervention enhanced small adenomas in the whole small intestine.

In the liver, cloudy apple juice- and cloudy apple juice plus B-PAE-treated animals resulted in an induction of Phase II enzymes (GST, QR, and TrxR). Further investigations identified a high concentration of polyphenol metabolites in the urine of animals treated with cloudy apple juice and cloudy apple juice plus B-PAE. This indicated that polyphenol metabolites may reach the liver after intestinal absorption. However, due to an overnight fasting prior to dissection, the protein content of liver cells of mice in the

water and B-PAE intervention groups was diminished. Several enzymatic activities such as CYP1A1, QR and TrxR were decreased as a result of this fasting effect. In contrast, placebo juice, CAJ and CAJ + B-PAE maintained hepatic protein contents due to the carbohydrate uptake from these drinks.

The possible mechanism of the development of adenomas was investigated using protein array technology. Proteomic analysis revealed that fasting effects increased phosphorylated ERK and the target iNOS in intestinal adenomas. Similarly, Cyclin D1 was reduced but only in the middle parts of the small intestine. B-PAE treatments reduced fasting associated effects by decreasing p-ERK, iNOS, and Cyclin D1.

Since only very low concentrations of metabolites of polyphenols were found in urine and in analogy to other comparable compounds, a direct passage into the intestine or hepato-biliary excretion can be assumed. Therefore these results might be attributed to procyanidins acting locally.

CAJ and B-PAE treatments did not affect inflammation markers such as nitric oxide content in urine or PGE₂ in serum. *In vivo* levels of apple polyphenols were not high enough to efficiently inhibit these inflammation markers.

Cyclin D1 was strongly reduced in the distal parts of the small intestine by CAJ and CAJ + B-PAE. As well as in a non-significant manner, beta-catenin was reduced in a similar way.

Overall, apple polyphenols from CAJ and B-PAE reduced adenoma development in the Apc^{Min/+} mouse model. This chemopreventive potential was dependent on the intestinal bioavailability. Control animals (12 h fasted) resulted in an increase of Erk phosphorylation and of iNOS expression. B-PAE-treated animals suppressed these fasting-associated effects compared to water controls as well as in the groups that had access to high-calorie apple juice.

Zusammenfassung

Das kolorektale Karzinom ist die zweithäufigste Krebsart in Europa, in den USA die dritthäufigste. Als ein Hauptrisikofaktor gilt die westliche Ernährung, die reich an gesättigten Fettsäuren und Fleisch sowie arm an Ballaststoffen ist. Im Laufe der Zeit zeigte sich, dass ein regelmäßiger Verzehr von Obst und Gemüse die Tumorzinzidenz senken kann. Eine der am häufigsten konsumierten Früchte in der westlichen Ernährung ist der Apfel. Verschiedene epidemiologische Studien sowie Fallkontrollstudien haben gezeigt, dass der regelmäßige Verzehr von einem oder mehr Äpfeln täglich das Risiko, an kolorektalem Karzinom zu erkranken, senken kann. Ein möglicher präventiver Effekt von Apfelsaft wurde bisher jedoch nicht vollständig untersucht.

Unsere vorangehenden Untersuchungen zeigten *in vitro* folgende biologischen Aktivitäten von Polyphenol-Extrakt aus Apfelsaft: Radikalfänger-Eigenschaften, Beeinflussung des Fremdstoffmetabolismus sowie antiinflammatorisches und antihormonelles Potential.

Darüber hinaus untersuchten wir den chemopräventiven Effekt von Apfelsaft am $Apc^{Min/+}$ Maus Modell für kolorektale Chemoprävention. Dieser Stamm entwickelt spontan in hohem Maße intestinale Adenome. Die Ergebnisse zeigten, dass trüber Apfelsaft und Apfel - Polyphenol - Extrakt signifikant die Tumorentstehung im Dünndarm der $Apc^{Min/+}$ Maus hemmen.

Dieser Mechanismus konnte bisher jedoch nicht vollständig geklärt werden.

In der aktuellen Studie wurde der mögliche molekulare Mechanismus in einem zweiten Tierversuch erforscht. Die Tiere erhielten Wasser (Kontrollgruppe), naturtrüben Apfelsaft, Placebo-Apfelsaft (Apfelsaft ohne Polyphenole), 0.2% B-PAE (procyanidinreicher Apfelsaftextrakt) und eine Mischung aus trübem Apfelsaft und 0.2% B-PAE. Sowohl die Gabe von Trübsaft als auch die von B-PAE reduzierten signifikant die Neubildung von Adenomen um 24% bzw. 34%. Dieser Befund kennzeichnet die Polyphenole als vorrangige chemopräventive Bestandteile dieses Modells.

Placebo-Saft und die Mischung aus Trübsaft und 0,2% B-PAE hemmten die Adenomentwicklung lediglich im nicht signifikanten Bereich (7% bzw. 17%). Die Zufuhr von Placebo-Saft erhöhte sogar die Bildung von kleinen Adenomen im gesamten Dünndarm.

In der Leber zeigte sich bei den mit Trübsaft und den mit dem Gemisch aus Trübsaft und B-PAE behandelten Tieren eine Induktion von Phase II Enzymen (GST, QR und TrxR).

Weitere Untersuchungen zeigten eine hohe Konzentration an Polyphenolmetaboliten im Urin der Tiere dieser Gruppen. Dies weist darauf hin, dass Polyphenolmetabolite nach Resorption im Darm in die Leber gelangen.

Durch Fasten über Nacht vor der Sektion war jedoch der Proteingehalt in der Leber in der Kontrollgruppe (Wasser) sowie der B-PAE Gruppe vermindert. Auch die Aktivität verschiedener Enzyme wie z.B. CYP1A1, QR and TrxR sank in Folge des Fastens. Im Gegensatz dazu blieb in den anderen Gruppen (Placebo-Saft, trüber Apfelsaft und trüber Apfelsaft mit B-PAE) auf Grund der Kohlehydratzufuhr durch diese Säfte der Proteingehalt in der Leber stabil.

Der mögliche Mechanismus des Adenomwachstums wurde mit Hilfe von Protein arrays untersucht. Proteomische Untersuchungen zeigten, dass Fasten die Phosphorylierung von ERK und die Expression des Zielproteins iNOS bei Darmadenomen steigerte. In ähnlicher Weise wurde Cyclin D1 reduziert, jedoch nur im mittleren Teil des Dünndarms. Die Behandlung mit B-PAE reduzierte fasten-assoziierte Effekte durch Abnahme von p-ERK, iNOS und Cyclin D1. Da kaum nachweisbare Konzentrationen von Polyphenolmetaboliten im Urin gefunden wurden, kann auch in Analogie zu vergleichbaren Substanzen eine direkte passage in den Dünndarm oder eine hepato-biliäre Exkretion angenommen werden. Somit können diese Ergebnisse möglicherweise einer lokalen Wirkung von Procyanidinen zugeschrieben werden. Entzündungsparameter wie der Stickoxyd Gehalt im Urin oder PGE₂ im Serum wurden durch Trübsaft und B-PAE nicht beeinflusst. Die *in vivo*-Spiegel der Apfelpolyphenole reichten nicht aus, um diese effektiv zu hemmen.

Cyclin D1 wurde durch Trübsaft und Trübsaft gemischt mit B-PAE in distalen Anteilen des Dünndarms stark reduziert. Wenn auch nicht signifikant, so wurde β -catenin in ähnlicher Weise reduziert.

Zusammenfassend kann man sagen, dass Apfel-Polyphenole aus trübem Apfelsaft und B-PAE das Adenomwachstum im Apc^{Min/+} Maus Modell hemmten. Das chemopräventive Potential war abhängig von der intestinalen Bioverfügbarkeit. Erhalten die Tiere ausschliesslich wasser (12 Std nüchtern) war die phosphorylierung bzw. expression von Erk und iNOS erhöht. Erhalten die Tiere B-PAE, waren diese Fasten-assoziierten Effekte reduziert im Vergleich zur Waserkontrolle, wie auch in den Gruppen, die Zugang zu hochkalorischen Apfelsaft hatten.

1 INTRODUCTION

1.1 Cancer Chemoprevention

1.1.1 Cancer Facts

Cancer, derived from the Latin word for crab, is a group of diseases characterised by uncontrolled cell growth and the spread of abnormal cells. Cancer is often linked to high mortality.

According to the World Health Organisation (WHO) in the year 2000, there were more than 10 million cases of cancer recorded worldwide. This is predicted to increase by 50% to the year 2020, reaching up to 15 million cases. In Europe and the United States, cancer is the second most common cause of death in the general population, and is the first cause of death in the 45-65 age range (www.who.org/cancer).

Cancer is a multifactorial illness that is caused by internal factors (genetic predisposition, hormones, immune conditions and mutations that occur from metabolism) and external factors (diet, tobacco, carcinogen exposure, radiation and infections) (Minamoto *et al.*, 1999).

1.1.2 Carcinogenesis

Carcinogenesis is a multistep and multifunctional phenomenon, in generally divided into initiation, promotion and progression. Only malignant tumors are able to be invasive and to metastasize while benign tumors are self-limited in their growth (Fidler, 1999). A schematic representation of multistage carcinogenesis is described in Fig.1 (left).

The process is initiated with the transformation of a normal cell into an initiated cell as a result from genetic damage. This step is a rapid and irreversible process involving extracellular and intracellular events. Environmental carcinogens, such as aflatoxins, polycyclic aromatic hydrocarbons (PAH) and nitrosamines are procarcinogens, which must be metabolically activated by Phase I enzymes in order to become electrophilic active carcinogens. If these electrophilic carcinogens interact with genomic DNA, carcinogen-DNA adducts are formed, which may lead to

oncogene activation or tumor suppressor gene inactivation. Metabolic activation is performed by Phase I enzymes (CYP450 and flavin-dependent monooxygenases) which involve oxidation, hydroxylation and reduction reactions. The CYP1A1 and CYP2A1 are examples of this enzyme family. Procarcinogens like PAH and N-nitrosamines are known to induce both enzymes forming intermediate carcinogens and therefore increasing cancer risk (Smith *et al.*, 1998). Reactive oxygen species (ROS) are also actively implicated in the metabolic activation of procarcinogens (Halliwell *et al.*, 2000; Rushmore and Kong, 2002). In addition, ROS and carcinogens can lead to epigenetic alterations like DNA methylation. Such epigenetic changes can strongly affect the regulation of gene expression (Cerdeira and Weitzman, 1997).

Tumor promotion comprises the selective expansion of initiated cells. Initiated cells undergo tumor promotion into preneoplastic cells which is considered to be relatively slow and reversible. Clonal progress of initiated cells produces a larger population of cells that are at risk of further genetic modifications and malignant conversion. This is attributed to growth factors, hormones and inflammation mediators (Greenwald, 2002). These factors influence cellular signaling, and processes like cell growth regulation, proliferation and differentiation may be affected (Kelloff, 2000). In addition, ROS formation may result in damage of DNA, proteins or lipids. However, oxidative stress is involved during progression as well (Breimer, 1990).

Progression is the last stage of tumor growth with neovascularisation processes and metastatic potential. Tumor progression comprises the extension of the malignant phenotype through additional mutations and genome instability. Progression provides the impetus for conversion from benign adenomas to infiltrative and finally metastasizing neoplasms (Harris, 1991).

Carcinogenesis prevention appears as a practical approach in order to reduce cancer-related mortality (Sporn and Suh, 2002). A schematic representation of the strategies for prevention is shown in Fig.1 (right).

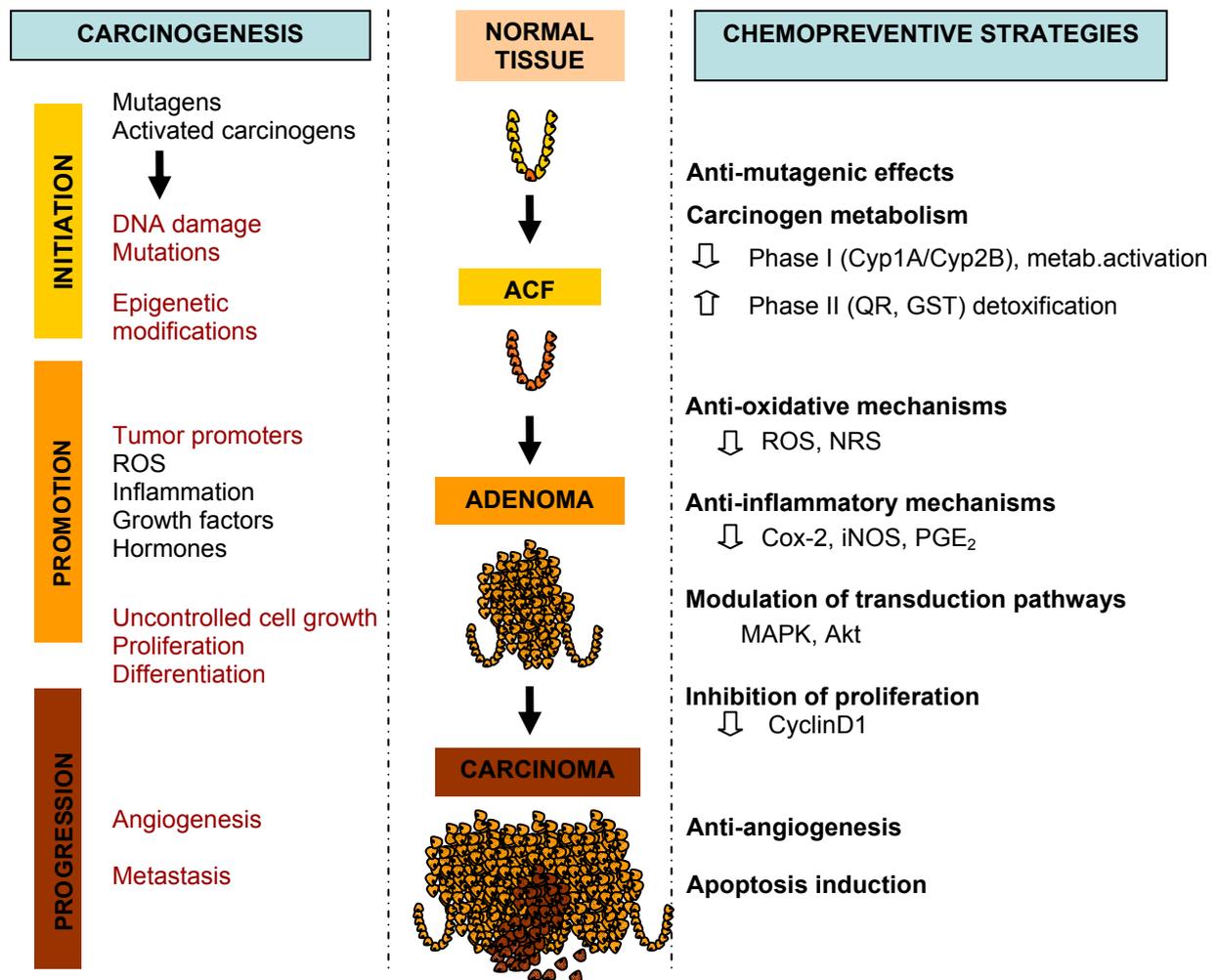


Fig. 1. Schematic representations of carcinogenesis (left) and mechanism of chemoprevention (right). See text for details. QR, NAD(P)H:quinone oxidoreductase; GST, glutathione-S-transferase; ROS, reactive oxygen species; RNS, reactive nitrogen species; COX, cyclooxygenase; iNOS, inducible nitric oxide synthase; PGE₂, prostaglandin E₂; MAPK, mitogen-activated protein kinase; Akt, protein kinase B. Adapted from (Gerhauser, 2008) and (Fodde *et al.*, 2001).

1.1.3 Chemoprevention

The aim of chemoprevention is the use of chemicals or dietary components to block, inhibit or reverse carcinogenesis (Sporn and Newton, 1979). Chemopreventive agents should have little or no toxicity, have a high efficacy, able to be orally administrable, have a known mechanistic action and low costs (Ferguson, 1994).

Depending on different stages along the natural history of disease, prevention can be roughly classified into three levels: primary prevention focusing on the prevention of onset of disease, secondary prevention aiming at early diagnosis and early treatment

if onset has occurred, and tertiary prevention which is at the last step trying to avoid recurrence, further progression, and complications (Zanker, 1999). Tab.1 describes each stage.

Tab. 1 Chemoprevention strategies. Adapted from (Zanker, 1999) and (Tsao *et al.*, 2004).

CHEMOPREVENTION STRATEGIES		POPULATION	EXAMPLES
PRIMARY	Avoidance of cancer-causing behaviour and exposure	Healthy	Smokers
SECONDARY	Cessation of exposure to carcinogenic agents of risk and screening to detect earlier stage malignancies	Individuals with premalignant lesions	Oral leucoplakia patients
TERTIARY	Administration of agents intended to prevent cancer development and to inhibit or delay the occurrence of metastases	Patients cured of their initial cancer or individuals definitively treated for their premalignant lesions	Oral cancer patients

As depicted in Fig. 1 (right), the process of carcinogenesis offers multiple targets for chemopreventive strategies. In the initiation stage, anti-mutagens either directly inactivate mutagens or block their metabolic activation.

Efficient inhibition of phase I enzymes to reduce metabolic activation may decrease DNA damage and therefore reduce cancer risk (Ioanides *et al.*, 1998).

Detoxification and therefore excretion will be completed *via* conjugation reactions (Phase II enzymes). Phase II enzymes (Glutathione S-transferases (GST), UDP-glucuronosyltransferases, sulfotransferases and N-acetyltransferases) involve covalent attachment of small polar endogenous molecules to form water-soluble compounds (Ioanides *et al.*, 1998). Detoxification through phase II enzymes is another preventive mechanism and may lead to decrease DNA damage (Goodman *et al.*, 2006).

Anti-oxidant mechanisms and possibilities to scavenge ROS contribute to an interesting prevention strategy which can affect all stages of carcinogenesis. A decrease in the oxidative stress status prevents cellular damage (Droge, 2002). Besides inactivation of ROS, alteration of tumor promoters plays an important role in the delay of carcinogenesis. Inflammation is a response triggered by damage to living tissues and is associated with multistage carcinogenesis through genetic and

epigenetic changes (Hussain and Harris, 2007). Induction of cyclooxygenase-2 (Cox-2) results in an increase of prostaglandin (PG) synthesis. PGs increase is strongly linked with apoptosis resistance and proliferation in epithelial tissues (Helliwell *et al.*, 2004; Jabbour *et al.*, 2002). Reactive nitrogen species (RNS) include multiple highly reactive molecules, which are mostly derived of nitric oxide produced by the inducible nitric oxide synthase (iNOS). Furthermore, RNS may also contribute to oxidative stress and Cox-2 induction (Klaunig and Kamendulis, 2004). Both Cox-2 and iNOS have been involved in the promotion stages mediating intracellular signalling like protein kinases and transcription factors like the activator protein-1 (AP-1) (Kundu and Surh, 2005).

A vast variety of cytoplasmic protein kinases are involved in relaying events in the cellular signalling. Components of upstream or cytoplasmic signalling networks include protein kinases, such as the family of proline-directed serine/threonine kinases named mitogen-activated protein kinases (MAPKs). Abnormal or improper activation or silencing of the MAPK pathway or its downstream transcription factors can result in uncontrolled cell growth, leading to malignant transformation (Surh, 2003). The Ras/MAP kinase cascade as a well-known example is important in tumorigenesis, leading to increase mitogenesis *via* induction of Cyclin D1 (Lavoie *et al.*, 1996). The inhibition of abnormal cell proliferation *via* modulation of cell cycle progression is one of the important strategies for chemoprevention (Alao, 2007).

In the progression stage, induction of apoptosis and inhibition of angiogenesis are important protective mechanisms against neoplastic transformation.

Multiple dietary and pharmacological agents have been investigated for chemopreventive potential (Kelloff, 2000).

According to their mechanism of action these agents can be classified as blocking or suppressing agents. Blocking agents are mostly related to inhibition of initiation processes, whereas suppressing compounds are considered to inhibit malignant expression of initiated cells (De Flora, 1998). Examples of well investigated chemopreventive compounds targeting various stages of carcinogenesis are summarized in Tab. 2. These agents have been shown to prevent cancer development in experimental models for chemoprevention (*in vitro* and *in vivo*).

Tab. 2 Carcinogenesis stages and chemoprevention strategy.

	CHEMOPREVENTIVE STRATEGY	EXAMPLE	COMPOUND	REF.
INITIATION	Phase I inhibition	Cyp1A2	Capsaicin	a
	Phase II induction	QR	Sulforaphane	b
	DNA damage prevention	UV DNA damage	EGCG	c
PROMOTION	antioxidant effects	Catalase	Vitamin C	d
	anti-tumor promoting effects	MAPK	Curcumin	e
	anti-hormonal effects	Aromatase	Genistein	f
	anti-inflammatory activity	Cyclooxygenases	Flavonoids	g
	inhibition of cell proliferation	Cyclin D1	Resveratrol	h
PROGRESSION	induction of apoptosis	Caspases	Quercetin	i
	inhibition of angiogenesis	VEGF, MMP	ω -3 PUFAs	j

References cited: ^a (Surh *et al.*, 1995) ^b (Zhang *et al.*, 1994) ^c (Wei *et al.*, 1999) ^d (van Haaften *et al.*, 2003) ^e (Mehta and Moon, 1991) ^f (Allred *et al.*, 2004) ^g (Kim *et al.*, 2004) ^h (Ahmad *et al.*, 2001) ⁱ (Wei *et al.*, 1994) ^j (Szymczak *et al.*, 2008).

1.2 Colorectal cancer development

1.2.1 Facts and statistics

Colorectal cancer (CRC) is a disease of the large bowel, including colon, rectum and appendix.

In western societies, CRC is the second most frequent malignancy. More than 940.000 cases occur annually worldwide and nearly 500.000 patients die from the disease each year (www.who.int). Colon and rectum cancers have a lower incidence in developing countries (Novak 2003).

More than 420.000 cases were estimated for Europe in the year 2008. In men, CRC was the second most common type cancer in incidence and the third in women. In terms of mortality, it was second among men and women with more than 220.000 deaths (World cancer report 2008, WHO). Mortality has increased 1.8% from 2004 to 2006 (Boyle and Ferlay, 2005; Ferlay *et al.*, 2007). Significant increase rates have been observed in East European countries such as in the Czech Republic and Slovakia. Industrial growth and poor CRC screening procedures may be associated with this increase (Center *et al.*, 2009).

According to the WHO 2008 World cancer report, CRC was the third cancer in mortality and incidence in the US during 2008 in both sexes. However, there is a tendency for a reduction. This might be attributed to early screening programs like colonoscopies, flexible colon sigmoidoscopies and faecal occult blood tests (Umar and Greenwald, 2009).

CRC aetiology has been thoroughly studied during the last decades. Age remains the most important risk factor for CRC. Although this disease can occur at any age, about 90% of diagnosed cases correspond to patients over 50 years old (American Cancer Society, www.cancer.org).

Only approximately 8% of CRC cases are defined as hereditary cancer syndromes (Weitz *et al.*, 2005). The two major syndromes are *Familial Adenomatous Polyposis* (FAP) and *Hereditary non polyposis colorectal cancer* (HNPCC) (Fig. 2).

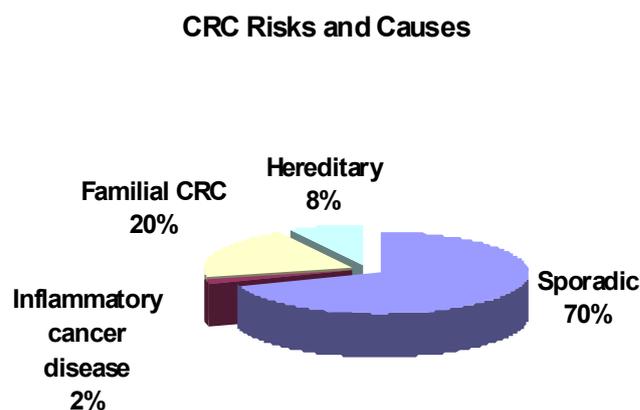


Fig. 2 CRC risk factors and causes. (Weitz *et al.*, 2005)

FAP is an autosomal-dominant disease characterized by development of multiple colonic adenomas at early age due to a mutation in the *Apc* gene (Grodén *et al.*, 1991). The incidence for FAP is estimated at about 1 in 8000 live newborns (Fearhead *et al.*, 2001). The clinical diagnosis of FAP depends upon the detection of hundreds to thousands of adenomatous polyps in the colon and rectum of affected patients. The polyps typically appear by the third decade of life but also in adolescence. Colonoscopy is suggested as screening procedure for FAP families. Untreated, CRC invariably develops by the early forties at the latest. The risk of developing CRC is reduced using prophylactic colectomy or proctocolectomy (Beech *et al.*, 2001). HNPCC or Lynch syndrome is an autosomal-dominant disorder caused by germline mutations of mismatch repair (MMR) genes (Lynch *et al.*, 1992). In

addition, HNPCC patients have microsatellite instability (Jass *et al.*, 2002; Liu *et al.*, 1996). Other minority syndromes like Flat adenoma, Cowden, and Peutz-Jeghers syndromes belong to the hereditary cancers as well (Lynch and de la Chapelle, 2003).

Presence of the mutated genes that cause familial colorectal cancer increases the risk of developing the disease. These mutations may be passed from one generation to the next or may be a product of a person's environment. This latter class does not really fulfil the hereditary CRC criteria (Weitz *et al.*, 2005).

A low percentage (2%) of CRC holds a direct relation with inflammation, because inflammation is directly associated with carcinogenesis (Coussens and Werb, 2002); (Itzkowitz and Harpaz, 2004).

In most of the cases, sporadic CRC is mainly influenced by environmental factors, with no familial or hereditary component. Lower penetrance genes combined with a Western-style diet contribute to the majority of sporadic CRCs (Heavey *et al.*, 2004).

1.2.2 Adenoma-carcinoma sequence

Development of CRC is a multi-step process and occurs through the accumulation of several discrete events. Originally described by Vogelstein (Fearon and Vogelstein, 1990) the adenoma-carcinoma sequence is developed by genetic mutations which bring histologic changes (Fig. 3).

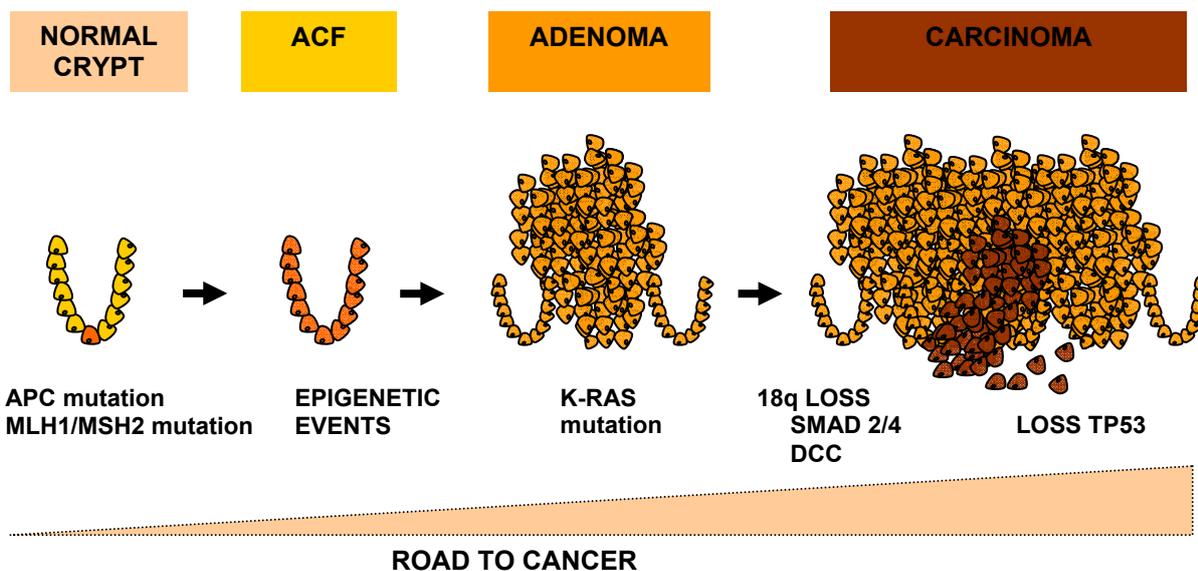


Fig. 3 A revision of the Vogelstein adenoma-carcinoma sequence. Adapted from McDonald *et al.* (2006). See text for details. ACF, aberrant crypt foci

An initial mutation in a stem cell within a crypt gives rise to dysplastic progeny, which induces the development of a monocryptal adenoma after successive replication cycles. More mutations are acquired as this process continues leading to genetic instability and ultimately to cancer (McDonald *et al.*, 2006). Several proteins are involved in this sequence.

One of the initial events in the development of CRC is Apc mutation. Approximately 80% of sporadic colon tumors show mutation in this gene (Powell *et al.*, 1992). The great majority of Apc mutations lead to a truncated protein. Lack of functional Apc protein leads to accumulation of β -catenin in the cytosol and its nuclear translocation (compare chapter 1.3.3). Downstream targets of Apc/ β -catenin include genes related with inflammation, proliferation, angiogenesis and oncogenes.

Histologically, the first recognisable manifestation of epithelial alterations are *aberrant crypt foci* (ACF). Apc mutations are still present at the ACF stage and they are related with the degree of dysplasia of these early lesions. ACF are putative precursors of colon cancer and have been described in mice after carcinogen induction (Bird, 1987).

Likewise, germ-line mutations of DNA MMR genes like MLH1 and MSH2 participate in the initial stages as well. Microsatellite instability is attributed to these mutations and is found in approximately 15% of CRC (Ionov *et al.*, 1993).

Another crucial mechanism during early stages is DNA methylation. Epigenetic events play an important role in gene regulation and DNA methylation modifies gene functions during cancer development (Hamilton, 1992). On one hand, CRC shows a decreased level of whole genomic methylation as one of the earliest molecular abnormalities (Makos *et al.*, 1992) which can lead to genomic instability and therefore facilitating or accelerating tumor progression (Rodriguez *et al.*, 2006). But on the other hand, like in the Apc gene, promoter hypermethylation results in a decreased expression of the protein (Esteller *et al.*, 2000).

The second step in the evolution towards intestinal cancer is acquisition of K-Ras mutations. This protein belongs to the low molecular weight GTPases and regulates cell growth, apoptosis, motility and differentiation. K-Ras activating point mutations occur in about 50% of human sporadic CRC cases (Malumbres and Barbacid, 2003). K-Ras mutations affect mostly specific codons (12-13,59-61) relevant to the

endogenous guanine triphosphatase activity, leading to the constitutive activation of the Ras/Raf/MEK/ERK signal transduction pathway (Cox and Der, 2002).

Further malignant progression towards the carcinoma stage involves loss of heterozygosity (LOH) of the chromosome 18q. In this region the tumor suppressor DCC and the transduction proteins SMAD4 and SMAD2 are located (Fearon *et al.*, 1990). SMAD2 and SMAD4 are pivotal proteins of the transforming growth factor- β (TGF- β) pathway which are involved in cell proliferation, differentiation, motility, adhesion or apoptosis (Massague, 2000). SMAD2 and SMAD4 mutations are usually found in CRC and are associated with its malignant progression (Fodde *et al.*, 2001; Riggins *et al.*, 1996). DCC (*deleted in colon cancer*) is a membrane protein involved in axon guidance but it is often linked to chromosomal instability and acts as a putative tumor suppressor (Cho and Fearon, 1995; Mueller *et al.*, 1998).

In most malignant cases, colorectal tumors are characterized by TP53 alterations. This tumor suppressor protein plays an important role in many cellular processes like DNA repair, apoptosis, cell cycle mutations, etc. TP53 mutations are closely associated with the transition from adenoma to carcinoma and such mutations occur in colorectal carcinomas (Baker *et al.*, 1989; Baker *et al.*, 1990).

1.2.3 Colorectal cancer chemoprevention

Pharmacological agents such as NSAIDs (non steroidal anti inflammatory drugs), e.g. aspirin and sulindac, as well as Coxibs (Cox inhibitors) have been intensively studied in CRC prevention. Nevertheless, dietary compounds with promising results in pre-clinical models have also attracted the interest of cancer prevention scientists.

In 1993, the CAPP-1 (Concerted Action Polyp Prevention) trial studied the preventive effects of aspirin in FAP patients; a significant reduction in the size of the largest recorded polyps was reported in patients who stayed in the trial for more than a year. Adverse effects were not described (Mathers *et al.*, 2003). A similar trial (CAPP-2) was designed for HNPCC patients but no inverse association between aspirin and colorectal adenomas was found. Moreover, almost no severe incidences were observed (Burn *et al.*, 2008).

Until now, two cohort and five case-control studies have been carried out with aspirin in sporadic CRC. Although aspirin reduced the risk of colon adenoma development,

gastrointestinal bleeding and gastric/duodenal ulcer have been described as adverse events. This is due to unspecific selectivity of aspirin which inhibits both Cox isoenzymes. Cox-1 is constitutive expressed and involved in gastrointestinal protection. Cox-2 on the other hand is an inducible protein related to inflammation and tumor promotion (Cuzick *et al.*, 2009). Other NSAIDs such sulindac showed unspecific Cox inhibition as well. Five small randomised trials have investigated sulindac in FAP patients; three described adenoma regression but the other two studies failed to find any effect. No specific adverse effects were indicated (Giardiello *et al.*, 2002; Herendeen and Lindley, 2003).

Celecoxib is more specific for Cox-2 inhibition than NSAIDs (Warner *et al.*, 1999). Therefore, Celecoxib was tested in 3 clinical trials. In a first randomised double-blind comparison of FAP patients, celecoxib caused a decrease in number of polyps and burden (Steinbach *et al.*, 2000). Further randomised double-blind comparisons in sporadic colorectal adenomas were the APC (*Adenoma Prevention with Celecoxib*) and the PreSAP (*Prevention of Sporadic Adenomatous Polyps*) trials. Both studies showed a reduced adenoma incidence but unexpected cardiovascular side effects were reported. Due to these adverse events, Celecoxib trials were suspended and the FDA (Food and Drug Administration in US) alerted for its increased risk of cardiovascular events (Arber, 2008).

Future clinical studies should focus on the examination of phytochemicals, which are considered generally to be of low hazard and well tolerated. Based on epidemiologic studies, dietary agents such as calcium, folate and polyphenols are now considered for clinical trials for CRC prevention.

Calcium supplementation has been studied as a promising CRC agent but contradictory results have been observed in both FAP patients and sporadic adenomas. Whereas colonic crypt fission was reduced in both FAP and sporadic cases, no significant changes were identified in terms of colonic mucosa proliferation in two large human studies. However, in two randomized double-blind comparisons, calcium supplementation suggested an inverse relation with adenoma recurrence in sporadic CRC patients (Sharma *et al.*, 2001). During 2003, a large double blind placebo controlled trial in patients with familial CRC cancer revealed a weak relation in adenoma recurrence. The authors indicated that no sufficient evidences are found to recommend calcium supplementation in CRC prevention (Weingarten *et al.*, 2005).

Likewise, epidemiologic and clinical studies indicate that dietary folate intake and blood folate levels might be inversely associated with sporadic colorectal cancer risk in terms of adenoma recurrence (Kim, 2003). During the last years, folate was supplemented in a large prospective cohort. Long term use resulted in a 30% CRC risk reduction. But contradictory results were obtained in a double-blind, placebo-controlled phase III trial which revealed that 1 mg folate per day had no effect to reduce the adenoma recurrence (Cole *et al.*, 2007).

Dietary polyphenolic compounds like curcumin have shown interesting results in a pharmacokinetic study. After 7 days treatment, Cox-2 and the M₁G DNA adduct (3-(2-deoxy-b-di-erythro-pentafuranosyl)-pyr[1,2-A]-purin-10(3H)one) were significantly reduced. Moreover curcumin was investigated in a small cohort of FAP patients. After 6 months, patients showed a reduction in polyp number and size. This could also be demonstrated in a similar study with quercetin (Cruz-Correa *et al.*, 2006). At the moment, other trials are recruiting patients for a phase II study of sporadic CRC with curcumin.

1.2.4 Animal models in colorectal cancer prevention

Animal models offer valuable preliminary data upon which human clinical trials can be based.

During the last years, several colorectal *in vivo* models have been developed. Among them, the carcinogen-injected azoxymethane (AOM) rodent model and the Apc^{Min/+} (Min) mouse model are the most common models in prevention studies for colorectal carcinogenesis studies (Femia and Caderni, 2008).

In the AOM model, dimethylhydrazine is metabolized to AOM and methylazoxymethanol (MAM). MAM is metabolised to methyldiazonium which can induce neoplastic transformation through DNA/RNA alkylation of colonic epithelial cells (Greene *et al.*, 1987). AOM-induced tumors are similar to human tumors in terms of histopathological characteristics. However, Apc mutations are rare, and they show lack of p53 mutations. Moreover, they hardly develop metastasis (Green and Hudson, 2005).

In contrast, Min mice hold a mutation in the Apc gene resulting in a protein truncated in position 850 (Moser *et al.*, 1990). This mouse homolog mutation also appears in patients with FAP. In addition, Apc716, Apc1309 and Apc1368 are other Apc mutated

mouse models generated depending on the mutation position. Similar to the human situation different mutations lead to diverse genotypes. For example, the Apc716 mouse develops 5-6 times more adenomas than Min mice (Oshima *et al.*, 1995).

1.3 Apc^{Min/+} (Min) mouse model

1.3.1 Wnt signaling pathway and targets

The Min mouse model holds an Apc punctual mutation leading to an inactive protein form. The Apc protein is involved in the Wnt signaling pathway, where a “Apc protein complex” regulates the canonical signal transduction (Fearhead *et al.*, 2001). The Apc mutation was firstly described in 1987 regardless to colon cancer. It is localized on the chromosome 5 (band 5q21-22) (Bodmer *et al.*, 1987). The human Apc gene has 8535 bp containing 21 exons and shares a 90% homology with the murine form. The most important regions of the Apc protein are the 15/20 amino acid region and the *armadillo* region. The first region allows β -catenin binding and the second contains multiple sites for phosphorylation (Fodde *et al.*, 2001). A schematic representation of the Wnt pathway can be observed in Fig. 4A and B.

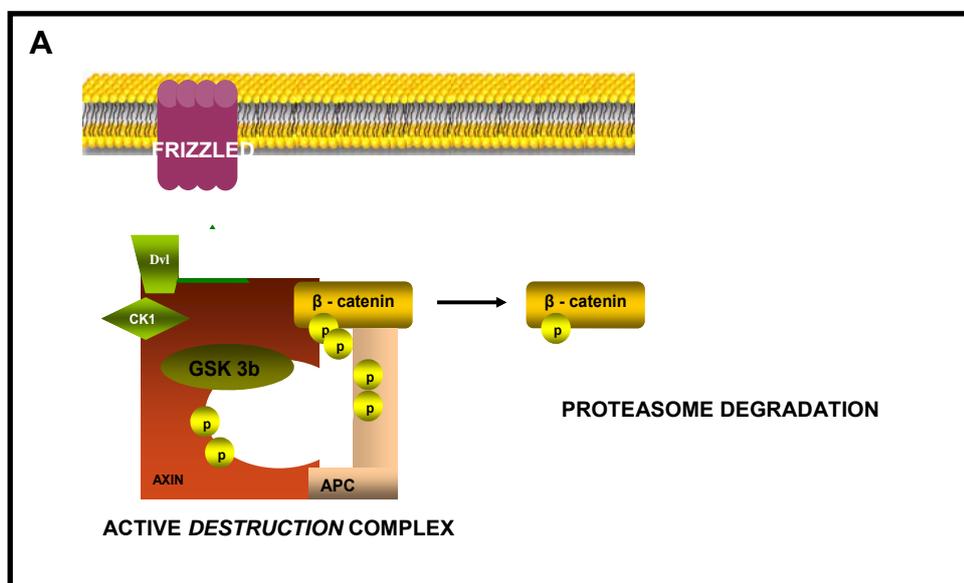


Fig. 4A. Schematic representation of Wnt signaling pathway. Without Wnt ligands the Apc complex remains active. DV1, *Disheveled-1*; CK1, Casein kinase-1; GSK3 β , Glycogen synthase kinase 3 beta; TCF/LEF, T-cell factor/Lymphoid enhancer-binding factor 1; Wnt, Wingless Int-1. Adapted from KEGGS database.

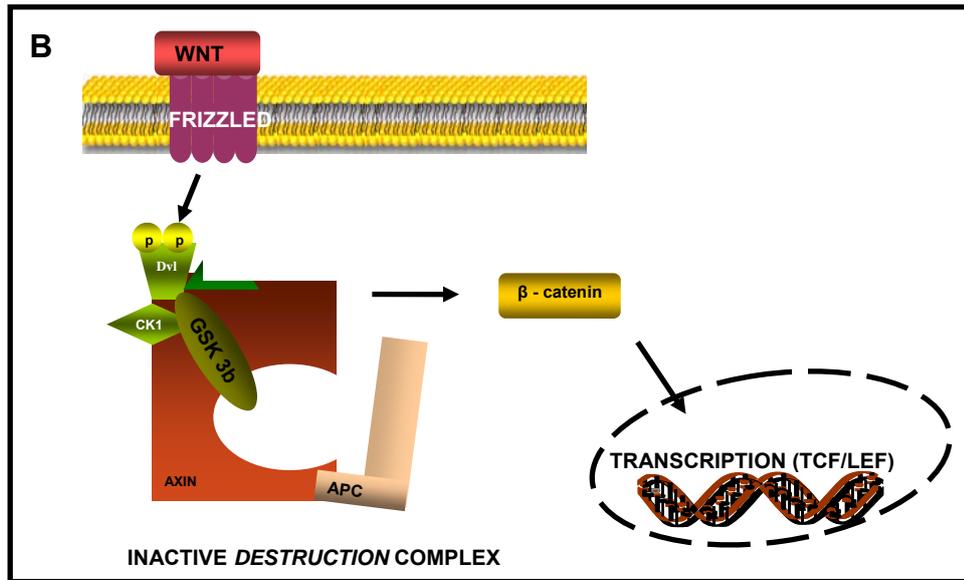


Fig. 4B. Schematic representation of Wnt signaling pathway. Wnt ligand binding inactivates the complex. DV1, *Disheveled-1*; CK1, Casein kinase-1; GSK3 β , Glycogen synthase kinase 3 beta; TCF/LEF, T-cell factor/Lymphoid enhancer-binding factor 1; Wnt, Wingless Int-1. Adapted from KEGGS database.

The canonical Wnt signaling pathway uses the Apc complex for the regulation of β -catenin target genes (Rubinfeld *et al.*, 1996). In the absence of Wnt ligands β -catenin is phosphorylated by GSK-3 β kinase and subsequently ubiquitinated. Finally, it is degraded by the proteasome (Orford *et al.*, 1997). In this situation, the Apc complex is active (Fig. 4 A).

In presence of Wnt ligands, the Frizzled receptor induces *Disheveled (DV1)* protein phosphorylation, including recruitment and inactivation of GSK-3 β kinase. Because β -catenin can not be phosphorylated, it is released and binds to DNA-binding proteins of the T-cell factor (TCF) family (Behrens *et al.*, 1996) (Fig.4 B). β -Catenin and TCF are the main cofactors of TCF-LEF (T-cell factor/ Lymphoid enhancer-binding factor 1). However, there are other important factors to activate specific target genes (Shitashige *et al.*, 2008). Different targets of the Wnt signaling pathway have been described. A representative list of TCF-LEF target genes is shown in Fig. 5.

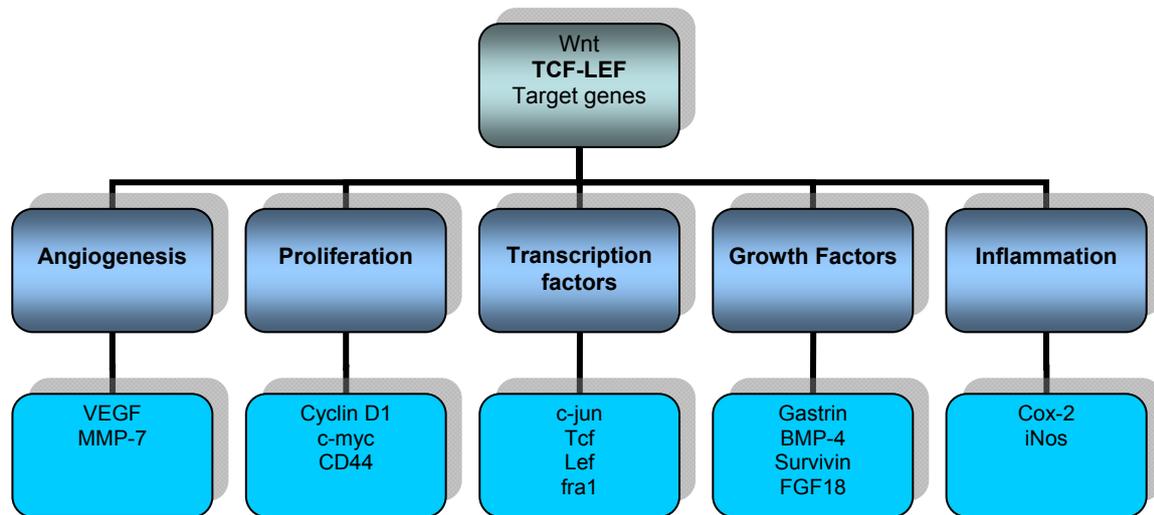


Fig. 5. Wnt target genes. Adapted from (Dihlmann and von Knebel Doeberitz, 2005)

In the Min mouse model, the Apc truncated protein results from a modification of thymine to an adenine nucleotide in codon 850. This short Apc protein is unable to maintain the Apc complex active, implying that β -catenin is always unphosphorylated and therefore released. Phenotypically, these mice will spontaneously develop adenomas in the SI and colon (Moser *et al.*, 1990).

1.3.2 Comparison between FAP and the Apc^{Min/+} mouse model

Based on the genetic similarity to FAP and many sporadic CRC, the Min mouse model is considered as a useful model for chemoprevention studies. When planning chemopreventive intervention studies in the Min mouse model one has to be aware of its limitations.

First studies using the Min mouse model proved that most of the adenomas were found in distal parts of the SI (small intestine), whereas in humans, tumors are principally found in the colon. This has been the principal critique to this model.

Recently, a histopathological study comparing tumors from FAP patients and from Min mice showed similarities in duodenal microadenomas. Because of these similarities the authors conclude that the Min mouse is a good model of FAP, in contrast to detractors (Preston *et al.*, 2008).

Also the occurrence of K-Ras and TP53 mutations differs between FAP and the Min mouse model. While K-Ras plays an important role in human CRCs there are no common K-Ras mutations in the Min mouse model (Shoemaker *et al.*, 1997).

Recently however, it has been reported that Min mice with Ras knockout developed increased numbers of intestinal tumors and had a decreased survival (van der Weyden *et al.*, 2008). Likewise, TP53 mutations which are common in human colonic adeno-carcinomas are not found in Min mice (Fazeli *et al.*, 1997).

Another controversy is the relation of PPAR γ in the human situation and the Min mouse model. Different effects have been observed with PPAR γ agonists. In human CRC cell lines such compounds induce growth inhibition linked with G₁ cell cycle delay (Sarraf *et al.*, 1998), whereas administration of PPAR γ ligands enhance polyp numbers in Min mice (Lefebvre *et al.*, 1999). Surprisingly, Apc mutation in combination with PPAR γ gene knockout enhanced tumor development (McAlpine *et al.*, 2006). This could be attributed to an increase in epithelial proliferation since similar results were obtained in Min mice with PPAR γ antagonists. Authors described an up regulation of Cyclin D1 and c-Myc genes (Fujisawa *et al.*, 2008).

Despite the critiques and controversies, the Min mouse model has been a suitable model for chemopreventive agents in pre-clinical evaluation.

1.3.3 Chemoprevention studies in the Min mouse model

Until the end of 2008, 294 different agents have been applied in Min mouse experiments including dietary and other chemopreventive compounds. This has been described in a systematic database of different Min mice experiments (Corpet and Pierre, 2003). The most commonly tested compounds have been Cox-2 inhibitors, NSAIDs, and dietary phytochemicals.

Cox-2 inhibitors can serve as a standard example of intervention studies using Min mice. Diet mixed with 500 ppm Celecoxib caused significant reductions in tumor multiplicity by 71%. Other pharmacological agents like NSAIDs showed similar inhibition efficiencies (Sulindac 160 ppm: 49% and piroxicam 200 ppm: 36%). Although aspirin showed a variation in polyp inhibition (24%-50%), this model seems to be satisfactory for the evaluation of such chemical agents.

In the database phytochemicals from dietary agents have been reported with different efficiency. Different percentages of adenoma reduction have been reported for plant phenolics: 0.1% curcumin (63%), 0.1 (+)-catechin: (71%) and 0.32 % Epigallocatechin-gallate (45%). Other agents such as resveratrol are still under

discussion due to contradictory results. Two studies have showed 24% and 70% tumor inhibition and another did not confirm this preventive effect (Sale *et al.*, 2005; Ziegler *et al.*, 2004). In addition, positive effects have been demonstrated for chemopreventive constituents of cruciferous vegetables: 0.06% Sulforaphane (50%) and 0.05 % PEITC (32%). Dietary fiber seems to be less effective than polyphenols in Min mice adenoma prevention: 7% White bran, 10% rye bran, 19% resistant starch and 6% fructo-oligosaccharides result in less than 12% tumor reduction. Moreover, 2.5 % inulin enhances tumor formation by 40%.

Tea is the most consumed beverage in the world aside from water. Green tea polyphenols have been extensively studied as cancer chemopreventive agent. A 0.08% and 0.16% green tea extract inhibited intestinal tumorigenesis in Min mice by 37% and 47%, respectively (Ju *et al.*, 2005). Further combination experiments with 80 ppm sulindac and 1.5% white tea provided greater tumor suppression than tea or sulindac treatment alone (Combination: 88%; white tea: 50%; sulindac: 50%) (Orner *et al.*, 2003).

1.3.4 β -catenin accumulated crypts as potential new biomarker

ACF lesions have been described as putative early preneoplastic lesions and have frequently been used for preclinical cancer chemoprevention studies. The number of ACF depends on the age of rodents, the doses of carcinogen and intervention time. In 5 week old rats treated with AOM (25 mg/ Kg BW), more than 200 ACF per colon can be identified. However, in the Min mouse model a lower frequency of ACF lesions are found. As an example, only 2-3 ACF per colon are found in 7 weeks old Min mice (Mori *et al.*, 2004). These lesions show an increased grade of dysplasia compared to AOM ACF lesions; thus they are referred as ACF_{Min}. ACF_{Min} are not elevated above the surrounding mucosa and its adequate identification depends on the homogeneity of blue methylene staining (Paulsen *et al.*, 2001). Overall, ACF_{Min} might not be satisfactory pre-neoplastic biomarkers in the colon.

Yamada *et al.* described a different type of lesions in the colon of Min mice which were designated as β -catenin accumulated crypts (BCAC). BCAC are regarded as independent lesions from ACF because they show excessive nuclear β -catenin staining. Moreover, BCAC have a higher grade of histological dysplasia and proliferative activity than ACF, thus these lesions are considered premalignant.

Approximately 10 BCAC per colon are found in 5 week old Min mice (Hata *et al.*, 2004; Yamada *et al.*, 2000). This represents an advantage compared to the lower number of adenomas in the colon of Min mice. The study of BCAC in the Min mice is interesting in order to focus on the question whether chemopreventive substances are still active in the colon and whether this situation could be compared to humans.

1.4 Health benefits of apples

1.4.1 Epidemiology facts.

Apples are the most important fruit produced in temperate climate regions of the world and play an important part in the U.S. diet. They are the second most popular fresh fruit consumed by Americans (USDA, US Dept. of Agriculture). The world apple production in the marketing year 2003/04 was estimated at 43 million tons (USDA, US Dept. of Agriculture). In Germany, apples are the most consumed fruits. During 2006, each family consumed a mean of 21.5 kg apples (ZMP Zentrale Markt- und Preisberichtsstelle GmbH; 2007).

Several studies have described the beneficial effects of apple products, especially those related to malignant diseases such as diabetes, cardiovascular diseases, obesity and cancer (Boyer and Liu, 2004).

Apple consumption and CRC risk has also been evaluated in epidemiological studies. The first study was performed in 1996. Deneo-Pellegrini observed a dose dependent CRC relative risk (RR, the risk of developing a disease) reduction (RR= 0.4; 95% CI= 0.25-0.66) in a case control study in Uruguay for people consuming 3 or more apples per week (Deneo-Pellegrini *et al.*, 1996).

During the last 5 years 3 epidemiological evaluations have been performed in Italy, USA and Scotland. In an Italian meta-analysis of case-control studies an inverse association between consumption of one or more apples/day and risk for colorectal cancer (OR= 0.8; 95 % CI = 0.71-0.90) was shown. Other cancers displayed an OR (odds ratio, ratio of the probability of occurrence of an event to the probability of the event not occurring) reduction as well (Gallus *et al.*, 2005). In the USA, evaluations from a large cohort study (Nurses' Health Studies) estimated a significant reduction in colorectal adenomas for the highest quintile of apple consumption (RR=0.83; 95 % CI=0.7-0.98) compared to the lowest quintile (Michels *et al.*, 2006). More recently, in

a case control study conducted in Scotland, significant reductions were reported in CRC risk in the highest quartiles of flavonols, quercetin, procyanidins and flavan-3-ols consumption. However, weaker evidence was shown when comparing highest versus lowest intakes of apples (OR=0.91; 95% CI=0.65-1.26) (Theodoratou *et al.*, 2007).

1.4.2 Apple juice components

Which components are responsible for the benefits? Apples constitute an important part of the human diet, as they are a source of dietary fibre, monomeric and dimeric carbohydrates, minerals and biologically active compounds, such as vitamin C (ascorbic acid) and certain phenolic compounds (Souci *et al.*, 2005).

Apples and apple juice (AJ) are rich sources of phytochemicals and a major contributor of flavonoids, antioxidants known for their protective effects. The content varies from 0.01 % to 0.1 % of the fresh weight (Heim *et al.*, 2002; Wojdylo *et al.*, 2008).

The most important polyphenols in apples are:

Phenol carbonic acids (1):

Chlorogenic acid (3-O-caffeoyl-D-quinic), and its isomers.

Flavonoids:

- FLAVAN-3-OLS (2):

Epicatechin, catechin and their polymeric structures.

- FLAVONOLS (3):

Quercetin and its mono- and diglycosides.

- DIHYDROCHALCONES (4):

Phloretin xyloglucoside and phloridzin.

The concentration of these phenolic compounds is strongly dependent on the variety of apples, maturity, area of cultivation and year of harvest (Alonso-Salces *et al.*, 2004; Guyot *et al.*, 2003).

Although part of the polyphenols may be lost during the AJ processing, procyanidins remain as the principal polyphenol class (Guyot *et al.*, 2003). Catechin oligomers,

known as oligomeric procyanidins (OPC) are the major compounds in apples and AJ. In apples, from 40% to 50% of the total polyphenols are procyanidins (monomers and dimers: 116-411 mg/kg and OPC: 388-1622 mg/kg). Moreover, procyanidins are also the main active compounds found in AJ and polyphenolic apples extracts. Industry commercializes clear AJ and cloudy apple juice (CAJ). While CAJ is a good source of procyanidins, clear AJ is very poor (Ozsmiansky, Wolniak et al. 2007). This has been also demonstrated analyzing CAJ extracts, which contain between 48-61% of the OPC. On the other hand, clear AJ extracts may contain similar total polyphenol amounts; although procyanidin content is lower (28-49%). They have the potential to exert strong anti-oxidative activity (Vanzani *et al.*, 2005).

Phenol carbonic acids are the second most abundant polyphenols in apples (45-384 mg/kg) and in CAJ (74 – 259 mg/l). These compounds are mainly found in the pulp and responsible for the characteristic acidic taste in some apple varieties and CAJ.

Amounts of flavonols (Apples: 116 – 411 mg/kg, CAJ: 46 – 124 mg/l) and dihydrochalcones (Apples: 20 – 155 mg/kg, CAJ: 14 – 87 mg/l) are also found in the apple peel. Both are involved in protection against the effects of solar radiation (Merzlyak *et al.*, 2008). Anthocyanins such as cyanidin-3-galactoside and other cyanidin glycosides are also found in red apples but in a low concentration (0-37 mg/kg) (Vrhovsek *et al.*, 2004). Calculated amounts were adapted according to a comprehensive review from Gerhauser (Gerhauser, 2008).

1.4.3 Chemopreventive potential of apples and apple compounds

Several *in vitro* and *in vivo* studies on apple polyphenols report mechanisms consistent with protection against colon cancer carcinogenesis. The chemopreventive potential is mainly due to polyphenols and pectin which can affect the overall process of carcinogenesis by several mechanisms.

Apple constituents and their chemopreventive effects have been reviewed recently (Gerhauser, 2008). Polyphenols are known to influence carcinogen metabolism. Pectins reduce the anti- mutagenic potential at the initiation stage and may induce DNA remethylation and histone deacetylases inhibition which may activate tumor suppressor genes.

Polyphenolic compounds such as OPC and quercetin conjugates are involved in anti-oxidative activities as scavenging O_2^- , OH^- and ROO^- radicals. Other known mechanisms in the promotion stage of carcinogenesis are anti-inflammatory effects such as interference with Cox-2 and NF- κ B. Furthermore, signal transduction pathways involved in tumor growth are another target of polyphenolic compounds. The most representative signalling pathways are MAPK and Wnt.

In the progression stage both, pectins and polyphenols are reported to block cell growth by inhibiting cell cycle-related proteins, they may even induce apoptosis.

All mentioned mechanisms have been discussed by Gerhäuser (Gerhauser, 2008).

1.4.3.1 *In vitro* CRC chemopreventive activity

At initiation stage, the modulation of Phase I and II carcinogen metabolism with apple polyphenols has been investigated in different *in vitro* systems. Inhibition of CYP1A1 activity by AJE has been reported by Pohl *et al.* In the Caco-2 cell line, a strong decrease was observed in Cyp1A at mRNA and protein expression and enzymatic activity by AJE treatment (Pohl *et al.*, 2006). In addition, detoxification enzymes like GSTs and UGTs were upregulated in LT 97 cell lines treated with polyphenolic apple extracts (PAE, 128 μ g/ml for 24 h). These cells were obtained from differentiated microadenomas which represents colonic preneoplastic lesions (Veeriah *et al.*, 2006).

Apple polyphenols have been shown to possess strong antioxidant activity *in vitro*, mainly associated with the phytochemical components rather than with the effect of vitamin C (Eberhardt *et al.*, 2000). In one study, when a reconstituted AJE (50-100 μ g/ml) mix was added to HT29 and Caco-2 cells, *tert*-butylhydroperoxide (TBH)-induced reactive oxygen species (ROS) levels were decreased after 24 h incubation. Modulation of menadione-induced DNA damage was mostly decreased in Caco-2 cells and less effected in HT-29. Quercetin conjugates, caffeic acid and chlorogenic acid were identified to be responsible for the preventive potential (Schaefer *et al.*, 2006). Other studies using the Comet assay method demonstrated that phenolic compounds from apple pomace (leftover material after juice extraction) decreased DNA damage in HT29 cells (McCann *et al.*, 2007).

The anti-inflammatory effects of apple polyphenols have also been investigated. Apple extracts inhibited nuclear factor kappaB (NF- κ B) in MCF-7 breast cancer cells

and human umbilical vein endothelial cells. NF- κ B is a transcription factor involved in the activation of inflammatory proteins, e.g. Cox-2 and iNOS (Davis *et al.*, 2006; Yoon and Liu, 2007). Moreover, epicatechin, phloretin and OPC inhibited Cox-1 activity from sheep seminal vesicle microsomes in a dose-dependent manner (Zessner *et al.*, 2008).

Fridrich *et al.* have described cell proliferation inhibition when HT29 cells were incubated with a polyphenolic apple extract. Furthermore, protein tyrosine kinase activity of the EGFR signalling pathway and PKC activity were effectively inhibited by the polyphenolic apple extract (Fridrich *et al.*, 2007b). Similarly, inhibition of cell growth was observed by a mix containing 78% polymeric procyanidins in the SW620 human metastatic colon carcinoma ($IC_{50} = 45 \mu\text{g/ml}$) (Gosse *et al.*, 2005). However, this can also be attributed to short chain fatty acids (SCFA) which are fermented from fiber and some polyphenols. These compounds have been described to inhibit cell proliferation *via* inhibition of histone deacetylases and induction of cell differentiation (Davis *et al.*, 2006).

At the progression stage, colon cancer preventive strategies include mechanisms to induce apoptosis. Polyphenolic apple extract induced apoptosis in HT29 cells with activation of caspase 3 and DNA fragmentation by 2 mg/ml PAEt (Kern *et al.*, 2007). *In vitro* experiments in B16 mouse melanoma cells and mouse mammary tumor cells revealed that apple procyanidins increased mitochondrial membrane permeability and cytochrome c release. In addition, activation of caspase-3 and caspase-9 was identified (Miura *et al.*, 2008). Also, SCFA are related to apoptosis induction (Sengupta *et al.*, 2006).

1.4.3.2 Colon cancer chemopreventive efficacy *in vivo*

Not many animal experiments using apple juice or apple polyphenols have been performed until now and most of them have focused on cancer prevention. Nevertheless, experiments related to neural and cardiovascular disorders have also been conducted (Aprikian *et al.*, 2002).

Several studies have focused on colon cancer prevention potential. In one study of AOM-induced aberrant crypt foci, rats received 0.01% of a procyanidin mixture (74.8 % procyanidins) in water. Hyperproliferative crypts and ACFs were 50% reduced in comparison with the water control (Gosse *et al.*, 2005).

In the rat model for chemically-induced colon carcinogenesis using DMH, Barth *et al.* compared treatments with clear AJ and CAJ. Although both drinks contained similar polyphenol amounts, CAJ pectin levels were 3 times higher. CAJ suppressed DMH induced genotoxic damage in mucosa cells of the distal colon. Both treatments reduced in similar manner the crypt cell proliferation index, but only CAJ reduced ACF (Barth *et al.*, 2005). In a second study, conducted to determine which fraction was causing these effects, CAJ was fractionated in a total polyphenol fraction and a cloud particle fraction consisting of proteins, fatty acids, polyphenols, and cell wall polysaccharides. As in the first experiment, CAJ results reduced ACF numbers but each fraction failed to reduce genotoxicity in colonocytes and ACF lesions. In contrast, crypt cell proliferation index was statistically significant reduced by CAJ, the total polyphenol fraction and the cloud particle fraction. Authors argued that the chemopreventive potential may be attributed to a combination effect of both fractions (Barth *et al.*, 2007).

Apple pectin has also been investigated for its chemopreventive potential. It is found at a range of 1-1.5% in apples. A study in Japan using the AOM-induced rat model showed a reduction in the multiplicity of colon tumors where diets were supplemented with 20% of either apple or citrus pectin. Only animals fed with apple pectin had a diminished tumor number (Ohkami *et al.*, 1995). Results were reproducible in a second animal experiment with the same set up. Similar results in terms of tumor number and incidence were observed. In addition, animals fed with 20% apple pectin had significantly reduced PGE₂ levels in distal colon mucosa (Tazawa *et al.*, 1997). This heteropolysaccharide acts as an anti-mutagen due to its ability to scavenge mutagens and to reduce mutagen concentration as a result of the increased fecal bulk (Gerhauser, 2008).

Other apple compounds like carbohydrates have been studied in Min mice. Enriched diets with 20% apple pomace were used as a source of resistant carbohydrates. After intervention, the treatment increased the mass of the SI. Moreover, the distal SI parts and colon showed increased cell proliferation and the crypt fission. Significantly, polyp number and polyp burden were augmented in all parts of SI. In the colon, polyp diameter and tumor burden was increased as well (Mandir *et al.*, 2008). Underlying mechanisms are not completely understood so far, but degradation of carbohydrates to short chain fatty acids and a subsequent increase in cell proliferation has been discussed.

Overall, pectin, fiber and polyphenols are the apple compounds mostly responsible for effects on the whole process of carcinogenesis.

1.5 Bioavailability

Biological efficacy *in vivo* is dependent on the bioavailability of dietary components, except for the gastrointestinal tract where local effects may occur.

Bioavailability is defined operationally and pharmacologically as the proportion of the compound administered orally that is absorbed from the GI tract. This represents the proportion of the compound that appears in plasma over time. The biological effects of polyphenols depend on their bioavailability and their chemical structure. This determines their rate and level of intestinal assimilation, and the nature of the metabolites circulating in the plasma (Scalbert and Williamson, 2000). The absorption of flavonoids in the intestine may be limited by the transport of flavonoids or their metabolites back to the intestinal lumen by ABC transporters (Walle *et al.*, 1999). A general overview of the bioavailability of polyphenols is showed in Fig. 6.

Usually, in the intestine many flavonoid aglycones tend to pass through epithelial cells where they are further conjugated by methylation, sulfation or glucuronidation (Day *et al.*, 2001; Spencer *et al.*, 2004) (Fig. 6, A1-A2).

In the SI, flavonoid glycosides can be deglycosylated by β -glucosidases including lactase phlorizin hydrolase which has specific activity towards flavonoid glycosides. The resulting flavonoid aglycon can be absorbed *via* passive diffusion through the enterocytes and also be conjugated (Crespy *et al.*, 2001) (Fig. 6, B). However, the sodium-dependent glucose transporter-1 (SGLT-1) might be implicated in the absorption of flavonoid glycosides. Flavonoids can be deglycosylated within the cell by cytosolic β -glucosidase and further conjugated (Walgren *et al.*, 2000) (Fig. 6, C1-C2).

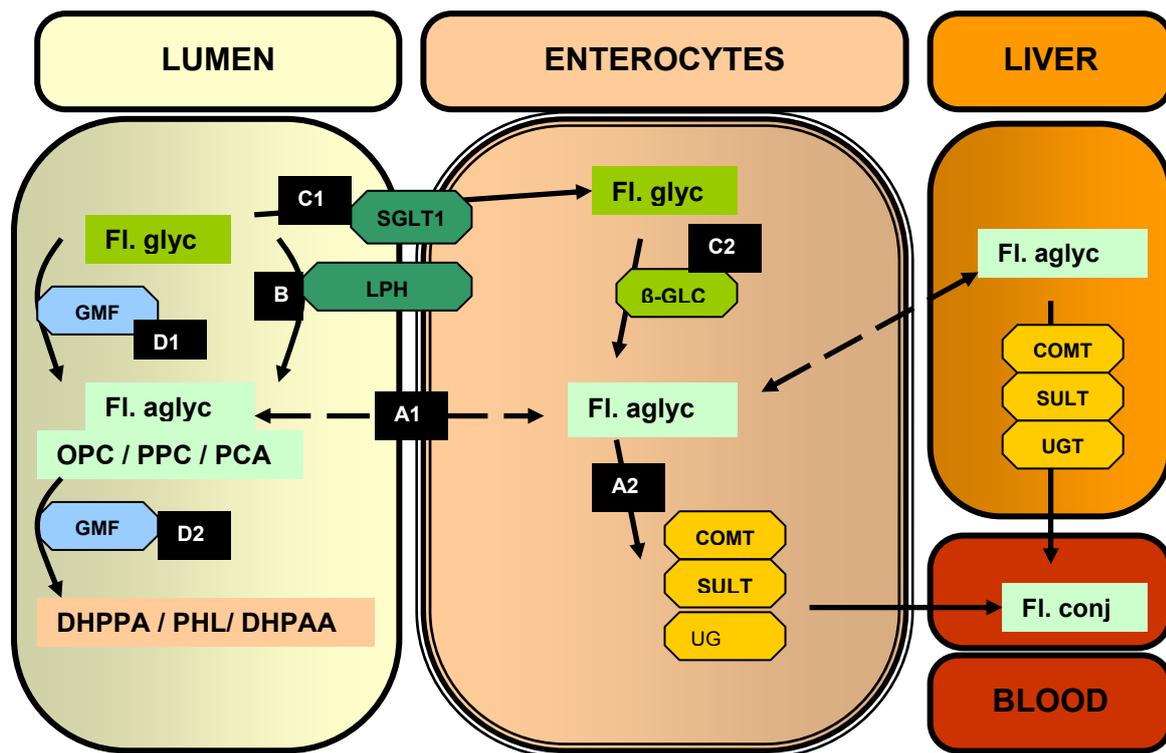


Fig. 6. Schematic representation of absorption, transport and metabolism of polyphenols.

Flav. aglyc., Flavonoid aglycones; Fl. glyc, Flavonoid glycosides; OPC, oligomeric proyanidins; PPC, polymeric procyanidins; PCA, Phenol carbonic acids; GMF, gut microflora; DHPPA, 3,4-dihydroxyphenyl propionic acid; PHL, phloroglucinol; DHPAA, 3,4-dihydroxyphenyl acetic acid; SGLT1, sodium-dependent glucose transporter 1; LPH, lactate phloridzin hydrolase; β -GLC, β -glucosidase; COMT, catecholamine orthomethyl transferase; UGT, UDP-glucuronyltransferase, SULF sulphotransferase; Fl. conj, conjugated flavonoid. Dashed lines indicate passive diffusion. Different absorption pathways: (A1-A2) Passive diffusion and conjugation of flavonoid aglycones, (B) Flavonoid glycosides can be hydrolyzed by LPH, (C1) transport of some flavonoid glycosides through the membrane by SGLT-1 and (C2) hydrolysis *via* β -GLC to release free flavonoid, (D) Effects of gut microflora. Schema adapted (Scalbert and Williamson, 2000).

Most phenol carbonic acids like chlorogenic acid cannot be absorbed in the SI due to the lack of esterase activities. But they can be hydrolyzed and metabolized by the gut microflora (Couteau *et al.*, 2001). Human intervention studies showed that half of the ingested chlorogenic acid is converted to hippuric acid in the colon, most likely by microbial metabolism (Olthof *et al.*, 2003) (Fig. 6, D1-D2).

Procyanidins have also been investigated but most experiments have been performed *in vitro*. Because of their molecular size, procyanidins are likely not as easily absorbed in the SI. Experiments on the *in vitro* absorption through a cell monolayer derived from Caco-2 cells indicated that radiolabeled procyanidin

monomer, dimer and trimer were absorbed in contrast to procyanidin polymers having an average degree of polymerisation of 7 (Deprez *et al.*, 2001). The same group reported that procyanidin polymers can be degraded by the colonic microflora *in vitro* into low-molecular-weight aromatic acids. The resulting metabolites might well be absorbed *in vivo* through the colon (Deprez *et al.*, 2000).

1.6 Aims of the project

Colorectal cancer (CRC) is the second most common cancer in the world. It is particularly common in western countries and its incidence is strongly associated with certain dietary patterns. Prevention strategies are required to reduce CRC risk in the population.

We previously demonstrated that the chemopreventive potential of apple polyphenolic extracts by several *in vitro* assays. Apple procyanidin extracts contained the strongest activities of all apple polyphenols.

The general aim of the present study was to characterize *in vivo* molecular mechanism underlying the chemopreventive effects of cloudy apple juice (CAJ) and polyphenolic apple extracts (PAE).

1- In our earlier experiments, CAJ and PAE intervention in Min mice significantly reduced adenomas numbers in comparison to water control. However, the mechanism remained unclear. In order to determine the possible mechanism involved in the transition from normal tissue to adenomas, gene expression patterns of Wnt signalling target genes were measured in normal mucosa in animals treated with CAJ and PAE in comparison with water control.

2- The previous *in vitro* experiments have identified procyanidins as strong chemopreventive compounds in CRC prevention. In the present work, a second Min mice experiment was conducted to identify which compounds might be responsible for the preventive effects.

Five treatments were tested, including CAJ, Placebo (AJ without polyphenols), B-PAE (procyanidin-rich extract), the combination of CAJ + B-PAE and water. From this animal experiment, adenomas were collected to study known signalling pathways

related with adenoma progression including: 1) phosphorylated proteins in the MAPK signalling pathway, 2) Wnt signalling pathway and target genes, and 3) proliferation markers. These experiments were performed using proteomic technology (Reverse phase protein arrays).

3- The previous *in vitro* experiments have suggested that PAEs might play a role in drug metabolism and have anti-inflammatory potential. To confirm them *in vivo*, in the present work, livers from the second animal experiment were used to measure activities of phase I and phase II enzymes, which are related with the initiation stage of carcinogenesis. Also, biomarkers such as PGE₂ in plasma and protein and NO in urine were measured to determine possible anti-inflammatory potentials.

2 MATERIALS AND METHODS

Chemicals

Acrylamide/Bisacrylamide	Roth, Karlsruhe
Agarose	Sigma, Steinheim
Ammonium persulfate	Roth, Karlsruhe
Boric Acid	Sigma, Steinheim
Bromophenol blue	Serva, Heidelberg
Bicinchoninic acid	Sigma, Steinheim
BSA protein standard	Sigma, Steinheim
BSA	Sigma, Steinheim
Dyethylenpyrocarbonate	Sigma, Steinheim
Dithiothreitol	Sigma, Steinheim
DMSO	Merck, Darsmtadt
Di-potassium hydrogen phosphate	Merck, Darmstadt
Ethanol	Sigma, Steinheim
Ethidiumbromide	Gibco, BLR. Eggenstein
Ethylacetate	Sigma, Steinheim
EDTA	Sigma, Steinheim
Formaldehyde	Sigma, Steinheim
Glycerol	Merck, Darmstadt
Glycine	Roth, Karlsruhe
Hexane	Sigma, Steinheim
Methanol	Karl Roth, Karlsruhe
3-(N-Morpholino)-propanesulphonic acid	Sigma, Steinheim
Perchloric-acetic acid	Calbiochem, San Diego, USA
Phosphoric acid	Sigma, Steinheim
Potassium Chloride	Merck, Darsmtadt
Potassium dihydrogen phosphate	Roth, Karlsruhe
2-Propanol	Sigma, Steinheim
Sodium dodecyl sulfate	Sigma, Steinheim
Sodium azide	Merck, Darmstadt
Sodium Chloride	Sigma, Deisenhofen

TEMED	Roth, Karlsruhe
Triton X-100	Sigma, Steinheim
Tris-Base	Roth, Karlsruhe
Tris-HCl	Roth, Karlsruhe
Tween-20	Sigma, Steinheim
Odyssey Blocking Buffer	Li-Cor, Bad Homburg
RNAse Zap	Ambion, Austin, USA
Urea	Sigma, Steinheim

Instrumentation

***In vitro* assays:**

SpectraMax 340 PC (Molecular Devices, Munich)

Fluorimeter Cytoflour 4000 (Perseptive Biosystems, Framingham, MA, USA)

Fluorimeter SpectraMax Gemini XS (Molecular Devices, Munich)

Saur Oxygen electrode (Saur, Reutlingen)

Liver enzymatic assays:

Ultraturrax homogenizer (IKA Labor Technik, Staufen)

Avanti Bioprocessing Centrifuge System (Beckman, Fullerton, USA)

Beckman Ultracentrifuge (Beckman, Fullerton, USA)

Animal dissection:

Heparinised capillaries (In vitro Diagnosticum, Berlin)

Hematocrite centrifuge, M 1101 (Compur, Munich)

Dissection microscope (Olympus, Planegg)

20x gauge needle (BD, Heidelberg)

RNA, DNA extraction and Polymerase Chain Reaction:

Micro-dismembrator (Sartorius, Göttingen, Germany)

Teflon containers (Sartorius, Göttingen, Germany)

RNA lysis buffer (Qiagen, Hilden, Germany)

RNeasy Qiagen kit (Qiagen, Hilden, Germany)

DNase assay kit (Qiagen, Hilden, Germany)

Gel Electrophoresis Chamber (Bio-Rad, München)
Electrophoresis power supplier Power Pac (Bio-Rad, München)
Biofuge centrifuge (Heraeus, Hanau, Germany)
Eppendorf spectrophotometer (Eppendorf, Hamburg, Germany)
Eppendorf PCR Thermocycler (Eppendorf, Hamburg, Germany)
Real time PCR, iCycler IQ5 (Bio-Rad, Munich)
Quartz cuvette (Steinbrenner, Wisenbach, Germany)
RNAse free water (Qiagen, Hilden, Germany)

Immunohistochemistry:

Tissue embedding system (Sakura Finnetek GmbH, Staufen)
Slide staining machine (Sakura Finnetek GmbH, Staufen)
Microtome RM 22 35 (Leica, Bensheim)
Embedding cassettes (Fischer Scientific, Houston, USA)
AxioSkop 2 MOT (Carl Zeiss, Göttingen)
Axiovision 4.0 (Carl Zeiss, Göttingen)

Infrared-based protein detection arrays with quantitative read out (IPAQ):

Odyssey fluorescence scanner (LI-COR, Lincoln, USA)
Qiagen dismembrator (Qiagen, Hilden, Germany)
3 mm stainless steel balls (Qiagen, Hilden, Germany)
Rocking shaker (Thermo-Fisher Waltmann, WA, USA)
Nitrocellulose membranes (Grace Bio-labs, Bend, OR, USA)
Piezo spotter (Biochip-Arrayer, Whaltman, MA, USA)
Genepix (Molecular devices, Munich, Germany)
Protein Mini-gel Wet Transfer System (Bio-Rad, Munich, Germany)
SDS-PAGE electrophoresis equipment (Bio-Rad, Munich, Germany)

PGE₂ ELISA:

Sucking Pump (Sartorius, Göttingen, Germany)
Scintillation counter PE2000 (Perkin Elmer, Whaltman, MA, USA)

2.1 Description of apple products for intervention

In collaboration with Dr. Will and Ms. Melanie Olk (Forschungsanstalt Geisenheim, Dept. of Wine and Drink research) a cloudy apple juice and polyphenolic enriched extracts were obtained. Apple (*Malus domestica*) varieties used for the CAJ06 and extracts are summarized in Tab. 3.

Tab. 3. Apple varieties used for CAJ06 and B-extracts.

CAJ 06	AE03-B	AE06-B
<i>Bohnapfel</i>	<i>Melrose</i>	<i>Bittenfelder</i>
<i>Maunzen</i>	<i>Granny Smith</i>	
<i>Winterrambour</i>	<i>Golden Delicious</i>	
<i>Tafelobst</i>	<i>Jonagold</i>	

CAJ06

For the preparation of CAJ06, 1000 kg of mixed varieties of cider and dessert apples (Tab. 3) were mashed with a cutting mill (Seepex). The mash was then squeezed with a horizontal press (Bucher HP-L 200). The juice is referred to as A-juice (A-CAJ). After centrifugation (Westfalia Tellerseparator SAR 3036), A-CAJ was shortly heated (85 °C, 30-40 s) and lastly stored in stainless steel tanks. The CAJ was filled in 0.75 l bottles and kept at 4 °C.

A clear apple juice (AS06-clear) was produced from the same harvest. CAJ06 was shortly incubated with an enzyme mix (50 mL/t Fructozym P, Erbslöh Geisenheim) then finned (10 g/hl Gelatin, 40 ml/hl Kieselsool, and 50 g/hl Bentonit, Erbslöh) and in the end separated and cross-flow filtered (Seitz, Mikro).

Placebo Juice

Placebo juice was obtained from the AS06-clear. Initially, a glass-chromatography column (Pilot 750x200 mm, Fa. Kronlab/YMC) was prepared with adsorbent SP70 resin. The column was firstly washed with distilled water and then AS06-clear was added to remove all polyphenolic compounds. The polyphenol-free eluate was filled up in 0.75 l bottles and kept at 4 °C. Placebo juice and CAJ have similar

characteristics except in polyphenolic content (CAJ: 452 mg/L), ascorbic acid and colloids. Such drinks contain always high amounts of potassium (Tab. 4).

Tab. 4. Comparison between CAJ06 and Placebo juice.

	Cloudy AJ 06	Placebo 06
Glucose g/l	18.9	23.7
Fructose g/l	69.2	71.4
Saccharose g/l	21.7	16.9
Sorbitol g/l	4.7	4.6
Polyphenols mg/l	452	0
Ascorbic acid mg/l	47	10.6
Na mg/l	8	7
Mg mg/l	51	48
Ca mg/l	49	49
K mg/l	1145	1146
Colloids mg/l	584	120

Data obtained from Dr. Will and Ms. Melanie Olk.

B-PAE06

For this extract, a lyophilized apple extract was obtained from apple pomace. 1200 Kg of *Bittenfelder* apples were processed with a Seepex cutting mill and processed as described above. The pomace was mixed 1:2 with demineralized water at 95 °C. This mix allows to set an optimal enzyme temperature (54°C). Afterwards, an enzymatic digestion was carried out for 2 h (pectinases and cellulases, 300 g/t, Erbslöh). After this step, the mixture (referred as B-juice) was decanted. Due to the higher amounts of pectin a second pectinase digestion was performed. The Polyphenols from 200 l of B-AS06 were retained on 15 l of adsorber resin SP 70 (Resindion, Italy) and packed into a chromatography column (Pilot 750x200 mm, Fa. Kronlab/YMC, Germany). Water-soluble constituents were removed with 50 l distilled water. Afterwards, polyphenolic compounds were eluted with 25 ml of 96% ethanol. The polyphenolic eluate was concentrated by evaporation using a rotary evaporator,

reconstituted in double-distilled water, and subsequently freeze-dried to 600 g yield extract.

B-PAE03

The production of this extract was similar to that of the AE06-B. However, 1200 kg from a mixture of different dessert apples was used. The polyphenols of 550 l from B-AS03 were extracted using a 55 l resin adsorbent P-495 (Bucher Alimentech, Auckland, New Zealand) in a Bucher-adsorber. Carbohydrates, organic acids and minerals were removed with 200 L distilled water. Elution was completed using 50 l 96% technical ethanol and the alcoholic extract was freeze dried in a polar phase. A yield of 260 g was obtained.

The quantitative determination of apple polyphenolic compounds was performed in 1 g/l diluted in 10% methanol (in water) using a 1090 HPLC/DAD system (Hewlett-Packard, Germany) prepared with 25 x 4.6 mm Aqua 5 µm C18 column and with a 4x3 mm C18 ODS pre-column (Phenomenex, Germany). Elution was performed in acetonitrile and 2 % acetic acid. Detection wavelengths were: 280 nm for flavonols and dihydrochalcones, 320 nm for phenol carbonic acids and 360 nm for quercetin derivatives. A summary of the polyphenol composition is shown in Tab. 5.

In CAJ, chlorogenic acid was identified as the major low molecular weight (LMW) component. Due to difficulties in analyzing oligomeric and polymeric procyanidins only LMW polyphenols were determined by CAJ.

Tab. 5. Comparison between B-PAE (mg/g) and CAJ (mg/l).

Low molecular weight polyphenol composition.

Polyphenols		mg/l	mg/g	mg/g
Sample-Name	Classification	CAJ06	B-PAE03	B-PAE06
Cumaroyl glucose		0.1	0.00	0.20
Chlorogenic acid		177.8	19.20	76.55
Cryptochlorogenic acid		5.3	3.00	5.02
Caffeic acid	(1)	0.2	4.00	0.69
3-Cumaroyl-China acid		4.5	3.00	0.70
5-Cumaroyl-China acid		5.5	5.50	0.16
4-Cumaroyl-China acid		41.8	3.80	52.13
Cumaric acid		0.4	4.20	0.57
Procyanidin B1		8.5	6.20	2.37
Catechin		6.9	2.70	4.25
Procyanidin B2	(2)	71.2	18.40	17.09
Epicatechin		36.3	17.70	14.27
Procyanidin C1		35.8	n.d.	13.55
Phloretin-2'-xyloglucoside		40.9	31.70	9.63
Dihydrochalcones	(3)	4.5	n.d.	0.68
Phlorizin		9.0	78.90	24.37
Quercetin-3-galactoside		1.3	8.10	2.82
Quercetin-3-glucoside		0.6	12.30	0.62
Quercetin-3-xyloside	(4)	0.3	18.10	1.35
Quercetin-3-arabinoside		0.4	3.50	0.00
Quercetin-3-rhamnoside		1.5	25.10	0.92
TOTAL		452.8	310.0	227.94

(1) Phenol carbonic acids (2) Flavan-3-ols (3) Dyhydrochalcones (4) Flavonols

Data obtained from Dr. Will and Ms. Melanie Olk

B-PAE03 was obtained from different apple mixes during 2003 and B-PAE06 was acquired from a single apple variety during 2006. Both B-PAE differ in composition and distribution. The HPLC analysis revealed less low molecular weight (LMW) polyphenol amounts for B-PAE06 in comparison to B-PAE03 (B-PAE03: 310 mg/l and B-PAE06: 227 mg/l). The B-PAE03 contained more flavonols and dihydrochalcones but less phenolcarboxylic acids. Similar amount of monomeric and dimeric procyanidins were found. CAJ06 contained more LMW compounds compared with both B-PAEs (CAJ: 452.8 mg/l). However, 50% of the present polyphenols were phenol carbonic acids. CAJ showed considerable amount of

monomeric and dimeric procyanidins as well as dyhydrochalcones. Lower concentrations of flavonols were identified (Tab. 5).

Since the polyphenolic B-PAE06 contents were different in comparison to the B-PAE03, 0.2% was prepared as a mixture of the two different extracts.

2.2 *In vitro* chemopreventive characterisation

(Under the supervision of Ms.Karin Klimo and Dr. Clarissa Gerhäuser)

All extracts were previously characterized in a battery of cell- and enzyme-based *in vitro* marker systems which include modulation of drug metabolism, anti-oxidant, radical-scavenging, anti-inflammatory and anti-tumor promoting activities. A short description of the assays is described in Tab. 6 (Gerhauser *et al.*, 1997).

Tab. 6 *In vitro* chemopreventive assays, mechanisms and principles (adapted from Gerhäuser *et. al.*, 2003)

ASSAY	MECHANISM MEASURED	PRINCIPLE	REFERENCE
DPPH	Radical scavenging potential	Photometric measure of free radical generation with DPPH reaction	(van Amsterdam, Roveri <i>et al.</i> 1992)
ORAC	Peroxy- or hydroxyl- radical abs. capacity	Induction of peroxy radical with AAPH using fluorescein as redox sensitive indicator molecule	(Cao and Prior 1999)
HXO/XO	Superoxide anion radical formation	Radical scavenging quantified by the rate reduction of XTT forming an orange formazan.	(Ukeda, Maeda <i>et al.</i> 1997)
Cyp1a1	Phase I enzyme induction	Dealkylation of CEC to fluorescent CHC	(Crespi, Miller <i>et al.</i> 1997)
QR	Carcinogen detoxification	NADPH-dependent menadiol-mediated reduction of MMT to a blue formazan	(Gerhäuser, You <i>et al.</i> 1997)
Aromatase	Anti-estrogenic activity	Aromatase mediated dealkylation of DBF substrate to a fluorescein monobenzylester	(Stresser, Turner <i>et al.</i> 2000)
COX-1	Anti-inflammatory activity	Monitoring oxygen consumption during conversion of AA to PGs	(Jang, Cai <i>et al.</i> 1997)

2.3 Min mouse intervention study

2.3.1 Intervention

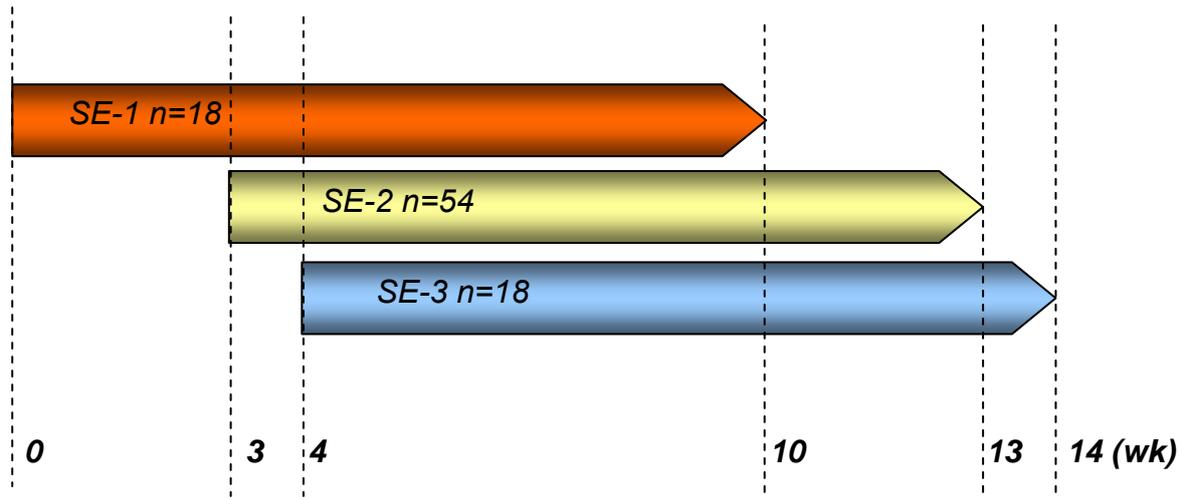
A total of 90 6-7 weeks old male C57BL/6J-Apc^{Min/+} mice were obtained from the Jackson Laboratory (Bar Harbor, ME, USA). The animal protocol (35-9185.81/G-134/06) was approved by the animal committee at the regional council in Karlsruhe.

Animals were randomly divided (with comparable initial body weights) in groups of 6. All groups were maintained of a controlled temperature and humidity environment with a 12-h light/dark cycle. Animals were provided free access to drinks and food but a special soy free diet was given to avoid a possible influence of soy compounds. After 5 days of adaptation, the intervention started for 10 weeks (Fig. 7 and 8). Five different interventions were compared the animal experiment. The animals received either tap water as a control, placebo juice, CAJ06, CAJ06 plus 0.2 % B-PAE or 0.2% B-PAE *ad libitum*, respectively. Liquid uptake was monitored daily and food uptake twice per week; moreover, all animals were weighed twice per week. Animals appearing sick were sacrificed immediately.

Since not all animals could be delivered a once, the intervention study was described in to three sub-experiments (SE) running in parallel (Fig. 8).



Fig. 7. Apc^{Min/+} intervention scheme (A: adaptation time).



Sub-experiment (SE) 1 = three intervention groups à 6 animals: Placebo, CAJ and CAJ + B-PAE

Sub-experiment 2 = nine intervention groups à 6 animals: 2x Water, 2x 0.2% B-PAE, 2x Placebo, 2x CAJ and 2x CAJ + B-PAE.

Sub-experiment 3 = three intervention groups à 6 animals: CAJ + B-PAE, Water and 0.2% B-PAE.

Fig. 8. Description of sub-experiments.

2.3.2 Dissection

Solutions and reagents:

PBS cold

Phos-STOP phosphatases inhibitors (Roche, Mannheim)

Complete proteases inhibitors (Roche, Mannheim)

Heparin (Sigma, Steinheim)

Indomethacine (Sigma, Steinheim)

70% Ethanol p.a.

PBS-buffered Formalin 4 %:

40 ml Formalin

4.4 g Na₂HPO₄ · 2H₂O

25.9 g Na₂HPO₄ · 12H₂O

Filling up to 1000 ml Millipore water

Mice were weighed before being euthanized. Animals were anaesthetised by CO₂ inhalation and sacrificed by heart puncture. Blood was extracted with a heparinised 20x needle. The hematocrit was determined using a hematocrit centrifuge with heparinised capillaries and centrifugation at 10.000 rpm for 10 min. Blood was splitted into two tubes, one with 10 Units of heparine for subsequent determination of antioxidant capacity (ORAC) and another with 30 µl 100 µM indomethacine for subsequent PGE₂ elisa assay. Both tubes were centrifuged at 10.000 rpm for 10 min. Serum samples were stored at - 80 °C until analysis.

Initially, the spleen and the liver were taken and directly weighed. Spleens were completely fixed in 4% PBS-formalin. Livers were cut in 2 pieces and one part was shock-frozen in liquid N₂ and the other fixed in PBS-formalin. SI was completely removed, washed with ice-cold PBS, GI tract was divided in 3 parts (*duodenum*, *jejunum* and *ileum*). Colons were prepared according to the BCAC protocol (Hata *et al.*, 2006) (compare to chapter 2.3.7). Each piece was opened longitudinally and flattened on paraffin section table and properly washed. Faecal rests and *cecum* were removed. During the tissue dissection remainders of fatty and mesenteric tissue were removed and discarded.

Each treatment was divided in to 3 groups (Groups; G1, G2 and G3; each n=30) and different experiments were planned. Fig. 9 illustrates a detailed plan.

Fig. 9. Experiment planning scheme. Each treatment was divided in to 3 groups (G1, G2 and G3). BCAC: b-catenin accumulated crypts. See text for description

Groups	Spleen Pathology	Liver pathology	Liver enz. assay	Swiss Rolls	Adenoma Prot. Exp.	Colon BCAC
G1	✓	✓	✓	✓	✗	✗
G2	✓	✓	✓	✓	✗	✓
G3	✗	✗	✗	✗	✓	✗

Histopathologic examination (G1 and G2)

Spleen and liver sections were formalin-fixed, paraffin-embedded cut into 3 μm slices and stained H&E. The histopathological examination was performed by Prof. Karbe (Wülfrath).

Liver enzymatic assays (G1 and G2)

Livers were removed and divide in 2 parts. One was snap-frozen in liquid N_2 and stored at -80°C before preparation of cytosolic and microsomal liver fractions.

Swiss rolls (G1 and G2)

All SI parts were fixed for 4 h with PBS-buffered formalin. After this time, number, location, and size of visible adenomas in the entire intestine were determined under the Olympus lighted magnifier. Adenomas were classified as small ($<1\text{mm}$), middle (1-3 mm) and large ($>3\text{mm}$). Finally, the tissue was rolled to swiss rolls, overnight dehydrated in 70% ethanol and embedded in paraffin (see section 2.8.1).

Adenoma protein expression (G3)

After opening the GI tract longitudinally, the mucosa was cleaned with ice cold PBS (containing phosphatases and proteases inhibitors tract). Adenomas were directly counted after the dissection and excised, snap-frozen in liquid N_2 and stored at -80°C until further analysis of protein expression and phosphorylation (see chapter 2.9). Due to the possible fast protein degradation and phosphatase activity, adenomas were only counted in total and not divided according to size.

β -catenin accumulated crypts (G2)

In collaboration with Prof. Mori and Dr. Yamada (Tumor Pathology Dept. Gifu University, Japan) murine colons were “en face” embedded for the examination of microlesions. Colons were removed and washed with cold PBS to remove faeces completely. Four mm diameter glass bars were inserted along the colon, and colons were incubated in buffered formalin for 5 min, then opened longitudinally, cleaned with PBS and flattened on a Whatman paper using two staples on each end. Fixation was performed overnight at room temperature. The next day, adenomas were excised and the colon was divided in 3 parts (proximal, middle and distal). Tissues were placed in histological sample nets and overnight dehydrated in 70 % ethanol (see chapter 2.8.3).

2.4 Clinical markers in serum

2.4.1 Serum protein determination

Reagents:

1 mg/ml BSA standard (Sigma, Steinheim)

Bio-Rad reagent (Bio-Rad, Munich)

Bio-Rad Protein Assay was used to determinate concentration of serum proteins in a 96 well plate format. The assay is based on the method of Bradford (Bradford, 1976). Bio-Rad reagent was diluted 1:5 in Millipore water and filtered through 0.45 μ m filter. Serum samples were diluted 1:200 and 1:400. A BSA standard was used as a reference.

Composition per well

190 μ l Bio-Rad reagents (diluted)

10 μ l serum sample or standard

Plates were incubated for 5 minutes at room temperature and then measured at 595 nm.

2.4.2 ORAC determination

Reagents:

Perchloric-acetic acid

0.5 N NaOH

2,2-Azobis-(2-amidinopropane) dihydrochloride (AAPH, Polyscience, USA)

Trolox (Calbiochem, USA)

ORAC Buffer:

0.75 mM Na₂HPO₂

0.75 mM KH₂HPO₄

0.21 μM Fluorescein

In order to precipitate proteins, 20 μl of 0.5 M perchloric-acetic acid was added to 20 μl of serum, mixed and centrifuged for 10 min at 4°C in an Eppendorf centrifuge. Afterwards, supernatants were neutralized with 1.2 μl 0.5 N NaOH and diluted 1:5 in ORAC buffer. For the ORAC assay, 20 μl were used for determination.

The decline of fluorescence was measured at 37 °C for 120 min until completion using a Cytofluor 4000 fluorescent microplate reader (excitation wavelength ex 530/25 nm, emission wavelength Em 585/30 nm). Results were expressed as ORAC units, where 1 ORAC unit equals the net protection of fluorescein produced by 1 μM Trolox.

Composition per well:

190 μl ORAC buffer

10 μl diluted serum

2.4.3 PGE₂ determination

Materials:

Supelco LC-18 Reverse phase extraction (Sigma, Steinheim)

PGE₂ ELISA Kit (R&D, Abingdon, UK)

Reagents:

^3H -PGE₂ conjugate 0.1 mCi/ml (Perkin Elmer, Waltham, MA, USA)

Ultra pure water (Cayman chemicals, Ann Arbor, MI, USA)

Acetonitrile (ACN)

15 % Ethanol

Hexane

Ethylacetate

RD5-39 Buffer (Elisa kit R&D kit)

This assay determines the PGE₂ content in serum. A preliminary extraction using a reverse phase extraction protocol (C₁₈ reverse phase columns) is required to avoid cross reactions with other serum components.

Serum was spiked with a radioactive tracer to determine the column extraction efficiency. 10 nCi [^3H]-PGE₂ were added to the serum sample as an internal standard (360 μl RD5-39 buffer, 40 μl serum and 20 μl 1:100 ^3H -PGE₂) and mixed well. Then, as initial CPM (counts per minute) measure, 20 μl were quantified in the scintillation counter.

Proteins were then precipitated by adding 20 μl 4 N HCl, mixing and cooling down for 15 minutes on ice and the serum mix was centrifuged at 13.000 rpm for 1 minute. Supernatant was afterwards subjected to solid phase extraction using C₁₈ columns which had been previously preconditioned (10 ml ethanol) and equilibrated (10 ml water). The supernatant was pipetted directly on to the C₁₈ column and slowly eluted under constant pressure conditions (1-2 drops per second). Columns were washed (water, 15% ethanol and hexane, 5 ml each) and analyte elution was carried out (4 mL ethylacetate). The eluate was kept at -80 °C until analysis.

Ethylacetate was evaporated under a nitrogen atmosphere and samples were resuspended in 250 μl of RD5-39 buffer. Recovery was calculated measuring 20 μl of the reconstituted sample in a scintillation counter and adjusted to the initial volume.

PGE₂ in plasma was quantitated using a PGE₂ immunoassay kit. The principle of the immunoassay is based on a competitive ELISA technique in which PGE₂ present in serum competes with a fixed amount of HRP-labelled PGE₂ for sites on a mouse monoclonal antibody plate. A negative control (NSB wells) and a blank (Bo) were pipetted together with the standard and serum.

NSB wells

150 µl R5-39 buffer
50 µl PGE₂ conjugate

Bo wells

100 µl R5-39 buffer
50 µl primary antibody solution
50 µl PGE₂ conjugate

Sample or Standard

100 µl STD or serum
50 µl R5-39 buffer
50 µl PGE₂ conjugate

The plates were incubated for 2 h at RT on an orbital shaker. Afterwards, each well was washed 4 times with 400 µl wash buffer and 200 µl of substrate solution were added. The plate was incubated for 30 min at RT, protected from light. Finally, 50 µl stop solution was added to each well. The O.D. was determined using the microplate reader system at 450 nm. A PGE₂ standard curve was created using a four parameter logistic curve-fit equation (Table curve software) and the corresponding PGE₂ serum concentration was then calculated for each sample.

2.5 Quantification of polyphenol metabolites and clinical markers in urine

2.5.1 HPLC-DAD determination

(collaboration with Jn.Prof. Elke Richling and Ms.Hannah Bergmann)

Materials:

0.45 µm Filters (Sartorius, Göttingen, Germany)
Bond Elut C18 Solid phase cartridges (Varian, Germany)
4.6 x 150 mm Atlantis columns (Waters, USA)

Reagents:

Phosphoric acid
Formic acid / Acetonitril (ACN)

Urine was filtered with a 0.45 µm filter, 1.8 ml urine was diluted in 200 µl 80% phosphoric acid and mixed. Afterwards, the diluted urine was centrifuged for 10 min at 10.000 rpm and 300 µl supernatant was added onto a C18 solid phase column and eluted with methanol. The polyphenol metabolites were measured with a HPLC-DAD using a 4.6 x 150 mm Atlantis column in a gradient of 1% formic acid (in water) in acetonitril in a flow of 0.8 ml/min. A final volume of 10 µl was injected and the polyphenols were detected using the respective absorption maximum.

2.5.2 Creatinine determination

Reagents:

10 mM Picric acid

100 mM Na OH

0.005 % Creatinine standard

The detection of creatinine was performed in a 96 well plate format. The assay is based on the method of Owen *et al.* (Owen *et al.*, 1954). The 100 mM NaOH solution was diluted in 10 mM picric acid in a relation of 1:5. Urine was diluted in a range of 1:5 and 1:10 and 10 µl were used for determination.

Composition per well

200 µl Reagent Na OH / picric acid (1:5)

10 µl Urine or creatinine standard

The absorbance was recorded at 492 nm with a SpectraMax 340 PC plate reader at 25 °C, measuring at 0 s and 60 s time points. The final value was calculated comparing the increment of absorbance between the creatinine standard and the sample. In order to obtain reliable results, the difference between the creatinine standard the diluted urine samples should be less than 10%.

2.5.3 Protein quantification

Reagents:

1 mg/ml BSA standard (Sigma, Steinheim)
Bicinchoninic acid solution (Sigma, Steinheim)
Copper solution (Sigma, Steinheim)
Trichloroacetic acid (TCA)

The bicinchoninic (BCA) method was used to determinate the protein concentration of urine in a 96 well plate format. The assay is based on the method of Smith *et al.* (Smith *et al.*, 1985). Copper was diluted in BCA in a 1: 50 relation. Urine was previously diluted 1:20 and 1:50 in water.

Proteins were precipitated using 980 μ l 10% TCA and 20 μ l diluted urine. Afterwards, samples were mixed, cooled down for 30 min on ice and centrifuged at 10.000 rpm for 10 min at 4°C. The protein pellet was resuspended in 480 μ l 10 % TCA and resuspended again. Samples were centrifuged at 10.000 rpm for 10 min at 4°C and the supernatant was discarded. Pellets were mixed in 450 μ l BCA-Copper solution (50:1) and 200 μ l per well were pipetted in duplicated in a 96 well plate A BSA standard from 1 μ g/ μ l to 0.0025 μ g/ μ l was used as a reference; as already described for the urine samples this standard curve was also precipitated in 10% TCA. Plates were incubated for 60 minutes at 37°C and then measured at 562 nm.

2.5.4 Determination of nitrite and nitrate levels

Reagents:

VCl₃ (Sigma, Steinheim)
1 N HCl (Prolabo, Strasbourg, France)
N-(1-Naphthyl) ethylenediamine dihydrochloride (NEDD) (Sigma, Steinheim)
Sulphanilamide (Sigma, Steinheim)
KNO₃ (Sigma, Steinheim)
NaNO₂ (Sigma, Steinheim)

VCl₃ solution:

400 mg VCl₃ in 50 ml 1 N HCl

Griess reagent:

0.1 % NEDD in water

Sulphanilamide (2% w/v) in 5 % Phosphoric acid (v/v)

Nitrite and nitrate levels of urine were measured following the reduction with VCl_3 (Miranda *et al.*, 2001). Urine samples were diluted 1:2 and 1:4 in water.

A nitrate standard solution (100 μ l) was serially diluted (from 200–1.6 μ M) in duplicate in a 96-well plate. After loading the plate with diluted urine (100 μ l), addition of VCl_3 (100 μ l) to each well to reduce nitrate to nitrite was rapidly followed by addition of the 100 μ l Griess reagent. Nitrite was measured in a similar manner except that samples and nitrite standards were only exposed to Griess reagents. The Griess reaction forms a chromophore from the diazotization of sulfanilamide by acidic nitrite followed by coupling with bicyclic amines such as N-1-(naphthyl) ethylenediamine. Diazonium products can be measured at 540 nm (Fig.10).

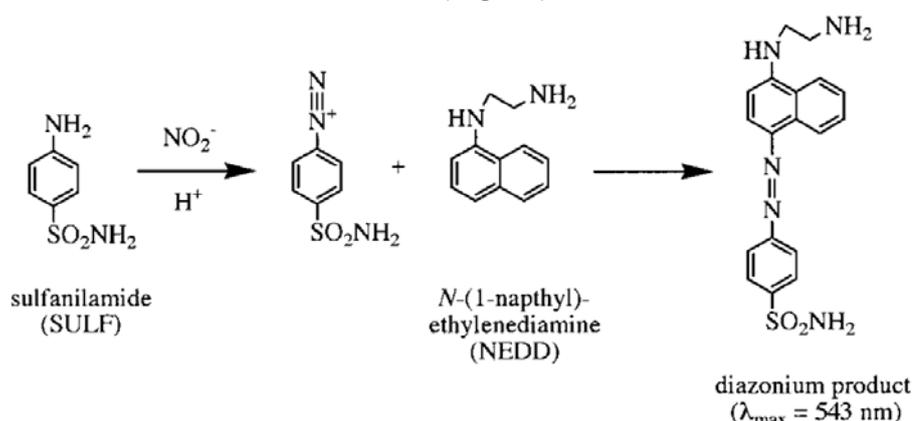


Fig. 10. Formation of diazonium products in acidic conditions (Miranda *et al.*, 2001).

 NO_2 determination (no incubation)100 μ l $NaNO_2$ standard (200-1.6 μ M) or urine sample100 μ l Griess reagentTOTAL Nitrite and nitrate determination (45 min 37 °C)100 μ l KNO_3 standard (200-1.6 μ M) or urine sample100 μ l Griess reagent100 μ l VCl_3

Each reaction was mixed in a 96 well plate and shaken before measure.

2.5.5 Carbohydrate determination

(Under supervision of Ms.Melanie Oik)

Reagents (R-Bio-pharm, Darmstadt):

BR-2: 100 mM Triethanolamine buffer (pH=7.6), 36.6 mM NADP and 100 mM ATP

BR-3: 320 U /ml Hexokinase and 160 U/ml glucose-6-phosphate dehydrogenase

BR-4: 700 U /ml phosphoglucose isomerase

The enzymatic detection of glucose/fructose was performed in a spectrophotometer in cuvette format at 25 °C. Urine was diluted 1:10 and 1:20, 100 µl were used for determination. All incubation steps were performed at RT and all absorbencies were recorded at 340 nm. Water was used as a blank.

Each diluted urine sample or water (100 µl) was incubated for 3 minutes with 1.9 ml Millipore water and 1 ml of reagent BR-2. Afterwards, the Absorbance (Abs_1) was measured. In the second step, 20 µl of BR-3 were pipetted in each sample, mixed and for 15 minutes incubated, and a second absorbance (Abs_2) was determined. In the last step, 20 µl of BR-4 were added, mixed and 15 minutes incubated, and a third absorbance was measured (Abs_3).

Determination of glucose was calculated using the following absorbance differences:

$$\Delta \text{ Abs-gluc} = (Abs_2 - Abs_1) \text{ sample} - (Abs_2 - Abs_1) \text{ blank}$$

Determination of fructose was calculated using the following absorbance differences:

$$\Delta \text{ Abs-fruct} = (Abs_3 - Abs_2) \text{ sample} - (Abs_3 - Abs_2) \text{ blank}$$

$$C \text{ (g/l)} = \frac{V \times MW}{\epsilon \times V \times d \times 1000} \times \Delta \text{ Abs (glucose or fructose)}$$

V = final volume (3.020 ml for glucose or 3.040 ml for fructose)

v = sample volume (0.1 ml)

MW= molecular weight of glucose or fructose (180.16 g/mol)

d = light path (1cm)

ϵ = Extinction coefficient of NADPH ($6.3 \text{ l} \times \text{mmol}^{-1} \times \text{cm}^{-1}$)

2.6 Liver Enzymatic activities

2.6.1 Preparation of cytosolic and microsomal protein fractions

Reagents:

Lysis buffer (pH=7):

60 mM KH_2PO_4
60 mM Na_2HPO_4
250 mM Sucrose
0.5% KCl

Microsome buffer (pH=7.4)

200 mM KH_2PO_4
10 mM MgCl_2

All steps were carried out under cold conditions to avoid protein degradation. Mice livers (approximately 1 g) were homogenized in 12 ml of cold lysis buffer in ultraturrax homogenizer and then centrifuged at 9000 g for 15 minutes. Lipids were discarded using a cotton filter and 10 ml supernatant was ultracentrifuged for 45 minutes at 35.000 rpm. A clear supernatant was collected for cytosolic proteins and aliquoted. Microsome pellets were resuspended in 1.5 ml of microsomes buffer and aliquoted.

Glutathione S-transferase (GST), quinone reductase (QR), thioredoxin reductase (TrxR) activities and glutathione (GSH) were determined in cytosolic fractions and Cyp1A1/Cyp2B1 in microsomal fractions. Protein determination of both fractions was performed using the BCA method.

2.6.2 Quantitative determination of liver protein content

The BCA method was used to determinate the protein concentration of cytosolic and microsomal fractions in a 96 well plate format. The same reactives were used as described before (see section 2.5.3). Protein lysates were diluted 1:15 and 1:30. A BSA standard was used as a reference.

Composition per well

190 µl BCA reagent + Copper (50:1 diluted)

10 µl serum sample or standard

Plates were incubated for 60 minutes at 37°C and then measured at 562 nm.

2.6.3 Glutathione S-transferases (GST)

GST activity was determined using cytosolic liver protein supernatants as source of GST and CDNB (1-chloro 2,4-dinitrobenzol) as a substrate (Keen *et al.*, 1976).

Reagents:

GSH stock (Sigma, Steinheim)

CDNB (Sigma, Steinheim) in EtOH

GST reaction mix:90 mM KH₂PO₄ / K₂HPO₄ Buffer pH = 6.5

0.9 mM CDNB

2.25 mM GSH

Per well:

20 µl diluted cytosol

180 µl GST reaction mix

The mix was incubated shortly at 37 °C. Liver cytosols were diluted 1:300 in lysis buffer (see 2.5.1) and 20 µl (approximately 30-80 µg prot) each were used for determination. The absorbance was recorded at 340 nm with a SpectraMax 340 PC plate reader at 37 °C, using its kinetic mode at 15 s intervals during a 15 min reaction time. The rate of conjugated CDNB formation was calculated using the extinction coefficient (ϵ) 9.6mM⁻¹ cm⁻¹ and a well diameter of 0.6 cm. After calculations, a factor of 35 was calculated. Data were normalized to protein concentration.

$$C \text{ (mmol/l)} = \frac{1}{\epsilon \times d \times 1000} \times \Delta \text{ Abs (Units/min)}$$

d = light path (0.6 cm, 200 µl end volume per well)

 ϵ = Extinction coefficient of CDNB (9.6 l × mmol⁻¹ × cm⁻¹)c (mmol/ml) = 175 × $\Delta \text{ Abs}$ (Units/min), then for 200 µ/well the factor is 35.

2.6.4 NAD(P)H: Quinone oxidoreductase (QR)

QR activity in liver cytosol was determined by measuring the kinetics of NADPH-dependent menadiol-mediated reduction of 3-(4,5-dimethyl-2-thiazyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) (Gerhauser *et al.*, 1997).

QR reaction mix:

0.08 % BSA

0.87 mM MTT

20 mM Tris/HCl

0.01% Tween-20

5 μ M FAD⁺

23 μ M NADP⁺

0.75 mM glucose-6-phosphate

Per well:

50 μ l diluted cytosol

200 μ l reaction mix

Shortly before measurement, the reaction mix was incubated at 37 °C and the substrate menadione and enzyme glucose-6 phosphate-dehydrogenase were added:
1.5 U of Glucose-6-phosphate-dehydrogenase
38 μ M Menadione (diluted in ACN)

Liver cytosol was diluted 1:30 in lysis buffer and 50 μ l each were used for determination. Plates were measured at 595 nm using a SpectraMax 340 PC plate reader each 30 s during a 15 min interval and the rate of MTT reduction was calculated using the molar extinction coefficient of 11,600 M⁻¹ cm⁻¹ and according to the well volume a diameter of 0.75 cm. Values were normalized to protein content.

After calculations, a factor of 29.4 was calculated

$$C \text{ (mmol/l)} = \frac{1}{\epsilon \times d \times 1000} \times \mathbf{Abs} \text{ (Units/min)}$$

d = light path (0.75 cm, 250 μ l end volume per well)

ϵ = Extinction coefficient of MTT (11.6 l \times mmol⁻¹ \times cm⁻¹)

c (mmol/ml) = 117.6 \times Δ **Abs** (Units/min), then for 250 μ l/ well the factor is 29.4.

2.6.5 Thioredoxin reductase (TrxR)

The measurement of TrxR activity was based on the NADPH-dependent reduction of 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) (Hill *et al.*, 1997). The activity was corrected for non-TrxR-dependent DTNB reduction by addition of auranofin, a TrxR inhibitor (Becker *et al.*, 2000). Liver cytosolic fractions (40-90 µg) were incubated in presence of auranofin (5 µM) or buffer. Plates were incubated at 37 °C for 20 minutes.

Reagents:

Auranofin (Sigma, Steinheim)

DTNB (Sigma, Steinheim)

Reaction mix

70 mM KH₂PO₄ Buffer

1.5 mM EDTA

0.004 % BSA

0.3 mM DTNB

0.2 mM NADPH

Per well:

25 µl diluted cytosol

225 µl reaction mix

The absolute Trx activity was calculated using with difference between absence of auranofin (total reductase enzymatic activity) and presence of auranofin (total reductase activity in absence of Trx activity).

A 10 min Kinetics was determined over 15 minutes taking one measure every 30 seconds at 412 nm. The rate of DTNB reduction was calculated using the molar extinction coefficient of 13,600 M⁻¹ cm⁻¹ and normalized to protein content. After calculations, a factor of 24.5 was calculated and according to the well volume a diameter of 0.75 cm. Data were normalized to protein concentration

$$C \text{ (mmol/l)} = \frac{1}{\epsilon \times d \times 1000} \times \Delta \text{ Abs (Units/min)}$$

d = light path (0.6 cm, 250 µl end volume per well)

ε = Extinction coefficient of DTNB (13.6 l × mmol⁻¹ × cm⁻¹)

c (mmol/ml) = 97.6 × Δ Abs (Units/min), then for 250µl/ well the factor is 24.4.

2.6.6 Glutathione (GSH)

Total liver glutathione was determined using 5,5'-dithiobis(2-nitrobenzoic acid) DTNB (Tietze, 1969).

Cytosol was deproteinized using 1% SSA. One cytosol volume was mixed with 2 volumes of SSA 1% and centrifuged for 5 minutes at 10.000 rpm. After deproteinization, cytosol was diluted 1:10 in water and 40 µl was used for the assay. GSH standards (from 800 pmol to 20 pmol) were prepared from 100 µM GSH stock solution (diluted in water).

Reagents:

GSH stock (Sigma, Steinheim)

DTNB (Sigma, Steinheim)

GSH Stock Buffer (pH= 7.5)

125 mM Na₂HPO₄

6.3 mM NaEDTA

GSH reaction mix:

16 mM Tris-HCl pH 7,4

0.61 mM Glucose 6 Phosphate

18.7 µM NADP⁺

0.57 mM DTNB

0.26 U Gluc.6 Phos. Dehydrogenase

0.24 U Glutathione Reductase

A 5 minutes kinetics was determined taking one measure every 30 seconds at 412 nm using a SpectraMax 340 PC plate reader nm. GSH content was calculated using the standard curve (10 µM to 0.25 µM GSH in stock buffer) and to normalized to protein concentration.

Per well:

40 µl diluted cytosol

170 µl reaction mix

2.6.7 Cyp1A1/Cyp2B1

Etoxyresorufin- and Pentoxyresorufin- O-deethylase (EROD and PROD) activities was monitored by the continuous spectrofluorimetric procedure of Prough (Prough *et al.*, 1978). Fresh microsomes were diluted in 1.5 ml microsomes buffer in order to homogenize. Afterwards, they were homogenized through a 20-gauge syringe.

Substrates:

7-Ethoxyresorufin, EROD (Invitrogen GmbH, Karlsruhe)

7- Pentoxyresorufin, PROD (Invitrogen GmbH, Karlsruhe)

For analysis, microsomes for analysis were diluted 1:16 in microsomes buffer and shortly mixed.

Cyp Buffer:

0.2 M Phosphate Buffer (microsomes buffer)

3.4 mM Glucose-6-phosphate

1.3 mM NADP⁺

5 U Gluc-6-phosphate dehydrogenase

EROD/PROD reaction mix:

2 μM 7-Ethoxyresorufin (in Cyp Buffer)

25 μM 7-Pentoxyresorufin (in Cyp Buffer)

Fluorescence intensity was measured using a microplate spectrofluorometer each 30 s during 10 min (excitation 522 nm and emission 530 nm). The rate of resorufin formation (nmol resorufin formed per minute) was determined by comparing the rate of increase (slope) in fluorescence to a resorufin standard curve (20 μM-0.2 μM).

$\text{nmol resorufin/min} = (\text{AFU/min}) / \text{slope standard}$

Data were normalized to protein content (nmol/mg/min)

Per well:

100 μl Reaction mix substrate 2 μM EROD or 25 μM PROD

100 μl diluted microsomes

2.7 Gene Expression experiments

2.7.1 Total RNA isolation and quantification

Buffers and solutions:

RNA Lysis Buffer (RLT Buffer) + 1% β -Mercaptoethanol

a) Tissue disruption

1 cm of colon and normal small intestinal tissue respectively was lysed using a micro-dismembrator S in a Teflon container. 600 μ l of 1% β -ME RLT lysis buffer (Qiagen, Hilden, Germany) was added to each sample, homogenized for 45 sec at 2500 rpm.

b) RNA purification

(Qiagen, Hilden, Germany)

600 μ l of 70% ethanol was added to the tissue lysate. 600 μ l mix was loaded carefully onto a Qiagen column and centrifuged for 15 s at 10.000 rpm. The eluate was discarded.

In order to avoid genomic DNA contamination a DNase digestion was applied. Each sample obtained 10 μ l of DNase I and 70 μ l DNA digestion buffer (RDD, DNase kit, Qiagen). This mix was pipetted directly onto the column membrane and incubated for 20 min at RT. After that, the column was washed with 350 μ l of wash buffer-1 (RW1 buffer, RNeasy kit) and centrifuged for 15 sec at 10.000 rpm. A second wash followed using 700 μ l of RW1 buffer and the samples were centrifuged again. The eluates were in both cases discarded. The column was washed with 500 μ l of RPE Buffer (RNeasy kit) and centrifuged for 2 minutes at 10.000 rpm. The eluate was again discarded. This last step was repeated once and then the column should be dry.

Finally, the RNA elution was completed with RNase free water (RNeasy kit). A first elution with 40 μ l was followed by another 25 μ l. Each elution was centrifuged for 1 min at 10.000 rpm. The RNA eluates were combined, then aliquoted and kept at - 80°C.

c) RNA quantification and determination of RNA integrity

Buffers and solutions:

Tris-Borate-EDTA (TBE):Buffer

90 mM Tris-base

90 mM Boric acid

2mM EDTA

1.2% Agarose Gel:

1.2 g Agarose

90 ml DEPC-H₂O

10 ml 1X Formaldehyde RNA Buffer

10X Formaldehyde (FA) RNA Buffer

200 mM MOPS

50 mM NaOAc

0.5 M pH 7.0 EDTA

1X Formaldehyde (FA) RNA

Buffer

100 ml 10X FA RNA Buffer

20 ml Formaldehyde 37%

880 ml DEPC-H₂O

RNA loading buffer

0.25% Bromophenol blue

4 mM EDTA

0.9 M formaldehyde 37%

20% Glycerol

30 % Formamide

4X FA gel buffer

Total concentrations of RNA were quantified using a 1:100 dilution in RNase free water in a quartz cuvette using an Eppendorf spectrophotometer. Absorbance at 260 nm indicates the RNA yield, which should be higher than 0.1 units. A ratio between A₂₆₀/A₂₈₀ indicates protein or phenol contamination and should not be lower than 1.8. This value was used as an index of RNA purity.

The integrity of RNA was tested using a denaturing agarose gel electrophoresis. 5 µg of RNA was mixed with RNA loading buffer, denatured at 65°C for 5 minutes and the mixture was cooled immediately for 2 minutes on ice. 1.2 % agarose in water was prepared by heating in a microwave oven for 5 minutes, avoiding boiling. After cooling down, 25 µl of EtBr (0.5 mg/ml) was pipetted to the gel mixture.

After loading the samples, the electrophoretic running was set at 75 V for 30 minutes. Finally the gel was photographed under UV light. Fig.11 shows an example of RNA integrity within different samples.

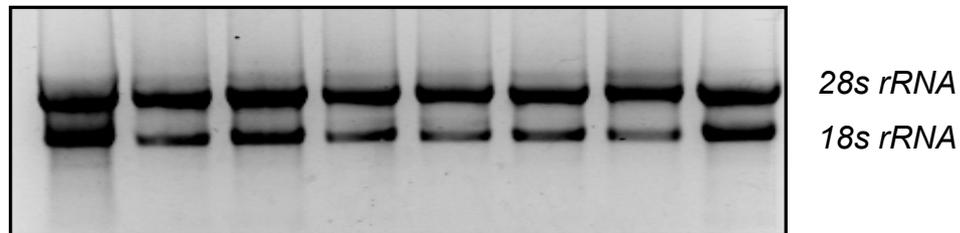


Fig. 11. 1.2 % RNA agarose gel. Examples of normal small intestine tissue RNA integrity.

2.7.2 RNA reverse transcription

Buffers and solutions:

DEPC-H₂O

Random Hexamer primer (Roche Diagnostics, Mannheim)

5 x Reaction-Buffer (Promega, Madison, WI, USA)

dNTP mix (10 mM each) (Eppendorf, Hamburg, Germany)

RNAse Inhibitor (Eppendorf, Hamburg, Germany)

M-MLV reverse transcriptase (Promega, Madison, WI, USA)

RT Reaction mix (per sample):

4 µl 5x Reaction-Buffer

1 µl dNTPs

0.5 µl Rnase Inhibitor

1 µl M-MLV reverse transcriptase

No RT reaction mix (per sample)

4 µl 5x Reaction-Buffer

1 µl dNTPs

0.5 µl Rnase Inhibitor

1 µl DEPC

For each sample 1 µg of RNA was reverse-transcribed. 1 µl of Random hexamer primers 1 µM were added and incubated at 70°C for 2 minutes. Afterwards, the samples were cooled down on ice. 6.5 µl of reaction mix was pipetted to each sample and incubated at 42°C for 1 hour to start the reverse transcription. The reaction mixture was stopped by heating at 95° C step for 5 minutes. The samples were then diluted with 20 µl of DEPC-H₂O.

2.7.3 Real Time PCR

Buffers and solutions:

10X Immobuffer (Bioline. London, UK)

5 u/μl IMMOLASE Taq. Polymerase (Bioline, London, UK)

50 mM MgCl₂ (Bioline. London, UK)

Eva-green (Biocat, Heidelberg)

Aqua-braun sterile water (Braun-Melsungen AG, Melsungen)

1mM Fluorescein (Bio-Rad, Munich)

50 mM dNTPS (Eppendorf, Hamburg)

100 μM Primers (Operon GmbH, Cologne)

100 μM Primers (Biomers GmbH, Ulm)

Eva-green(in aqua-braun water diluted)

10 X Immobuffer

1.6 mM dNTPs

5 mM MgCl₂

10 X Eva-green

20 μM Fluoresceine

PCR mix (25 μl per sample):

12.375 μl Eva-green mix

0.125 μl Immolase Taq. pol.

8.5 μl Aqua Braun water

1μl Forward primer

1 μl Reverse primer

2 μl cDNA

A set of interesting genes was selected, especially those related to inflammation, proliferation and angiogenesis.

Each primer pair was tested in a standard curve to determine efficiency of amplification. The Real Time PCR program was also adjusted for each gene. See Tab. 7 for a general scheme of the programs.

Tab. 7. General Real Time PCR program

1 step	95 °C (6 min)	hot start Taq.pol activation	
2 step	95°C (30 s)	Denaturation	<i>n</i> Cycles
3 step	55-60°C (30 s to 60 s)	Annealing	
4 step	72 °C (30 to 60 s)	Extension	
5 step	72°C (5 min)	Final extension	
6 step	57 °C (1 min)	Gap step	
7 step	gradient 55°-94°C (10 s)	Melting curve	80 Cycles
8 step	hold 4 °C		

The standard curve dilution series were adjusted for every single gene. A list of used primers is shown in the Tab.8.

Tab. 8 Selected primers and characteristics

PRIMER	Gene code	Forward 5'-3'	Reverse 5'-3'	Amplicon (bp)	Conc. (nM)	Annealing (°C)	Source
β-actin	NM_007393	CCC AGA GCA AGA GAG GTA TC	CGC AGC TCA TTG TAG AAG G	110	400	55	(1)
Cycloph. E	NM_019489	AGG TCC TGG CAT CTT GTC CAT	GAA CCG TTT GTG TTT GG TCC A	94	400	57	(3)
GAPDH	XM_132897	TGA AGC AGG CAT CTG AGG G	CGA AGG TGG AAG AGT GGG AG	102	400	59	(3)
Cox-1	NM_008969	ACC TAC AAC TCA GCG CAT GAC TAC	CAG AAG CTG AAC ATC TGG TAA CTG TT	147	300	59	(1)
Cox-2	NM_011198	CCT GCT GCC CGA CAC CTT CA	AGC AAC CCG GCC AGC AAT CT	139	400	57	(2)
Cyclin D1	NM_007631	CGA GAA GTT GTG CAT CTA CAC TGA	TGA GCT TGT TCA CCA GAA GCA	83	300	57	(1)
c-Myc	AH_005318	ATC AGC AAC AAC CGC AAG TG	TCC TCC TCT GAC GTT CCA AGA	74	400	55	(2)
VEGF	NM_009595	GAT AGA GTA CAT CTT CAA GCC GTC C	GTT TAA CTC AAG CTG CTG CCT CGC C	122	400	56	(4)

The following oligonucleotide sequences were obtained: (1) (Chen, Hao et al. 2004), (2) (Bondesen, Mills et al. 2004), (3) (RTP primer database) (4) Thesis Dr. Elisabeth Bertl.

Quantification of PCR products is based on the determination of Ct values. The Ct value is defined as the point at which the fluorescence of the amplified sample crosses the threshold is called the Ct.

Eva-green is a dye that binds the minor groove of double stranded DNA. When Eva-green dye binds to double stranded DNA, the intensity of the fluorescent emission increases. As more double stranded amplicons are produced, Eva-green dye signal will increase. Every sample was pipetted in triplicates and the final Ct value was calculated as a mean.

2.7.4 Relative expression calculations

The methods used to calculate the efficiency and gene expression quantification were done using the *Pfaffl* method (Pfaffl, 2001). In order to get precise and reproducible results, the reactions should have efficiency as close to 100% as possible. At this efficiency the template doubles after each cycle during exponential amplification.

The slope of the standard curve can be used to establish the exponential amplification and efficiency of the PCR reaction by the subsequent equation:

$$\text{PCR Efficiency} = 10^{-1/\text{slope}}$$

The ratio (gene fold induction) is established using the following formula:

$$\text{ratio} = \frac{(E_{\text{target}})^{\Delta\text{CP}_{\text{target}}(\text{control} - \text{sample})}}{(E_{\text{ref}})^{\Delta\text{CP}_{\text{ref}}(\text{control} - \text{sample})}}$$

Fig. 12. Mathematical method to calculate PCR efficiency and ratio. Adapted from Pfaffl et al. 2001

2.8 Protein expression experiments

Reagents for antigen retrieval:

Citrate Buffer (pH=6.0)

10 mM Sodium citrate

0.05 % Tween-20

Adjusted with 1 N HCl

Tris-EDTA Buffer (pH=9.0)

10 mM Tris-base

1 mM EDTA

0.05 % Tween

Other reagents:

Diaminobenzidine (DAB) Substrate

1.5 mM DAB, in Tris-HCl Buffer

pH=7.6

0.03 % H₂O₂

10x PBS Buffer pH=7.2:

77mM Na₂HPO₄

27 mM NaH₂PO₄

9% NaCl

Vector M.O.M. Immunodetection Kit PK-2200 (Vector Inc., Burlingame, CA,USA)

Hematoxyline (Appllichem GmbH, Darmstadt)

DAPI (Molecular probes, Carlsbad, USA)

SuperFrost® microscope slides (Menzel Gmbh, Braunschweig)

Eukitt (Kindler, Freiburg)

2.8.1 Formalin-Fixed Paraffin Embedded Preparation (collaboration with the Dept. of Pathology, Heidelberg University)

Embedding in paraffin (Paraffin ParaplastR Plus) and cutting of liver, spleen, small intestinal and colon tissue was performed the in Dept. of Pathology of Heidelberg University (Ms. Karin Rebholz). Samples were fixed overnight in 4% formalin, dehydrated in increasing ethanol solutions (70%-80%-90%-100% 2 h each), incubated 3 times during 2 h in 100% anhydrous ethanol, and incubated in Xylene 3 times at 1 h. Tissues were embedded in paraffin blocks for 1 h at 60°C. Blocks were cut by serial sections of 3 µm thickness using a microtome (Leica RM2235), placed on microscope slides SuperFrost® and allowed to dry overnight at 37°C. Slides were kept at 4 °C until use.

The deparaffinization and Hematoxylin-Eosin (H&E) staining processes was performed as described in Tab.9.

Tab. 9. Deparaniffinization and H&E staining steps

<u>Deparaffinization</u>	<u>Hematoxylin-Eosin staining</u>
Xylol 3 x 5 min	Hematoxylin 5 min
100 % Ethanol 2 min	Destilled water 5 s
100 % Ethanol 2 min	1 % Eosin 1 min
80 % Ethanol 1.5 min	Destilled water 5 s
70 % Ethanol 1 min	70% Ethanol 5 s
50 % Ethanol 1 min	96% Ethanol 5 s
Destilled water 30 s	100 % Ethanol 2 x 2 min
PBS/TBS 5 min	Xylol 2 x 2 min
	Eukitt

2.8.2 Immunohistochemistry (IHC) protocol

(Performed by Ms. Renate Steinle)

A general IHC protocol is described below. Antibodies, incubations and all characteristics are summarized in the Tab. 10.

Firstly, activation of tissue antigens is needed. The antigen retrieval buffer breaks the protein cross-links formed by formalin fixation and thereby uncovers hidden antigenic sites. Slides were heated up to 95°C without boiling in a microwave oven. Slides were cooled down for 30 min at 40°C in PBS/TBS, shortly washed in distilled water and 5 min in PBS/TBS at RT. Endogenous peroxidases were blocked for 15 min with 3% H₂O₂-PBS and then washed in PBS/TBS 3 times for 5 min. Tissue sections were blocked according to each primary antibody. Between incubations or blocking steps slides were washed 2 times 5 min in PBS/TBS. Then, the respective primary antibody was added (See Tab.10 for blocking and primary/secondary antibody conditions). After washing 3 times for 5 minutes in PBS and slides were incubated with a biotintylated secondary antibody. The protein staining was performed using the ABC reagent (Vectastain®ABC Elite®, Vector MOM Kit), where the avidin binds with high affinity to secondary antibody coupled to biotintylated horseradish peroxidase.

Tab.10 Selected antibodies and immunohistochemistry conditions.

Antibody	Company	Antigen retrieval	Blocking	Incubation 1 st Ab	Incubation 2 nd Ab
Cox-2 Sc-1747	Santa Cruz	Citrate Buffer	20 min RT PBS 1% BSA	1:280 1 h RT Anti goat	1:100 30 min RT Anti goat
Cyclin D1 Sc-753	Santa Cruz	Tris-EDTA	20 min RT PBS 1% BSA	1:70 1h RT Anti rabbit	1:200 60 min RT Anti rabbit
i-NOS Sc-650	Santa Cruz	Citrate Buffer	30 min RT PBS 1% BSA 0.1 % casein	1:300 4°C o/n Anti rabbit	1:400 4°C o/n Anti rabbit
Ki67 NCL-Ki67P	Thermo	Citrate Buffer	30 min RT PBS 1% BSA	1:200 30 min RT Anti rabbit	1:300 60 min RT Anti rabbit
β-catenin BD-610154	BD	Citrate Buffer	60 min RT MOM	1:700 o/n 4 °C Anti mouse	1:300 30 min RT Anti mouse

After washing for 5 min in PBS, ABC reagent was added for 30 min. The peroxidase is then developed by the DAB substrate incubated for 5 min to produce brownish colorimetric end products. Secondary antibody cross reactivity was checked using a negative control without first antibody. Meyers' hemalaun staining was performed for 5 s and washed with flowing tap water during 3 min. Sections were dehydrated through changes in ethanol solutions with increasing concentrations (50%- 70%- 100%, 5 min each), incubated for 20 min in xylol, and mounted in cover-slides using Eukitt® solution. Slides were examined using an AxioSkop 2 MOT microscope.

2.8.3 BCAC Immunostaining (collaboration with Prof. Mori and Dr. Yamada, Gifu University Japan)

All colon sections were flat in paraffin embedded using a small metal tamper. Immunohistochemistry was performed on 4- μ m-thick paraffin-embedded sections from all 3 segments of the colon. Deparaffinization and rehydration was done as described before. Citrate buffer was used for the antigen retrieval.

Immunodetection of β -catenin was carried out using a Vector® M.O.M.™ which detects mouse antigens in mouse tissue reducing the background. This was performed using the M.O.M blocking reagent for 60 min at RT. Slides were washed in PBS 2 times for 5 minutes. β -catenin primary antibody 1:750 (in PBS) was added and incubated overnight in a humid chamber. The following day slides were washed 3 times for 5 min in PBS.

For DAB staining, endogenous horseradish peroxidase activity was blocked by treatment with 3% H₂O₂ (in methanol) solution. The protein staining was carried out with a secondary biotinylated anti-mouse diluted 1:300 in PBS and incubated 30 min at RT. Slides were washed in PBS 3 times for 5 minutes and incubated with the ABC reagent. Staining was performed with a DAB solution for 4-5 min was used as substrate for the peroxidase enzyme. Hemamlaun and dehydration was performed as described in section 2.8.2. Immunoreactivities were regarded as positive if the apparent stainings were detected in cytoplasm and/or nuclei for determining BCAC.

For fluorescent immunohistochemistry, after the primary antibody incubation slides were washed in PBS 3 times for 5 minutes. Anti-mouse Rhodamine-labeled (TRITC) secondary antibodies (Dako, Japan) were incubated for 30 min in the dark at RT and

washed 3 times for 5 minutes. A counterstained was performed with 1:1000 DAPI (in PBS) and incubated for 5 min at RT. Slides were washed 3 times for 5 minutes again.

All slides were examined using the AxioSkop 2 MOT microscope whereas fluorescent staining was visualized using an F-View II firewire fluorescence camera.

2.9 Reverse phase protein arrays (RPPA) (collaboration with Dr.

Ulrike Korf and Heiko Mannsperger, Dept. Genome Analyses, DKFZ)

Reagents:

T-Per (Pierce, Rockford, IL, USA)

NP-40 (Sigma, Steinheim)

BCA kit (Pierce, Rockford, IL, USA)

Whatman buffer (Whatman, London, UK)

Fast green FCF (Sigma, Steinheim)

IRDye™700DX-labeled secondary antibody (LI-COR, Lincoln, USA)

Odyssey blocking buffer (LI-COR, Lincoln, USA)

0.02% NP40 in PBS

FCF staining buffer (in Millipore H₂O):

0.005 % Fast Green FCF in 10% acetic acid

30% EtOH

2.9.1 Protein adenoma lysates and determination of protein content

Tumors were weighed and taken up in 12 volumes (v/w) of T-PER buffer in Eppendorf tubes. Lysis was made with a Qiagen dismembrator using steel balls, 30 Hz for 4 min. Protein lysates were centrifuged at 13.000 rpm for 12 min at 4 °C. Supernatants were pipetted into fresh tube and ultracentrifuged for 30 minutes at 30.000 rpm at 4°C.

Supernatants were collected and the protein concentration was determined with the BCA method (see section 2.6.2). A BSA standard was used as a reference.

For determination, 10 μ l of tumor lysate and 190 μ l BCA reagent was pipetted per well. Plates were incubated for 60 minutes at 37°C and then measured at 562 nm.

2.9.2 Selection of monospecific antibodies

Reagents:

Polyacrylamide (Roth, Karlsruhe)

Bromophenol blue (Serva, Heidelberg)

Ammonium persulfate (APS) (Sigma, Steinheim)

Sodium dodecyl sulphate (SDS) (Merck, Darmstadt)

β -Mercaptoethanol (β -ME) (Sigma, Steinheim)

Stacking gel

8%-10% (v/v) Polyacrylamide

25% (v/v) Tris-HCl 0.5 M, pH 8.8

1% (v/v) SDS 10%

1% (v/v) APS 10%

0.1% TEMED

SDS-loading buffer

10% (w/v) SDS

0.3 M Tris-HCl, pH 6.8

50% (v/v) Glycerol

0.5% (w/v) Bromphenol blue

5% (v/v) β -ME

Running gel

10%-15% (v/v) Polyacrylamide

25% (v/v) Tris-HCl 0.5 M, pH 6.8

1% (v/v) SDS 10%

1% (v/v) APS 10%

0.1% TEMED

Runing Buffer 5x,pH 8.3

120 mM Tris-base

1.25 M Glycine

5% (w/v) SDS

Blotting buffer10x, pH 8.5

250 mM Tris-base

2 M Glycine

Blotting buffer 1x (in distilled H₂O)

10% blotting buffer

20% methanol

TBS-T 10X pH=8.0

0.2 M Tris

1.36 M NaCl

TBS 1X pH=8.0

10% TBS-T 10x

0.1% Tween-20

A reliable and precise detection of reverse protein arrays is dependent on the specificity and selectivity of the first antibody. All antibodies used (Tab. 11) have been previously tested in Western blot in order to determine their monospecificity.

Proteins (25 µg) were mixed with 1x SDS-loading buffer, heated for 5 min at 95 °C before loading and separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). An upper 8-10% polyacrylamide stacking gel concentrates the samples in the gel. The lower resolving gel had a 10-15% polyacrilamyde concentration, depending on the size of the proteins of interest. The electrophoresis was run at 100 V in 1x protein running buffer.

Proteins were transferred from the polyacrylamide gel onto a PVDF membrane using a protein mini-gel wet transfer System. PVFD membranes were soaked shortly in methanol and washed with PBS. The sandwich was prepared with a sponge pad, two sheets of Whatman filter, the PVDF membrane, the polyacrylamide gel, two sheets of Whatman filter paper and a sponge pad. This sandwich was placed in the anode part of the cassette, the cassette was filled with 1x blotting buffer and the proteins were transferred at 4°C at 100 V for 1.30 h.

After the transfer, the membrane was washed in PBS buffer and incubated for 1 h in Odyssey® blocking buffer. Primary antibody was added to Odyssey blocking buffer at the appropriate concentration (range 1:500 to 1:5000) and incubated for 60 min at RT on a platform shaker. The membrane was subsequently washed in PBS-T 4 times for 5 min and then exposed to the IRDye™700DX-labeled secondary antibody (1:8000) diluted in Odyssey blocking buffer for 1 h at RT. After that, the membrane was washed in PBS-T 4 times for 5 min and a last 5 min PBS wash was performed to remove residual Tween-20.

Signals were detected using the Odyssey NIR scanner and protein intensities were calculated with the Odyssey 2.0 software following manufacturer's instructions. Examples of western blots are given in the next figure, showing examples of a western blot and an RPPA for c-Myc (Fig. 12b).

Tab. 11 List of used antibodies

Antibody	Company	Catalogue nr	1 st Ab Dilution ^a
pERK1/2	RnD	AF1018	1:2000
ERK	SC	sc-94	1:5000
pMek	SC	M7683	1:2000
Mek	BD	610122	1:5000
pp38	CST	9215	1:2000
p38	SC	sc-728	1:5000
pAKT	CST	4056 and 4058	1:2000
Akt	SC	sc-1619	1:5000
pSTAT3	CST	9131	1:2000
Stat3	SC	sc-482	1:2000
Stat5	SC	sc-853	1:2000
cyclinD1	CST	2922	1:500
β -catenin	CST	9562	1:500
c-myc	SC	sc-788	1:1000
NfKB	SC	sc-109	1:1000
PCNA	SC	sc-7907	1:1000
iNOS	SC	sc-650	1:500
PKC alpha	ABC	ab32376	1:1000

SC: Santa Cruz; CST: Cell Signalling technologies; RnD: RnD Systems; ABC: Abcam; BD: Beckton Dickinson. ^a Dilution for WB only (1:300 for arrays)

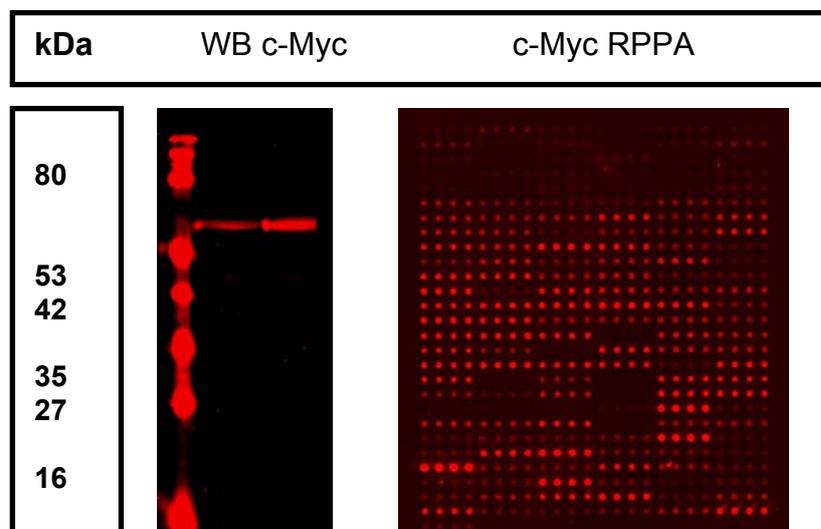


Fig. 12b. Examples of c-Myc (64 kDa.) using the Odyssey system and Min mice adenoma lysates; kDa: Kilodalton; WB: Western blot; RPPA: Reverse phase protein array.

2.9.3 Protein spotting onto nitrocellulose slides

Protein lysates were adjusted to 2 µg/µl and diluted 1:1 with Whatmann array buffer and a volume of 25 µl of each sample was pipetted in a 394 well plate.

Clear supernatants were spotted with a Piezo spotter on nitrocellulose membranes. All samples were spotted in quadruplicate and three drops (333 pl per drop, 2 ng/spot) were delivered per spot. The spotting needle was washed with PBS two times after each spotting. Of each slide, two sub-arrays were printed resulting in eight spots per sample and slide. In addition, a BSA calibration curve between 1 µg/µl and 100 pg/µl and Whatman array buffer as negative control were also spotted. All the system was working at 4 °C. Spotted slides were kept dark at -20°C until analysis.

2.9.4 Infrared-based protein array quantification

Tumor protein arrays and analysis have been described by Loebke et al. (Loebke *et al.*, 2007).

Slides were blocked at 4°C overnight with a 1:3 mixture of Odyssey blocking buffer/PBS. Incubations with primary antibody were performed for 2 h at RT in Odyssey blocking buffer with a 1:300 dilution for all antibodies. After washing in PBS containing 0.02% NP40 (w/w) 4 times for 12 minutes, a 1:10.000 dilution of the IRDye™700DX-labeled secondary antibody in PBS containing 0.02% NP40 was applied for 30 min at RT. The slides were rinsed and dried at room temperature, scanned with the Odyssey NIR scanner, and signal intensities were detected with the Odyssey 2.0 software following manufacturer's instructions.

Fast green FCF was used to normalize signals to protein content. FCF binds to connective proteins. The slides were rinsed for 2 min with PBS containing 0.02% NP40 and incubated with FCF staining buffer protected from light for 1 h at RT. Then, the slides were properly washed in Millipore water and destained for 2 h with 30% ethanol (in acetic acid). A final washed was performed with Millipore water for 1 minute. Samples were scanned at 700 nm.

The average intensity of each single spot was determined (pixels/cm²). The mean intensity of the control spots was determined and subtracted from the mean intensity of the sample spots to correct for background and/or noise due to unspecific antibody

binding. Intensity values were calculated using Genpix software and statistics were calculated using Sigma Plot 10.0.

2.10 Statistical analysis

Differences in food consumption, liquid uptake and body weight were determined using a multiple linear regression analysis for the Y-axis and the slope (ADAM statistics program, DKFZ), collaboration with Dr. W. Rittgen (Department of Biostatistics, DKFZ).

Tumor values, clinical parameters, gene and protein expression experiments were evaluated by one-way ANOVA variance analysis. ANOVA on ranks was performed with non-normal data distribution. Both were calculated with SIGMA-STAT 2.0.

3. RESULTS

In extension of our previous studies, the aim of the presented project was to reinvestigate the colon cancer-preventive potential of apple juice and apple juice extracts under more defined conditions.

3.1 Inhibition of intestinal tumorigenesis in Min mice

3.1.1 Characterisation of apple juices and extracts for intervention

We have demonstrated previously that polyphenolic extracts from apple juice are biologically active in terms of radical scavenging, influence drug metabolism and possess anti-inflammatory and anti-hormonal potential *in vitro* (Zessner *et al.*, 2008).

In order to demonstrate whether CAJ and PAE are *in vivo* chemopreventive we have performed a first Min mouse experiment. In this previous animal experiment, a CAJ (AS04) and a mix of two PAEs were used. This PAE mix consisted of a 1:1 combination of AE03B and PAE04 to include a wide range of polyphenolic compounds. CAJ04 and 0.2% PAE significantly reduced tumor multiplicity in the SI by 38% and 40%, respectively (Gerhäuser 2007, Forum Deutsche Krebsgesellschaft).

Although the results obtained were interesting, the possible mechanisms remained unclear. Therefore, as presented here, a second Min mouse experiment was planned. As intervention groups, the following treatments were used: water as a control, placebo juice, CAJ, 0.2 % B-PAE and a combination of CAJ plus 0.2 % B-PAE. The placebo juice (apple juice without polyphenols) was included to determine whether the polyphenols are the main chemopreventive compounds in apple juice. In addition, one CAJ and a mix of two different B-PAE (B polyphenolic apple extract) were used. B-PAEs (B-polyphenolic apple extract) are lyophilized apple extracts obtained from apple pomace after an enzymatic digestion. Apple pomace is known as a rich source of procyanidins and digestion facilitates the extract of compounds bound to the cell membrane. We decided to use this procyanidin rich extract due to the strong *in vitro* chemopreventive activities reported by Zessner *et al.* (Zessner *et al.*, 2008). The combination of CAJ and 0.2% B-PAE was intended to act as a "functional food" and hence to investigate whether such combination increases the chemopreventive potential.

Quantification of high molecular weight (HMW) and low molecular weight (LMW) components of B-PAE03 and B-PAE06 showed a difference in terms of oligomeric and polymeric procyanidins. PAE04 and B-PAE03 contained more procyanidins than B-PAE06 (PAE04: 48%, B-PAE03:46% and B-PAE06:35%).

PAE04, B-PAE03 and B-PAE06 as representative of *Bittenfelder* CAJ were tested in a set of *in vitro* bioassays to define *in vitro* bioactivity (Tab. 12).

Tab. 12. Summary of potential cancer chemopreventive activities of PAEs.

Sample	DPPH	X/XO	ORAC _{ROO}	Cyp1A	QR Induction	Aromatase	Cox-1
	IC ₅₀ [μg/ml] ^a	IC ₅₀ [μg/ml] ^a	[units] ^b	IC ₅₀ [μg/ml] ^a	CD [μg/ml] ^c	IC ₅₀ [μg/ml] ^a	% Inhibition (100 μg/ml)
PAE04	8.7	17.1	2.4	11.5	1.3	5.9	62
B-PAE03	18.6	18.2	1.8	2.4	1.8	5.4	69
B-PAE06	12.1	26.5	1.8	5.4	1.6	4.3	70

^a IC₅₀: half maximal inhibitory concentration. Concentration that reduces the effect by 50%

^b ORAC Units are corresponding to fluoresceine protection against radicals in comparison with 1μM Trolox. The test was done at 1μg/ml.

^cCD: Concentration required to double the specific activity of quinone reductase.

Briefly, both B-PAE03 and B-PAE06 were especially active in modulation of drug metabolism as well as anti-inflammatory and anti-hormonal potential *in vitro*. Three assays were used to test antioxidant capacity and radical scavenging potential. In the DPPH and the superoxide anion scavenging assay, both B-PAE06 and PAE04 demonstrated strong radical scavenging compared to B-PAE03. Due to quercetin and its conjugates B-extracts possessed higher potential in inhibiting Cyp1A activity. In general, oligomeric and polymeric procyanidins resulted in strong aromatase inhibition in all extracts. All three extracts showed high anti-inflammatory activity with similar percentages of inhibition in the Cox-1 assay. This is attributed mostly to epicatechin.

PAE04 and B-PAE03 content was different in terms of procyanidin concentration and therefore radical scavenging activity (DPPH and X/XO assays) was better compared to the B-PAE06. Differences might be explained by comparatively high percentages of carbohydrates in the B-extracts (PAE04: 7.5%, B-PAE03: 22.0% and B-PAE06: 40.1%) and consequently reduced polyphenols content (PAE04: 48%, B-PAE03:46% and B-PAE06:35%).

Overall, these results are mainly similar to the prior *in vitro* experiments and agree with the data provided by Zessner *et al.*

As described in Section 2.3 (Material and methods) in the present study, we planned to compare the effects of B-PAE, CAJ, CAJ+ B-PAE vs. water and polyphenol free placebo juice in terms of tumor development, various clinical parameters, potential biomarkers for chemoprevention and liver enzyme activities. Five groups of 18 male Min mice each (separated in 3 sub-experiments) received the intervention drinks instead of drinking water for a period of 10 weeks.

3.1.2 Drink and Food consumption and weight development

3.1.2.1 Liquid and food consumption

During the intervention period, liquid and food consumption was documented daily to calculate the polyphenol dose per day for each treatment and to monitor if treatments interfere with the energetic balance, respectively. Both parameters were calculated as a mean \pm standard error of mean (SEM) per treatment. Statistical analysis was performed using multiple linear regression analysis for the Y-axis and the slope.

All animals drank similar quantities with an average daily liquid consumption of 2.9 ml (Water: 3.0 ± 0.3 ml/day, B-PAE: 3.0 ± 0.3 ml/day, placebo: 2.8 ± 0.8 ml/day, CAJ: 2.9 ± 0.8 ml/day and CAJ + B-PAE: 2.9 ± 0.6 ml/day). CAJ- and CAJ + B-PAE- treated animals consumed slightly less liquid than the other groups.

A small difference was observed for food uptake. On average, the animals of the water and B-PAE groups consumed about 3 g chow per day (Water: 3.14 ± 0.33 mg/day and B-PAE: 2.96 ± 0.32 mg/day). Food consumption of the placebo (2.6 ± 0.3 mg/day), CAJ (2.7 ± 0.3 mg/day) and CAJ + B-PAE (2.7 ± 0.2 mg/day) was reduced by 10%.

Overall, at the end of the intervention period, no significant differences between treatments were observed. Nevertheless, water and B-PAE groups drank more and consumed more chow than the groups receiving CAJ, placebo juice or CAJ + B-PAE. This is probably due to the additional calories provided by the carbohydrate content of the juice-based liquids (Fig. 13 A and B).

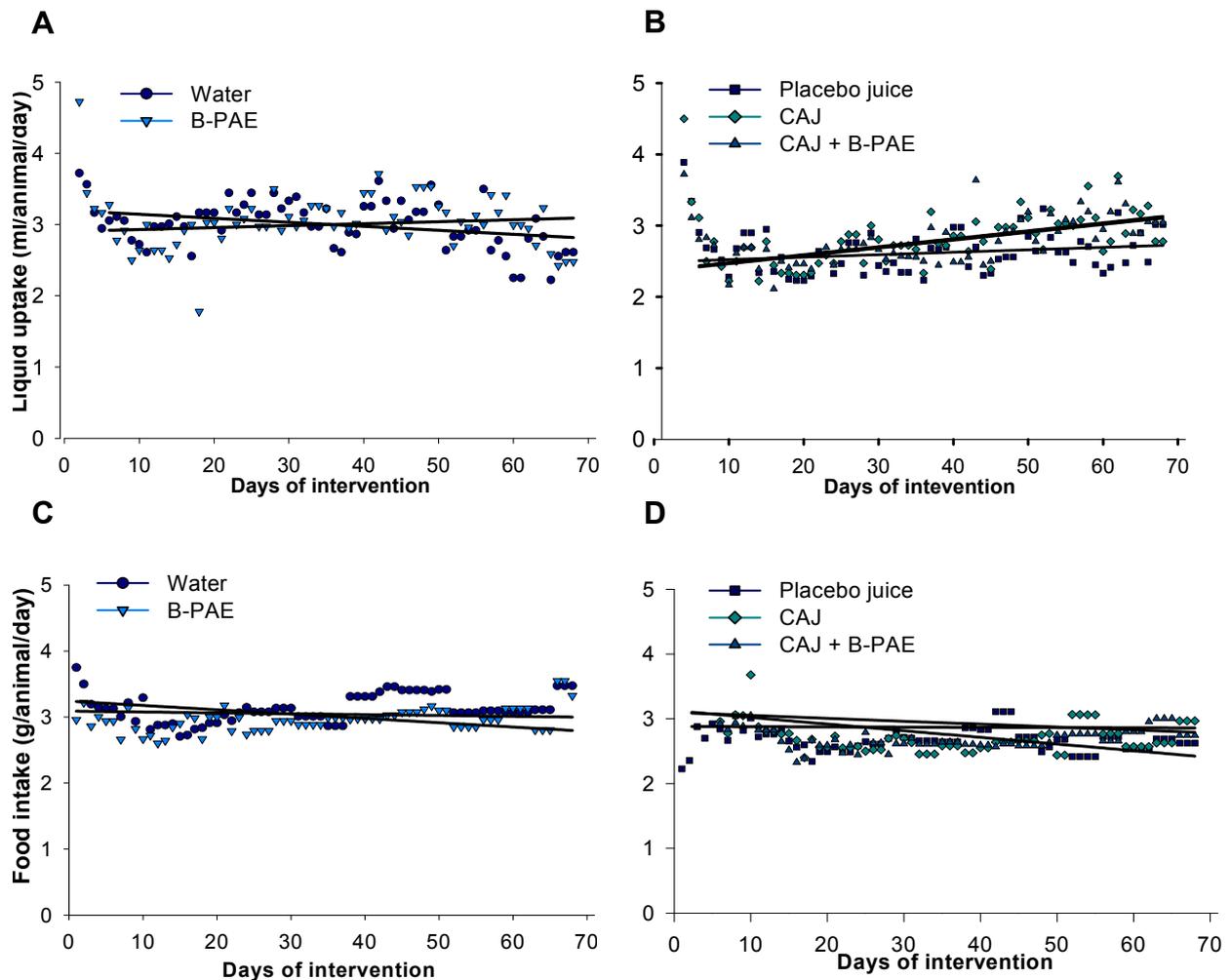


Fig. 13. Daily average intake of liquid (A,B) and food (C,D) during 10 weeks intervention.

Multiple linear regression analysis for Y-axis and the slope. Mean (n=15). The first days of adjustment to the intervention liquids were excluded from the analyses.

3.1.2.2 Body weight

Body weights (BW) were measured per animal twice a week and $BW \pm SEM$ was calculated per treatment average. In the Min mouse model, as a result of the intestinal adenoma development and subsequent intestinal haemorrhages the BW can decrease during intervention. Then, a higher weight may indicate better health condition and could suggest that treatments are well tolerated.

Initially, animals of the water and B-PAE groups started the intervention with similar BW (average \pm SEM; Water: 23 ± 0.2 g and B-PAE: 23 ± 0.3 g). Both treatments resulted roughly in similar body weight changes during the first 40 days of intervention (Water: 24.4 ± 0.3 g and B-PAE 24.3 ± 0.4 g). In contrast, from day 40 of intervention the water group started to increase the BW more slowly, and a loss of weight during the last 14 days was observed (Fig. 14 A). On the other hand, the BW of the B-PAE

group increased until the end of the intervention (Water: 24.6 ± 0.4 g and B-PAE: 25.6 ± 0.4 g). In the last week of intervention, the animals of the water group weighed significantly less than those of the B-PAE (Fig. 14 A).

Placebo, CAJ and CAJ + B-PAE groups started the intervention with similar BW (average \pm SEM; Placebo: 22.6 ± 0.3 g, CAJ: 22.8 ± 0.3 g and CAJ + B-PAE: 22.2 ± 0.2 g). Due to the high liquid of consumption and reduced chow intake, they initially lost about 3% during the first 5 days. Then, the liquid and food consumption normalized. Up to day 30 of intervention all animals gained weight over time similar daily increases (average \pm SEM day 30: Placebo: 22.9 ± 0.3 g, CAJ: 23 ± 0.3 g and CAJ + B-PAE: 23 ± 0.2 g). Between day 30 and 70, the average BW of the placebo group was slightly lower than that of CAJ and CAJ + B-PAE groups. However, ANOVA statistical analysis showed no significant difference in weekly BW gain among the Placebo, CAJ and CAJ + B-PAE treatments throughout the study. After intervention for 10 weeks, the CAJ group had higher BW in comparison to water, placebo juice and CAJ + B-PAE groups (Placebo: 24.4 ± 0.50 g, CAJ: 25 ± 0.4 g and CAJ + B-PAE: 25.2 ± 0.4 g). These differences were not statistically significant (One-way ANOVA with Tukey multiple comparison) (Fig.14 B).

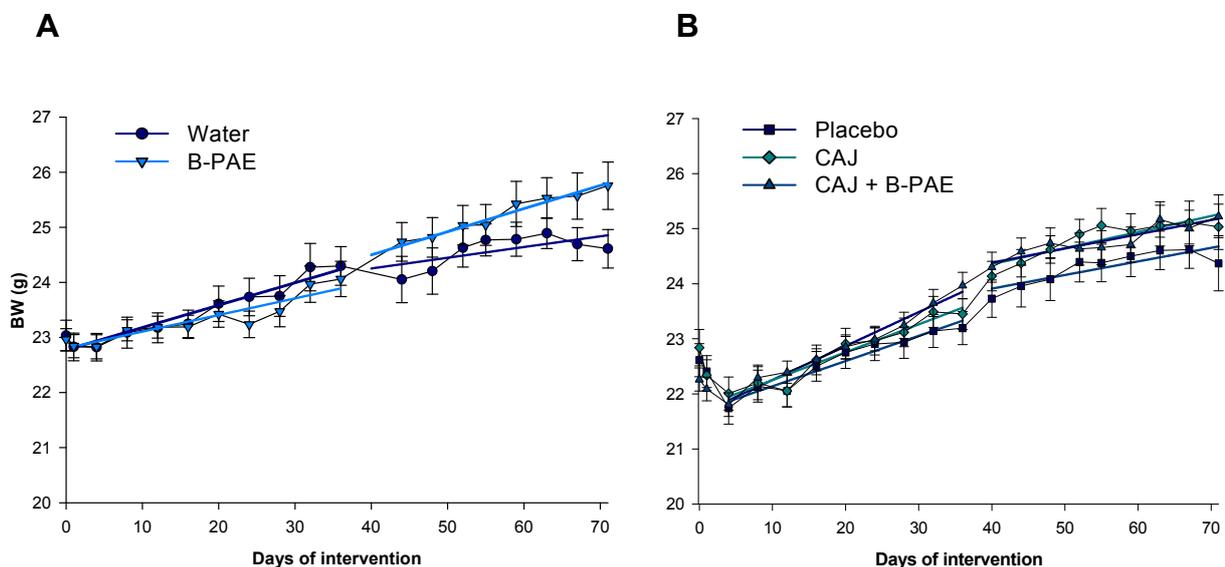


Fig. 14. Average body weights (BW). (A) Water and B-PAE, (a) $p=0.058$ and (b) $p=0.038$ Student's *t*-test comparison and (B) Placebo, CAJ and CAJ + B-PAE, One-way ANOVA with Tukey multiple comparison test. Mean \pm SEM ($n=15$).

3.1.2.3 Daily polyphenol consumption

The mean intake of polyphenols was calculated for each treatment based on the daily mean drink uptake and the total polyphenolic content of CAJ and B-PAE. Values were

normalized by dividing with the average of BW per treatment. Due to difficulties in analyzing oligomeric and polymeric procyanidins, only LMW polyphenols were estimated for CAJ.

Both CAJ and B-PAE 0.2% were given a similar dose (B-PAE: 70.8 mg/kg BW and CAJ: 55.1 mg/kg BW), whereas in the CAJ + B-PAE group animals received nearly twice the quantity of polyphenols (119.3 mg/kg bw) (Tab. 13).

Tab.13. Polyphenol dose during intervention. Data were calculated without procyanidins (PCs) for CAJ.

Treatment	Avg. daily dose [mg/kg bw]	Polyphenol content [mg/l]	Mean daily liquid intake [ml]	Mean B.W. [g]
B-PAE (0.2 % in water)	70.8	562	3.1	24.2
CAJ	55.1 + PCs ^a	452	2.9	23.8
CAJ + B-PAE (0.2%)	119.3 + PCs	1014	2.8	23.8

^aPC: Procyanidins

3.1.3 Multiplicity, distribution and size

Min mice developed multiple intestinal neoplasias in their intestinal tracts within several weeks after birth in a range from 22 to 84 adenomas. As expected, tumors were mostly located in the SI and few were found in the colon. For adenoma scoring, the SI was divided in 3 parts: Int-1 (Proximal SI), Int-2 (Middle SI) and Int-3 (Distal SI). Adenoma multiplicity was determined to investigate whether treatments influence the development from normal tissue to adenomas. Moreover, the size was important to characterize if treatments affect adenoma progression.

Adenoma numbers in SI

In the SI, in the water group 52.1 ± 3.8 adenomas were counted (mean \pm SEM). In contrast, 0.2% B-PAE and CAJ interventions resulted in 34.3 ± 2.4 and 39.6 ± 3.0 adenomas, respectively. Overall, B-PAE and CAJ significantly reduced ($p < 0.05$ by ANOVA) total adenoma number by 24% and 34%, respectively. In the placebo juice

and CAJ+B-PAE groups, 48.7 ± 3.4 and 43.3 ± 2.8 adenomas were scored, correspondingly. This resulted in a non-significant 6% and 17% reduction (Tab.14).

Adenoma numbers in the colon

In the colon, only a few adenomas were found (Water: 1.9 ± 0.3 ; B-PAE: 1.2 ± 0.3 ; Placebo: 1.4 ± 0.3 ; CAJ: 1.6 ± 0.3 ; CAJ + B-PAE: 1.1 ± 0.2). There was no significant difference. Due to the small numbers in colonic adenomas, calculation of % inhibition does not provide meaningfully data (Tab.14).

Tab. 14. Adenoma number, localization and percentage of inhibition in SI and colon

	Treatment	n	Small Intestine (SI)			Total SI	Colon
			Int-1	Int-2	Int-3		
1	Water	17	7.1 ± 0.6^a	18.2 ± 1.9	26.8 ± 2.2	52.1 ± 3.8	1.9 ± 0.33
2	0.2 % B-PAE	17	4.6 ± 0.5 35%*^b	11.1 ± 0.9 39%*	18.7 ± 1.5 30%*	34.3 ± 2.4 34%*	1.2 ± 0.31 30%
3	Placebo	16	6.5 ± 0.8 9%	16.5 ± 1.5 11%	25.8 ± 2.5 4%	48.7 ± 3.4 6%	1.4 ± 0.31 25%
4	CAJ	18	5.3 ± 0.5 25%	13.1 ± 1.1 27%	21.1 ± 2.1 21%	39.6 ± 3.0 24%*	1.6 ± 0.36 16%
5	CAJ + 0.2 % B-PAE	18	6.2 ± 0.5 13%	15.2 ± 1.1 16%	22.4 ± 2.0 16%	43.3 ± 2.8 17%	1.1 ± 0.25 40%

^a Mean \pm SEM

^b percentage of inhibition in comparison to the water control

* $p < 0.05$ One-way ANOVA with Tukey's multiple comparisons test (in red numbers)

Total adenoma numbers in GI tract

Box-plots can be useful to display differences between populations. This graphic representation summarizes the following statistical measures: median and the percentiles. The percentiles measure the spread or dispersion around the median of a data set and they are represented as upper and lower whiskers. The upper whisker is the 25th percentile and the lower the 75th percentiles. The box itself contains the middle 50% of the data.

Overall, in the GI tract, the water group developed 53.9 ± 3.6 adenomas. In contrast, 0.2% B-PAE and CAJ interventions resulted in 35.6 ± 2.4 and 41.2 ± 3.0 adenomas, respectively. Treatments with CAJ and B-PAE 0.2% significantly suppressed tumor incidence compared with water control by 22% and 33% ($p < 0.05$ by ANOVA). Correspondingly, CAJ + B-PAE intervention inhibited tumor load by 17% and placebo juice treatment resulted in an insignificant 7% reduction in adenoma numbers (Fig.15).

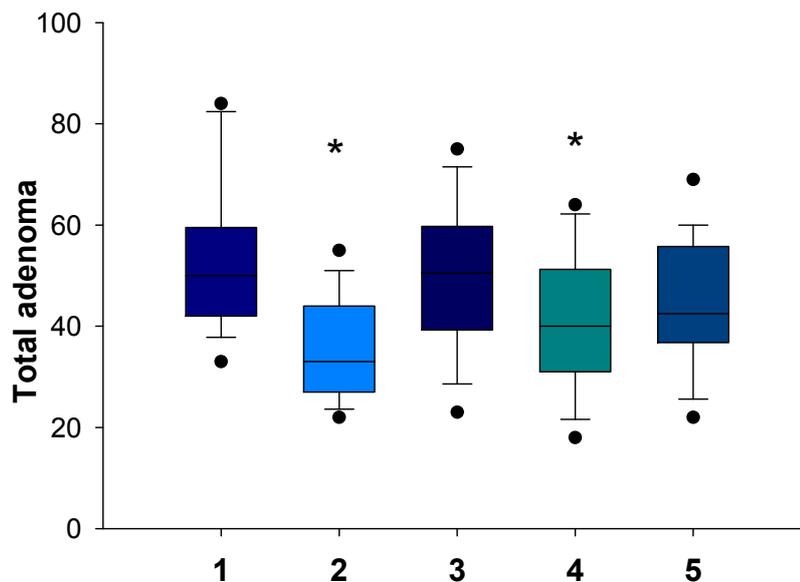


Fig. 15. Box plot of total adenoma distribution in all GI tract. * $p < 0.05$ One-way ANOVA with Tukey's multiple comparisons test. Treatments: (1) Water; (2) B-PAE 0.2%; (3) Placebo; (4) CAJ and (5) CAJ + B-PAE 0.2 %.
Box: interquartile range, line: median, whiskers: 25th and 75th percentile and dots: outliers

Adenoma distribution in the SI

With respect to the distribution of the adenomas in the SI, approximately one half was found in Int-3 (Fig. 16). In each segment, adenoma values were reduced by B-PAE treatment in comparison with the water control (Int-1: control 7.1 ± 0.6 , B-PAE 4.6 ± 0.5 ; Int-2: control 18.2 ± 1.9 , B-PAE 11.1 ± 0.9 and Int-3: control 26.8 ± 2.2 , B-PAE: 18.7 ± 1.5). This resulted in a significant adenoma reduction in all SI segments of 35%, 39% and 30%, respectively ($p < 0.05$ by ANOVA). The highest inhibition was observed in Int-2 (Tab.14 and Fig.16).

CAJ-treatment reduced adenoma counts in all SI segments as well (Tab. 14) representing a 25%, 27% and 21% inhibition of adenoma numbers, correspondingly. Although each part was reduced in a non-significant manner, a significant ($p < 0.05$ by

ANOVA) 24% tumor inhibition was observed in total SI. Again the highest inhibition was observed in Int-2 (Fig. 16). Placebo and CAJ + B-PAE led to non-significant reductions.

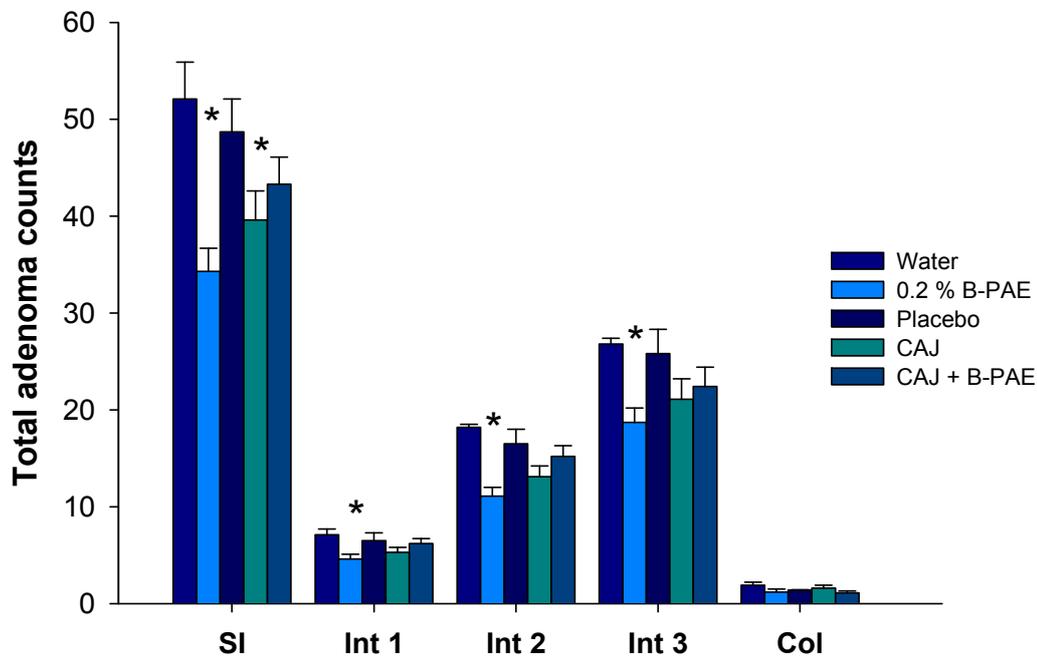


Fig. 16. Adenoma multiplicity and localization in SI and Colon. * $p < 0.05$ One-way ANOVA with Tukey's multiple comparisons test. Mean \pm SEM. Int-1: Proximal SI; Int-2: Middle SI; Int-3: Distal SI.

Influence on adenoma size

In order to discriminate between tumor size, adenomas were classified as small (<1 mm), medium (1-3 mm) and large (>3 mm). According to this distribution most adenomas were of medium size (Water: 80%, B-PAE: 63%, Placebo: 61%, CAJ: 68% and CAJ + B-PAE: 73%). CAJ and B-PAE intervention significantly reduced medium size adenomas (1-3 mm) by 39% ($p < 0.05$) and 49%, respectively ($p < 0.05$) (water: 43 ± 5.0 ; CAJ: 28 ± 3.5 ; B-PAE: 22 ± 3.0). Placebo and CAJ + B-PAE treatments also decreased medium size adenomas by 32% and 25% (Placebo: 29.5 ± 3.3 ; CAJ + B-PAE: 32 ± 3.2), but this decrease was not statistically significant (Fig. 17).

Interestingly, intervention with placebo juice significantly increased ($p < 0.05$ by ANOVA) the total number of small adenomas (<1 mm) by 49% (water: 9.7 ± 1.5 ; placebo: 17.8 ± 2.0), whereas CAJ and B-PAE slightly increased small size adenomas by 14% and 21%, correspondingly. Nevertheless, this increase was not statistically significant (Fig. 17).

In animals receiving B-PAE and CAJ, we detected less medium and large size adenomas in all SI. Moreover, small (< 1mm) adenoma numbers were augmented in distal parts of SI in comparison to the control group. In CAJ + B-PAE treated animals we observed more large adenomas in proximal SI, compared with the control (control 1.0 ± 1.2 and CAJ + B-PAE 1.9 ± 1.0).

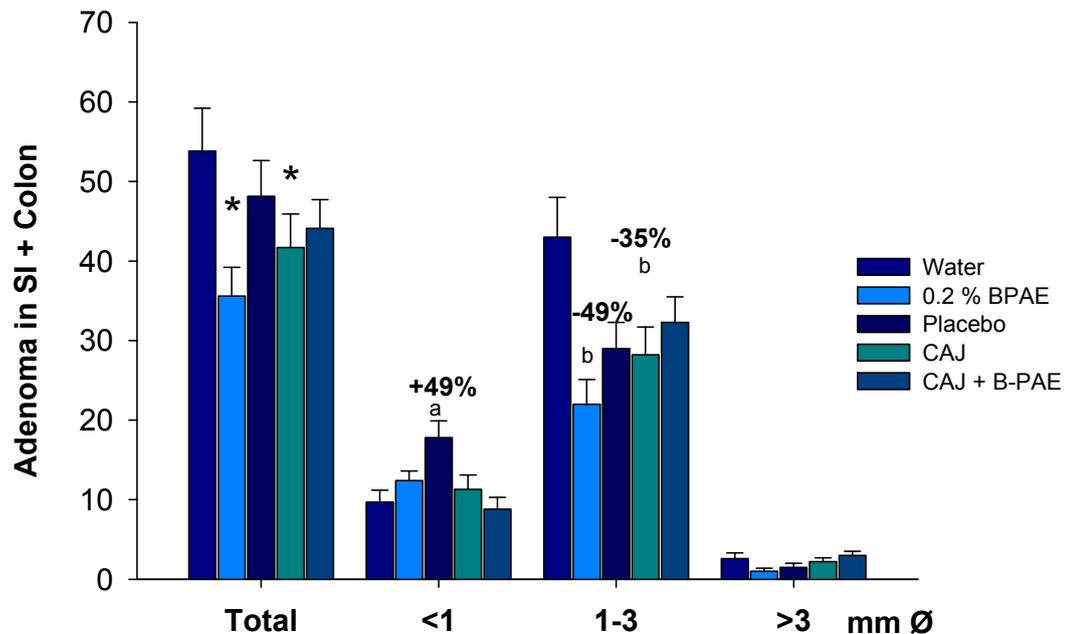


Fig. 17. Adenoma size in SI and colon. Mean \pm SEM. (a) * $p < 0.05$ One way ANOVA with Dunn's multiple comparisons test, (b) $p < 0.05$ One-way ANOVA with Tukey's multiple comparisons test. <1: Small size adenomas; 1-3: Middle size adenomas; >3: large size adenomas.

3.1.4 Study of colon microadenomas

Only few adenomas were found in the colon. Therefore, an evaluation of the effect of the intervention was difficult. To solve this problem, we investigated the possible effects of CAJ and B-PAE treatments on colonic microlesions. As mentioned in the introduction (chapter 1.3.4), colonic microadenomas are found in high frequency and regarded as preneoplastic lesions in the Min mouse colon. So far, no chemopreventive substance has been shown to reduce such preneoplastic lesions in the Min mouse model. A microadenoma compresses surrounding crypts and shows positive reactivity for β -catenin in the cytoplasm (Hata *et al.*, 2006). An example of colon microlesions is shown in the Fig. 18.

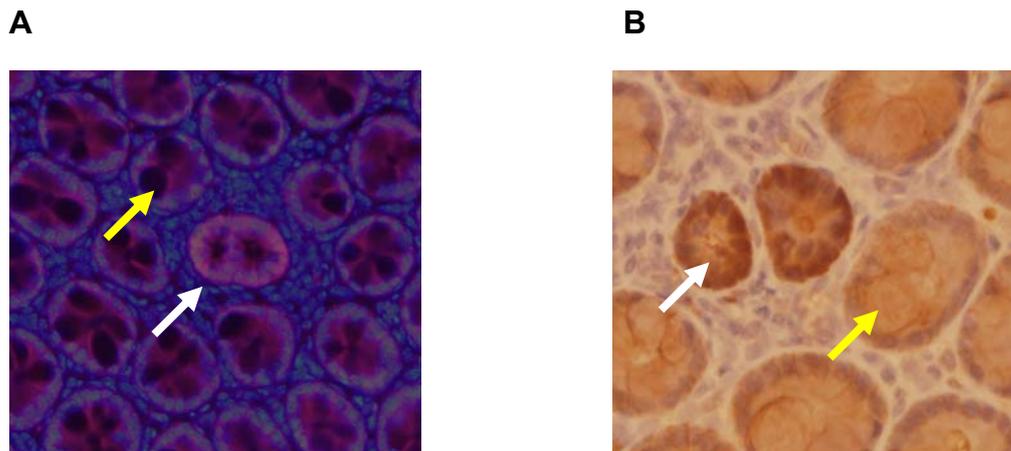


Fig. 18. Min mouse colon microlesions (β -Catenin accumulated crypts). White arrows indicate microlesions and yellow arrows show normal appearing crypts. A) β -catenin immunohistochemistry with fluorescence detection and B) β -Catenin with colorimetric immunostaining.

In the colon, normal crypts have a membrane β -catenin staining and no signs of dysplasia (yellow arrow Fig. 18). On the other hand, BCACs show a nuclear hyperchromatic staining of β -catenin and a higher grade of dysplasia. This represents an activation of the Wnt pathway which is involved in carcinogenesis progression stages.

Immunohistochemistry experiments confirm that such colonic lesions also overexpress for other Wnt signalling targets such as Cyclin D1 and iNos. Moreover, colonic lesions were positive for the Ki67 proliferation marker (Fig. 19).

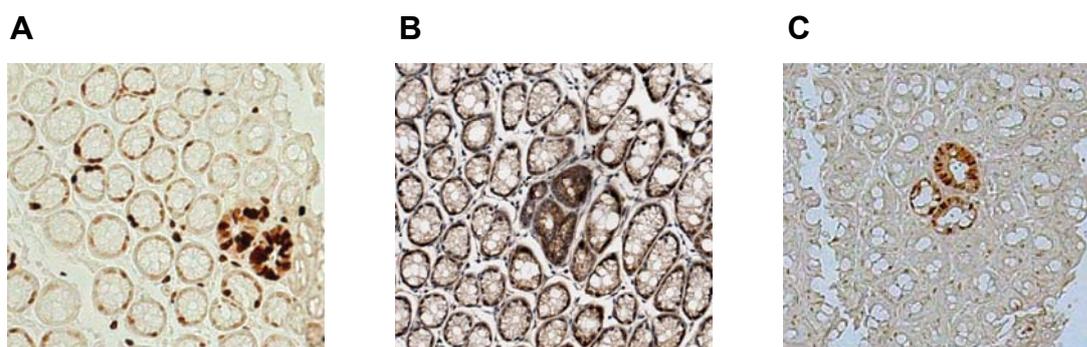


Fig. 19. Min mouse colon microlesions. DAB immunohistochemistry detection of A) Ki67 as proliferation marker; B) iNos; C) Cyclin D1.

The highest number of microlesions was observed in colon preparations of the water group (average \pm SEM; Water: 71.5 ± 12.1 microlesions/cm²). In animals of the B-PAE and placebo treatment groups, 46.1 ± 10.3 and 32.3 ± 7.7 microlesions/cm² were

counted, respectively. Both intervention groups demonstrated the strongest inhibition in numbers of colonic microlesions (Placebo: 55 % and B-PAE: 35% reduction); however, there were no significant differences between treatments (Fig. 20).

Almost similar microadenoma numbers/cm² were detected in colon preparations of CAJ and CAJ + B-PAE treatments (CAJ: 62.1 ± 12.1 microlesions/cm² and CAJ + B-PAE: 55.7 ± 6.3 microlesions/cm²). Both treatments did not significantly inhibit microlesion numbers compared to the water group (CAJ: 22% and CAJ + B-PAE: 13.3%).

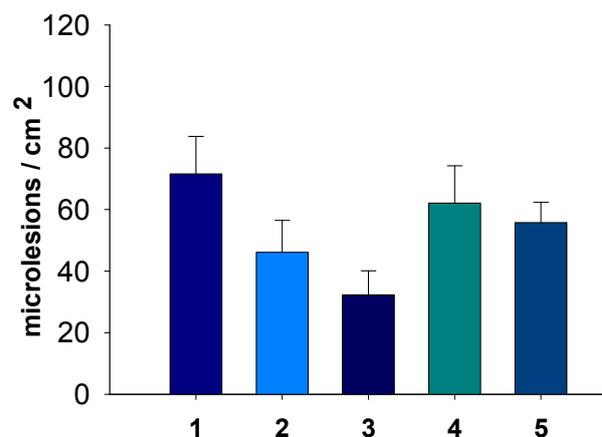


Fig. 20. Min mice colon microlesions in the different treatment groups. Total microlesions. Mean ± SEM. Treatments: (1) Water; (2) B-PAE 0.2%; (3) Placebo ;(4) CAJ and (5) CAJ + B-PAE 0.2%.

Microlesion distribution

Each colon was divided in 3 segments (proximal, middle and distal). In water treated animals, comparable numbers of microlesions were identified in all segments (Prox: 23.5 ± 4.9; Middle: 20.7 ± 3.0 and Distal: 27.4 ± 9.8 microlesions/cm²).

In the proximal part of the colon, we counted 23.5 ± 4.9 microlesions/cm². Intervention with placebo juice and CAJ strongly reduced numbers of preneoplastic lesions to 6.7 ± 2.0 and 7.4 ± 2.0, respectively. B-PAE and CAJ + PAE also reduce microlesions multiplicity to 11.0 ± 2.1 and 17.2 ± 3.0, correspondingly. Overall, statistically significant reductions ($p < 0.05$ by ANOVA) were found by Placebo (71%) and CAJ (68%) treatments. Although not statistically significant, reductions were also found in the CAJ + B-PAE (53%) and B-PAE (27%)- treated groups (Fig. 21 A).

In the middle part, 20.7 ± 3.0 microlesions/cm² were found in colon samples of the water-treated controls. Only intervention with placebo juice reduced the number to

11.6 ± 1.2 microlesions/ cm^2 . However, this reduction (43.8%) was not statistically significant. In all other intervention groups, similar levels of microlesions/ cm^2 compared with the water group were counted (B-PAE: 18.5 ± 5.7 , CAJ 20.7 ± 6.2 , CAJ + B-PAE: 17.5 ± 2.5). This resulted in non statistically significant reductions (Fig. 21 B).

In the distal part of the colon, 27.4 ± 9.8 microlesions/ cm^2 were identified in water-treated animals. Only in the placebo group with 13.5 ± 3.6 microlesions/ cm^2 and in the B-PAE group with 13.7 ± 3.0 microlesions/ cm^2 , the numbers of lesions were reduced. In contrast, CAJ treatment increased preneoplastic lesions to 32.7 ± 4.8 (20% in comparison with the water group). Treatments with CAJ + B-PAE resulted in 27.2 ± 5.3 microlesions/ cm^2 . This represents a reduction by about 50% for placebo and B-PAE treatments (data were not statistically significant). On the other hand, for the CAJ + B-PAE treatment no inhibition was found (Fig. 21 C).

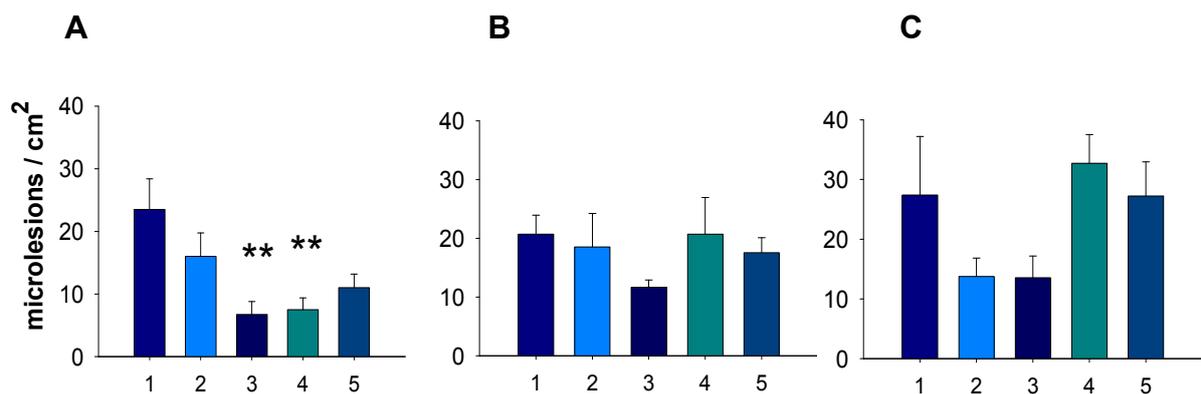


Fig. 21. Min mice colon microlesions in different segments. Mean \pm SEM. A) Proximal part of the colon; B) Middle part; C) Distal. * $p > 0.05$ One-way ANOVA with Tukey's multiple comparisons test (in comparison to water control). Treatments: (1) Water; (2) B-PAE 0.2%; (3) Placebo; (4) CAJ; (5) CAJ + B-PAE 0.2%.

3.2 Pathologic analyses

3.2.1 Spleen weights

It has been well documented that Min mice display enlarged spleen and decreased hematocrit values. This is attributed to adenoma progression and the subsequent intestinal bleeding. Therefore, Min mice present anemia, and extramedullary hematopoiesis takes place in the spleen, increasing its weight. Fig. 22 summarizes average spleen weights and hematocrit values.

In all Min mice we observed enlarged spleen weights in comparison to wild-type (Wt) mice (mean \pm SEM; Wt: 2.81 ± 0.2 mg/g BW). The most increased spleen weights were observed for the water-, CAJ + B-PAE- and placebo treatment groups (water 14.7 ± 1.0 mg/g BW, Placebo: 14.0 ± 2.3 mg/g BW and CAJ + B-PAE: 17.5 ± 1.2 mg/g BW). Compared to Wt, this is a 5-, 5- and 6-fold induction, respectively. However, B-PAE and CAJ treatment groups showed less increased spleen weights (B-PAE: 9.8 ± 1.0 mg/g BW and CAJ: 13.0 ± 1.1 mg/g BW). This corresponds to an approximately 3- and 4-fold induction compared to Wt spleens. Taking water treatments as a reference, a significant reduction ($p < 0.05$ by ANOVA) was observed for the B-PAE treatment group. In addition, CAJ reduced the spleen weight in a non-significant manner (Fig. 22 A).

3.2.2 Hematocrit values

Hematocrit values in Wt animals were approximately $40 \pm 0.4\%$. Animals of the water-, CAJ + B-PAE- and placebo group showed the lowest average hematocrit values (Water: $27.64 \pm 1.3\%$, Placebo: $25.8 \pm 1.1\%$ and CAJ + B-PAE: $25.4 \pm 0.8\%$). B-PAE and CAJ treatments increased the hematocrit value (B-PAE: $30.8 \pm 1.4\%$ and CAJ: $28.8 \pm 1.2\%$) in comparison with the water control group. Like for the spleen weights, there was a statistically significance ($p < 0.05$) comparing hematocrit values of the B-PAE treated group with water treated control. Furthermore, CAJ treatment also increased the hematocrit but this result was not statistically significant (Fig. 22 B).

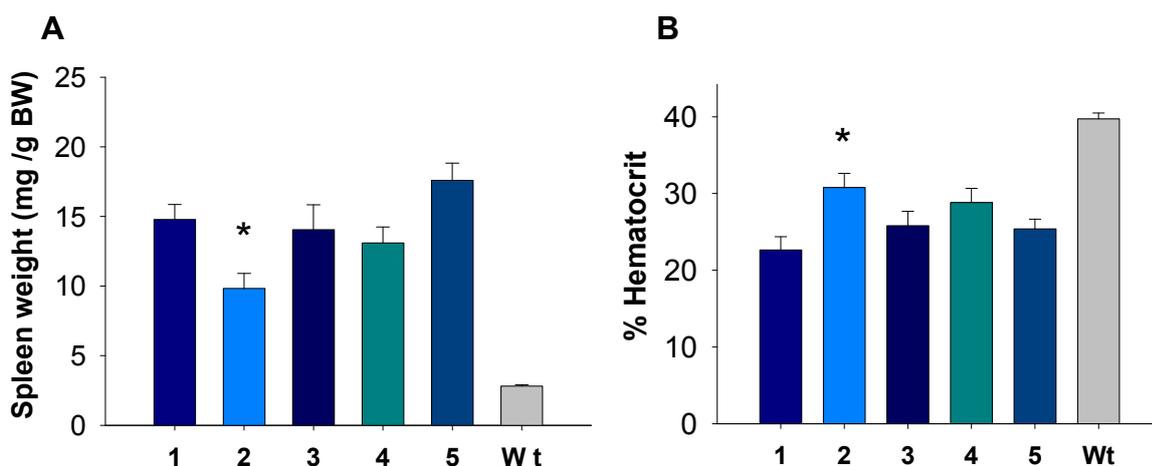


Fig. 22. Changes in A) Spleen Weight and B) Hematocrit values * $p < 0.05$ One-way ANOVA with Bonferroni's multiple comparisons test (in comparison with water control). Treatments: (1) Water; (2) B-PAE 0.2%; (3) Placebo; (4) CAJ; (5) CAJ + B-PAE 0.2% and Wt (Wild type, taken from previous Min mouse experiment performed by Dr. Lydia Pan).

3.2.3 Histopathological analysis

(collaboration with Prof. Karbe, Wülfrath)

Animals were randomly selected for the following histological examinations:

Liver:

Necrobiotic foci

Extramedullary hematopoiesis

Lobular pattern

Cytoplasmatic vesicles

Glycogen content

Nuclear morphology

Spleen

Extramedullary hematopoiesis

Liver

Liver slices were stained with H&E to determine differences at the histo-morphological level (necrobiotic foci, extramedullary hematopoiesis and lobular pattern) and at the cytoplasmatic level (cytoplasmatic vesicles, glycogen and nuclear morphology).

Cytoplasmatic vesicles are a marker for liver stress and toxicity. No signs of toxicity were observed in this study for all treatments. Since the animals were kept in metabolic cages for 12 h before termination of the experiment and were therefore food deprived, all animals displayed reduced glycogen liver reserves. To determine associated stress through mutagenic substances, the nuclear morphology was observed. In H&E liver staining, these nuclei are recognized for its increased volume with strong hematoxylin staining. This is mainly a result of polyploidy. All Min mice showed normal nuclear morphology and no differences between treatments were identified (Tab. 15).

Another pathological examination to investigate the effects after substance exposure is the formation of an increased lobular structure in the liver. Generally, this is associated with liver hypertrophy which can be identified in an increase of Kupffer cells. Unexpectedly, some water control animals had symptoms of liver hypertrophy. However, all animals from the B-PAE treated group contained signs of hypertrophy but no adverse effects through metabolic exposure were reported (Tab 15).

Necrobiotic foci are another morphological structure to examine putative liver stress through compound exposure but no differences were observed for all treatments. All animals presented high extramedullary hematopoiesis which is associated to GI bleeding (Tab. 15).

Tab. 15. Histopathologic liver examination.

Groups	Necrobiotic foci	E.H grade	Lobular pattern
Water	4/4	3/4	3/4
0.2 % B-PAE	3/6	4/6	6/6
Placebo	5/8	5/8	3/4
CAJ	4/4	4/4	2/4
CAJ + B-PAE	5/9	6/9	4/9

Different animal numbers were used for pathological analysis.

E.H. (extramedullary hematopoiesis: erythroid, myeloid and megakaryocytes foci)

Spleen

In the spleen, almost all animals revealed strong extramedullary hematopoiesis (EH). The increase in mean spleen mass is consistent with EH, possibly secondary to intestinal bleeding. The EH grade was determined detecting hematopoietic foci in the spleen, which are clumps of erythroid and myeloid precursors together with megakaryocytes.

For this parameter, 12 animals per group were examined. For the evaluation, each spleen was rated in a range of 0 (low grade of EH) to 5 (high grade of EH) and then an average was calculated. B-PAE treatment resulted in a reduced grade of extramedullary hematopoiesis. One placebo group also showed a reduced grade (Tab.16). So far, the difference between both placebo groups can not be explained.

Tab. 16. Histopathologic spleen examination.

Groups	n	E.H. grade
Water	6	4.5 / 5
	6	4.1 / 5
0.2 % B-PAE	6	3.2 / 5
	6	3.5 / 5
Placebo	6	4.6 / 5
	5	3.5 / 5
CAJ	6	4.8 / 5
	6	4.2 / 5
CAJ + B-PAE	6	4.5 / 5
	6	4.6 / 5

Grades: From 0 (low grade) to 5 (high grade)

EH (extramedullary hematopoiesis)

3.3 Urine analyses

3.3.1 Urine volume

Urine was collected to determine the bioavailability of apple polyphenols. This analysis is very useful to characterize which polyphenols may act locally in the SI or if they are absorbed and further conjugated in the liver. Local or systemic effects can be responsible to inhibit adenoma development in SI.

After finishing the intervention, a set of Min mice from each experimental group were housed in rodent metabolism cages to collect urine for different determinations. During this time, animals had only access to intervention drinks, but not food. It is worth pointing out that animals were fasted overnight for 12 h before collecting urine samples. Therefore, data were analyzed independently for groups with fasted (Water vs. B-PAE) and non-fasted animals (Placebo vs. CAJ and CAJ + B-PAE). The difference is because non-fasted animals received sugars as a source of energy from their drinks, whereas fasted animals did not received such carbohydrates.

As observed in Fig. 23, urine volumes were affected by treatments (mean \pm SEM; Water: 0.97 ± 0.08 ml, B-PAE: 0.79 ± 0.09 ml, Placebo: 4.8 ± 0.31 ml, CAJ: 4.4 ± 0.21 ml and CAJ + B-PAE: 3.9 ± 0.06 ml). Urine volumes of the placebo, CAJ and CAJ + B-

PAE groups were increased up to 4-fold ($P < 0.001$ by ANOVA) in comparison with the water and B-PAE groups (Fig. 23). Since the liquid uptake was not monitored during the period in the metabolic cages, it is not clear whether the increased urine volume is due to increased consumption of these carbohydrate- containing drinks (to prevent being hungry).

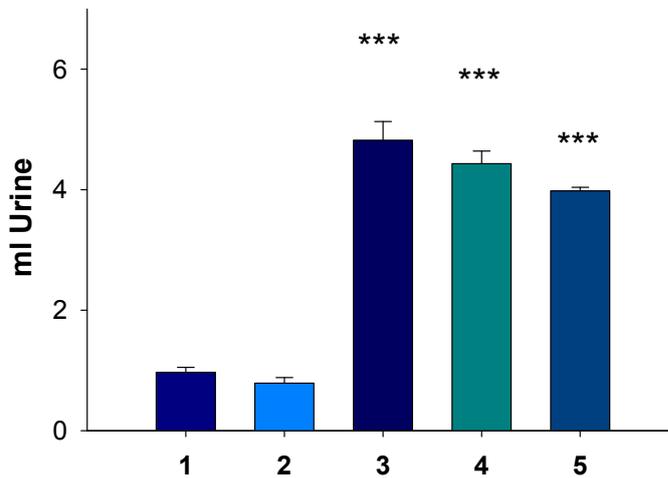


Fig. 23. Urine volume. Urine was collected over night. 3 mice were allocated per cage (Mean \pm SEM) *** $p < 0.001$ One-way ANOVA with Bonferroni's multiple comparisons test (in comparison to water control). Treatments: (1) Water ;(2) B-PAE 0.2%; (3) Placebo; (4) CAJ and (5) CAJ + B-PAE 0.2%.

3.3.2 Minerals

Mineral and carbohydrate analyses were performed to better understand differences in urine volume. Dietary potassium intake can lead to hyperkalemia, which increases urine excretion as a physiological response to remove excess of potassium ions. The renin-angiotensin system in the kidney is involved in this process and may increase urine volumes (Kacsoh et al., 2000).

It has been reported that AJ contains up to 1.1 g/L of potassium (Jarman *et al.*, 2001). Potassium and calcium ions were determined using atomic absorption spectrometry. All data were calculated as mean \pm SEM and normalized to mg creatinine per treatment.

In the urine of placebo- treated mice, 0.87 ± 0.18 mg Ca^{2+} /mg creatinine was detected. In urine of CAJ- and CAJ + B-PAE- treated animals concentrations were increased up to 1.17 ± 0.15 Ca^{2+} /mg creatinine and 1.33 ± 0.42 mg Ca^{2+} /mg creatinine. However, values showed a high SEM.

Potassium analysis resulted in similar urinary values for all three intervention groups, 11.9 ± 0.9 K⁺/mg creatinine for the placebo group, 11.3 ± 0.5 K⁺/mg creatinine for the CAJ treated animals and 11.9 ± 1.1 K⁺/mg creatinine for the mice receiving CAJ + PAE. Overall, there were no statistically significant differences between treatments in both Ca²⁺ and K⁺ determination (Tab.17). Water and B-PAE treatment groups were not determined because of limited urine availability.

Tab. 17. Ca²⁺ and K⁺ determination in urine

Treatment	Urine		
Measure	Ca ²⁺ (mg/mg creat)	K ⁺ (mg/mg creat)	n
Placebo	0.87 ± 0.18^a	11.9 ± 0.9	6
CAJ	1.17 ± 0.15	11.3 ± 0.5	4
CAJ+B-PAE	1.33 ± 0.42	12.1 ± 1.1	6

^a Mean \pm SEM. Data normalized to creatinine (creat).

3.3.3 Carbohydrates

As already mentioned, glucose and fructose are the most common sugars in CAJ and placebo drink. Drinks contain approximately 20 g/L glucose and 70 g/L fructose. As in diabetes, higher amounts of sugars can induce polyuria. We performed a urinary carbohydrate analysis for groups receiving a juice-based intervention.

In the group treated with placebo juice, we measured 2.7 ± 2.0 mg glucose/mg creatinine in the urine. Intervention with CAJ + B-PAE and CAJ reduced the glucose concentrations in the urine to 0.61 ± 0.06 mg glucose/ mg creatinine and to 2.1 ± 1.4 mg glucose/ mg creatinine, respectively.

Normalized fructose levels in urine were higher than glucose concentrations. In placebo treated animals, 11.5 ± 6.1 mg fructose/mg creatinine were observed. With mice treated with CAJ and CAJ + B-PAE, concentrations of 8.7 ± 4.4 and 4.4 ± 1.7 mg fructose/mg creatinine were measured, respectively.

All differences were not statistically significant and values showed a high standard error (Tab. 18). Again, urine of water and B-PAE treated animals was not analyzed due to limited availability.

Tab.18. Glucose and fructose determination in urine

Treatment	Urine			
	Assay	Glucose (mg/mg creat.)	Fructose (mg/mg creat.)	n
Placebo		2.7 ± 2.1 ^a	11.5 ± 6.1	5
CAJ		2.1 ± 1.4	8.7 ± 4.4	3
CAJ+B-PAE		0.6 ± 0.06	4.4 ± 1.7	5

^a Mean ± SEM. Data normalized to creatinine (creat)

3.3.4 Analysis of phenolic compounds and metabolites

(collaboration with Jn.Prof. Elke Richling and Ms. Hannah Bergmann)

Quantification of apple juice polyphenols and metabolites by HPLC separation coupled with UV detection (HPLC/UV) was very useful to study the bioavailability of the phenolic apple compounds. If polyphenols are found in urine, that implies absorption in the liver, possibly effects in plasma, and systemic distribution. The HPLC/UV technique enabled the identification of 8 polyphenols and their metabolites in urine from Min mice (epicatechin, quinic acid, 4-hydroxy-phenylacetic acid, ferulic acid, 3,4-di-hydroxy-phenylacetic acid, caffeic acid and isoferulic acid). Data were given in mean ± SEM and in µg polyphenol/mg creatinine (Fig. 24).

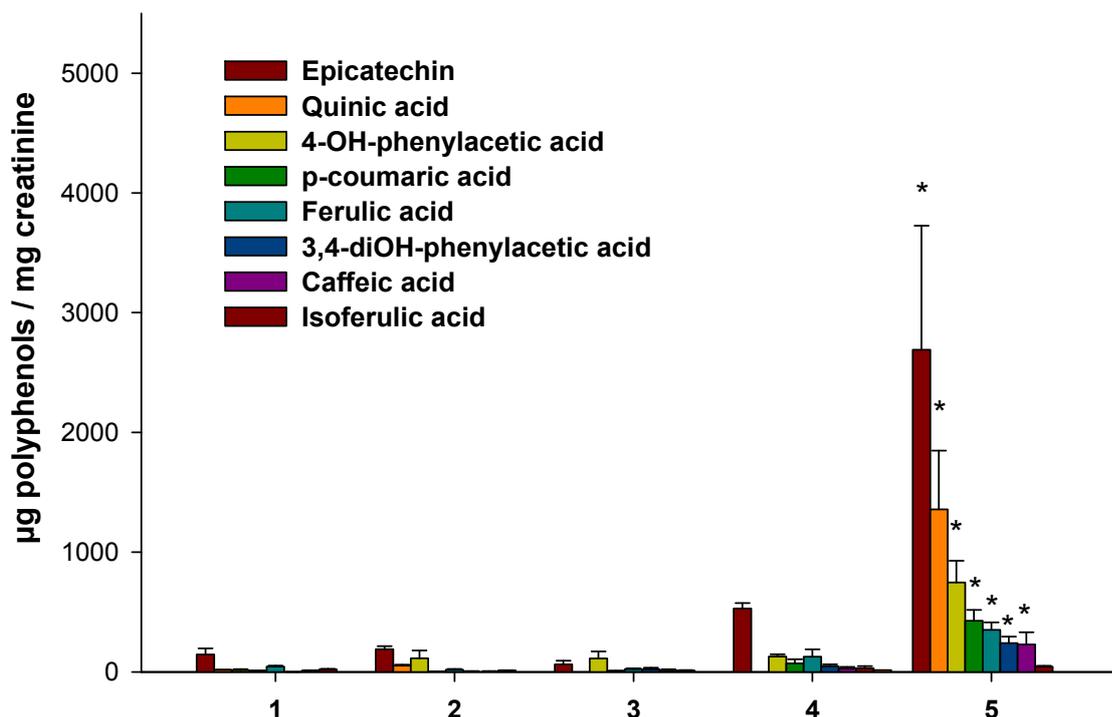


Fig. 24. Concentration of polyphenols and metabolites in urine of Min mice. Mean \pm SEM.

Data normalized to creatinine. Treatments: (1) Water; (2) B-PAE; 0.2%; (3) Placebo; (4) CAJ and (5) CAJ + B-PAE 0.2%. * $p < 0.05$ One-way ANOVA with Tukey's multiple comparisons test. (Each group $n=4$).

All 8 compounds were detected in urine collected from all groups, although at different concentrations. Only in urine of mice treated with CAJ + B-PAE we identified a statistically significant increase ($p < 0.05$ by ANOVA) in 7 polyphenols and metabolites compared with rest of groups (in $\mu\text{g polyphenols/mg creatinine}$; epicatechin: 2690.1 ± 1035.9 , quinic acid: 1358.2 ± 489.9 , 4-hydroxy-phenylacetic acid: 747.0 ± 181.6 , ferulic acid: 458.54 ± 89.6 , 3,4 di-hydroxy-phenylacetic acid: 350.1 ± 64.6 , caffeic acid: 240.5 ± 55.6 and isoferulic acid: 230.8 ± 50.1). In the urine of CAJ-treated animals, urinary excretion of 5 metabolites could be detected in considerable amounts compared to water control (in $\mu\text{g polyphenols/mg creatinine}$; epicatechin: 530.9 ± 44.5 , 4-hydroxy-phenylacetic acid: 128.0 ± 19.2 , ferulic acid: 71.1 ± 34.2 and 3,4 di-hydroxy-phenylacetic acid: 126.2 ± 30.4). All other polyphenols were detected at lower concentration (Fig. 24).

In urine of water-, placebo juice- and B-PAE- treated animals, low amounts of all compounds and metabolites, respectively, were found. Only epicatechin was detected

in appreciable amounts (in μg polyphenol/mg creatinine; Water: 146.2 ± 50.1 , placebo: 190.0 ± 26.1 and B-PAE: 63.3 ± 30.4). Small amounts of quinic acid (in μg polyphenol/mg creatinine: 52.8 ± 7.6) and 4-hydroxy-phenylacetic acid (in μg polyphenol/mg creatinine: 114.0 ± 65.3) were found in urine from B-PAE animals. Since all 8 metabolites were detected of very low amounts in urine of water and placebo treated mice, these results indicate that some polyphenols and metabolites may be derived from food uptake (Fig. 24).

Overall, intervention with CAJ + B-PAE resulted in higher urinary excretion of all analysed metabolites in comparison with all other treatments. In urine from the CAJ group, these compounds were also detected but approximately at 5 fold lower concentrations compared to CAJ + B-PAE. Therefore, these results imply that in the CAJ + B-PAE group a part of the high molecular weight polyphenols such as procyanidins are absorbed and metabolized in the liver. In contrast, when the animals received CAJ or B-PAE, the majority of polyphenols may remain in the GI tract.

3.3.5 Urine inflammatory markers

3.3.5.1 Nitric oxide (NO)

Measurement of NO in biological fluids has attracted attention in the field of inflammation biomarkers. Upregulation of iNOS, and the consequent overproduction of NO, has been implicated as a promoter of several diseases including cancer (Itzkowitz and Yio, 2004; Wink *et al.*, 1998). Detection of NO in urine is associated with inflammation and its increase is detected in colon cancer patients (Groschel *et al.*, 1992). We were interested to study whether the anti-inflammatory *in vitro* potential of apple juice is also detected *in vivo*. After detecting polyphenols in urine of CAJ and CAJ+B-PAE we investigated the possible effect on NO production. Moreover in Min mice, administration of iNOS inhibitors or iNOS gene knock out results in suppression of intestinal polyposis in all GI tract (Ahn and Ohshima, 2001).

Total NO detection ($\text{NO}_2^- + \text{NO}_3^-$) revealed similar urinary levels in all treatment groups (μmol total NO / mg creatinine; Water: 2.20 ± 0.1 , B-PAE: 2.03 ± 0.18 Placebo: 2.09 ± 0.30 , CAJ: 1.90 ± 0.11 and CAJ + B-PAE: 2.38 ± 0.32). Treatments did not

significantly affect urinary nitrite excretion and fasting effects were not observed (Tab. 19).

3.3.5.2 Protein content

Proteinuria is associated with nephrotoxicity and common in diabetes and inflammation. In addition, it has been suggested as a paraneoplastic phenomenon in CRC (Roumen and Wijnen, 1998). Protein was detectable in the urine of all animals independent of the intervention. Normalized levels in the water control group were in the range of 4.0 ± 0.3 μg protein/ mg creatinine. Unexpectedly, intervention with B-PAE significantly increased urinary protein levels by 35% to 6.2 ± 0.7 μg protein/ mg creatinine. Intervention with placebo juice, CAJ and CAJ + B-PAE did not influence protein levels in the urine, indicating that polyphenol consumption as such may not directly be responsible for the observed effects in the B-PAE group (Tab. 19). Rather, the combination of fasting and polyphenol consumption may lead to higher urinary protein excretion.

Tab. 19. Effect of treatments on urine parameters

Treatment	Assay			
	$\text{NO}_2^- / \text{NO}_3^-$ (nmol /mg creat.)	n	Protein (μg /mg creat.)	n
Water	2.20 ± 0.11^a	14	4.0 ± 0.30	12
0.2 % B-PAE	2.03 ± 0.18	17	$6.2 \pm 0.68^*$	14
Placebo	2.09 ± 0.30	17	4.8 ± 0.51	16
CAJ	1.90 ± 0.11	15	3.8 ± 0.48	15
CAJ+B-PAE	2.38 ± 0.32	17	3.7 ± 0.44	17

^a Mean \pm SEM. Data normalized to creatinine (creat)

* $p=0.01$ Student's t-test comparison versus water control.

3.4 Plasma analyses

3.4.1 ORAC

The HPLC experiments revealed that some metabolites derived from CAJ and B-PAE were detectable in urine. It was of interest to study whether these phenolic compounds exert systemic anti-oxidant effects in the plasma. Therefore, an ORAC assay with plasma from Min mice was performed. Data were normalized to protein content.

As summarized in Tab. 20, all treatments resulted in similar plasma ORAC values in the range of 11-12 ORAC units/ μg protein. No statistically significant differences were observed (Tab. 20). Determination of protein concentration in plasma resulted in a range of 35.8-37.0 mg/ml for water, placebo, CAJ and CAJ + B-PAE treatments groups. B-PAE treatment increased in a non-significant manner the plasma protein levels to 42 mg/ml (Tab 20). According to Green *et. al*, C57BL/6J male mice contain about 52.0 ± 1.0 mg/ml of protein in serum (Green *et. al*, 1966).

Tab. 20 Effect of treatments on protein concentration and antioxidant capacity (ORAC) in plasma

Treatment	Determination		
	protein (mg/ml)	ORAC (units/ μg)	n
Water	37.0 ± 2.4^a	11.8 ± 0.5	17
0.2 % B-PAE	42.0 ± 1.3	11.0 ± 0.9	13
Placebo	36.5 ± 1.4	11.9 ± 0.9	16
CAJ	35.8 ± 1.6	11.3 ± 0.5	18
CAJ+B-PAE	37.0 ± 1.0	12.1 ± 1.1	17

^aMean \pm SEM. ORAC data normalized to protein (μg)

3.4.2 PGE₂

We next tested the hypothesis whether components of CAJ or B-PAE may inhibit COX activity as another indication of anti-inflammatory activity (Wu, 2005). PGE₂ are synthesized by the microsomal prostaglandin synthase E (mPGE-synthase). The rate-

limiting enzymes in PGE₂ synthesis are Cox-1 and Cox-2 enzymes. For that reason, detection of PGE₂ in plasma is a good biomarker to detect influences on Cox activity. Data were given in pg PGE₂/ μg protein. PGE₂ was quantified using an ELISA-based method. PGE₂ levels were normalized to protein levels. As summarized in Tab. 21, PGE₂ plasma levels were in the range of 119-137 pg/μg. Overall, no statistically significant difference was measurable (Tab. 21).

Tab. 21 Effect of treatments on PGE₂ levels in plasma

Treatment	Assay	
	PGE ₂ (pg/μg prot)	n
Water	118.9 ± 16.2 ^a	18
0.2 % B-PAE	119.0 ± 16.6	16
Placebo	136.9 ± 16.9	16
CAJ	148.7 ± 19.5	15
CAJ+B-PAE	121.4 ± 21.8	16

^a Mean ± SEM. Data normalized to protein (μg)

3.5 Influence on liver enzymatic activities

Certain dietary ingredients may modulate biotransformation of carcinogens. Biotransformation is the process whereby a substance is changed from one chemical to another (transformed) by a chemical reaction within the body (Rushmore and Kong, 2002). The liver is the first organ for biotransformation.

Enzymatic activities were determined to analyze whether the *in vitro* chemopreventive potential of CAJ and B-PAE may be relevant in an *in vivo* system. We focused our experiments on Phase I enzymes (Cyp1A1 and Cyp2B1), Phase II enzymes (GST, QR and TrxR) and glutathione (GSH) levels. These parameters were analyzed in liver microsomes (Phase I enzymes) and cytosolic preparations (Phase II enzymes and GSH). Data were normalized to the respective protein content.

3.5.1 Liver weights

As already described, all animals were housed in metabolic cages with free access to drinks. In this section, fasted vs. non-fasted animals were compared independently. The first observation associated with fasting was a statistically significant reduction of liver weight, which is indicative of glycogen degradation (fasted (water and B-PAE) 0.042 ± 0.005 g/g BW vs. not-fasted (placebo juice, CAJ and CAJ + B-PAE) 0.052 ± 0.005 g/g BW). Liver weights were approximately 20% reduce. This reduction was strongly associated with a decrease in protein concentration (fasted: 5.87 ± 0.09 and not fasted: 7.02 ± 0.01 mg/ml) (Fig. 25).

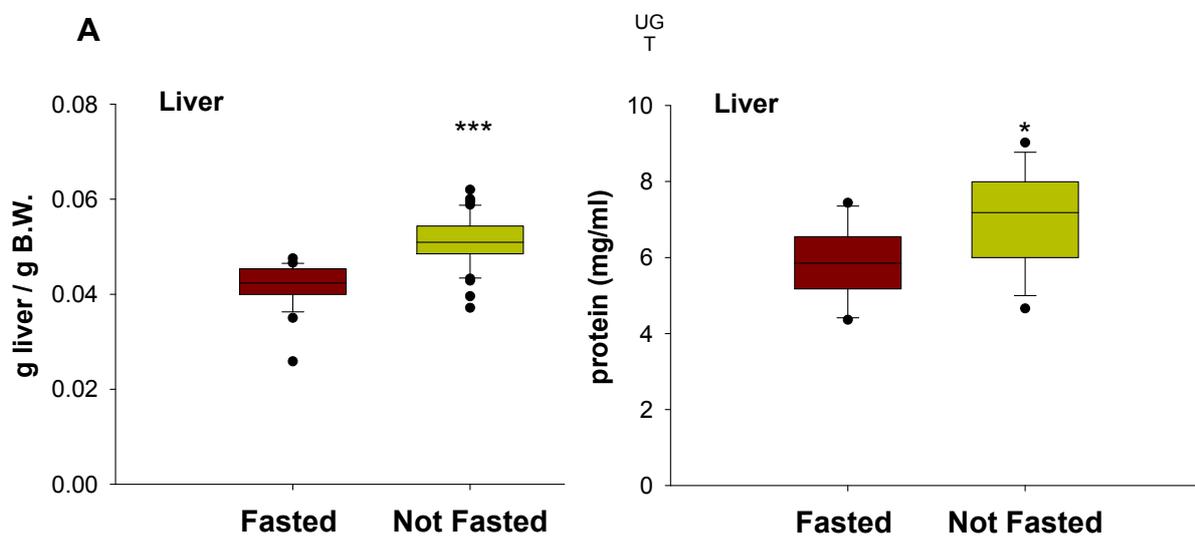


Fig. 25. Effects of fasting on A) liver weights (g liver/ g BW) and B) protein content. (mg/ml)

*** $p < 0.001$ and * $p < 0.05$ Student's t-test comparison. Box: Interquartile range; line: median; whiskers: 25th and 75th quartile; dots: outliers. Fasted group: water, B-PAE; Not-fasted group: Placebo, CAJ and CAJ + B-PAE.

3.5.2 Phase I enzymes

Metabolic activation is performed by cytochrome P450 (Cyp) proteins (phase I enzymes). This family of proteins acts as monooxygenases. Apple polyphenols such as quercetin have been described *in vitro* as extremely potent inhibitors of Cyp1A activity (Zessner *et al.*, 2008). Therefore, it was of interest to measure the effect of our interventions on hepatic Phase I enzyme activities. As shown in Fig. 26, fasting had a striking effect on Cyp1A activity. In the fasted group, the mean microsomal activity (18.4 ± 7 nmol/min/mg) was about 50% lower than in the not-fasted groups ($40.5 \pm$

14.5 nmol/min/mg; $p < 0.001$ Student's t-test). Within the two groups, we did not detect statistically significant differences (Tab. 22). In contrast to Cyp1A activity, Cyp2B1 was not influenced by fasting. Also, no statistically significant changes were measurable within the fasted and not-fasted groups, respectively (Tab. 22).

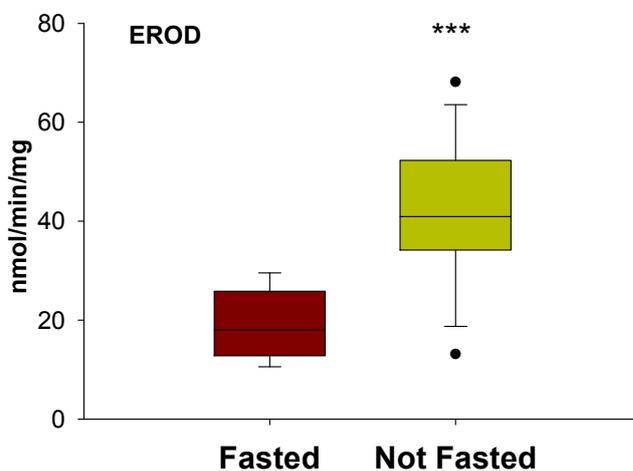


Fig. 26. Fasting effects on hepatic Cyp1A1 activity.). *** $p < 0.001$ Student's t-test comparison.

Tab. 22. Effects of treatments on liver microsomal enzyme activities

Treatment	Liver microsomal activity		
	EROD (Cyp1A1) nmol/ min /mg	PROD (Cyp2B1) nmol/ min /mg	n
Water	17.7 ± 3.4 ^a	8.2 ± 1.3	4
0.2 % B-PAE	21.3 ± 3.1	10.5 ± 1.3	4
Placebo	40.7 ± 6.4	8.9 ± 0.8	6
CAJ	41.2 ± 5.2	7.6 ± 0.7	6
CAJ+B-PAE	45.8 ± 9.1	7.7 ± 0.9	4

^a Mean ± SEM. Data normalized to protein (mg)

3.5.3 Phase II enzymes

Cytosolic liver fractions were used to determine the activity of GST, QR and TrxR enzymes (summary in Tab. 23).

Tab. 23. Effects of treatments on liver cytosolic enzyme activities

Treatment	Liver cytosolic activities			n
	GST ^a nmol/ min /mg	QR nmol/ min /mg	TrxR nmol/ min /mg	
Water	1740.3 ± 305.3 ^b	33.6 ± 3.3	10.0 ± 1.6	4
0.2 % B-PAE	2448.1 ± 553.6	25.8 ± 2.0	12.7 ± 3.2	4
Placebo	1837.4 ± 248.0	40.1 ± 4.7	18.1 ± 1.3	6
CAJ	1990.0 ± 306.4	43.4 ± 4.4	20.9 ± 1.9	6
CAJ+B-PAE	2235.3 ± 221.4	49.9 ± 3.4	25.3 ± 1.5 *	4

^a GST: Glutathione-S- transferase; QR: NAD(P)H: Quinone oxidoreductase; TrxR: Thioredoxin reductase

^b Mean ± SEM. Data normalized to protein (mg)

*p=0.02 Student's t-test comparison (in comparison to placebo).

3.5.3.1 Glutathione-S-transferases (GST) activity

GSTs catalyze the addition of aliphatic, aromatic, or heterocyclic free radicals as well as epoxides and arene oxides to GSH (Hayes and McLellan, 1999). B-PAE treatment increased hepatic GST activity using chlorodinitrobenzene as a substrate from 1740.3 ± 305.3 nmol/min/mg (water control) to 2448.1 ± 553.6 nmol/min/mg (Tab. 23). Even though this is a 29% increase, differences were not statistically significant. Intervention with the placebo juice resulted in GST cytosolic activities of 1837.4 ± 248.0 nmol/min/mg. Treatment with CAJ and CAJ + B-PAE further increased GST activities up to 1990.0 ± 306.4 and 2235.3 ± 221.4 nmol/min/mg, respectively. A trend for induction can be observed but this increase was not statistically significant. CAJ + B-PAE induced GST activity by 20% compared to placebo juice (Tab. 23). GST activities were not affected by fasting.

3.5.3.2 NADPH: quinone reductase activity (QR)

QR are flavoproteins catalyzing a two-electron reduction with preference for short-chain acceptor quinones. For the water control group, we measured an activity of 33.6 ± 3.3 nmol/min/mg based on the menadione-mediated reduction of MTT. QR activity was reduced to 25.8 ± 2.0 nmol/min/mg by B-PAE intervention. This indicates a 24% reduction but data were not statistically significant. In the placebo group, we detected a QR activity of 40.1 ± 4.7 nmol/min/mg. CAJ and CAJ+B-PAE intervention increased QR activities up to 43.4 ± 4.4 and 49.9 ± 3.4 , correspondingly. Like with the GST activities, CAJ + B-PAE induced 20% the QR activity in a non-significant manner (Tab. 23). Similar to Cyp1A microsomal activity, fasting significantly reduced QR activity by about 30% from 47.7 ± 10.4 nmol/min/mg in the not fasted groups to 31.1 ± 7.0 nmol/min/mg in the fasted groups (Fig. 27).

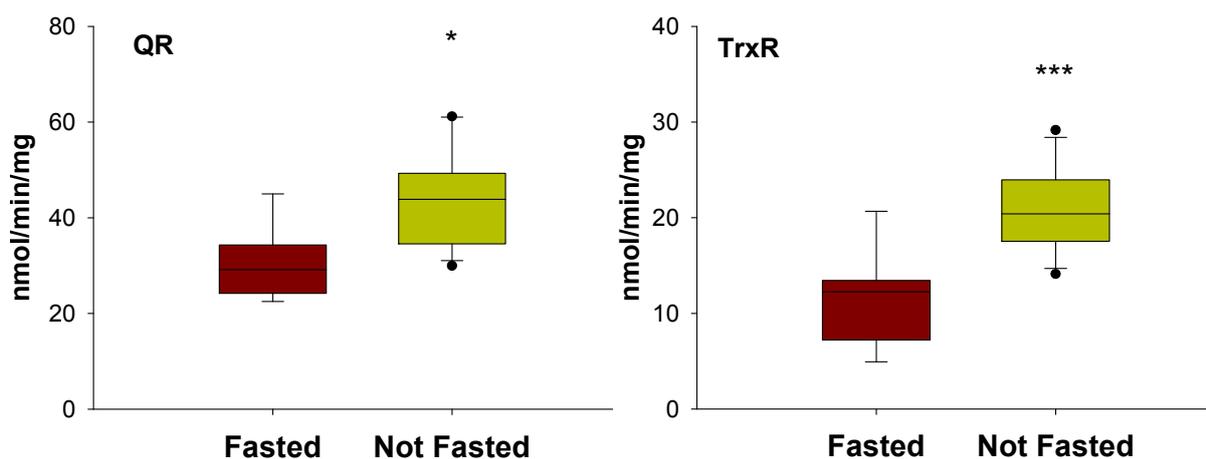


Fig. 27. The impact of fasting on liver QR and TrxR activities. A) QR enzyme activity; B) TrxR enzyme activity. Data normalized to mg of protein *** $p < 0.001$ and * $p < 0.05$ Student's t-test comparison. Fasted groups: water, B-PAE; not-fasted groups: placebo juice, CAJ, CAJ + B-PAE.

3.5.3.3 Thioredoxin Reductase (TrxR)

TrxR are an oxidoreductase enzyme family and catalyse the NADPH-dependent reduction of the redox protein thioredoxin. They play a role in several cellular processes such as oxidative stress, cell growth, apoptosis, ascorbate recycling and are involved in several human diseases (Mustacich and Powis, 2000). TrxR activity in the liver was not affected by B-PAE treatment (12.7 ± 3.2 nmol/min/mg) compared to the water control (10.0 ± 1.6 nmol/min/mg). Placebo juice- treated animals showed a

liver TrxR activity of 18.1 ± 1.3 nmol/min/mg, and CAJ intervention resulted in 20.9 ± 1.9 nmol/min/mg TrxR activity. Compared to placebo, CAJ + B-PAE treatment (25.3 ± 1.5 nmol/min/mg) statistically significantly induced TrxR activity by 39% ($p < 0.05$ Student t-test) (Tab. 23). Overall, the three not-fasted groups had an about 50% higher hepatic TrxR activity (average 21.0 ± 4.5 nmol/min/mg) than fasted groups (average 11.5 ± 4.6 nmol/min/mg) (Fig. 27).

3.5.3.4 Hepatic glutathione (GSH) levels

Glutathione (γ -L-Glutamyl-L-cysteinylglycine) plays an important role in redox regulation. GSH can be oxidized to GSSG which protects cells from oxidative stress due to free radicals (Hayes and McLellan, 1999). GSH levels are reported as mean \pm SEM and normalized to protein concentration. We determined total GSH levels with dithionitrobenzene (DTNB) as a measure of indirect antioxidant effects of apple components. As observed with several enzyme activities, fasting significantly reduced hepatic GSH levels by about 35% from 149.4 ± 24.1 nmol GSH/mg prot in the not-fasted groups to 97.2 ± 15.5 nmol GSH/mg prot in the fasted group ($p < 0.001$ Student's t-test, Fig. 28).

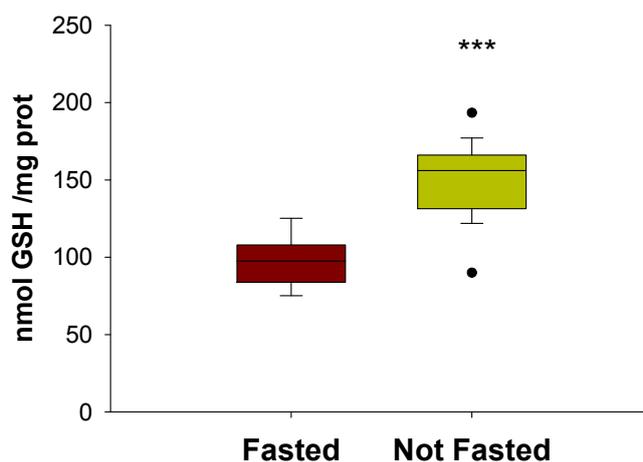


Fig 28. Effects of fasting on hepatic GSH levels. Data normalized to mg of protein

*** $p < 0.001$ Student's t-test comparison.

Tab. 24. Effects of treatments on liver GSH levels

Treatment	GSH nmol GSH / mg	n
Water	99.8 ± 5.4 ^a	4
0.2 % B-PAE	94.6 ± 10.9	4
Placebo	155.8 ± 9.5	6
CAJ	138.5 ± 11.0	6
CAJ+B-PAE	159.3 ± 12.7	4

^a Mean ± SE. Data normalized to protein (mg)

Within the fasted and not-fasted groups, treatments did not affect glutathione levels (Tab. 24).

3.6 Mechanisms in normal mucosa

As previously described, we had performed a first Min mouse experiment investigating the effects of CAJ04 and a 1:1 mix of an A- and B-PAE on adenoma development in Min mice (Gerhäuser, Forum DKG 2008). From this experiment, 1 cm each of normal tissue derived from the three SI segments (Int-1, Int-2 and Int-3; proximal, middle, distal) and the colons were collected for gene expression analyses. Also, swiss rolls were prepared from all segments.

Briefly, in this previous experiment, the total number of adenoma in the water control was higher than in the present experiment (total number of adenomas in the SI; 1. experiment: 64.7 ± 7.2, 2. experiment: 52.1 ± 3.8, compare Tab. 14). Importantly, CAJ04 and PAE significantly reduced tumor multiplicity in the SI by 38% and 40%, respectively. Highest adenoma reduction was observed in the middle (CAJ04: 39% and PAE: 41%) and the distal part of the SI (CAJ04: 52% and PAE: 34%, Tab. 25).

Tab. 25. First Min mouse experiment: Treatment effects in GI and adenoma localisation.

Treatment	Small Intestine (SI)				Total SI	Colon
	n	Int-1	Int-2	Int-3		
Water	12	7.6 ± 1.3	22.8 ± 2.6	34.3 ± 4.6	64.7 ± 7.2	1.3 ± 0.3
AS04	12	6.3 ± 1.4	13.9 ± 1.6 39 %*	20.2 ± 2.6 52 %*	40.3 ± 5.0	1.8 ± 0.4
PAE 0.2%	12	5.4 ± 2.1	11.0 ± 4.3* 41 %	22.5 ± 1.5 34 %	38.9 ± 2.9	0.9 ± 0.2

GI was divided in 4 parts Int-1, Int-2 and Int-3 and colon. Adenoma multiplicity in the SI and colon parts. (*) $p < 0.05$ (Kruskal-Wallis One way ANOVA on Ranks). Mean ± SEM.

After demonstrating that PAE and CAJ prevent adenoma development in the Min mouse model, we were interested in identifying which genes are involved in the progression from normal mucosa to adenoma. Therefore, we measured gene expression by quantitative RT-PCR. Genes related to proliferation (Cyclin D1 and c-Myc), inflammation (Cox-2), and angiogenesis (VEGF and MMP7) were investigated in these normal tissue samples (not containing adenoma).

3.6.1 Determinations at the transcriptional level

RNA was extracted from normal mucosa. Gene expression was quantified by the Sybr-Green method, based on the computation of Ct values (Bio-Rad, iCycler). GAPDH, β -actin and Cyclophilin E were used as housekeeping genes in order to obtain more reliable results. Ct (Cycle threshold) values for the housekeeping genes varied from 23 to 28. In quantitative RT-PCR, Ct values represent the cycle when the PCR increases exponentially the synthesis of the amplicon and the detected fluorescence increases over the detection threshold. Consequently, higher Ct values mean less mRNA expression and lower values imply a higher expression.

Cyclin D1 mRNA was detectable in all GI tract (Ct values from 26 to 30). However, mRNA of c-Myc, VEGF and MMP7 were expressed at very low levels (Ct > 32, relative expression data not shown). Because this implies a higher error in the ratio calculations, c-Myc, VEGF and MMP7 were not further investigated.

Cyclin D1 mRNA expression was analyzed in mucosal samples of all SI and Colon. In Int-1 segments, CAJ04 and PAE treatments resulted in an increase of expression (average ratio ± SEM; Water: 1.00 ± 0.05, CAJ: 1.44 ± 0.06 and A-PAE: 1.76 ± 0.22).

In Int-2 parts, expression in samples of water and CAJ04 treated animals was similar, but PAE intervention reduced Cyclin D1 gene expression by 30% (Water: 1.00 ± 0.13 , CAJ: 1.23 ± 0.18 and PAE: 0.68 ± 0.09). In Int-3 parts, the normalized ratio was close to 1 for water- and CAJ- treated animals whereas PAE treatment reduced Cyclin D1 mRNA expression by 25% (Water: 1.00 ± 0.20 , CAJ: 0.98 ± 0.10 and PAE: 0.74 ± 0.08). This trend was also observed in the colon (Water: 1.00 ± 0.19 , CAJ: 0.91 ± 0.13 and PAE: 0.68 ± 0.07). Overall, Cyclin D1 was slightly reduced in the distal regions of the GI albeit non-significantly (Fig. 29). However, results did not correlate with the tumor yield in the SI.

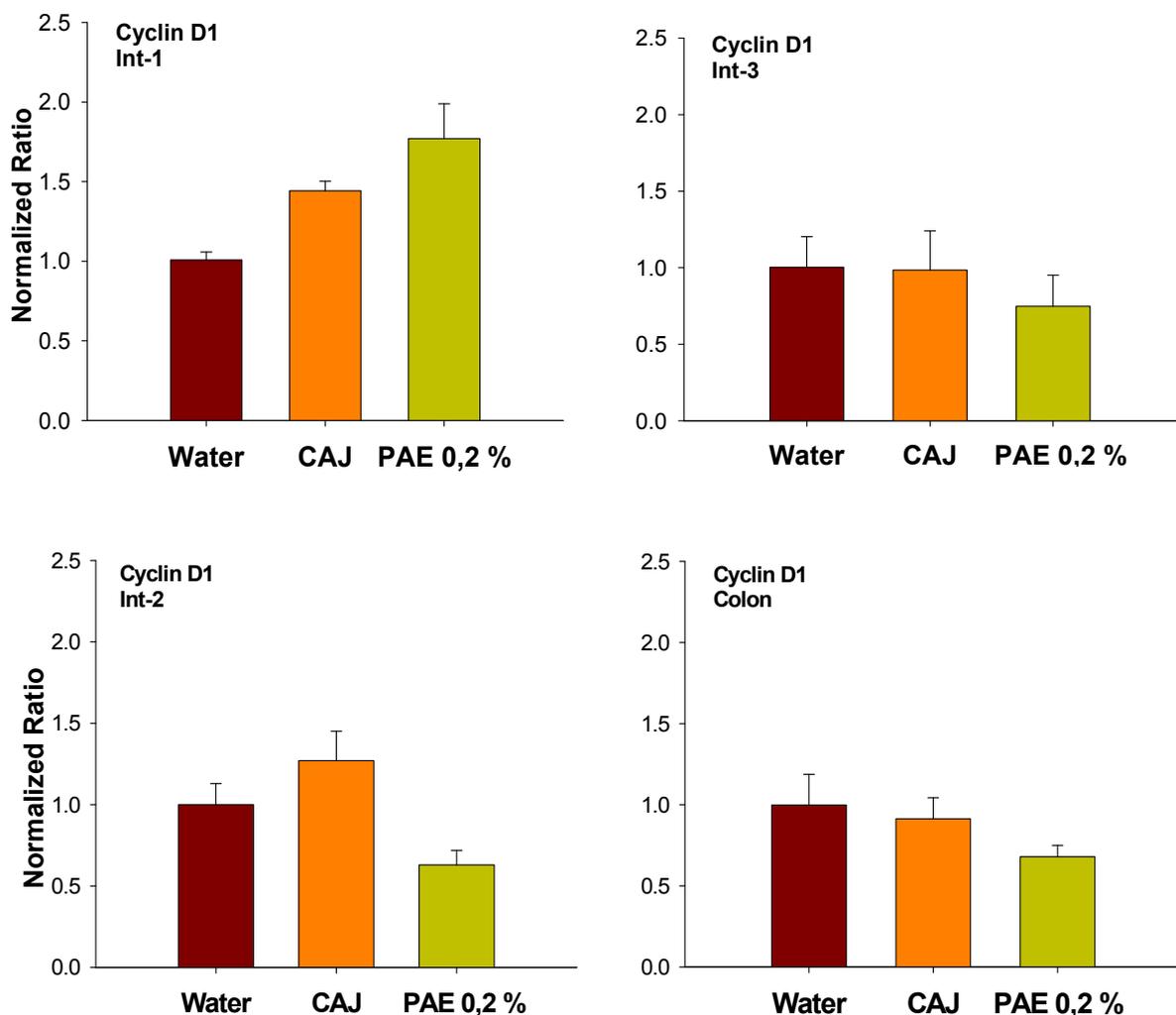


Fig. 29. Cyclin D1 mRNA expression (qPCR) in SI segments and colon. Values were normalized using GAPDH, β -actin and Cyclophilin E as housekeeping genes. Mean \pm SEM (n=6).

Analysis of Cox-2 mRNA by quantitative RT-PCR resulted in high Ct values (>32). These values were shown in all SI segments indicating low expression. In the colon,

lower Ct values were observed (from 28 to 30). Both intervention using CAJ04 and PAE reduced in a non-significant manner Cox-2 relative mRNA expression by 30%-43% (Water: 1.00 ± 0.12 , CAJ: 0.57 ± 0.10 and PAE: 0.69 ± 0.13) (Fig. 30).

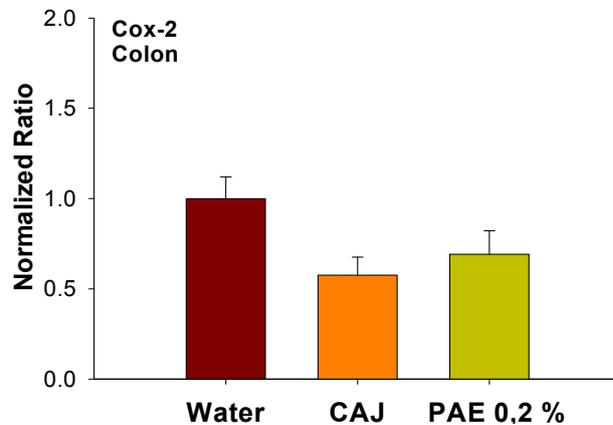


Fig. 30. Cox-2 gene expression (qPCR) in colon. Ratios were normalized using GAPDH, β -actin and Cyclophilin E housekeeping genes. Mean \pm SEM (n= 6).

3.6.2 Determination at the protein level

In order to investigate Cox-2 and Cyclin D1 protein expression, immunohistochemical (IHC) experiments were performed in normal mucosa. In comparison to qRT-PCR, this technique is less sensitive but allows to assess the expression profile of the specific proteins and to evaluate tissue localisation.

As shown in Fig. 31A, Cox-2 protein was detected in the lamina propria and the stroma, whereas it was absent in the epithelia (crypts and villi). There was no difference in Int-3 that could be related to the treatment with CAJ03 and PAE. Cyclin D1 expression was limited to nuclei in the crypts (Fig. 31 B). Expression was not influenced by the treatment. In Int-2 segments no changes Cyclin D1 and Cox-2 expression were observed (data not shown).

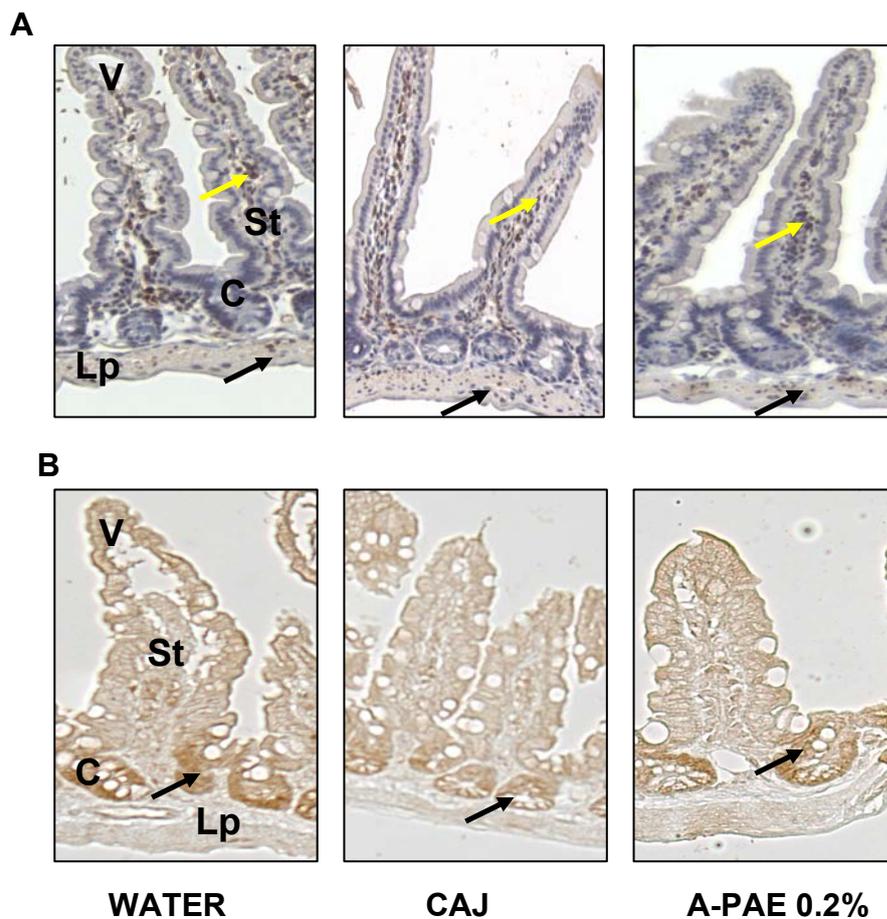


Fig. 31. IHC detection of Cox-2 and Cyclin D1 in SI segment Int-3 (normal tissue). (A) Cox-2 non-epithelial staining: Lamina propria (black arrows) and stroma (yellow arrows). (B) Cyclin D1: nuclear staining in the crypts (black arrows). C: Crypt; Lp: Lamina propria; St: Stroma and V: Villi. Original magnification 200x.

Since all these experiments with normal mucosa samples were not very informative, we thought of a technology which would enable us to quantitatively compare protein expression and/or post-translational modifications in larger sample sets in a high-throughput manner. In cooperation with Dr. Ulrike Korf and Heiko Mannsperger (DKFZ), we were able to apply the method of RPPA (reverse phase protein array) to our tumor samples of these second Min mouse experiments.

3.7 Proteomic analysis in tumor tissue

3.7.1 Principle of reverse phase protein arrays

RPPA (Reverse phase protein array) technology is a new useful tool for monitoring protein abundance and turnover of posttranslational modifications on a large scale, and is ideal for hypothesis-based post-translational analyses in cell signalling (Spurrier *et al.*, 2008).

With RPPA, protein lysates are spotted on a nitrocellulose membrane and the protein of interest is detected by incubation with a specific primary antibody. The captured primary antibody is then subsequently visualized by a secondary antibody which is coupled with a near-infrared dye. The final read-out is performed using an infrared scanning system (Fig. 32).

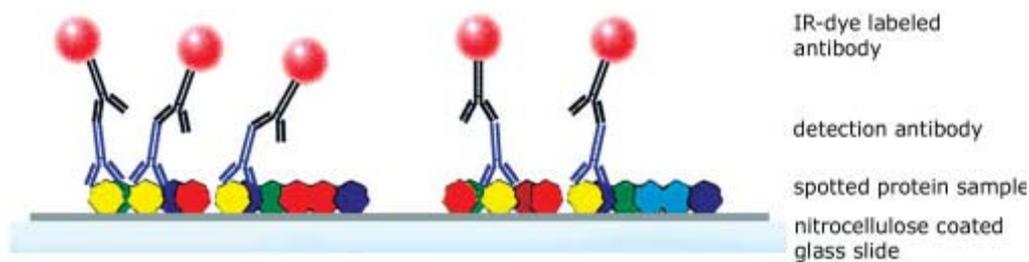


Fig. 32 Schematic illustration of RPPA (Loebke *et al.*, 2007).

This technique allows protein quantification with high reproducibility and an error rate of <5% (Loebke *et al.*, 2007). In comparison, a total standard deviation of 20–35% is commonly reported for quantitative Western blotting (Koller and Watzig, 2005). On the other hand, immunohistochemistry can provide valuable information regarding abundance of cellular protein antigens in tissue. However, immunohistochemistry has been reported to be subjective, nonquantitative and subject to processing variability (e.g., formalin fixation artefacts) (Mighell *et al.*, 1998).

Proteomic studies based on RPPA were performed to identify which proteins are involved in adenoma development and to elucidate mechanisms involved in the reduction of adenoma development by apple juice intervention. A set of protein arrays were prepared using ultracentrifuged lysates of adenomas derived from Int-2 and Int-3. Only these parts are shown since here we observed the strongest inhibition in adenoma multiplicity in comparison to the water control. To avoid bias because of

fasting effects, treatments were independently analyzed (Fasted: Water vs. B-PAE and not- fasted: Placebo vs. CAJ and CAJ + B-PAE).

3.7.2 Protein expression of Proliferating Cell Nuclear Antigen (PCNA)

PCNA is an auxiliary protein of DNA polymerase that is specifically expressed in cells during the S phase of the cell cycle and it is used as a marker for cellular proliferation (Kelman, 1997).

As indicated in Fig. 33, intervention with B-PAE on the one hand, and CAJ and CAJ + B-PAE on the other hand did not significantly decrease cell proliferation, based on PCNA protein expression. B-PAE treatment reduced expression by 20% compared to the water control in Int-3, whereas similar PCNA levels were observed in Int-2 (Fig. 33). CAJ and CAJ + B-PAE non-significantly increased PCNA expression in adenomas of Int-2 by 31 and 32%, respectively, in comparison to the placebo group. In contrast in Int-3 samples, we observed a reduction of PCNA expression by about 25% (Fig. 33).

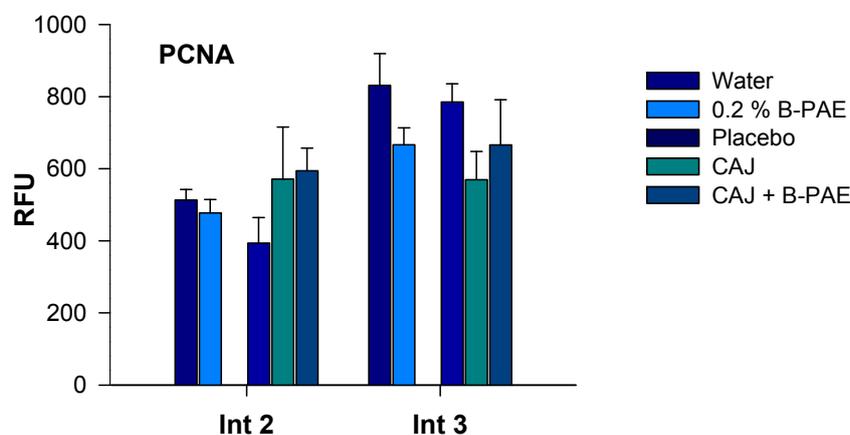


Fig. 33. PCNA expression in adenoma tissue. Signals were expressed in Relative Fluorescence Units (RFU). Int-1 was not calculated due to low protein concentration of the prepared lysates.

3.7.3 Wnt signalling pathway

3.7.3.1 β -catenin

β -catenin is a key component of the Wnt signalling pathway. As already mentioned in chapter 1.3.1, inappropriate activation of the Wnt signalling pathway is considered to be an initiating event in the transformation of intestinal epithelial cells (Dihlmann and von Knebel Doeberitz, 2005).

Using the RPPA technology strong expression levels of β -catenin were found in the adenoma samples. In Int-2 adenoma samples, neither B-PAE nor CAJ nor CAJ + B-PAE treatment resulted in significant changes in β -catenin expression. In Int-3 samples, B-PAE had also no influence on β -catenin levels (Fig. 34). However, intervention with CAJ and CAJ + B-PAE non-significantly reduced β -catenin expression by 49% and 57% in comparison with the placebo juice group ($p=0.07$ and $p=0.13$ ANOVA Bonferroni test).

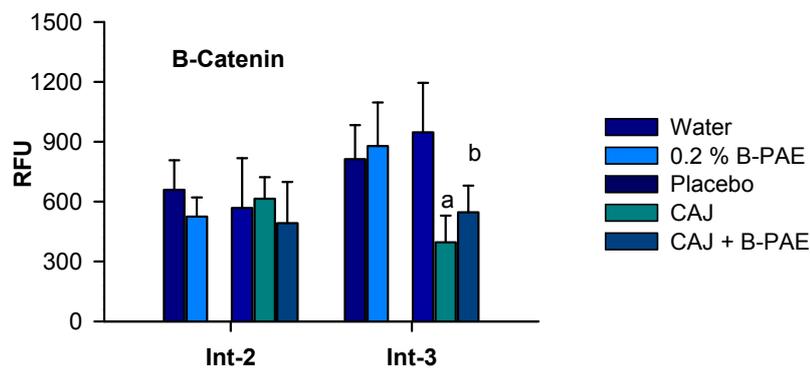


Fig. 34. β -Catenin protein expression in adenoma tissue. Signals were expressed in Relative Fluorescence Units (RFU). Mean \pm SEM. (a) $p=0.07$ and (b) $p=0.13$ One-way ANOVA with Bonferroni's multiple comparisons test (comparison with placebo juice group).

3.7.3.2 Cyclin D1

Cyclin D1 is a target of Wnt signalling. It was therefore of interest to analyze whether the effects on β -catenin expression could be translated to effects on Cyclin D1 expression. In comparison with the water control, B-PAE treatment did not significantly reduce Cyclin D1 expression in adenoma samples of Int -2 and Int-3 (Fig. 35). However in Int-2 adenomas, a trend of a reduction was observed by B-PAE intervention (Fig. 35). In all three non-fasted groups (Placebo, CAJ and CAJ + B-PAE),

Cyclin D1 expression was reduced by approximately 50% in comparison with the water control in a non-significant manner ($p=0.16$, Student t-test). This effect was not seen in samples of Int-3. In Int-3 adenomas, water and placebo control Cyclin D1 levels were in a similar range. Interestingly, in comparison with the placebo juice group, intervention with CAJ or CAJ + B-PAE significantly reduced Cyclin D1 expression by 70% and 83%, respectively ($p<0.05$ by ANOVA). This reduction might be a consequence of reduced β -catenin levels as described in chapter 3.7.3.1.

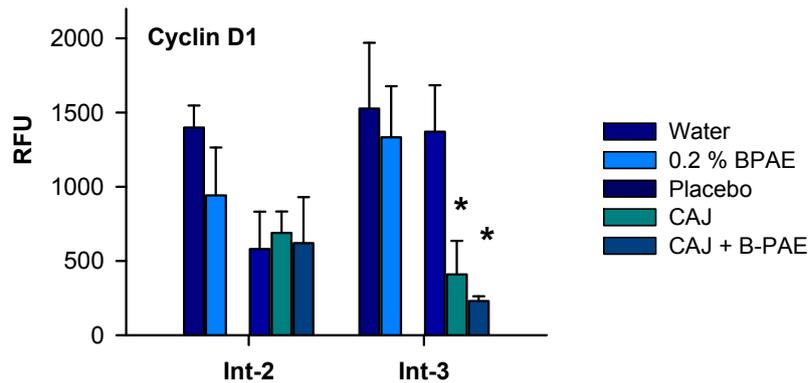


Fig. 35. Protein expression and expression of Cyclin D1 in adenoma tissue. Signals were expressed in Relative Fluorescence Units (RFU). Mean \pm SEM. * $p<0.05$ One-way ANOVA with Bonferroni's multiple comparisons test (comparison to placebo).

3.7.4 Mitogen activated protein kinase (MAPK) pathway

The MAPK pathway mediates signal transduction from cell membrane receptors to downstream transcription factors, leading to cellular responses such as cell proliferation, growth, apoptosis, motility and survival (Dhillon *et al.*, 2007) (Fig. 36).

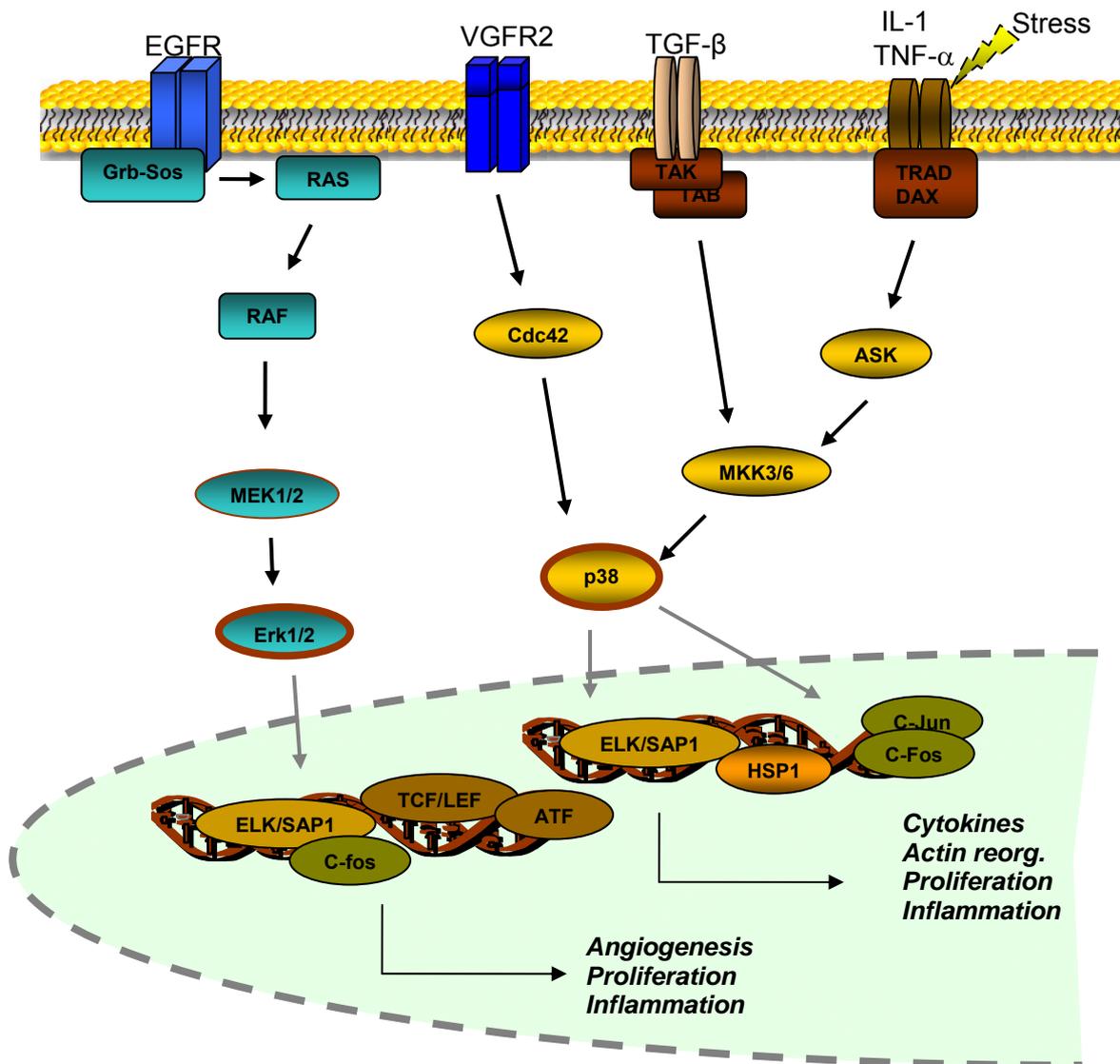


Fig. 36. General scheme of the MAPK and p38 signalling pathway. ATF, activating transcription factor; Cdc42, cyclin-dependent kinase 42; EGFR, Epidermal growth factor receptor; ERK1/2, Extracellular-regulated kinase 1/2; Grb-Sos, GTPase son of sevenless; HSP1, heat shock protein-1; IL-1, Interleukin-1; MEK, Mitogen-regulated kinase; Raf, rapidly growing fibrosarcoma or rat fibrosarcoma; Ras, rous avian sarcoma homologue; TAK, TGF- β activated kinase; TCF/LEF, T-cell factor/Lymphoid enhancer-binding factor 1; TAB, TAK binding protein; TGF- β , Transforming growth factor-beta; TNF, Tumor necrosis factor; TRAD/DAX, Tumor necrosis factor receptor-1/Dosage-Sensitive Sex Reversal; VGFR-2, Vascular growth factor receptor-2. Adapted from KEGGS database.

3.7.4.1 MEK and ERK

MEK and ERK proteins are crucial proteins in the EGFR signaling pathway. When MEK or ERK are phosphorylated, the pathway is active and activates genes related to proliferation, angiogenesis and inflammation (Fig. 36). In the Min mouse model, phosphorylated ERK is increased in adenomas (Khor *et al.*, 2008). Based on this information, we were interested whether apple juice intervention may reduce MAPK signaling and therefore reduce adenoma growth. Phosphorylation of MEK and ERK as a downstream target was analysed by RPPA, using antibodies specific for the phosphorylated forms of MEK and ERK. Specificity was tested by Western blotting (data not shown). For comparison, antibodies for the un-phosphorylated forms of MEK and ERK were utilized.

In fasted groups, administration of B-PAE reduced MEK1/2 phosphorylation in adenomas of Int-2 and Int-3 with borderline significance (Int-2: 25%, $p=0.10$ and Int-3: 29%, $p=0.09$) compared to the water control (Fig. 37 A). This reduction was specific for the phosphorylated form of MEK, as total MEK levels were not changed by the intervention with B-PAE (Fig. 37 B). In contrast, in the non-fasted groups, neither phosphorylation nor expression of the non-phosphorylated form of MEK was influenced by intervention with CAJ and CAJ + B-PAE (Fig. 37 A+B).

In adenomas of the placebo juice-treated animals, MEK phosphorylation was about 50% higher than in adenomas of the water control group. But both reductions were not statistically significant. These data may imply that fasting and fasting-associated stress activates the MAPK signaling pathway. However, B-PAE treatment prevented this activation (Fig. 37 A)

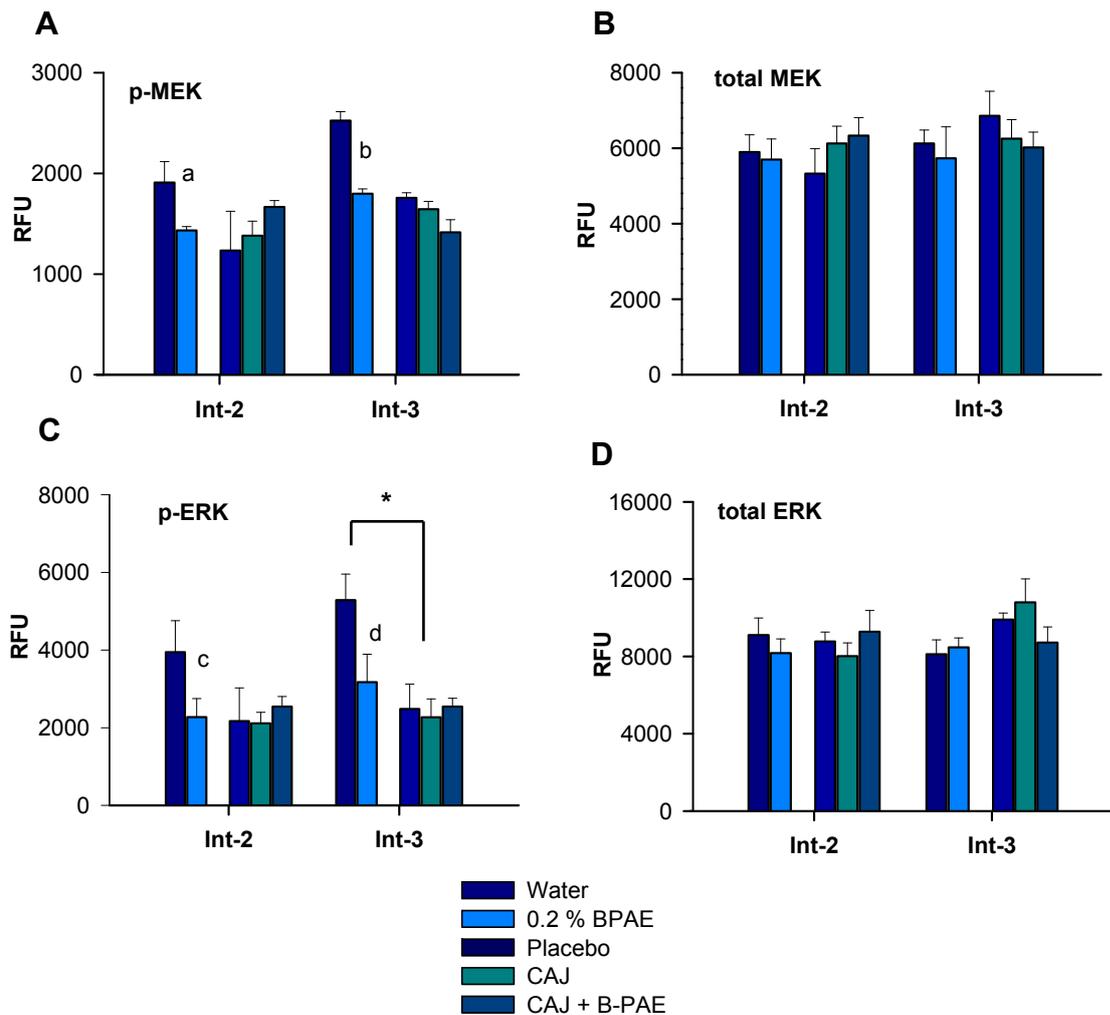


Fig. 37. Phosphorylation and protein expression of MEK and ERK in adenoma tissue. Signals were expressed in Relative Fluorescence Units (RFU). (A) Detection of p-MEK, (B) unphosphorylated MEK, (C) p-ERK and (D) unphosphorylated ERK. Mean \pm SEM; (a): $p = 0.1$; (b) $p = 0.09$; (c) $p = 0.10$; (d) $p = 0.053$ and $*p = 0.038$ Student's t-test comparison (in comparison with water control).

To confirm the role of the MAPK signaling pathway, we further analysed ERK phosphorylation and expression as a downstream protein. In adenomas of the B-PAE-treated group, p-ERK levels were (borderline) significantly reduced in comparison to water control (Int-2: 42%, $p=0.10$ and Int-3: 40%, $p=0.053$, Student's t-test) (Fig. 37 C). Highest expression was detected in samples of the water control group. Again, these effects were specific for a reduction of phosphorylation, since expression of the unphosphorylated form was not changed by intervention (Fig. 38 D). Similar ERK phosphorylation levels were found in adenomas of the CAJ- and CAJ + B-PAE-treated animals compared to the placebo control (Fig. 37 C). Also, total ERK levels were not affected (Fig. 37 D). In comparison to the placebo group, ERK phosphorylation was about 2-fold increase in adenomas of the water control group in

both Int-2 and Int-3 (Int-2: $p=0.16$ and Int-3: 0.038 ; Student's t-test). Like with the MEK results, these data may indicate that fasting and fasting-associated stress activates the MAPK signaling pathway. Again, this activation was prevented by B-PAE intervention.

3.7.4.2 iNOS

iNOS is induced in inflamed tissues and generates nitric oxide (NO) that can promote mutagenic changes like DNA oxidization and protein nitrosylation (Hussain and Harris, 2007). iNOS is a downstream target of the MAPK pathway (Surh, 2003).

As shown in Fig. 38, protein levels of iNOS were higher in adenomas of water treated mice in comparison to B-PAE treatment. B-PAE treatment decreased iNOS protein expression in Int-2 and Int-3 tumors (Int-2: 54%, $p=0.035$ and Int-3: 28%, $p=0.14$, Student's t-test). CAJ and CAJ + B-PAE treatments did not significantly reduce iNOS levels compared to the placebo juice group.

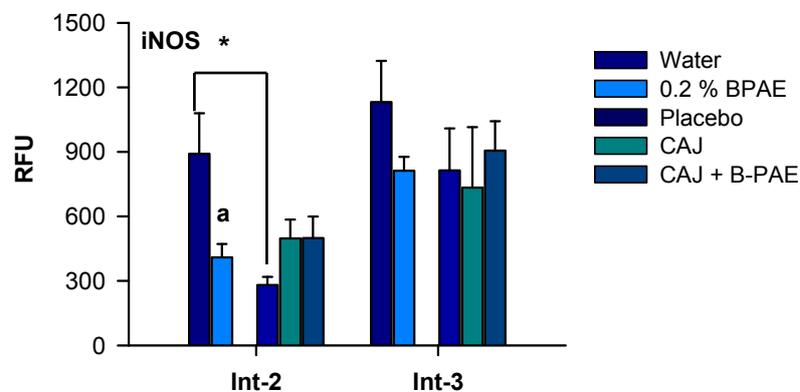


Fig. 38. Protein expression of iNOS in adenoma tissue. Signals were expressed in Relative Fluorescence Units (RFU). Mean \pm SEM. (a) $p = 0.035$ t-test and * $p = 0.018$ Student's t-test comparison (in comparison with water control).

As a downstream effect of activation of the MEK/ERK signaling pathway, expression of iNOS was significantly upregulated in adenomas of the water in comparison with the placebo juice group especially in Int-2. Again, this result may be due to fasting-induced stress ($p= 0.018$ Student's t-test). Intervention with B-PAE normalized elevated iNOS expression detected in the water control group to levels similar to those in the CAJ and CAJ + B-PAE group (Int-2). A similar trend was observed in Int-3 adenomas (Fig. 38).

Cox-2 is another protein with an important role in inflammation and promotion of tumor growth (Buchanan and DuBois, 2006; Wang and Dubois, 2006). However, due to difficulties in finding a suitable specific antibody, the RPPA technology could not be used to analyse Cox-2 expression.

3.7.4.3 p38

The p38 signalling pathway is activated in response to a wide range of cellular stress stimuli and cytokines (Olson and Hallahan, 2004).

In contrast to the ERK pathway, when p38 protein levels were measured, neither expression nor phosphorylation was affected. Similar low intensities in all groups were found for p-p38 (Fig. 39 A and B).

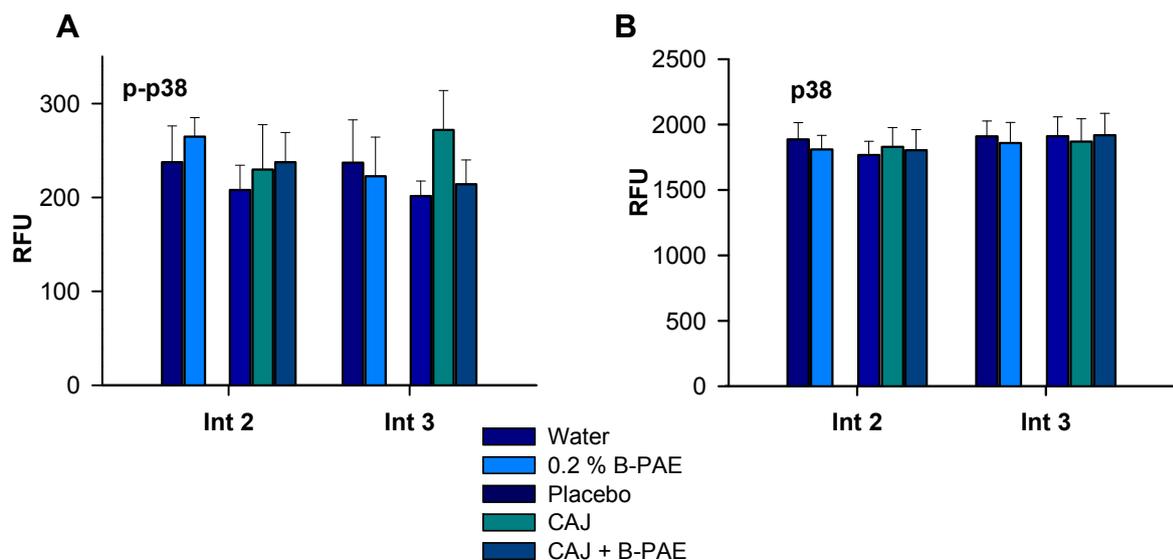


Fig. 39. p38 phosphorylation and protein expression and in adenoma tissue. Signals were expressed in Relative Fluorescence Units (RFU). Mean \pm SEM.

3.7.5 Summary of all proteomic results investigated by RPPA

In addition to proteins of the Wnt- and MAPK signalling pathways mentioned above, we analysed the expression and/or phosphorylation of AKT, STAT3, STAT5, c-Myc and PKC. None of these proteins were significantly regulated by B-PAE treatment in comparison to the water control (Tab. 26), or by CAJ or CAJ + B-PAE in comparison with the placebo juice control (Tab. 27).

Tab. 26 Protein phosphorylation and expression values after B-PAE normalized to control (Water)

Protein	INT-2		INT-3	
	Water	B-PAE 0.2%	Water	B-PAE 0.2%
p-ERK	1.00 ± 0.21 ^a	0.58 ± 0.12	1.00 ± 0.13	0.60 ± 0.14
ERK	1.00 ± 0.10	0.90 ± 0.08	1.00 ± 0.09	1.04 ± 0.06
p-MEK	1.00 ± 0.11	0.75 ± 0.02	1.00 ± 0.06	0.71 ± 0.02
MEK	1.00 ± 0.08	0.97 ± 0.09	1.00 ± 0.06	0.94 ± 0.14
p-p38	1.00 ± 0.16	1.11 ± 0.08	1.00 ± 0.19	0.94 ± 0.18
p38	1.00 ± 0.07	0.96 ± 0.08	1.00 ± 0.06	0.97 ± 0.08
p-Akt	1.00 ± 0.09	0.88 ± 0.05	1.00 ± 0.45	0.98 ± 0.07
Akt	1.00 ± 0.14	0.78 ± 0.01	1.00 ± 0.13	1.11 ± 0.15
pSTAT3	1.00 ± 0.06	0.88 ± 0.05	1.00 ± 0.16	0.98 ± 0.07
STAT3	1.00 ± 0.14	0.78 ± 0.01	1.00 ± 0.13	1.11 ± 0.15
STAT5	1.00 ± 0.08	0.79 ± 0.10	1.00 ± 0.10	1.05 ± 0.10
β-catenin	1.00 ± 0.22	0.80 ± 0.15	1.00 ± 0.21	1.08 ± 0.27
Cyclin D1	1.00 ± 0.11	0.67 ± 0.23	1.00 ± 0.29	0.87 ± 0.23
c-Myc	1.00 ± 0.08	0.87 ± 0.08	1.00 ± 0.32	1.09 ± 0.11
PCNA	1.00 ± 0.06	0.93 ± 0.07	1.00 ± 0.11	0.80 ± 0.06
iNOS	1.00 ± 0.21	0.46 ± 0.07*	1.00 ± 0.17	0.72 ± 0.06
PKC	1.00 ± 0.09	0.79 ± 0.11	1.00 ± 0.14	0.95 ± 0.09

^a Mean ± SEM, (n=6).

*p<0.05 t-test (Student's t-test in comparison to water control group). **Bold** numbers indicate borderline significance.

Tab. 27. Protein phosphorylation and expression values after CAJ and CAJ + B-PAE treatments normalized to control (Placebo)

Protein	INT-2			INT-3		
	Placebo	CAJ	CAJ + B-PAE0.2%	Placebo	CAJ	CAJ + B-PAE 0.2%
p-ERK	1.00 ± 0.39 ^a	0.97 ± 0.13	1.17 ± 0.12	1.00 ± 0.26	1.00 ± 0.39	1.03 ± 0.09
ERK	1.00 ± 0.06	0.91 ± 0.08	1.06 ± 0.13	1.00 ± 0.26	0.92 ± 0.19	1.00 ± 0.39
p-MEK	1.00 ± 0.32	1.12 ± 0.12	1.35 ± 0.12	1.00 ± 0.03	0.94 ± 0.04	0.81 ± 0.07
MEK	1.00 ± 0.12	1.15 ± 0.09	1.19 ± 0.09	1.00 ± 0.10	0.91 ± 0.07	0.88 ± 0.06
pp38	1.00 ± 0.13	1.10 ± 0.23	1.14 ± 0.15	1.00 ± 0.08	1.35 ± 0.21	1.06 ± 0.13
p38	1.00 ± 0.03	1.04 ± 0.08	1.02 ± 0.09	1.00 ± 0.08	0.98 ± 0.12	1.00 ± 0.09
p-Akt	1.00 ± 0.03	1.12 ± 0.06	1.00 ± 0.07	1.00 ± 0.05	0.86 ± 0.06	0.93 ± 0.07
Akt	1.00 ± 0.23	1.26 ± 0.13	1.05 ± 0.21	1.00 ± 0.18	0.68 ± 0.17	0.72 ± 0.12
pSTAT3	1.00 ± 0.12	0.89 ± 0.07	1.00 ± 0.12	1.00 ± 0.15	0.73 ± 0.06	0.86 ± 0.14
STAT3	1.00 ± 0.22	1.08 ± 0.14	0.95 ± 0.18	1.00 ± 0.21	0.79 ± 0.16	1.01 ± 0.13
STAT5	1.00 ± 0.23	1.15 ± 0.10	0.89 ± 0.17	1.00 ± 0.16	0.62 ± 0.13	0.82 ± 0.10
β-catenin	1.00 ± 0.44	1.08 ± 0.19	0.87 ± 0.36	1.00 ± 0.26	0.42 ± 0.14	0.58 ± 0.14
Cyclin D1	1.00 ± 0.43	1.19 ± 0.25	1.07 ± 0.53	1.00 ± 0.23	0.30 ± 0.16*	0.17 ± 0.02*
c-myc	1.00 ± 0.14	1.03 ± 0.13	0.96 ± 0.14	1.00 ± 0.31	0.75 ± 0.45	0.82 ± 0.22
PCNA	1.00 ± 0.18	1.45 ± 0.37	1.51 ± 0.16	1.00 ± 0.06	0.73 ± 0.10	0.85 ± 0.16
iNOS	1.00 ± 0.14	1.77 ± 0.31	1.78 ± 0.36	1.00 ± 0.24	0.90 ± 0.35	1.15 ± 0.17
PKC	1.00 ± 0.17	1.05 ± 0.09	0.78 ± 0.12	1.00 ± 0.13	0.81 ± 0.18	0.91 ± 0.12

^a Mean ± SEM, (n=6).

*p<0.05 ANOVA (Bonferroni test in comparison to placebo juice group). **Bold** numbers indicate borderline significance.

4. DISCUSSION

Colorectal cancer (CRC) is the second leading cause of cancer-related deaths in western societies. The progression of the disease from benign adenoma through early carcinoma to malignant metastatic cancer requires years or even decades. Due to a long asymptomatic period preceding detection, the disease is mostly threatening.

The chance of long-term survival for colon, rectal, or other cancers improves significantly upon early detection. Although early detection is a key contemplation in the fight against cancer, ideally, researchers and clinicians would like to prevent cancer from developing.

In recent years, it has been suggested that diet contains many compounds that can inhibit CRC, at least in animal models (Corpet and Pierre, 2003; Corpet and Tache, 2002). This is also supported by epidemiological studies in humans (Donaldson, 2004). It has been estimated that improvement of diet and exercise could reduce the number of new cancer cases by 30–40% (WCF/AICR, 2007).

A WHO/FAO report recommends as a population-wide intake goal the consumption of a minimum of 400g of fruit and vegetables per day for the prevention of chronic diseases such as cancer (WCF/AICR, 2003). Different countries have initiated health programs such as the *5-a-day* program, which recommends eating 5 or more portions of a variety of fruit and vegetables. One of the servings can be a 100% fruit juice. However, the role of juices in cancer prevention has not been completely investigated.

Dietary components aim to promote health and prevent diseases such as CRC are rapidly increasing in popularity. To show convincingly that these compounds really are chemopreventive, their efficacy in animal models should be first demonstrated, and their mechanism of action in cancer prevention should be understood.

The main objective of this thesis was to investigate which mechanisms are involved in the prevention of adenoma formation by AJ and PAE the Min mouse model. Therefore, the work has focused on the initiation and the promotion stage of carcinogenesis. Proteomic and transcriptional profiles of adenomas and normal

tissue were investigated after intervention using different techniques in order to identify proteins/genes and pathways involved in the inhibition of adenoma formation. Before termination of the experiment, animals were kept in metabolic cages for 12 h to collect urine and feces for subsequent analyses. During this time period, animals had access to their respective intervention drinks. As a consequence, two groups (water control and B-PAE) were kept under fasting conditions, whereas the remaining three groups consumed calories in the form of carbohydrates provided by placebo juice and CAJ, respectively. Since fasting effects apparently influenced several end points addressed in this study, the potential impact of its effects will be discussed.

4.1 Inhibition of intestinal tumorigenesis

4.1.1 Effect of apple polyphenols on adenoma development

In our previous first animal experiment both CAJ04 and PAE treatments reduced adenoma development in the small intestine (SI). These findings have been confirmed by the present animal experiment using CAJ06 and B-PAE interventions. In the SI, CAJ06 resulted in a lower inhibitory effect on adenoma numbers than CAJ04 (CAJ04: 40% and CAJ06: 24%). Similar inhibition percentages were caused by B-PAE and PAE (PAE: 39% and B-PAE: 34%) (**Tab. 14 and Tab. 25**).

Adenoma number

The highest inhibition of the adenoma numbers was observed by B-PAE treatments. As already explained, B-PAE is obtained from an apple pomace enzymatic digestion, and consequently the content of oligomeric and polymeric procyanidins is increased. Since intervention with B-PAE resulted in the highest percentage of adenoma inhibition (34%), this may indicate the role of procyanidins as active chemopreventive compounds *in vivo* (**Tab. 14 and Fig. 15**).

Our previous animal experiment and the present animal study confirm that procyanidin-rich PAE are chemopreventive in the Min mouse model. So far, the *in vivo* effect of PAE has only been demonstrated in the AOM model. Although both are models for CRC prevention, it is important to point out that the AOM model is a chemically induced colon cancer model. Nevertheless, 0.01% of an apple

procyanidin fraction resulted in a 50% reduction of hyperproliferative crypts and ACF. This represents a satisfactory effect at low procyanidin concentration (Gosse *et al.*, 2005). However, Barth *et al.* have suggested that the chemopreventive potential of CAJ in the AOM-model might be a synergistic effect of the procyanidins and cloud particle fractions (Barth *et al.*, 2007). But as already mentioned, the effect of dietary fiber in the Min mouse model seems to have a lower effect on adenoma reduction (Corpet and Pierre, 2003). In the present study, we may therefore speculate that polyphenols are the main chemopreventive compounds.

Although CAJ- and B-PAE-treated animals received nearly a similar polyphenol dose, CAJ potential was weaker than B-PAE intervention. A difference may be determined by analyzing the mean degree of polymerisation (DPm) of procyanidins. Generally, CAJ or commercial AJ contain a DPm of 4-6. But this value increases to 8 in B-PAE03 due to the enzymatic extraction. An increased DPm might result in a more efficient chemopreventive potential. Unexpectedly, the CAJ04 used for the first Min mouse experiment showed a DPm of 13. Hence, the differences in DPm between CAJ04 and CAJ06 might be the reason of the weaker potential in adenoma inhibition of the CAJ06 used for the present work.

Isolated OPC have been identified in our earlier investigations as important active compounds of apples (Zessner *et al.*, 2008). Other *in vitro* experiments have demonstrated the chemopreventive potential of oligomeric procyanidins in colorectal, prostate, breast and oesophageal cancer cell lines (Nandakumar *et al.*, 2008).

Although we did not examine dysplastic crypts in the SI, we can assume that initiated crypts will develop adenomas over time. Therefore, the tumor multiplicity indicates whether treatments took effect at the initial stage of the adenoma-carcinoma sequence. As already described, two events are involved in the initiation of the adenoma-carcinoma sequence, Apc mutations and the subsequent TCF/LEF targets, and epigenetic modifications. For the first possibility, Kern *et al.* described that TCF/LEF reporter gene assay showed no inhibition after AP treatment. The authors conclude that PAE does not mediate growth-stimulating effects in HT29 *via* the Wnt signaling pathway (Kern *et al.*, 2005). Detailed transduction mechanisms will be discussed in section 4.6.

In contrast, PAE incubation has recently been reported to cause demethylation in the DNA repair gene hMLH1 in the RKO CRC cell line. In addition, strong inhibition of the

methyltransferases DMNT-1 and DMNT-3b was observed. Both results were related to the diminished cell viability of RKO cells (Fini *et al.*, 2007). Taking in consideration both investigations, epigenetic events may play a crucial role during initiation stages in the Min mice.

Another evidence for the efficacy of polyphenols at initial stages is the effect of placebo intervention. Interestingly, placebo juice treatment decreased adenoma numbers in the SI by 7%. This result indicates that polyphenols are the main active compounds in CAJ and B-PAE treatments (**Tab. 14 and Fig. 15**).

CAJ + B-PAE treatments did not result in a synergistic effect of CAJ and B-PAE intervention. A dose of 120 mg/kg resulted in a less inhibitory potential but in a higher bioavailability. HPLC analysis identified higher amounts of catechins and polyphenol metabolites in CAJ + B-PAE-treated animals. In contrast, no polyphenol metabolites were identified in the urine of B-PAE-treated animals (**Tab. 13 and Fig. 24**). This finding may implicate that a reduced local effect is happening in the SI for CAJ + B-PAE intervention, which in turn reduces the effect of adenoma inhibition. Other reported intervention experiments using polyphenolic compounds in the Min mouse model have caused less inhibitory effects. As an example, interventions with 2% rutin or 2% quercetin, suppressed about 15% of adenomas in the SI. Similar results were observed with 20% freeze-dried cherries. Both authors argued in each discussion that such an effect may be dependent on the bioavailability of the polyphenols, and therefore less local effects may occur (Kang *et al.*, 2003; Mahmoud *et al.*, 2000).

Overall, a kind of dose-response effect was observed when comparing all treatments. Water and placebo juice interventions resulted in almost similar number of adenomas in the SI. The most effective adenoma inhibition was observed for CAJ and B-PAE treatment groups. In the CAJ + B-PAE intervention group, higher amounts of polyphenols did not cause an increase in adenoma inhibition as compared to B-PAE and CAJ treatments (**Tab. 14**). However, these effects were dependent on the bioavailability.

In a Min mouse experiment, EGCG in drinking water in the range of 0.02% to 0.32% inhibited small intestinal tumorigenesis from 37% to 47%. The authors discussed that a saturation trend of the adenoma inhibition was observed as the concentration of EGCG increases in a dose-dependent manner (Ju *et al.*, 2005). Other previous

experiments demonstrated similar results with green tea extracts (Suganuma *et al.*, 2001).

Adenoma size

Both B-PAE and CAJ intervention groups confirmed the results of our previous animal experiment. As in the first animal experiment, treatments with CAJ and B-PAE reduced middle-sized (1–3 mm) adenomas and increased the small ones (<1 mm). This value represents the adenoma progression during the intervention. Our results revealed that CAJ and B-PAE polyphenolic components block the adenoma size (**Fig. 17**). Procyanidins might be responsible for reducing adenoma size, and thus anti-proliferative effects should be associated. The possible anti-proliferative mechanisms will be discussed in section 4.6.

However, the present study shows that placebo juice treatments statistically significantly increased small-size adenomas by 50% in comparison to the water control group. Taken together, these findings indicated that placebo juice intervention might have an effect in the reduction of adenomas. Placebo juice contains a high concentration of carbohydrates and lower amounts of pectin (**Tab. 4 and Fig. 17**).

It has been reported that higher-sucrose diets in the Min mouse model enhanced tumor development and epithelial cell proliferation in the SI. IGF-I and insulin serum levels were increased, suggesting a relevant mechanism involved in the adenoma development (Wang *et al.*, 2009). IGF-I and insulin effects are strongly linked to inhibition of apoptosis and cell cycle activations in intestinal epithelia (Giovannucci, 2007).

Pectin might be a second candidate to explain this increase in adenoma size in the SI by placebo treatment. But in one Min mouse experiment, resistant carbohydrates from bran wheat doubled the occurrences of adenomas of the proximal SI. Moreover, when the source of carbohydrates was obtained from apple pomace, a 2-fold increase was observed in the whole SI. The authors suggested the possible role of fermentation products as tumor enhancers (Mandir *et al.*, 2008). In contrast, as already described in the introduction, other positive effects have been described with dietary fiber such as white or rye bran. The possible preventive effects are also discussed in the next subchapter (see 4.1.2)

4.1.2 Effects of apple polyphenolic compounds in the colon

Based on the multistep carcinogenesis in the colon, it is suggested that there are at least two distinct stages for colon tumorigenesis in Min mice: macroscopic tumors and microadenomas. Therefore, additional events are accordingly required for evolution from microadenomas to macroscopic tumors (Yamada *et al.*, 2005). Moreover, a higher microflora biotransformation occurs in the colon, thus increasing the pro-oxidant environment (Sanders *et al.*, 2004).

In the colon, similar adenoma numbers were detected in all treatment groups. All animals developed a range from 0 to 3 adenomas, and therefore we decided to investigate microlesions, which are considered to be putative premalignant lesions and are found in high frequency.

Placebo juice intervention significantly reduced proximal colon microlesion numbers compared to water control by 71% (**Fig. 21 A**). In addition, placebo treatments resulted in non-significant microlesion reduction in middle and distal colon (**Fig. 21 B and C**). The putative effects of the placebo juice require an explanation. Inhibitory effects of placebo seem to remain in the whole colon. Again two possibilities may be considered, carbohydrates or pectin.

Since carbohydrates, such as glucose or saccharose, have been described to increase colonic proliferation in Min mice, these compounds might have no relation to the inhibitory potential of placebo juice (Mandir *et al.*, 2008).

On the other hand, as already described, CAJ contains 4 times more pectin than placebo and is linked to cloud particles. Pectin is able to pass through the SI more or less intactly and subsequently colonic microflora can degrade the pectin and liberate short-chain fatty acids (SCFA) that may have an influence on the mucosa (Harris and Ferguson, 1993). Placebo juice has no cloud particles but analytics have identified free pectin, which might be efficiently degraded by colonic microflora.

But the effects of pectin and SCFA on CRC remain controversial. Pre-clinical models have been used to test its protective potential. In one reported Min mouse experiment, 10% pectin-enriched diets did not inhibit intestinal tumorigenesis but rather increased the numbers of adenomas slightly (Jacobasch *et al.*, 2008). However, in the AOM model, 10 % pectin in the diet increased the SCFA content 4-

fold in cecum. In addition, ACF were significantly suppressed by intervention (Rao *et al.*, 1998). The role of SCFA is still controversial because no consistent results have been obtained neither in animal models and nor in human intervention studies. Differences may be attributed to the colonic microflora, composition of basal diets and duration of intervention (Pool-Zobel, 2005).

It was difficult to identify the possible effect of placebo intervention. Therefore, an analytical identification in feces would be very valuable to identify SCFA in the colon.

Until now, there is no evidence that chemopreventive agents reduce microlesions in Min mouse colon. But in the AOM-induced rat model, and after carcinogen injection, the rats develop microlesions in the colon. In this model, the dietary agents myricitrin and sesaminol glucosides significantly reduced microlesions in the colon (Asano *et al.*, 2007; Sheng *et al.*, 2007).

4.2 Pathologic analyses

In B-PAE-treated animals, a smaller spleen weight and a higher hematocrit percentage were observed in comparison to water controls. Both results indicated reduced intestinal haemorrhages, which are associated with the lower total adenoma number and size reduction in comparison to water control. These observations are in line with the low grade of extramedullary hematopoieses (E.H.) in the spleen of the B-PAE intervention. B-PAE polyphenolic compounds prevented paraneoplastic effects of adenoma development (**Fig. 22 and Tab.16**).

In our first animal study, a positive correlation ($r = 0.91$) between spleen weight and tumor load and a negative correlation between hematocrit values and tumor load ($r = -0.82$) were calculated. In the present study, this correlation could not be confirmed. This might be explained as an effect of fasting.

Combination of CAJ and B-PAE slightly increased spleen weights in comparison to the placebo intervention. This may be related to the E.H. grade, which was slightly increased in the histopathological analysis of the spleen. Moreover, CAJ + B-PAE intervention showed a hematocrit percentage similar to that of the placebo controls (**Fig. 22 and Tab. 16**). It has been suggested that regular consumption of polyphenols reduces the absorption of non-haem iron due to the chelating

characteristics of the polyphenols (Mennen *et al.*, 2005). *In vitro* studies with the Ca-Co2 cell line have demonstrated that iron absorption was reduced in the baso-lateral layer after a grape seed extract incubation (Kim *et al.*, 2008).

Since no signs of toxicity were observed in CAJ + B-PAE-treated animals, the higher polyphenol doses in the CAJ + B-PAE treatment may reduce iron absorption. Subsequently, the hematocrit percentage is diminished, and to compensate the reduced absorption, the spleen starts processes of extramedullary hematopoiesis.

So far, little is known about the toxicology of apple polyphenols. However, when Applephenon® (an extract from unripe apples) was given to rats in a dose of 2000 mg/kg, neither acute nor subchronic toxicity was detected (Shoji *et al.*, 2004). Our administered dose was 10 times lower for the CAJ + B-PAE treatments than in the experiment with Applephenon®, for that reason we can assume that the given dose was not toxic for the animals. In addition, histo-pathological analysis did not show toxicity in the liver.

4.3 Urine analyses

4.3.1 Bioavailability of CAJ and CAJ + B-PAE

As shown in **Fig. 24**, CAJ treatments slightly increased epicatechin concentrations in urine. This increase indicated that a small proportion of the catechin pool passed through the epithelial intestinal barrier and was metabolized in the liver. In contrast, HPLC analysis has revealed that CAJ + B-PAE components were highly absorbed and metabolized in the liver. The consequence of a high polyphenol intake has been described by Silberg *et al.* In rats, high flavonoid doses resulted in the saturation of the intestinal secretion of conjugates, which might improve the bioavailability (Silberberg *et al.*, 2006).

The major degradation products of procyanidins are epicatechin, 4-OH-phenylacetic acid and 3,4-diOH-phenylacetic acid. OPC and polymeric procyanidins are mostly degraded to catechin monomers by depolymerisation. Such monomers might be oxidized to 4-OH-phenylacetic acid and 3,4-diOH-phenylacetic by the gut microflora (Couteau *et al.*, 2001).

The rest of the detected metabolites, i.e. quinic acid, p-coumaric acid, ferulic acid and caffeic acid, were also identified in the urine of CAJ + B-PAE-treated animals. Ferulic and isoferulic acids might be degraded by the colonic microflora from epicatechin and further metabolised in the liver to m-coumaric acid and caffeic acid (Gonthier *et al.*, 2003). Quinic acids are mostly degraded from chlorogenic acid due to the esterase activity of the gut microflora. But there also reports of p-coumaric, ferulic and caffeic acid detected in urine as a degradation compound of chlorogenic acid (Gonthier *et al.*, 2006).

A good way to determine the absorption and metabolism of colon chemopreventive substances is the analysis of ileostomy fluids. Our network collaborators (WG Richling) have developed this method which consists of ileostomy patients drinking CAJ and the subsequent collection of ileostomy fluids in bags at various points in time. Polyphenols and metabolites are determined by HPLC (Kahle *et al.*, 2005).

In the first ileostomy experiment, after ingestion of 1 L of CAJ, a range of 0 to 33% of the initial quantity was recovered in the ileostomy bags with maximum excretion at 2 h (Kahle *et al.*, 2005). Interestingly, identified polyphenols in ileostomy fluids still displayed antioxidant activity after SI passage (Gerhauser *et al.*, 2008). Further studies with ileostomy patients showed that the majority of procyanidins (90%) were detected in the ileostomy bags, demonstrating that substantial amounts of these compounds in fact reach the colon under physiologic circumstances. But other metabolic products of polyphenols, such as phenol carbonic acids metabolites, were also detected (Kahle *et al.*, 2007).

The present study confirms for the first time in a pre-clinical model that apple polyphenols such as catechins and metabolites are in part absorbed by the SI and the colon.

4.3.2 Protein content in plasma and urine.

Unexpectedly, B-PAE intervention significantly increased the protein concentration in urine compared to water control animals by 35%. Likewise, serum protein content in B-PAE-treated animals was 20% higher in comparison to water control (**Tab. 19 and 20**). Both findings may be associated with the higher hematocrit value in comparison

to the rest of the treatments (**Fig. 22**). Higher hematocrit implies a lower serum volume and therefore an increase in serum protein concentration (Cirillo *et al.*, 1992).

A possible increase of high blood pressure might be due to the paraneoplastic effect of less adenoma numbers instead of being a direct consequence of B-PAE intervention. Polyphenols and metabolites were not detected in the urine of B-PAE-treated animals due to the very low bioavailability. This implies insignificant plasma availability and consequently no systemic effects (**Tab. 14 and Fig 24**).

Also in urine, protein content was increased by B-PAE treatments. Generally, protein in urine is a sign of inflammation and possible renal failure. Although in our previous work no protein concentration was determined in urine of PAE-treated animals, histopathological kidney analysis did not reveal any signs of renal failure associated with a higher hematocrit. The hypothesis that B-PAE intervention might result in adverse effects in the kidney absorption tubules as a result of an increase of protein content in urine was not contemplated because several reports indicated that polyphenolic compounds reduce proteinuria and associated effects. It has been described that in rats with renal failure induced by arginine, green tea diminished toxicity by reducing total NO in urine (Yokozawa *et al.*, 2003). Also, red wine polyphenols reduced high blood pressure and proteinuria in an induced hypertension mouse model (Jimenez *et al.*, 2007).

Physiologically, in the B-PAE intervention, the protein content in urine might increase as a result of the augmented protein content in serum. Higher protein content in serum might increase kidney reabsorption, and therefore higher protein content in urine was detected. It is important to mention that the rest of the interventions groups showed a decrease of serum protein content in comparison to normal Wt values as a result of intestinal bleeding. In contrast, B-PAE intervention restores protein concentration in serum due to lower intestinal haemorrhages (**Fig. 22 and Tab. 20**).

Overall, our findings revealed that as a result of strong paraneoplastic effects in the Min mouse, determination of protein in urine should only be interpreted cautiously as an inflammation marker.

Another remarkable finding is the reduction of glucose and fructose in urine of CAJ + B-PAE-treated animals. The increase of carbohydrates in placebo juice treatments suggested that polyphenols caused a decrease of glycosuria (**Tab. 18**). A frequent

model used for obesity or noninsulin-dependent mellitus diabetes is the Zucker rat. In this rat strain, lyophilized apples suppressed glycosuria and proteinuria. The authors suggested that these beneficial consequences might be caused by an improvement of lipid plasma levels and the limitation of oxidative damage in the kidneys (Aprikian *et al.*, 2002). Other reported *in vivo* experiments have shown that tea catechins inhibit the carbohydrate digestive enzymes α -amylase, intestinal sucrase, and α -glucosidase in the intestines of rats. This suggests that glucose production may be decreased in the gut, in turn lowering glucose and insulin concentrations (Kobayashi *et al.*, 2000).

4.4 Enzymatic activities

4.4.1 Effects of fasting in liver weight

In the GI tract and the liver, phase I and phase II enzymes are especially important for defense against environmental carcinogens due to constant exposure of toxins through ingested food. Additionally, these enzymes are abundant in epithelial tissues and can be modulated by dietary compounds found in the GI tract (Yang *et al.*, 2001).

After finishing the intervention, the first observation was a significant reduction in liver weight of fasted animals. On the one hand, the histo-pathological analysis revealed a glycogen reduction in all animals. But on the other, we have observed a decrease of protein liver content in fasted mice. Therefore, protein degradation occurred during the first 12 h of food deprivation (**Fig. 25A and B**).

During starving, the growth hormone (GH) rises causing an insuline decline, and glucose homeostasis is maintained. Moreover, glucagon avoids hypoglycemia by inducing gluconeogenesis and glycogenolysis and initiating hepatic glucose release (Klover and Mooney, 2004). All these biochemical changes are strongly regulated by the PGC-1 α protein, which is increased during fasting (Yoon *et al.*, 2001).

In a reported *in vivo* experiment, mice were fasted for 12, 24 and 72 h and microarray gene expression was used to determine differences after fasting. During the first 12 h of fasting, carbohydrate, amino-acid and lipid metabolic pathways were upregulated. Moreover, gene expression analysis revealed a strong induction of the

phosphoenolpyruvate carboxykinase-1 (pepck1), malate-aspartate shuttle and a consequent upregulation of the TCA cycle. after 12 h the authors also detected an induction of unfolded-protein response and of the metallothionein-1 which are related signs of oxidative stress (Sokolovic *et al.*, 2008).

On the other hand, Sokolovic *et al.* have pointed out that after 12 h of fasting, many proteins related to amino-acid metabolism are strongly activated. Similarly, according to a review of Finn *et al.*, during short term fasting, proteolysis is activated to supply amino acids as a source of energy. In hepatocytes, during fasting, protein degradation is regulated by the low levels of insulin and the increased levels of glucagon, IGF-I and glucocorticoids. Finn *et al.* have pointed out that in this process of protein catabolism, chaperons may facilitate the degradation of cytosolic proteins (Finn and Dice, 2006).

4.4.2 Phase I enzymes

CYP1A1 and CYP2B1 play critical roles in the metabolic activation of carcinogenic compounds leading to toxicity and CRC (Oyama *et al.*, 2004). It has been demonstrated that diets rich in meat increase CRC risk. The consumption of meat cooked at high temperatures forms PAH and heterocyclic aromatic amines that increase CYP1A activity (Sugimura, 2002). Similarly, the consumption of red meat induces CYP2B activity through N-nitrosamines (Bingham *et al.*, 2002).

In the present work, CYP1A1 hepatic enzymatic activity was dramatically reduced after fasting, whereas no difference was observed for CYP2B1 (**Fig. 26**). These findings agree with previous published studies. Decreases in CYP1A activities during starvation have been reported in rats (Dixon *et al.*, 1960; Ma *et al.*, 1989). However, totally different enzymatic activity results were reported in rats that were fasted for 12 h. In this experiment, CYP1A1 remained unchanged but CYP2B1 was significantly induced after fasting (Brown *et al.*, 1995). It is important to point out that our results differ from caloric restriction (CR) experiments. CR experiments in fasted rats showed an increase of both CYP1A1 and CYP2B1 activities (Chou *et al.*, 1993).

The main reason of CYP1A1 reduction might be associated with liver protein degradation after fasting. But the influence of oxidative stress might help to reduce its activity. Sokolovic *et al.* have shown that 12 h fasting was able to cause initial stages

of oxidative stress in the liver. In rabbits, liver-induced inflammation revealed that CYP1A1 was strongly downregulated in the absence of change of protein expression (El-Kadi *et al.*, 2000).

4.4.3 Phase II enzymes

Both QR and TrxR phase II enzymes were significantly reduced after fasting. In addition food deprivation had a striking effect on reducing GSH levels (**Fig. 27 and 28**).

The reduction of TrxR enzymatic activity might be related to Txnip. This protein is a negative regulator of TrxR and induces its degradation. Txnip is known to maintain glucose homeostasis during fasting by preventing excess glucose uptake or metabolism. Hepatic overexpression of Txnip causes elevated serum glucose levels and decreased ketone bodies (Chutkow *et al.*, 2008).

Other reported experiments have shown GSH depletion in the rat liver after 18 h of fasting. In addition, GST liver activity remained unchanged. Moreover, refeeding of mice restored GSH liver levels (Leeuwenburgh and Ji, 1996; Szkudelski *et al.*, 2004). These findings are in line with our results.

So far, no direct relation between QR and fasting has been demonstrated. However, Van den Bosch *et al.* investigated the effects in 24 h-fasted rats in Phase II intestinal enzymes. Gene expression experiments using cDNA array technology validated with qRT-PCR showed no difference in Phase II intestinal enzymes, whereas GST and GSH were strongly reduced. A possible justification would be that during starving dietary electrophiles are not present and consequently the detoxification system may be downregulated (van den Bosch *et al.*, 2007).

It is remarkable that all CYP1A1, QR, TrxR and GSH were significantly decreased after fasting. On the one hand, it is possible that the detoxification system is downregulated since there is no need to detoxify. But on the other, fasting may reduce total protein content in the liver.

Effects of CAJ and B-PAE in phase II enzymes

Treatments with CAJ and CAJ + B-PAE induced GST and QR in the liver in a non-significant manner. Interestingly, CAJ + B-PAE intervention resulted in a statistically significant increase of Trx enzyme activity. These results were strongly associated with the bioavailability because HPLC analysis revealed that CAJ + B-PAE components are highly absorbed and metabolized in the liver. As result of this absorption, the activity of the investigated phase II enzymes was induced (**Fig. 24 and Tab. 23**).

Other reported experiments have demonstrated similar effects. Hepatic QR and GST enzymatic activities were upregulated in rats treated with apple, grape and black currant juices. In contrast, neither a carbohydrate drink nor an orange juice resulted in the induction of the hepatic phase II enzymes (Breinholt *et al.*, 2003). Together with our experiment,s this indicates that the polyphenols are the main compounds involved in phase II enzyme induction.

Although in different tissues, similar results were observed by our collaborators from Jena (Pool-Zobel and Wöflf). They reported an increase of GST and UDP genes in LT97 cells with PAE incubations (Veeriah *et al.*, 2008). However, in the DMH-initiated rat model, CAJ treatments showed no difference in GST levels in normal colon mucosa (Barth *et al.*, 2005).

The possible mechanism of induction might be *via* Nrf2 and the transcription of antioxidant responsive elements (ARE). The AREs are regulatory sequences found on promoters of several phase II detoxification genes that are inducible by xenobiotics and antioxidants (Dinkova-Kostova *et al.*, 2002).

The Nrf2 activation is mediated by the repressor protein Keap1, which acts by directly interacting with Nrf2, thus preventing its nuclear accumulation. The activation occurs whenever this interaction and repression is disrupted, allowing Nrf2 to translocate and accumulate in the nucleus. It has been also demonstrated that Nrf2 activation is mediated by mechanisms that lead to its stabilization or the upregulation of Nrf2 gene expression at the transcriptional level, such as MAPK or PI3K activation (Na and Surh, 2008).

In our experiments, due to the relatively high absorption, the putative candidate for this effect in the CAJ and CAJ + B-PAE treatments is epicatechin. Other *in vivo* bioavailability experiments have demonstrated that epicatechin is relatively well absorbed in the SI and its metabolites are found in plasma after 2 h administration (Feng, 2006).

In vivo experiments with green tea showed similar findings (Embola *et al.*, 2002; Khan *et al.*, 1992; Maliakal *et al.*, 2001). EGCG and other catechins might be absorbed in the liver and be responsible for detoxification–gene activation. Green tea polyphenols activate MAPK and PI3 signalling pathways, leading to activation of Nrf2 and ARE with subsequent induction of phase II enzymes. But it has been discussed that green tea polyphenols may interact with cysteine residues present in Keap1, thereby inducing Nrf2 dissociation and nuclear activation of the ARE (Na and Surh, 2008).

Other *in vivo* experiments have demonstrated that polymeric black tea polyphenols induce phase II enzymes *via* Nrf2 in mouse liver and lungs through PKC and PI3-pathways (Patel and Maru, 2008). This suggests that different black and green tea polyphenols induce Nrf2 activation by different pathways.

Furthermore, polyphenol degradation products such as ferulic and coumaric acids are able to induce phase II enzymes (Yeh and Yen, 2006). In the CAJ + B-PAE treatment group, such compounds might help to induce phase II enzymes in a synergistic way. Nevertheless, lower amounts of such compounds were detected in urine of the CAJ intervention group. These low concentrations might not be sufficient to induce detoxification enzymes. Other apple compounds such as chlorogenic acid and quercetin have been described to be involved in Nrf2 activation as well (Eggler *et al.*, 2008; Feng *et al.*, 2005).

4.5 Mechanisms in normal mucosa

We have already mentioned the importance of Apc mutations as one of the initial events in the progression of carcinogenesis. In the Min mouse model, after the third week and due to the loss of Apc heterozygosity, any area of intestinal mucosa might presumably develop polyps. However, most of the adenomas start to be detectable at 6–8 weeks of age. Whenever normal mucosa of Min mice was compared with Wt

mice, longer villi were found in the ileum at 12 weeks. Moreover, immunologic changes such as increases of CD8⁺ cells, Ig A and PGE₂ at 5 weeks of age are increased in normal mucosa (Kettunen *et al.*, 2003).

Therefore it was of interest to determine whether TCF/LEF target genes were involved in early stages of adenoma development. For this purpose, the normal mucosa of Min mice of the first animal experiment was investigated.

In the distal parts of the SI and the colon, Cyclin D1 was non-significantly reduced by PAE treatments. Nevertheless, there was no direct relation with adenoma inhibition since CAJ intervention showed the strongest tumor inhibition and yet no association with Cyclin D1 was observed (**Tab. 25 and Fig. 29**). These results might indicate that Cyclin D1 is not important for the early stages but may be involved as a secondary event. In fact, Cyclin D1 is not essential for the development of intestinal tumorigenesis because *Apc*^{Min/+} *cyclin D1*^{-/-} mice still develop adenomas, albeit at a lower frequency (Hulit *et al.*, 2004). On the other hand, it has been observed that Cyclin D1 is upregulated in middle and large size adenomas. Therefore, Cyclin D1 expression may be involved as a secondary event and an important factor in adenoma establishment and growth. The same authors have suggested that Cyclin D1 is not a direct target of Wnt signalling pathway. This was demonstrated using promoter reporter constructs to measure increases of cyclin D1 expression (Sansom *et al.*, 2005).

In the Min mouse, other TCF/LEF target genes such as c-myc play an important role at initiation stages. C-Myc was initially identified as an oncogene but has also been associated with homeostatic mucosa maintenance. Conditional intestinal c-myc deletion resulted in a crypt loss within weeks (Muncan *et al.*, 2006). Interestingly, simultaneous deletion of c-myc and *Apc* in the intestinal epithelia rescued phenotypes of perturbed differentiation, migration, proliferation and apoptosis, which occur on deletion of *Apc* (Sansom *et al.*, 2007).

In the colon, qPCR experiments allowed detecting a 50% reduction in Cox-2 gene expression after CAJ and PAE treatments. However, both results were not statistically significant (**Fig. 30**).

In one animal experiment, Chen *et al.* observed a Cox-2 gene reduction in the colonic mucosa in 23 weeks old Wt in comparison to Min mice (Chen *et al.*, 2004). In

the present study, the possible overexpression of Cox-2 in normal mucosa may be reduced by CAJ and PAE treatments and therefore may decrease inflammation in the large bowel. Nevertheless, there was no relation with colon tumors. Due to the low number of adenomas in the colon, it would be valuable to analyze whether the treatments reduced the microlesions in the colon.

Moreover, in the DMH-induced rat model treated with CAJ, Barth *et al.* did not find any associated Cox-2 reduction in the colonic mucosa (Barth *et al.*, 2007).

Wnt targets are required to keep the integrity and renewal of the mucosa. But low levels are needed to preserve the homeostasis. So far, in Min mice, the mechanism of regulation in the normal mucosa is not fully understood. The effects of CAJ and PAE in normal mucosa remain to be elucidated.

4.6 Mechanistic investigations in adenomas

Since the results from the previous animal experiment in normal mucosa were not clear, the aim of the present work was to elucidate the cellular mechanisms in adenomas.

As already discussed, the liver was strongly affected by food deprivation. Therefore, the difference between fasted (B-PAE and water groups) and non-fasted (Placebo, CAJ and CAJ + B-PAE groups) mice adds another factor that we would like to explain.

It has become clear that the intestinal epithelium is an important metabolic site and to a great extent responsible for the first-pass metabolism of nutrients and xenobiotics. Prolonged fasting has several effects that may modulate the physical forces acting on the gut mucosa. First, the intermittent switch to postprandial motility patterns is lost, along with changes in volume, density, and composition of the luminal contents. Second, the mucosa itself becomes atrophic, with shortened villi in the small bowel mucosa (Inoue *et al.*, 1993; Martins *et al.*, 2001). It has been suggested that this change in the mucosa may happen via ROS action (Brownlee *et al.*, 2007).

4.6.1 Fasting effects and Cyclin D1 expression

Cyclin D1 is a Wnt target gene activated through nuclear β -catenin in CRC cell lines (Tetsu and McCormick, 1999). In addition, its expression is increased in Min mice adenomas. As already mentioned, crossing Min mice with *cyclin D1*^{-/-} mice reduced the intestinal tumor number in the SI and the colon (Hulit *et al.*, 2004). Cyclin D1 forms a protein complex and functions as a regulatory subunit of CDK4 or CDK6, (Cyclin-dependent kinases) the activity of which is required for cell cycle G1/S transition (Alao, 2007). In Int-2, fasting-associated effects increased Cyclin D1 expression in a non-significant manner by approximately a half (**Fig. 35**). Interestingly, *in vivo* experiments in normal mucosa of fasted mice performed by Sokolovic revealed that different cell cycle and apoptosis genes were strongly downregulated in normal mucosa. The authors described a robust decrease in Cyclin D1 expression after 12 h fasting. Consequently, CDCK4, Cyclin E and Rb proteins were also downregulated. This indicated an overall slow-down of the cell turnover. The authors argued that this decreased turnover might be associated with a proportional contraction of all components in the SI (Sokolovic *et al.*, 2007).

Totally different results were observed in the present thesis. Our findings imply that after 12 h food deprivation, adenomas trend to activate cell cycle and to increase cell turnover.

Effects of treatments in Cyclin D1

Food deprivation almost did not affect Cyclin D1 expression in Int-3. But CAJ and CAJ + B-PAE interventions reduced its expression by 3 and 4 times, respectively, thereby reducing fasting-associated effects. Although not statistically significant, a similar pattern was found for β -catenin in Int-3 segments (**Fig. 35 and 34**).

A feasible elucidation of the protective mechanism is difficult due to the food deprivation. But a possible speculation might be that β -catenin reduction is associated with polyphenol bioavailability in both CAJ and CAJ + B-PAE interventions (**Fig. 24**).

Our data also suggested that some low molecular compounds might penetrate inside the enterocytes and interact with proteins. These absorbed compounds might be

responsible for β -catenin reduction by CAJ and CAJ+B-PAE treatments. Moran *et al.* described in Min mice experiment in which 0.1% carnosol from rosemary significantly reduced adenomas by nearly 50%. A cytosolic effect was described in which treatments restored both E-cadherin and β -catenin to the enterocyte membranes in a similar way as in Wt mice. Phenolic diterpenes such as carnosol are known for their high permeability, and therefore intracellular effects could easily take place (Moran *et al.*, 2005).

4.6.2 Fasting effects in MEK/ERK phosphorylation

EGFR transduction is actively involved in controlling proliferation, survival and migration of epithelial cells. Physiologically, gastrointestinal mucosa is a rapidly self-renewing tissue where enterocyte differentiation and migration are needed and controlled by EGFR activity (Stappenbeck and Gordon, 2000). Promotion and progression steps of CRC are stimulated by EGFR activity (Mendelsohn and Baselga, 2000).

After 12 h of fasting, we observed a strong reduction in both MEK and ERK phosphorylation (**Fig. 37**). Both results implied that a decreased EGFR transduction signal affects transcription factors such as Elk-1, c-fos and CREB. The AP-1 transcription factor is a heterodimer formed by c-jun and c-fos. This protein is actively involved in CRC development by regulating cell proliferation, differentiation, transformation and/or apoptosis. Moreover, c-fos is positively regulated by Elk-1 transcription factors. Furthermore, Elk-1 induces TCF-LEF genes, whereas CREB acts as a repressor of β -catenin (Milde-Langosch, 2005). A detailed target promoter analysis may be important to elucidate possible pathway connection. A transcriptional regulator of Cyclin D1 is the AP-1 binding domain and consequently activated by ERK phosphorylation. Therefore, food deprivation may be responsible for Cyclin D1 increase *via* ERK phosphorylation (**Fig. 35 and 37**).

Sokolovic *et al.* have described that after 12 h of fasting, mainly amino-acid and carbohydrate metabolism-related genes are strongly affected. One of the most affected metabolic pathways is the glutamine synthesis. After 12 h of food deprivation, preservation of cytosolic glutamine levels is a principal metabolic change because enterocytes use glutamine as a primary source of energy (Sokolovic *et al.*,

2007). Moreover, glutamine administration reduces gut mucosal atrophy. Interestingly, *in vitro* experiments with rat intestinal epithelial cells demonstrated an increase in ERK phosphorylation after 4 h of glutamine incubation. The authors further discussed that the connection between glutamine and ERK activation may be relevant as an early fasting adaptation stage to maintain the integrity of the intestinal mucosa (Larson *et al.*, 2007). On the other hand, one of the known biological processes in the small intestine during fasting is the increase of oxidative stress (Jonas *et al.*, 2000). Moreover, it has been reported that ERK signalling together with c-Jun N-terminal kinase (JNK) are activated in the intestine during oxidative stress (Zhou *et al.*, 2005).

Nevertheless, these findings have been reported in normal mucosa. In the present study, fasting increased proliferation in adenomas through p-ERK. As already explained, one reason might be a possible increase of glutamine as a source of energy and oxidative stress.

Suppression of fasting effects by B-PAE intervention

B-PAE treatment is shown to reduce half ERK and MEK phosphorylation in adenomas compared to water control in a non-significant manner and consequently the derived effects of 12 h fasting (**Fig. 37**). Significance may be affected due to a low animal number, and thus increasing the number of samples might result in a statistically significant difference. These results are in line with earlier investigations from Gossé *et al.* In the AOM-induced rat model, a procyanidin-rich fraction (0.01% in drinking water) reduced ACF/colon by 50% and also decreased ERK phosphorylation.

Although it is difficult to describe a possible molecular mechanism due to fasting, OPC and polymeric procyanidins reduce fasting-associated effects. B-PAE contains mostly polymeric and oligomeric procyanidins that can be responsible for this EGFR pathway inhibitory potential. In addition, flavonols in B-PAE identified by HPLC analysis can modulate signal transduction.

Our speculations were supported by *in vitro* experiments. HT29 cells stimulated with EGF in the presence of low PAE concentrations (from 0.6 to 6 nM) significantly reduced ERK phosphorylation (Kern *et al.*, 2005). The same group described that

PAE significantly inhibited EGFR signal transduction in the human colon cancer cell line HT29. Pure compounds such as dimeric procyanidins and quercetin glucosides were found to be the most active polyphenols (Fridrich *et al.*, 2007b). Further investigations with trimeric and dimeric procyanidins isolated from grapes significantly reduced EGFR autophosphorylation in the human vulva carcinoma cell line A431 (Fridrich *et al.*, 2007a).

Overall, B-PAE treatments reduced fasting-associated effects in adenomas by reducing ERK phosphorylation.

4.6.3 iNOS upregulation and fasting

Two key proteins during inflammation processes in adenoma development are Cox-2 and iNos. Both are increased in adenomas and are putative targets for chemoprevention (Hull *et al.*, 1999; Scott *et al.*, 2001).

Similar to ERK and MEK expression, during fasting, iNOS was inhibited in Int-2 and Int-3 approximately by a half and a third, respectively (**Fig. 37 and 38**). So far, no association between inflammation and fasting has been investigated in the SI, but it has recently been described that LPS-induced liver inflammation in rats is increased by fasting. Expression of Cox-2 and iNOS was enhanced by fasting, and feeding declined this upregulation (Adams *et al.*, 2009).

iNOS promoter contains a AP-1, NF- κ B, CREB and C/EBP binding domains. All these transcription factors result in a complex regulation of the gene. But p-ERK is one of the upstream regulators of iNOS. The activation of iNOS *via* ERK transduction has been demonstrated in melanoma epithelial cell lines. Blocking EGFR signalling through MEK inhibitors, iNos expression was subsequently downregulated. The same effects were obtained knocking down B-raf by RNAi. Despite the connection between MAPK activation and iNOS expression, authors also discussed that other factors might be involved (Ellerhorst *et al.*, 2006). Nevertheless, the MAPK cascade is an upstream activator of NF- κ B (Seger and Krebs, 1995). In fact, evidence exists in several cell types for the involvement of NF- κ B in mediating the regulation of iNOS by ERK (Kim *et al.*, 2007; Pergola *et al.*, 2006).

Overall, this result indicated that fasting increased the inflammation process in adenomas. A general conclusion of fasting effects in adenomas is the increased

expression of Cyclin D1 and iNOS *via* p-ERK. As already mentioned in the last section, this might be related to the use of glutamine as an energy source and possible oxidative stress in epithelial tissues.

Effects of B-PAE in iNOS expression

Similar to p-ERK, we have observed that B-PAE intervention reduced iNOS expression compared to water control. The increment of iNOS expression during fasting is downregulated by B-PAE treatments. Similar to the comparison of fasted vs. non-fasted animals, both p-ERK and iNOS were reduced in a similar fashion, comparing B-PAE- and water-treated animals (**Fig. 37 and 38**).

One of the aims of the present study was to investigate into the effects of CAJ and PAE treatments in inflammation. We decided to investigate NO and PGE₂ synthesis as inflammation biomarkers. As already described, iNOS is expressed in Min mice adenomas and therefore NO production increases the oxidative status. Moreover, NO production activates Cox-2 expression.

The mechanism that links PGE₂ and adenoma development was described by Pai *et al.* PGE₂ transactivates EGFR activating downstream proteins thus promoting CRC growth and gastrointestinal hypertrophy (Pai *et al.*, 2002). On the other hand, PGE₂ can also induce the Wnt pathway through accumulation of β -catenin in the nucleus via Akt or G α subunits (EP receptor) (Buchanan and DuBois, 2006). Therefore, it was of interest to determine whether CAJ and B-PAE treatments might reduce NO and PGE₂ production in urine and serum, respectively. Both biomarkers resulted in similar values for all treatments (**Tab. 19 and 21**).

The similar PGE₂ levels in serum indicated a lower effectivity in Cox enzymes inhibition *in vivo*. Our previous *in vitro* results demonstrated that PAE inhibited Cox activity with an IC₅₀ of 400 μ g/ml, which is a high concentration. Epicatechin, phloretin, trimeric and tetrameric procyanidins were described as the main inhibitory compounds of PAE (Zessner *et al.*, 2008). Other more efficient polyphenolic compounds such as tricetin potently inhibited *in vitro* Cox enzymes with an IC₅₀ value of 1 μ M. Similarly to B-PAE, tricetin inhibited adenoma number by 33%, but PGE₂ levels in plasma were reduced by 40% (Cai *et al.*, 2005).

Since iNOS was one of the affected targets, NO determination was planned as a putative biomarker in urine. But NO detection depends on concentration, short half-life, and high reactivity with other chemical compounds such as superoxide radicals, thiols, and haem proteins. This difficulty can be avoided by analyzing PGE₂ and NO directly in adenomas after the snap-frozen collection, and more valuable information would be obtained.

The increased inflammation due to the fasting effects is reduced by B-PAE intervention in adenomas. A general overview of all possible mechanisms and pathways is summarized in **Fig. 40**.

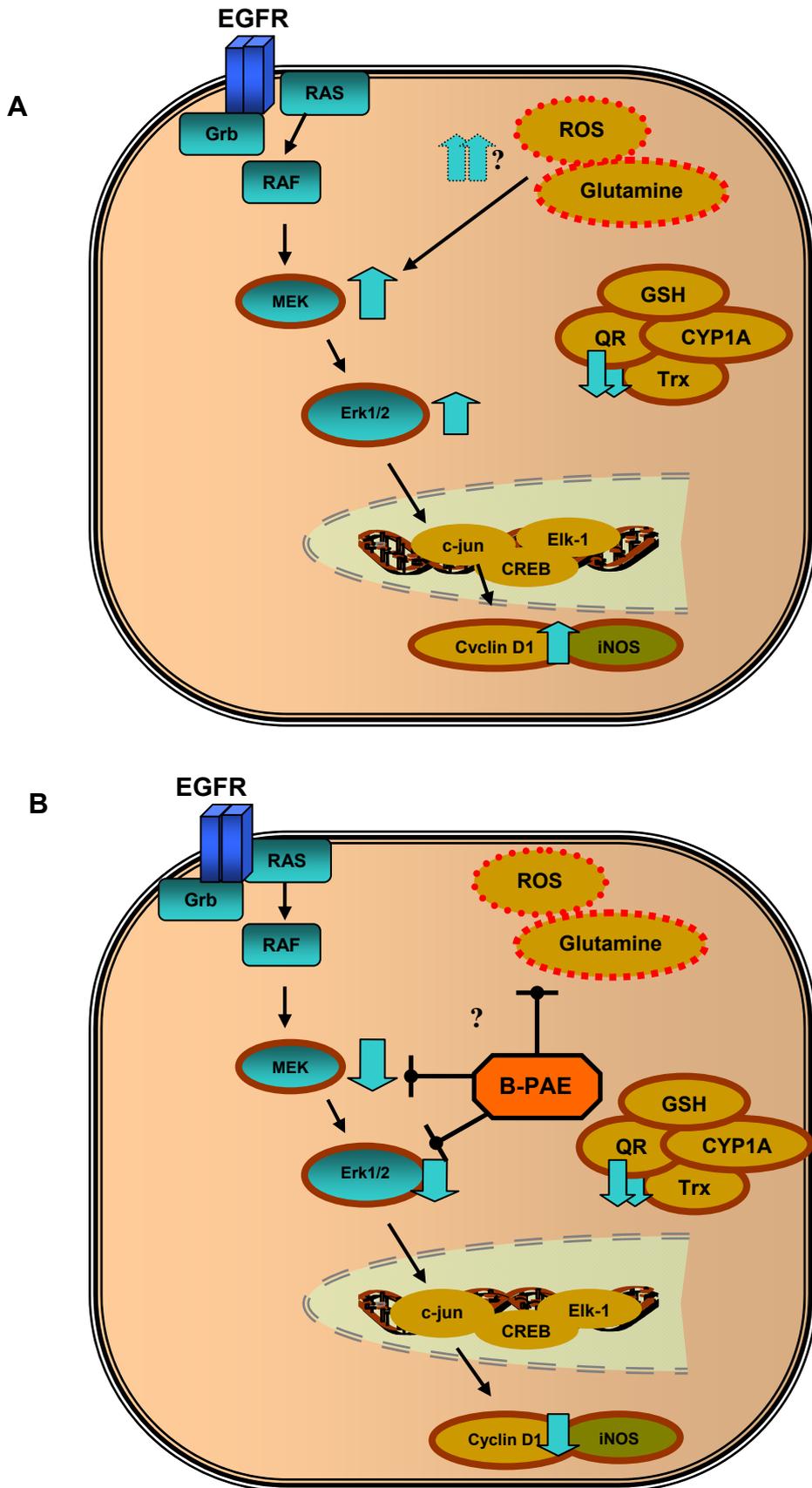


Fig. 40. Putative mechanism for A) fasted animals and B) fasted animals receiving B-PAE treatment

4.7 From pre-clinical models to the human situation

The role of fruit juices in cancer prevention is still an on-going question. Many studies have addressed fruit juices and modulation of oxidative status.

In one study, our collaborators (WG Rechkemmer/Briviba) used two different polyphenolic-rich juices in human subjects. Antioxidant parameters such as plasma malondialdehyde and oxidative DNA damage in lymphocytes were reduced. One juice contained a mixture of apple, mango and orange juice and the other was rich in anthocyanin-providing aronia, blueberries, and boysenberries, while both juices contained similar polyphenol amounts (Bub *et al.*, 2003). Likewise, another intervention with rich polyphenolic fruit juices caused comparable antioxidant effects. Moreover, GST and GSH levels were increased and the DNA-binding activity of NF- κ B was reduced (Hofmann *et al.*, 2006; Spormann *et al.*, 2008). Improved antioxidant capacity against hydroxyl radicals was detected in serum of volunteers who drank 150 ml CAJ (Ko *et al.*, 2005). Further comparable results were obtained in one human intervention study after 1 h CAJ ingestion in terms of serum DPPH (Chrzczanowicz *et al.*, 2008). In contrast, our collaborators (WG Briviba) have reported that consumption of 1 Kg of apples did not affect plasma parameters like antioxidant capacity, endogenous DNA strand breaks and inhibition of H₂O₂-induced DNA damage in lymphocytes. But, DNA from lymphocytes was protected from iron damage and the oxidized pyrimidines were diminished (Briviba *et al.*, 2007).

In a recent human intervention study with patients with colorectal adenomas, fruit juice was associated with reduced adenoma risk. A RR of 0.74 (95% CI = 0.58-0.96) was described, similar to vegetables or fruits (Wu *et al.*, 2009). In a further human intervention study with 10 % freeze-dried berries, when patients with CRC and/or polyps were taking 60 g of a berry extract, proliferation and angiogenesis biomarkers were significantly reduced. Moreover, apoptosis was enhanced (Stoner *et al.*, 2008).

Targeting inflammation in CRC has provided interesting results in FAP patients. Together with celecoxib, NSAIDs and sulindac have been also the focus of human studies. Unfortunately, both sulindac and aspirin usage have been related to bleeding events. In a randomized clinical trial with a flavonoid mix in patients with acute bleeding from internal haemorrhoids, treatments prevented bleeding and a reduced risk of relapse (Ho and Seow-Choen, 2000). Recently, it has been reported that a

PAE in combination with aspirin reduced rat gastrointestinal injury in terms of decreased acute and chronic injury (D'Argenio *et al.*, 2008).

We have presented the chemopreventive potential of CAJ and B-PAE in Min mouse as a pre-clinical model. Both treatments have caused positive results in initiation and promotion stages of colon carcinogenesis. Moreover, fasting-associated effects were in some cases suppressed. This indicates that dietary compounds positively affected the development of CRC. B-PAE was described as a potent chemopreventive agent. This might be attributed to the procyanidin content, which has been widely investigated in cancer chemoprevention. On the other hand, CAJ is presented as a source of secondary plant compounds, which demonstrates that diet is one of the major factors affecting CRC. Other western-associated malignancies such as cardiovascular and neuronal diseases might also be prevented with rich polyphenolic diets. To date, one of the most popular rich polyphenolic drinks is green tea. Therefore, clinical trials with green tea have started some years ago. In the same way, we propose CAJ and PAE as chemopreventive agents, which should therefore be taken into consideration for clinical trials and human intervention studies.

4.8 General conclusions

To date, there is a huge interest in finding new compounds and molecules that could prevent the development of several malignant diseases. Our aim was to determine the *in vivo* signalling pathway involved in cancer prevention.

1- One of the purposes of the present work was to determine mechanistic effects of CAJ and PAE in normal mucosa from our previous animal experiment. PAE intervention reduced Cyclin D1 expression in the distal SI and Cox-2 expression in the colon. Nevertheless, both reductions were statistically not significant, and no relation with the adenoma numbers was observed. Further investigations are necessary to study the effects of CAJ and PAE at the initiation stage of adenoma formation.

2- In the present work, the possible effect of apple polyphenolic compounds in adenomas was studied in a second animal experiment. First, the results indicated that polyphenols are the active compounds since placebo treatment only inhibited adenoma development in animals by 7%. Second, apple polyphenols might act as

chemopreventive compounds in a dose-response manner because treatments with CAJ + B-PAE showed less adenoma inhibition compared to CAJ and B-PAE (B-PAE: 34%, CAJ06: 24% and CAJ + B-PAE: 17%). Third, the highest inhibition was shown with B-PAE intervention. This indicated that procyanidins are major active compounds. These findings confirmed previous *in vitro* experiments performed in our work group. All these results have a strong relation to the bioavailability of apple polyphenols. Further possible effects of placebo juice intervention in the small intestine and the colon remain unclear and require additional investigations.

3- In B-PAE (procyanidin-rich) intervention, mechanistically investigations in adenomas might indicate that ERK signalling is one of the possible transductions pathways modulated by oligomeric and polymeric procyanidins. The poor bioavailability of B-PAE compounds and a consequently local effect in the small intestine could be the reason to block EGFR transduction signalling.

CAJ inhibitory potential might be reduced due to the lower amount procyanidins. But other monomeric polyphenols may also be responsible for an intracellular effect. In the distal SI, potential targets are β -catenin and Cyclin D1. However, due to unexpected fasting conditions in both treatments, these assumptions remain speculative.

4- Another of the aims of the present work was to determine the active potential of CAJ and B-PAE in terms of drug metabolism and anti-inflammatory capacity. CAJ- and CAJ + B-PAE-treated animals induced QR and GST in a non-significant manner. Moreover, CAJ + B-PAE intervention caused a statistically significant increase of TrxR enzymatic activity. These results are strongly related to the bioavailability. The induction of phase II enzymes agrees with our previous *in vitro* experiments and it is for the first time in mice reported. Inflammatory effects in urine and plasma could not be demonstrated. Interestingly, B-PAE treatment reduced iNOS expression in adenomas compared to water control.

5- Reductions of GSH content as well as CYP1A1, QR and TrxR may be explained as a consequence of protein reduction in the liver, being in turn as a consequence of unexpected fasting effects. In adenomas, fasting effects increase proliferation (Cyclin D1) and inflammation (iNOS) *via* p-ERK. These findings were suppressed by B-PAE intervention.

Finally, these findings strongly suggest that CAJ and PAE contain promising chemopreventive compounds that should be further explored for prevention of CRC in human intervention studies and clinical trials.

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PUBLICATIONS AND POSTER PRESENTATIONS

Publications:

Cancer chemopreventive potential of apple juice — Results of a short-term human intervention study with ileostomy patients.

C. Gerhäuser, K. Klimo, K. Kahle, **A. Garreta-Rufas**, R. Steinle, W. Scheppach and E. Richling. European Journal of Cancer Supplements, Volume 6, Issue 3, March 2008.

Identification of 3-hydroxy- β -damascone and related carotenoid-derived aroma compounds as novel potent inducers of Nrf2-mediated Phase 2 response with concomitant anti-inflammatory activity.

C. Gerhäuser, K. Klimo, W. Hümmer, J. Hölzer, A. Petermann, A. Garreta-Rufas, F.D. Böhmer, P.Schreier. Molecular Nutrition and Food Research (accepted).

Natural cloudy apple juice and a polyphenol-enriched apple extract prevent intestinal adenoma formation in a genetic model for colon cancer prevention.

L. Pan, **A. Garreta-Rufas**, Zessner H., Will F., Klimo K., Frank N., Dietrich H., Becker H., Gerhauser, C. (in preparation)

Mechanistic investigations in the Apc^{Min/+} mouse model by cloudy apple juice and a procyanidin-enriched apple extract.

A. Garreta-Rufas, H. Mannsperger, M. Olk, R.Steinle, K. Klimo , Frank N., F. Will, Dietrich H., E. Richling, U. Korf, C. Gerhauser (in preparation).

Poster presentations:

Cloudy apple juice and a polyphenolic apple extract prevent adenoma development in the the Apc^{Min/+} mouse model.

Antonio Garreta-Rufas, R. Steinle, K. Klimo, M.Olk, F. Will, H. Dietrich and C.Gerhäuser.

DKFZ PhD student meeting. Weil der Stadt, Germany. July, 2007.

Apple juice intervention reduces adenoma development and ERK 1/2 phosphorylation in the Apc^{Min/+} mouse model.

Antonio Garreta, Heiko Mannsperger, Melanie Olk, Renate Steinle, Karin Klimo, Norbert Frank, Frank Will, Annemarie Poustka, Ulrike Korf and Clarissa Gerhäuser. Poster presentation AACR 2008, San Diego, 12-16.4.2008

Cancer chemopreventive potential of apple juice: Results of a short-term human intervention study with ileostomy patients.

Clarissa Gerhäuser, Karin Klimo, Katrin Kahle, **Antonio Garreta-Rufas**, Renate Steinle, Wolfgang Scheppach and Elke Richling. Poster presentation AACR 2008 San Diego, 12-16.4.2008

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