Review

Prevention of colon carcinogenesis by apple juice *in vivo*: Impact of juice constituents and obesity

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It is estimated that 75 – 85% of all chronic diseases are linked to lifestyle-related and environmental factors. The development of colon cancer is positively associated with obesity and inversely associated with the intake of dietary fibre, fruit and vegetable. Apple juice is the most widely consumed fruit beverage in Germany. It contains a specific spectrum of polyphenols and other components that may reduce the risk of colon cancer. Epidemiologic studies suggest an inverse correlation between apple consumption and colon cancer risk, although the mechanisms for these observations are not clear. The present review summarizes the preventive potential of apple juices and different apple constituents on biomarkers related to colon carcinogenesis with special focus on the *in vivo* evidence and the cancer promoting condition of obesity. However, under the cancer promoting condition of obesity, apple juice did not show cancer-preventive bioactivity. In our experiments a cancer-preventive bioactivity of apple juice is lacking in rats under the cancer-promoting condition of obesity. To further investigate, whether this lack of efficacy observed in obese rats might be representative for obese individuals human intervention studies on high risk groups such as obese or diabetic individuals are of interest and will be conducted.

Keywords: Aberrant crypt foci / DNA damage / Dyslipidemia / Proliferation / Zucker rat

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1 Introduction

Estimates of the cancer burden in Europe for 2006 demonstrate that colorectal cancer is the second most common form of cancers with 412 900 incident cases and the second leading cause of cancer death [1]. The cause of colon cancer has been linked to high caloric food intake, low physical activity and resulting obesity (reviewed by [2]). Consistent epidemiological findings suggest, that a high dietary intake of fruits and vegetables is associated with a reduced risk of cancer and that specific phytochemicals may be responsible for the observed preventive effect [3]. According to recent epidemiological data colorectal cancer risk [4] and the risk of colorectal advanced adenoma recurrence [5] could be significantly modulated by dietary intake of polyphenolic flavonoids. Apples are a very significant source of flavonoids in European countries and there are several lines of evidence suggesting that apple constituents have chemopreventive activities [6]. A consistent inverse association between apple intake ($\geq$1 apple/day *vs.* <1 apple/day) and risk of colon cancer is reported by Gallus *et al*. [7] who analysed data from multicenter case-control studies conducted in Italy including 1953 incident cases of colorectal cancer. In the Nurses' Health Study including overall 34 467 women and 1720 prevalent cases of adenoma of the distal colon and rectum an inverse correlation of apple consumption with diagnosis of intestinal polyps was reported. Those women in the highest quintile compared to those in the lowest quintile of apple consumption had an OR of 0.83 (95% CL, 0.7 – 0.89) for colorectal adenomas [8].

Apples contain polyphenols and several classes such as phenolic acids, flavan-3-ols, flavonols and dihydrochalcones have been found. Among these classes the main apple polyphenols are chlorogenic acid, quercetin glycosides, procyanidins, phlorizin and phloretin-2′-*O*-xyloglycoside [9]. Beside the apple, apple juice might also contribute to

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Abbreviations: AC, aberrant crypts; **ACF,** aberrant crypt foci; **a.l.,** ad libitum; **CB,** control beverage; **CF,** cloud fraction, **CleA,** clear apple juice; **CloA,** cloudy apple juice; **DMH,** 1,2-dimethylhydrazine; **IGF-1,** insulin-like growth factor 1; **MDA,** malondialdehyde; **p.f.,** pair fed; **PF,** polyphenol fraction

Table 1. Mechanisms of cancer-preventive activity of apple, apple juice extracts and constituents

Mechanism of cancer-preventive activity	Reference
Antioxidant activity (scavenge free radicals and reduce oxidative stress)	[42, 43, 57, 86 – 91]
Inhibition of cell proliferation	[46, 61, 84, 92]
Induction of tumour suppressor gene expression	[93, 94]
Induction of cell-cycle arrest	[44, 95]
Induction of apoptosis	[44, 60, 83, 94 – 98]
Modulation of signal transduction pathways	[44, 80 – 82, 99, 100]
Induction of enzyme expression and activity related to toxicological defence and stress response Phase II enzymes, Glutathione transferase, UDP-glucuronosyltransferases	[46, 101, 102]
Inhibition of enzyme expression and activity Phase I enzymes, Cyclooxygenase 1, Histone-deacetylase	[57, 103, 104]
Inhibition of cell adhesion and invasion	[61, 105]
Prevention of DNA damage	[42, 102, 105]
Enhancement of immune function and surveillance	[106]

the daily polyphenol intake. For example juices of 'traditional' German cider apple cultivars contain up to 970 mg/L of total polyphenols [10].

Many recent studies have focussed on the chemopreventive biological mechanisms of apple, apple juice extracts and single constituents *in vitro* (Table 1). Although these *in vitro* data are convincing it is difficult to directly extrapolate these effects to an *in vivo* situation which would further require the consideration of factors which determine bioavailability and metabolism. The general ability of the mammalian intestine to absorb polyphenols has already been shown *in vivo* and *in vitro* (reviewed by [11]). Recent studies performed with ileostomy patients have demonstrated that the acute consumption of 1 L of cloudy apple juice (CloA) resulted in an up to 33% recovery of the orally applied polyphenols in the ileostomy bags. This indicates that the majority of the polyphenols are either absorbed in upper compartments of the intestine or metabolized [12]. In contrast to the general low gastrointestinal recovery of apple polyphenols the larger polymeric procyanidins are less efficiently absorbed along the gastro-intestinal tract and 90% of these ingested procyanidins could be found in the ileostomy bags after the consumption of 1 L of CloA [13]. In summary bioavailability studies strongly indicate that apple polyphenols and their metabolites display bioactivity either systemically after absorption of the original polyphenol or its metabolites or locally in the colon lumen.

Obesity is a major risk factor for colon cancer. Meta-analyses of prospective studies showed that a 5 kg/m^2 increase in BMI is associated with increased risk of colon cancer in both, men and women, but with a stronger association in men [14, 15]. Despite this observational link of obesity and colon cancer risk, the obesity-related carcinogenic pathways are complex and not fully understood. In recent years obesity-associated insulin resistance formed the core of these pathways as it underlies several metabolic perturbations, including hyperinsulinemia, hypertriglyceridemia, hyperglycemia, increased plasma levels of free fatty acids and cholesterol, all shown to have a positive correlation with colon cancer risk [16, 17]. In colonocytes increased concentrations of insulin may directly or indirectly by the elevation of free insulin-like growth factor 1 (IGF-1) levels induce a mitogenic effect. Exposure of colonocytes to glucose, triglycerides and free fatty acids may lead to metabolic perturbations, alterations in cell signalling pathways and oxidative stress [2]. Further obesity-associated changes of proinflammatory adipokines such as resistin and leptin have been implicated in the pathogenesis of inflammatory bowel disease and the subsequent development of colon cancer [18], whereas adiponectin might have anti-cancer properties through its antiproliferative and proapoptotic effects [19]. Therefore, the prevention or amelioration of obesity and/or obesity-associated hormonal and metabolic derangement by dietary manipulation might subsequently reduce the risk of colon cancer.

Beside their direct anti-carcinogenic properties the protective activity of polyphenols could be extended to beneficial effects on metabolic disturbances such as obesity, obesity-associated insulin resistance and dyslipidemia. Several intervention studies indicate that apples and different polyphenols also found in apples have anti-obesity and antidiabetic effects by modulating lipid and glucose metabolism. In obese Zucker rats supplementation with 20% lyophilized apples lowered plasma cholesterol and LDL cholesterol as well as triglyceride accumulation in heart and liver. Further the heart concentration and urinary excretion rate of malondialdehyde (MDA) was lowered in obese Zucker rats by apple supplementation, suggesting improved protection against lipid peroxidation [20]. In streptozotocin-induced diabetic rats an acute gavage of procyanidins (250 mg/kg bw) reduced blood glucose levels probably due to the insulin-mimetic activity of procyanidins [21]. Additionally, polyphenols such as phlorizin, which reduce intestinal absorption of glucose, may avoid the development of insulin resistance and improve insulin sensitivity by preventing prolonged hyperglycemia [22, 23].

In this paper, we focus on *in vivo* studies examining the chemopreventive properties of apple juice and its fractions under normal body weight condition and under the cancer-promoting condition of obesity. This paper summarizes pre-

Table 2. HPLC analysis of polyphenolic compounds in the cloudy (CloA) and clear (CleA) apple juices from harvest 2002 or 2005[a)]

Polyphenols	CloA 2005 (mg/l)	CloA 2002 (mg/l)	CleA 2002 (mg/l)
Procyanidin B1	8.5	5.8	4.2
Procyanidin B2	14.4	20.9	15.5
Procyanidin C1	3.9	nd	nd
(+)-Catechin	11.2	nd	nd
(−)-Epicatechin	15.5	17.9	17.5
∑ Flavan-3-ols	53.5	44.6	37.2
Phloretin-2′-*O*-xyloglucoside	62.6	59.8	60.9
Phloridzin	25.1	19.7	23.9
Phloretin glucoside unknown	7.3	nd	nd
Phloretin-2′-*O*-galactoside	nd	6.3	6.0
∑ Dihydrochalcone derivatives	95	85.8	90.8
Chlorogenic acid	149.9	155.9	154.0
Crypto-chlorogenic acid	12.0	nd	nd
Caffeic acid	5.3	0.6	2.3
Cumaroyl glucose	1.5	nd	nd
3-Cumaroyl quinic acid	2.6	1.9	1.9
4-Cumaroyl quinic acid	52.9	76.7	75.1
Coumaric acid	1.0	nd	nd
∑ Phenolic acids	225.2	235.1	233.3
Quercetin-3-*O*-galactoside	1.2	1.7	1.9
Quercetin-3-*O*-glucoside	0.6	1.0	1.1
Quercetin-3-*O*-xyloside	0.6	nd	nd
Quercetin-3-*O*-arabinoside	0.8	nd	nd
Quercetin-3-*O*-rutinoside	nd	0.5	0.8
Quercetin-3-*O*-rhamnoside	1.7	2.5	3.2
∑ Flavonols	4.9	5.7	7.0
Total polyphenol (HPLC)	378.6	371.2	368.3

a) Data represent the mean of four independent HPLC measurements. Not detected (nd). Reprinted with permission from Barth *et al.* [31]. Copyright (2007) American Chemical Society.

vious *in vivo* studies, which evaluated the anti-carcinogenic properties of apple juice and apple juice fractions by using the rodent model of 1,2-dimethylhydrazine (DMH)-induced colon cancer. These studies were further extended by recent investigations of the cancer-preventive efficacy of apple juice under the cancer-promoting influence of obesity, performed in a newly established obesity – colon cancer model.

2 Study 1: The cancer-preventive potential of cloudy apple juice is higher than that of clear apple juice (CleA) in DMH-induced colon cancer

The aim of the first study was the investigation of cancer-preventive mechanisms of apple juice *in vivo* [24]. The clear (CleA) and the CloA were produced and analytically characterized by the Research Institute Geisenheim, Institute of Enology and Beverage Technology. According to HPLC-based quantification, both juice preparations contained comparable amounts of total polyphenols (Table 2; CloA and CleA of the harvest 2002). The CloA provided higher quantities of procyanidins B1/B2 as well as pectin [24]. Further CloA contained the heterogeneous cloud fraction (CF), which is lacking in the CleA. The CF consisted of lipids, proteins, polyphenols and cell wall polysaccharides [24]. Male F344 rats were randomly assigned to three groups (n = 30/group; 1, control (Cont) water; 2, CloA; 3, CleA). One week after starting the juice intervention half of each group (n = 15) received i.p. injections of either DMH (20 mg/kg body wt) or 0.9% NaCl four times at 1 wk intervals. All animals were killed 3 wk after the last injection. Several biomarkers associated with colon cancer, such as colonocyte DNA damage, epithelial hyperproliferation and tumour related gene expression, have been investigated.

With this experimental setup we showed, that both juice preparations significantly decreased epithelial hyperproliferation compared to water controls with a significantly higher efficacy of CloA compared to CleA (Fig. 1B). Additionally CloA but not CleA significantly reduced DMH-induced DNA damage and the aberrant crypt foci (ACF) size compared to water controls (Figs. 1A, D). After DMH and/or juice intervention there were no changes in transcript levels of colonic cyclooxygenase isoforms (COX-1, COX-2) or glutathione-associated enzymes (γGCS, GST-P and GST-M2) (for data see [24]).

These results clearly indicated that primarily the CloA exhibited chemopreventive activity in DMH-treated F344 rats. From these data it was not possible to evaluate the relative impact of single constituents or subfractions as determinants for the anticancer effects of the complex CloA. As

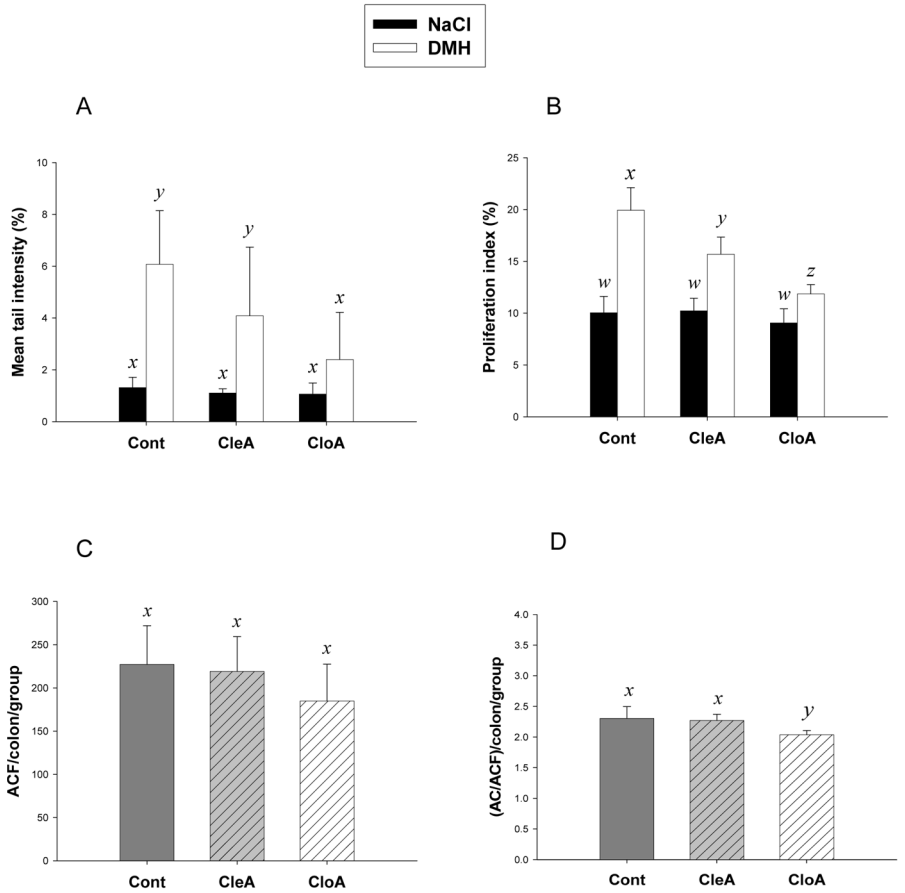


Figure 1. Bars represent means ± SD. Bars with different superscripts are significantly different, $p < 0.05$, $n = 5$). Biomarkers of colon cancer in F344 rats drinking CloA, CleA or water (Cont): (A) comet assay results of genotoxic DNA damage expressed as the mean tail intensity analysed in 150 mucosa cells of the distal colon on three slides (two-way ANOVA and Newman – Keuls multiple group comparison tests); (B) proliferation index in the distal colon expressed as the percentage of bromodeoxyuridine (BrdU)-positive cells determined within 25 randomly chosen crypts per animal; (C/D) analysis of ACF total number (C) and size (aberrant crypts (AC)/ACF) (D) in the distal colon mucosa of DMH-treated animals (NaCl-treated control animals do not develop ACF and were therefore not included in statistical evaluation, one-way ANOVA and Newman – Keuls multiple group comparison tests). Reprinted from [24] with permission from Oxford University Press.

both juices contained comparable concentrations and types of polyphenols, we hypothesized that nonpolyphenolic compounds such as pectin or constituents of the complex CF might be responsible for the stronger cancer-preventive effect by CloA. Pectin is the major soluble fibre present in CloA. Pectin is rapidly fermented by intestinal bacteria releasing distinct spectra of SCFAs [25], which exert a trophic function on colonic mucosa [26] and further influence mucosal proliferation and apoptosis in the colon [27, 28]. As the pectin dosage provided by the CloA was comparably low and provided less than 2.5% of the dosage used by others [29, 30] it is more likely that the CF determined the higher bioactivity of CloA compared to CleA lacking this fraction. This suggestion led to the experimental design of the second study, where the major fractions of the apple juice, the polyphenol and the CF, were applied in the animal model of DMH-induced colon carcinogenesis.

3 Study 2: The cloud and the polyphenol fractions (PFs) are less efficient when compared to cancer-preventive properties of cloudy apple juice

The aim of the second study was to identify the fractions in the CloA, which accounted for the cancer-preventive effect [31]. The CloA was fractionated into a total polyphenol (monomeric and polymeric) and a heterogeneous CF consisting of 24% proteins, 48.6% fatty acids, 18% polyphenols (supposed procyanidins [32, 33]) and 7.4% cell wall polysaccharides. The general study design as well as the selection of analysed parameters was similar to the previous study by Barth *et al*. [24]. In the present study four treatment groups ($n = 12$/group; 1, CleA; 2, PF, 3, CF; 4, PF + CF) and a control group receiving tap water ($n = 24$) were included. One week after starting with the respective

Table 3. Biomarkers of colon cancer in F344 rats[a)]

	Cont NaCl	Cont DMH	CloA	PF	CF	PF + CF
Mean tail intensity (%)	2.0 ± 0.6*x*	7.7 ± 2.4*y*	3.3 ± 1.3*z*	6.5 ± 2.9*y*	5.8 ± 2.1*y*	4.8 ± 1.6*y*
Proliferation index (%)	7.5 ± 1.5*x*	14.9 ± 1.9*y*	9.4 ± 1.0*x*	12.4 ± 1.7*z*	11.6 ± 1.0*z*	12.4 ± 1.5*z*
ACF number/colon/group	0	149.2 ± 13.0	127.2 ± 15.3	142.7 ± 58.4	140.0 ± 29.8	137.7 ± 33.6
ACF size/colon/group	0	2.1 ± 0.2	2.1 ± 0.2	2.1 ± 0.3	2.1 ± 0.2	2.1 ± 0.3

a) Data are the mean ± SD. Values in a common row with different letters are significantly different ($p < 0.05$; $n = 6$, one-way ANOVA; Newman–Keuls multiple group comparison tests; NaCl-treated animals do not develop ACF and were therefore not included in statistical evaluation concerning ACF number and size). All animals except those of Cont NaCl group were treated with DMH. ACF, controls receiving tab water (Cont), CloA, PF, CF. Reprinted with permission from Barth *et al.* [31]. Copyright (2007) American Chemical Society.

intervention half of the control group ($n = 12$) received i.p. injections of the vehicle solution (NaCl 0.9%) while the other half of the controls and all other groups were treated with DMH (20 mg/kg body wt) four times at 1 wk intervals. The fractions were dissolved daily in water at concentrations equivalent to the CloA polyphenol (371.2 mg/L quantified by HPLC) and cloud content (750 mg/L).

According to the first study [24] the cancer-preventive effects of CloA were confirmed as the intervention led to a significant reduction of DMH-induced DNA damage and hyperproliferation. In addition a reduction of ACF number by trend compared to the DMH-treated control group (Table 3) has been observed. The epithelial hyperproliferation was further significantly decreased by all fractions (CF, PF, CF + PF) whereas ACF and DNA damage remained unchanged (Table 3).

Extending the results of the juice intervention (study 1) these data show that CloA had a higher cancer-preventive potential than a separate or combined intervention with PF and CF and further, besides PF, identified CF as an additional bioactive fraction of CloA. As based on these results it has been suggested that the cloud particles, which are composed of a nucleus of proteins surrounded and completed by polysaccharides, might serve as vectors transferring entrapped polyphenols into the colon under protection from absorption in the small intestine, so that these polyphenols can exert their chemopreventive activity in the colon lumen [31]. Since the same CloA was used for studies 1 and 2 a loss of bioactivity of CloA due to longer storage is one possible reason for the lack of efficiency to reduce ACF size in study 2 compared to study 1. It has also to be considered that in study 1 ACF size was reduced only by 0.3. The grater variance between animals of one group in study 2 compared to study 1 (mean SD for ACF size in study 1, 0.13; in study 2, 0.24) might prevent significant reduction of ACF size in study 2.

Based on the demonstrated cancer-preventive effect of CloA in normal weighing rats we were further interested whether CloA exerts also anticancer activity under the cancer-promoting condition of obesity. Therefore, an animal model was established which resembles the obesity-associated promotion of colon cancer to further examine the complex underlying biological mechanisms.

4 Study 3: Obesity significantly promoted the appearance of preneoplastic aberrant crypt foci in DMH-treated Zucker rats

Previous animal studies have shown that diet induced [34, 35] and genetically determined obesity [36] led to an increased number of chemically induced ACF, whereas dietary restriction reduced the number of total ACF [37]. There has been considerable debate in the past decade addressing the hypothesis that obesity-associated disarrayed metabolic and hormonal plasma parameters, including insulin, triglycerides, glucose and free fatty acids, may promote ACF development and colon cancer. Therefore, beside the establishment of an obesity-colon cancer model the aim of this study was to identify the major obesity-associated parameters correlating with ACF-development. Further, we were interested whether a supposed change of ACF number or size might be a result of obesity-related modulation of early events in carcinogenesis, such as DNA-damage or colonocytes hyperproliferation.

The Zucker rat was used as a well established genetic model representative for early-onset obesity in humans [38]. Female lean and obese Zucker rats fed ad libitum (a.l.) were chosen to determine the obese genotype effect upon colon cancer initiation and promotion. Further, to selectively control an increased body weight on the background of similar energy intake we have included an additional Zucker obese group, which was pair fed (p.f.) to the lean a.l. group. By this feeding regimen an ~30% food restriction was induced when compared to the obese a.l. group. Half of each group was treated by i.p. injections of either DMH (15 mg/kg body wt, $n = 11–12$/group) or 0.9% NaCl ($n = 12$/group) four times at 1 wk intervals. Compared to the DMH-dosage applied in F344 in previous studies the 25% reduction of the dosage in Zucker rats has been performed in accordance to the strategy recommended by Raju and Bird [39]. Five weeks after the last injection animals were sacrificed after over night fasting. Metabolic and endocrine plasma parameters such as triglycerides, free fatty acids, MDA, cholesterol, glucose, insulin, IGF-1 and adipokines including resistin, adiponectin and leptin were analysed. In this context the product of lipid peroxidation

Table 4. Final body weight, energy intake and biochemical blood parameters in lean and obese Zucker rats fed a.l. or p.f.[a)]

	Lean	Obese p.f.	Obese a.l.
Final body weight (g)	248 ± 12.6*x*	328.6 ± 20.1*y*	409.5 ± 33.6*z*
Energy intake (kJ/d)	224.7 ± 13.0*x*	223.4 ± 0.1*x*	318.6 ± 2.9*y*
Triglycerides (mg/dL)	55.8 ± 14.2*x*	137.6 ± 58.1*y*	366.0 ± 289.4*z*
Cholesterol (mg/dL)	79.73 ± 17.0*x*	315.4 ± 35.9*y*	340.0 ± 40.1*y*
Free fatty acids (mmol/L)	0.8 ± 0.2*x*	1.0 ± 0.3*y*	1.4 ± 0.4*z*
Glucose (mg/dL)	104.8 ± 9.9*x*	223.8 ± 130.8*y*	140.3 ± 25.9*y*
Insulin (ng/mL)	0.5 ± 0.1*x*	42.5 ± 33.7*y*	23.0 ± 17.8*y*
IGF-1 (ng/mL)	1025.8 ± 184.6*x*	671.3 ± 207.3*y*	992.0 ± 172.9*x*
Resistin (ng/mL)	10.2 ± 2.2*x*	12.3 ± 2.6*y*	9.5 ± 2.4*x*
Adiponectin (μg/mL)	6.0 ± 0.8*x*	6.9 ± 2.3*x*	5.8 ± 1.0*x*
Leptin (ng/mL)	2.4 ± 1.0*x*	66.1 ± 15.3*y*	71.1 ± 13.2*y*
MDA (μM)	1.4 ± 0.3*x*	3.0 ± 0.5*y*	4.3 ± 1.1*z*

a)Data are the means ± SD. Values in a common row with different letters are significantly different ($p < 0.05$; $n = 24/23$, effects of group (lean, obese p.f, obese a.l.) and DMH treatment and the interaction between these two factors were analysed by two-way ANOVA; DMH treatment did not affect weight, energy intake or plasma parameters and therefore effects were reanalysed by one-way ANOVA pooling data of DMH and NaCl-treated animals, Tukey – Kramer post-hoc test, pooled parameters are presented). Reproduced with permission from Koch *et al.* [34].

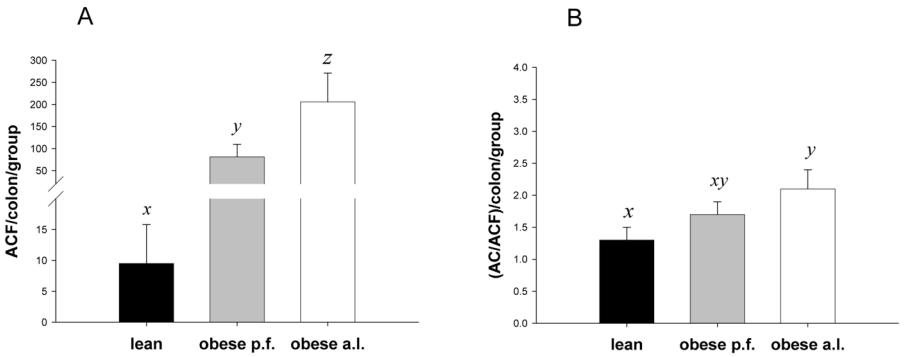
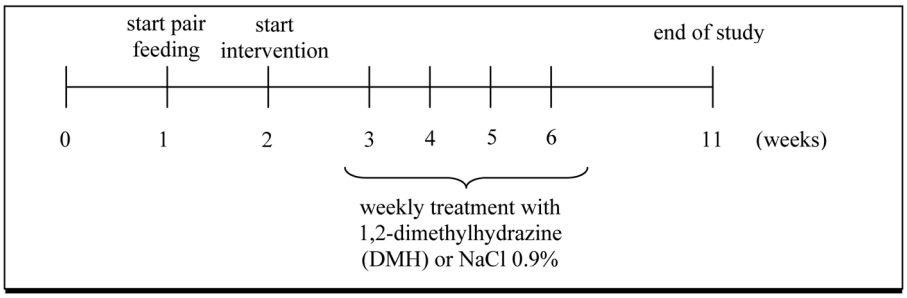


Figure 2. Biomarkers for colon cancer in lean and obese Zucker rats fed a.l. or p.f.: analysis of ACF total number (A) and size (AC/ACF) (B) in the distal colon mucosa of DMH-treated animals (NaCl-treated control animals do not develop ACF and were therefore not included in statistical evaluation). Bars represent means ± SD. Bars with different superscripts are significantly different ($p < 0.05$; $n = 6/5$; one-way ANOVA; Tukey – Kramer post-hoc test). Reproduced with permission from Koch *et al.* [34].

MDA serves on the one hand as a biomarker for oxidative stress [40] and on the other hand may also be involved in tumour genesis itself by formation of DNA adducts with potential mutagenic effects [41].

Food restriction by the pair feeding strategy could not prevent the development of obesity and the increase in obesity-associated plasma parameters (Table 4). This means that the mean body weight significantly differ between the Zucker lean a.l. and the obese p.f. groups although energy intake was identical. Under a.l. conditions the obese genotype resulted in a significantly higher number and size of DMH-induced ACF compared to the lean genotype. Food restriction seemed to be a potent protective factor, thus reducing the number of ACF but not the ACF size (Fig. 2) in the obese p.f. group. Although group differences regarding the ACF number in the distal colon were significant, no changes in DNA damage or epithelial proliferation rate could be detected in this context (for data see [34]). The plasma concentration of triglycerides, MDA and free fatty acids were lowest in lean, intermediate in obese p.f. and highest in the group of obese a.l. Zucker rats thus following the pattern of ACF number in these groups (Table 4). Cholesterol, insulin, glucose and leptin plasma levels were only dependent on the genotype and irrespective of food intake higher in obese when compared to lean Zucker rats, while IGF-1 levels were significantly reduced in obese p.f. compared to lean and obese a.l. Zucker rats (Table 4). Further, single regression analysis between ACF and selected hormonal and metabolic parameters showed that all parameters except free fatty acids, IGF-1 and glucose significantly correlate with ACF number and size (Table 5). A stepwise multiple regression was conducted to determine specific combinations of metabolic and endocrine factors associated with obesity that further significantly correlate with the number and size of ACF [34]. ACF number was associated with total energy intake, body weight and cholesterol and ACF size with body weight and cholesterol. When excluding these dominant predictors (body weight, energy intake



Assay	Treatment	Intervention	Lean a.l.	Obese p.f.	Obese a.l.
- DNA damage	NaCl	CB	6	6	6
- Proliferation					
- Plasma parameters		CloA	6	6	6
- DNA damage	DMH	CB	6	6	6
- Proliferation					
- Aberrant crypt foci		CloA	6	6	6
- Plasma parameters					

Figure 3. Experimental study design including the number used for the respective assays of lean and obese Zucker rats fed a.l. or p.f. drinking CloA or CB. Colonic mucosa of the distal colon was used for all tissue-based assays.

Table 5. Regression analysis in the context of the Zucker rat experiment with the number and size of ACF as dependent variables[a)]

	ACF number		ACF size	
Independent variable for simple regression	R^2 adj.	*p*	R^2 adj.	*p*
Body weight (g)	0.760	<0.0001	0.530	0.0006
Energy/wk (MJ)	0.755	<0.0001	0.433	0.0024
Cholesterol (mg/dL)	0.662	<0.0001	0.596	0.0002
MDA (μM)	0.600	0.0002	0.361	0.0064
Triglycerides (mg/dL)	0.443	0.0021	0.348	0.0075
Free fatty acids (mmol/L)	0.121	0.0935	0.006	0.3112
Insulin (ng/mL)	0.217	0.0340	0.286	0.0157
IGF-1 (ng/mL)	−0.066	0.9098	−0.065	0.8764
Glucose (mmol/L)	−0.032	0.4880	−0.066	0.9241

a) Reproduced with permission from Koch *et al.* [34].

and cholesterol) MDA was the strongest determinant of ACF number but not of ACF size. Further regression analysis clearly showed that MDA plasma concentration significantly correlated with triglycerides and body weight [34].

In summary obesity modulation of ACF in Zucker rats correlated stronger with parameters related to lipid metabolism than with those related to glucose metabolism. Additionally, as based on functional relevance of these plasma parameters and partially based on regression analysis we suggest that triglycerides, free fatty acids, MDA, IGF-1 and cholesterol might be the major factors, which determine at least part of the obesity-related effects on colon carcinogenesis in the Zucker rat. Besides early events of carcinogenesis, which were unaffected by obesity, such as DNA damage and colonocyte hyperproliferation, other cellular parameters such as the colonocyte apoptosis rate might be candidates among the underlying mechanisms responsible for the observed differences regarding the preneoplastic ACF.

After this animal model was established and major determinants for ACF were identified we used this model for an intervention trial with CloA.

5 Study 4: Cloudy apple juice was ineffective in preventing DMH-induced colon carcinogenesis in the Zucker rat

To investigate the anticancer activity of CloA under the cancer-promoting influence of obesity the Zucker rat [34] was used in the next experiment for a CloA intervention trial. The general study design remained unchanged and beside colon cancer biomarkers, those systemic parameters,

Table 6. Final body weight, energy and polyphenol intake and biochemical blood parameters in lean and obese Zucker rats fed a.l. or p.f.[a)]

		Lean	Obese p.f.	Obese a.l.
Final body weight (g)	CB	241.6 ± 16.7*x*	332.4 ± 19.5*y*	398.6 ± 30.8*za*
	CloA	244.2 ± 13.7*x*	333.7 ± 31.8*y*	440.5 ± 34.3*zb*
Energy intake food (kJ/d)	CB	192.1 ± 10.0*x*	190.5 ± 0.2*x*	275.2 ± 22.8*ya*
	CloA	189.3 ± 10.3*x*	193.6 ± 0.2*x*	302.4 ± 15.3*yb*
Energy intake drink (kJ/d)	CB	22.6 ± 1.8*x*	36.0 ± 8.0*y*	20.2 ± 2.6*x*
	CloA	21.8 ± 1.8*x*	33.8 ± 11.3*y*	21.6 ± 1.3*x*
Polyphenol intake (mg/kg body wt)	CB	0	0	0
	CloA	20.1 ± 1.4*x*	24.0 ± 7.6*x*	11.9 ± 0.8*y*
Triglycerides (mg/dL)	CB	86.2 ± 48.8*x*	123.3 ± 59.8*ya*	233.1 ± 125.2*za*
	CloA	83.2 ± 26.0*x*	269.3 ± 114.6*yb*	517.4 ± 291.6*zb*
Cholesterol (mg/dL)	CB	87.9 ± 12.6*x*	337.7 ± 40.2*ya*	341.7 ± 41.0*y*
	CloA	90.2 ± 7.6*x*	271.9 ± 48.8*yb*	296.4 ± 65.3*y*
Free fatty acids (mmol/L)	CB	0.8 ± 0.2*x*	1.0 ± 0.2*y*	1.1 ± 0.2*y*
	CloA	0.8 ± 0.2*x*	1.2 ± 0.2*y*	1.1 ± 0.3*y*
IGF-1 (ng/mL)	CB	1067.2 ± 107.7 x	742.3 ± 152.2*y*	1141.5 ± 248.4*x*
	CloA	1118.3 ± 140.1*x*	830.4 ± 169.3*y*	1116.6 ± 153.4*x*
MDA (μM)	CB	1.8 ± 0.4*x*	3.8 ± 0.7*y*	5.1 ± 0.8*z*
	CloA	2.0 ± 0.2*x*	3.8 ± 1.8*y*	5.4 ± 0.9*z*

a) Data are the means ± SD. Values of one parameter in a common row and column with different letters are significantly different ($p < 0.05$; $n = 12$; effects of group (lean, obese p.f, obese a.l.), DMH treatment and intervention with CloA and the interaction between these three factors were analysed by three-way ANOVA, DMH treatment did not affect weight, energy intake or plasma parameters and therefore effects were reanalysed by two-way ANOVA pooling DMH and NaCl-treated animals, Tukey – Kramer post-hoc test, pooled parameters are presented). For comparison of lean, obese p.f. and obese a.l. *x*, *y* and *z* were used and for CB and CloA *a* and *b*.

Table 7. Biomarkers of colon cancer in NaCl-treated lean and obese Zucker rats fed a.l. or p.f.[a)]

		Lean	Obese p.f.	Obese a.l.
Mean tail intensity (%)	CB	3.1 ± 1.3*x*	5.2 ± 1.8*x*	3.5 ± 1.4*x*
	CloA	3.0 ± 1.2*x*	8.2 ± 6.7*xy*	4.3 ± 3.3*xy*
Proliferationn index (%)	CB	7.2 ± 4.3*x*	6.2 ± 5.5*x*	3.0 ± 1.0*x*
	CloA	8.1 ± 2.3*x*	4.2 ± 2.1*x*	4.9 ± 2.1*x*

a) Data are the means ± SD. Values of one parameter in a common row and column with different letters are significantly different ($p < 0.05$; $n = 6/5$; effects of group (lean, obese p.f, obese a.l.), DMH treatment and intervention with CloA and the interaction between these three factors were analysed by three-way ANOVA Tukey – Kramer post-hoc test; here presented are only data from NaCl-treated animals.

which have been identified as major (promoting) determinants for ACF in the previous study were analysed. Half of each treatment group ($n = 6$) either received CloA (harvest 2005, polyphenol content is shown in Table 2) or an isocaloric control beverage (CB), which was equilibrated for carbohydrates, minerals, vitamin C and acid supply according to the composition of the CloA. Intervention lasted for 9 wk starting 1 wk before the first DMH/NaCl 0.9%-injection as schematically shown in Fig. 3. All animal experiments were performed in accordance with the local ethic committee (no. 35-9185.81/G-52/05).

Obese a.l. Zucker rats receiving CloA weighed significantly more than those receiving CB due to a higher energy intake by food (Table 6). However, in this regard there was no detectable difference in lean and obese p.f. Zucker rat groups. Obese p.f. Zucker rats drinking CloA and CB consumed more liquid and therefore liquid derived calories than lean and obese a.l. Zucker rats. Overall the polyphenol intake *per* kg body wt was the same in lean and obese p.f. Zucker rats but was significantly lower in obese a.l. Zucker rats (Table 6).

In contrast to the earlier studies (studies 1 and 2) in the F344 rats the CloA showed no cancer-preventive effect in the Zucker rat model independent of genotype or energy intake. DNA damage, epithelial proliferation rate and ACF number and size were not modulated by the CloA in comparison with CB (Fig. 4 for DMH-treated animals, Table 7 for NaCL-treated animals).

Further, plasma concentrations of free fatty acids, IGF-1 and MDA were unchanged by CloA intervention compared to the respective CB groups. The plasma triglyceride level of lean Zucker rats remained constant in CB and CloA groups but was significantly elevated in both CloA obese Zucker rat groups compared to CB (Table 6). Only the cho-

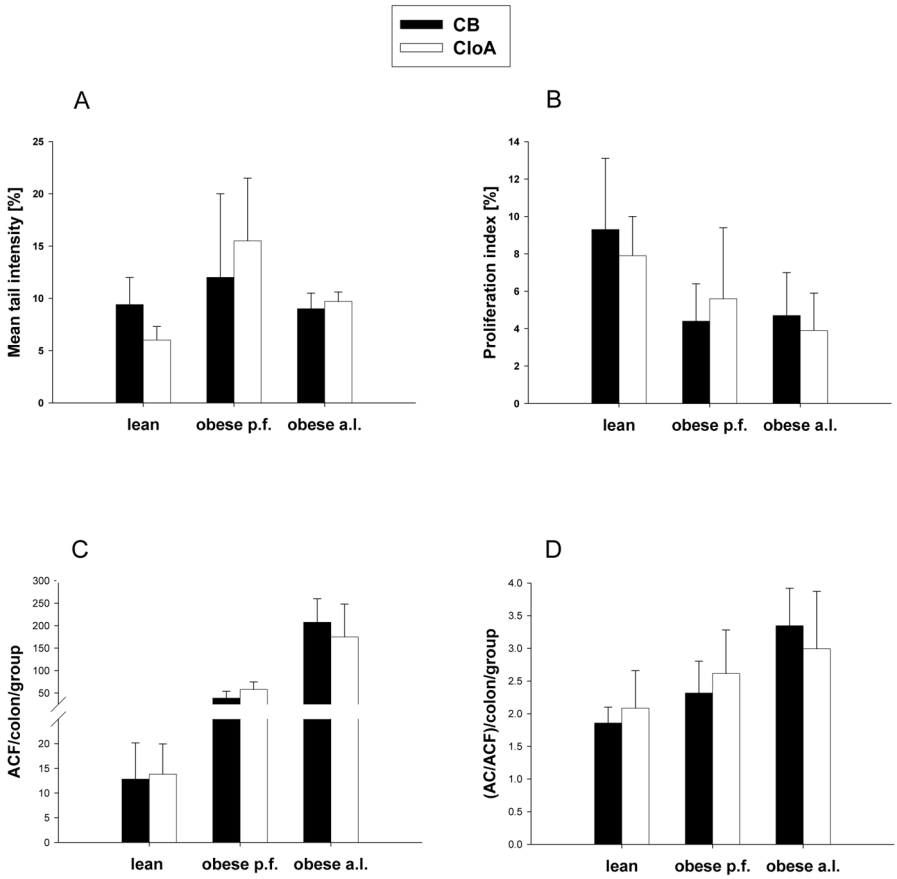


Figure 4. Bars represent means ± SD. Bars with different superscripts are significantly different ($p < 0.05$; $n = 6/5$). Biomarkers of colon cancer in DMH-treated lean and obese Zucker rats fed a.l. or p.f. drinking CloA or CB: (A) comet assay results of genotoxic DNA damage expressed as the mean tail intensity analysed in 150 mucosa cells of the distal colon on three slides (two-way ANOVA; Tukey – Kramer post-hocc test); (B) proliferation index in the distal colon expressed as the percentage of bromodeoxyuridine (BrdU)-positive cells determined within 25 randomly chosen crypts per animal (two-way ANOVA; Tukey – Kramer post-hocc test); (C/D) analysis of ACF total number (C) and size (AC/ACF) (D) in the distal colon mucosa (one-way ANOVA; Tukey – Kramer post-hocc test).

lesterol plasma concentration was significantly decreased by CloA compared to CB in the obese p.f. group but not in lean or obese a.l. Zucker rats (Table 6).

Our previous studies [24, 31] have consistently shown that CloA has cancer-preventive properties in normal weight F344 rats. Meanwhile these *in vivo* results received considerable mechanistical backing by various *in vitro* studies showing antioxidant [42, 43], proapoptotic [44, 45] and antiproliferative [44 – 46] activities by different apple constituents.

However, CloA showed no cancer-preventive effect in Zucker rats, independent of energy intake or genotype. As quantity and quality of polyphenols vary greatly depending on apple varieties, growth conditions, plant nutrition, storage and processing (reviewed by [6]), a different spectrum of apple constituents than in the juice used in earlier studies [24] might explain the lack of cancer-preventive properties in Zucker rats. However, as the total polyphenol content as well as the sum of procyanidins, the most promising anticancer apple constituents [44, 45], were even higher in the CloA 2005 as compared to the CloA of the harvest 2002 [24] (Table 2), a lower polyphenol content of the CloA 2005 could be excluded as a cause of the observed inactivity. Another limitation of comparing the Zucker obese with Zucker lean and F344 is reflected by the observed variations in fluid intake characteristics which implicated a lower polyphenol dosage (mg/kg body wt) in obese a.l. Zucker rats when compared to the Zucker lean or the F344 rats used in previous experiments [24, 31]. Beside the potential impact of varying dosage and juice composition, also the genetic background of rat strains might contribute to the observed different susceptibility to cancer-prevention and also chemically induced colon cancer. Although DMH is one of the most potent and reliable carcinogens for the selective induction of colonic tumours in rodents, differences in susceptibility to DMH have been observed in differ-

ent inbred mice [47, 48]. Comparing the mean ACF number *per* colon of F344 rats (227 ACF) [24] and of lean Zucker rats (12 ACF) leads to the suggestion that these rat strains vary in susceptibility to colon cancer induction and might also differ in the response to chemoprevention by apple juice.

DMH requires metabolic activation to exert its carcinogenic action. It is mainly metabolized by cytochrome P4502E1 (CYP2E1) to methylazoxymethanol subsequently decomposed to methyldiazonium radikals, which are efficient alkylating agents for a variety of cellular biomolecules including DNA. The DNA-adduct O^6-methylguanine is mainly responsible for colon carcinogenesis [49]. Therefore it is hypothesized, that DMH colon carcinogenesis may be either inhibited or promoted depending on whether CYP2E1 activity is induced or inhibited [50]. In addition to being highly inducible by its substrate, CYP2E1 has been reported to be elevated in diverse nutritional conditions like fasting [51] and obesity [52], though in obese Zucker rats CYP2E1 activity is reduced compared to lean rats [53, 54]. The rate of drug metabolism further varies highly between rat strains [55] and apple constituents have also be shown to modulate cytochrome P450 enzymes activity [56, 57]. So variability in CYP2E1 activity due to differences in rat strains, nutritional status and chemopreventive compounds may influence DMH metabolism and subsequently the formation of DNA-adducts in lean and obese Zucker rats and F344 rats. This might provide one possible explanation for the difference in ACF number and the absence of chemopreventive action of CloA in Zucker rats compared to F344 rats. However, as DNA strand breaks were quite similar between F344 and Zucker rats and also between lean and obese Zucker rats a variation in DMH bioactivation might not primarily be responsible for differences in ACF formation between these groups [58].

Beside variability in DMH metabolism also variable rates of proliferation and apoptosis of colon epithelial cell long after carcinogen exposure, and induction of a variety of mutations may explain the different susceptibility to DMH. Whereas rates of cell proliferation appear to be inconsistent with cancer susceptibility there is increasing evidence that points to apoptosis as a critical factor for the susceptibility to DMH-induced colon tumorigenesis [59]. Despite the differences between rat strains concerning DMH-induced apoptosis also resistance to apple constituent-induced apoptosis might be critical for ACF formation and could be one explanation for the absent of chemopreventive properties of CloA in Zucker rats compared to F344 rats. Apple polyphenols have been shown to induce apoptosis in tumour cell *in vivo* and *in vitro* [44, 60, 61].

Further, we have used 48 homozygote obese *fa*/*fa* Zucker rats and 24 heterozygote lean *Fa*/*fa* Zucker rats for the respective experimental groups (for genotyping details see [34]). Although the *fa* allele was classified as a recessive allele [62], there is increasing evidence that *Fa*/*fa* heterozygotes have their own unique phenotype, which is usually intermediate between homozygote wild type and homozygote mutant Zucker rats [63, 64]. So heterozygote lean female Zucker rats displayed significantly elevated body weights, percentage of body fat, and serum leptin levels compared to *Fa*/*Fa* homozygotes [65]. Confirmatory results were reported by Heo *et al*. [66]. They showed that *Fa*/*fa* Zucker rats had higher epididymal fat pad weights and twofold higher leptin levels in adipose tissue. Therefore it remains to be clarified whether CloA would exert a chemopreventive effect in homozygote lean *Fa*/*Fa* Zucker rats comparable to F344 rats, which would indicate a *fa* allele effect on cancer-preventive efficacy by CloA. As the *fa* allele is associated with higher body fat there might also exist a promoting impact of adipocyte-related factors on the formation of ACF in heterozygotes, which could not be compensated by apple juice constituents.

The leptin plasma concentration is closely related to the amount of adipose tissue and leptin may be one factor that contributes to the higher colon cancer risk associated with obesity as leptin promotes proliferation [67], motility and invasiveness in human colon cancer cells and in premalignant epithelial cells [67 – 69]. Therefore, the higher leptin plasma concentrations in *Fa*/*fa* heterozygote lean compared to homozygote lean Zucker rats as described by others [65] might be an ACF-promoting determinant, which could prevent a cancer-preventive effect by CloA in heterozygote lean which are still receptive to leptin.

Except of plasma cholesterol none of the plasma parameters previously predicted as possible ACF determinants, namely triglycerides, free fatty acids, MDA, IGF-1 and neither body weight nor energy intake were lowered by CloA intervention in the Zucker rats. This might be another explanation why ACF number or size was not reduced by CloA. Some studies found a positive association between cholesterol plasma levels and risk of colorectal adenomatous polyp and colon cancer development [17, 70]. Further, feeding animals with high-cholesterol diets resulted in increased serum cholesterol levels, number of azoxymethane-induced colon cancers and excretion rates of bile acids and neutral sterols with the feces [71]. In contrast to these findings, other studies reported that low cholesterol levels increase colon cancer incidence [72, 73]. It has been suggested that the inverse association may be either due to the metabolic effects of undiagnosed cancer on serum cholesterol levels [74, 75] or due to a higher catabolic activity from cholesterol to several bile acids, which are then excreted into the intestine via the bile and/or a higher excretion rate of nonabsorbed cholesterol in feces (reviewed by [76]). Luminal sterols are thought to be an important factor in the etiology of colon cancer [77, 78]. Therefore, an increase in sterols in the lumen of the intestine, regardless of their origin, may result in an increased risk of colon cancer. The plasma cholesterol levels of obese p.f. and a.l. Zucker rats were significantly elevated compared to lean

Zucker rats. Although the plasma cholesterol levels were lowered by CloA compared to CB, this did not modify ACF number in obese p.f. Zucker rats. The fact, that only cholesterol but neither the plasma levels of triglycerides, free fatty acids, MDA, IGF-1 nor body weight and energy intake, all previously predicted as possible ACF determinants, were changed by CloA compared to CB, might be a further explanation of the lacking efficacy of CloA in reducing ACF number or size.

6 Summary and conclusions

As based on the general preventive characteristics of fruits, apples contain bioactive components, which are primarily protective against colonic cancer by directly influencing cellular parameters in colonocytes or colon derived cell lines. However, the main bioactive apple ingredient(s) responsible for these effects *in vivo* remain to be identified. Although potent candidates could already be identified *in vitro* (*e.g.*, procyanidins, dihydrochalcones), the majority of *in vivo* studies have indicated the limited potential of a single substance or subfractions of the complex juice to achieve the observed *in vivo* activities of the complex combination of bioactive constituents in apple juice [24, 31]. Again these studies all emphasize the higher benefit of the whole food.

The presented strategy established for the investigation of apple juice chemopreventive activity might serve as a model strategy for further determination of bioactivity of different fruit varieties and constituents. This strategy has been developed by a consortium of scientists. It covered analytical characterization of apple juice constituents [10, 79], the evaluation of their *in vivo* bioavailability [12] combined with metabolism studies [13], and the mechanistical analysis of *in vitro* bioactivity using different cell based as well as cell-free assay systems [42, 43, 46, 57, 80 – 84]. Additionally it included *in vivo* investigation of cancer-preventive activity in a genetically [85] and chemically [24, 31] induced rodent model of colon carcinogenesis. As obesity bears major cancer promoting parameters (*e.g.*, proinflammatory cytokines, growth factors), we have extended the latter model by aspects of obesity to bring the model closer to the human situation. The pathways and molecular targets which mediate the observed cancer-promoting activity of obesity-related aberrated endocrine or metabolic factors might be identical but oppositely regulated to those pathways and targets modulated by cancer-preventive dietary constituents. This might explain the lack of bioactivity of apple juice as observed in this newly established animal model but could also reflect the general limitations of comparing results derived from different animal models with varying susceptibility towards tumour inducers or cancer-preventive agents. We suggest that this lack of efficacy might be representative for obese individuals who do not at all or only partly benefit from nutritional doses of bioactive food compounds to improve their individual health status including efficient cancer-prevention. As this hypothesis could not be derived solely from *in vitro* studies or *in vivo* animal experiments, further research is warranted on whole food cancer-preventive benefits in human intervention studies focussing on high risk groups such as obese or diabetic individuals.

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