

Calcium carbonate suppresses haem toxicity markers without calcium phosphate side effects on colon carcinogenesis

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Abstract

Red meat intake is associated with increased risk of colorectal cancer. We have previously shown that haemin, haemoglobin and red meat promote carcinogen-induced preneoplastic lesions, aberrant crypt foci, in the colon of rats. We have also shown that dietary calcium phosphate inhibits haemin-induced promotion, and normalizes faecal lipoperoxides and cytotoxicity. Unexpectedly, high-calcium phosphate control diet-fed rats had more preneoplastic lesions in the colon than low-calcium control diet-fed rats. The present study was designed to find a calcium supplementation with no adverse effect, by testing several doses and types of calcium salts. One in vitro study and two short-term studies in rats identified calcium carbonate as the most effective calcium salt to bind haem in vitro and to decrease faecal biomarkers previously associated with increased carcinogenesis: faecal water cytotoxicity, thiobarbituric acid reactive substances. A long term carcinogenesis study in dimethylhydrazine-injected rats demonstrated that a diet containing 100 µmol/g calcium carbonate did not promote aberrant crypt foci, in contrast with previously tested calcium phosphate diet. The results suggest that calcium carbonate, and not calcium phosphate, should be used to reduce haem-associated colorectal cancer risk in meat-eaters. They support the concept that the nature of the associated anion to a protective metal ion is important for chemoprevention.

MESH Keywords Animals ; Biological Markers ; Calcium Carbonate ; administration & dosage ; pharmacology ; Calcium Phosphates ; administration & dosage ; adverse effects ; Colon ; drug effects ; Colonic Neoplasms ; chemically induced ; prevention & control ; Diet ; adverse effects ; veterinary ; Dietary Supplements ; Feces ; chemistry ; Female ; Heme ; toxicity ; Meat ; adverse effects ; Rats ; Rats, Inbred F344

Author Keywords Red meat ; Haem ; prevention ; calcium ; colon cancer ; Calcium ; Colorectal cancer ; Lipid peroxidation

Introduction

Red and processed meat consumption increases the risk of colorectal cancer, according to meta-analyses of epidemiological studies [1 , 2]. In its 2007 report, the World Cancer Research Fund panel judges that “the evidence that red meat and processed meat are a cause of colorectal cancer is convincing” [3]. The panel thus recommends: “Limit intake of red meat and avoid processed meat”. Several meat components have been speculated to explain that meat-eaters are at a higher risk for colorectal cancer. Meat cooked at a high temperature contains mutagenic heterocyclic amines that induce colon, mammary and prostate tumours in rodents and monkeys [4]. However, heterocyclic amines might not play an important role in colorectal cancer incidence, since (i) chicken intake is a major contributor of heterocyclic amines intake, but it is not associated with the risk [5], and (ii) doses of heterocyclic amines that induce cancer in animals are 1000 to 100,000 times higher than doses ingested by humans [6]. Red meat enhances the formation of putative carcinogenic N-nitroso compounds in human and rodent faeces [7 –9]. N-nitroso compounds level is associated with the promotion of colon carcinogenesis by cured meat [10]. But N-nitroso compounds brought into rat faeces by a bacon-based diet do not initiate or promote preneoplastic lesions in rat colon [11]. Red meat also contains haem, the iron-bearing prosthetic group of myoglobin. Intake of haem iron is associated with an increased risk of colon cancer among women of the Iowa Women’s Health Study [12]. Intake of black pudding, a blood sausage over-loaded with high haem iron, is associated with increased risk of colorectal cancer among women of the Swedish Mammography Cohort [13].

Experimental animal studies support the hypothesis that haem iron promotes colorectal carcinogenesis [14]. We have shown that dietary haem, in the form of haemin (haem stabilized by a chlorine atom, also called Ferriprotoporphyrin IX chloride), haemoglobin or red meat, promotes putative precancerous lesions, aberrant crypt foci (ACF) and mucin-depleted foci (MDF), in the colon of rats given a low-calcium diet [15 –17]. This haem-induced promotion is associated with increased lipoperoxidation and cytotoxicity of faecal water, and linked to urinary 1,4-dihydroxynonane mercapturic acid (DHN-MA) excretion, a fat peroxidation biomarker [18]. The addition of calcium phosphate to the diet inhibits haemin-induced lipoperoxidation, cytotoxicity and promotion of carcinogenesis in rats [17], and we can explain the lack of effect of meat diets on colon cancer carcinogenesis in previous study [19] by a high concentration of calcium in the diet. The addition of calcium phosphate to beef meat diet fully prevents red meat promotion of colon carcinogenesis, but, unexpectedly, the rats given the no-meat high-calcium phosphate high fat diet had more ACF and more MDF than the rats given the no-meat low-calcium phosphate diet [16]. In the absence of meat, dietary calcium phosphate appears to promote carcinogenesis in this rodent model, which precludes its use in human volunteers.

The present study was designed to determine a calcium supplementation with no adverse effect by testing different types of calcium salts, and finding the minimum effective calcium dose.

Materials and Methods

Animals and Diets

Eighty five Fischer 344 female rats were purchased at four weeks of age from Iffa Credo (St.Germain l'Arbresle, France). Animal care was in accordance with the guidelines of the European Council on animals used in experimental studies. The animal colony and staff got official agreement #31-121 for animal studies by French government.

Short-term studies

Two short-term studies were designed to find the most effective calcium salt and the minimum effective calcium dose to suppress early biomarkers of haem toxicity. Rats were housed individually in metabolic cages. The room was kept at a temperature of 22°C on a 12-h light-dark cycle. After 5 days of acclimatization to the AIN76 powdered diet, rats were given the experimental diets. Body weights and food intake were monitored at the beginning, and at the end of each study. **In the first study**, forty rats were randomly allocated to 8 dietary groups (5 rats per group) for one week. Faeces were collected at day 7 and were frozen at -20°C. Animals were killed 7 d after the start of the experimental diets. **In the second study**, twenty five rats were randomly allocated to 5 dietary groups (5 rats per group) for two weeks. Faeces were collected at day 14 and were frozen at -20°C. Animals were killed 14 d after the start of the experimental diets.

Long-term study

A three-month study was designed to test the hypothesis that the minimum effective dose of the most effective calcium salt, selected from short-term studies results, does not promote carcinogenesis in rats. Twenty rats were housed by pairs in stainless steel wire bottomed cages. The room was kept at a temperature of 22°C on a 12-h light-dark cycle. Rats were allowed 7 d of acclimatization to the room and to the control diet, before being injected i.p. with the carcinogen 1,2 dimethylhydrazine (Sigma chemical, St.Quentin, France; 190 mg/kg body wt) in NaCl (9 g/L). In most published studies, several carcinogen injections are given to rats. We reasoned here that the first shot initiates carcinogenesis, but that the following shots would promote carcinogenesis, blurring diet-induced promotion. We thus chose a single-shot protocol, following Glauert [20]. Seven days later, rats were randomly allocated to two groups of ten, and allowed free access to calcium carbonate-based diets for 94 d. We chose to initiate all rats with the carcinogen, since the study was designed to show dietary promotion of carcinogenesis.

Diets

Experimental diets shown in Table 1 were based on a modified standard AIN-76 diet [21], prepared and formulated in a powdered form by UPAE (INRA, Jouy-en-Josas, France). For the first short-term study, high-beef meat diets were formulated to contain 60% meat (w/w, freeze-dried, 0.6 mmol haem/g of meat), and dibasic calcium phosphate was included in all diets at a low concentration of 20 µmol/g. To determine the minimum effective dose of calcium phosphate, the effect of seven concentrations of calcium was tested in seven groups of rats given this low-calcium beef meat diet supplemented with calcium phosphate (doses range: 33 to 250 µmol/g, diet names: Phos33 ... Phos250). To compare the efficacy of calcium phosphate with other salts, the effect of two calcium salts was tested in two groups of rats given calcium carbonate (Carb250) or calcium gluconate (Gluc250) at the highest tested dose (250 µmol/g). For the second short-term study, the effect of calcium carbonate was tested in five groups of rats given a haemoglobin diet, supplying 0.6 mmol/g haem to mimic the 60% beef meat diet, supplemented with calcium carbonate (doses range: 33 to 155 µmol/g, diet names: Carb33 ... Carb155). For the long-term carcinogenesis study, two experimental diets were formulated to contain two levels of calcium carbonate (33 and 100 µmol/g, diet names: Control Diet and Carb100) added into the control low calcium phosphate diet.

Preparation of Faecal Water

Faecal pellets were collected for 24 h under each cage (one rat/cage, short-term studies ; two rats/cage, long-term study), thus leading to five samples per diet. Freeze-dried faeces were used to calculate dry faecal mass and to prepare faecal water by adding 1 ml of sterilized water to 0.3 g of dry faeces. Samples were then incubated at 37°C for one hour, stirring thoroughly every 20 min, followed by centrifugation at 20 000 g for 10 min. The aqueous phase was re-centrifuged at the same speed and duration and the subsequent supernatant (faecal water) collected and stored at -20°C until use.

TBARS and Haem Assay

Thiobarbituric acid reactive substances (TBARS) were measured in faecal water according to Ohkawa et al. [22], exactly as previously described, and results are given as malondialdehyde (MDA) equivalent. [15]. Haem contents of freeze-dried faeces and of faecal water were measured by fluorescence according to Van den Berg et al. [23] and Sesink et al. [14], respectively, as already described [15]. Both TBARS and heme in faeces were measured on faecal samples from both short-term studies.

In vitro experiment

Ability of five different calcium salts (1- calcium phosphate, 2-calcium chloride, 3-calcium gluconate, 4-calcium carbonate, 5-calcium lactate) to bind and to precipitate haem from bovine haemoglobin solution was studied *in vitro*. Haemoglobin (Sigma chemical, St.Quentin, France) 0.36 mmol was acidified at pH 1.4 with HCl 2 mol and then heated one hour at 105°C to dissociate haem from haemoglobin. This solution was centrifuged at 20 000 g for 10 min. Supernatant with free haem was collected.

Six concentrations of each of five calcium salts were prepared (25, 50, 75, 100, 125, 150 mmol). According to Sesink [24], one ml haemoglobin added to one ml calcium salt was incubated for 90 min at 37.5°C in the dark and mixed every 10 min. The mixture was centrifuged for 2 min at 10 000 g . Supernatant (100 µl) was assayed for remaining haem after 20-folds dilution in distilled water, by absorbance measure at 400 nm with a Lambda 2 spectrophotometer (PYE Unicam-PU8600 UV/VIS-spectrophotometer Philips). Different haemoglobin concentrations were prepared as standard solutions: 1/1 (100%), 1/5, 1/10, 1/20, and 1/50 in water. The assay was repeated three times and mean values recorded.

ACF Assay

Rats were killed by CO₂ asphyxiation in a random order at day 94 of the long-term study. Colons were excised from rats immediately post mortem, flushed with cold Krebs solution (Sigma chemical, St.Quentin, France), opened longitudinally and fixed flat between two sheets of filter paper in 10% formalin (Sigma chemical, St.Quentin, France), marked with a two-digit blinding code. Colons picked up in random order were stained for 6 min in a 0.05% filtered solution of methylene blue [25]. Numbers of ACF per colon, and number of crypts in each ACF, were counted under light microscope at x40 magnification in duplicate by two readers, blinded for the origin of the colon.

Statistical Analysis

Results were analyzed using Systat 10 software for Windows, and reported as mean ± SD. Long-term study data were analyzed by Student's t test, except ACF data that were analyzed by two-way analysis of variance (ANOVA: dietary group and reader). Short-term study data were considered firstly using one-way ANOVA. If a significant difference was found between groups ($P < 0.05$) then pairwise comparisons were made with fisher LSD test multiple comparison test.

The meta-analysis of the relation between calcium carbonate and colon cancer in carcinogen-injected rats was done as a sub-analyses of a study that we published in 2005 [26]. From this precedent meta-analysis, we selected studies where calcium carbonate had been fed to experimental groups of rats. The studies were homogeneous (Q Cochran $P > 0.05$), we thus used the "Fixed Effects" model, using EasyMA DOS software (2001 version) to compute data.

Results

Weight and food intake during *in vivo* studies

Final body weight of rats was 107 ± 2 g after 7 d on experimental diets in the first short-term study, without significant differences between groups. Food intake was not significantly different (data not shown), but Gluc250-fed rats tended to eat less than the other groups (Gluc250: 7.2 ± 2.0 g/d, all other groups: 9.1 ± 1.0 g/d, $P = 0.09$). No difference between groups body weight and diet intake was observed in the second short-term study or in the long-term study. Mean body weights were respectively 166 ± 2 g after 14 d, and 201 ± 8 g after 94 d on the experimental diets.

First short-term study: TBARS of Faecal Water

Haem can induce the formation of peroxy radicals in fats, which may be cytotoxic and cleave DNA *in vivo* . This might explain the promoting effect of haem on colon carcinogenesis. Lipid peroxidation was thus measured in faecal water by TBARS assay. Calcium can prevent haem-induced promotion of colon carcinogenesis by precipitating haem in the colonic content, thus haem concentration in faecal water was measured.

Haem concentration in faecal water from beef meat fed rats was significantly reduced in rats given 150 and 250 µmol/g calcium phosphate ($P < 0.01$; Figure 1.A), but not in rats given lower levels of calcium phosphate. Dietary calcium carbonate and gluconate 250 µmol/g produced the same effect on haem than calcium phosphate (Figure 1.A). Surprisingly, haem-induced lipid peroxidation measured by TBARS level was not significantly reduced by the calcium phosphate diets, but only the high-calcium carbonate diet decrease TBARS level in faecal water ($P < 0.01$; Figure 1.B).

In vitro study

Any tested concentration of calcium carbonate higher than 25 mMol was much more effective to precipitate haem than any other tested calcium salt. Proportion of trapped haem was more than three times higher with calcium carbonate than with calcium phosphate (100 mMol) ($P < 0.001$; Figure 2).

Second short-term study: TBARS of Faecal Water

Beef meat was replaced by haemoglobin in the second study diets, since we have demonstrated previously that they produce similar effects on colon carcinogenesis and on faecal water peroxidation [15]. The high-calcium phosphate diet (250µmol/g) did not prevent haem toxicity in the 7-d study, in contrast with our previous 50-d study [17]. We suggest this duration was too short to reduce TBARS level, thus we chose the second short-term study to last for 14-d. As calcium carbonate was the only effective salt against haem-induced lipid peroxidation *in vivo* and the only effective salt to precipitate haem *in vitro*, this salt was used to determine the smallest effective calcium dose.

Haem concentration was lower in faecal water from rats given a diet containing at least 80 µmol/g of calcium carbonate ($P < 0.001$; Figure 3.A). Accordingly, diets containing at least 80 µmol/g of calcium carbonate reduced haem-induced lipid peroxidation of faecal water ($P < 0.001$; Figure 3.B).

ACF Assay

We chose to test if a diet containing 100 µmol/g calcium carbonate would promote colon carcinogenesis, because the second short-term study showed that a 80 µmol/g calcium carbonate diet reduces faecal TBARS concentration, and that 110 µmol/g seemed twice more potent than 80 µmol/g to bind faecal haem. In contrast with a calcium phosphate diet [16], the consumption of a diet containing 100 µmol/g calcium carbonate for 94 days did not increase significantly the number of ACF per colon, 101 days after a dimethylhydrazine injection (Figure 4).

Discussion

These data confirm that dietary calcium reduces faecal water lipoperoxides level, a biomarker linked with haem-induced carcinogenesis promotion. They also establish that the type of calcium salt is paramount in this protection, and that calcium carbonate is effective without the promoting side effect of calcium phosphate.

Red meat promotes ACF and MDF in the colon of carcinogen-induced rats, and promotion by meat is associated with faecal water haem, cytotoxicity and TBARS [15, 16]. The mechanism of promotion by haem is not known, and may be linked to lipid peroxidation. We have explored the effect of faecal water from beef-fed rats on normal (Apc +/+) and premalignant colonic cells (Apc Min/+). Results show that Apc mutated cells survive haem-induced faecal lipoperoxides, notably 4-hydroxynonenal (HNE) that is toxic to normal cells. Selection of mutated cells by cytotoxic lipoperoxides may thus explain haem-promotion of colon carcinogenesis [27]. Calcium added on top of beef-meat or haemin diets normalizes the number of ACF and MDF at the same low level as in control group [17]. Calcium phosphate precipitates haem in the gut, and little haem remains available to induce lipid peroxidation [17, 24]. The trapping of haem by calcium thus abolishes carcinogenesis promotion. In this study, we established that different calcium salts are able to precipitate haem *in vitro* and in faecal water of haem-fed rats and by consequence are able to reduce faecal water peroxidation (Figures 1 and 2). We observed that only high concentrations of calcium phosphate were able to precipitate haem (Figure 1A). Furthermore the ability of the three tested salts (phosphate, carbonate and gluconate) to trap haem was very similar in high-calcium diets (Figure 1A). However, only calcium carbonate decreased significantly the level of lipid peroxidation in faecal water (Figure 1B). So, to screen more precisely the efficiency of different calcium salts, we measured the *in vitro* trapping of haem: Only calcium carbonate precipitated haem significantly. In conclusion, this study shows for the first time a difference of efficiency between calcium salts *in vitro* (figure 2) and *in vivo*, in the trapping of haem and the limitation of peroxidation (figure 1B). We have no explanation for the relatively high TBARS level in controls and in rats given high doses of calcium phosphate or gluconate: we speculate the beef meat in first study may have contained already oxidized fat or oxidizing agents different from haem iron (Figure 1B).

Our previous study showed that rats eating a high-calcium control diet had more ACF and more MDF than rats eating a low-calcium control diet [16]. This promotion was observed with calcium phosphate in a high-fat diet context, but was not seen in a low-fat diet context without a clear explanation for the high-fat/low-fat discrepancy [17]. Bull et al. also showed that the promotion of tumours by calcium phosphate was more important in 30%-fat diet-fed rats than in 3%-fat diet-fed rats [28]. Phosphate, not calcium, might be the promoting nutrient, an issue already discussed by Bruce [29]. Since our *in vitro* study indicated that calcium carbonate was more effective than calcium phosphate to trap haem (Figure 2) and since the first *in vivo* study indicated that a high concentration of calcium carbonate was able to precipitate haem and to limit peroxidation *in vivo*, we determined the lower effective dose of calcium carbonate in a second short-term study (Figure 3). We showed that 80 µmol of calcium carbonate/g of diet is enough to trap haem and to reduce peroxidation. The first aim of this study was to identify the lower dose and type of calcium sufficient to limit the effect of haem in the colon without side effects. We thus tested the effect of an addition of 100 µmol of calcium carbonate/g of diet on the promotion of colon carcinogenesis at the level of ACF (Figure 4). In contrast with the promoting effect of calcium phosphate at 250 µmol/g [16], we did not observe a promoting effect of calcium carbonate on ACF. This study shows for the first time that a calcium salt can inhibit the toxic effect of haem in the colon without side effect on colon carcinogenesis promotion.

To confirm that calcium carbonate does not promote carcinogenesis in rats, we made a meta-analysis of published data on calcium carbonate and colon carcinogenesis (Figure 5). Our previous meta-analysis study shows that calcium (all salts) modestly decreases tumour incidence in rats and that some calcium salts are more protective than others [26]: calcium lactate, but not calcium phosphate, reduces chemically-induced carcinogenesis. Figure 5 shows data from a meta-analysis of 11 studies of high calcium carbonate diets on colon carcinogenesis in chemically-initiated rats; the combined relative risk from all of these studies was 0.983 (95% confidence interval: 0.902; 1.071, $p = 0.69$), compared with low calcium controls. This meta-analysis confirms the lack of promoting side-effect by calcium carbonate on colon carcinogenesis. These results on rodents are coherent with two randomized, double-blinded, placebo-controlled trials. Calcium carbonate supplementation was associated with a significant - though moderate - reduction in the risk of recurrent colorectal adenomas in the Calcium Polyp Prevention Study [30]. In contrast, daily supplementation of calcium carbonate (with vitamin D) for seven years to 36,282 postmenopausal women from 40 Women's Health Initiative centres had no effect on the incidence of colorectal cancer among postmenopausal women [31]. Altogether, these results suggest that calcium carbonate supplementation to neutralize haem does not bear any risk. However, chelation of dietary heme iron bears the theoretical risk of iron deficiency leading to anaemia: recommendations should take this in account.

The study also supports the concept that nutrients like calcium may inhibit the toxicity associated with the excess of another nutrient like haem. This concept differs from the classical chemoprevention concept, since calcium carbonate does not reduce carcinogenesis in rats that are not given dietary haem. This specific calcium preventive effect, by interaction, should be looked for in a cohort study by crossing haem and calcium intake with adenoma or cancer risk.

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Abbreviations

ACF : aberrant crypt foci

MDF : mucin-depleted foci

TBARS : thiobarbituric acid reactive substances

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Figure 1

Effect of diets on haem and TBARS in faecal water after the first short term study (A) Haem in faecal water. (B) TBARS (MDA equivalents) in faecal waters as marker for lipid luminal peroxidation. * Significantly different from Phos20 ($P < 0.01$, by fisher LSD test). Data are means, bars are standard deviations ($n = 5$ in each group). Note: Phos20 to Phos250 are low-calcium beef diet supplemented with calcium phosphate from 20 $\mu\text{mol/g}$ to 250 $\mu\text{mol/g}$. Carb250 and Gluc 250 are low-calcium beef diet supplemented respectively with calcium carbonate and calcium gluconate at 250 $\mu\text{mol/g}$.

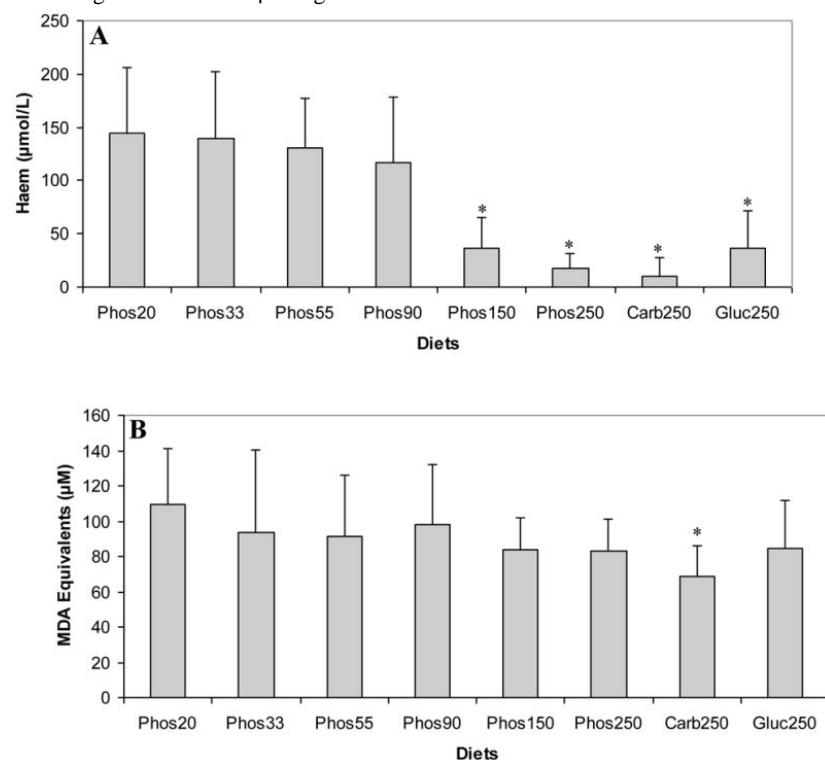


Figure 2

Effect of calcium salts on haem solubility in vitro. Haem (0.36 mM) was incubated with six different concentrations of calcium salts (150, 125, 100, 75, 50, 25 mMol). Data are means, bars are standard deviations (n = 3). * Significantly different from other salts.

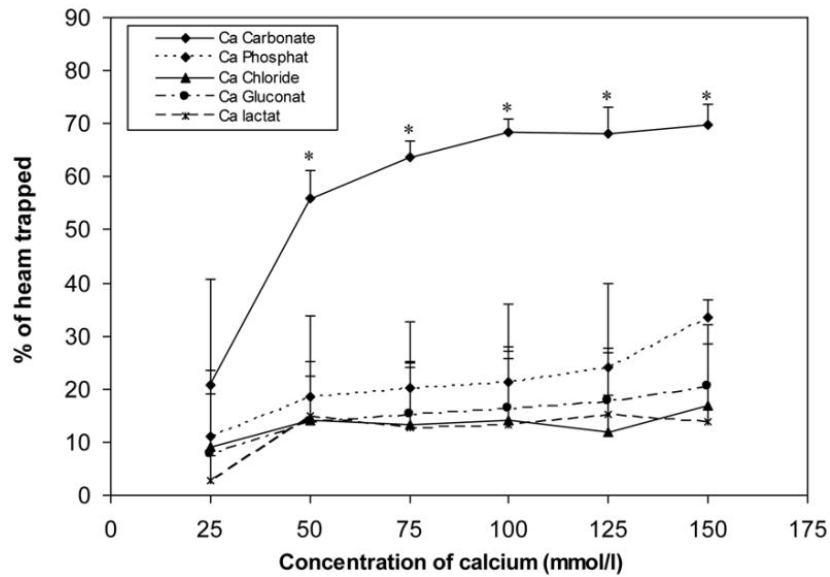


Figure 3

Effect of calcium carbonate-based diets (30 to 155 $\mu\text{mol/g}$) on faecal water values after the second short term study **A**. Haem concentration in faecal water ($\mu\text{mol/L}$) **B**. TBARS concentration in faecal water, expressed as $\mu\text{mol/L}$ MDA equivalents. * Significantly different from Carb30 by fisher LSD test ($P < 0.05$). Data are mean, bars are standard deviation (n=5 in each group). Note: Carb33 to Carb155 are low-calcium haemoglobin diet supplemented with calcium carbonate from 33 $\mu\text{mol/g}$ to 155 $\mu\text{mol/g}$.

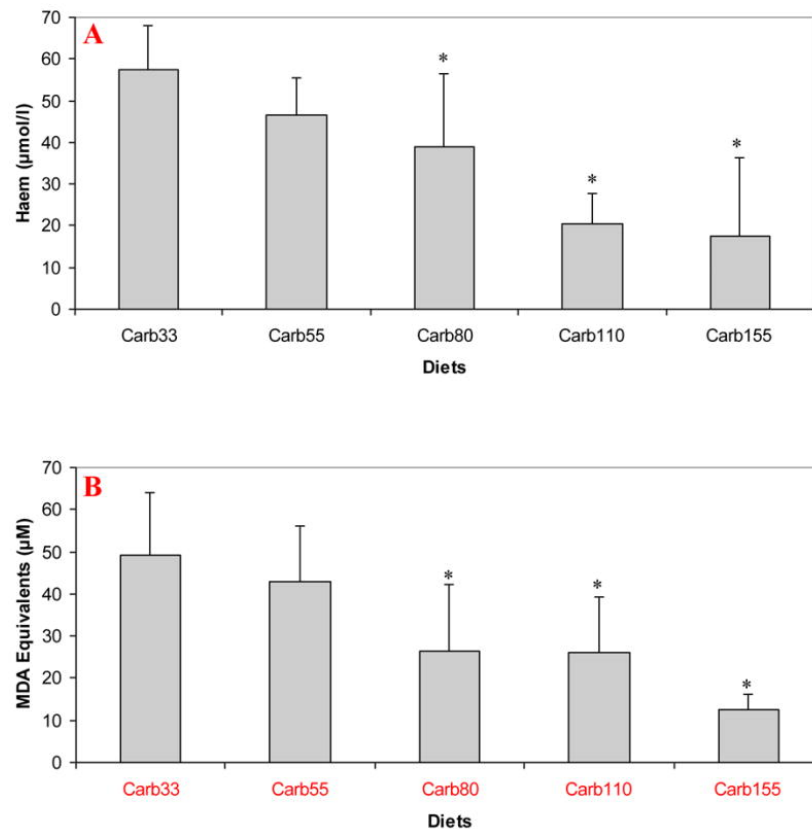


Figure 4

Effect of calcium salts in diet on putative precancerous lesions (ACF) per rat colon 101 d after the injection of dimethylhydrazine. Hatched bars are results of this study with 100 $\mu\text{mol/g}$ of calcium carbonate. Solid bars are previous results with 250 $\mu\text{mol/g}$ of calcium phosphate (Pierre *et al* 2008 [16]). Results are expressed after normalization of the Control Diet groups to 100. * Significantly different control group (CD). Note: The Control Diet was a low-calcium haemoglobin diet supplemented with calcium carbonate at 33 $\mu\text{mol/g}$. Carb100 and CaPhos250 are low-calcium haemoglobin diet supplemented respectively with calcium carbonate (100 $\mu\text{mol/g}$) and calcium gluconate (230 $\mu\text{mol/g}$).

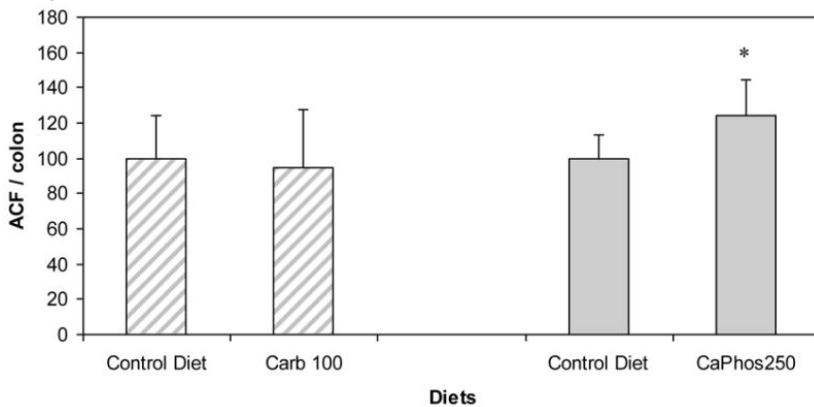


Figure 5

Meta-analysis of the relation between calcium carbonate and colon cancer in carcinogen-injected rats. The common relative risk with 95% confidence was calculated from 11 studies. * * Solid black circles correspond to the study specific relative risk. The open white circle represents the common relative risk. Horizontal lines are 95% confidence intervals. References are indicated between brackets

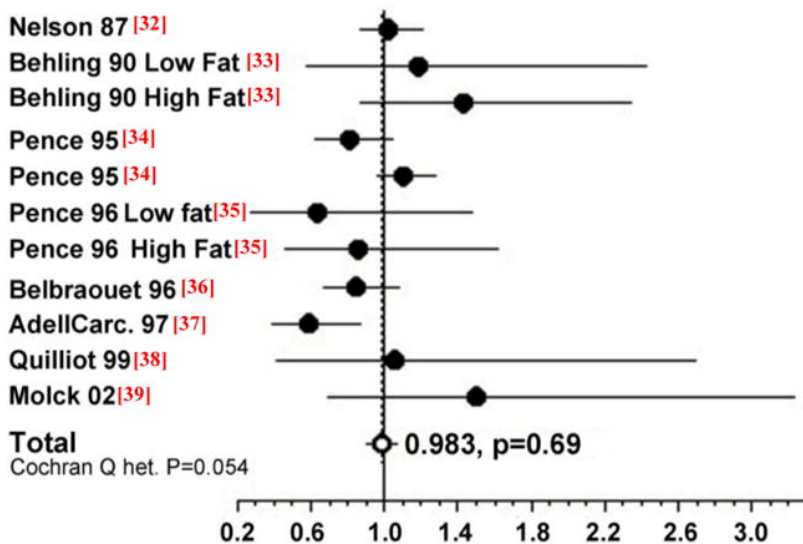


Table 1

Formulation of diets (g/100g)^c

	First short-term study: Beef meat-based diets								Second short-term study: Casein-based diets					Long-term study	
	Phos20	Phos33	Phos55	Phos90	Phos150	Phos250	Carb250	Gluc250	Carb33	Carb55	Carb80	Carb110	Carb 155	Control Diet (Carb33)	Carb100
B Beef	60.0	60.0	60.0	60.0	60.0	60.0	60.0	60.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Haemoglobin	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.63	0.63	0.63	0.63	0.63	0.0	0.0
Safflower Oil	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0
Lard	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0
Casein ^a	5.9	5.9	5.9	5.9	5.9	5.9	5.9	5.9	59.0	59.0	59.0	59.0	59.0	59.0	59.0
Corn Starch	6.0	6.0	6.0	6.0	6.0	6.0	6.0	6.0	7.0	7.0	7.0	7.0	7.0	7.0	7.0
Sucrose	12.8	12.6	12.3	11.9	11.1	9.7	10.6	2.5	16.0	15.8	15.5	15.2	14.8	16	15
Cellulose	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5	5
Methionine	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3
Mineral mix ^b	3.5	3.5	3.5	3.5	3.5	3.5	3.5	3.5	3.5	3.5	3.5	3.5	3.5	3.5	3.5
Vitamin mix ^b	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Choline bitartrate	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
Ca. phosphate	0.2	0.4	0.7	1.1	1.9	3.3	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
Ca. carbonate	0.0	0.0	0.0	0.0	0.0	0.0	2.5	0.0	0.3	0.5	0.8	1.1	1.5	0.3	1.0
Ca. gluconate	0.0	0.0	0.0	0.0	0.0	0.0	0.0	10.6	0.0	0.0	0.0	0.0	0.0	0.0	0.0

^a Low-calcium casein^b AIN76 mix, but 500 g/kg of dibasic calcium phosphate replaced by sucrose in mineral mix^c Phos20 to Phos250 are low-calcium beef diet supplemented with calcium phosphate from 20 µmol/g to 250 µmol/g. Carb250 and Gluc 250 are low-calcium beef diet supplemented respectively with calcium carbonate and calcium gluconate at 250 µmol/g. Carb33 to Carb155 are low-calcium haemoglobin diet supplemented with calcium carbonate from 33 µmol/g to 155 µmol/g. Control diet and Carb100 low-calcium haemoglobin diet supplemented with calcium phosphate from 33 µmol/g to 100 µmo