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Intestinal tumour chemoprevention with the antioxidant lipoic acid stimulates the growth of breast cancer

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ABSTRACT

Objective: Breast and intestinal cancers chemoprevention would significantly impact on cancer care. Hence, we assessed the chemopreventive efficacy of the antioxidant lipoic acid (LA) in mice overexpressing a wild-type Her2/neu, as an animal model of breast cancer, and in APCmin mice for intestinal cancer.

Methods: Mice were randomised at weaning, and were treated with LA for lifetime. Tumour incidence, growth rate and histopathology were analysed on an individual tumour basis.

Results: LA efficiently chemoprevented tumour appearance in APCmin mice. Strikingly, though, LA doses, that were chemopreventive in APCmin mice ($\geqslant$300 μg/day), increased breast cancer growth in Her2/neu mice. Even in experimental groups, where LA overall reduced tumour risk (80 μg/day), LA consistently stimulated the growth rate of established breast tumours. Breast and colon tumours incidence was unaffected by LA, indicating no significant impact of LA on tumour initiation and no protection from mutations driving tumour progression.

Conclusions: Stimulation of breast cancer growth and inhibition of intestinal tumours by LA indicate that diverse growth control mechanisms are modulated by LA in different organs. Concern is raised about the use of LA for cancer chemoprevention.

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1. Introduction

Despite evidence that lifestyle and diet affect tumour incidence,[1–3] tumour chemoprevention in man using dietary antioxidants remains an unmet goal.[4,5] Higher intakes of vegetables and fruits have been associated consistently, although not universally, with reduced risk of cancer at several sites.[1] As a consequence, numerous classes of vegetable compounds, most of which with antioxidant properties, have been proposed for chemopreventive strategies.[1–3,6]

However, prospective clinical trials largely failed to confirm the benefit suggested by pre-clinical or retrospective studies.[5,7] Rather to the contrary, β-carotene was shown to increase lung cancer incidence in cigarette smokers.[8] Long-term vitamin C, vitamin E, selenium and garlic administration had no beneficial effects on gastric cancer,[9] and vitamin E significantly increased the risk of head-and-neck cancer.[10]

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Sporadic tumours in man tend to accumulate driving genetic changes over the lifetime, and, as a consequence, their incidence increases progressively with age.[11] On the other hand, most frequently used pre-clinical tumour models, e.g. subcutaneous inocula of fully transformed cells, are characterised by a rapid outgrowth of artificially induced tumour masses. Even several genetically modified animals, i.e. transgenic mice hosting a mutated Her2/neu,[12] demonstrate tumour incidence and growth rates that are unrealistically high and tumour latencies that are too short compared with man. Finally, causal mutations (e.g. engineered viral oncogenes) may not reflect those commonly encountered in human tumours. This may bias chemopreventive strategies towards mechanisms of tumour development that may not correspond to those of human cancer.

At variance with the models described above, the overexpression and amplification of a wild-type Her2/neu (c-erbB2, ERBB2) protooncogene are deeply implicated in the development of human breast cancer.[13] The overexpression of a germ-line Her2/neu leads to the development of neoplastic mammary foci after a considerable latency[14] and upon the accumulation of additional activating mutations.[15] Correspondingly, the APCmin (multiple intestinal neoplasia) mouse is a close model of familial adenomatous polyposis (FAP), an autosomal dominant condition characterised by the rapid development of numerous colorectal adenomatous polyps that require additional mutations to progress.[16]

A protective role of antioxidants on breast and colon cancers has been proposed.[1,2] α-Lipoic acid (LA) and its reduced dithiol form are potent antioxidants naturally present in animal and plant cells[17,18] (Fig. 1). LA recycles vitamins C and E, and increases intracellular glutathione concentrations,[17,18] reducing oxidative stress *in vivo*. LA induces cell cycle arrest and apoptosis in transformed cells, while protecting their normal counterparts.[19] Moreover, it can be safely administered to patients for extended periods of time, and it is widely used as a nutritional supplement. Hence, LA has been proposed as an effective agent in cancer chemoprevention.

However, caution should be exerted on oversimplified models of tumour development and on 'universal' chemoprevention strategies. Organ-dependent effects have been reported for colorectal,[20] lung, breast and prostate[5] cancers. These findings suggest that the key tumour growth regulatory pathways may critically differ in different organs. Indeed, breast cancer is largely oestrogen-dependent,[13] whereas oestrogens protect from intestinal cancer.[21] Moreover, breast cancer can be insensitive to growth stimulatory pathways, such as COX-2, that play a major role in colon cancer.[22,23] Hence, in this work we investigated a potential chemopreventive role of LA in animal models of breast and intestinal cancers.

2. Materials and methods

2.1. *Her2/neu mice*

FVB/N TgN(MMTVneu)202Mul transgenic mice carrying a wild type rat neu under the transcriptional control of the mouse mammary tumour virus promoter/enhancer (jaxmice.jax.org/strain/002376.html) were purchased from the Jackson Laboratory. Her2/neu female progenies were randomly assigned (ticket drawing) at weaning to the treatment groups. All treatments were tested in at least four separate experiments that were conducted over three years of study.

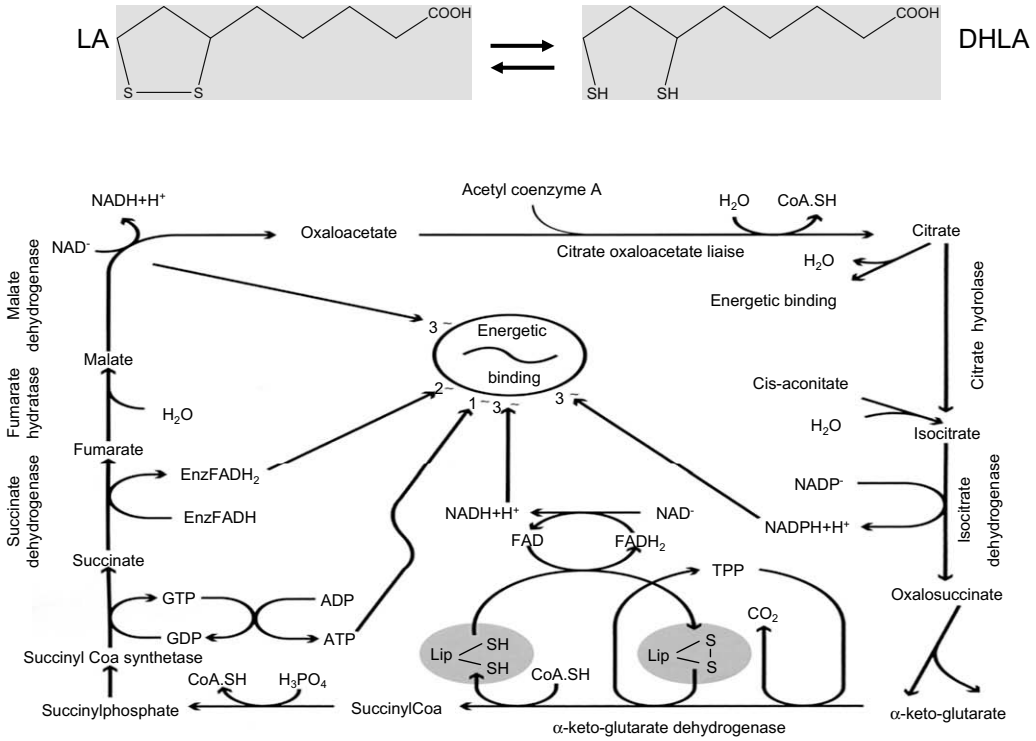

Fig. 1 – Lipoic acid structure and metabolic cycle.

Treated animals were evaluated weekly on an individual basis. The first day when a mammary mass was detected was recorded as the 'latency' of that specific tumour. Growth curves of individual breast tumours were obtained by weekly measurements of tumour volumes (shortest diameter^2 × longest diameter/2).[24] Tumour growth curves were normalised on a group-by-group basis, according to the corresponding mean tumour latency. Average tumour growth curves were subsequently computed (Supplementary Fig. S1).

2.2. *APCmin mice*

Male C57BL/6J-*Min*/+ mice were purchased from the Jackson Laboratory (jaxmice.jax.org/strain/002020.html) and were bred and genotyped as described.[25] *Min*/+ female mice were randomly assigned at weaning to each of the treatment groups. All treatments were tested in at least four separate experiments that were conducted over three years of study. Tumour numbers and diameters were recorded at autopsy (Supplementary Fig. S2). Individual mouse tumour burdens were computed by summing-up the volumes of all detected tumours.

2.3. *Tissue sampling and tumour scoring*

Her2/neu and APCmin mice were sacrificed by ether inhalation at the completion of the 9th and the 6th month of age, respectively, or when critically ill. Macroscopic mammary tumours in Her2/neu mice were excised, fixed in formalin, embedded in paraffin and examined (M.P. and R.L.) after haematoxylin and eosin staining.[14] In APCmin animals, the entire colon and small intestine were quickly removed, rinsed in saline and spread on Petri dishes (Supplementary Fig. S2). Intestinal segments were fixed in formalin, stained with methylene blue and examined under a stereo dissecting microscope to record tumour number and diameter.[26] Intestines were subsequently embedded in paraffin; sections were stained with haematoxylin and eosin[26] and examined (M.P. and R.L.).

Procedures involving animals were conducted in compliance with institutional guidelines and with national (D.L. No. 116, G.U., Suppl. 40, February 18, 1992; Circolare No. 8, G.U., July, 1994) and international laws and policies (UKCCCR Guidelines for the Welfare of Animals in Experimental Neoplasia; EEC Council Directive 86/609, OJ L 358. 1, December 12, 1987; Guide for the Care and Use of Laboratory Animals, United States National Research Council, 1996).

2.4. *Lipoic acid*

Synthesised LA with a purity $\geqslant$99% was provided by Antibiotics (Milano, Italy). A single batch of LA was used throughout this work. LA was provided with either drinking water or solid food, to assess a possible effect of administration mode on LA action. LA was provided from weaning and was administered over the mouse lifetime. As a mouse drinks approximately 5 ml of water a day, drinking-water solutions were prepared to provide 80, 300 or 500 μg LA daily, respectively. Stock solutions of LA (333–2000x) were prepared in 1 N NaOH. Solid diet pellets containing 1 g/kg or 0.1 g/kg LA were made by Rieper (Vandoies, Italy) (www.rieper.com). The expected daily intake of solid food is approximately 3 g for a 20 g mouse. Therefore, the expected intake of LA was 3 mg a day for the 1 g LA/kg diet or 0.3 mg a day for the 0.1 g LA/kg diet, respectively.

2.5. *Statistical analysis*

At weaning, each female Her2/neu or *Min*/+ mouse was allocated to one of the different treatment arms (12–22 mice *per* arm across all experiments). The randomisation procedure (ticket drawing) encouraged a balance across groups over time to prevent time-dependent effects. Heterogeneity in tumour characteristics among groups was detected with the Kruskal–Wallis test. Differences between each treated group and control mice were assessed with the Mann–Whitney test (www.elegans.swmed.edu/~leon/stats/utest.html). As tumour volume values followed normal distributions (unpublished observation), the student *t*-test (www.physics.csbsju.edu/stats/) was used to compare means of tumour volumes. Hazard ratios (HR) and ^{2} of cumulative tumour incidence curves were computed (www.graphpad.com/). The effects of treatment and time on tumour growth were evaluated by two-way ANOVA for repeated measurements. *Post hoc* analyses were performed using the Bonferroni test for pairwise multiple comparisons (5% significance level). Rates of tumour growth of the different experimental groups were compared with Manova/F test statistics. As unequal numerousness across groups prevents the use of ANOVA tests, imputation of missing data-points was performed. To prevent bias in subsequent comparisons, random values were generated whose mean, standard deviation and standard error corresponded to those of the respective observational data (manuscript in preparation). Input data never exceeded 35% of the total data-points.

3. Results

3.1. *Her2/neu mice*

Antioxidants may protect DNA from structural damage by free radicals, thereby reducing the chances of additional oncogene mutations/tumour progression. If this were the case, LA was expected to both lower tumour incidence and to slow-down tumour progression. However, other regulatory mechanisms, besides oncogenic DNA mutations, e.g. DNA methylation,[27,28] transcription factor activation[29] and stimulation of constitutive growth stimulatory pathways,[30–32] can drive tumour progression. An influence of antioxidants upon these tumour promoting mechanisms[33–36] was expected to lead to a significant delay (or anticipation) in tumour appearance, without affecting tumour incidence. Hence, we determined the impact of different doses of LA on tumour incidence and growth rates on continuously treated mice.

3.1.1. *Tumour frequency*

Overall, 316 breast tumours were analysed. Tumour numbers *per* mouse did not significantly differ between treated and untreated groups (range 1.8–2.2) (Supplementary Table S1). As tumour development in Her2/neu mice requires additional oncogenic mutations,[15] this indicated that LA does not effec-

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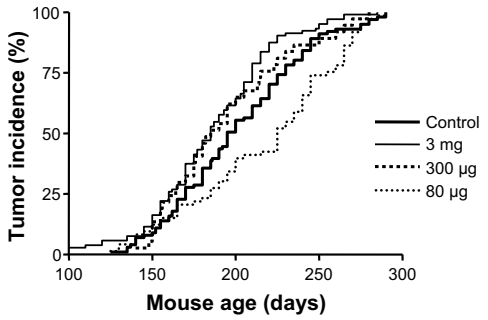

b

LA / day	χ^2	P value	Median Control	Median Treated	Hazard Ratio	95% CI of Hazard Ratio
3 mg	19.280	<0.0001	200	179	1.967	1.651 to 3.706
300 µg	4.446	0.0350	200	185	1.466	1.033 to 2.450
80 µg	6.201	0.0128	200	225	0.7079	0.494 to 0.919

Fig. 2 – Incidence over time of breast tumours in Her2/neu mice. (a) Cumulative incidence curves were constructed as described in Section 2. Data on 115 tumours were computed in control mice, 76 in 80 µg LA/day, 39 for 300 µg LA/day and 86 tumours for 3 mg LA/day. (b) Statistical analysis. Hazard ratios were computed from the cumulative incidence curves. χ^2 values (www.graphpad.com/) are indicated.

tively protect from DNA mutations, and does not play a significant role in tumour initiation (Supplementary Table S1). The trend towards a lower tumour incidence in mice treated with 3 mg LA was correlated to their shorter lifespan (Fig. 2 and data not shown).

3.1.2. *Tumour latency*

Animals were evaluated weekly on an individual basis, and the time of appearance of each tumour mass was recorded as its 'latency'. Tumour location was also recorded, to allow to construct individual tumour growth curves (Supplementary Fig. S1) (see below).

Control mice demonstrated an average tumour latency of 207 ± 4 days (range: 135–300 days), in excellent agreement with historical data (205 days)[14] (Supplementary Table S1). Mice treated with 80 µg LA showed a tendency to a delayed tumour appearance (+11.2 days *versus* controls) (Mann–Whitney; $P = 0.056$), suggesting effectiveness of LA as anti-tumour promoter at early stages of tumour development. On the other hand, a significantly shorter latency was demonstrated by mice treated with 3 mg LA/day (–18.7 days *versus* controls) (Mann–Whitney; $P = 0.012$), indicating that high doses of LA have a promoting effect on breast tumour growth. Mice treated with 300 µg LA/day showed a consistent tendency to a shorter tumour latency (–13.2 days *versus* controls).

3.1.3. *Tumour incidence over time*

Cumulative incidence curves were obtained (Fig. 2A). A delay in tumour appearance was observed in mice treated with 80 µg LA/day. However, markedly anticipated tumour occurrence was recorded in the groups treated with higher LA doses (300 µg and 3 mg).

Eighty micrograms LA was associated with hazard ratios (HR) of 0.7079 (confidence intervals, CI: 1.033–2.450) *versus* controls ($P = 0.0128$) (Fig. 2B). On the other hand, a strikingly higher tumour risk (HR = 1.967; CI: 1.651–3.706) was demonstrated for 3 mg LA *versus* controls (^{2} analysis; $P < 0.0001$) and for 300 µg LA *versus* controls (HR = 1.466; CI: 1.033–2.450) (^{2} analysis; $P = 0.035$). Hence, high doses of LA have a significant, negative impact on the course of breast neoplastic disease in Her2/neu mice.

3.1.4. *Tumour growth rates*

The spreading of tumour appearance over lifetime (Supplementary Fig. S1b) prevented a direct comparison of tumour growth rates across control and treated groups. Hence, the mean tumour latency was calculated in each experimental group (Supplementary Fig. S1c–g) (Supplementary Table S1). Mean latency dates were utilised as zero time-points for grouping tumour growth curves (Supplementary Fig. S1c–g). Average tumour growth curves were subsequently computed.

Two-way ANOVA comparison of tumour growth curves showed that both treatments (80 mg LA or 3 mg LA) and time had significant effects on tumour growth in treated animals *versus* controls ($P < 0.0001$; $P < 0.0001$) (Fig. 4 and Supplementary Fig. S3). Bonferroni *post hoc* analyses confirmed that a treatment with 80 mg LA significantly delayed (Fig. 4A), whereas that with 3 mg LA significantly accelerated (Fig. 4B) tumour growth.

Notably, though, the growth rate of macroscopic tumours did not appear to differ across different LA-treated groups, even when LA proved to be overall protective for tumour development (80 µg/day) (Figs. 3 and 4). To clarify the impact of LA on established tumours, the growth rate component of the curves described above was analysed, upon time-zero normalisation (Fig. 5). A comparison across all experimental groups (Fig. 5 and Supplementary Fig. S3a–d) was associated with an *F*-test statistic of 8.5 ($P < 0.001$), rejecting the null hypothesis of equality. Pairwise comparisons of treated groups *versus* controls (using corrections for multiple comparisons) demonstrated a similarly higher-than-control rate of growth for tumours treated with 80 µg or 3 mg LA/day.

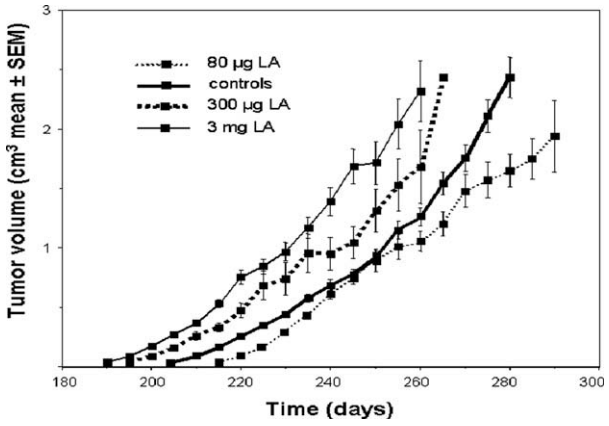

Fig. 3 – Growth curves of breast tumours in Her2/neu mice. Growth curves were latency normalised as described in Section 2 and in Supplemental Fig. S1, and computed as average *in vivo* growth rates of spontaneous tumours. Untreated animals (thick solid line); 80 µg LA in drinking water (thin dotted line); 300 µg LA in food pellets (thick dotted line); and 3 mg LA in food pellets (thin solid line). SEM are shown as vertical bars.

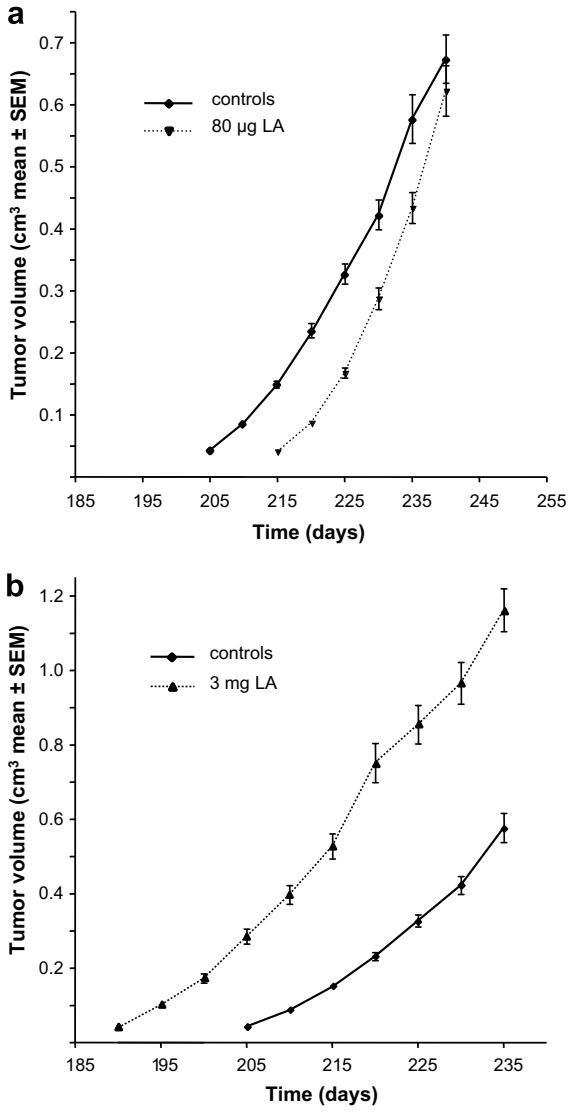

Fig. 4 – Statistical analysis of breast tumour growth curves. Latency-normalised growth curves were analysed by two-way ANOVA as described: (a) 80 µg LA (dotted line) *versus* controls (solid line). Eighty micrograms LA significantly delayed tumour growth compared with controls. Both treatment and time had a significant effect on tumour growth (two-way ANOVA; $P < 0.0001$) and (b) 3 mg LA (dotted line) *versus* controls (solid line). Three milligrams LA significantly increased tumour growth compared with controls. Both treatment and time had a significant effect on tumour growth (two-way ANOVA; $P < 0.0001$).

3.1.5. *Histopathology*

Areas of hyperplasia and dysplasia were detected in breasts from Her2/neu mice (Fig. 6). Both untreated and treated mice developed nodular mammary adenocarcinomas with a solid morphology, similar to that of human solid ductal carcinoma *in situ*. Apoptotic and proliferative indexes, as well as cytopathological appearance, tumour architecture and angiogenesis, did not significantly differ between control and treated groups.

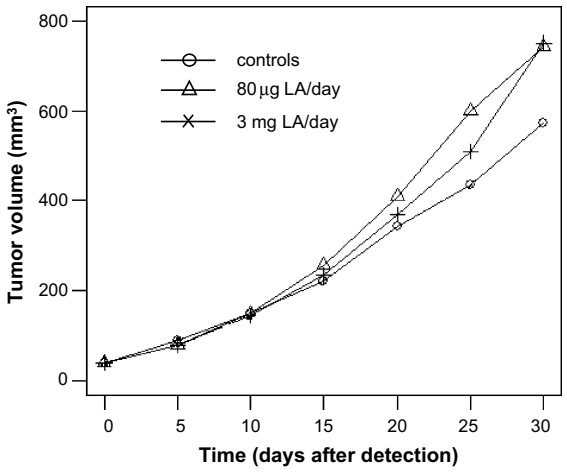

Fig. 5 – Statistical analysis of the growth rate component of the breast tumour growth curves. Tumour volume medians were computed for each time-point as described in Section 2. Latency-normalised growth curves were analysed by two-way ANOVA as described. Time 0: average tumour latency in each experimental group, normalised to a common time of origin for all growth curves; time 5–30: days after tumour detection.

3.2. *Adenomatous polyposis coli (APC)min mice*

3.2.1. *Tumour frequency*

The incidence and volume of macroscopic gastrointestinal tumours were quantified at autopsy. A total of 905 tumours were analysed. No significant differences in tumour incidence were observed across groups (range of means: 10.7–15.6 tumours *per* mouse) (Fig. 7 and Supplementary Table S2). A trend towards lower tumour frequencies at higher dosages of LA was correlated to a reduction of tumour size, with smaller tumours being likely to fall below the detection limit at autopsy (Fig. 7 and Supplementary Table S2). Hence, similarly to Her2/neu mice, LA did not appear to effectively protect from DNA mutations, nor played a significant role in tumour initiation.

3.3. *Tumour volume*

Average tumour volumes were significantly smaller in the groups treated with 500 µg or 3 mg LA/day, as compared with control animals ($P = 0.032$; $P = 0.021$, respectively) (Fig. 7 and Supplementary Table S2). This demonstrated effectiveness of LA treatments in chemopreventing intestinal tumour growth.

3.4. *Tumour burden*

Tumour burden was significantly reduced (39–44%) in mice treated with 3 mg LA (0.173 cm^3) (Mann–Whitney; $P = 0.04$) (Fig. 7 and Supplementary Table S2). Treatment with 500 µg LA also significantly reduced tumour burden *versus* controls (0.157 *versus* 0.282 cm^3, respectively) (Mann–Whitney; $P = 0.004$). A trend towards smaller tumour burdens was observed in mice treated with 300 µg LA, confirming a broad efficacy of LA in chemopreventing intestinal cancer.

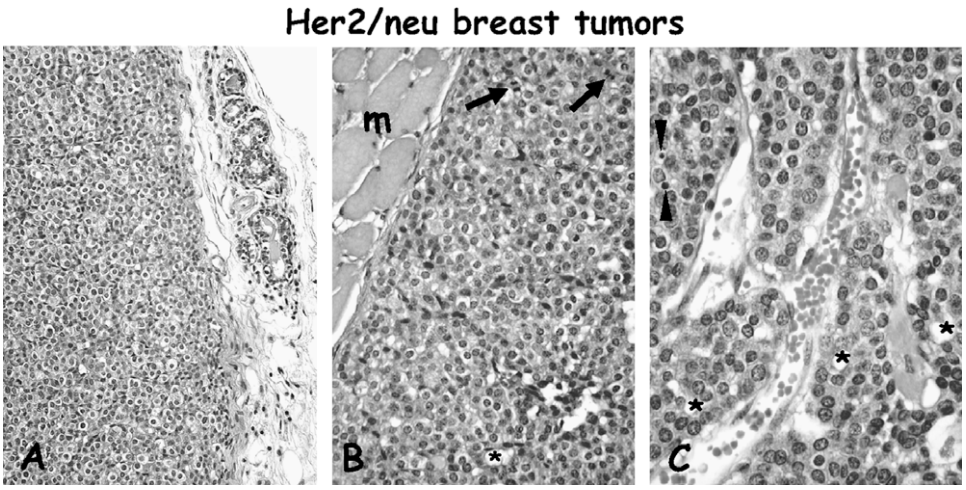

Fig. 6 – Histopathological analysis of Her2/neu mammary tumours; H&E staining. Both treated and untreated mice developed nodular mammary adenocarcinomas with a typical solid morphology, similar to the human 'solid' ductal carcinoma *in situ* (DCIS). (A) Solid carcinomatous proliferation; non-neoplastic mammary tissue is present (upper right corner). (B, C) Few luminal spaces can be found within mainly solid tumour proliferations (*). Muscular tissue adjacent to tumour (m). Mitotic (arrows) and apoptotic (arrowheads) figures.

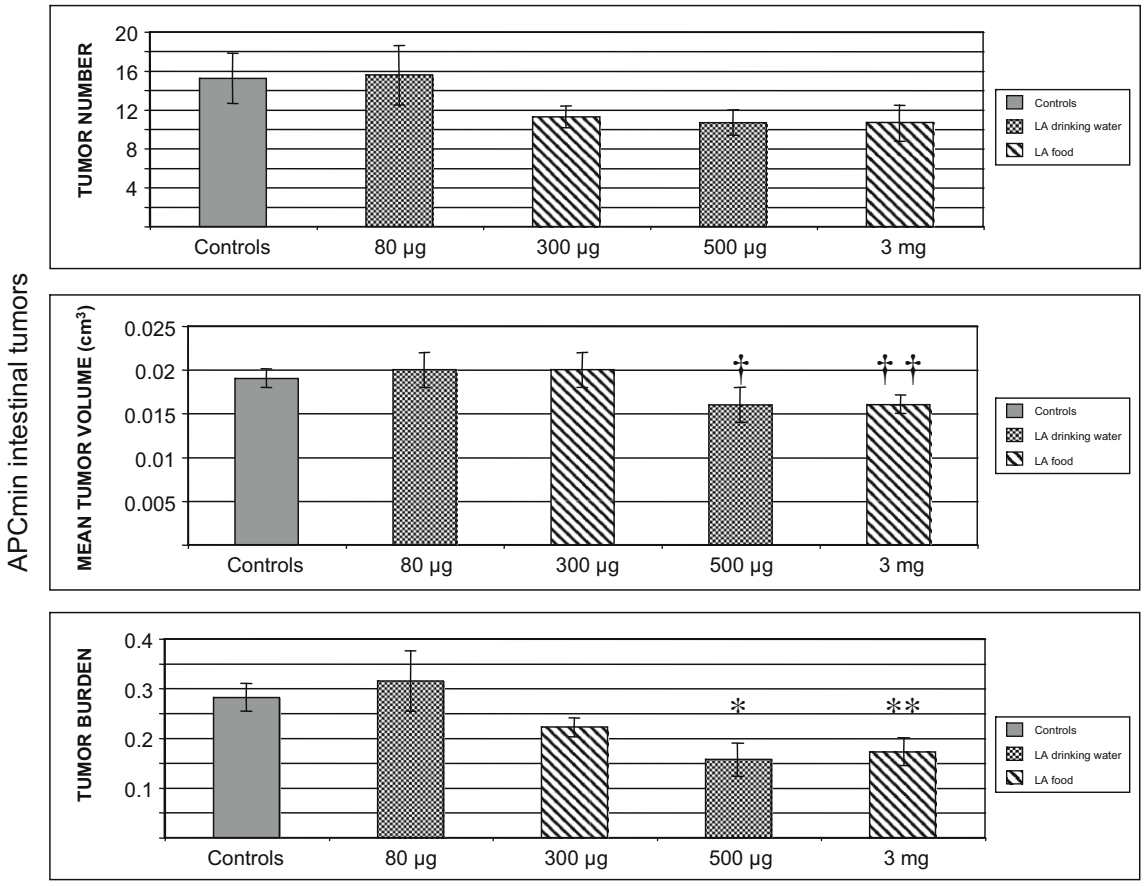

Fig. 7 – Tumour growth in APCmin mice. (top) Number of tumours *per* mouse. (mid) Average tumour size. (bottom) Average tumour burden *per* mouse. Data on tumours from untreated animals and from those treated with LA in water or in food pellets are shown as indicated in the panels. †Average tumour volume 516 µg LA *versus* controls $P = 0.032$ (t-test) and †† Average tumour volume 3 mg LA *versus* controls $P = 0.021$ (t-test). *Average tumour burden 516 µg LA *versus* controls $P = 0.004$ (Mann–Whitney) and **average tumour burden 3 mg LA *versus* controls $P = 0.04$ (Mann–Whitney).

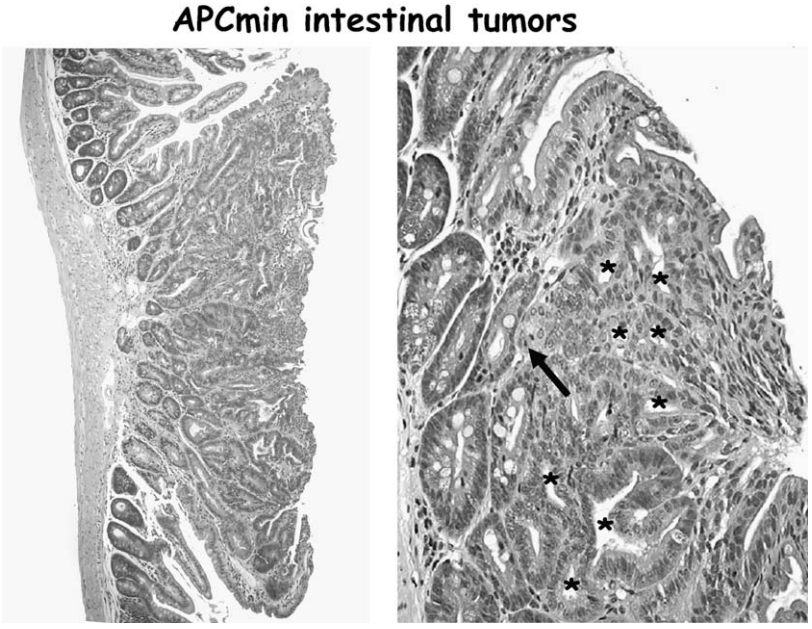

Fig. 8 – Histopathological analysis of APCmin intestinal tumours; H&E staining. Representative examples of polyps from small intestines of APCmin mice. (A) Intestinal polyp with neighbouring normal villous epithelium. (B) Dysplastic glands (*) in an adenomatous polyp devoid of goblet mucus-secreting cells, with areas of palisade arrangement of elongated nuclei; a mitotic figure is shown (arrow). The neighbouring normal mucosal epithelia contain goblet cells and enterocytes with normal appearance.

3.5. *Histopathology*

Areas of hyperplasia and dysplasia were detected (Fig. 8). Apoptotic figures and metaphases were quantified. Cytopathological appearance, nuclei heterogeneity, capsule, blood vessel number and diameter were recorded. None of these indexes did significantly differ between control and treated groups.

4. Discussion

Breast and colon tumours are the leading causes of cancer-related mortality in the western world[13] (info.cancerresearchuk.org/cancerstats/geographic/world/?a=5441), and effective chemoprevention would significantly impact on cancer care.[2,37] Better pre-clinical models, with higher predictive value for human studies, may significantly help towards this goal. In this study, Her2/neu overexpressing mice and APCmin mice were used to assess the chemopreventive efficacy of LA. These strains develop breast cancer or intestinal tumours with significant similarities to the course of these diseases in man, and were shown to be useful for drug-based chemoprevention, e.g. tamoxifen[38] or NSAIDS,[39] respectively, in close parallel with results obtained in man.

In this work, we used these mouse models in a large-scale study to define the impact of the antioxidant LA in tumour chemoprevention. Mice were treated daily with doses of LA that correspond to those routinely used in man (e.g. 200–1800 mg/day for a 70 kg individual). The treatment was started at weaning, i.e. well before the onset of the neoplastic lesions.[14,38] To optimise the chances of observing an effect of LA on tumour growth, the treatment was continued over the mouse lifetime.

Antioxidants may protect DNA from free radicals, and reduce chances of oncogene mutations. Hence, antioxidants' use may lower tumour incidence, and slow-down tumour progression. Tumour incidence was not modified by any of the LA doses utilised. As tumour appearance in both Her2/neu and APCmin mice requires additional activatory mutations,[15,40,41] our findings indicate that LA may not significantly protect from DNA damage. The absence of detectable differences in the differentiation/architecture of the neoplastic lesions in treated *versus* control Her2/neu and APCmin mice is consistent with this scenario.

Strikingly, LA had opposite effects on colon *versus* breast, as it inhibited the former, but stimulated the latter. Of interest, even in Her2/neu mice, where LA showed a positive chemopreventive effect (80 μg/day), the growth rate of macroscopic tumours was higher than in controls. These findings show that LA has a growth-stimulatory effect on established breast tumours. The overall protective effect/delay in tumour appearance observed in 80 μg LA-treated mice suggests that low doses of LA at early phases of tumour development may impinge on diverse growth control mechanisms (see below).

Pharmacokinetic factors, e.g. higher overall doses of LA to the intestine as opposed to breast, may have had a role in organ-specific effects. However, the most dramatic divergence in the effects of LA on tumour growth (stimulation for breast, inhibition for the intestine) was observed at the highest LA dosages, which are expected to provide adequate supply of LA to both organs. Moreover, corresponding results were ob-

tained with different administration modes and doses, ruling out trivial supplementation artefacts. Hence, additional factors appear to play a role, among them a diversity of regulatory networks in the two tumour types.[21–23] A modulatory role of LA on tumour growth, but not on tumour incidence is, indeed, consistent with interference with tumour growth regulatory mechanisms unrelated to oncogenic DNA mutations. Redox levels exert a direct regulatory role in cell growth,[42] gene transcription,[43–46] signal transduction[47] and cell metabolism.[48] DNA methylation is also affected by the redox equilibrium.[35,36] Mutation-independent, ubiquitous growth stimulatory platforms, e.g. tetraspanin webs, are functionally linked to redox equilibria,[33] and can play an important stimulatory role on the growth of transformed cells.[30–32]

Our findings raise concern on the impact of LA on breast cancer growth, suggesting caution on its use for tumour chemoprevention. A broader relevance of our findings for other antioxidants should be interpreted with caution, given the complexity of the metabolic effects of LA. As an example, LA, similarly to vitamin C, can have both antioxidant and prooxidant effects,[49] which may considerably complicate the interpretation of our findings. However, the differences in response of breast cancer *versus* colon cancers to LA treatment in animal models are remarkably consistent with organ-dependent effects that have emerged from clinical studies in man, e.g. for epigallocatechin-3-gallate,[50] resveratrol[51] and curcumin,[52] indicating that further enquiry into the broader issue of antioxidants in cancer prevention is needed.

Conflict of interest statement

None declared.

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Guarantor statement: The guarantor, SA, accepts full responsibility for the work and/or the conduct of the study, had full access to the data, and controlled the decision to publish.

Contributors: All authors contributed to the intellectual development of this paper, to the organisation of the study and to the analysis of the data. SA and CR had the original idea for the study. CR and ADL performed the *in vivo* studies. RLS, RL and MP performed the pathological analysis. SA analysed the overall data, wrote the first draft paper, and is the guarantor. LA provided statistical expertise and critical corrections to the manuscript. CP provided critical corrections to the manuscript.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ejca.2008.08.021.

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