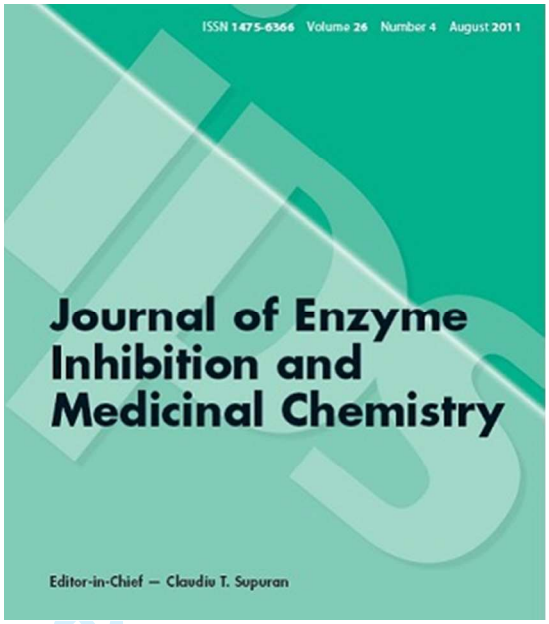

SYSTEMIC ALKALINISATION DELAYS PROSTATE CANCER CELL PROGRESSION IN TRAMP MICE

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Keywords:	Tumour microenvironment, chemoprevention, alkalinisation, prostate tumour, TRAMP mice

SYSTEMIC ALKALINISATION DELAYS PROSTATE CANCER CELL PROGRESSION IN TRAMP MICE

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ABSTRACT

The microenvironment of solid tumours is extremely acidic and this condition arises since the precancerous stage. This acidic milieu could therefore provide a useful target for both prophylactic and therapeutic approaches. In TRAMP transgenic mice, an *in vivo* model of prostate adenocarcinoma, oral administration of alkaline water was devoid of unwanted side effects, and when started from an early age was as effective as NaHCO3 in significantly delaying tumour progression, while when started when prostate tumours were already present a non statistically significant trend in the same direction was detected. These findings indicate that the use of alkalinizing drugs should be considered for chemoprevention and, in association with standard chemotherapy, for treatment of human prostate adenocarcinoma.

Keywords: tumour microenvironment; chemoprevention; alkalinisation; prostate tumour; TRAMP mice.

INTRODUCTION

During tumour development, the microenvironment becomes progressively acidic due to different and often concomitant mechanisms: local hypoxia resulting from poor blood perfusion, increased flux of carbons through fermentative glycolysis, and the release by cancer cell of lysosome content into the extracellular matrix (ECM) (1-3). Indeed, extracellular acidosis in human solid tumours can reach pH values as low as 6.5. Likely, adaptation of cancer cells to an acid microenvironment occurs early during cancer progression, as pre-cancer cells undergo a metabolic switch in ATP generation, from oxidative phosphorylation to glycolysis. Since the latter pathway provides a much lower energy gain, transformed cells greatly increase their glucose uptake to meet their amplified metabolic requirements, resulting in intracellular lactate accumulation and the excretion of H^+ by proton transporters (4), causing progressive acidification of the extracellular milieu. This acidified habitat supports cancer cells with a stabilized glycolytic phenotype, which in turn leads to sustained generation of metabolic acids, even in well- oxygenated conditions, and to selection of cancer cells resistant to acid-mediated apoptosis (5). It has been hypothesised that these mechanisms lead to a competitive advantage of cancer cells toward normal bystander cells, that cannot survive in an increasingly acidic microenvironment (1, 2). Besides being toxic to normal cells, acidosis can stimulate invasion and metastatization by degrading and remodelling the ECM, increasing angiogenesis through the release of vascular endothelial growth factor, and inhibiting the immune response (1, 5-10).

It is likely that targeting the driver functions that confer selective advantages to tumour cells can be a suitable alternative approach for cancer therapy. The reversal of pH gradient in cancer cells is increasingly considered as a hallmark of virtually all cancers, and a potential target for new anti-tumours therapies (11). In particular, alkalinizing treatment with existing molecules such as proton pump inhibitors (PPIs) and buffers, such as $NaHCO_3$, citrate or TRIS has been a proposed for human therapy (2). This approach has been supported by a clinical study on companion animals with spontaneous tumours in which the PPI lansoprazole, administered at high dose and combined

with a water alkalizer, has proven effective in enhancing tumour response to metronomic chemotherapy (12), and by two clinical trials, in either osteosarcoma (13) or metastatic breast cancer patients (14), where the administration of the PPI esomeprazole either improved the local effect of neoadjuvant chemotherapy or prolonged the time to progression and the overall survival rate in treated patients. Coming to the effect of alkalizer agents as monotherapy, it has been demonstrated that $NaHCO_3$ inhibits human mammary and prostate metastases in mouse xenograft models (15). In a recent paper, the oral administration of a commercially available water alkalizer significantly reduced tumour growth in a syngeneic melanoma mouse model (16). However these results should be confirmed in a more physiological model, i.e. in transgenic animals spontaneously developing tumours.

TRAMP mice are the best model available so far for pharmaceutical studies on prostate carcinoma, since 100% of these animals display spontaneous multistage prostate carcinogenesis, with histological and molecular features similar to those present in human prostate cancer (17, 18). Not surprising, the TRAMP model has been used to successfully test the chemopreventive efficacy of several natural anticancer agents such as green tea, grape, garlic, cabbage, tomato, hop (19-25). The efficacy of microenvironment alkalization in such a model was assessed by Ibrahim-Hashim and co-authors, which found that $NaHCO_3$ in drinking water prevented the onset of prostate cancer in transgenic TRAMP mice, albeit it was ineffective in treating established tumours (26). Despite this excellent proof-of-principle demonstration, the authors themselves correctly stated that the administration of this regimen to humans would be unadvisable, due to the unwanted side effects resulting from sustained intake of high doses of $NaHCO_3$. Moreover, sodium bicarbonate can't be considered the ideal buffer for tumour treatment and prevention for many reasons. These include first that $NaHCO_3$ is for sure not a potent buffer molecule inasmuch as it can reach no more than pH 8.5 in a water solution. Moreover, it is unbalanced in term of electrolyte equilibrium, containing exclusively Na, and therefore exposing to many potential side effects in prolonged treatment regimens, including cardiovascular and renal dysfunctions. Lastly, at the concentration proposed in

the Ibrahim-Hashim's paper it would result disgusting when used for oral administration, independently from the disease condition. We have therefore tested in the same TRAMP model the anti-tumour effect of a water alkalinizer (Alkawater) through which the best pH condition can be reached in water solution (from pH 9.0 to 10.0) depending on the tumour and systemic pH; moreover, the taste of alkalinized water is comparable to that of either tap and mineral water and the solution is balanced in term of electrolytes, containing Na+, K+, Ca+ and Mg+. The results have shown that the administration of alkaline water was devoid of any unwanted side effect, and when started from an early age was as effective as NaHCO3, significantly delaying tumour progression, while when started when prostate tumours were already present showed a trend in the same direction.

MATERIALS AND METHODS

Cell culture and reagent

Tramp C1 prostate carcinoma cell lines (ATCC, Rockville, MD, USA) were cultured in DMEM containing 10% FCS, glutamine and penicillin/streptavidin.

The alkaline stock solution contained 6% NaCl and 9% KOH and was diluted 1:1000 in tap water to obtain a drinking solution at pH 9,5, and 1:300 for a solution at pH 10,5.

$NaHCO_3$ was dissolved in tap water at the concentration of 200 mM, as previously described (26).

Animals and anti-acid treatment

Animal studies and research protocols were reviewed and approved by the Ethics Committee of the IRCCS San Martino-IST and were conducted in accordance with the current Italian regulations and guidelines for the care and use of laboratory animals (D.L. 26/2014).

For in-vivo studies we used two different models, the spontaneous prostate tumour model developing in the transgenic TRAMP mice and xenograft model with TRAMP C1 cells.

TRAMP mice were maintained in heterozygosity by crossing C57Bl/6 TRAMP females with C57Bl/6 wild type males (Charles River Laboratories, Calco, Italy), and transgene verification was carried out when newborn mice reached three weeks of age using DNA obtained from tail clipping as previously described (18). Transgene-positive mice were then randomly divided into four groups, each supplied with different types of water: the "control" group of 30 mice was administered with tap water; the "prevention" group of 59 mice with alkaline water at pH 9.5, starting at 4 weeks of age; the "therapy" group of 37 mice with alkaline water at pH 10.5, starting at 12 weeks of age; the "bicarbonate" group of 34 mice with $NaHCO_3$, starting at 4 weeks of age. These time points were chosen accordingly with the progression of cancer in TRAMP mice, where high grade prostatic intraepithelial neoplasia (PIN) or well-differentiated prostate cancer are present by 10-12 weeks of age (17). All animals were monitored daily for signs of suffering and, starting from 20 weeks of age, were checked by palpation for the development of precocious neuroendocrine tumours. 26% of TRAMP mice of all groups displayed rapidly growing and very aggressive poorly differentiated neuroendocrine tumours that developed very early (24 to 29 weeks of age) respect to our chosen end point (32 weeks of age). These animals were not considered in our analysis, since these tumours are not comparable to the human pathology, their growth arise independently from atypical hyperplasia or other epithelial lesions (27) and represent an extremely rare and advanced stage of carcinomas (28). We instead took into the account neuroendocrine tumours that develop later, as consequence of stochastic events related to malignant progression (29). Throughout the experiment, mice were fed with food and water *ad libitum* and water consumption was recorded. Mice were sacrificed by CO_2 inhalation at 32 weeks of age and subjected to accurate necropsy. Body weight (bw) was registered before sacrificed, the entire urogenital (UG) apparatus, consisting of emptied bladder, urethra, seminal vesicles and prostate was excised and weighed. Seminal vesicles and testes were then removed and the prostate was fixed in 4% neutral buffered formalin and processed for histology and immunohistochemistry. Bladder was maintained to orient samples during embedding, so that sectioning were done starting from the dorsal part.

For the xenograft model six 6-week-old C57/Bl6 male mice (Charles River Laboratories) were injected subcutaneously (sc) in the right flank with $6x10^6$ TRAMP C1 cells suspended in 0.1 ml of PBS, a dose that is tumorigenic in 100% of syngeneic animals (30). The animals were then randomly divided into two groups and either fed with pH 9.5 alkaline water, or with regular tap water. The mice were then regularly palpated to assess tumour latency. Tumour growth was recorded measuring nodule size with a calliper three times a week; when a nodule reached the size of 250 mm^3, all the animals were sacrificed and tumours were excised, measured, weighed and formalin fixed for further analyses.

Istology and immunohistochemical analysis

For histology and immunohistochemistry analysis, samples were fixed in 4% neutral buffered formalin, embedded in paraffin and cut to obtain 3-4μm thick sections. Slides were then either stained with haematoxylin and eosin (H&E) for pathological analysis or processed for immunohistochemistry. All the evaluations were done in blind and scored independently by two investigators. Samples were examined for the presence of low or high grade PIN, well-differentiated adenocarcinoma (AK), phyllodes-like tumour (PHY), and neuroendocrine tumour (NE) (18, 29).

Immunohistochemistry was performed using rabbit monoclonal anti-Ki67 or rabbit polyclonal anti-CD3 (Abcam, Cambridge, UK). Primary antibodies were diluted 1:150 and 1:100 respectively in PBS containing 0,1%Tween20 and 1%BSA, and incubated for 1 hour at RT. After washing the slides were incubated with a biotinilated anti-rabbit secondary antibody (Pierce, Thermo Fisher Scientific, Waltham, MA, USA) followed by peroxidase-conjugated streptavidin (BioSPA Biochemical, Milano, Italy). Samples were then stained using the Vectastain DAB Kit (Vector Laboratories, Burlingame, CA USA). To quantify proliferation, 5 randomly selected fields on each H&E-stained sections, from four different animals, were blindly photographed, with an oil-immersion 100x objective. Positive nuclei were counted and expressed as percentage of the total

nuclei present in each field.

Statistical analysis

All statistical analyses were done with the IBM SPSS Statistic Software version 20 or 2x2 contingency table calculator (http://vassarstats.net/tab2x2.html).

RESULTS

Alkaline water administration delays prostate adenocarcinoma progression in TRAMP mice

To evaluate toxicity of the treatment, we initially administered alkaline water at pH 10.5 to 5 C57/Bl6 male mice for two months, and observed no signs of toxicity. Similarly, we did not register any significant difference in body weight when treated mice were compared with control mice receiving tap water (data not shown).

Afterward, we started to assess the therapeutic effect of alkaline water administration. Water consumption varied among the groups, with the $NaHCO_3$-treated animals that doubled their intake (13.2 ± 1.47 ml/day/animal), compared to control group (7.25 ± 0.43 ml/day/animal). Conversely, the water intake for both the "prevention" and "therapy" groups was slightly lower (5.8 ± 1.12 ml/day/animal and 5.2 ± 0.66, respectively).

At the established experimental end point (32 weeks of age), we sacrificed and thoroughly necropsied all animals. We found no animals with evidence of oedema or abnormal organ size, in target and non-target organs, however we observed hydronephroses in 11 out of the 29 (38%) animals fed with $NaHCO_3$.

The animals in the "prevention" group displayed a significant (P=0.017) decrease in the UG/bw ratio with respect to the animals in the "control" group, while no difference with the "control" group was found in the animals belonging to the "therapy" group (P=0.26); the mice in the

"bicarbonate" group showed a sharp decrease in the UG/bw ratio with respect to controls (P=0.001). (Fig. 1).

All collected tumours were then examined for both histological and immunohistochemical analyses. Tissue sections, stained with H&E, were evaluated in a blind assay by two different researchers. In order to evaluate the progression of the disease we recorded the different type of lesions found on each slide (Fig. 2).

Overall tumour incidence was 100% in all groups but comparison between "control" and "prevention" groups demonstrated that our treatment produced a decrease in the incidence of high grade PIN from 83 to 29,5% (P=0.001), and a corresponding increase in the incidence of low grade PIN from 4,17 to 36,4% (P=0.001) (Fig. 3a). Also the administration of alkaline water with the "therapy" scheme affected PIN progression, and albeit statistical significance was not achieved, there was a clear reduction in the high to low PIN ratio, as compared to the "control" group (Fig. 3b). The "bicarbonate" group, showed a significant decrease, with respect to "control" group, in the incidence of high grade PIN (from 83 to 52% P=0.020), and a corresponding increase in the incidence of low grade PIN (from 4,17 to 44% P=0.01).

As it regards AK incidence, we found with respect to "control" group a reduction of 12.8% in the "prevention" group, of 46.7% in the "therapy" group and of 61.6% in the "bicarbonate" group.

We detected no statistically significant difference in PHY or NE incidence among the groups.

Immunohistochemical analyses evidenced no changes in tumour cell proliferation, measured by Ki67 staining, as well as in the distribution and amount of tumour infiltrating T cells between treated group and controls (data not shown).

Alkaline water administration delays tumour growth in xenotransplants of an androgen independent prostate cancer cell line

C57/Bl6 wild type male mice injected s.c. with TRAMP C1 syngeneic cells, were randomly divided into two groups that received either tap water or alkaline water at 9.5 pH, and tumour growth was

monitored (Fig. 4). The growth of tumour nodules in treated mice was delayed respect to control mice. The delay was statistically significant till day 14 from cell injection. Histology on tumours collected from the mice at the end of experiment, showed no differences in the histological types between the two groups (data not shown). Also, immunohistochemistry did not show any difference in tumour cell proliferation and tumour infiltration by T cells between the two groups (data not shown).

DISCUSSION

The effect of alkaline water treatment confirms that the alkalinisation of the microenvironment has a prophylactic effect on prostate cancer progression, as already observed by Ibrahim-Hashim (26) and co-authors with $NaHCO_3$ using a small number of animals. We have followed this therapeutic strategy in a much larger cohort of mice and also using a different alkalinizing agent. We have found that oral administration of alkaline water or $NaHCO_3$ to TRAMP mice, beginning at 4 weeks of age, are equal in inducing a delay in the progression of prostate adenocarcinoma, with reduction in the incidence over time of both high grade PIN and AK. Confirming previous results with $NaHCO_3$, both the alkalinizing agents we tested induced the growing tumours to retain a more differentiated low grade PIN for longer.

However, the preventive treatment with alkaline water has the advantage to be devoid of the long-term unwanted side effects of so a high dose of $NaHCO_3$. As a matter of fact, we have found hydronephrosis in a significant percentage of $NaHCO_3$-treated mice. In these animals an excess of water intake was also detected, suggesting possible long-term impact on blood pressure, and on cardiac and renal function. Instead, no tissue or organ impairment was detected in the two groups of mice treated with alkaline water. Indeed, the administration of high dose of $NaHCO_3$ regiment to humans would be unadvisable (26), making alkaline water a safer alternative.

The effect of the “therapy” scheme was less significant, however there was a clear trend toward a delay in PIN progression and a reduction in AK incidence. This conclusion is supported by the

results obtained with xenografts, a model of early tumour, which showed that alkaline water treatment delays tumour growth. These results are comparable to those obtained with $NaHCO_3$ treatments in a mouse model of mammary tumour (3). These findings are encouraging, since no therapeutic effect was detected when $NaHCO_3$ was administered to TRAMP mice with the same scheme (26).

It is of note that PHY tumours and NE tumours did not respond to the alkalinizing treatments. It remains to be elucidated whether these tumours do not rely on an acidic microenvironment for their growth, or if they have escape mechanisms that allow them to counter the effect of alkalinizing agents. However, the prostate tumours in humans are almost all adenocarcinomas, the NE tumour accounting for about 4% of the total (28) and the PHY tumours of the prostate being very rare (31), therefore alkaline water may be considered a promising low-cost therapeutic approach that should be taken into account for prophylaxis and, combined with standard chemotherapy, for treatment of human prostate cancer. The few clinical trials available to date suggest that the anti-acidic/alkalinizing approach may well represent an efficient way to implement the existing anticancer therapies (32, 33).

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Declaration of interest

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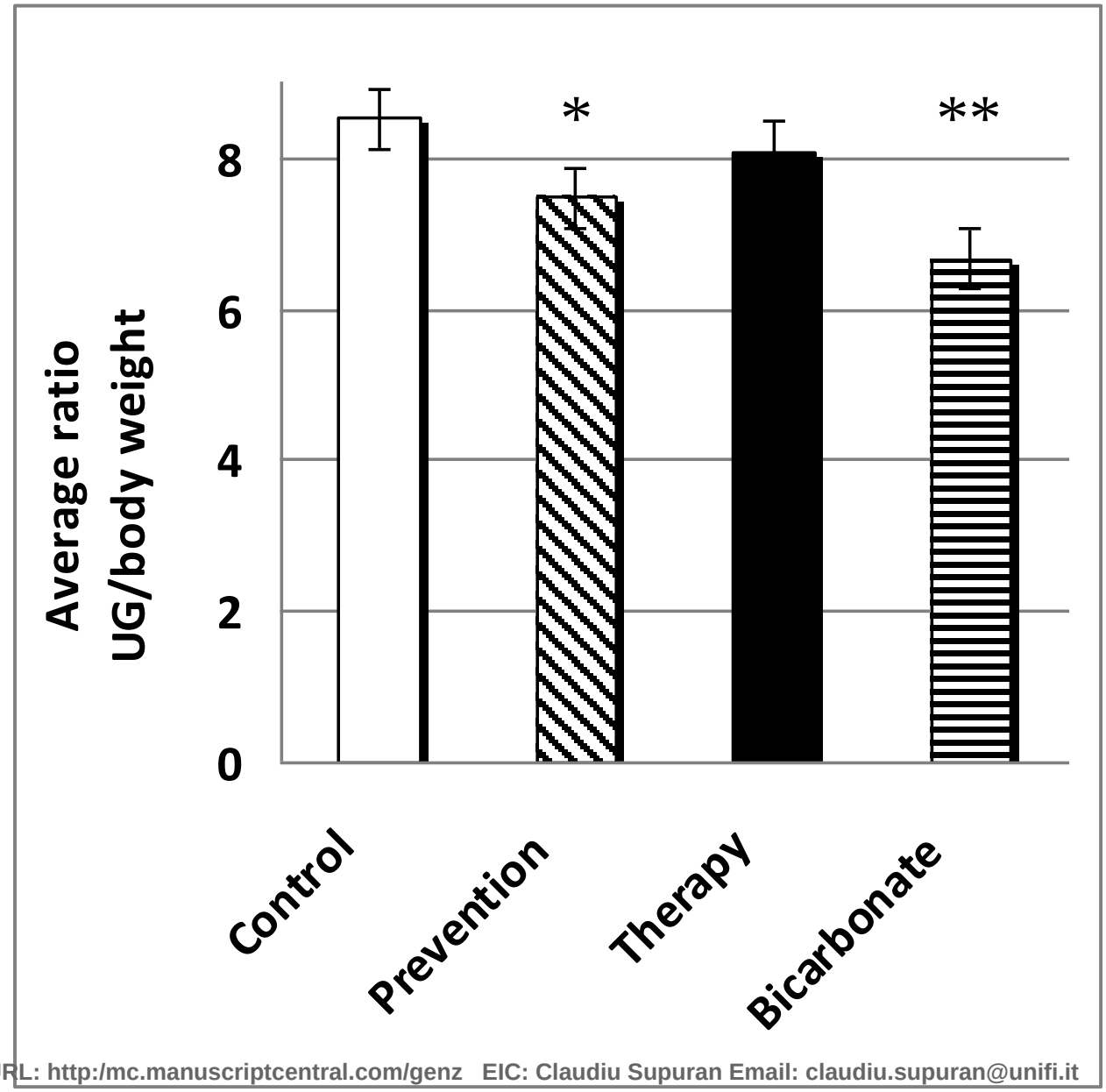
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UG/body weight
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Control
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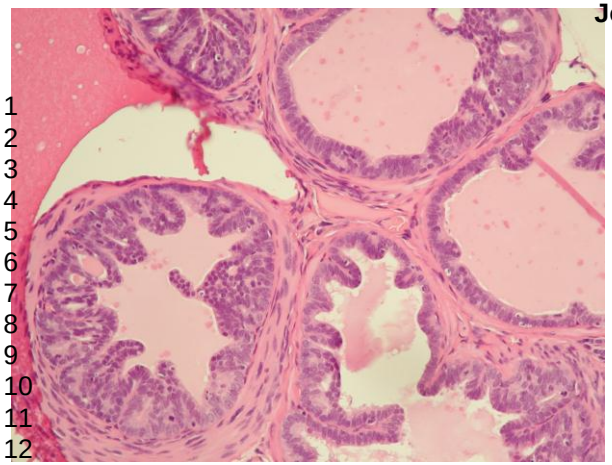
Therapy
Bicarbonate



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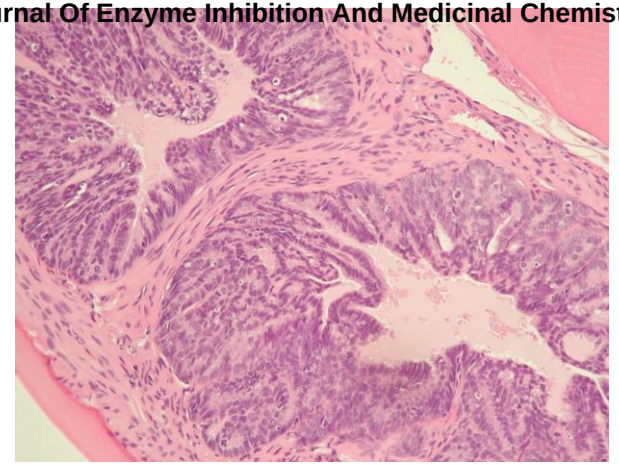

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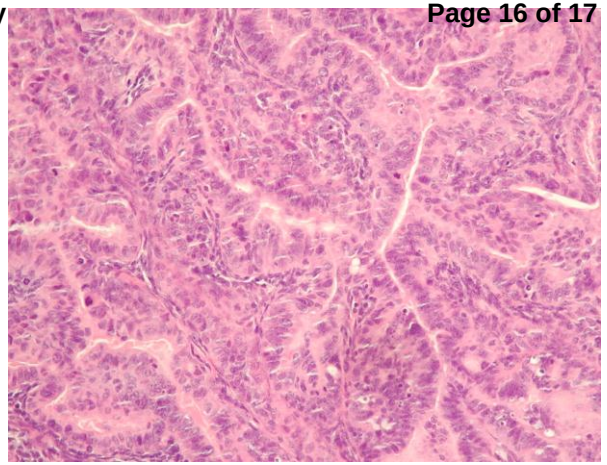
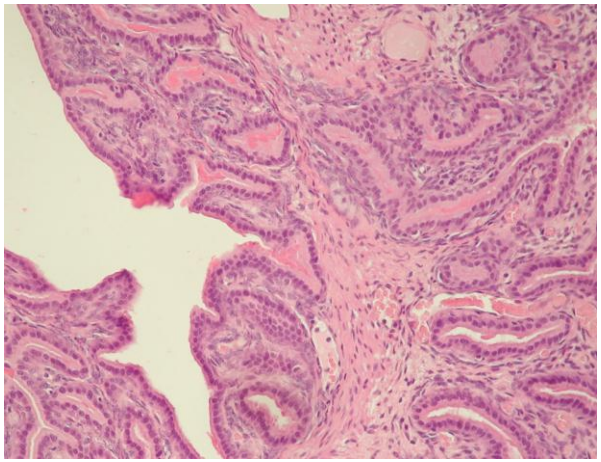
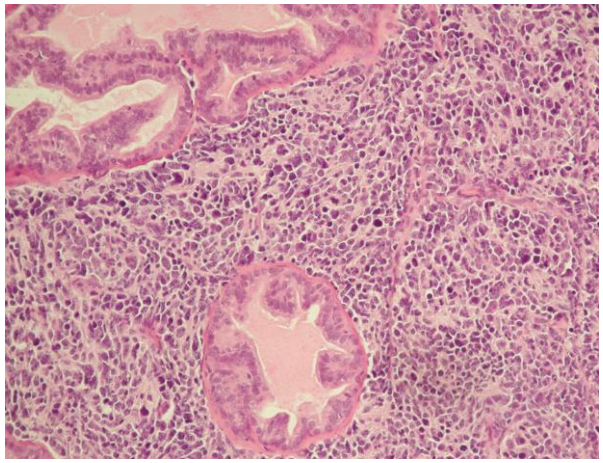


Adenocarcinoma

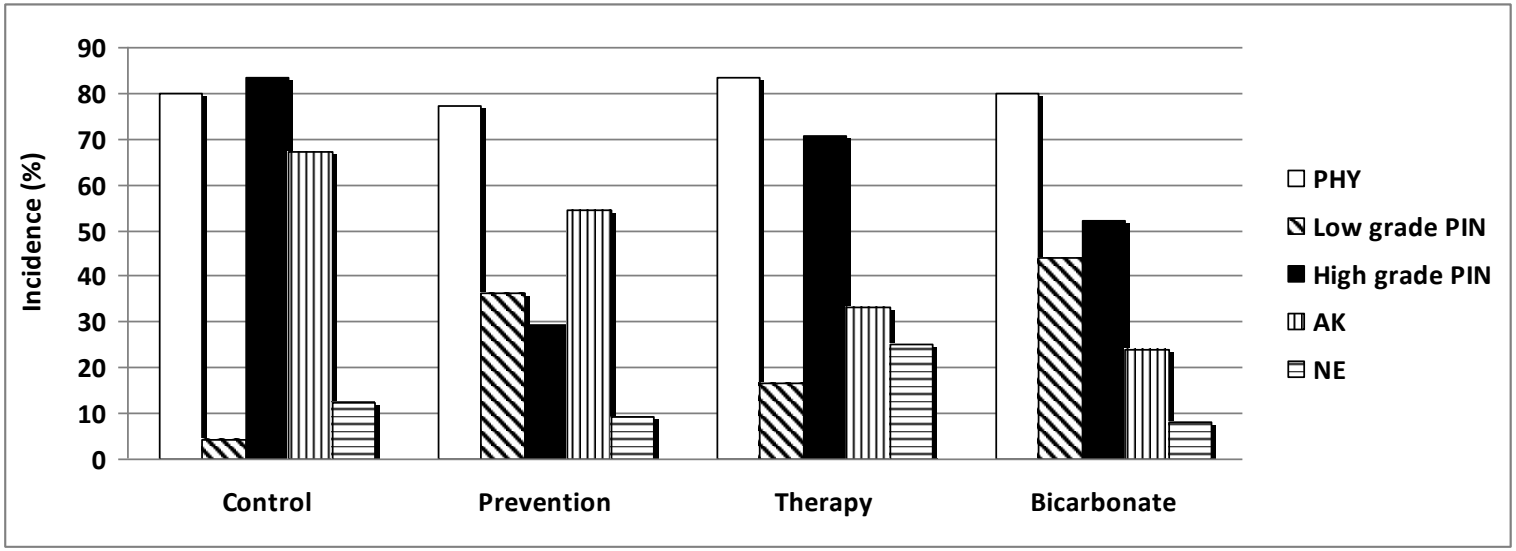


Phyllodes-like tumour

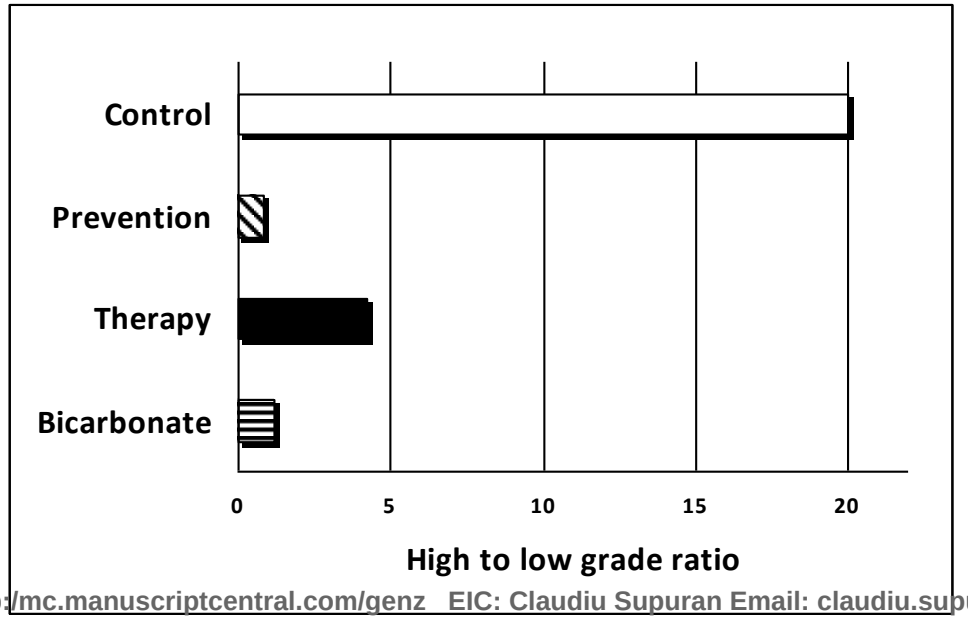


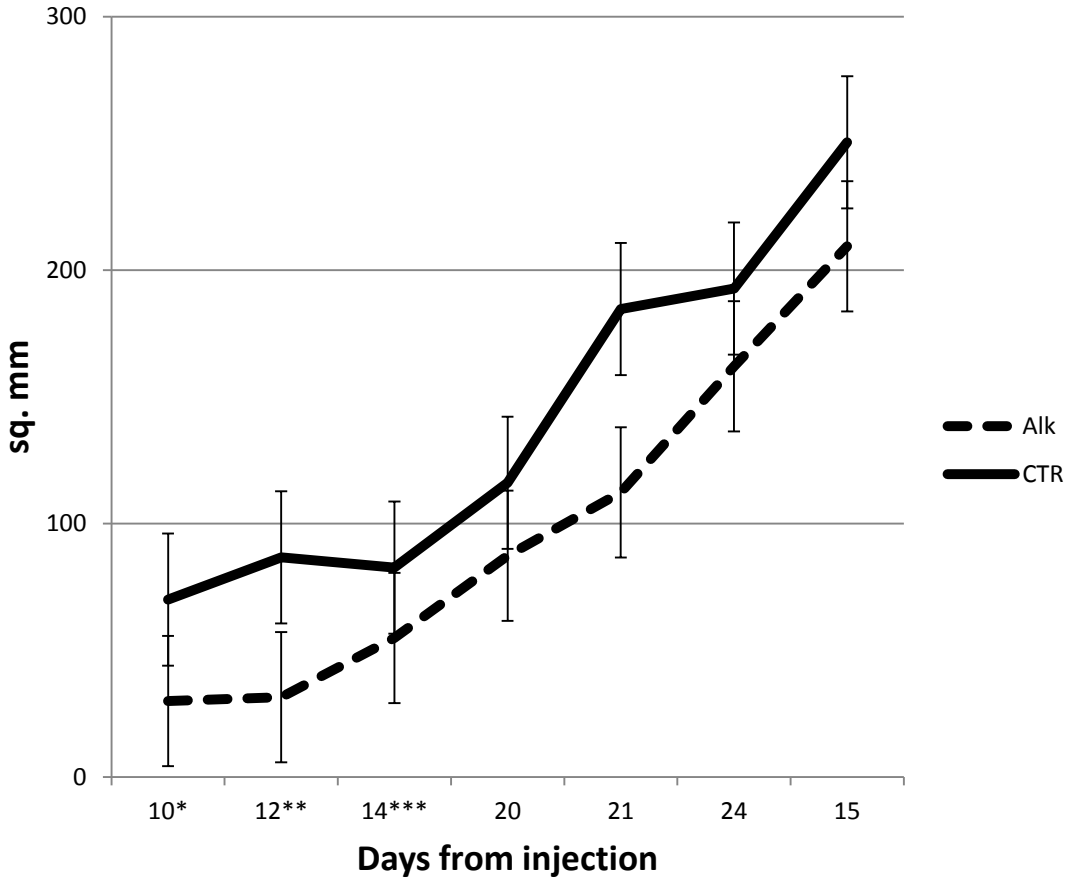
Neuroendocrine carcinoma

A


Incidence (%)
90
80
70
60
50
40
30
20
10
0
Control
Prevention
Therapy
Bicarbonate
PHY
Low grade PIN
High grade PIN
AK
NE

B


Control
Prevention
Therapy
Bicarbonate
0
5
10
15
20
High to low grade ratio


300
200
100
0
sq. mm
10*
12**
14***
20
21
24
15
Days from injection
Alk
CTR