

## Experimental research

# Evaluation of the potential benefits of alkaline drinking water on tumor development reveals vascular protective effects

Raquel García-Gómez<sup>1</sup>, Ignacio Prieto<sup>1</sup>, Sara Amor<sup>2</sup>, Gaurangkumar Patel<sup>1</sup>, María de la Fuente<sup>2</sup>, Miriam Granado<sup>2</sup>, María Monsalve<sup>1</sup>

<sup>1</sup>Instituto de Investigaciones Biomédicas Alberto Sols (CSIC-UAM), Madrid, Spain

<sup>2</sup>Faculty of Medicine, Universidad Autónoma de Madrid (UAM), Madrid, Spain

**Submitted:** 14 February 2021

**Accepted:** 1 June 2021

Arch Med Sci Civil Dis 2021; 6: e84–e102

DOI: <https://doi.org/10.5114/amscd.2021.109241>

Copyright © 2021 Termedia & Banach

**Corresponding author:**

María Monsalve PhD  
Instituto de Investigaciones  
Biomédicas Alberto  
Sols (CSIC-UAM)  
Madrid, Spain  
E-mail: [mpmonsalve@iib.uam.es](mailto:mpmonsalve@iib.uam.es)

## Abstract

**Introduction:** The proposed benefits of alkaline water generally lack experimental supporting evidence.

**Material and methods:** We tested the effects of alkaline water on tumor development in C57BL/6 mice. To recapitulate early tumor development, mice were fed a high-fat diet and treated with DEN (50 µg/l) and TCPOBOP (0.5 µg/g) for 24 weeks.

**Results:** Cell proliferation was significantly lower in animals receiving alkaline water, and VEGFR2 staining was higher in the vasculature, suggesting less advanced disease stages. B16-V5 cells were injected subcutaneously or through the tail vein to generate primary tumors or lung metastatic nodules, respectively. Subcutaneous tumors of mice exposed to alkaline water showed a lower proliferative index and vasculature with a better-preserved intima layer and structure. The number of lung metastatic nodules was lower in mice exposed to filtered water. The vascular effects were tested in a rat model of hypertension (spontaneously hypertensive rat – SHR). Following 12 weeks of alkaline water consumption, the aortic rings had an enhanced vasodilatory response to a nitric oxide donor (nitroprusside – NTP), and several inflammatory markers were reduced in blood and heart tissue.

**Conclusions:** These results indicate that alkaline water could have a protective effect on vascular function, reduce inflammation, and in the context of tumor development, reduce the incidence of metastasis.

**Key words:** water, filter, tumor, vasculature.

## Introduction

The chemical composition of drinking water is dependent on many factors such as the season [1], the relative volume of rainwater fallen [2], the underlying regional soil [3], and the water treatment processes [4], among others. Indeed, the chemical composition of samples taken from the same place can vary from one year to the next and, consequently, the evaluation of how regular consumption of any given tap drinking water can affect our health is a complex, challenging and even daunting task. The increasing and highly variable presence of pollutant by-products of human activity such as metals [5], microplastics [6], other persistent organic compounds and xenobiotics in general [7], further complicates the general picture.

Nevertheless, it is evident that the quality of water, like the quality of food, plays a key role in human health [8], and a growing concern over the quality of drinking water has fueled the consumption of bottled water [9]. Initiatives and regulations aiming for a reduction in the use of plastic [10] along with ample evidence indicating the presence of potentially toxic plastic derived compounds in bottled water [11] have, in turn, boosted the interest in safer alternatives, mainly the use of carbon filter systems, that have been shown to significantly reduce the concentration of several potentially hazardous chemicals in the water [10], and alternative Tetra Pak packaging, although the use in Tetra Pak of polyethylene also poses potential health risks [12]. By the same token, the chemical composition of bottled and filtered water varies widely, and the adequate evaluation of the impact of any given water composition on human health is difficult. Thus, whereas a large number of studies have evaluated how different nutrients or nutritional regimes impact our health [13], studies on water are comparatively few and generally very limited in scope.

Water filtering systems for use in the home (point-of-use water filters) can be grouped into four basic types: those that remove solid particles, including bacteria, but do not affect water chemistry [14]; those that decrease the total concentration of ions using reverse osmosis, which are used widely in coastal regions [15]; those that are based on activated charcoal, which are the most commonly used in western countries and are particularly effective in removing  $\text{Cl}^-$  and organic volatile compounds, but do not remove inorganic salts [16]; and systems that aim to “correct” the unnaturally low pH commonly found in sanitized tap water and are relatively new additions to the market. They increase the pH of the water either by filtering or by electrolyzing water.

Most studies on the potential health benefits of alkaline water have focused on gastrointestinal disorders; in particular, it has been recommended for patients with gastric acidosis [17], and accumulated evidence has led to the approval of alkaline electrolyzed water apparatuses as medical devices in Japan. Less scientific attention has been paid to other claimed potential benefits for users in the absence of abdominal complaints, derived from an expected improvement in whole-body redox balance. In particular, the potential of alkaline water to impact tumor development has been widely debated but few meaningful scientific studies have been conducted on animal models or humans. A 2016 systematic review by Fenton and Huang [18] found “...a lack of evidence for or against diet acid load and/or alkaline water for the initiation or treatment of cancer”. A more

recent study reported that alkaline water could inhibit the growth and proliferation of a cultured breast cancer cell line [19].

In the present study, we aimed to determine the impact of the daily intake of filtered alkaline water (Alkanatur) versus tap water, from the city of Madrid, on tumor development in mice. Three different studies were conducted: melanoma B16-V5 cells were injected subcutaneously or through the tail vein to generate primary tumors or lung metastatic nodules, respectively. Also, to evaluate the impact of alkaline water on a model that recapitulates the early stages of tumor development, non-alcoholic steatohepatitis (NASH) was induced in mice using a combination of high-fat diet and exposure to two tumor promoting agents. The results indicate that Alkanatur alkaline water could have a relevant effect on tumor development over tap water, in particular on cell proliferation and on the preservation of the vascular structure and function, which might have a positive impact on the incidence of metastasis.

## Material and methods

### Mice

Male C57BL/6 mice were used in this study. The animals were bred and housed at the Animal Facility of the Instituto de Investigaciones Biológicas Alberto Sols (CSIC-UAM). Animal experimental protocols were approved by the Institutional Animal Care and Use Committee of the IIB, the CSIC and the Consejería de Medio Ambiente de la Comunidad de Madrid (PROEX 317/15). All procedures conformed to the Declaration of Helsinki. All animals received humane care according to the criteria outlined in the “Guide for the Care and Use of Laboratory Animals” prepared by the National Academy of Sciences and published by the National Institutes of Health (No. 86-23 revised 1985).

Following weaning, the animals were split into two experimental groups: one group was given free access to tap water (non-filtered, non-sterilized) available at the institute, and a second group was given the same water that had been previously filtered using the Alkanatur alkaline ionized water filter system. Water was changed daily until the animals were sacrificed. At 12 weeks of age, the animals were exposed to the tumor development protocols described below. Each experimental group included 8 animals in order to guarantee at least 6 per group for statistical analysis.

### Rats

Male Wistar Kyoto (WYK) and spontaneously hypertensive (SHR) rats were used in this study. The animals were bred and housed at the Animal Facility of the UAM Faculty of Medicine. Animal ex-

perimental protocols were approved by the Institutional Animal Care and Use Committee and the Consejería de Medio Ambiente de la Comunidad de Madrid (PROEX 221/19). All procedures conformed to the Declaration of Helsinki. All animals received humane care according to the criteria outlined in the "Guide for the Care and Use of Laboratory Animals" prepared by the National Academy of Sciences and published by the National Institutes of Health (No. 86-23 revised 1985).

Following weaning, the 6-week-old rats were split into two experimental groups: one group was given free access to tap water (non-filtered, non-sterilized) available at the institute, and a second group was given the same water that had been previously filtered using the Alkanatur alkaline ionized water filter system. Water was changed daily until the animals were sacrificed, at 12 weeks of treatment. Each experimental group included 6 animals. Weight gain and systolic blood pressure were monitored every 2 weeks.

Mean arterial blood pressure (MBP) measurements were performed every 2 weeks by tail-cuff plethysmography using a Niprem 645 blood pressure system (Cibertec, Madrid, Spain). For that purpose, rats were placed in a quiet area ( $22 \pm 2^\circ\text{C}$ ) and habituated to the experimental conditions. Before measurements, rats were prewarmed to  $34^\circ\text{C}$  for 10–15 min. Then, the occlusion cuff was placed at the base of the tail and the sensor cuff was placed next to the occlusion cuff. Next, the occlusion cuff was inflated to 250 mm Hg and deflated over 20 s. Five to six measurements were recorded in each mouse and the mean of all measurements was calculated each day per animal.

The rats were sacrificed by decapitation after an intraperitoneal injection of sodium pentobarbital (100 mg/kg). Blood was collected and plasma and PBMCs were separated using a Ficoll gradient.

#### Experimental primary tumor growth

A total of  $0.5 \times 10^6$  mouse melanoma B15-V5 cells were injected subcutaneously into the back of 12-week-old male mice, and 12 days later they were euthanized by cervical dislocation and tumors were dissected and fixed in 4% paraformaldehyde for histological analysis. Tumor volume was measured using calipers and calculated from the formula  $4/3 \times \pi \times \text{width} \times \text{length} \times \text{height}$ .

#### Experimental metastasis assay

A total of  $0.5 \times 10^6$  B15-V5 cells were injected into the lateral tail vein of 12-week-old male mice, and 12 days later mice were euthanized and lungs were dissected and fixed with Bouin's Solution (Sigma-Aldrich, St Louis, MO) for histological analysis.

#### Cell culture

B16-V5 murine melanoma cells originally provided by Dr. MS Soengas (CNIO, Madrid) were cultured in DMEM with 10% FBS, 2 mM glutamine and antibiotics.

#### Induction of hepatocarcinoma

12-week-old male mice were fed a high-fat diet (HFD) (TD88137; Harlan, Barcelona, Spain) to induce liver steatosis. The animals were simultaneously treated with two carcinogens: 1,4-bis-[2-(3,5-dichloropyridyloxy)] benzene (TCPOBOP), which was injected intraperitoneally (ip) at a dose of  $0.5 \mu\text{g/g}$  once per week; and diethylnitrosamine (DEN), which was administered at a dose of  $50 \mu\text{g/l}$  in the drinking water. The animals were euthanized at 36 weeks of age and livers were dissected and fixed with Bouin's Solution (Sigma-Aldrich) for histological analysis.

#### Vascular reactivity

After sacrifice, aortas were collected and cut into 2 mm segments. Each segment was prepared for isometric tension recording in a 4-ml organ bath as previously described [20].

In brief, the organ bath contained modified Krebs-Henseleit solution (KHB) at  $37^\circ\text{C}$  (mM): NaCl 115; KCl 4.6;  $\text{KH}_2\text{PO}_4$  1.2;  $\text{MgSO}_4$  1.2;  $\text{CaCl}_2$  2.5;  $\text{NaHCO}_3$  25; glucose 11. The solution was equilibrated with 95% oxygen and 5% carbon dioxide to a pH of 7.3–7.4. Two fine (100  $\mu\text{m}$  diameter) steel wires were passed through the lumen of the vascular segment; one wire was fixed to the organ bath wall and the other was connected to a strain gauge for isometric tension recording (Universal Transducing Cell UC3 and Statham Microscale Accessory UL5; Statham Instruments, Inc, Oxnard, CA, USA). This arrangement enables the application of passive tension in a plane perpendicular to the long axis of the vascular cylinder. The changes in isometric force were recorded using a Power Lab data acquisition system (AD Instruments). An optimal passive tension of 1 g was applied to the vascular segments and then they were allowed to equilibrate for 60–90 min. Before beginning the experiment, the vascular segments were stimulated with potassium chloride (100 mM) to determine the contractility of smooth muscle. Any segments which failed to contract at least 0.5 g were discarded. The segments were then washed with fresh modified KHB solution and allowed to stabilize.

Once stabilized, the response to cumulative doses of the vasoconstricting agents ET-1 ( $10^{-10}$  to  $10^{-7}$  M) (Sigma-Aldrich) and Ang-II ( $10^{-11}$  to  $10^{-6}$  M) (Sigma-Aldrich) was recorded. Contraction response was evaluated as the percentage of the

contraction produced in response to 100 mM KCl (maximum response).

To evaluate the response to vasorelaxing agents, the stabilized segments were pre-contracted with U46619 ( $10^{-8}$  M to  $10^{-6}$  M), a stable analogue of thromboxane A<sub>2</sub> (Sigma-Aldrich), and when the contraction reached a stable level, the response to cumulative doses of ACh ( $10^{-9}$  to  $10^{-4}$  M) (Sigma-Aldrich), and NTP ( $10^{-9}$  to  $10^{-5}$  M) (Sigma-Aldrich) was monitored. The relaxation response was evaluated as the percentage of the active tone measured upon exposure to  $10^{-5}$  M NTP (maximum response).

### Hematoxylin-eosin staining

Tissue sections were fixed in 10% buffered formalin, embedded in paraffin, and 4- $\mu$ m sections were cut, de-waxed and hydrated. Sections were stained for 3 min with Harris' hematoxylin and 2 min with eosin (H&E). Finally, the slides were dehydrated and mounted with DPx Mountant (Sigma-Aldrich). Images were acquired with a Nikon E90i microscope equipped with a DS-Fi1 camera (Nikon, Tokyo, Japan) and analyzed using ImageJ software (NIH).

### Immunohistochemistry

Fixation and staining were performed using the Vectastain ABC Kit and the Peroxidase Substrate Kit DAB (both from Vector Laboratories, Burlingame, CA), following the manufacturer's instructions. Samples were incubated with anti-Ki67 (RM-9106-S1; Thermo Scientific, Wilmington, DE) or anti-F4/80 (MCA497; AbD Serotec, Oxford, UK) primary antibodies and then with the corresponding secondary antibodies linked to alkaline phosphatase, and finally developed. Images were acquired and analyzed as for H&E staining.

### Immunofluorescence

Fixation, staining and analysis procedures were as previously described [21]. Antibodies were smooth muscle  $\alpha$ -actin (SMA)-Cy3 (Sigma-Aldrich) and vascular endothelial growth factor receptor 2 (VEGFR2) (Cell Signaling Technology, Danvers, MA). VEGFR2 immunofluorescence was detected by incubation with a fluorescent secondary antibody (FITC, Sigma-Aldrich). Samples were then stained with DAPI (Invitrogen Corp., Carlsbad, CA), mounted and visualized using a Nikon A1R confocal microscope or a Zeiss LSM 700 and analyzed with ImageJ to determine the positive area versus total tissue area.

### Protein extraction and western blotting (WB)

Whole cell extracts were prepared from heart as previously described [22]. Proteins were separated using 10–12% SDS-PAGE gels and trans-

ferred to PVDF Amersham Hybond-P membranes (GE Healthcare) by semidry transference using the TransBlot SD cell system (Bio-Rad). The specific antibodies used were: 4-Hydroxy-2-nonenal (HNE) Antibody (Alpha Diagnostic Int.), iNOS (PA5-16524, Invitrogen), IL-1 $\beta$  (H-153, Santa Cruz Biotechnology Inc.). Quantitation of Red Ponceau staining of the total protein transferred was used as the loading control. ImageJ software was used to analyze western blots.

### Gene expression analysis

Heart tissue samples were homogenized in the presence of 1 ml of Trizol reagent and total RNA was isolated following the manufacturer's instructions. cDNA was synthesized from total RNA preparations by reverse transcription of 1  $\mu$ g of RNA using the MMV reverse transcriptase, in a final volume of 20  $\mu$ l. The mixture was incubated at 37°C for 45 min at then cooled for 2 min at 4°C. The resulting cDNA was used as a template for subsequent qPCR. The primers used are listed below. Each 10  $\mu$ l of PCR reaction mixture included 1  $\mu$ l of cDNA, 5  $\mu$ l of Mastermix qPCR (Cultek, Dutscher Group) and primers (0.3  $\mu$ M). Samples were run in triplicate in a Mastercycler RealPlex<sup>2</sup>, Eppendorf).  $\beta$ -actin was used as a loading control. TNF- $\alpha$  Forward 5'-ATGGGCTCCCTCATCAGT-3' TNF- $\alpha$  Reverse 5'-CAAGGGCTCTTGATGGCAGA-3' TGF- $\beta$  Forward 5'-CTGTACGCTGTCAGGCTCTC-3' TGF- $\beta$  Reverse 5'-CCAGGTGGAAGTTCTGCGAT-3' iNOS Forward 5'-TGCACAGAATGTTCCAGAATCCC-3' iNOS Reverse 5'-TTGGACTTGCAAGAGATATCCG-3' IL-1 $\beta$  Forward 5'-GCCAACAAGTGGTATTCTCCATGAGC-3' IL-1 $\beta$  Reverse 5'-TTGTCACCCCGGATGGAATG-3' IL-10 Reverse 5'-TTGTCACCCCGGATGGAATG-3' IL-10 Forward 5'-GCTCAGCACTGCTATGTTGC-3' Arg-1 Reverse 5'-GTAGCCGGGGTGAATACTGG-3' Arg-1 Forward 5'-GGACATCGTGATACATCGGCT-3' IL-6 Reverse 5'-TGAAGTCTCTCTCCGGACTT-3' IL-6 Forward 5'-GAGACTTCCAGCCAGTTGCC-3' IL-4 Reverse 5'-TCATTACGGTGCAGCTTCT-3' IL-4 Forward 5'-TCCACGGATGTAACGACAGC-3' IF- $\gamma$  Reverse 5'-ACACGTTCTGGTGCTTCCAA-3' IF- $\gamma$  Forward 5'-CGGGAGTGAGCTTTGATGA-3'

### ELISAs

Circulating levels of IL-6, IL-1 $\beta$  and TNF- $\alpha$  were analyzed in plasma samples using ELISA testing kits (RAB0480-1K, RAB0311-1KT, RAB0278-1KT from Merck) following the instructions of the manufacturer. Values were standardized to total plasma protein levels.

### Statistical analysis

Data are expressed as mean  $\pm$  SD. Statistical significance was evaluated using a two-tailed un-

paired *t* test. Values were considered statistically significant at  $p < 0.05$ .

## Results

### Non-alcoholic fatty liver disease

The first protocol aimed to evaluate the initial stages of tumor development. We chose a model for the development of hepatocellular carcinoma (HCC) induced by a combination of HFD and the administration of two carcinogens: TCPOBOP (0.5 µg/g) administered ip weekly and DEN (50 µg/l) added to the drinking water. This model shares characteristics with hepatocellular carcinoma in humans, which can progress from non-alcoholic liver disease (NAFLD). Importantly, the liver is also a central metabolic organ and, as such, is particularly sensitive to agents both beneficial and detrimental derived from the diet. The diet and carcinogens treatment started when the animals were 12 weeks old and the animals were sacrificed at 36 weeks of age. The animals were divided from weaning into two groups, one drinking tap and the other alkaline filtered water. The water was changed daily until the mice were sacrificed.

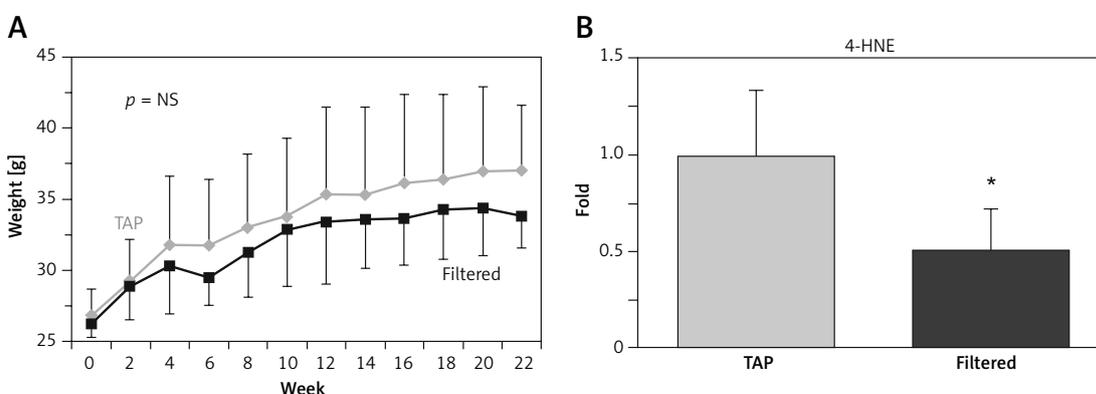
Follow-up of weight gain during treatment did not show significant differences among the groups (Figure 1 A). Initial macroscopic evaluation of the livers failed to show the presence of tumors in any of the mice (Figure 2 A, right panel). There were also no evident differences between the two groups in the color of the liver, which would suggest relevant differences in the level of steatosis (Figure 2 A, right panel). However, histological analysis of liver sections showed clear development of NAFLD in both groups, as revealed by H&E staining, with the presence of macro- and microsteatosis as well as destruction of the parenchyma that suggested the presence of advanced fibrosis and inflammation, both characteristics of NAFLD (Figure 2 A, left panel). Nota-

bly, this preliminary evaluation suggested a lower grade of steatosis and better preservation of the parenchyma in the mice exposed to filtered water (Figure 2 A, left panel).

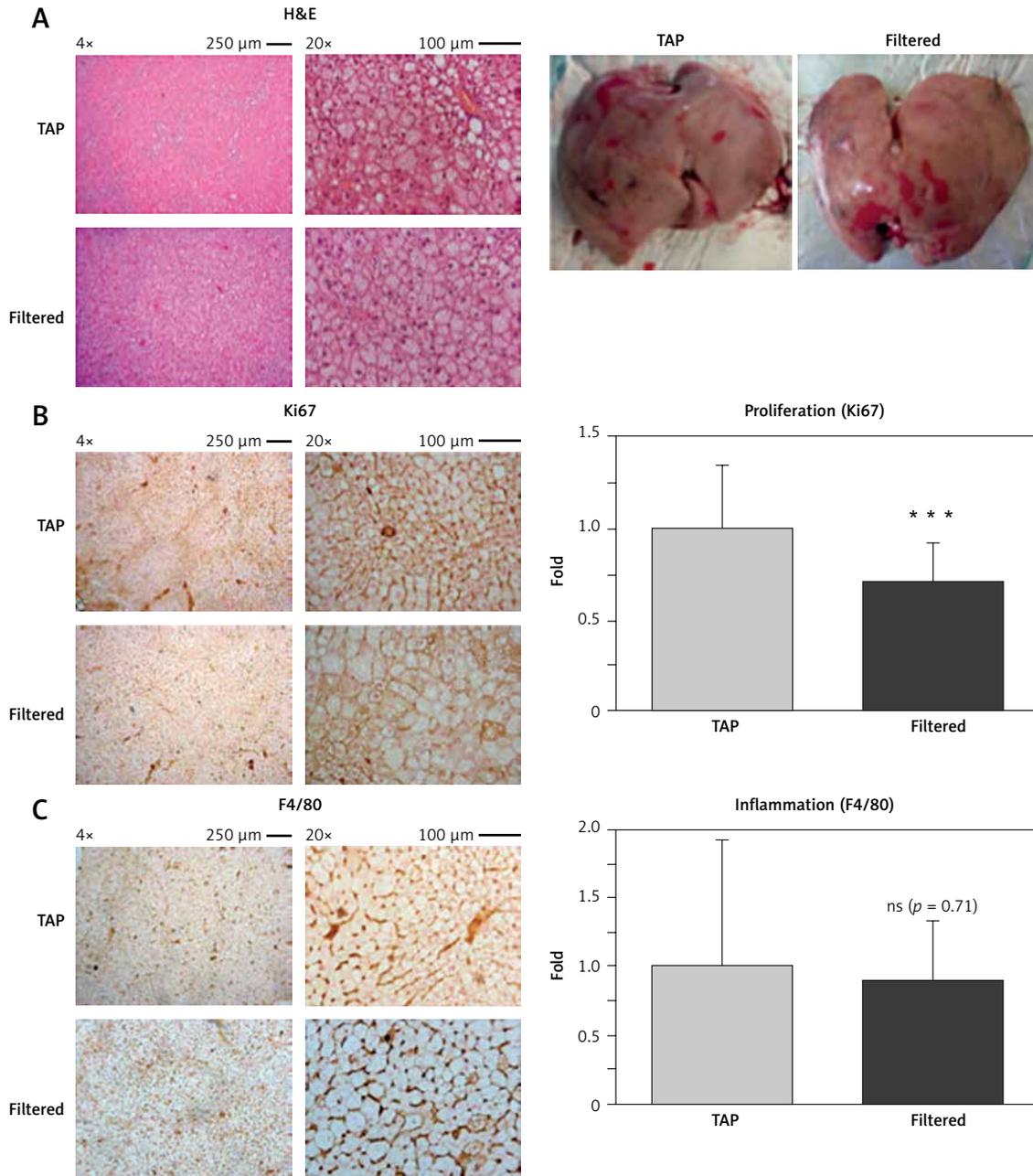
To measure the pathological impact of these differences, we determined the level of fibrosis by immunofluorescence using an antibody directed against αSMA, which labels myofibroblasts, and we also assessed the inflammatory status using an antibody directed against the protein F4/80, which labels macrophages and resident Kupffer cells. We found that mice treated with filtered water had, on average, lower levels of both αSMA (Figures 2 D–F) and F4/80 (Figure 2 C) staining, although the differences did not reach statistical significance. Inflammation is normally associated with high levels of oxidative stress and subsequent increase of modified proteins. We used as a surrogate marker of oxidative stress the evaluation of the levels of proteins modified by 4-HNE by WB and found that the levels of 4-HNE modification were significantly lower in mice exposed to filtered water (Figure 1 B).

Aiming to determine the relevance of these findings, we next analyzed liver cell proliferation rates using an antibody directed against the proliferation antigen Ki67. The total levels of Ki67-positive nuclei/total number of nuclei is a good indicator of the general level of proliferation and includes not only hepatocytes, which proliferate as part of a tissue regenerative response, but also myofibroblasts and activated immune cells (both resident and infiltrated). We found that the proliferation indices were significantly lower in the liver of mice exposed to filtered water than those administered tap water (Figure 2 B), suggesting that the pathology in the former is less advanced.

The entrance of nutrients and toxins into the liver parenchyma occurs through the vascular tree, and the pathological changes that characterize NAFLD are typically first evident in the liver vasculature; these impact the access and process-

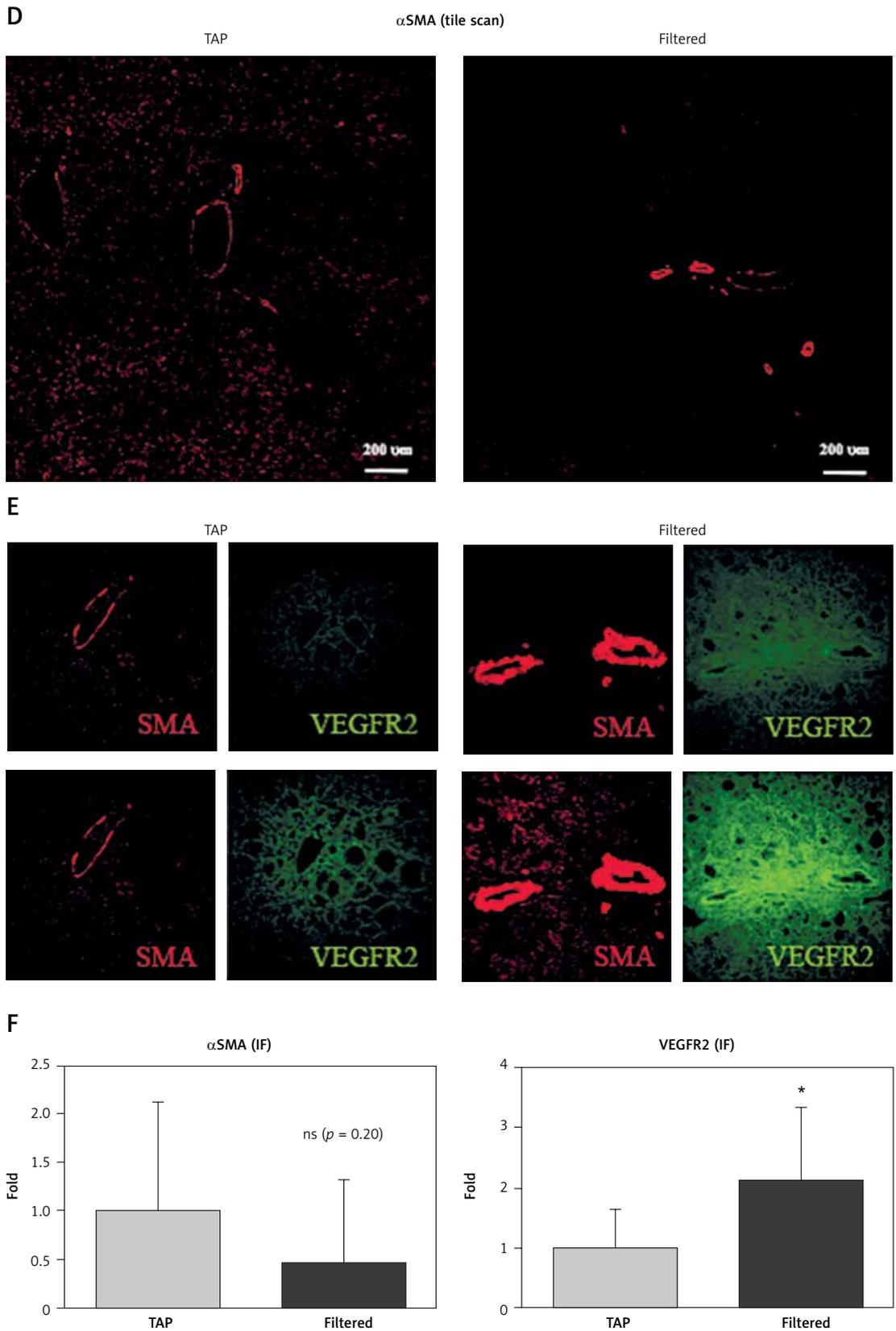


**Figure 1.** NAFLD mice treated with tap or filtered water. **A** – Weight gain over the 24-week period of diet and administration. **B** – WB analysis of 4-HNE levels in liver extracts. Red Ponceau staining was used as a loading control. Data are mean ± standard deviation. \* $p < 0.05$



**Figure 2.** Diet induced NAFLD in mice treated with tap or filtered water. **A** – Left, H&E staining of liver histological sections. Right panel. Whole-liver images. **B** – Left, IHQ Ki67 staining of liver histological sections. Right, quantitative analysis of Ki67<sup>+</sup> cells. **C** – Left, IHQ F4/80 staining of liver histological sections. Right, quantitative analysis of F4/80<sup>+</sup> cells

Data are mean ± standard deviation. \**p* < 0.05; \*\**p* ≤ 0.01; \*\*\**p* ≤ 0.005. ns – non-significant.



**Figure 2.** Cont. **D** – IF SMA staining of liver histological sections. Maximal projection images of tissue section tile scans, acquired with a confocal microscope. **E** – IF αSMA (red) and VEGF2 (green) staining of liver histological sections. **F** – Quantitative analysis of SMA (left) and VEGF2 (right) IF

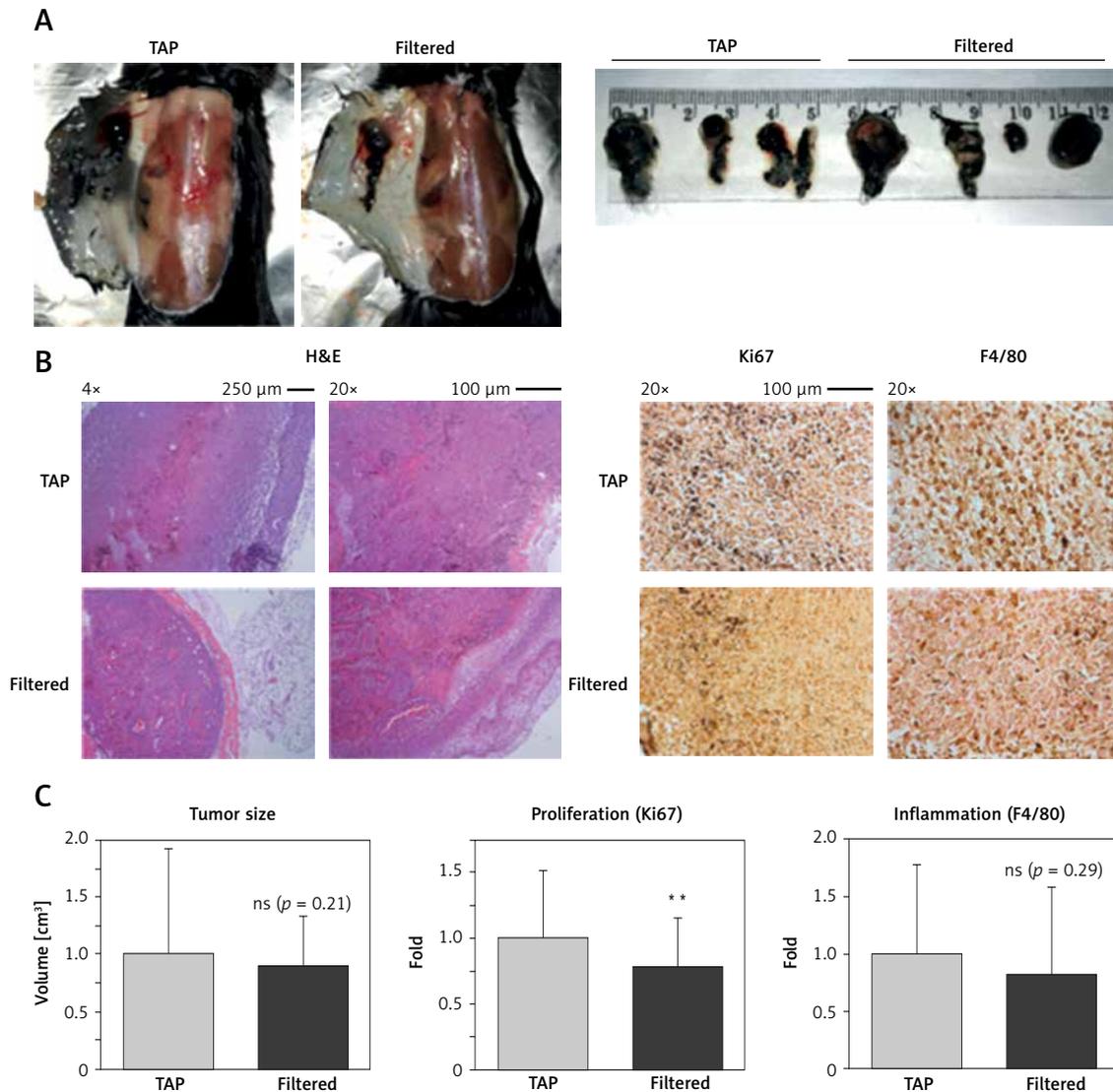
Data are mean ± standard deviation. \* $p < 0.05$ ; \*\* $p \leq 0.01$ ; \*\*\* $p \leq 0.005$ . ns – non-significant.

ing of nutrients by hepatocytes. Accordingly, we sought to evaluate vascular dysfunction in livers by immunofluorescence using an antibody directed against VEGFR2, a specific marker of vascular endothelial cells. VEGFR2 is the main receptor for the angiogenesis factor VEGF-A. The labeling of VEGFR2 was compared with that of  $\alpha$ SMA, which labels the vascular media layer (Figures 2 E, F). We found that compared with animals on filtered water, the liver vasculature of animals on tap water was more dilated, a characteristic of NAFLD, and had significantly lower levels of VEGFR2 immunoreactivity, suggesting the presence of vascular dysfunction (Figures 2 E, F), another feature that contributes to the development of the disease. Taken together, these results suggest that the animals exposed to alkaline water show less ad-

vanced NAFLD, which could have an impact on the development of HCC.

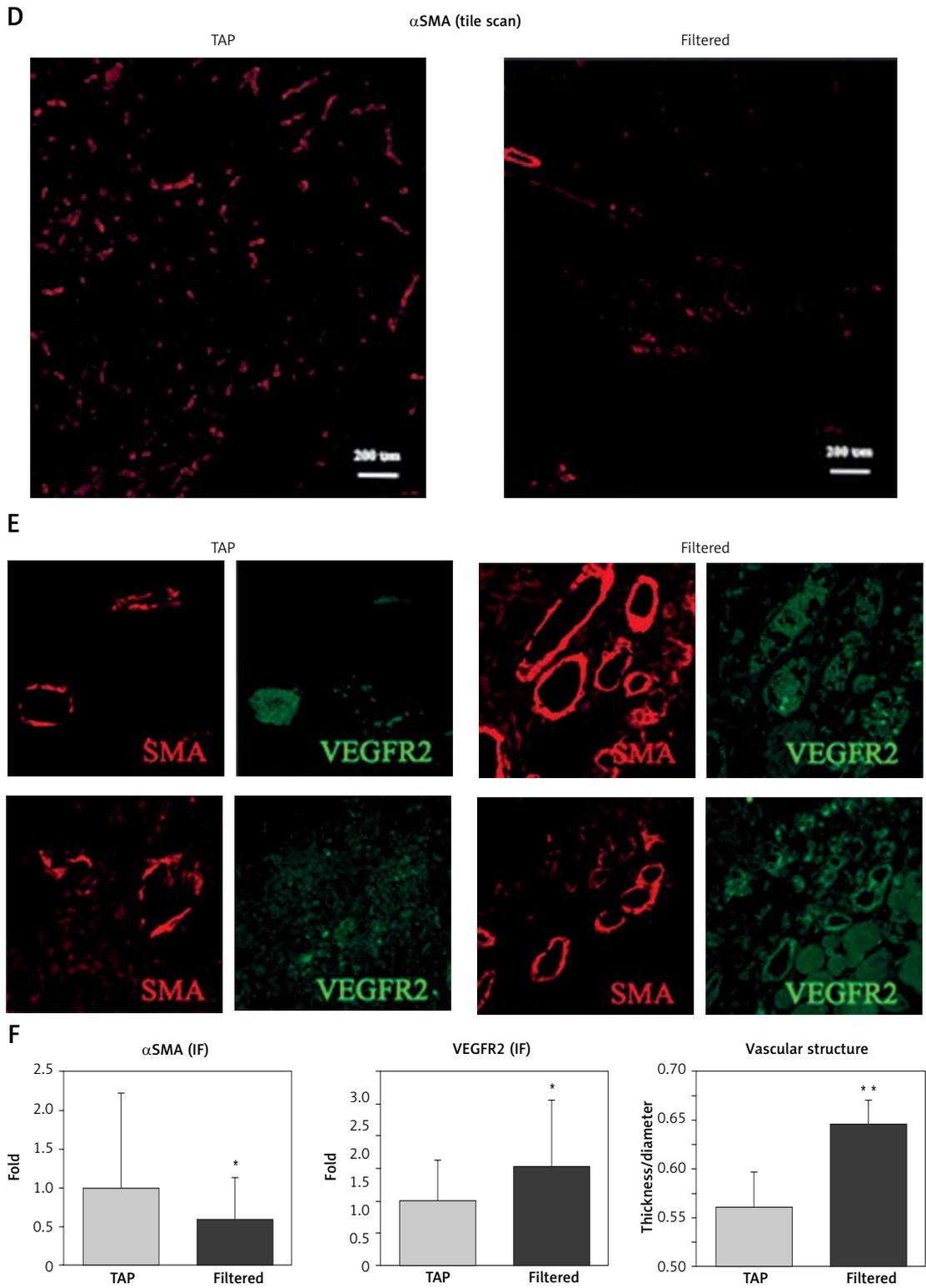
### Primary tumors

To further investigate whether the proliferative capacity of tumor cells *in vivo* was different between mice exposed to tap or filtered water from weaning, 12-week-old mice were subcutaneously injected with melanoma B16-V5 cells. These cells proliferate very rapidly *in vivo*, forming large primary tumors in less than 2 weeks. Twelve days after injection, the mice were sacrificed and the tumors were dissected and measured. No significant differences were found for tumor volume between the two groups ( $p = 0.21$ ), although there was non-significantly greater mean volume in the group with access to filtered water (Figures 3 A, C).



**Figure 3.** Primary tumors derived from B16-V5 injected subcutaneously in mice treated with tap or filtered water. **A** – Left, representative images of open mice with subcutaneous tumors before tissue resection. Right, whole tumors and scale. **B** – Left, H&E staining of tumor histological sections. Right, IHC Ki67 and F4/80 staining of tumor histological sections. **C** – Quantitative analysis of tumor size (left), Ki67+ cells (center) and F4/80+ cells (right)

Data are mean  $\pm$  standard deviation. \* $p < 0.05$ ; \*\* $p \leq 0.01$ ; \*\*\* $p \leq 0.005$ . ns – non-significant.



**Figure 3.** Cont. **D** – IF SMA staining of tumor histological sections. Maximal projection images of tissue section tile scans, acquired with a confocal microscope. **E** – IF  $\alpha$ SMA (red) and VEGF2 (green) staining of tumor histological sections. **F** – Quantitative analysis of SMA (left), VEGF2 (center), and vascular structure (right) as derived from IF images

Data are mean  $\pm$  standard deviation. \* $p < 0.05$ ; \*\* $p \leq 0.01$ ; \*\*\* $p \leq 0.005$ . ns – non-significant.

Qualitative analysis of histological tumor sections by H&E staining suggested that the tumors of mice exposed to filtered water were more encapsulated and were less hemorrhagic than those exposed to tap water (Figure 3 B), which might be consistent with a lower tendency to promote metastatic processes. To test that possibility, we first measured proliferation by Ki67 labeling. We noted that the tumors of mice in the filtered water group had a significantly lower grade of proliferation than those in the tap water group (Figures 3 B, C), which could suggest less aggressive tumors. Since tumor development is normally associated with an inflammatory process, we analyzed the level of inflammatory infiltrate using an antibody directed against F4/80. We failed to detect significant differences between the groups, although the mean values were lower in the group of mice treated with filtered water (Figures 3 B, C).

We next evaluated the level of angiogenesis in the tumors, a characteristic associated with tumor developmental stage and the likelihood of metastasis [23]. We examined the vascular media layer using an antibody to  $\alpha$ SMA, and analyzed the positive area per section. We found that the level of vascularization was significantly lower in mice treated with filtered water than with tap water (Figures 3 D–F). We then analyzed the quality of the vascular structures using the endothelial marker VEGFR2. We found that, consistent with the findings in the liver, VEGFR2 expression was significantly higher in the animals treated with filtered water, suggesting a better vascular structure (Figures 3 E, F). The reduced level of vascularization could be consistent with a larger tumor size and reduced risk of metastasis.

To more directly evaluate the degree of vascular frailty, which is strongly associated with higher risk of metastasis [24], we analyzed the structure of the tumor vasculature by measuring the largest and smallest diameters of the vessels, and used the ratio of smallest/average diameter as an index of vascular quality. We noted that the vascular structure was significantly more circular in the mice treated with filtered water (Figures 3 F), a characteristic reported to be associated with a lower risk of metastasis and an improved redox balance [25, 26].

### Metastatic lung nodules

Based on these findings, we next tested the capacity of B16-V5 melanoma cells to form metastatic lung nodules in mice. The animals were divided from weaning into two groups, one drinking tap and the other alkaline filtered water. The water was changed daily until the mice were sacrificed. At 12 weeks of age mice were injected intravenously with B16-V5 melanoma cells and animals

were sacrificed 12 days later, when lung nodules were already detectable in most mice. Following lung dissection, the lungs were extracted and the total number of nodules was counted. The results showed that the total number of nodules was significantly lower in animals on filtered water than in those on tap water (Figures 4 A, E), suggesting that mice treated with filtered water are more resistant to the formation of metastasis.

Structural analysis of the tissue sections using H&E staining (Figure 4 B) and evaluation of cell proliferation in the nodules as determined by Ki67 staining (Figure 4 C) did not show differences among the groups (Figure 4 B). Importantly, lungs from animals treated with filtered water also showed a lower grade of inflammatory infiltrate, as determined by staining with an F4/80 antibody (Figures 4 D, F).

Finally, we analyzed the vasculature in the lungs, since the presence of endothelial dysfunction is a key facilitating factor not only in the dissemination of cells from the primary tumor but also in the nesting of the circulating tumor cells [27]. We used antibodies directed against  $\alpha$ SMA and VEGFR2 for the analysis of the tissue samples. In accord with the previous findings in the liver and in the primary tumor model, we found no differences in the tissue level of fibrosis ( $\alpha$ SMA) and a significantly lower level of angiogenesis (evaluated as the area covered by SMA + VEGFR2) in animals treated with tap water (Figures 4 G–I), suggesting a lower level of tumor-associated angiogenesis in agreement with the lower number of metastatic nodules in the mice treated with filtered water. These findings are likely relevant for the migration of circulating tumor cells into tissues, and hence the formation of metastatic lung nodules.

In sum, all of these data are consistent with a model in which the consumption of filtered (Alkanatur) water better preserves vascular function in the tumors and hence the response of the organism to tumor developmental process, both primary and metastatic, and could also have an impact on the general development of NAFLD.

### Vascular function

In order to evaluate more directly the impact of the intake of alkaline water on the vasculature we decided to test its effect on a well-established model of hypertension, spontaneously hypertensive Wistar (SHR) rats and the normotensive normal Wistar rats (WKY). SHR and Wistar rats were exposed from weaning to tap water or filtered alkaline water for 12 weeks. During this period, the rats' weight and mean blood pressure were monitored every 2 weeks. We found that SHR drinking filtered water had gained significantly

more weight than those drinking tap water after 4–6 weeks of treatment, but these differences were no longer significant at 8 weeks of treatment. No significant differences in weight were found in WKY (Figure 5 A). Mean blood pressure increased from 4 to 12 weeks of treatment in both SHR and WKY rats, but no significant differences were found between rats drinking tap or filtered alkaline water (Figure 5 B).

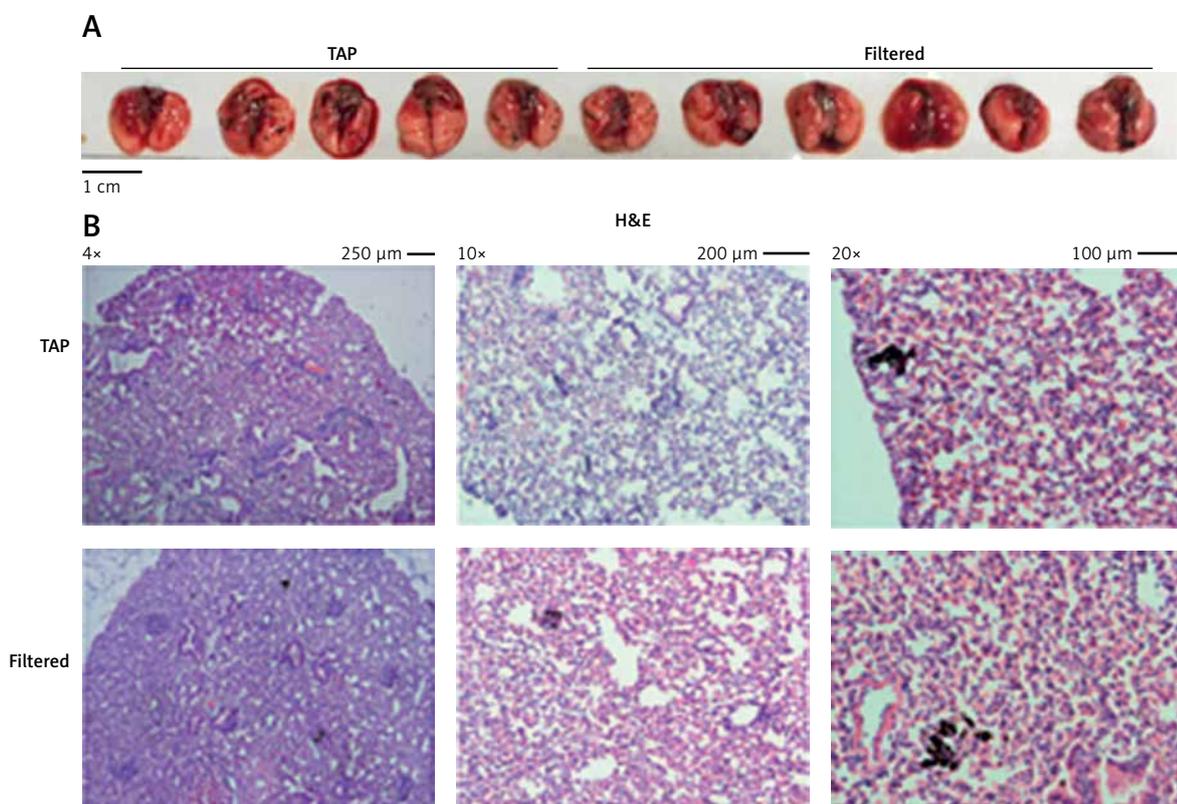
Then we evaluated vascular reactivity in aortic rings. We first tested the vasodilatory response to nitric oxide using increasing doses of the NO donor NTP in rings previously hypercontracted. Endothelium-independent relaxation in response to NTP elicited a more pronounced vasodilatory response in both WKY and SHR rats treated with filtered water, with the average differences reaching statistical significance at  $10^{-6}$  M NTP in SHR rats (Figure 6). The endothelium-dependent relaxation was determined subjecting the hypercontracted aortic segments to ACh dose-response curves, but no significant differences could be identified (Figure 7).

Enhancement in the response to NTP could also be associated with reduced contraction of smooth muscle cells in response to vasoconstrictors. So, we next tested in fully dilated aortic rings the response to AgtII (Figure 6). Lower contraction in response to

AgtII was noted for both WYK and SHR rats in the alkaline water group, but the differences did not reach statistical significance. Similar results were found for the response to ET-1 (Figure 7).

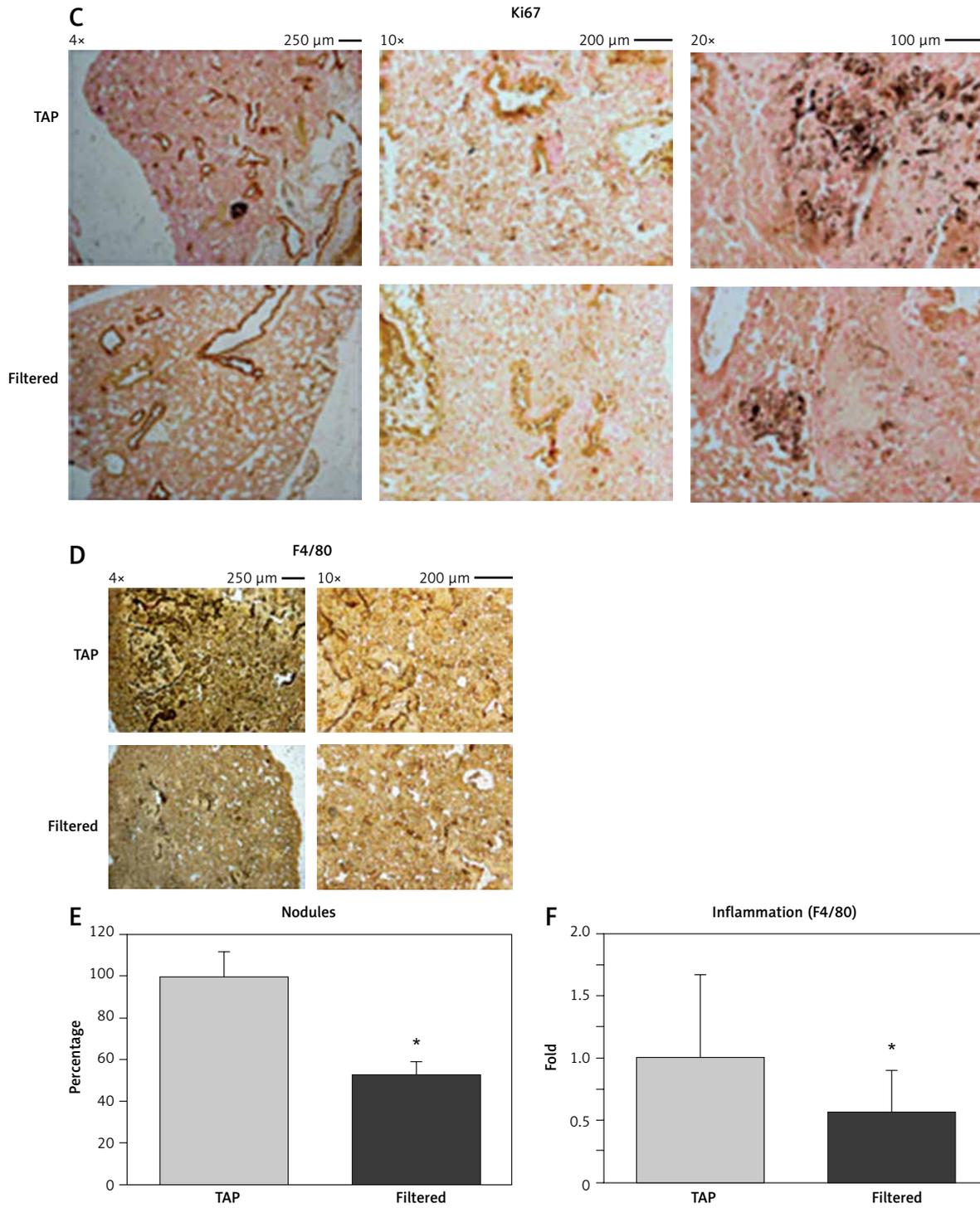
Since hypertension is generally associated with a state of low-grade chronic inflammation, we tested the inflammatory status in the rats by analyzing the circulating levels of three pro-inflammatory cytokines, IL-1 $\beta$ , IL-6 and TNF- $\alpha$ . As previously reported, SHR rats showed higher levels of all three cytokines and in the scope of our study these differences reached statistical significance for IL-1 $\beta$ . Alkaline water consumption resulted in the reduction of the levels of all the cytokines tested in SHR rats, reaching statistical significance for IL-1 $\beta$ . In contrast, in WKY rats, alkaline water did not significantly alter the levels of these cytokines (Figure 8 A).

In order to further corroborate the significance of these results, we analyzed the gene expression levels of several inflammatory mediators in the heart, TNF- $\alpha$ , TGF- $\beta$ , IF- $\gamma$ , IL-1 $\beta$ , IL-4, IL-6, IL-10, iNOS, Arg-1. Consistently with previous findings, we observed higher levels in SHR than in WKY, a difference that was significant for TNF- $\alpha$  and IL-1 $\beta$ . Consumption of alkaline water did not significantly alter the levels of any of the



**Figure 4.** Metastatic lung nodules derived from B16-V5 injected iv in mice treated with tap or filtered water. **A** – Whole lung images. **B** – H&E staining of lung histological sections

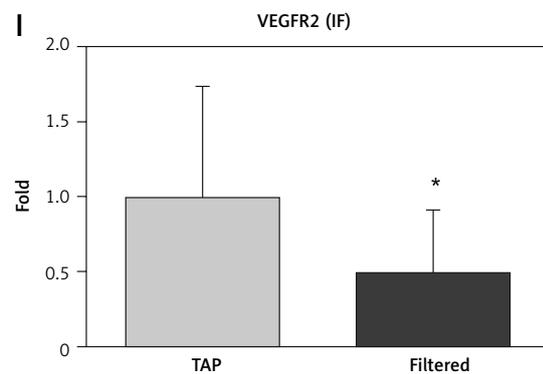
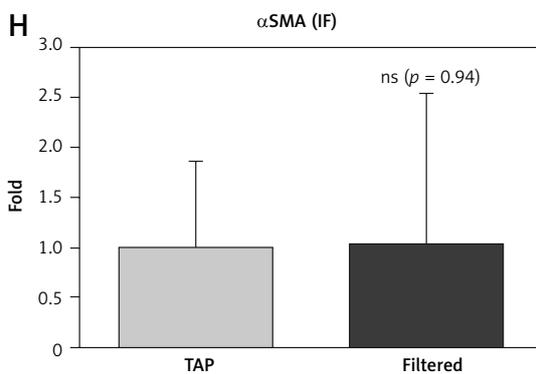
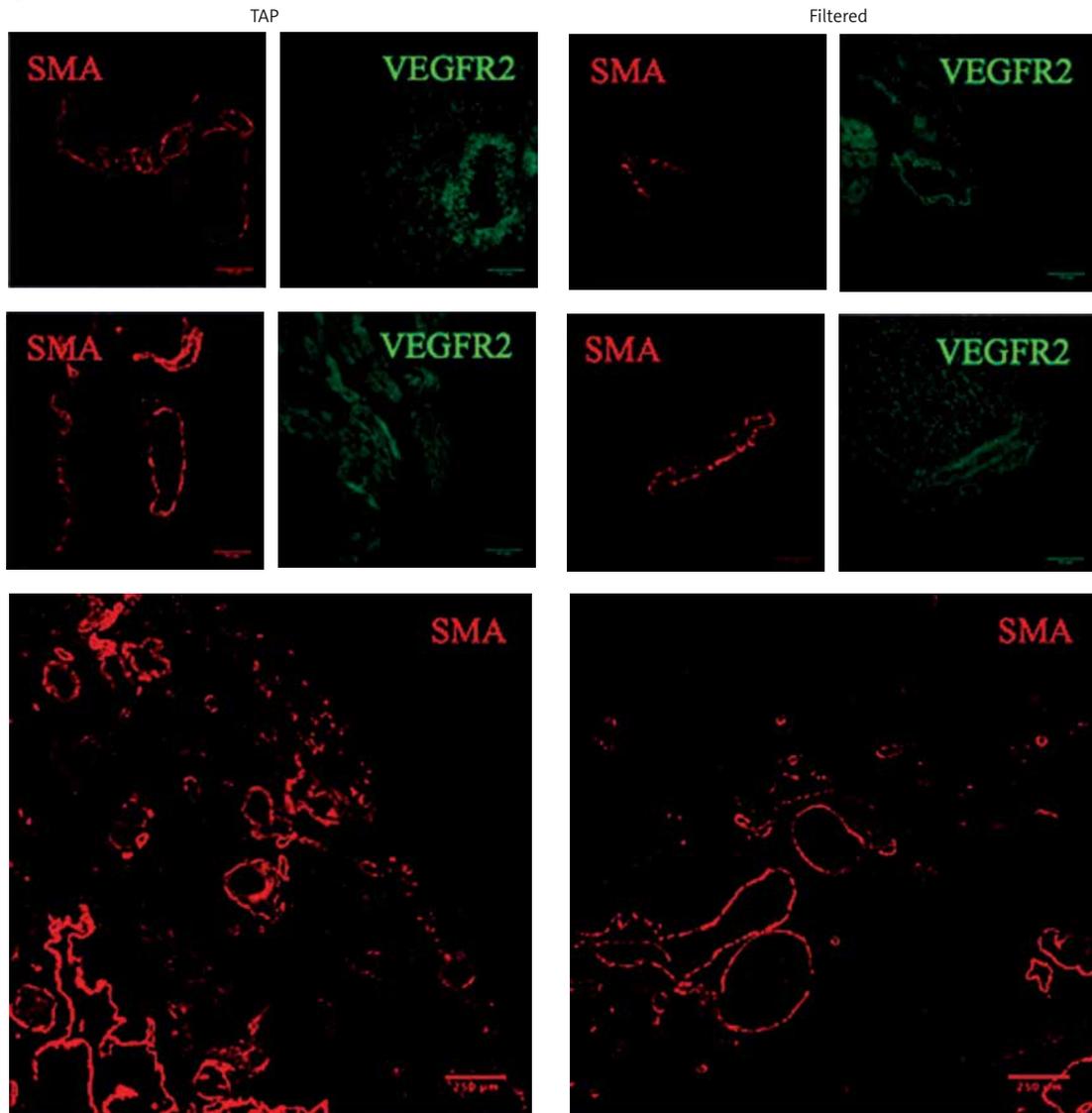
Data are mean  $\pm$  standard deviation. \* $p < 0.05$ ; \*\* $p \leq 0.01$ ; \*\*\* $p \leq 0.005$ . ns – non-significant.



**Figure 4.** Cont. **C** – IHQ Ki67 staining of lung histological sections. **D** – IHQ F4/80 staining of lung histological sections. **E** – Quantitative analysis of lung nodules. **F** – Quantitative analysis of F4/80+ cells

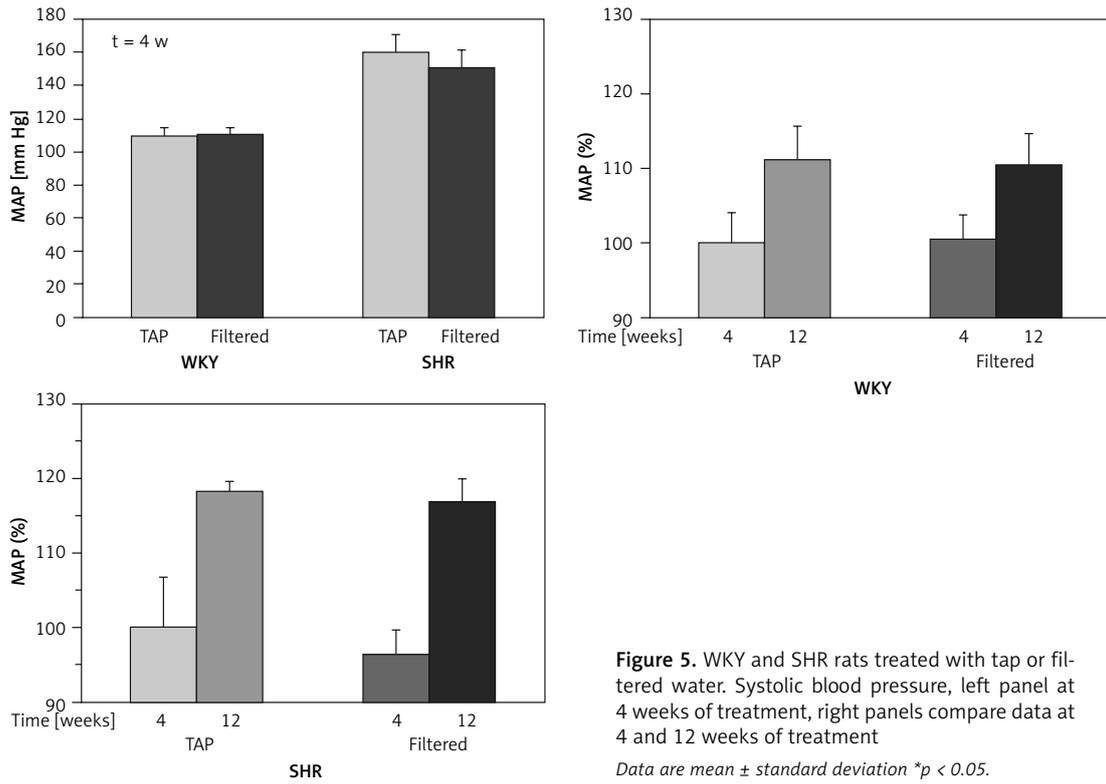
Data are mean ± standard deviation. \* $p < 0.05$ ; \*\* $p \leq 0.01$ ; \*\*\* $p \leq 0.005$ . ns – non-significant.

G

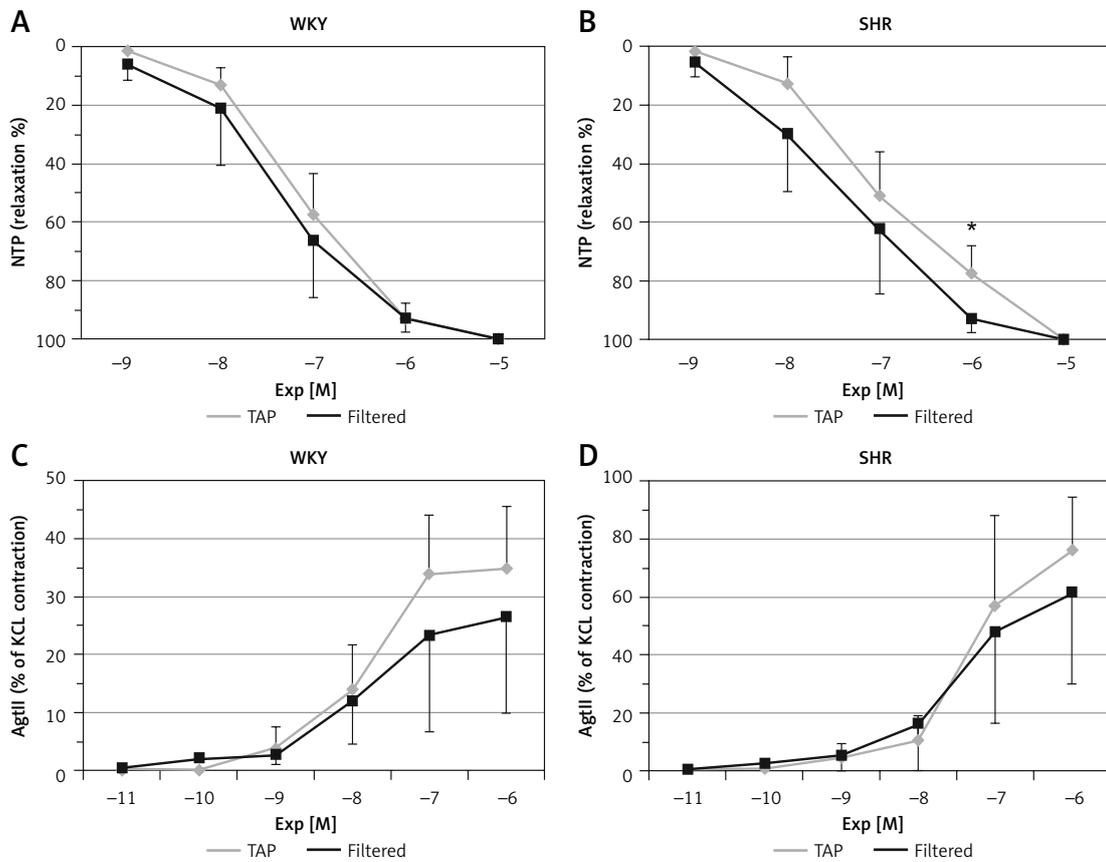


**Figure 4.** Cont. **G** – Top, IF SMA (red) and VEGF2 (green) staining of lung histological sections. Bottom, IF  $\alpha$ SMA staining of tumor histological sections. Maximal projection images of tissue section tile scans, acquired with a confocal microscope. **H** – Quantitative analysis of SMA. **I** – Quantitative analysis of VEGF2

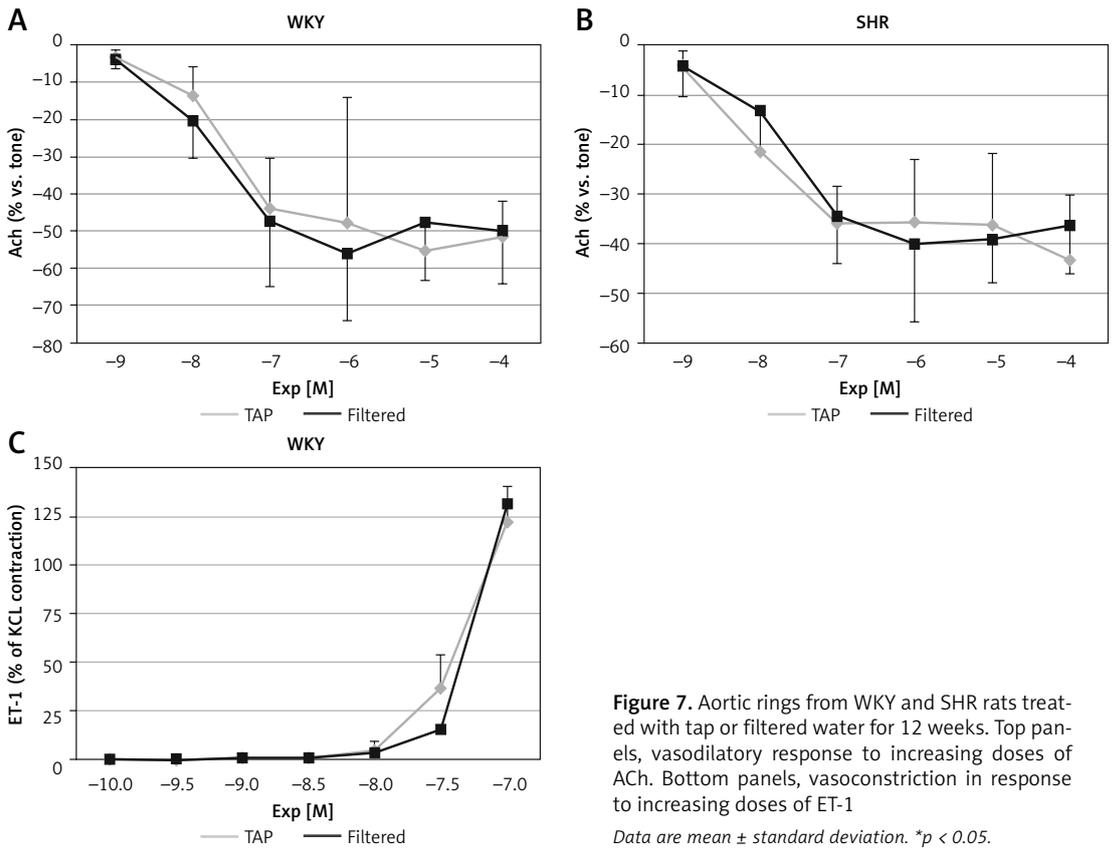
Data are mean  $\pm$  standard deviation. \* $p < 0.05$ ; \*\* $p \leq 0.01$ ; \*\*\* $p \leq 0.005$ . ns – non-significant.



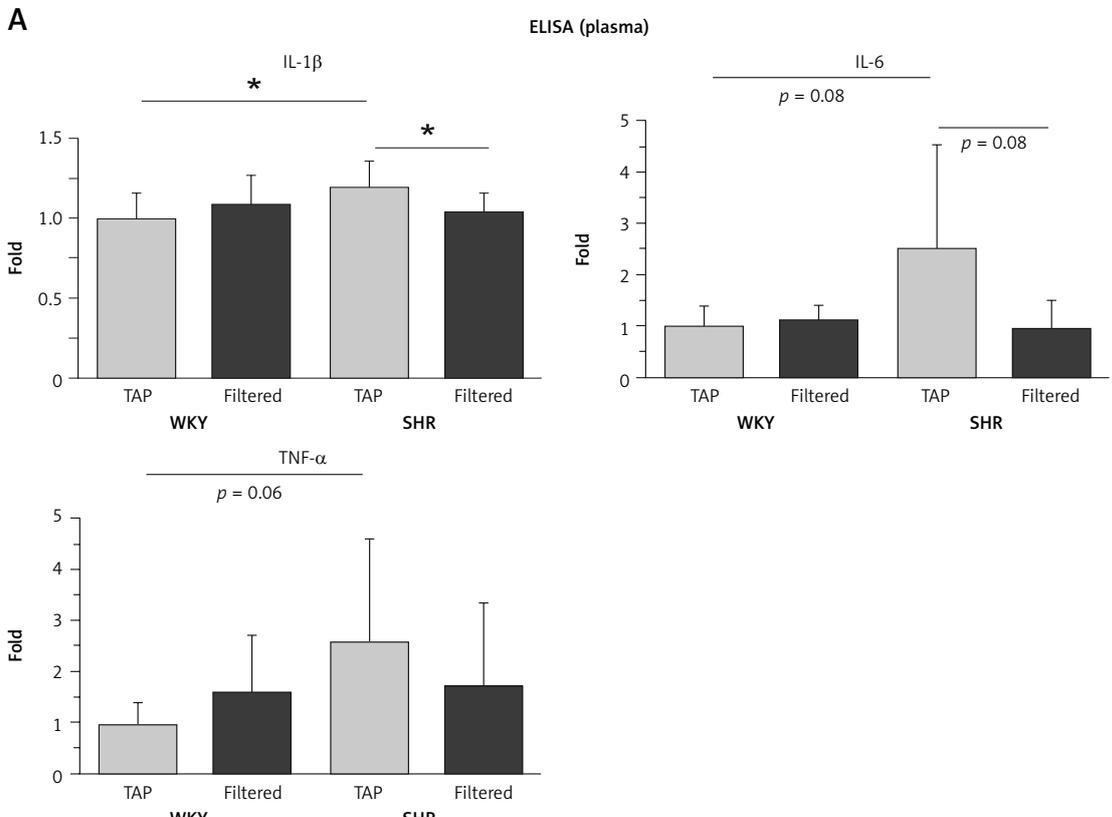
**Figure 5.** WKY and SHR rats treated with tap or filtered water. Systolic blood pressure, left panel at 4 weeks of treatment, right panels compare data at 4 and 12 weeks of treatment  
Data are mean ± standard deviation \**p* < 0.05.



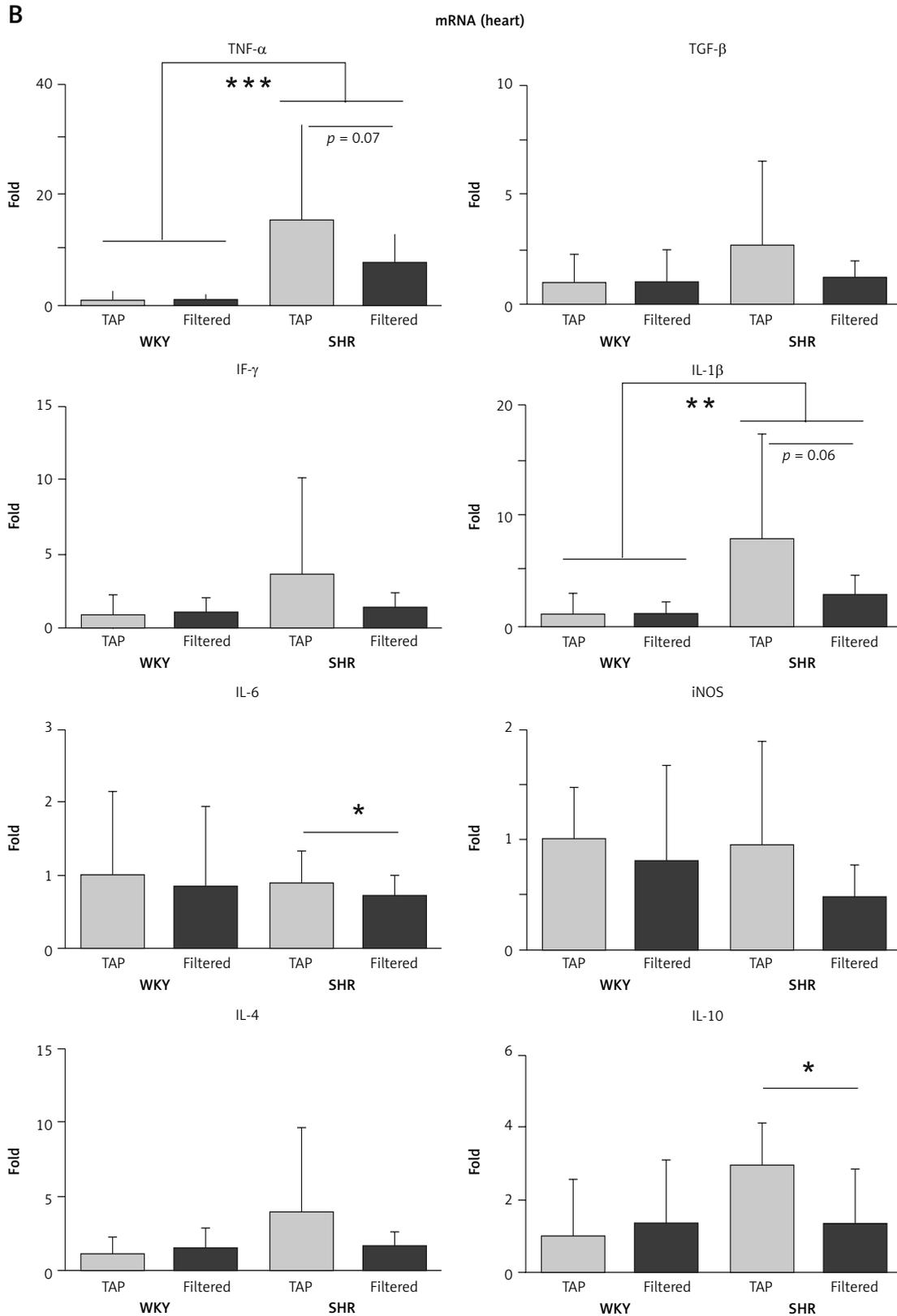
**Figure 6.** Aortic rings from WKY and SHR rats treated with tap or filtered water for 12 weeks. Top panels: vasodilatory response to increasing doses of NTP. Bottom panels: vasoconstriction in response to increasing doses of AgtII  
Data are mean ± standard deviation. \**p* < 0.05.



**Figure 7.** Aortic rings from WKY and SHR rats treated with tap or filtered water for 12 weeks. Top panels, vasodilatory response to increasing doses of ACh. Bottom panels, vasoconstriction in response to increasing doses of ET-1  
Data are mean ± standard deviation. \**p* < 0.05.

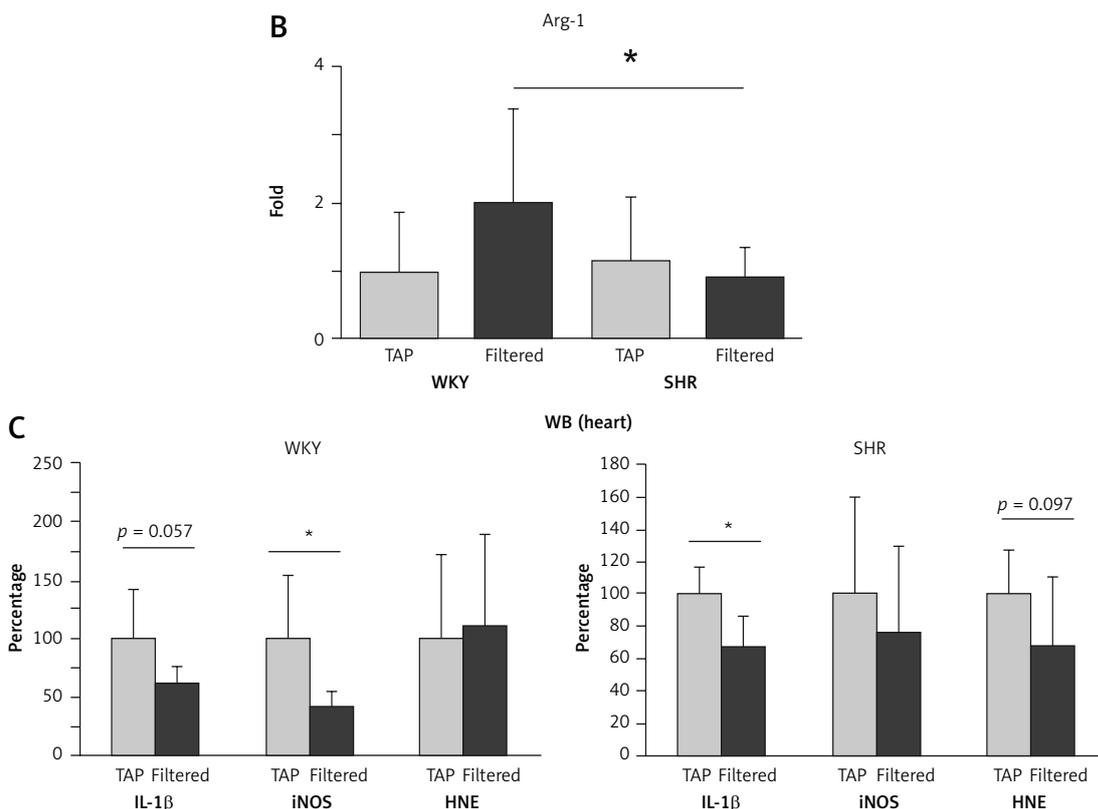


**Figure 8.** Inflammatory profile in WKY and SHR rats treated with tap or filtered water for 12 w. A – ELISA analysis of inflammatory cytokines in plasma samples  
Data are mean ± standard deviation. \**p* < 0.05.



**Figure 8.** Cont. **B** – qRT-PCR analysis of inflammatory genes in heart tissue samples

Data are mean  $\pm$  standard deviation. \*,  $p < 0.05$ .



**Figure 8.** Cont. **B** – qRT-PCR analysis of inflammatory genes in heart tissue samples. **C** – Western blot analysis of pro-IL-1 $\beta$ , iNOS and HNE modified proteins in heart tissue samples

Data are mean  $\pm$  standard deviation. \* $p < 0.05$ .

mRNAs tested in WKY rats. In contrast, in SHR rats there was a general decrease in all the inflammatory molecules, but the differences only reached statistical significance for IL-6 and IL-10 (Figure 8 B).

Aiming to validate the significance of these results, we tested the levels of pro-IL-1 $\beta$  and iNOS by western blotting in heart tissue. We found that alkaline water consumption significantly reduced the levels of iNOS in WKY and of pro-IL-1 $\beta$  in SHR (Figure 8 C).

Since reduced inflammation is normally associated with reduced levels of oxidants, we evaluated the oxidative status of the heart tissue, analyzing by western blotting the presence of proteins modified with HNE, but consumption of alkaline water did not result in a significant reduction in the formation of HNE adducts in WKY or SHR rats (Figure 8 C).

In sum, these data suggest that rats drinking alkaline water have reduced levels of inflammatory mediators, and could indicate a general reduced level of inflammation.

## Discussion

Global weather change is significantly reducing the average yearly rainwater precipitation in countries such as Spain [28] and has a negative

impact on the quality of tap water from municipal water supplies [29]. Indeed, rising concerns over the quality of tap water has increased the domestic use of both bottled water [30] and point-of-use watering filtering or purifications systems [31]. However, investigations into the impact of these devices on human health are scarce. In particular, there is a paucity of studies on the potential health promoting effects of alkaline water on human health [32, 33], with controversial results [18], and most have focused on its role in the amelioration of gastric reflux [17, 34]. Cancer is now recognized as a complex disease in which development of the nutritional and metabolic status of the affected subject plays an important role [35]. As a consequence, many studies aim to evaluate of how different macro- or micronutrients, diet regimens or nutritional supplements affect cancer development [36]. Studies that focus on water in this context are comparatively rare. Only very recently, a growing body of literature focusing on water contaminants is bringing into focus the role of water quality in human health and in particular, in cancer. Perhaps not surprisingly, the main conclusion is that water is important for many things, including cancer.

The commercial devices developed to increase the pH of tap water to produce “alkaline” water

vary widely in their characteristics and capabilities, which, together with variability in water sources, makes general conclusions on the use of “alkaline” water hard to draw. Accordingly, it is paramount to highlight the importance of testing each device and water type individually. Indeed, the main limitation of the present study is the evaluation of a single filtering type device and a single tap water source.

We induced tumor development processes in mice using protocols that allowed us to study early tumorigenesis, primary tumor development and also metastasis. We found that in all three experimental protocols used, mice that had access to filtered water showed a less advanced disease stage. In particular, the most consistent finding was that the quality of the vascular structure was significantly better in animals consuming filtered water.

We first studied initial tumorigenesis in a model for the development of HCC, in which we induced liver steatosis with HFD and then facilitated tumor development through the administration of two teratogens. We found that animals in both groups developed advanced NAFLD, but the animals with access to filtered water had lower cell proliferation rates, and better preservation of the intima vascular layer. Similarly, the metastasis model showed a lower number of nodules and reduced inflammation.

We concluded that the evaluation of the effect of alkaline water on tumor development using three complementary mouse models, for initiation, primary tumor development and metastasis, suggests that the most evident and significant difference among the animals drinking tap water and alkaline water was in the vasculature. While tumors disrupt vascular stability in animals drinking alkaline water, vascular structures seemed to be better preserved, with reduced thickening of the media layer, better endothelial coverage and reduced tortuosity, characteristics that could be the underlying cause of the observed reduced formation of metastatic nodes. In view of these results, we decided to test more directly the impact of alkaline water consumption in the vasculature.

To that end we used a rat model of spontaneous hypertension and treated these rats and their normotensive controls for 12 weeks with alkaline or tap water. We found that consumption of alkaline water improved the vasodilatory response to nitric oxide in hypertensive rats, and this change was associated with the reduction of some inflammatory markers, in particular the circulating levels of IL-1 $\beta$ , the mRNA levels of IL-6 and IL-10 in the heart, and the protein levels of pro-IL-1 $\beta$  also in the heart, supporting the notion that the consumption of alkaline water could have some

beneficial effects on hypertension that are associated with an improved vasodilatory response and a reduction in the inflammatory status.

In conclusion, the effects on human health derived from the regular consumption of alkaline water are still a matter of controversy, largely due to a lack of relevant scientific studies evaluating them. Originally developed as a complementary treatment for dyspepsia acidosis, it has been presumed to have beneficial effects for other conditions, with claims lacking a scientific basis to sustain them. In view of the general interest in its potential benefits, it is therefore necessary to adequately evaluate the extent of its impact on human health.

Altogether, these results suggest that the use of water filters may have beneficial effects on the different stages of tumor development and also underscore the role played by the vasculature and the preservation of a good vascular structure in these processes. They also suggest that the main mediator of the beneficial effects of this filtering system might be the vascular cells and the immune system. At this stage we do not have data to propose the potential mechanisms involved. It is, however, perhaps not surprising that endothelial cells, and immune cells which are directly exposed to the bloodstream, are strongly influenced by the water that the animals drink. Similarly, the intestinal epithelium is likely to be strongly influenced, as has been observed when different diets or nutrients are evaluated.

In conclusion, our study not only supports the potential beneficial effects of the use of a water filtering system, but also highlights the urgent need for similar studies that foster the discussion within the scientific community on what is and what is not important in the water that we drink.

### Conflict of interest

The authors declare no conflict of interest.

### References

1. Serpa D, Nunes JP, Keizer JJ, Abrantes N. Impacts of climate and land use changes on the water quality of a small Mediterranean catchment with intensive viticulture. *Environm Pollution* 2017; 224: 454-65.
2. Shammil M, Rahman MM, Bondad SE, Bodrud-Doza M. Impacts of salinity intrusion in community health: a review of experiences on drinking water sodium from coastal areas of Bangladesh. *Healthcare* 2019; 7: 50.
3. Huang L, Wu H, van der Kuijp TJ. The health effects of exposure to arsenic-contaminated drinking water: a review by global geographical distribution. *Int J Environm Health Res* 2015; 25: 432-52.
4. Ding S, Deng Y, Bond T, Fang C, Cao Z, Chu W. Disinfection byproduct formation during drinking water treatment and distribution: a review of unintended effects of engineering agents and materials. *Water Res* 2019; 160: 313-29.

5. Iyare PU. The effects of manganese exposure from drinking water on school-age children: a systematic review. *Neurotoxicology* 2019; 73: 1-7.
6. Novotna K, Cermakova L, Pivokonska L, Cajthaml T, Pivokonsky M. Microplastics in drinking water treatment – current knowledge and research needs. *Sci Total Environ* 2019; 667: 730-40.
7. Domingo JL, Nadal M. Human exposure to per- and poly-fluoroalkyl substances (PFAS) through drinking water: a review of the recent scientific literature. *Environ Res* 2019; 177: 108648.
8. Ashton J. Water is the staff of life and at the heart of public health. *J R Soc Med* 2019; 112: 516-8.
9. Allaire M, Mackay T, Zheng S, Lall U. Detecting community response to water quality violations using bottled water sales. *PNAS* 2019; 116: 20917-22.
10. Wang MH, He Y, Sen B. Research and management of plastic pollution in coastal environments of China. *Environ Pollut* 2019; 248: 898-905.
11. Zuccarello P, Ferrante M, Cristaldi A, et al. Exposure to microplastics (< 10 µm) associated to plastic bottles mineral water consumption: the first quantitative study. *Water Res* 2019; 157: 365-71.
12. Park EJ, Han JS, Park EJ, et al. Repeated-oral dose toxicity of polyethylene microplastics and the possible implications on reproduction and development of the next generation. *Toxicol Lett* 2020; 324: 75-85.
13. de-Magistris T. Nutrition, choice and health-related claims. *Nutrients* 2020; 12: 650.
14. Bielefeldt AR, Kowalski K, Schilling C, Schreier S, Kohler A, Scott Summers R. Removal of virus to protozoan sized particles in point-of-use ceramic water filters. *Water Res* 2010; 44: 1482-8.
15. Albergamo V, Blankert B, Cornelissen ER, et al. Removal of polar organic micropollutants by pilot-scale reverse osmosis drinking water treatment. *Water Research* 2019; 148: 535-45.
16. Buzalaf MAR, Levy FM, Rodrigues MHC, de M. Bastos JR. Effect of domestic water filters on water fluoride content and level of the public water supply in Bauru, Brazil. *J Dent Child* 2003; 70: 226-30.
17. Tanaka Y, Saihara Y, Izumotani K, Nakamura H. Daily ingestion of alkaline electrolyzed water containing hydrogen influences human health, including gastrointestinal symptoms. *Med Gas Res* 2019; 8: 160-6.
18. Fenton TR, Huang T. Systematic review of the association between dietary acid load, alkaline water and cancer. *BMJ Open* 2016; 6: e010438.
19. Al Zahrani SM, Omar UM, Rahimulddin SA, et al. Antiproliferative and apoptotic effects of the natural alkaline water (Zamzam) in breast cancer cell line MCF-7. *J Cancer Res Ther* 2019; 15: 1098-104.
20. Amor S, García-Villalón AL, Rubio C, et al. Effects of age and caloric restriction in the vascular response of renal arteries to endothelin-1 in rats. *Exp Gerontol* 2017; 88: 32-41.
21. Zambrano A, García-Carpizo V, Gallardo ME, et al. The thyroid hormone receptor  $\beta$  induces DNA damage and premature senescence. *J Cell Biol* 2014; 204: 129-46.
22. Monsalve M, Wu Z, Adelmant G, Puigserver P, Fan M, Spiegelman BM. Direct coupling of transcription and mRNA processing through the thermogenic coactivator PGC-1. *Mol Cell* 2000; 6: 307-16.
23. Paduch R. The role of lymphangiogenesis and angiogenesis in tumor metastasis. *Cell Oncol* 2016; 39: 397-410.
24. Blazejczyk A, Papiernik D, Porshneva K, Sadowska J, Witrzyk J. Endothelium and cancer metastasis: Perspectives for antimetastatic therapy. *Pharmacol Rep* 2015; 67: 711-8.
25. Cantelmo AR, Conradi LC, Brajic A, et al. Carmeliet, inhibition of the glycolytic activator PFKFB3 in endothelium induces tumor vessel normalization, impairs metastasis, and improves chemotherapy. *Cancer Cell* 2016; 30: 968-85.
26. García-Quintans N, Sánchez-Ramos C, Prieto I, et al. Oxidative stress induces loss of pericyte coverage and vascular instability in PGC-1 $\alpha$ -deficient mice. *Angiogenesis* 2016; 19: 217-28.
27. Follain G, Osmani N, Azevedo AS, et al. Hemodynamic forces tune the arrest, adhesion, and extravasation of circulating tumor cells. *Development* 2018; 145: 33-52. e12.
28. Morote ÁF, Olcina J, Hernández M. The use of non-conventional water resources as a means of adaptation to drought and climate change in semi-arid regions: South-Eastern Spain. *Water* 2019; 11: 93.
29. Wu D, Zhou Y, Lu G, et al. The occurrence and risks of selected emerging pollutants in drinking water source areas in Henan, China. *Int J Environ Res Public Health* 2019; 16: 4109.
30. Doria MF. Bottled water versus tap water: understanding consumers' preferences. *J Water Health* 2006; 4: 271-6.
31. Pérez-Vidal A, Díaz-Gómez J, Castellanos-Rozo J, Usaquén-Perilla OL. Long-term evaluation of the performance of four point-of-use water filters. *Water Res* 2016; 98: 176-82.
32. Shin DW, Yoon H, Kim HS, et al. Effects of alkaline-reduced drinking water on irritable bowel syndrome with diarrhea: a randomized double-blind, placebo-controlled pilot study. *Evid Based Complement Altern Med* 2018; 2018: e9147914.
33. Chycki J, Kurylas A, Maszczyk A, Golas A, Zajac A. Alkaline water improves exercise-induced metabolic acidosis and enhances anaerobic exercise performance in combat sport athletes. *PLoS One* 2018; 13: e0205708.
34. Weinsheim DT, Lesser H, Sataloff DRT. Unforeseen consequence of alkaline water therapy for laryngopharyngeal reflux. *Ear Nose Throat J* 2019; 97: 384-5.
35. Kerschbaum E, Nüssler V. Cancer prevention with nutrition and lifestyle. *Visc Med* 2019; 35: 204-9.
36. Solheim TS, Vagnildhaug OM, Laird BJ, Balstad TR. Combining optimal nutrition and exercise in a multimodal approach for patients with active cancer and risk for losing weight: rationale and practical approach. *Nutrition* 2019; 67-68: 110541.