

Study of plasma homocysteine, folic acid and vitamin B₁₂ levels for radiation exposed transplanted solid Ehrlich tumors

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ABSTRACT

Background: Alterations of systemic metabolic parameters are always present vitamins in cancer in addition radiotherapy may increases and complicates these disorders. The analysis of each biochemical metabolic alterations may give more understanding to biochemistry of malignancy and improving the degree of radiotherapy. The current study aimed to follow up the changes of folate, vitamin B₁₂ and homocysteine in normal and malignant mice with and without radiation exposure, as folate and B₁₂ metabolism play a role in hypermethylation of certain genes including tumor suppressor and apoptotic genes of cancer. **Materials and Methods:** The study was conducted through six groups of mice, normal, malignant, normal and malignant exposed gamma radiation with different fractionated doses (0.7 and 1.2 Gy).

Results: Comparisons between the results of all groups revealed that: folate and vitamin B₁₂ were lower and homocysteine was higher in gamma irradiated malignant and gamma irradiated nonmalignant mice in comparison with normal control and malignant mice. Folate and vitamin B₁₂ levels were also lower whereas homocysteine was higher in malignant mice in comparison with normal control group. **Conclusion:** These results denoted that increased homocysteine or/and decreased folate and vitamin B₁₂ in the two malignant exposed groups may have a role in the primary tumor regression leading to the recommendation that preventing up taking of folate and vitamin B₁₂ either as supplements of land nutrients rich with subsequent increase in homocystein may be beneficial through its toxic effect in stopping or minimizing tumor progression. The disturbance of vitamins may be due to the metabolic alterations associated with tumor development.

Keywords: Folic acid, homocystein, vitamin B₁₂, malignancy, radiotherapy

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INTRODUCTION

Vitamin B₁₂, folic acid and vitamin B₆ have a number of interrelated biological roles that make them potentially important agents in cancer ⁽¹⁾. However folate, vitamin B₁₂ and homocysteine are essential for methyl group metabolism and DNA methylation ⁽²⁾.

Folate is a water-soluble vitamin naturally found in green leafy vegetables, cereals, legumes, and fruits. It plays an important role in DNA synthesis, integrity, and stability. Folate

deficiency usually causes defective DNA repair and chromosomal fragile site expression, leading to chromosomal breaks and micronucleus formation ⁽³⁾. The role of folate and its synthetic form folic acid in cancer development and progression is highly controversial ^(3,4,5).

Choi and Mason ⁽⁶⁾ and Blount et al. ⁽⁷⁾ Stated that folate deficiency leads to ovarian cancer through two mechanisms: inducing misincorporation of uracil into DNA, thus disrupting DNA integrity and DNA repair; and by altering DNA methylation, which can alter

expression of critical tumor suppressor genes and proto oncogenes. In addition Kim (5) suggested that dietary intake and blood levels of folate appear to be inversely related to the risk of several malignancies, in particular colorectal cancer, although the strength, specificity and magnitude of this association have not been consistent. It is biologically plausible that polymorphisms or gene-environment interactions rather than folate intake alone would have an impact on breast Cancer risk (8,9).

Vitamin B₁₂, folic acid and vitamin B₆ as coenzymes in the synthesis of pureness and thymidylate for DNA synthesis. When these nutrients levels are insufficient, the initiation of cancer is facilitated by reduction of thymidylate synthesis, resulting in an increased incorporation of uracil in DNA repair, and neoplastic transformation (1,10). In addition the increased chromosome breakage associated with low intake of folate, vitamin B₁₂ or homocysteine has been demonstrated (11). While high vitamin B₁₂ levels were associated with the risk of subsequent diagnosed cancer, mostly within the first year of follow-up (3,12). The levels of plasma folate and vitamin B₁₂ are likely to be associated with the progress of liver cancer (13).

Homocysteine is a sulfur-containing amino acid produced as an intermediate of methionine metabolism by one of two pathways: remethylation or transsulfuration abnormalities of these pathways leads to hyperhomocysteinemia, which is associated with an increased risk of many disorders including vascular and neurodegenerative diseases, autoimmune disorders, birth defects, diabetes, renal disease, osteoporosis, neuropsychiatric disorders, and cancer (12).

Homocysteine is remethylated back to methionine folate-dependent reaction homocysteine acquires a methyl group from N-5-methyltetrahydrofolate with the help of methionine synthase which requires vitamin B₁₂ for its functionality, and the reaction also involves recycling of tetrahydrofolate¹⁴. Folate, which is pivotal for cell proliferation, Low plasma folate levels are also linked to cancer via DNA methylation which is an important for normal genome regulation and development.

Homocysteine is recycled to methionine with the help of methionine synthase¹⁵.

The aim of the present study is to evaluate the change of folic acid, vitamin B₁₂ and homocysteine in normal, malignant, normal irradiated and malignant irradiated mice in order to throw a light on the responsibility of these parameters on tumourgenesis and the effect of radiotherapy on their levels.

MATERIALS AND METHODS

Experimental design

Forty eight (48) male Blab C mice, age was about 3 months were used in the present study, they were obtained from national institute of cancer (Egypt) and their weight ranged from 18-20 gm., each 8 mice were randomly used in plastic cages where they watered and fed after acclimation for two weeks. The exposure was extended for four weeks (twice/week), the dose exposure each time was 0.7 and 1.2 gray respectively.

They were classified into six equal groups:

- Control group with no treatment.
- Ehrlich malignant group where the tumor was transplanted in thigh region of mice in the dose of 2×10^6 cells/mice.
- Normal radiation exposed group (0.7 Gray).
- Normal radiation exposed group (1.2 Gray).
- Malignant radiation exposed group (0.7 Gray).
- Malignant radiation exposed group (1.2 Gray).

Irradiation protocol

Gamma-irradiation of mice was carried out at the National Centre for Radiation Research and Technology (NCRRT), Egyptian Atomic Energy Authority, using Gamma Cell40, Canada biological irradiator furnished with a Caesium 137 source, at dose rate 0.713 rad/sec.

The irradiated groups were exposed to fractionated radiation dose of 0.7 and 1.2 gray twice/week for 4 weeks, after 8 days of Ehrlich transplantation. The whole body of the irradiated groups were exposed to a cumulative

radiation dose equal to 5.6 and 9.6 gray respectively.

Blood Sampling

At the end of experiment: 14 days acclimation, 8 days post transplantation and 28 days irradiation, blood samples were collected by sacrificing. Sera were separated and stored at -20°C for the analysis of homocysteine, Vitamin B₁₂, and folic acid.

Chemicals

Homocysteine levels in serum were determined using rat homocysteine Elisa kit purchased from cusabio ESB – E13376s, vitamin B12 and folic acid level serum was determined using rat Elisa kit purchased from cusabio catalog no.MBS-701134.

The size of the tumor measured two times weakly for 4 weeks, and the size of the tumor calculated from the following equation:

Size of tumor = $a \times b \times c = c^3$ (cubic centimeter)

Where a= length of the tumor

b = width of the tumor

c = height of the tumor

Anesthetic procedures and handling of animals were approved by and complied with the ethical guidelines of Medical Ethical Committee of the National Research Centre in Egypt (Approval number: 14077, RF-133, 4-2015).

Statistical analysis

The obtained data were presented as mean \pm SD. one-way analysis of variance (ANOVA) was carried out using statistical package program (COSTAT).The statistical comparison among the groups for each parameter were performed using Duncan's multiple range tests, A probability level of $p < 0.05$ was considered.

RESULTS

Table 1- 3 summarizes the data of homocysteine, folic acid and vitamin B₁₂ respectively in the six groups of mice, normal,

malignant, normal and malignant exposed gamma radiation with different fractionated doses (0.7 and 1.2 Gy).

The Ehrlich tumors groups examined significant increase of homocysteine compared to the healthy control group. Simultaneously both doses of exposed malignant groups comprised significant increase in the same parameter compared to their corresponding nonmalignant exposed groups (healthy exposed groups). When we compare both doses of nonmalignant (healthy) exposed groups with healthy control, and both doses of malignant exposed groups with malignant control, we noticed significant increase of homocysteine levels of healthy exposed and malignant exposed groups with their corresponding. Also there were statistical change observed between the two fractionated doses of 0.7 and 1.2 gray of both groups in the healthy exposed groups with each other and in the malignant exposed groups with each other.

To the contrary of homocysteine, folic acid showed significant decrease comparing malignant group and healthy control group and both doses of malignant exposed and their related of nonmalignant (healthy) exposed groups. Simultaneously the two exposed nonmalignant (healthy) groups and the two exposed malignant groups experienced significant decrease folic acid compared to healthy control and malignant group respectively, with no significant changes between the two exposed groups of nonmalignant (healthy) exposed groups with each other, or in malignant exposed groups with each other.

Regarding Vitamin B₁₂, significant decrease in malignant groups compared with healthy control group. In addition both malignant exposed groups compared with their corresponding healthy exposed groups (0.7 and 1.2 gray), showed significant decrease of vitamin B₁₂. At the same time the level of Vitamin B₁₂ were lower in the two malignant exposed groups compared with malignant non exposed group. Also there were significant changes between the two healthy exposed groups and healthy control

group.

From the above results it is clear that, malignancy and radiation had a critical effect on the levels of the three parameters: folic acid, Vitamin B₁₂ and homocysteine.

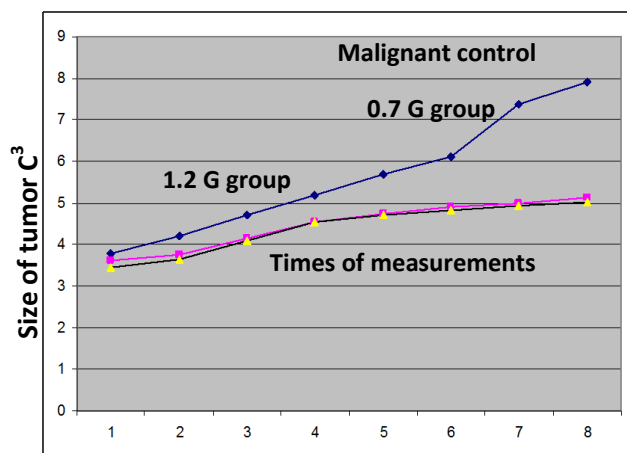


Figure 1. Histogram representing tumor size (c³) of malignant untreated control and irradiated groups (0.7 and 1.2 gray).

Table 2. Mean Values \pm SD of serum folic acid (ng/L) in normal control (Unexposed and irradiated 0.7 and 1.2 gray) and malignant groups (Unexposed and irradiated 0.7 and 1.2 gray).

Group	Healthy $\bar{x} \pm SD$	Malignant $\bar{x} \pm SD$	Probability
Non Exposed	14.32 \pm 0.69 ^a	8.10 \pm 0.51 ^c	P<0.02
Exposed to 0.7G	10.20 \pm 0.50 ^b	6.96 \pm 0.22 ^d	P<0.02
Exposed to 1.2 G	9.85 \pm 0.49 ^b	6.60 \pm 0.31 ^d	P<0.02

DISCUSSION

This study was designed to assess folic acid, vitamin B₁₂ and homocysteine on primary tumor and tumor in situ, also to investigate role of radiotherapy on their levels. Therefore we began radiotherapy on the 8th day of Ehrlich transplantation as the tumor was visualized and we are able to calculate its dimension and noticed its regression.

The etiology of neoplastic transformation is a complex process where several causes play different roles in tumor development and tumor progression. Despite the many researches concerned risk factors influencing

Figure 1 shows tumor growth rate of implanted tumor group untreated and treated groups (size by c³) was markedly decreased in malignant exposed groups as compared to malignant control group as a factor of time.

Table 1. Mean Values \pm SD of serum homocysteine ((nmol/L) in normal control (unexposed and irradiated 0.7 and 1.2 gray) and malignant groups (unexposed and irradiated 0.7 and 1.2 gray).

Group	Healthy $\bar{x} \pm SD$	Malignant $\bar{x} \pm SD$	Probability
Non Exposed	3.69 \pm 0.19 ^f	4.84 \pm 0.20 ^e	P<0.02
Exposed to 0.7G	5.22 \pm 0.21 ^d	6.51 \pm 0.23 ^b	P<0.02
Exposed to 1.2 G	5.78 \pm 0.21 ^c	7.08 \pm 0.21 ^a	P<0.02

Table 3. Mean Values \pm SD of serum vitamin B₁₂ (pg/L) in normal control (Unexposed and irradiated 0.7 and 1.2 gray) and malignant groups (Unexposed and irradiated 0.7 and 1.2 gray).

Group	Healthy $\bar{x} \pm SD$	Malignant $\bar{x} \pm SD$	Probability
Non Exposed	382 \pm 41.95 ^a	272 \pm 30.23 ^{bc}	P<0.02
Exposed to 0.7G	307.5 \pm 46.68 ^b	227 \pm 26.46 ^{de}	P<0.02
Exposed to 1.2 G	245 \pm 30.91 ^{cd}	205 \pm 24.39 ^e	P<0.02

carcinogenesis in human and experimental animals and in vivo and in vitro studies, the results are in consistent. Metabolic alterations, chromosome breakage, disrupting DNA integrity and DNA repair ^(6,7) had been proved to influence beginning and progress of neoplasm.

Therefore in the present study we choose three metabolic parameters which are closely related to each other and their relation with the treatment and prognosis of neoplasms are controversies.

Zhang *et al.* ⁽¹⁶⁾ for example, showed that folic acid and vitamin B₁₂ had no significant effect on colorectal invasive cancer or breast cancer among women. In the contrary Ingild *et al.* ⁽¹⁷⁾

observed significant decrease of folate and significant increase of homocysteine in cervical cancer which was in accordance with the current data.

In cancer patients, the plasma folate level is expected to be low because tumor cells must draw folate from the blood for *de novo* purine synthesis (18,19).

Blount *et al.* (7) concluded that folate deficiency leads ovarian cancer through different mechanisms; in an earlier study MC Cully (20) found that: Treating animals with homocysteine thiolactone causes increase necrosis in transplanted malignant neoplasms which supports our findings.

The relation between these three parameters may be due to folate and cyanocobalamine (B₁₂) dependent reactions in the metabolic pathways of homocysteine. The enzyme methylene tetrahydrofolate reductase [MTHFR] converts 5-10 methylene tetrahydrofolate to 5 methyl hydrofolate in the presence of NADPH. In turn 5 methyl tetrahydrofolate serves as a substrate for vitamin B₁₂ dependent methionine synthetase, and an elevated homocysteine level is associated with common defect of this enzyme (2).

Radiotherapy, chemotherapy, immunotherapy as well as surgical interference are the main methods for cancer treatment. In order to simulate radiotherapy, fractionated dose of radiotherapy was used for 4 weeks in the present study.

Although ionizing radiation had positive effect on malignant tumor, it has negative effect on other healthy tissues and minimizing the dose as possible is necessary. Therefore in the present study we exposed the animals to two different fractionated doses to compare their effects on tumor regression and the parameters levels.

Induced cancer in the present study caused significant increase of homocysteine and significant decrease of folate and vitamin B₁₂ (tables 1-3). The decrease of vitamins may be due to the metabolic alterations associated with tumor development or/and increase cell surface receptors. Folic acid receptors are elevated in cancer (21). These folate receptors have been

recognized for their potential role in cellular folate uptake (22).

Hyperhomocysteinemia noticed in malignant mice (table 1) most probably is secondary to the observed hypovitaminemia (23) found that total homocysteine was correlated negatively with the level of folate, vitamin B₁₂ and B₆, i.e decrease of vitamin B₁₂ and folic acid associated with hyperhomocysteinemia.

Exposure to fractionated radiation dose (0.7 and 1.2 Gy) in healthy mice caused, like in malignancy, significant decrease in folic acid and vitamin B₁₂ with significant increase of homocysteine. Vitamins are heterogeneous collection of small organic molecules that usually function in enhancing the utilization of other nutrient and in the maintenance of tissue structures. Since ionizing radiation had a destructive effect on the animal organs and tissues and there is negative relation between homocysteine and the two vitamins, the results concerning ionizing radiation was associated with disturbance in the oxidant- antioxidant ratio with increase production of free radicals which may affect vitamin metabolism. The disturbance in vitamin metabolism may offer another explanation for increase of homocysteine and decrease vitamins.

Regarding these tested parameters radiotherapy (exposure of the malignant mice to the two chosen dose of radiation) in the present study followed the same trend of isolated malignancy or radiation. These results are in coincidence with the study of Marianne *et al.* (24) who noticed rapid and persistent decrease in vitamin B₁₂ after radiotherapy in rectal cancer patients.

Homocysteine has an inverse relation with folate, low folate levels result in a high plasma homocysteine. Increased plasma homocysteine has also been shown to be closely related to cancer. Recent advances have proven that there is advanced-stage cancer cells might secrete homocysteine because it is high concentration might also be cytotoxic to the cancer cells. Therefore, it may be important for proliferating cells to maintain an optimum homocysteine concentration (15).

Ingvald *et al.* ⁽¹⁷⁾ observed significant decrease of folate and significant increase homocysteine in cervical cancer survivors after radiotherapy which are in accordance with our results. Similarly BRIAN *et al.* ⁽²⁵⁾ agreed with our results as they concluded that, the use of supplement of antioxidants during chemotherapy and radiotherapy should discouraged because of the possibility of tumor protection and reduced survival.

Also Eliana *et al.* ⁽²⁶⁾ found that evaluation of homocysteine concentrations during chemotherapy is extremely important because their levels increase during chemotherapy treatment since this is preferable.

In other words, can we control the tumor invasion by decrease intake of these two vitamins with consequent increase of homocysteine. As mentioned before malignant cells had high folate receptors and folate supply lead to increase cellular folic acid uptake; this, in its turn, increase DNA formation and mitotic division of cancer cells leading to flaring up of cancer cells, therefore, theoretically and according to these results for inhibition of cancer cells in situ before or after radiotherapy, the prevention of folic acid to reach the malignant cells may be beneficial. This prevention may be through either decrease intake of folic acid or blocking folate receptors as a part of immunotherapy of malignant tissues.

Low folic acid was accompanied with high homocysteine, which have cytotoxic effect on malignant cells ⁽²⁰⁾. Unfortunately this compound has also a hazardous effect on normal cells; intravenous injection of high level of homocysteine (200 Iu) lead to abundant cell death in the ganglion cells layer ⁽²⁸⁾.

The role of folate, and a water-soluble B-vitamin, in cancer development and progression remains highly controversial. The bases of this issue are whether folic acid (the synthetic form of folate) and/or high folate intake from dietary and supplemental sources and blood concentrations in general can increase cancer risk ⁽³⁰⁾.

However all these undesirable effects of hyperhomocysteine on other normal tissues

may be ignored compared with its lethal effect on cancer cells and therefore low folate and vitamin B₁₂ intake was recommended. Also we must differentiate between the role of these parameters in cancer incidence (where uptake of folic acid and vitamin B₁₂ prevent cancer incidence), and their role in treatment of cancer.

Conflicts of interest: Declared none.

REFERENCES

1. Ames BN (2001) DNA damage from micronutrient deficiencies is likely to be a major cause of cancer." *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis*, **475(1)**: 7-20.
2. Brustolin S, Giugliani R and Felix TM (2010) Genetic of homocysteine metabolism and associated disorders. *Brazilian Journal of Medical and Biological Research*, **43**: 1-7.
3. Aune ED, Deneo-Pellegrini H, Ronco A, Boffetta P, Acosta G, Mendilaharsu M, De Stefani E (2011) Dietary folate intake and the risk of 11 types of cancer: a case-control study in Uruguay. *Annals of Oncology*, **22(2)**: 444-451.
4. Miller JW and Ulrich CM (2013) Folic acid and cancer-where are we today? *The Lancet*, **381(9871)**: 974-976.
5. Kim YI (2008) Folic acid supplementation and cancer risk: point. *Cancer Epidemiology Biomarkers & Prevention*, **17(9)**: 2220-2225.
6. Choi SW and Mason N JB (2000) Folate and carcinogenesis: An integrated Schemc. *J Nutr*, **13**: 129-132.
7. Blount BC, Mack MM, Were CM., Macgregor J, Hiatt RA and Wang G (2007) Folate deficiency causes uracil misincorporation into human DNA and chromosome breakage Implication for cancer and neuronal damage. *Proc Natl Acad Sci, USA*, **94**: 3290-3295.
8. Dlewis SJ, Harbord RM, Harris R, Smith G (2006) Meta-analyses of observational and genetic association studies of folate intakes or levels and breast cancer risk. *J Natl Cancer Inst*, **98**: 1607-1622.
9. AL Lissowska J R, Gaudet MM, Brinton LA, Chanock SJ, and Peplonska B, (2007) Genetic polymorphisms in the one-carbon metabolism pathway and breast cancer risk: a population- based case- control study and meta-analyses. *Int J Cancer*, **120**: 269-2703.
10. Van Poppel G and H Van Den Berg (1997) Vitamins and cancer. *Cancer letters*, **114(1)**: 195-202.
11. Fenech M (1999) Micronucleus frequency in human lymphocytes is related to plasma vitamin B12 and homocysteine. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis*, **428(1)**: 299-304.
12. Larsson SC, Giovannucci E, Wolk A (2006) Folate intake, MTHFR polymorphism, and risk of oesophageal, Gastric,

- and pancreatic cancer: Ameta-analysis. *Gastroenterology*, **131**: 1271 – 1283.
13. Cui LH, Quan ZYU, Pio JM, Zhang TT, Jiang MU, Shin MH , Choi DJS (2016) Plasma Folate and Vitamin B12 Levels in Patients with Hepatocellular Carcinoma . *Int J Mol Sci*, **17(7)**: 1032.
 14. Locasale JW (2013) Serine, glycine and one-carbon units: cancer metabolism in full circle. *Nat Rev Cancer*, **13**: 572–583.
 15. Hasan T, Arora R, Bansal AK, Bhattacharya R, Sharma GS, Singh LRk (2019) Disturbed homocysteine metabolism is associated with cancer. *Experimental & Molecular Medicine*, **51**: 21.
 16. Zhang SM, Cook NR, Albert CM, Gaziano JM, Buring JE, Manson JE (2008) Effect of Combined Folic Acid, Vitamin B6, and Vitamin B12 on Cancer Risk in WomenA Randomized Trial. *JAMA*, **300(17)**: 2012–2021.
 17. Ingvild Vistad, Gunnar B. Kritensen, Sophie D. Fossa, Alv A. Dahi and Lars Markrid (2009) Intestinal malabsorption in long-term survivors of cervical cancer treated with radiotherapy. *International Journal of radiation oncology. Biology physics*, **73(4)**: 1141-1147.
 18. Ehrlich M (2002) DNA methylation in cancer: too much, but also too little. *Oncogene*, **21**: 5400–5413.
 19. Zhang D, Wen X, Wu W, Guo Y and Cui W (2015) Elevated homocysteine level and folate deficiency associated with increased overall risk of carcinogenesis: meta-analysis of 83 case-control studies involving 35,758 individuals. *PLoS ONE*, **10**: e0123423.
 20. Mc Cully KS (1976) Homocysteine thiolactone metabolism in malignant cells. *Cancer Res*, **36**: 3198-3202.
 21. Antony AC, Verma RS, Unune AR, La Rosa GA (1989) Identification of a Mg²⁺-dependent protease in human placenta which cleaves hydrophobic folate-binding proteins to hydrophilic forms. *Journal of Biological Chemistry*, **264(4)**: 1911-1914.
 22. Kane MA, and Waxman DS (1990) Role of folate binding proteins in folate metabolism. *Pathology Reviews*, Springer, 39-48.
 23. Ropinson K, Arheart K , Refsum H , Brattstrom L , Boers G , Ueland P , Ruppa, PP , Palma – Reis R . , Meleady R , Daily L . , Witteman, J , and D Gralamy (1998) low circulating folate and vitamin B6 concentrations risk factors for stroke , peripheral vascular disease and coronary artery disease . *Circulation*, **97**: 437-443.
 24. Guren MG, Schneeded J, Tveit KM, Ueland PM, Nexø E, Duelan S (2004) Biochemical signs of impaired Cobalamin status during and after radiotherapy for rectal cancer. *Int J Radiat Oncol Biol Phys*, **60 (4, 3)**: 807-813.
 25. Brian D, Lawenda KM, Kelly EJ, Ladas SM, Sagar AV, Jeffery BB (2008) Should supplemental antioxidant administration be avoided during chemotherapy and radiation therapy? *JNCI: Journal of the National Cancer Institute*, **100(11)**: 773–783.
 26. Yamashita EK, Teixeira BM, Yoshihara RN, Yoshi RKK, Alves BCA, Cehrke FS, Vilas-Boa VA, Correia JA, et al. (2014) Systemic chemotherapy interferes in homocysteine metabolism in breast cancer patients. *Journal of Clinical Laboratory Analysis*, **28**: 157-162.
 27. Moore P, Elsherbeny A, Roon P, Schoenlein, PV, Ganapathy V, and Smith SB(2001) Apoptotic cell death in the mouse retinal ganglion cell layer is induced in vivo by the excitatory amino acid homocysteine. *Exp Eye Res*, **73**: 45-57.
 28. Kim YI (2018) Folate and cancer: a tale of Dr. Jekyll and Mr. Hyde? *The American Journal of Clinical Nutrition*, **107 (2)**: 139–142.

