# Elevated physiological levels of folic acid can increase *in vitro* growth and invasiveness of prostate cancer cells

Lars F. Petersen*, Nigel T. Brockton†, Ashraf Bakkar*‡, Shuhong Liu*, Jing Wen§, Aalim M. Weljie§ and Tarek A. Bismar*¶**

*Department of Pathology and Laboratory Medicine, University of Calgary and Calgary Laboratory Services, †Division of Population Health Research, Alberta Health Services – Cancer Care, Calgary, Alberta, Canada, ‡Department of Biochemistry, Faculty of Dentistry, Sinai University, Sinai, Egypt, §Department of Biological Sciences, University of Calgary, ¶Southern Alberta Cancer Research Institute, Tom Baker Cancer Centre, Calgary, Alberta, and **The Segal Cancer Centre, Montreal, Quebec, Canada

## OBJECTIVES

• To investigate the effects of different folic acid concentrations on the growth and invasiveness of prostate cancer cell lines.
• To determine if observed changes are correlated with changes in levels of the potential prostate cancer biomarker, sarcosine, a byproduct of folate metabolism.

## MATERIALS AND METHODS

• The prostate cancer cell lines PC-3, LNCaP and DU145 were cultured in media containing 4, 20 or 100 nM of folic acid and assayed for growth over 9 days by counting viable cells at 3-day intervals, or for invasion by passage through a Matrigel-coated transwell membrane.
• Cells grown in the different folic acid media were collected and subjected to metabolomic analysis by gas chromatography and mass spectrometry to measure levels of intracellular sarcosine.

### What's known on the subject? and What does the study add?

Evidence has emerged identifying folic acid supplementation as a potential risk factor for cancer development or progression. Long-term folic acid supplementation has been shown to increase the risk of prostate cancer development by three-fold. Sarcosine is a byproduct of folate metabolism and has been proposed as a biomarker for aggressive prostate cancer phenotypes.

We looked at the effects of physiologically relevant levels of folic acid on *in vitro* prostate cancer cell growth and invasion, and demonstrated that higher levels can have the effect of increasing both of these biological processes. We also show that these changes toward a more aggressive phenotype are not linked to increased sarcosine levels, however other metabolic pathways may be involved.

## RESULTS

• The results show that higher levels of folic acid can increase cell growth in PC-3 and LNCaP prostate cancer cell lines, and may also increase the invasive capacity of PC-3, LNCaP and DU145 cells.
• We did not observe a correlation between increased invasion from higher folic acid concentrations and levels of sarcosine, but there were significant changes in other metabolites in cells grown in higher levels of folic acid.

## CONCLUSION

• These findings suggest that folic acid has an important and potentially negative role in prostate cancer progression.

## KEYWORD

prostate cancer, folic acid, invasion, metabolomics, sarcosine
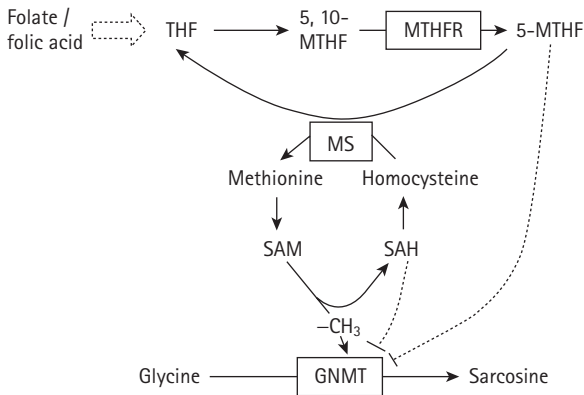
## INTRODUCTION

Prostate cancer is the most commonly diagnosed cancer and one of the most common causes of cancer death in men in North America [1]. Although certain risk factors for prostate cancer, including age and ethnicity, are not modifiable, it is important for men to consider what environmental factors can potentially reduce their risk, and recent studies have suggested that diet can play a critical role in this regard [2–4].

Folate is an essential dietary nutrient for healthy cell growth and division because of its role in the synthesis of purine nucleotides for DNA replication [5] and because it is a methyl donor for DNA, proteins and amino acids [6]; indeed, chemotherapeutic agents have been developed to exploit the sensitivity of tumour cells to folate deficiency [7]. Reduced folate intake has been associated with changes in DNA hypomethylation [8,9] and consequent changes in the expression of important genes involved in carcinogenesis [10]. Adequate folate nutrition helps maintain appropriate nucleotide pools; folate deficiency could lead to misincorporation of uracil and an increased risk of several cancers such as colon [11] or breast cancer [12]; however,

FIG. 1. *A schematic overview outlining the role of folate/folic acid in sarcosine synthesis. THF, tetrahydrofolate; 5,10-MTHF, 5,10-methylene tetrahydrofolate; MTHFR, methylene tetrahyrdofolate reductase; Hcy, homocysteine; MS, methionine synthase; SAM, S-adenosylmethionine; SAH, S-adenosylhomocysteine; GNMT, glycine N-methyltransferase. Modified from Luka, 2008 [38].*



## MATERIAL AND METHODS

### CELL CULTURE AND REAGENTS

Human prostate carcinoma cell lines, LNCaP, PC-3 and DU145, were obtained from the American Type Culture Collection (Manassas, VA, USA). Cell cultures were maintained according to the provider's instructions, and all cell lines were authenticated by on-site DNA (STR) profiling using an Applied Biosystems 3130 Genetic Analyzer with AmpF/STR Identifiler PCR Amplification Kit from Applied Biosystems (Life Technologies Corp., Carlsbad, CA, USA). Briefly, these cells were cultured in RPMI-1640 medium (11875-093, Life Technologies) supplemented with 10% fetal bovine serum (FBS) or folic acid-free RPMI-1640 (27016-021, Life Technologies) supplemented with indicated amounts of folic acid and 10% FBS.

### GROWTH ASSAY

The LNCaP, PC-3 and DU145 cells were cultured for 72 h in folic acid-free RPMI-1640 media with 10% FBS. Cells were seeded in 6-well plates at a density of $10^4$ cells/well and cultured in folic acid-free growth media, supplemented with folic acid to final concentrations of 4, 20 or 100 nM. After 3, 6 and 9 days of culture, the cells were trypsinized and washed, stained with trypan blue, and counted using a haemacytometer to assess growth and viability.

### INVASION ASSAY

We assessed the invasiveness of LNCaP, PC-3 and DU145 in response to different concentrations of folic acid in the growth media. Cell invasion experiments were performed with 8-μm porous transwell chambers precoated with BD Biosciences Matrigel (BD Biosciences, San Jose, CA, USA, catalogue no. 354480) according to the manufacturer's instructions. Briefly, cell lines were cultured in folic acid free RPMI-1640 growth media supplemented with 4, 20 or 100 nM of folic acid for 72 h, and then starved for 24 h in the same media without FBS. The serum-starved cells were diluted to a density of $1.5 \times 10^5$ cells/mL and 500 μL of cell suspension was placed in the upper compartment of the transwell chambers, in 24-well culture plates containing 750 μL of RPMI with 10% FBS as a chemoattractant in the lower compartment. Cells were

studies have also suggested that increased folate, or the synthetic folic acid supplements, have a potentially harmful role in these cancer types [13–18], as well as in prostate cancer [19]. These studies raise important public health questions, as the mandatory fortification of grain products with folic acid (0.14 mg per 100 g grain) has been in implementation since 1998 [20] and, according to data from the National Health and Nutrition Examination Survey, this contributes to approximately 140 μg of folic acid intake from fortified grain products across all quintiles of folate intake [21]. This programme of fortification was implemented to reduce the risk of neural tube defects in pregnant women [22,23], but the impact on cancer incidence and progression is unclear and may differ between cancer sites.

In one epidemiological study, Figueiredo *et al.* [19] reported that men taking a folic acid supplement (1 mg per day) over a 10-year period had a threefold higher risk of developing prostate cancer. Interestingly, those in the placebo control group with higher dietary or plasma folate levels had an inverse risk of prostate cancer, underscoring a potential difference between the metabolism of dietary folate and folic acid supplements, and the risk of prostate carcinogenesis associated with each.

In addition to its role in DNA synthesis and methylation, folate (in the form of 5-methyl tetrahydrofolate [5-MTFH]) is the upstream methyl group donor for the generation of S-adenosylmethionine (SAM), the universal methyl donor for the methylation of several metabolic substrates [24]. One such substrate, sarcosine, has recently been identified as an *in vivo* biomarker for aggressive prostate cancers [25]. Sreekumar *et al.* [25] have also shown in *in vitro* models that high sarcosine levels are associated with increased invasive capacity in prostate cancer cell lines. Folate and folic acid are important contributors to the one-carbon pool that is required for sarcosine biosynthesis via glycine N-methyltransferase (GNMT), as outlined in Fig. 1 [26]. There are complex relationships between the inhibition of methyltransferases and other enzymes in the folate metabolic pathways [27,28]. Consequently, it is plausible that a higher folate status could lead to increased sarcosine production in certain tissues. The epidemiological data linking folic acid supplementation with prostate cancer risk is inconsistent, subject to bias and confounding; there has been little work done to analyse these mechanisms under controlled conditions.

In the present study, we observe the effect of increased levels of folic acid on prostate cancer cell lines, *in vitro*, focusing on cell growth and invasiveness. Furthermore, we address the hypothesis that higher concentrations of folic acid will result in an increase in intracellular sarcosine levels, corresponding to an increase in prostate cancer cell invasion, and we present data to describe the global metabolomic profiling of prostate cancer cells grown in different levels of folic acid.

| Folic acid concentration | Prostate cancer cell line | | | | | | | | |
| | LNCaP | | | PC-3 | | | DU145 | | |
| | Total invaded | Total migrated | % Invasion | Total invaded | Total migrated | % Invasion | Total invaded | Total migrated | % Invasion |
| 4 nM | 38.7 | 722.3 | 5.4 | 104 | 212.8 | 48.9 | 97.3 | 531.2 | 18.3 |
| 20 nM | 62.3 | 631 | 9.9 | 152.3 | 227.7 | 66.9 | 312.5 | 617.2 | 50.6 |
| 100 nM | 255.3 | 1038 | 24.6 | 164.5 | 223.2 | 73.7 | 354.8 | 520.1 | 68.2 |

*Values are presented as the mean from three replicates of a representative experiment.*

incubated for 24 h (PC-3 and DU145) or 48 h (LNCaP), after which invasion through the Matrigel membrane was assessed. The invasive cells that had crossed to the underside of the membrane were fixed and stained in 1% crystal violet in 95% ethanol. Membranes were mounted on slides, imaged at 40× objective and counted in five different fields per sample. The number of invasive cells was compared with the number of cells that had migrated through an uncoated chamber to give percent invasion (Table 1, Fig. 3A).

## METABOLOMIC PROFILING

Samples for metabolomics analysis (≈1 million cells/sample) were first extracted using the procedure described by Bligh and Dyer [29]. The aqueous phases were dried under vacuum and subsequently derivatized. Briefly, 30 mL methoxyaminehydrochloride (20 mg/mL in pyridine) was added and incubated in a shaker at 37 °C for 3 h. Trimethylsilylation was then performed with 30 mL of N-methyl-N-trimethylsilyltrifluoroacetamide for 1h at 37 °C. Samples were diluted (1:5) with hexane before gas chromatography (GC) and mass spectrometry (MS) analysis. Analysis was carried out using Waters GCT Premier mass spectrometer (Waters Corp., Milford, MA, USA), and for all analyses the injector temperature was 275 °C. Helium was used as the carrier gas and maintained at a constant flow of 1.2 mL/min. The MS was operated in a range of 50–800 m/z. Derivatized aqueous extracts were analysed using a 30 m DB5-MS column in the splitless mode. The initial column temperature of 80 °C was held for 1 min, then the temperature was ramped at 12 °C per min to 320 °C and this temperature was held for 8 min. The sarcosine to

alanine ratio was monitored using ions from sarcosine eluting at 6.11 min (m/z 116, 147, 190, 218) and alanine at 5.71 min (m/z 116, 147, 190, 218, 233). Metabolites were identified using the NIST library and the GOLM metabolite database [30].

## STATISTICAL ANALYSIS

Data are presented as means (SD). Statistical significance was determined with Student's *t*-test unless otherwise stated. A *P* value of < 0.05 was considered to indicate statistical significance. Multivariate statistical analysis was performed using SIMCA-P (v. 12.0, Umetrics). In these cases, principal component analysis was used first to assess outliers, and then orthogonal partial least squares discriminant analysis (OPLS-DA) was used to assess which peaks from the GC-MS data were altered among treatment groups.

## RESULTS

### FOLIC ACID INCREASES GROWTH OF SOME PROSTATE CANCER CELLS

The growth rate of LNCaP and PC-3 cells increased at higher concentrations of folic acid (LNCaP: 4 nM vs 100 nM, *P* = 0.003; 20 nM to 100 nM, *P* = 0.024. PC-3: 4 nM vs 20 nM, *P* = 0.03; 4 nM vs 100 nM, *P* = 0.003; 20 nM vs 100 nM, *P* = 0.02); however, the proliferation rate of DU145 cells did not show a dose-dependent response to increased folic acid concentration. The growth curves for each cell line are shown in Fig. 2.

### FOLIC ACID INCREASES INVASIVENESS OF PROSTATE CANCER CELLS

For all three cell lines tested, there was a significantly greater proportion of cells

invading across the Matrigel matrix between the 4 nM and 100 nM folic acid groups (Fig. 3B) (LNCaP, *P* = 0.025; PC-3, *P* = 0.033; DU145, *P* = 0.015). Although statistically insignificant, we also observed an increase in percent invasion between the 20 nM and 100 nM folic acid groups in each cell line (Fig. 3A).

### INTRACELLULAR SARCOSINE LEVELS IN PROSTATE CANCER CELLS ARE NOT SIGNIFICANTLY AFFECTED BY INCREASED FOLIC ACID

To determine whether the increased invasive behaviour of our prostate cancer cell lines was attributable to an increase in sarcosine production in these cells, we performed metabolomic analysis on samples of LNCaP, PC-3 and DU145 that had been grown in 4, 20 or 100 nM folic acid media for one week. Sarcosine levels are expressed as a ratio of total sarcosine peaks to total alanine peaks (alanine levels do not change in relation to invasion in prostate cancer cells) [25]. LNCaP, PC-3 and DU145 all showed a subtle, but statistically insignificant, increase in intracellular sarcosine levels with increasing levels of folic acid (Fig. 4A). We similarly assessed extracellular sarcosine levels in the media from cells grown in the different folic acid concentrations, and likewise found no significant change in the ratio of total sarcosine to total alanine (data not shown).

To evaluate if the lack of sarcosine response to folic acid was indicative of a larger lack of global metabolic changes, we performed an untargeted metabolite analysis. In this experiment, GC-MS spectra from all samples were analysed for common peaks, with a total of 594 features (peaks) profiled. Any metabolite can give rise to one or more features because of derivitization procedures

*FIG. 2. Growth of prostate cancer cells in media containing varying concentrations of folic acid. LNCaP, PC-3, or DU145 cells were cultured in folate-free RPMI-1640 media supplemented with folic acid to the indicated concentrations (4, 20 and 100 nM folic acid). Cells were counted on days 3, 6 and 9, and viability was determined using trypan blue exclusion. Experiments were conducted in triplicate. Data are mean (SD) values from one experiment performed in triplicate; three independent experiments were performed in triplicate with similar results. *P < 0.05.*

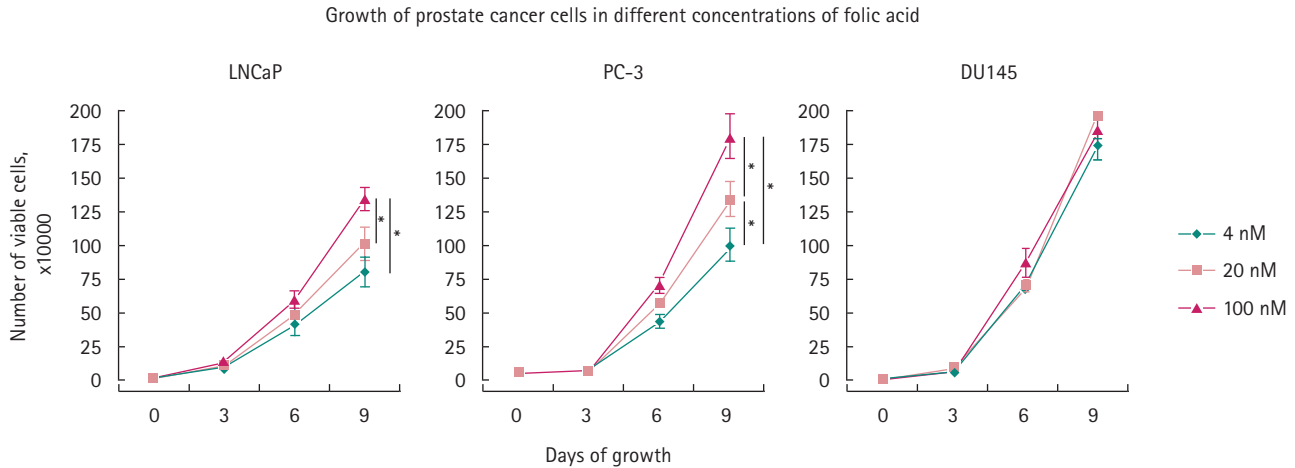Growth of prostate cancer cells in different concentrations of folic acid



*FIG. 3. Invasion of prostate cancer cells grown in media containing varying concentrations of folic acid. (A) The invasiveness of LNCaP, PC-3 and DU145 cells cultured in the indicated concentrations of folic acid media was ascertained by culturing cells in invasion chambers coated with Matrigel for 24–48 h, after which cells that had invaded and passed through a porous membrane were fixed, stained and counted. Percent invasion was calculated by dividing the total number of invaded cells by the total number of cells that migrated across an uncoated chamber membrane in the same folic acid conditions. (B) Representative micrographs of fixed and stained cells that invaded through Matrigel in the indicated concentrations of folic acid media. Experiments were performed in triplicate. Data are mean (SD) values from one experiment performed in triplicate; three independent experiments were performed in triplicate with similar results. *P < 0.05.*
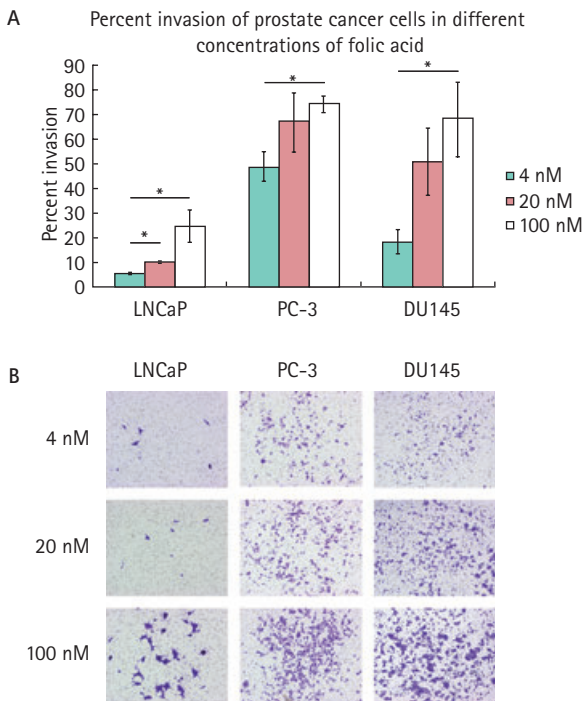


or isomeric heterogeneity. Multivariate statistical analysis was performed using OPLS-DA analysis on a subset of the most important 187 peaks, indicating a significant global difference between the samples treated with 4, 20 and 100 nM folic acid (Fig. 4B, $P = 0.006$). Several of the most significant peaks contributing to the differences between groups were identified, one of which was glycine, a key metabolite in sarcosine formation (Fig. 1). The levels of glycine were clearly highest in the cells grown in 100 nM folic acid, compared with cells grown in the 4 nM and 20 nM groups (Fig. 4C; LNCaP, $P = 0.018$; PC-3, $P < 0.001$) The similarity between the 4 nM and 20 nM treated groups was also evident in the global profile and, to further investigate the differences between the high and low levels of folic acid treatment, another model was built comparing only the 4 nM and 100 nM treated groups (Fig. 4D). In this case, there was a clear difference between the global metabolite profiles. Table 2 shows a list of the metabolites that were most significantly altered between these two groups.

## DISCUSSION

The mandatory fortification of grain products with folic acid was introduced in 1998 to reduce the incidence of neural tube defects in babies [22,23], but there is increasing concern that there may be unanticipated consequences of this apparent public health success [13–18]. In particular,

there is mounting evidence to suggest that dietary supplementation with folic acid may have negative health effects for patients with existing adenomas [17], or may increase the risk of some types of cancers [19], To our knowledge, there have been no studies performed to assess the direct effect of folic acid on prostate cancer cell invasion, *in vitro*. Consequently, in the present study, we investigated whether increased folic acid concentrations were associated with increased proliferation of prostate cancer cell lines and whether increased folic acid concentration increased the invasive capacity of these cells. We also investigated whether increased folic acid is associated with elevated levels of intracellular sarcosine, which has previously been linked to aggressive prostate cancer phenotypes *in vivo* [19].

We chose 4 nM, 20 nM, and 100 nM final concentrations of folic acid, as these represent typical low, average and high physiological serum levels, respectively, and have been previously shown to have an effect on cell growth and viability of cultured lymphocytes [31]. Other studies have indirectly shown that hyper-physiological folic acid levels (standard RPMI-1640 containing 2.3 μM of folic acid) dramatically increase the growth rates of LNCaP, DU145, and PC-3 prostate cancer cells compared with 'low' (1 nM folic acid) and 'physiological' (25 nM folic acid) levels [32,33]. The growth assay results of the present study in LNCaP and PC-3 cell lines support these *in vitro* studies, and provide direct evidence to suggest that prostate cancer cells grow faster in a high, physiologically relevant folic acid environment. This is particularly significant because several chemotherapeutic agents, including Methotrexate and 5-Fluorocil, have been developed to inhibit the metabolism of folate in rapidly dividing cancer cells [7].

A key trait of aggressive cancers is the metastatic capabilities of cancer cells, which are able to escape the primary tumour site by invading blood vessels and travelling to secondary sites. In the present study, we showed that prostate cancer cells are significantly more invasive at a high physiological level of folic acid (100 nM) compared with a folic acid-deficient state (4 nM). A possible explanation for these results is that folic acid deficiency is linked to genomic instability and DNA damage in

FIG. 4. Metabolomic profiling of prostate cancer cells grown in different concentrations of folic acid. (**A**) Intracellular levels of sarcosine were measured after metabolomic profiling of samples of LNCaP, PC-3 and DU145 cells grown in media with 4, 20 or 100 nM of folic acid. Sarcosine levels are expressed as a ratio of sarcosine : alanine for each sample. (**B**) Multivariate statistical analysis of global metabolite profiles from groups treated with different levels of folic acid. Scores plot from OPLS-DA analysis showing overall global metabolite relationships among treatment groups. Each point represents an individual sample, and its position is determined by a combination of the underlying intracellular metabolite concentrations. Samples are coloured by folic acid concentration (red squares, 4 nM; green circles, 20 nM; blue diamonds, 100 nM). (**C**) Representative bar plot of relative glycine concentrations by folic acid concentration and per cell line. (**D**) OPLS-DA scores plot of 4 nM and 100 nM samples, coded as in (**A**). Data are mean (SEM) values. *P < 0.05.
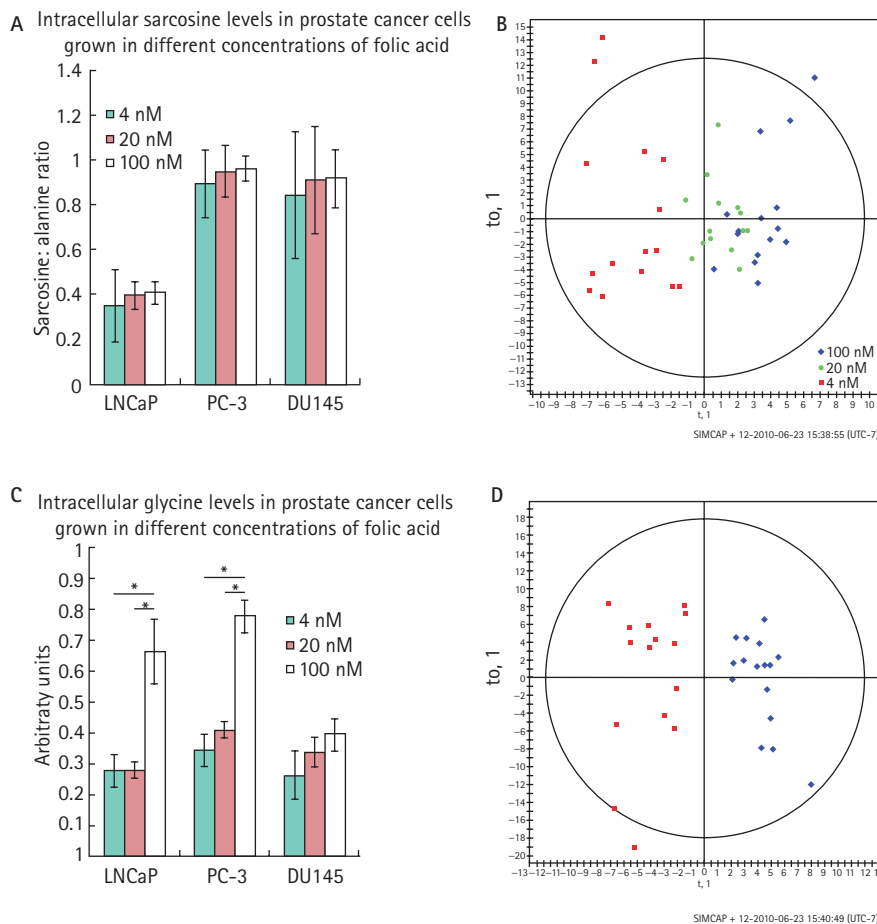


lymphocyte cells, whereas high levels of folic acid or folate derivatives have been shown to alleviate these negative effects on DNA stability [34–36]. In the context of folic acid fortification and supplementation, therefore, a more important comparison is between the groups of cells grown in typical physiological levels of folic acid (20 nM) and those grown in high levels. Here we also document a higher proportion of invasive cells in high folic acid, but this difference was not statistically significant in any of our cell lines when cultured in folic acid concentrations of 20 nM and 100 nM.

However, combined with cell growth results showing significantly increased growth with higher folic acid concentrations, these data suggest that increased concentrations of folic acid make these cell lines more aggressive than low concentrations. The lack of consistency in DU145 proliferative activity compared with that of LNCaP and PC3 may be attributable to differences in the genetic composition and base growth rates among those cell lines.

A recent study suggested that elevated levels of sarcosine detected in the urine

TABLE 2 *Metabolites significantly altered by increased levels of folic acid, as identified by multivariate statistical analysis on global metabolic profiles of prostate cancer cells grown in 4 and 100 nM folic acid*

| Metabolite | KEGG ID* | Up/Down |
|---|---|---|
| Glycine | C00037 | + |
| Glycerol-3-phosphate | C00093 | + |
| Adenosine-5-monophosphate | C00020 | + |
| Glyceric acid-3-phosphate | C00597 | + |
| Guanine | C00242 | + |
| Cholesterol | C00187 | + |
| alpha-Lactose | C00243 | + |
| D/L-Lactic acid | C00186/C00256 | + |
| L-Tryptophan | C00078 | + |
| β-Hydroxybutyric acid | C01089 | + |
| Stearic acid | C01530 | + |
| Raffinose | C00492 | + |
| Malic acid | C00149 | + |
| a-Sorbitol | C00794 | + |
| N-acetylaspartic acid | C01042 | − |
| Benzoic acid | C00180 | − |
| Octanoic acid | C06423 | − |
| Adenine | C00147 | − |

*KEGG IDs for each metabolite are provided as a reference for potential metabolic pathways that may be changed through alteration of these metabolites.* + *indicates a significant increase in each metabolite in the 100 nm groups compared with 4 nm groups;* − *indicates a significant decrease. Metabolites are ordered by significance.*

of patients with prostate cancer were associated with a more aggressive tumour phenotype [25]. Sarcosine is formed by the methylation of glycine, catalysed by GNMT; the direct methyl group donor is SAM. The predominant pathway for regeneration of SAM, in humans, is via the remethylation of homocysteine to methionine, for which 5-MTFH is the methyl donor; betaine-homocysteine methyltransferase expression is limited to hepatic and renal tissues in humans [37], therefore, we might expect higher folate availability to facilitate increased sarcosine production. However, the results of the present study do not support a significant correlation between folic acid levels and intracellular sarcosine in our prostate cancer cell lines. There are two explanations that could account for these results. First, despite the requirement for methylated folate moieties for the generation of SAM, the methyl donor for sarcosine production from glycine, 5-MTFH, actually inhibits GNMT activity, at least in hepatic tissues [26,38]. Second, there is some evidence that some methytransferases act in a tissue-specific manner [39].

Although there is currently no information on GNMT enzymatic activity in prostate tissue, GNMT has been identified as a tumour susceptibility gene for prostate cancer, and was found to be underexpressed in ≈ 80% of prostate cancer tissues [40]. Interestingly, our metabolomic analysis indicated that glycine was significantly increased in cells grown in high levels of folic acid, which is contrary to that which we would expect if sarcosine was being produced at higher levels, and may suggest a disruption in the sarcosine synthesis pathway. It is worth noting, however, that glycine is involved in a number of other metabolic reactions, and the static concentrations determined could also be influenced by a number of other pathways.

The discovery of sarcosine as a potential biomarker for aggressive prostate cancers has excited professionals in the field [41], and has also sparked some controversy, with two very recent studies showing that sarcosine detected in urine samples is not significantly increased in metastasized castration-resistant prostate cancers or

cancers with high Gleason scores [42,43]. Although the sarcosine results in the present study are in the context of folic acid metabolism, we also did not observe a correlation between intracellular sarcosine levels and invasion *in vitro* in our prostate cancer cells. Sreekumar *et al.* [44], however, maintain that sarcosine levels, expressed relative to alanine levels, are increased in biopsy-positive individuals.

Our metabolomic profiling indicated several other metabolites that are significantly altered between low and high folic acid levels in these prostate cancer cell lines, which could be of potential importance in prostate cancer progression because many of these metabolites represent important components of canonical metabolic pathways. Some of the potential pathways altered by increased folic acid levels are presented in Table S1, and the interconnectivity of these pathways and the metabolites therein are illustrated in Fig. S1.

In summary, the results of the present study indicate that physiologically high levels of folic acid are associated with increased growth of the prostate cancer cell lines LNCaP and PC-3 and increased invasive capacity of LNCaP, PC-3, and DU145. These results are relevant from a public health perspective, but more work will need to be done before we can conclude that fortification of food with folic acid, or dietary folic acid supplementation is a cause for concern in the context of cancer formation or progression. In particular, one theme that is beginning to emerge is the difference in biological activity between the synthetically produced folic acid (which is more stable but needs to be reduced and methylated to become metabolically active), and naturally occurring forms of folate (specifically forms that are already methylated) that are obtained from consumption of unfortified food sources [19]. Appropriate *in vivo* models in which both synthetic and natural folic acid supplementations are used will shed more light on this issue.

## REFERENCES

1 **Jemal A, Siegel R, Ward E, Hao Y, Xu J, Thun MJ.** Cancer statistics, 2009. *CA Cancer J Clin* 2009; **59**: 225–49

2 **Mazhar D, Waxman J.** Diet and prostate cancer. *BJU Int* 2004; **93**: 919–22

3 **Shirai T, Asamoto M, Takahashi S, Imaida K.** Diet and prostate cancer. *Toxicology* 2002; **181–182**: 89–94

4 **Zlotta A.** Weighing the data on diet and prostate cancer. *Can Urol Assoc J* 2008; **2**: 516–7

5 **James SJ, Basnakian AG, Miller BJ.** *In vitro* folate deficiency induces deoxynucleotide pool imbalance, apoptosis, and mutagenesis in Chinese hamster ovary cells. *Cancer Res* 1994; **54**: 5075–80

6 **Lucock M.** Is folic acid the ultimate functional food component for disease prevention? *BMJ* 2004; **328**: 211–4

7 **Bertino JR.** Cancer research: from folate antagonism to molecular targets. *Best Pract Res Clin Haematol* 2009; **22**: 577–82

8 **Rampersaud GC, Kauwell GP, Hutson AD, Cerda JJ, Bailey LB.** Genomic DNA methylation decreases in response to moderate folate depletion in elderly women. *Am J Clin Nutr* 2000; **72**: 998–1003

9 **Yi P, Melnyk S, Pogribna M, Pogribny IP, Hine RJ, James SJ.** Increase in plasma homocysteine associated with parallel increases in plasma S-adenosylhomocysteine and lymphocyte DNA hypomethylation. *J Biol Chem* 2000; **275**: 29318–23

10 **Jhaveri MS, Wagner C, Trepel JB.** Impact of extracellular folate levels on global gene expression. *Mol Pharmacol* 2001; **60**: 1288–95

11 **Choi SW, Mason JB.** Folate status: effects on pathways of colorectal carcinogenesis. *J Nutr* 2002; **132**: 2413S–8S

12 **Lajous M, Lazcano-Ponce E, Hernandez-Avila M, Willett W, Romieu I.** Folate, vitamin B(6), and vitamin B(12) intake and the risk of breast cancer among Mexican women. *Cancer Epidemiol Biomarkers Prev* 2006; **15**: 443–8

13 **Kim Y.** Does a high folate intake increase the risk of breast cancer? *Nutr Rev* 2006; **64**: 468–75

14 **Kim YI.** Role of folate in colon cancer development and progression. *J Nutr* 2003; **133**: 3731S–9S

15 **Ulrich C, Potter J.** Folate and cancer-timing is everything. *JAMA* 2007; **297**: 2408–9

16 **Ulrich CM, Potter JD.** Folate supplementation: too much of a good thing? *Cancer Epidemiol Biomarkers Prev* 2006; **15**: 189–93

17 **Cole BF, Baron JA, Sandler RS** *et al*. Folic acid for the prevention of colorectal adenomas: a randomized clinical trial. *JAMA* 2007; **297**: 2351–9

18 **Kim YI.** Folate, colorectal carcinogenesis, and DNA methylation: lessons from animal studies. *Environ Mol Mutagen* 2004; **44**: 10–25

19 **Figueiredo JC, Grau MV, Haile RW** *et al*. Folic acid and risk of prostate cancer: results from a randomized clinical trial. *J Natl Cancer Inst* 2009; **101**: 432–5

20 **Pfeiffer CM, Johnson CL, Jain RB** *et al*. Trends in blood folate and vitamin B-12 concentrations in the United States, 1988–2004. *Am J Clin Nutr* 2007; **86**: 718–27

21 **Yeung L, Yang Q, Berry RJ.** Contributions of total daily intake of folic acid to serum folate concentrations. *JAMA* 2008; **300**: 2486–7

22 Prevention of neural tube defects: results of the Medical Research Council Vitamin Study. MRC Vitamin Study Research Group. *Lancet* 1991; **338**: 131–7

23 **Wald N.** Folic acid and the prevention of neural tube defects. *Ann N Y Acad Sci* 1993; **678**: 112–29

24 **Brosnan JT, Jacobs RL, Stead LM, Brosnan ME.** Methylation demand: a key determinant of homocysteine metabolism. *Acta Biochim Pol* 2004; **51**: 405–13

25 **Sreekumar A, Poisson LM, Rajendiran TM** *et al*. Metabolomic profiles delineate potential role for sarcosine in prostate cancer progression. *Nature* 2009; **457**: 910–4

26 **Luka Z, Mudd SH, Wagner C.** Glycine N-methyltransferase and regulation of S-adenosylmethionine levels. *J Biol Chem* 2009; **284**: 22507–11

27 **Takata Y, Huang Y, Komoto J** *et al*. Catalytic mechanism of glycine N-methyltransferase. *Biochemistry* 2003; **42**: 8394–402

28 **Beagle B, Yang TL, Hung J, Cogger EA, Moriarty DJ, Caudill MA.** The glycine N-methyltransferase (GNMT) 1289 C->T variant influences plasma total homocysteine concentrations in young women after restricting folate intake. *J Nutr* 2005; **135**: 2780–5

29 **Bligh EG, Dyer WJ.** A rapid method of total lipid extraction and purification. *Can J Biochem Physiol* 1959; **37**: 911–7

30 **Hummel J, Strehmel N, Selbig J, Walther D, Kopka J.** Decision tree supported substructure prediction of metabolites from GC-MS profiles. *Metabolomics* 2010; **6**: 322–33

31 **Kimura M, Umegaki K, Higuchi M, Thomas P, Fenech M.** Methylenetetrahydrofolate reductase C677T polymorphism, folic acid and riboflavin are important determinants of genome stability in cultured human lymphocytes. *J Nutr* 2004; **134**: 48–56

32 **Yao V, Bacich D.** Prostate specific membrane antigen (PSMA) expression gives prostate cancer cells a growth advantage in a physiologically relevant folate environment *in vitro*. *Prostate* 2006; **66**: 867–75

33 **Yao V, Berkman CE, Choi JK, O'Keefe DS, Bacich DJ.** Expression of prostate-specific membrane antigen (PSMA), increases cell folate uptake and proliferation and suggests a novel role for PSMA in the uptake of the non-polyglutamated folate, folic acid. *Prostate* 2010; **70**: 305–16

34 **Ni J, Lu L, Fenech M, Wang X.** Folate deficiency in human peripheral blood lymphocytes induces chromosome 8 aneuploidy but this effect is not modified by riboflavin. *Environ Mol Mutagen* 2010; **51**: 15–22

35 **Wang X, Thomas P, Xue J, Fenech M.** Folate deficiency induces aneuploidy in human lymphocytes in vitro-evidence using cytokinesis-blocked cells and

probes specific for chromosomes 17 and 21. *Mutat Res* 2004; **551**: 167–80

36 **Wang X, Fenech M.** A comparison of folic acid and 5-methyltetrahydrofolate for prevention of DNA damage and cell death in human lymphocytes in vitro. *Mutagenesis* 2003; **18**: 81–6

37 **Sunden SL, Renduchintala MS, Park EI, Miklasz SD, Garrow TA.** Betaine-homocysteine methyltransferase expression in porcine and human tissues and chromosomal localization of the human gene. *Arch Biochem Biophys* 1997; **345**: 171–4

38 **Luka Z.** Methyltetrahydrofolate in folate-binding protein glycine N-methyltransferase. *Vitam Horm* 2008; **79**: 325–45

39 **Rowling MJ, McMullen MH, Chipman DC, Schalinske KL.** Hepatic glycine N-methyltransferase is up-regulated by excess dietary methionine in rats. *J Nutr* 2002; **132**: 2545–50

40 **Huang YC, Lee CM, Chen M *et al.*** Haplotypes, loss of heterozygosity, and expression levels of glycine N-methyltransferase in prostate cancer. *Clin Cancer Res* 2007; **13**: 1412–20

41 **Couzin J.** Biomarkers. Metabolite in urine may point to high-risk prostate cancer. *Science* 2009; **323**: 865

42 **Struys EA, Heijboer AC, van Moorselaar J, Jakobs C, Blankenstein MA.** Serum sarcosine is not a marker for prostate cancer. *Ann Clin Biochem* 2010; **47**: 282

43 **Jentzmik F, Stephan C, Miller K *et al.*** Sarcosine in Urine after Digital Rectal Examination Fails as a Marker in Prostate Cancer Detection and Identification of Aggressive Tumours. *Eur Urol* 2010; **58**: 12–8

44 **Sreekumar A, Poisson LM, Rajendiran TM *et al.*** Re: Florian Jentzmik, Carsten Stephan, Kurt Miller *et al.* Sarcosine in Urine after Digital Rectal Examination Fails as a Marker in Prostate Cancer Detection and Identification of Aggressive Tumours. *Eur Urol* 2010; **58**: 12–8. *Eur Urol* 2010; **58**: e29–30; author reply e2–2

Correspondence: Tarek A. Bismar, University of Calgary Faculty of Medicine, Department of Pathology and Laboratory Medicine, Rockyview General Hospital, Calgary Laboratory Services, 7007, 14 Street SW, Calgary, Alberta T2V 1P9, Canada.
e-mail: tarek.bismar@cls.ab.ca

Abbreviations: **SAM**, S-adenosylmethionine; **GNMT**, glycine N-methyltransferase; **FBS**, fetal bovine serum; **GC**, gas chromatography; **MS**, mass spectrometry; **OPLS–DA**, orthogonal partial least squares discriminant analysis; **5–MTFH**, 5-methyl tetrahydrofolate.

## SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article.

FIG. S1. Interconnecting canonical metabolic pathways that are potentially altered with increased concentrations of folic acid in prostate cancer cells. Metabolites highlighted in green are increased, and those highlighted in red are decreased in cells grown in 100 nM folic acid compared with cells grown in 4 nM folic acid.

Table S1 Potential canonical metabolic pathways altered with increased concentrations of folic acid.

Please note: Wiley-Blackwell are not responsible for the content or functionality of any supporting materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.