Folate metabolism in the human colon: cellular responses identified through *in vitro* studies

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Abstract

Low folate intake influences the risk of not only neural tube defects and vascular diseases, but also colorectal tumours. Folate forms have different roles in the one-carbon metabolism. This includes DNA synthesis and methylation reactions. Folate is present as 5'-methyltetrahydrofolate (MTHF) in the diet and plasma, while in supplements the synthetic form is present, known as folic acid (pteryol glutamic acid, PGA). In animal studies, PGA seemed to have dual modulatory effects on colorectal carcinogenesis depending on the timing and dose of intervention. In this thesis the effect of different folate forms and dosages in the colon were studied in vitro using a nutrigenomics approach. The DNA microarray technique was applied to study the expression pattern of thousands of genes, and assess known and identify new processes affected by folate. Gene expression analysis revealed that high PGA concentrations increased energy metabolism and lowered iron metabolism related genes in the human colorectal cell line, HT29. Further investigation showed that intracellular iron concentration was decreased in three human colorectal cell lines. This effect on iron metabolism was studied in an existing human randomized, placebo-controlled trial. After six months of folate supplementation the serum ferritin, but not the colonic iron, was decreased. We also studied the difference between PGA and MTHF exposure in HT29 cells, using a physiological concentration range of both folates. A dose dependent effect was found for both folates. The normal serum values of PGA increased genes involved in the folate metabolism, compared to identical MTHF concentration. Supplemental concentrations of MTHF affected protein synthesis, endoplasmatic reticulum/Golgi and cancer related genes, compared to identical PGA concentrations. More specifically, high MTHF increased differentiation, adhesion and cell viability; all known cancer related processes. This may suggest a more beneficial effect of MTHF as compared to PGA. However we need to further investigate the effect of PGA and MTHF on iron metabolism, cancer related processes or energy metabolism in humans. Hopefully this will lead to more insight in the effect of folate supplementation with regard to colorectal carcinogenesis.

Contents

1

Introduction

For more than 70 years folate, vitamin B11, has been an important vitamin in nutrition research (1). Several epidemiological studies have shown that low folate intake increases the risk of not only neural tube defects and vascular diseases, but also colorectal tumours (2,3). At the present time in nutrition research, molecular biology and genetics are being applied in combination with epidemiology and physiology (4). Recently the mapping of the human genome was accomplished and new DNA technologies are developing rapidly, for example transcriptomic techniques, such as DNA microarray technology (5-7). This technology allows scientists to investigate the expression of thousand of genes simultaneously, to gain insight in entire pathways and identify new ones (4,7,8).

Folate is involved in several known processes, such as proliferation, apoptosis and differentiation and these processes are altered in carcinogenesis. Therefore, these processes were studied. In addition, we wanted to identify new processes in which folate is involved. To extrapolate the *in vitro* identified effects to *in vivo*, these effects will be studied in a folate intervention trial. Ideally, the outcome of these studies would provide insight into cancer related processes, which in turn would provide much needed early nutrient sensitive biomarkers.

In the research presented here, the effect of folate and its metabolites on gene expression in the human colon was investigated. This was studied via a nutrigenomics approach using the transcriptomic technique DNA microarrays. This was accomplished through:

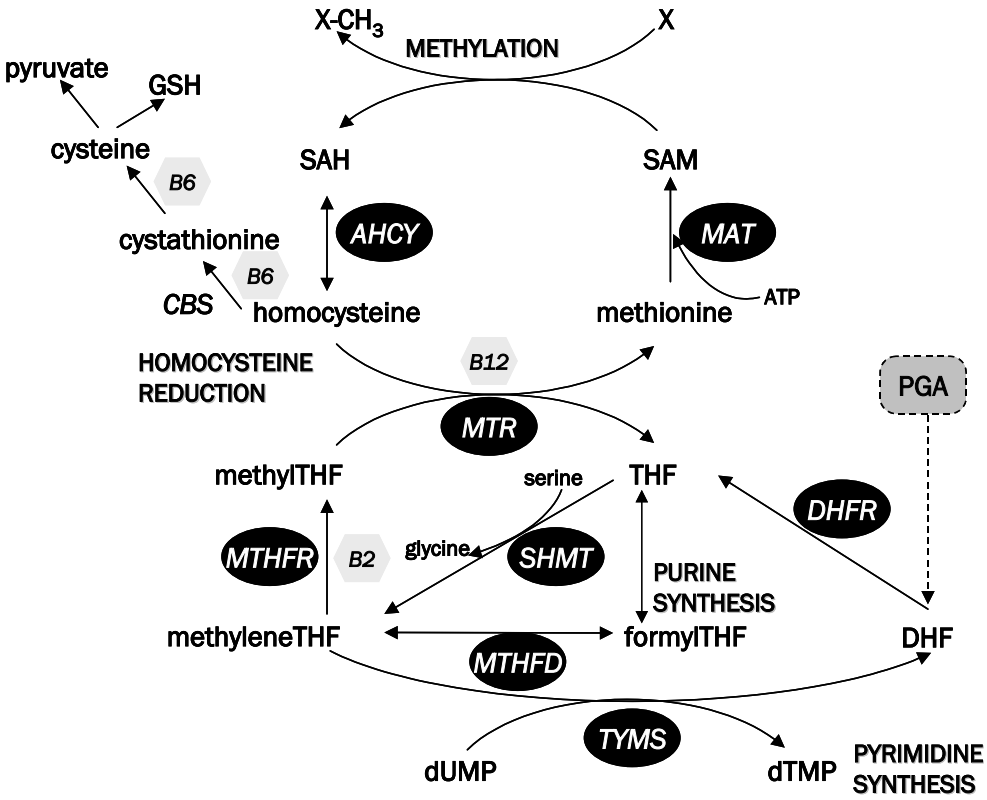
- Optimising the DNA microarray technique used for our studies
- Investigating effect of folate on proliferation, apoptosis and differentiation and identifying new processes *in vitro*
- Investigating the difference between different forms of folate
- Extrapolating the effects found *in vitro* to *in vivo* using a human folate intervention trial

In this introduction the biological role of folate in healthy cells and in relation to colorectal carcinogenesis is discussed. Also, an overview of literature on folate research in nutrigenomics and *in vitro* studies is presented. Finally, the rationale and the outline of this thesis are addressed.

Folate related processes and metabolites

Folate is an essential B-vitamin for many physiological functions. Various folate forms have different roles in the one-carbon metabolism necessary for cell homeostasis, such as DNA synthesis, methylation reactions and the reduction of homocysteine (3,9). In the one-carbon metabolism other B-vitamins also play an important role as cofactors for enzymes, such as vitamin B12 for methionine synthase and vitamin B2 for 5,10'-methyltetrahydrofolate reductase (MTHFR) (10,11) (Figure 1.1). A shortage

Figure 1.1 Schematic representation of folate metabolism (adapted from Scott et al (10))



*<u>Abbreviations:</u> **AHCY:** S-adenosylhomocysteine hydrolase; **ATP:** adenosine triphosphate; **B2:** vitamine B2, riboflavine; **B6:** vitamine B6, pyridoxine; **B12:** vitamine B12, cobalamine; **CBS:** cystathionine beta-synthase; **DHF:** dihydrofolate; **DHFR:** dihydrofolate reductase; **GSH:** gluthatione; **THF:** tetrahydrofolate; **MAT:** methionine adenosyltransferase; **MTHFR:** 5,10-methylenetetrahydrofolate reductase; **MTHFD:** methylenetetrahydrofolate dehydrogenase; **MTR:** 5-methyltetrahydrofolate-homocysteine -methyltransferase/methionine synthase; **PGA:** folic acid, pteryol glutamic acid; **SAH:** S-adenosyl homocysteine; **SAM:** S-adenosyl methionine; **SHMT:** serine hydroxymethyltransferase; **TYMS:** thymidylate synthase; **X:** DNA, RNA, lipids, proteins, neurotransmitters*

of folates leads to impaired activity of these metabolic processes. Cells that divide rapidly demand extra high levels of nucleotides. Folate serves as a cofactor in both pyrimidines

and purines syntheses. The conversion of uracil into thymidine is part of DNA synthesis (Figure 1.1). When this reaction works insufficiently, the dUMP/dTMP ratio is increased and uracil is incorporated into the DNA. This can lead to mutations in DNA and ultimately DNA strand breaks; both processes are known to be involved in cancer. Another event in carcinogenesis is the increased gene expression of oncogenes due to global DNA hypomethylation, thus leading to increased cellular proliferation (2). Hypomethylation of the CpG islands of the promoter regions of oncogenes occurs when the SAM pool, the universal methyl donor (12), is diminished (Figure 1). At the same time hypermethylation of the CpG islands of the promoter regions of tumour suppressor genes can occur (13). The expression of a number of other genes can be influenced by this aberrant DNA methylation, for example genes needed for differentiation (14). Loss of differentiation is often associated with carcinogenesis (15).

Multiple markers to investigate folate response are known and new ones are still being developed, all serving their own needs and with their own pros and cons (Table 1.1) (16). These help us to gain insight in the processes in which folate is involved. Folate depletion does not occur in a symmetric fashion among various tissues in the body, therefore folate measurement in the tissue of interest may provide additional information. The same is true for homocysteine levels in serum or plasma. Homocysteine can be used as a functional measure of folate respons, although homocysteine levels are increased by folate and/or vitamine B12 depletion (16,17). Another functional measure is S-adenosyl methionine (SAM) and S-adenosyl homocysteine (SAH), increase of SAM and decrease of

Table 1.1 Overview folate identified pathways

Biomarker	Type marker	Pros and cons
Homocysteine	functional assessment	Sensitive indicator for folate and vitamin B12 depletion, gender dependent
SAM/SAH	functional assessment	Functional reflection of folate status, correlates with methyl acceptors, only represents single tissueplasma SAH looks promising biomarker, but not SAM
Genomic DNA methylation	functional assessment	Emerging as sensitive folate status biomarker, quantitative method, genomic hypomethylation also due to methionine, choline and vitamin B12 deficiency
Uracil DNA misincorporation	functional assessment	Biomarker of folate depletion especially in cell lines; under development
Gene expression	nutrigenomics	Under development, tissue specific, identification of underlying molecular mechanism

SAH is generally correlated to the extent of DNA methylation (Figure 1.1). The methylation of CpG islands by SAM and/or SAH, can be directly measured by enzymatic semiquantitative assays or with a new quantitative liquid chromatography/mass spectrometry method (16). The last functional measurement is uracil misincorporation into DNA (Figure 1.1).

In this thesis we did not only study the effect of folate exposure on proliferation, apoptosis and differentiation, but also on intracellular folate metabolites. This was done because separate animal studies showed that folate deficiency and folic acid (pteryol glutamic acid, PGA) supplementation correlates with plasma folate status, but does not correlate with the mucosal folate status, SAM, SAH and SAM/SAH ratio (18-20).

In tumour prone animals, both folate deficiency and elevated PGA intake increased intestinal neoplasia (21-23). In humans, one study investigated the expression level of folate related enzymes and their association with the outcome of colorectal cancer. They showed that the mean gene expression levels of reduced folate carrier, folylpolyglutamate synthase, γ-glutamyl hydrolase and thymidilate synthase were significantly higher in the tumour biopsies compared with the normal mucosa (24). Several observational epidemiologic studies have investigated the effect of folate on colorectal carcinogenesis (2,13). Effect of PGA supplementation on colorectal carcinogenesis has only been examined in a limited number of small, prospective intervention trials. These few folate interventions are conducted using 2-20mg PGA per day, with or without B12, in patients with colorectal polyps or adenomas. PGA supplementation in these studies led to decreased global DNA hypomethylation, increased SAM, decreased tumour suppressors expression and decreased cell proliferation (25-31). The relative roles of various folate turnover mechanisms are not fully defined, nor are the consequences of changes in folate intake or turnover (32). A fundamental issue is which biological samples are most predictive of the response to the bioactive food component in the target tissue (8). For example, colonic mucosal concentrations of folate seems to be predicted accurately by blood measurements of folate status, although only among individuals ingesting physiological quantities of folate (33,34).

In conclusion, B-vitamins play an important role in the one-carbon metabolism. Folate, vitamin B11, is needed for DNA synthesis, methylation reactions and reduction of homocysteine. There is an accumulating body of evidence suggesting that a low folate status predisposes to the development of colorectal cancers. Mechanisms in which folate is involved are uracil misincorporation and aberrant DNA methylation affecting the gene expression. Few folate interventions are conducted. Those conducted all used high concentrations of PGA, with or without B12, in patients with colorectal polyps or adenomas. Only one of these studies investigated the effect on global DNA methylation, but none explored the effect on DNA uracil misincorporation. High and low folate status have an effect on DNA methylation and uracil misincorporation in the colon; other processes in which folate is involved remain unclear.

In dietary supplementation the oxidised form folic acid (PGA) is used, while in the diet the natural reduced folates are present; mainly 5-methyltetrahydrofolate (MTHF) and formyltetrahydrofolate. PGA contains only a single glutamate, and exhibits greater bioavailability than natural occurring folate (9,13,35). Once transported into cells, folic acid is converted to tetrahydrofolate and is chemically identical to natural food folates (9,13), which can be further converted to MTHF. MTHF is the primary form of folate entering human circulation from the intestinal cells (13,36). Until now studies investigating the difference between PGA and MTHF only studied the bioavailability (37-39) or the lowering of homocysteine (39-43). MTHF can restore endothelial function in hypercholesterolemic patients, probably by affecting cellular oxidative metabolism (44). In patients with coronary artery disease, 5 mg PGA for 6 weeks or intra-arterial 5-MTHF (achieving a plasma concentration of at least 1 mM), both improved flow-mediated dilatation, but only PGA lowered homocysteine concentration (45). Another study concluded that supplementation of PGA is not more efficient than MTHF in preventing human lymphocyte genomic instability (46). In the colon cancer cell line Caco-2 exposure to supraphysiological concentrations of PGA or MTHF decreased proliferation in all the MTHF conditions compared to PGA conditions was found (47).

There seems to be a difference between the bioavailability of PGA and MTHF. Until now only high concentrations of both PGA and MTHF are used in in vitro and in vivo studies. The difference in response to these folates is not well investigated, and especially not in the colon.

Not all processes in which folate plays a role are clarified and understood (32). To gain more insight on the effect of folate, a kinetic mathematical model has been made to explore folate pathways, but focusing merely on purine synthesis (most of the enzymes are addressed in Figure 1) (48). As mentioned before, most of the folate research has focused on DNA stability and, to a lesser extent, on proliferation and apoptosis (28,49). In pigs and mice with a low folate status, genes differentially expressed belonged to a wide variety of pathways, e.g. G-protein signalling, apoptosis and adhesion (18,50). Folate is also required for the cytochrome C reduction in mitochondria (9).

Multiple markers of folate exposure and response are known, these help to investigate the processes in which folate is involved. New processes in which folate is involved are appearing in literature, but these need to be further explored, new ones to be found and the relevance established.

Nutrigenomics

Nutritional genomics or nutrigenomics is defined as the interaction between nutrition and an individual's genome or the response of an individual to different diets (8). Technologies in science are changing rapidly since the discovery of DNA and the recent mapping of the human genome (5-7). The emerging science nutrigenomics is the application of high-throughput genomics tools such as transcriptomics, proteomics or metabolomics in nutritional research (51). New genomic technologies have made it possible to investigate the biological effects of nutrients on global gene expression and to gain insight in entire pathways (5). Genomics makes use of advanced technologies such as DNA microarrays (5-7,52). DNA microarrays consist of tens of thousand of genes. Using these microarrays for analysis of variation between RNA samples, gene expression profiles are obtained. This technique is called transcriptomics (53,54). To date, a limited number of studies have examined the effect of folate status on gene expression (18).
In pig livers and mouse colon with a low folate status, differentially expressed genes were identified using DNA microarrays. They belonged to different pathways, such as translation/transcription, immune response, G-protein signalling, apoptosis, lipid metabolism, cell adhesion and many other pathways (18,50). A transcriptomic study of colonic rat mucosa (55) and nasopharyngeal epidermoid carcinoma cells (56) found that folate depletion did not affect the expression of any folate related genes, neither did the former two studies. Only one study found down regulation of folate related genes. Folate

transporter was down regulated in folate deficient human primary T-lymphocytes (57). This study also reported a difference in expression of DNA repair, cell cycle and mitochondrial genes. Folate depletion induced the up regulation of immune related genes and down regulation of cell adhesion related genes in young rats (55). In old rats folate depletion induced up regulation of caspases and down regulation of immune related genes (55). Folate depletion in nasopharyngeal epidermoid carcinoma cells induced down regulation of H-cadherin (cell adhesion) by hypermethylation of H-cadherin promotor (56). Although in all studies extreme differences in folate concentration were used, only a relative small number of genes responded.

We should not accumulate DNA microarray datasets alone to identify the underlying mechanism. Firstly, the technical and biological variations should be investigated under the experimental conditions used. Secondly, to support the gathered data, they should be coupled to functional assays to define markers of dietary response (4), such as apoptosis (57) and proliferation (28). If this is not possible, other techniques to verify the obtained biomarkers should be used, such as quantitative RT-PCR (18,50), ELISA (50) or Western blot (50).

In conclusion, a nutrigenomics approach using transcriptomic techniques can be used to identify the underlying molecular mechanism of metabolic and physiological responses to a nutrient. Only a few studies have used a nutrigenomics approach for investigating the effects of folate on gene expression. Most of those did not verify their findings with other techniques, such as quantitative RT-PCR. They found cell cycle, immune response and cell adhesion genes to be affected. None of these studies have used human colon cell lines or physiological relevant folate concentrations.

In vitro studies

Although epidemiological data strongly suggest a link between dietary folate intake and colorectal cancer risk, studies investigating potential mechanisms of DNA instability have been limited because of the lack of suitable *in vitro* cell culture models (58). Culture models have proved valuable in the investigation of proto-oncogene expression, growth factor production, proliferation, and angiogenesis (58). The use of *in vitro* models has several advantages over *in vivo* models. Firstly, individual components of the diet can be investigated separately or combined (59). Secondly, exposures can be done under highly controlled conditions. Thirdly, it is possible to use cell lines that represent the different

stages in colorectal carcinogenesis (59). Fourthly, *in vitro* models are less expensive and time consuming than *in vivo* models (60). In order to identify processes of folate in the human colon, three different human colon epithelial cell lines were used as a model in this thesis; HT29, Caco-2 and CCD841CoTr. The most widely used *in vitro* models for intestinal epithelia are HT29 and Caco-2 cell lines (59-61). Both cell lines are human colon adenocarcinoma derived cell lines (59,61). Caco-2 cells can be differentiated to develop apical villi and express proteins typical of small intestine (61) and HT29 can be differentiated to a mucus secreting cell (62). CCD841CoTr is an SV-40 immortalized primary cell line. Ideally this cell line should mimic as closely as possible its counterpart *in vitro*, although SV40 transformation itself will affect cell behaviour. The three cell lines grow continuously, while primary cultures have very stringent culture requirements and, at present can only be maintained for a few days (60). The advantage of primary cultures lies in the direct comparability with healthy tissue. These three cell lines selected represent a sufficient diversity and acceptance for gaining insight into the effect of folate on the human colon. Until now, i*n vitro* models used in folate research are primary human lymphocytes (63), the human acute promyelocytic leukaemia cell line HL60 and the Chinese hamster ovary cell line CHO (2). In most of the studies using these cell lines the markers used in response to folate were dUMP/dTMP ratio, DNA strand breaks or DNA hypomethylation (2,58,63). *In vitro* studies show that inhibition of folate metabolism increases the insertion of uracil into DNA, although these results have not been obtained in human studies (16,64,65). A small number of studies have used a nutrigenomic approach to investigate the effect of PGA on cell lines (55-57), but none of them have used human colon cell lines.

The results below have been obtained using more traditional approaches. In immortalised colon epithelial cells folate, deficiency (1-10 ng/mL PGA) induces uracil misincorporation, DNA hypomethylation and inhibits DNA excision repair, compared to cells grown in 4000 ng/mL PGA (58). In colon cancer cell lines Caco-2 and HCT116 10,000 ng/mL PGA inhibits the expression and activation of epidermal growth factor receptor (EGFR) (47). EGFR plays a critical role in regulating the development and progression of colorectal cancer (47,66). In Caco-2, grown in 0.4 ng/mL PGA compared to 4000 ng/mL PGA reduced homocysteine remethylation (67). In both Caco-2 and HCT116 grown in 1000 ng/mL PGA compared to folate deficiency intracellular folate concentration increased (68). While folate deficiency increased intracellular SAM, SAH and SAM/SAH ratio in HCT116, these were all decreased in Caco-2 (68). Folate deficiency had no effect on

global DNA methylation in both cell lines, but Caco-2 had a decreased DNA methyltransferase activity (68). In HCT-116, both 10,000 ng/mL PGA and MTHF in HCT116 specifically inhibits basal and serum-induced activation of the EGFR promoter, which could partly be attributed to alterations in methylation. (66). Serum-induced activation of c-fos promoter activity was unaffected by MTHF (66).

For several reasons as stated below, we added vitamin B12 to all our experimental conditions at the normal serum concentration of 500 pg/mL. Vitamin B12 is a cofactor for methionine synthase (10,11) and often added to folate supplementation because folate alone can mask vitamin B12 deficiencies (10). Furthermore, vitamin B12 deficiency may mask changes in response to different folate concentrations. In the human intervention study, which we will use to extrapolate our *in vitro* findings, supplementation will contain both folate an vitamin B12. In normal culture media folate is present at a concentration of 4000 ng/mL. The same is true for vitamin B1, B3, B4, B5 and B6, while the concentration of vitamin B2 is 400 ng/mL. Vitamin B12 is not present in normal culture media and has not been supplemented in the former *in vitro* studies.

In conclusion, in vitro models used in folate research are, for the most part, non-colon derived cell lines and compare a supraphysiological PGA concentration to folate depletion in absence of vitamin B12. Markers of folate response investigated in these studies are dUMP/dTMP, strand breaks, SAM/SAH or DNA hypomethylation.

Due to the complexity of the human colon and the limited knowledge of extrapolation from *in vitro* to *in vivo,* one would like to test the *in vitro* found markers *in vivo*. This was possible as the research presented in this thesis is part of a multidisciplinary project that has been conducted to clarify the role of folate and related B-vitamins in colorectal carcinogenesis (69). A meta-analysis of human observational studies on folate intake and risk of colorectal adenomas was conducted. Furthermore, a Dutch case-control study was used to investigate the folate, vitamin B2 and B12 intake and MTHFR, TS and SHMT1 polymorphisms association to colorectal adenoma risk (70). In this study, the promoter methylation of six selected tumor suppressor genes and DNA repair genes in adenoma tissue was also analysed in relation to folate intake and *MTHFR C677T* genotype. In a human folic acid and vitamin B12 intervention study, DNA uracil misincorporation and promoter methylation of these genes were compared between an intervention and a

placebo group (71). The combined results of these relatively small studies, included in the thesis of Dr. Maureen van de Donk, suggest that a potential adverse effect of folic acid should be considered, especially when administered after colorectal neoplastic lesions have been established.

Rationale of this thesis

The studies described in this thesis were performed to identify processes of folate response in the human colon. In this thesis a nutrigenomics approach using the transcriptomic technique, DNA microarrays, was used for the *in vitro* identification of processes of folates and its metabolites. This will provide us with clues about the mechanism that underlies the effect of folate on the human colon. As shown in this introduction, there is an accumulating body of evidence suggesting that a low folate status possibly predisposes to the development of colorectal cancers. Cancer related mechanisms in which folate is involved are uracil misincorporation and aberrant DNA methylation, affecting gene expression. Few folate interventions are conducted using high dosages of PGA, with or without B12, in patients with colorectal polyps or adenomas. PGA supplementation in these studies led to decreased global DNA hypomethylation, increased SAM, decreased tumour suppressors expression and decreased cell proliferation. The bioavailability of PGA seems to be greater than the bioavailability of MTHF, although homocysteine decrease by PGA or MTHF gave conflicting results. *In vitro* models used in folate research are for the most part non-colon derived cell lines and used to compare supraphysiological PGA concentration to folate depletion in absence of B12. Markers of folate response investigated in these studies are dUMP/dTMP, strand breaks, SAM/SAH or DNA hypomethylation. New pathways, for example, cell cycle, immune response and cell adhesion, in which folate is involved are appearing in literature; however these need to be further explored and new ones to be found. Only a few *in vitro* studies have used a nutrigenomics approach to investigate the molecular mechanism of responses to folate, although none of them used human colon cell lines. Little is known about the extrapolation of *in vitro* to *in vivo*.

In summary this led to the following research questions:

1. What is the effect of PGA supplementation on folate metabolites and cancer-related processes, such as cell proliferation, apoptosis and differentiation?
2. Moreover, which unknown processes are affected by PGA supplementation?
3. Is this depending on which human colon cell line is used?
4. Can these similar effects be found with MTHF supplementation?
5. Can the *in vitro* results be verified *in vivo*, in humans?

We conducted three *in vitro* studies, in the first we compared a normal to a high PGA exposure of all three human colon cell lines. In the second we compared a normal to a high PGA and MTHF exposure in HT29 cells. In the third *in vitro* study we compared the difference between PGA and MTHF exposure in HT29 cells using a normal to high concentration range of both folates. In all three studies the nutrigenomics approach was used combined with physiological assays or other verification methods, such as quantitative RT-PCR. A human folate and vitamin B12 intervention study was performed (FOCO-trial, Van den Donk *et al.* (71)), as part of this multidisciplinary project. This study was used to test whether *in vitro* findings could be verified *in vivo* for the markers of response obtained in the first *in vitro* study.

Outline of this thesis

This thesis describes the nutrigenomics analyses of the response of long term (three week) folate exposures of human colon cell line(s). Chapter 2 describes the technical aspects and evaluation of the technical and biological variation in our nutrigenomics approach used in the other chapters. Chapter 3 describes the study carried out with three cultured human colon cell lines exposed to low and high PGA. In this study, the nutrigenomics approach was combined with more traditional physiological assays. The approach in Chapter 3 provided us with additional leads about the mechanism that underlies the effect of folate on the human colon, specifically the effect of folate on the energy metabolism. In Chapter 4, the most responsive cell line, HT29, and effects of not only PGA but also MTHF were investigated. Chapter 5 describes an exposure study of HT29 cells including a supplemental, but still physiological concentration range of PGA and MTHF. In Chapter 6 the markers of response obtained from the study described in Chapter 3 were used to relate *in vitro* data to *in vivo* by investigating these markers in a human folate and vitamin B12 intervention study (71). In Chapter 7, the findings of all

studies are summarised, technical aspects and possible application of the identified processes are discussed using the results of the studies described in previous chapters, and future research directions are mentioned.

2

The Intraclass Correlation Coefficient applied for evaluation of data correction, labeling methods and rectal biopsy sampling in DNA microarray experiments

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Abstract

We show that the Intraclass Correlation Coefficient (ICC) can be used as a relatively simple statistical measure to assess methodological and biological variation in DNA microarray analysis. The ICC is a measure that determines the reproducibility of a variable, that can easily be calculated from an ANOVA table. It is based on the assessment of both systematic deviation and random variation, and facilitates comparison of multiple samples at once. We used the ICC first to optimize our microarray data normalization method and found that the use of median values instead of mean values improves data correction. Then the reproducibility of different labeling methods was evaluated and labeling by indirect fluorescent dye incorporation appeared to be more reproducible than direct labeling. Finally, we determined optimal biopsy sampling by analyzing overall variation in gene expression. The variation in gene expression of rectal biopsies within persons decreased when two biopsies were taken instead of one, but did not considerably improve when more than two biopsies were taken from one person, indicating that it is sufficient to use two biopsies per person for DNA microarray analysis under our experimental conditions. To optimize the accuracy of the microarray data, biopsies from at least six different persons should be used per group.

Introduction

DNA microarray technology is becoming a commonly and widely used hybridization based technique in research and clinical applications (53). The technique originated from the need to address the functions of the large numbers of genes that have been identified in large scale sequencing programs. The use of high precision robots allowed researchers to immobilize tens of thousands of DNAs on extremely small areas of a solid base, generating high density DNA microarrays. Finally, the advent of fluorescent technology made it possible to address the microarrays in a relatively simple, safe and accurate manner. All of this has led to a great increase in the throughput of analysis of biological molecules and samples. Applications of DNA microarrays range from genetic mapping studies and mutational analysis to genome wide monitoring of gene expression (72).

The technology was first used for assessment of cellular mRNA expression levels. The goal of this application is to determine gene expression of the "whole" genome in any cell or tissue sample of interest (73). For this, isolated (m)RNA is labeled and hybridized to the DNA microarray. After washing and scanning of the DNA microarray, a raw fluorescence data image is obtained which should represent overall gene expression level of the original sample. Usually, not every gene is represented on the DNA microarray and hybridization results are influenced by multiple factors, namely the labeling method, hybridization conditions, the sequence of the gene and target features. Since in most cases not absolute expression levels, but differences in expression between samples are determined, problems can be minimized by direct comparison of two samples, labeled with different fluorophores (e.g. Cy3 and Cy5) on one array (54). To compare multiple samples (each separately labeled with e.g. Cy5) a common reference sample is labeled (labeled with Cy3) and can be used in each hybridization. In the ideal situation the reference represents each spotted gene on the DNA microarray, but in practice this is a pool representing each investigated sample.

For expression analysis to be efficient and reliable, reproducible laboratory protocols and validated procedures for data normalization are required (74). Labeling procedures are constantly optimized and new procedures are being developed, primarily to reduce the amount of input material that is required (75-77). Critical to the success of these protocols is that they are reproducible. Therefore, it is striking that no simple, standard protocol is available to assess the reproducibility. The last step before comparing the gene expression of different samples is data normalization, which is performed on the obtained raw fluorescent data. Data normalization makes use of the Cy3 reference

sample images and allows directly comparison of the Cy5 sample values on different slides (54,78). This is a widely used approach, but again, no simple standard method is available to assess whether data normalization is performed correctly. Different methods have been reported to address the way data normalization and labeling methods are performed (79,80). These methods range from simple linear correlation coefficient (R^2) analysis to sophisticated statistical methods (79-81). When evaluating different labeling methods, one is interested in the systematic deviation (i.e. difference in the amount of labeled RNA samples), which preferably should be as small as possible. However, when the R2 is used in DNA microarray data analysis, its feature to be insensitive to the systematic deviation makes it unsuitable for the purpose of assessing reproducibility (82). Another limitation of the R2 is that it only facilitates the comparison of two samples at the same time. An attractive alternative is the use of the Intraclass Correlation Coefficient (ICC). This is a relatively simple statistical procedure used to determine the reproducibility of a measurement of a variable (83-85). This correlation is based on Variance Components Analysis and measures the homogeneity within groups relative to the total variation. The ICC is large when there is little variation within the groups compared to variation among group means, where groups consist of replicate measurements. A small ICC occurs when within-group variation is large as compared to between-group variability, indicating that some unknown variable has introduced non-random effects in the different groups. The maximum value of the ICC is one and the minimum value is theoretically zero (81,83-85). The ICC is routinely used in epidemiological studies to address the test-retest reliability, validity of questionnaires, inter-laboratory concordancy and correlation of plasma/tissue levels to disease status. Having seen how the ICC is utilized for reliability, reproducibility and validation analysis (86-88), we decided to investigate whether it can be used to assess technical variation in DNA microarray technology, and, more specifically, to assess the reproducibility of sample RNA labeling methods and to optimize data normalization. We decided to extend its use to assessment of biological variation. To this end, the within- and between-person variation in gene expression in small biological samples was estimated. The analysis of gene expression in biological samples is used for clinical as well as epidemiological studies. Since human material is often scarce, it is necessary to determine how many biopsies should be taken to acquire sufficient accuracy in the assessment of the tissue that is analyzed.

In this paper, we show that the ICC is a relatively simple statistical measure that can be used to estimate methodological and biological variation as exemplified by addressing the validity of our data normalization procedure, by comparing the reproducibility of different labeling methods and by analyzing variation of gene expression in human rectal biopsies.

Material and Methods

Sample preparation

Caco-2 and HT29 cells (ATCC, Manassas, VA, USA) were inoculated in 75cm^2 culture flasks at 40,000 cells/cm^2. Cells were grown at 37°C in air with 5% CO2 and 100% relative humidity in Dulbecco's modified Eagle's medium (Sigma, Zwijndrecht, The Netherlands) supplemented with NaHCO3 (3.7 g/L, Sigma), non-essential amino acids (1x, ICN, Zoetermeer, The Netherlands), FCS (5%, Invitrogen, Breda, The Netherlands), penicillin (5000 U, Sigma) and streptomycin (5 mg/L, Sigma). Culture media were replaced every second day. Cells were split at 70-80% confluence with a ratio 1:5 and seeded for subcultures. Cells were harvested at 90% confluence.

Persons with intestinal complaints visiting the hospital for a colonoscopy were asked to participate in this study. If the colonoscopy findings showed no visible symptoms, a rectal biopsy was taken. In total 27 persons were recruited at the colonoscopy visit, five subjects donated multiple biopsies (n=4-6) and twenty-two individuals gave one biopsy. The biopsies taken had an average size of 7 mm^3, an average weight of 1,2 mg and gave an average yield of 13 µg of total RNA. The biopsies were lyophilized and grinded before use. Total RNA from cultured cells and tissue was extracted using the TRIZOL according to the supplier (Invitrogen). RNAs, that were not used for mRNA isolation, were purified using the RNeasy protocol (QIAquick RNeasy kit, QIAgen, Leusden, The Netherlands). mRNA was isolated from total RNA by poly(A)+ selection using oligod(T) sephadex (mRNA purification kit, Pharmacia, Roosendaal, The Netherlands). Concentrations were determined spectrophotometrically at A_{260nm} and all samples were checked after 1 hour incubation at 37°C on 1% TAE/agarose gels for absence of degradation.

Direct labeling

The RNA labeling protocol is based on Schena et al. (89). Either total RNA or mRNA was labeled by incorporation of either Cy3-dCTP or Cy5-dCTP during reverse transcription. A combination of HT29 and Caco-2 mRNA (1.0 µg) or total RNA (40.0 µg) was used for the

labeling. In short, 1.0 µg of sample poly(A)$^+$ RNA or 40.0 µg total RNA was mixed with 1.0 ng control luciferase mRNA (Promega, Leiden, The Netherlands), 2.0 µg oligo-dT$_{21}$ primer (Isogen, Maarssen, The Netherlands) and/or 150.0 ng random hexamers (Invitrogen) in a final volume of 13.5 µL, heated for 3 min at 65°C (RNA denaturation) and 10 min at 25°C (primer annealing), and immediately put on ice. Then, a reverse transcription reaction was performed for 2 hours at 37°C in a final volume of 25 µL. The reaction mixture contained the RNA template with the annealed primer, 1x first-strand buffer (Invitrogen), 10 mmol/L dithiothreitol, 0.5 mmol/L dATP, 0.5 mmol/L dGTP, 0.5 mmol/L dTTP, 0.04 mmol/L dCTP, 0.04 mmol/L Cy3-labeled dCTP (or Cy5-labeled CTP, Amersham), 15 U of RNase OUT (Invitrogen), and 150 U of SuperScript II reverse transcriptase (Invitrogen). The labeled cDNA obtained was purified by an ethanol precipitation performed at room temperature. The pellet was dried and dissolved in 10 µL TE, pH 8.0 (10 mmol/L Tris-HCl and 1 mmol/L EDTA). After a 3-min boiling step, the cDNA was immediately put on ice, and 2.5 µL of 1 mol/L NaOH was added. The cDNA was then incubated for 10 min at 37°C to break down the remaining RNA. To neutralize the pH, 2.0 µL of 1 mol/L HCl and 2.5 µL of 1 mol/L Tris-HCl (pH 6.8) were added. Finally, an additional ethanol precipitation at room temperature was performed, and the resulting cDNA pellet was dissolved in 25 µL hybridization buffer containing 5x SSC, 0.2% SDS, 5x Denhardt's solution, 50% (vol/vol) formamide, and 0.2 mg/mL denatured herring sperm DNA. Prior to hybridization, the labeled cDNA was heated (improves cDNA dissolving) for 3 min at 65°C and spun for 2 min at 12,000 *g* to remove undissolved debris.

Indirect labeling

This RNA labeling protocol is based on the protocol from Henegariu et al. (90). In the reverse transcription step aminoallyl dUTP was incorporated and afterward chemically coupled to Cy5 monofunctional dye. A combination of HT29 and Caco-2 mRNA (1.0 µg) or total RNA (40.0 µg) was used for the labeling. For biopsy material 12.5 µg total RNA was used for the labeling. In short, 1.0 µg of sample poly(A)$^+$ RNA, 12.5 µg or 40.0 µg total RNA was mixed with 1.0 ng control luciferase mRNA, 2.0 µg oligo-dT primer (21-mer) and/or 150 ng random hexamers in a final volume of 12.75 µl, heated for 3 min at 65°C (RNA denaturation) and 10 min at 25°C (primer annealing), and immediately put on ice. Then, a reverse transcription reaction was performed for 2 hours at 37°C in a final volume of 25 µL. The reaction mixture contained the RNA template with the annealed primer, 1x first-strand buffer, 10 mmol/L dithiothreitol, 0.5 mmol/L dATP, 0.5 mmol/L

dGTP, 0.5 mmol/L dCTP, 0.3 mmol/L dTTP, 0.2 mmol/L aminoallyl dUTP (Sigma), 15 U of RNase OUT, and 150 U of SuperScript II reverse transcriptase. The obtained cDNA was purified by an ethanol precipitation performed at room temperature. The pellet was dried and dissolved in 10 µL TE, pH 8.0. After a 3-min boiling step, the cDNA was immediately put on ice, and 2.5 µL of 1 mol/L NaOH was added. The cDNA was then incubated for 10 min at 37°C to break down the remaining RNA. To neutralize the pH, 2.0 µL of 1 mol/L HCl and 2.5 µL of 1 mol/L Tris-HCl (pH 6.8) were added. An ethanol precipitation at room temperature was performed, and the resulting cDNA pellet was dissolved in 10 µL 0.1 mol/L sodium bicarbonate buffer (pH 9.3). The chemical coupling took place for 30 min at room temperature by adding 10 µL 5 mmol/L Fluorolink Cy5 monofunctional dye (Pharmacia) to the cDNA. An ethanol precipitation was performed at -20°C for at least 2 hours, and the resulting cDNA pellet was dissolved in 100 µL Millipore filtered water. All cDNAs were purified using the PCR purification protocol (QIAquick PCR purification kit, QIAgen). Finally, an additional ethanol precipitation at room temperature was performed, and the resulting cDNA pellet was dissolved in 25 µL hybridization buffer. Prior to hybridization, the labeled cDNA was heated for 3 min at 65°C and spun for 2 min at 12,000 *g* to remove undissolved debris.

Microarrays construction

An in-house produced subtracted cDNA library, enriched for genes which are expressed in differentiated and undifferentiated Caco-2 cells (Peijnenburg et al. unpublished results), were printed on silylated slides (CEL Associates, Houston, TX, USA) using a PixSys 7500 arrayer (Cartesian Technologies, Durham, NC, USA). Arrays were spotted by passive dispensing using quill pins (Chipmaker 3, Telechem, Sunnyvale, CA, USA), resulting in a spot diameter of 0.12 mm at a volume of about 0.5 nL. After printing, microarrays were allowed to dry at room temperature for at least 3 days. Free aldehyde groups were blocked with $NaBH_4$ according to the method of Schena et al. (89). The microarrays, used for the validation of the normalization method and assessment of different labeling methods, contained 1152 spotted genes in duplicate. For the assessment of biological variation the microarrays that were used contained 2304 single spotted genes.

Microarray hybridization

Prior to hybridization, microarrays were prehybridized in hybridization buffer at 42°C for several hours. After prehybridization, slides were rinsed twice in Millipore filtered water,

once in isopropanol, and dried by centrifugation (2 min, 470 g). The hybridization was performed in a Geneframe (1 x 1 cm^2, 25 µL hybridization volume; Westburg, Leusden, The Netherlands). A 1:1 (vol/vol) mixture of Cy3- and Cy5-labeled cDNAs was hybridized to each array. Arrays were hybridized overnight at 42°C in a humid hybridization chamber. After hybridization, slides were washed at room temperature, first in 1x SSC/0.1% SDS (5 min) and subsequently in 0.1x SSC/0.1% SDS (5 min) and 0.1x SSC (1 min) and then dried by centrifugation (2 min, 470 *g*).

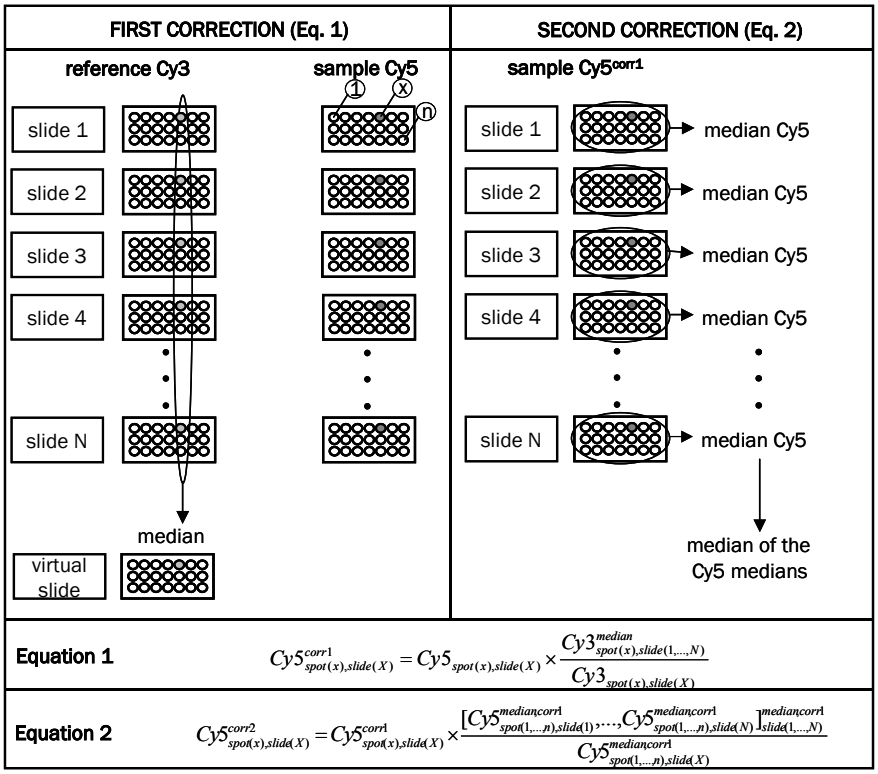
Microarray scanning

Microarrays were scanned using a confocal laser scanner (ScanArray 3000, General Scanning, Watertown, MA, USA) containing a GHeNe 543 nm laser for Cy3 measurement and a RHeNe 633 nm laser for Cy5 measurement. Scans were made with a pixel resolution of 10 µm, a laser power of 90% and a photomultiplier tube voltage of 80%. The software package ArrayVision (Version 7.0, Imaging Research, Ontario, Canada) was used for image analysis of the TIFF files, as generated by the scanner. Density values of each spot, multiplied by the area and the background (surrounding entire template) were collected and stored for further data processing in Microsoft Excel (Windows, Microsoft).

Experimental set-up and data-normalization

All arrays were hybridized with a Cy5-labeled sample cDNA and a Cy3-labeled reference cDNA. For validation of the normalization method and the assessment of different labeling methods, the reference cDNA was a mixture of directly labeled mRNA of HT29 and Caco-2 cells. A mixture of indirectly labeled total RNA from rectal biopsies, HT29 and Caco-2 cells was used as reference cDNA for assessment of biological variation. The reference cDNA was pooled after fluorescent labeling and subsequently subdivided and hybridized on all arrays. The reference hybridization signals of the spots should under ideal circumstances be identical on each slide. In practice this signal will differ due to (i) fluctuations in the amount of DNA spotted and (ii) variations in the hybridization conditions within a slide, between slides and between different experiments (random variation). For these reasons corrected sample hybridization signals, $Cy5^{corr1}_{spot(x),slide(X)}$, were calculated for each spot x on slide X according to equation in Figure 2.1 of the first correction. Where N is the total number of hybridized slides, $Cy5_{spot(x),slide(X)}$ and $Cy3_{spot(x),slide(X)}$ are the measured Cy3 and Cy5 hybridization signals of spot x on slide X,

Figure 2.1 Procedure for data normalization, divided into a first (random error) and second (systematical error) correction.



First correction (Equation. 1) is accomplished by using the median value of the Cy3 signal (virtual slide) for each spot separately, while second correction (Equation 2) makes use of the median of the overall Cy5 signal.

and $Cy3^{median}_{spot(x),slide(1,...,N)}$ is the median of the hybridization values of spot x on all slides hybridised in the experiment. To correct for differences in labeling efficiency between samples and for inaccuracies in the amount of sample mRNA used in the labeling reaction (systematic labeling deviation), sample hybridization values were also corrected for the median Cy5 signal according to equation in Figure 2.1 of the second correction. Where n is the total number of spots on the array, $Cy5^{median,corr1}_{spot(1,...,n),slide(X)}$ is the median of the Cy5 signals of all spots on slide X after the first correction, and $[Cy5^{median,corr1}_{spot(1,...,n),slide(1)},...,Cy5^{median,corr1}_{spot(1,...,n),slide(N)}]^{median,corr1}_{slide(1,...,N)}$ is the median of the median Cy5 signals of all hybridized slides after correction 1. Before applying the corrections described by equations 1 and 2, first a signal intensity threshold was set. All spots with a Cy3 or Cy5 signal lower than once the average background of the Cy3 and Cy5 scans, were excluded from data analysis.

Statistical analysis

The log of the normalized value for each Cy5 spot (in case of duplicate spots the mean normalized value) was taken, and the ICC for each labeling method was determined. The Variance Components analysis was performed in SPSS 10.0 for Windows. The ICC was obtained from Mean Squares (MS) of the two-way random effects model:

$$ICC = \frac{MS_b - MS_w}{MS_b + (N-1)MS_w}$$

where MS_b is the between-spots Mean Square (spot 1,...,n), MS_w is the within-spots Mean Square (spot 1,...,n in slide 1,...,N) and N is the number of replicates.

In the text-output of SPSS a "Single Measure Intraclass Correlation (ICC_1)" and an "Average Measure Intraclass Correlation" were obtained. The single measure ICC was used for calculation of the ICC's of different number of repeats (i), by the following formula;

$$ICC_i = \frac{1}{1+\left(\lambda / i\right)} \quad \text{where } \lambda \text{ is } \quad \lambda = \frac{1 - ICC_1}{ICC_1}$$

The value of ICC tends to be slightly smaller than one. The closer the ICC is to one, the more similar samples are (83-85).

Results

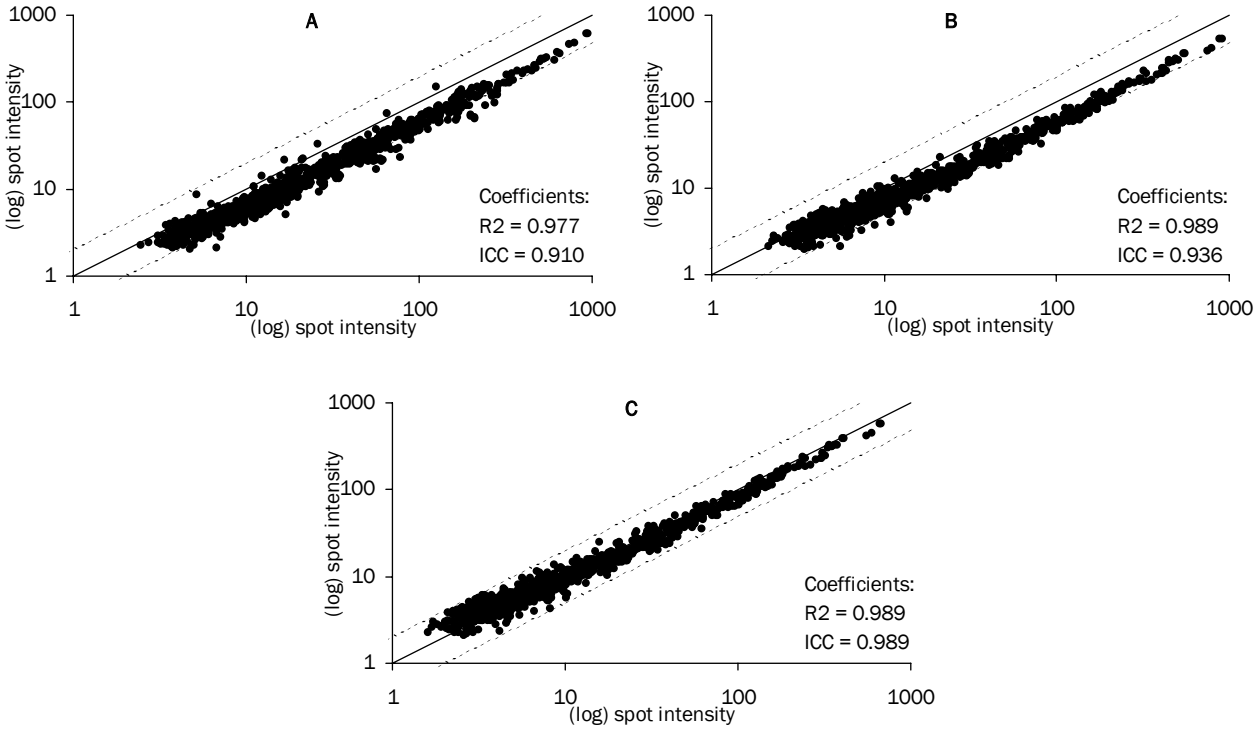
Assessment of Intraclass Correlation Coefficient characteristics

The ICC is a measure that can be used to quantify the reproducibility of a variable. At the same time, it is a measure of the homogeneity within groups of replicate measurements relative to the total variation between groups. As opposed, R^2 is a measure of linear association between two variables. One feature of R^2 is that it disregards systematic error, this implies that the R^2 observes the average of the cloud of points as if it was on the x=y line.

To gain insight in characteristics of the ICC for the analysis of DNA microarrays, a dataset was generated by labeling a sample two times independently with Cy5. This was mixed afterwards with Cy3 labeled RNA from the same reference pool, and hybridized to separate but identical slides. From this microarray dataset three scatter plots were made (Figure 2.2). The first scatter plot was obtained from the raw microarray data (Cy5 values, Figure 2.2A). Correcting this data for the random error (Figure 2.1, Equation 1) resulted in

the second scatter plot (Figure 2.2B). The third scatter plot (Figure 2.2C) was obtained with the data of the second scatter plot after adjusting this for the systematic error (Figure 2.1, Equation 2). Subsequently the R^2 and ICC were calculated for each scatter plot. The uncorrected raw microarray dataset rendered the smallest ICC and R^2 of all three scatter plots (ICC = 0.910 and R^2 = 0.977). After correcting the data for the random error (Figure 2.1, Equation 1) both ICC and R^2 improved (ICC = 0.936 and R^2 = 0.989). In contrast, when correcting for the systematic error (Figure 2.1, Equation 2) only the ICC improved (0.936 versus 0.989). This shows that the ICC is sensitive for both the random variation and the systematic deviation, while the R^2 is only sensitive for the random variation. Thus the ICC can be used to assess systematic deviation, whereas the R^2 cannot. Since the number of replicates is not limited for calculation of the ICC, more than

Figure 2.2 Analysis of sensitivity of Intraclass Correlation Coefficient (ICC) to random variation and systematic deviation.



A DNA microarray data set of two hybridizations with the same sample were used to analyze R^2 and ICC. The scatter plots compare the spot intensities of 1054 genes that are represented on each microarray. The raw microarray data (A) was first normalized by correcting with the Cy3 reference, resulting in scatter plot (B). These data were further normalized by adjusting for the systematic error, resulting in scatter plot (C).

2 samples can be compared at once. The R^2 can only be calculated for two samples at the same time, and thus only allows comparison for each scatter plot separately. Because the ICC is sensitive for both random variation and systematic deviation, it is a useful tool to obtain insight in both technical and biological variation.

Assessment of technical variation: data normalization

Different normalization methods haven been described for DNA microarray datasets (78,79,87,91). Working with Cy5/Cy3 ratios corrected by the mean or median Cy5 signal on each array is often used for DNA microarray data normalization (74,79). To assess the best method, correcting based on the mean or median signal we performed a hybridization experiment, applied the normalization steps (Figure 2.1, Equation 1 and 2) with either the mean or median and calculated the ICC for each method. Five arrays were hybridized with identical samples labeled with Cy5 independently and a Cy3-labeled reference cDNA was used to allow comparison between the arrays. When correcting the data for the random error (Figure 2.1, Equation 1) with the mean or median Cy3 signal of each spot, no difference in ICC was observed. If subsequently correction for the systematic error (Figure 2.1, Equation 2) is performed with the mean or median value of the overall Cy5 signal, a ΔICC_{mean} of 0.030 (SD 0.017) and ΔICC_{median} of 0.037 (SD 0.012) was obtained. Correction for the systematic error with median value gave a slight improvement compared to the mean value. Since use of the median value for the correction has the advantage that outliers will not disturb the corrections, median values were used for both corrections in the data normalization in all further experiments.

Assessment of technical variation: labeling methods

Various different labeling methods have been described for microarray hybridization experiments (75-77,89). These methods can be divided in direct or indirect labeling methods. In the direct labeling method the fluorescent dye is incorporated during the cDNA synthesis, while in the indirect labeling method coupling of fluorescent dye to cDNA occurs afterwards. Since the fluorescent dyes are very bulky, indirect labeling may provide an advantage since less bulky nucleotide modifications can be used, which are likely to improve reverse transcriptase function and will result in a higher insertion of the modified nucleotides and longer cDNA strands. We compared the quality and reproducibility of oligo-dT primed direct labeling and indirect labeling using either mRNA or total RNA as input material. In addition, for labeling of mRNA, also a combination of

oligo-dT and random hexamer was tested in the indirect as well as in the direct labeling method. This approach aims at a higher labeling efficiency, and cannot be used for total RNA since it would also result in labeling of ribosomal RNA. For an overview of the methods tested, see Table 2.1. In the indirect labeling method, aminoallyl nucleotides were incorporated during the cDNA reaction followed by reactive dye coupling. We also tested biotine nucleotide incorporation followed by streptavidine-dye coupling. However, in our hands the biotine-streptavidine based technique resulted in very poor signal to noise ratios, due to high background signals. Therefore this method was not taken along in further evaluation (data not shown).

Each labeling method was performed five times, independently, using Cy5 fluorescent dye. All methods were hybridized to separate but identical microarrays. The labeling methods were compared by using the Cy3-labeled reference cDNA, which was simultaneously hybridized on each array. To assess both random and systematic labeling deviation, only the first normalization step was performed (Figure 2.1, Equation 1). To assess only the random labeling variation, both normalization steps were performed (Figure 2.1, Equation 1 and 2). As a measure for reproducibility, the ICC was calculated for each method. As a measure for quality, the average signal spot intensity, background and signal to noise ratio were calculated for each method (Table 2.1).

Table 2.1 Assessment of different RNA labeling methods.

Methods	ICC^{corr1}	ICC^{corr2}	signal	background	signal/noise
D,O,M*	0.973	0.983	38.76	6.20	6.25
D,OR,M*	0.985	0.991	73.97	7.12	10.39
D,O,T*	0.971	0.980	80.04	6.33	12.65
I,O,M*	0.951	0.988	12.00	1.14	10.53
I,OR,M*	0.978	0.993	51.04	1.32	38.67
I,O,T*	0.956	0.981	26.41	1.14	23.17

*The Intraclass Correlation Coefficient (ICC), average signal spot intensity, background and signal to noise ratio of different labeling methods using mRNA or total RNA as starting material are given. *D: directly labeling; I: indirectly labeling; O: oligo dT primer; R: random hexamer primer; M: mRNA; T: total RNA;* ICC^{corr1}*: ICC after first correction and* ICC^{corr2}*: ICC after both corrections (systematic deviation)*

In all three direct labeling methods a smaller ICC was obtained compared to the ICC of indirect labeling methods when both corrections were applied, while a higher ICC was obtained of the directly labeling methods when only the first correction was applied. The random labeling variation seems to play a greater role in the direct labeling methods, while the systematic deviation seems to play a bigger role in the indirect labeling methods (Table 2.1).

mRNA labeled with a combination of oligo dT and random hexamer primers had a better reproducibility and signal to noise ratio compared to mRNA labeled with only oligo dT primers, for both direct and indirect labeling method. The ICC for total RNA tended to be smaller than the ICC for mRNA. Based on these results it was decided to use the indirect labeling method in all subsequent experiments. The use of mRNA in combination with oligo dT and random hexamer primers gave the best results and is the preferred method if sufficient amounts of RNA are available. However, total RNA was used in subsequent experiments, since only limited amounts of RNA could be obtained from the rectal biopsies that were used and further mRNA isolation would result in too little input material.

Assessment of biological variation

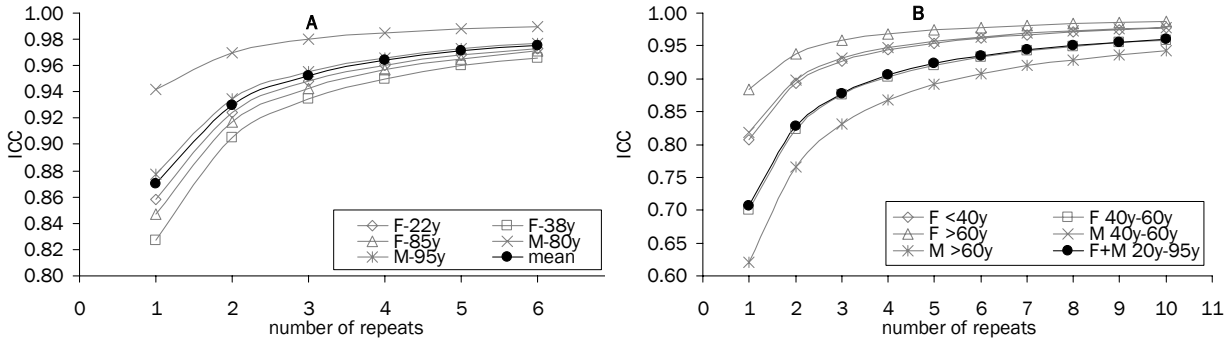
The ICC was also applied to optimize human rectal biopsy sampling by investigating the variation in gene expression in rectal biopsies between and within persons. Since this question is targeted at assessment of random variation (difference in gene expression) and not the systematic deviation, both data corrections (Figure 2.1, Equation 1 and 2) were performed on the microarray data set. Multiple biopsies (n= 4-6) were taken from five different healthy persons for the within-person variation analysis. From twenty-two persons with intestinal complaints, but without visible symptoms, one rectal biopsy was obtained to assess the variation in gene expression of biopsies from different subjects. Total RNA was labeled using the indirect labeling method and hybridized to separate but identical microarrays in combination with identical reference cDNA on all slides. To determine the within-person variation, all biopsies of one individual were used separately in SPSS to obtain two ICCs, namely the single and average measured value. Using these, the ICCs were calculated ranging from one to six biopsies, for each of the five individuals separately (Figure 2.3A). An average ICC of 0.870, of the five individuals, was found if one biopsy per person was taken. It should be noted that a variation in gene expression in one biopsy can only be obtained by calculation in relation to gene expression data of multiple biopsies. If two biopsies are used for analysis of variation, the ICC becomes 0.930. Additional biopsies lead to a slightly increased ICC, but the biggest increase in ICC is found in the step from one to two biopsies.

Often in microarray data analysis an R^2-threshold of 0.9 is used (75-77) and in epidemiological studies for within-person analysis an ICC-threshold of 0.9 is used (88).

For the ICC this means that the variance due to between-subject variations is at least 90% of the total variance.

The ICC of two biopsies is above 0.9. Therefore it can be concluded that at least two rectal biopsies per person should be used when working with DNA microarrays. To establish if the ICC can be improved by pooling biopsies of a person, the following analysis was done. The ICC was obtained from two individuals, with two biopsies per individual, these were used separately or the values were averaged per individual (N=4 or N=2, ICC_4 versus ICC_2). This was also performed for three individuals, with two biopsies per individual, these were used separately or the values were averaged per individual (N=6 or N=3, ICC_6 versus ICC_3). Using the mean of two biopsies gave an improved ICC compared to the single biopsies (ΔICC 0.026 SD 0.009). The reliability of the microarray data of biopsies from different persons also becomes greater when the number of biopsies increases (Figure 2.3B). When combining all the subjects, biopsies from four different persons give an ICC above 0.9. This is well within the resolution of the analysis, since biopsies from 21 different persons are necessary to obtain an ICC that is identical to the technical variation (0.981, Table 1 (indirect labeling of total RNA)). By dividing the subjects into groups, by age and gender, more homogeneous groups are created. In three cases this resulted in improved ICC's, except in the group of male subjects which are older than 60 years; there for unknown reasons biopsies from six different persons are needed to give an ICC above 0.9 (0.908). By pooling all data, the diversity in this group (M >60 y) is concealed by the uniformity of the other groups.

Figure 2.3 Assessment of variation in human rectal biopsy overall gene expression.



Within-subject variation of rectal biopsy gene expression from five different persons (3A) and between-subject variation of rectal biopsy gene expression (3B). These variations were obtained from 2304 different DNA microarray signals. F: female, M: male, y: years

Discussion

Assessment of Intraclass Correlation Coefficient characteristics

The purpose of this study was to investigate whether the Intraclass Correlation Coefficient can be used as statistical method to optimize DNA microarray experiments. We attempted to evaluate whether the ICC can be used as a simple tool for the assessment of technical and biological variation in microarray experiments. We demonstrated that the ICC is sensitive for both systematic deviation and random variation. Therefore we used the ICC to test our normalization method, to assess different labeling methods and to optimize human rectal biopsy sampling.

Assessment of technical variation: data normalization

Data normalization by co-hybridization of a separately labeled reference sample, is widely used and accepted in DNA microarray analysis (74,79); in most cases mean values are used. We compared data normalization performed with mean or median values. Since the ICC is sensitive for both random and systematic deviation, it could be established that the median value, rather than the mean value, of overall Cy5 values should be used for correction for the systematic error (Figure 2.1, Equation 2), in data normalization. The normalization step is based on the assumption that there are only minor differences in overall gene expression between samples, and that there is symmetry in expression levels of up- and down-regulated genes. The assumption that there are only minor differences in overall gene expression levels between samples is not valid in each experimental set-up. First of all, the hybridized arrays should contain a large number of genes. Furthermore, the arrays should not contain a pre-selected group of genes which is expected to hybridize stronger to one sample than to the other. Finally, the total number of different RNA messengers expressed in the samples should be equal. When in one sample a lower variation in the type of RNA messengers is expressed compared to the other, the hybridization signals will be relatively high for the sample where a lower number of different mRNA transcripts are expressed, assuming that equal amounts of RNA are used in the reverse transcription reaction. For these exceptions, another method for the second data correction step should be used, for example normalization based on a set of ‘housekeeping’ genes or normalization based on a set of spiked controls, each with their own disadvantages.

Assessment of technical variation: labeling methods

Total or mRNA that was indirectly labeled gave a better reproducibility and quality as compared to direct labeling, though a larger systematic error was found. The reproducibility and quality was increased when mRNA was reversibly transcribed with the combination of oligo dT and random hexamer, for the direct as well as indirect labeling. Theoretically the reproducibility (ICC) has a maximum value of 1. In most papers a reproducibility for labeling methods of 0.9 and higher is considered to be sufficient (75-77). In view of the above, a good reproducibility was obtained for all labeling methods, particularly for the methods which use the indirect labeling method. The low background of the indirect labeling method can be ascribed to the removal of excess dye which is accomplished by a column, in contrast to precipitation in the direct labeling method. Adjusting for the systematic error led to enhanced ICCs, especially for the indirect labeling methods. This can be due to efficiency differences of the chemical coupling of the dye. An increase of the coupling time might overcome this problem.

Assessment of biological variation

As expected the between-person variation was found to be greater than the within-person variation in human rectal biopsies. No difference was found between male and female rectal biopsies. The within-person variation in gene expression can be attributed to a difference in composition of the biopsies. The biopsies taken can vary in the blood and muscle content, but also in the constitution of the epithelium layer consisting of enterocytes, goblet, endocrine and gut associated lymphatic tissue cells. The above mentioned difference can also have an effect on the between-person variation. Variation in the biopsies can also be due to the fact that the rectum might be affected by disease, despite the absence of visible symptoms in colon or rectum.

To decrease variation, two biopsies per subject should be taken for analysis. This was determined for individuals separately (Figure 2.3A), but also when multiple biopsies from different persons were combined into multiple groups and analyzed, the mean of two biopsies gave an improved ICC compared to using the single biopsies (ΔICC 0.026 SD 0.009). From the results presented in Figure 2.3B, where a single biopsy per person was used for gene expression analysis, it can be determined that four persons per group are required to obtain a sufficiently homogeneous sample, based on an ICC cutoff of 0.9. However, by separating persons in groups, we found that in some cases (males of over 60 years) four biopsies seemed not sufficient. This is in agreement with Hwang et al. (92),

who found in DNA microarray analysis of bone marrow samples from different lymphoid leukemia subtypes, that seven persons per group are required to separate distinct disease states or other physiological differences with statistically significant reliability.
We also have used this dataset to determine the minimum sample size in microarray experiments. Using the same threshold as Hwang (0.95) we found that a minimum of seven subjects per subgroup should be used for this dataset.
In view of all the data we suggest that if the group that is sampled is diverse or not well characterized, as is generally the case in intervention trials or cohort studies, the overall cutoff should be increased to 0.95. This implies that, if one biopsy per person is analyzed for gene expression, at least eight different persons per group are needed. This number can be reduced to a minimum of six persons if two or more biopsies per person are sampled and averaged.
One should take also into account that six repeats are considered to be enough in our laboratory conditions but this value has to be determined by each experimentator as it may depend on several local factors (array quality, probe labeling, hybridization conditions, scanning of the slides, etc.).

We showed that the ICC can be used for assessment of technical and biological variation in microarray experiments. After evaluation of the technical variation we recommend that the indirect labeling should be used and whenever possible mRNA should be taken as input material with the combination of oligo dT and random hexamer primers. Assessing the biological variation of human rectal biopsies revealed that two biopsies per person and at least six persons, in total per group, should be analyzed when studying gene expression for example in human dietary intervention trials.

3

Iron and cell turnover response of human colon epithelial cell lines to different steady state folic acid conditions

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Abstract

Folate, a water-soluble B-vitamin, is a cofactor in one-carbon metabolism and is essential for DNA synthesis, amino acid interconversion, methylation and, consequently for normal cell growth. To determine cellular responses to chronic differences in extracellular folate, we analysed steady state effects on homeostasis in three human colon epithelial cell lines at two folic acid concentrations. In 100 ng/mL folic acid, all cell lines had an higher intracellular 5-methyltetrahydrofolate, compared to 10 ng/mL folic acid. HT29 and CCD841CoTr had a higher proliferation and apoptosis rate with lowered differentiation, while Caco-2 showed no marked differences. cDNA microarray analysis confirmed the differences in cell turnover and cellular responses between the cell lines. In HT29 cells exposed to 10 ng/mL folic acid, genes related to iron metabolism were down regulated. This was confirmed by lowered intracellular iron levels in the 100 ng/mL compared to 10 ng/mL folic acid exposure in all cell lines. In conclusion, the chronic exposure of cells to 10ng/mL folate results in a higher iron content, but cell turnover response to folic acid seems to be cell line specific, in line with different *in vivo* responses as observed in animal studies.

Introduction

Folates are important cofactors in a large number of metabolic processes, such as amino acid interconversion, nucleotide synthesis and methylation. For this variety of metabolic processes, different C1-forms of tetrahydrofolate are present in the cell. For example, 10-formyltetrahydrofolate is the C1-donor in de novo biosynthesis of purines. In the conversion reaction of uracil into thymidine by thymidilate synthase, 5,10-methylenetetrahydrofolate is the C1-group donor (93,94). 5,10-methylenetetrahydrofolate is also part of the methylation cycle, which supplies cells with S-adenosyl methionine (SAM). SAM is the universal methyl donor for a wide range of substrates, such as lipids, hormones, chromosomal DNA and proteins (10). A shortage of folates leads to impaired activity of these metabolic processes, resulting in several diseases, possibly including colon cancer (9,95,96). Although the essential role of folate in basic cellular processes was largely established over 30 years ago and has been extended by more recent folate depletion studies (6,9), little information is available on cellular adaptation to different extracellular steady state levels of folic acid. This information is essential to allow evaluation of effects of chronic sub optimal and elevated folic acid levels. Due to the use of functional foods, multivitamin pills and folic acid fortification or a combination, we are increasing our folic acid levels (97). These elevated folic acid levels need attention, since the dose and timing of supplementation may be essential in colon carcinogenesis (21,23).

For analysis of long-term effects of folic acid, we studied three different human colon cell lines. We anticipated that cell line specific responses could occur and that the use of multiple cell lines would allow us to identify shared characteristics. We chose to use HT29 and Caco-2, because these are the most commonly used human colon epithelial cell lines. Since these cell lines are of tumour origin, we also incorporated a SV40 immortalized human colon cell line, CCD841CoTr, in our studies. To achieve a steady state condition, a three weeks exposure time was used. The cells were exposed to pteryolmonoglutamic acid (PGA), the folate used in fortified foods and supplements. Normally serum folate levels are in the range of 3-10 ng/mL, due to supplementation values of 100 ng/mL can be obtained (57,97). Therefore we exposed to cells to 10 ng/mL and 100 ng/mL PGA. To ensure that vitamin B12 deficiency would not disturb our results, a normal human serum level of 500 pg/mL vitamin B12 was present during each exposure. To obtain the largest folate variation possible that is still relevant to dietary conditions we used these two exposure levels. This is in contrast to most studies, which

used full folate depletion and/or supraphysiological folate concentrations (4000 ng/mL) (55-57), and do not include vitamin B12.

The aim of this study was to determine shared responses of human colon epithelial cell lines to long term exposure to two different physiological relevant folic acid concentrations. This was done by analysis of folate metabolism, cell turnover and gene expression by using cDNA microarray technology (73,98).

Experimental procedures

Cell culture

Three human colon epithelial cell lines HT29, Caco-2, and CCD841CoTr (passage number 21, 26 and 26) (ATCC, Manassas, VA, USA) were grown, in duplicate, on Dulbecco's modified Eagle's medium (DMEM) without folic acid (Invitrogen, Breda, The Netherlands), supplemented with 10 ng/mL or 100 ng/mL pteroyl glutamic acid (PGA) for 3 passages. Cells were seeded in 75cm^2 culture flasks at respectively 80,000 and 25,000 cells/cm^2, to avoid difference in cell density at the moment of harvesting, and grown at 37°C in air with 5 % CO_2 and 100 % relative humidity. The growth medium was supplemented with $NaHCO_3$ (3.7 g/L, Sigma), non-essential amino acids (1x, ICN, Zoetermeer, The Netherlands), FCS (5%, Invitrogen), penicillin (5000 U, Sigma) streptomycin (5 mg/L, Sigma) and methylcobalamin (vitamin B12; 500 pg/ml, Sigma). Culture media were replaced every second day. Cells were split at 70-80 % confluence and harvested at 90 % confluence. Total RNA was extracted using TRIZOL according to the supplier (Invitrogen). mRNA was isolated from total RNA by poly(A)$^+$ selection using oligod(T) Sephadex (mRNA purification kit, Pharmacia, Roosendaal, The Netherlands). Concentrations were determined spectrophotometrically at A_{260nm} and all samples were checked on 1% TAE/agarose gels. Samples were stored at -80°C for cDNA microarray analysis. From seven flasks of each exposure, the cells were trypsinised and the cell pellets were stored at -80°C for folate, iron, SAM and SAH determination. Before storage an aliquot was taken from each cell pellet, and used to determine the protein content (in duplicate) using the Bio-Rad Detergent Compatible (DC) protein assay (Bio-Rad, Veenendaal, The Netherlands). MTHFR was genotyped at 677bp and 1298bp position of each cell line using PCR/RFLP method (99). This resulted for HT29 in 677 CT and 1298 AC genotype, Caco-2 in 677 CC and 1298 CC genotype, and CCD841CoTr in 677 CT and 1298 AA genotype (data not shown).

Analysis of folate metabolism

5-methyl tetrahydrofolate concentrations were measured in duplicate using the HPLC method of Konings et al. (100). Twenty volumes (200µL) of extraction buffer (CHES/HEPES 50 mM, pH 7.85 with mercaptoethanol and ascorbic acid) was added to the cells. The mixture was heated for 5 minutes in a boiling water bath and cooled. To each extract 50 µl of rat plasma conjugase was added and incubated for 4 hours at 37°C. This mixture was treated further according to Konings et al (100). The size of the affinity column was adapted to the sample volume. S-adenosyl methionine (SAM) and S-adenosyl homocysteine (SAH) were determined in duplicate using the HPLC method of Melnyk et al. (101). Instead of coulometric detection, UV-detection at 260 nm was used. The method was adapted for cultured cells by addition of a thaw-freeze cycle for three times, at the beginning of the procedure. Cell lysis by thaw-freezing cycle was checked on an aliquot by microscopical observation of tryphan blue incorporation. The coefficient of variation for multiple injections (n=10) of the same sample within a run was 4.7% for SAH and 3.4% for SAM.

Analysis of parameters for cell turnover and metabolic activity

5-Bromo-2'-deoxy-uridine (BrdU) incorporation into the DNA of growing cells was measured (n=36) as a marker of proliferation (BrdU Labelling and Detection Kit II, Boehringer Mannheim, Roche Diagnostics, Almere, The Netherlands). Reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) by mitochondrial succinate dehydrogenase was used as a marker for metabolic activity (viability) of the cells (n=36) as described by Huveneers Oorsprong et al. (102). Intracellular adenosine triphosphate (ATP) production was determined (n=30), as a marker of cellular energy status, by CellTiter-Glo Luminescent Cell Viability Assay (Promega, Leiden, The Netherlands). Caspase 3 and 7 activities were measured (n=36) as a marker of apoptosis (Apo-One Homogeneous Assay, Promega). After each assay, an aliquot was taken from every sample and used to determine the protein content by Bio-Rad DC protein assay (Bio-Rad). Total protein was used to correct for differences in cell numbers between samples. E-cadherin was measured as a marker of differentiation. Whole-cell lysates were prepared by 5 min boiling of cell pellets with 1 mL 200 mM sucrose, 20 mM Tris pH7.4, 1 mM DTT and protease inhibitors (Roche). Protein concentration was determined using Bio-Rad Detergent Compatible protein assay (Bio-rad). Determination of actin and E-cadherin protein expression was achieved by SDS-PAGE (Criterion Precast system, Bio-

rad) and Western blotting. Proteins were transferred to Protran Nitrocellulose membrane (Schleicher and Schuell, 's Hertogenbosch, The Netherlands) before immuno detection with antibodies directed against actin (Santa Cruz, 1:200, SC-10731) and E-cadherin (DAKO, 1:200, M3612). Horseradish peroxidase conjugated anti-goat (V8051) and anti-mouse (W4021) (Promega; 1:7500 and 1:2500) were used for the color detection with TMB stabilized substrate for horseradish peroxidase (Promega). Densometric determination of actin and E-cadherin bands was performed with Imagemaster 1D (Pharmacia).

Analysis of gene expression

cDNA microarray analysis (array construction, mRNA labelling and hybridisation) was performed as described by Pellis *et al.* (98). Indirect labelling of mRNA was used, by incorporation of aminoallyl dUTP in the cDNA and subsequent chemical coupling to Cy5 monofunctional dye. A standard reference sample, consisting of a pool of mRNA from all exposed cell lines, was used and coupled to Cy3 monofunctional dye. Before hybridisation, the Cy3- and Cy5-labeled samples were mixed 1:1 (v/v) and hybridised to a cDNA microarray. This cDNA microarray contains 1056, duplicate, Caco-2 derived cDNAs (Peijnenburg et al. unpublished results) and 192 control sequences and known genes selected for functional relevance in folate metabolism, folate transport, DNA repair and carcinogenesis. All cDNAs were sequenced and annotated by means of BLAST searches in NCBI. Redundant clones were removed from the dataset, leaving 1026 unique annotations. Density values multiplied by the area of each spot, and the background (surrounding entire template) were collected using Arrayvision (Imaging Research, Ontario, Canada). Before data correction, background subtraction was performed. If the variation was less than 30% the median value was taken from the duplicate clones of the duplicate microarrays, otherwise this clone was discarded from further analysis. These values were used for principle component analysis (GeneMath, Applied Maths, Belgium), to find variances between treatments.

Analysis of intracellular iron

Intracellular iron concentration was analysed, in triplicate, using Atomic Absorption Spectrometry (Perkin Elmer SIMAA 6100, with graphite furnace and Zeeman background correction). Sample preparation was done for the cell lines as follows, 0.5 mL Millipore filtered water was added to the cell pellet and mixed and the cell suspension was

sonicated for 15 minutes. A 10 μL aliquot was stored at -20°C for protein concentration (in duplicate) using the Bio-Rad Detergent Compatible (DC) protein assay (Bio-Rad, Veenendaal, The Netherlands). Cells suspensions were digested after addition of 1.0 mL 70% HNO_3 (Baker) by a 6 hours incubation at 65°C, followed by an overnight-incubation at room temperature and a subsequent 6 hours incubation at 65°C. 1.0 mL Millipore filtered water was added and additionally incubated at 65°C overnight. Mixtures were sonicated for 5 min and an 11-time dilution was prepared. Finally, the atomic absorption was measured at 248.3 nm. The coefficient of variation for multiple injections (n=10) of the same sample within a run was 4.1%.

Statistical analysis

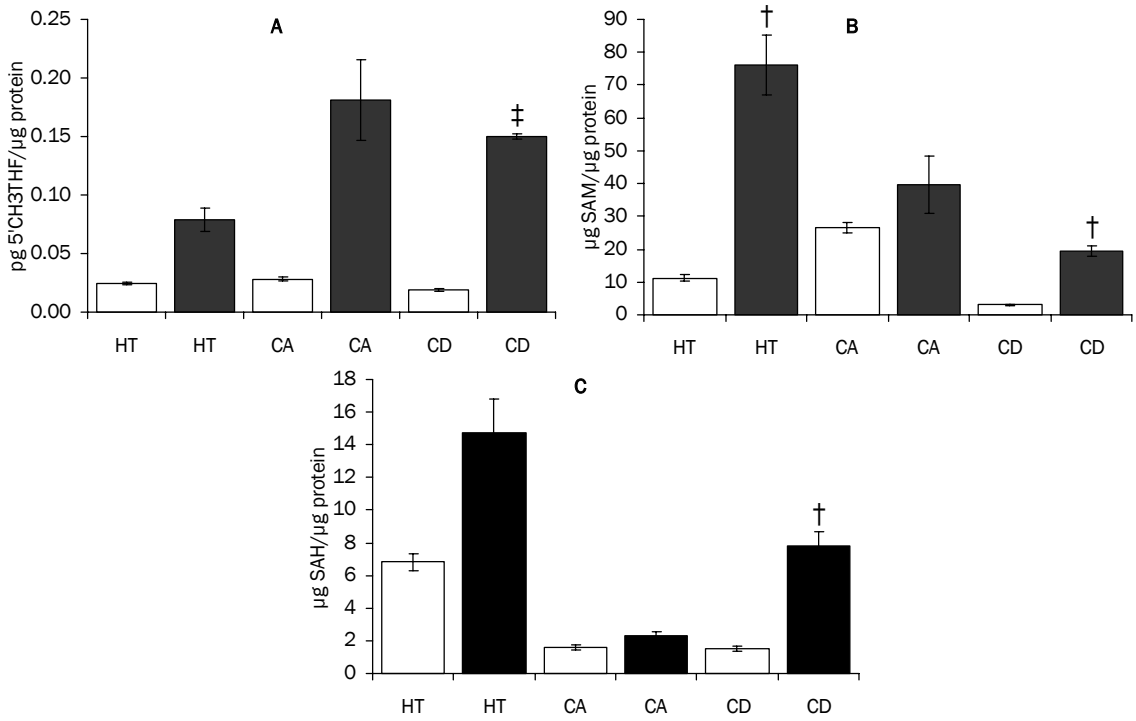
Statistical comparisons were made using an unpaired, two-tailed Student's t-test with a confidence level of 95%.

Results

Analysis of folate metabolism

Intracellular 5-methyltetrahydrofolate levels for the three different cell lines after adaptation to 10 ng/mL and 100 ng/mL are shown in Figure 3.1A. The 10 ng/mL folic acid exposure resulted in similar intracellular 5-methyltetrahydrofolate levels. The 100 ng/mL folic acid exposure resulted in higher intracellular 5-methyltetrahydrofolate concentration as compared to the 10 ng/mL folic acid exposure in all cell lines. However, the increases ranged from 3.2 fold in HT29, 6.4 fold in Caco-2 and a statistical significant 8.0 fold in CCD841CoTr. Higher intracellular SAM levels were observed for all cell lines in the 100 ng/mL folic acid exposure compared to the 10 ng/mL folic acid exposure (Figure 3.1B). A significant increase was found in HT29 and CCD841CoTr, namely 6.8 and 6.5, compared to a 1.5 fold increase in Caco-2 cells. Also higher intracellular SAH levels were observed for all cell lines in the 100 ng/mL folic acid exposure, a 1.5-fold increase was found in Caco-2, a 2.2-fold increase in HT29 and a significant 5.3-fold increase in CCD841CoTr (Figure 3.1C).

Figure 3.1 Folate metabolite concentrations

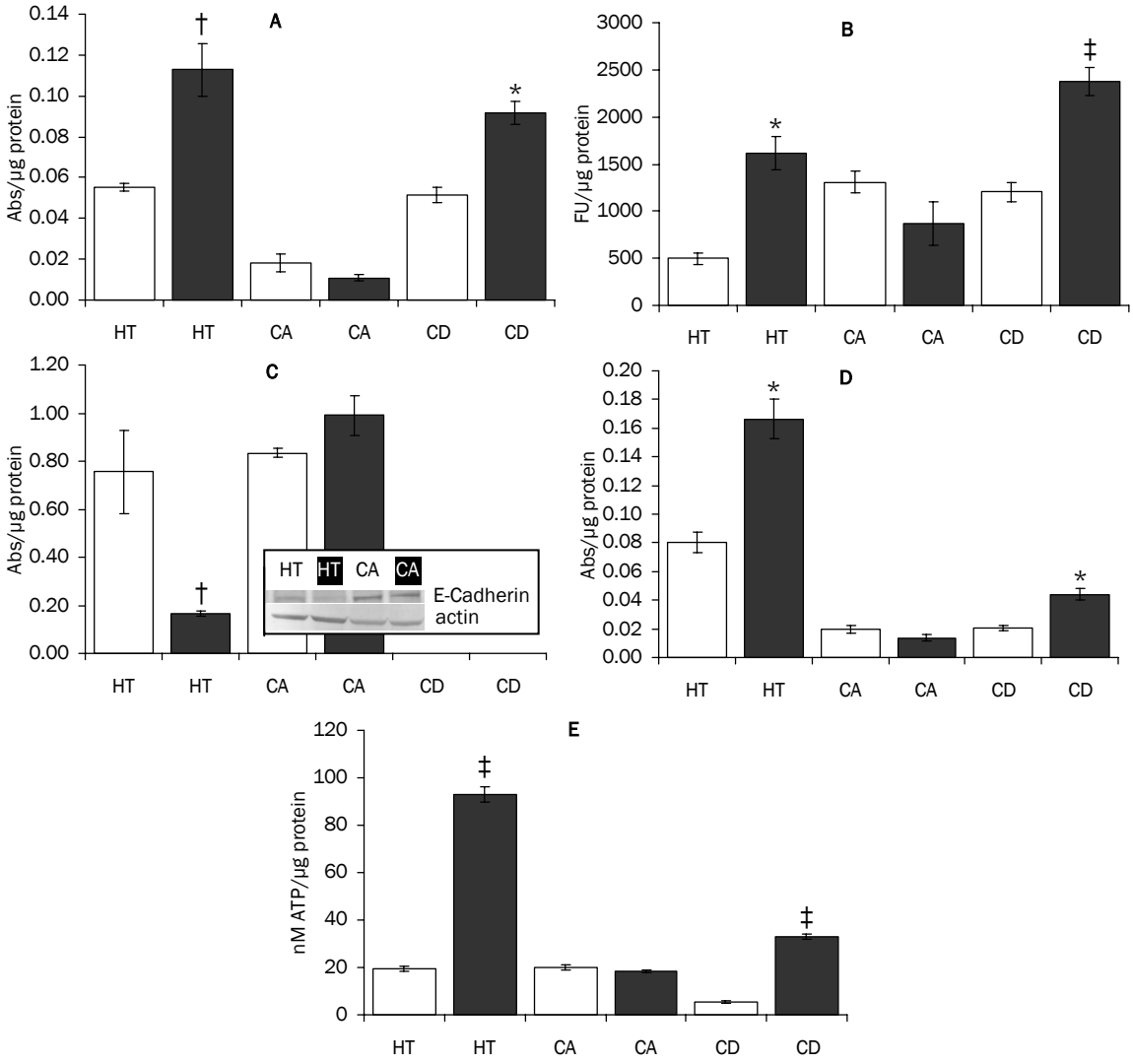


Intracellular 5-methyltetrahydrofolate (panel A), S-adenosyl methionine (SAM, (panel B)) and S-adenosyl homocysteine (SAH, (panel C)) were measured using HPLC. Values are normalized for protein content. Data are expressed as the mean ± SD (n=2). p-value †<0.05 and ‡<0.001, white bars: 10 ng/mL folic acid, black bars: 100 ng/mL folic acid, HT; HT29, CA: Caco-2, CD: CCD841CoTr.

Analysis of parameters for cell turnover and metabolic activity

During growth at 10 ng/mL and 100 ng/mL folic acid, a 2.4 fold higher growth rate was observed at 100ng/mL folic acid for all cell lines. The growth rates in standard DMEM, which has a supra physiological folic acid concentration of 4000 ng/mL, were found to be in between that of 10 and 100 ng/mL folic acid. Growth in the absence of folic acid, but with 5% serum, which provides a final folic acid concentration of 2 ng/mL, resulted in growth arrest after the first passage (data not shown). The increased growth rate coincided with a higher proliferation (Figure 3.2A) and apoptosis (Figure 3.2B) rate for both HT29 and CCD841CoTr at the 100 ng/mL folic acid condition. The difference in the proliferation rate was 2-fold for HT29 ($p<0.05$) and 1.8-fold fold for CCD841CoTr ($p<0.01$). HT29 cells showed a 3.2-fold higher rate of apoptosis at 100 ng/mL folic acid concentration ($p<0.01$), whereas CCD841CoTr showed a 2.0-fold higher rate of apoptosis

Figure 3.2 Cell turnover and metabolic acitivity



*Functional Assays (panel A) BrdU incorporation in DNA (n=36), (panel B) caspase 3 and 7 activity (n=36), (panel C) differentiation marker E-cadherin (n=2), (panel D) MTT conversion (n=36), (panel E) ATP content (n=30). Values are normalized for protein content, except figure C. Date are expressed as the mean ± SD. p-values *:<0.01, †<0.05 and ‡<0.001, white bars: 10 ng/mL folic acid, black bars: 100 ng/mL folic acid, HT: HT29, CA: Caco-2, CD: CCD841CoTr.*

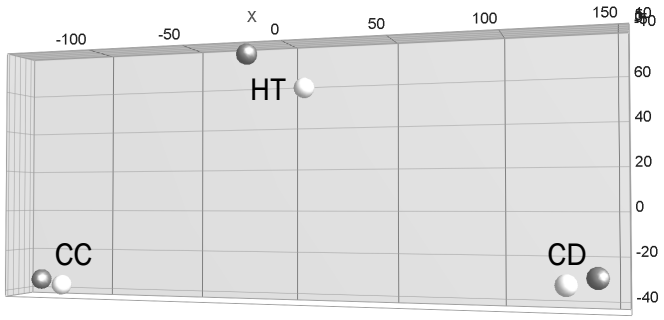
($p<0.05$). In contrast, Caco-2 showed the highest rate of both proliferation and apoptosis at 10 ng/mL folic acid, although the difference in proliferation and apoptosis rates between the two folic acid concentrations were not significant. Differentiation was analysed by determination of E-cadherin protein expression using immunoblotting. In the 100 ng/mL folic acid exposure a 4.6 fold lower E-cadherin protein level was found for HT29 as compared to the 10 ng/mL folic acid exposure (Figure 3.2C). E-cadherin expression was slightly higher in Caco-2, in CCD841CoTr E-cadherin protein levels were too low to be detected. Metabolic activity was analysed by MTT conversion and ATP

levels. Both HT29 and CCD841CoTr displayed a 2.1-fold ($p<0.01$) higher MTT conversion at the 100 ng/mL folic acid condition, although the overall level was higher for HT29 (Figure 2D). There was no difference observed for Caco-2 between both folic acid conditions. Intracellular ATP levels were 6.3-fold higher ($p<0.001$) at 100 ng/mL folic acid for CCD841CoTr and 4.8-fold for HT29, although absolute ATP levels were higher in HT29 (Figure 3.2E). No difference in ATP levels due to the different folic acid concentrations was observed for Caco-2 cells.

Analysis of gene expression

Hierarchical clustering (data not shown) as well as principal component analysis (PCA) (Figure 3.3) of total gene expression patterns revealed that the basal differences between cell lines were greater than their individual responses to different concentrations of folic acid. The PCA plot also indicates that the quantitative gene expression response to 10 ng/mL and 100 ng/mL folic acid differs between cell lines. In HT29 the effect on gene expression was the largest, while Caco-2 gave the smallest response of the three cell lines that were investigated. This is in agreement with the number of genes (16 for HT29, 4 for CCD841CoTr and 2 for Caco-2) that were 2 fold and greater differentially expressed, between the 10 and 100 ng/mL folic acid exposures. This difference in responsiveness is in agreement with the response seen in the cell turnover assays. Ten genes were higher expressed in HT29 at the 100 ng/mL folic acid

Figure 3.3 Principle Component Analysis



Principle Component Analysis of the total gene expression patterns in three human colon cell lines exposed to 10 ng/mL and 100 ng/mL folic acid. Each dot represents an averaged duplicate micoarray, in all cell lines the light dot represents 10 ng/mL folic acid and the dark dot represents 100 ng/mL folic acid.

Table 3.1 Genes differentially expressed (ratio ≥ 2) at different folic acid exposures.

#	gene abbreviation	HT29	Caco-2	CCD841CoTr
1	SPINK1	**2.4 (120)**	1.2 (140)	1.0 (32)
2	CDH3	**2.2 (8)**	1.0 (8)	-1.1 (4)
3	SAT	**2.1 (70)**	1.0 (40)	-1.1 (40)
4	CARP	**2.1 (30)**	-1.1 (20)	1.2 (22)
5	DKK1	**2.4 (30)**	1.0 (40)	-1.2 (30)
6	SOX4	**2.4 (30)**	1.0 (12)	-1.1 (6)
7	AGR2	**2.2 (180)**	1.1 (350)	1.1 (4)
8	FGA	**2.8 (14)**	-1.1 (20)	-1.2 (14)
9	APOB	**2.1 (100)**	1.2 (12)	1.2 (18)
10	TI-227H	**2.3 (100)**	1.3 (80)	1.0 (40)
11	ANPEP	1.1 (2)	**2.1 (4)**	-1.1 (4)
12	FOLR1	1.2 (12)	1.1 (8)	**2.4 (28)**
13	GOT2	-1.2 (34)	-1.4 (28)	**2.1 (40)**
14	CGI-85	-1.1(12)	-1.1 (10)	**2.0 (26)**
15	ANXA13	**-5.6 (60)**	-1.4 (30)	-1.2 (6)
16	FTL	**-2.2 (1200)**	-1.1 (560)	-1.4 (800)
17	ALDH1A1	**-2.1 (20)**	-1.3 (36)	-1.4 (4)
18	B2M	**-2.4 (400)**	-1.1 (90)	-1.6 (600)
19	SLC40A1	**-2.1 (20)**	-1.1 (8)	-1.1 (4)
20	SLC7A7	**-2.1 (40)**	-1.0 (30)	-1.1 (20)
21	LLD-57492	-1.1 (3)	**-2.0 (8)**	-1.1 (5)
22	CDH17	-1.1 (3)	-1.1 (12)	**-2.1 (8)**

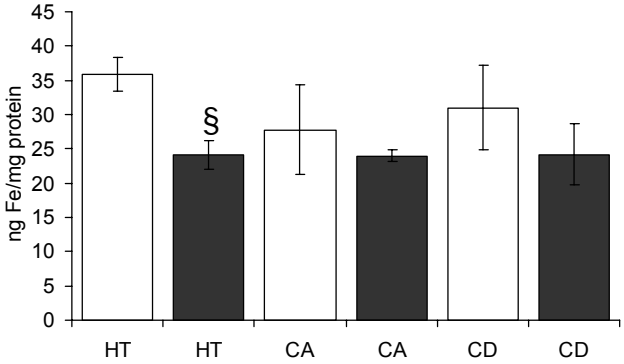
The ratio of 100 ng/mL to 10 ng/mL folic acid is given and a ratio greater than two is indicated in bold. A negative ratio implies a higher expression at 10 ng/mL folic acid. The numbers between brackets indicates expression levels and shown as signal divided by ten times the background.

condition (Table 3.1). These genes were not differentially expressed in either Caco-2 or CCD841CoTr. The same is true for the one gene of Caco-2 and three genes of CCD841CoTr that were 2 fold higher expressed at 100 ng/mL folic acid, with the exception of glutamic-oxaloacetic transaminase 2 (GOT2) which was higher expressed in CCD841CoTr 100 ng/mL folic acid and showed a reverse differential expression in Caco-2, although not 2 fold. This was also the case for the genes that were 2 fold or lower expressed at the 100 ng/mL folic acid exposure as compared to the 10 ng/mL folic acid exposure. In HT29 six genes were found to be higher expressed at 10 ng/mL folic acid and one in Caco-2 and CCD841CoTr, while no similar genes were found between cell lines.

Since the largest response was observed for HT29, we examined the function of the differentially expressed genes of HT29 in more detail. The genes that are clearly differentially expressed between the folic acid concentrations can be distributed into two subsets (Table 3.2). One subset consists of genes that are related to cell turnover (proliferation, apoptosis and differentiation). The expression pattern of these genes is in agreement with the results obtained in the functional assays.

The second subset of differentially expressed genes with lower expression at 100 ng/mL folic acid, have a function in iron metabolism. These are the iron transporter SLC40A1 and ferritin light chain. Ferritin plays a role in cellular iron storage and is a known marker of differentiation (103). To confirm the effect of folic acid on iron metabolism in human colon epithelial cell lines, we decided to determine intracellular iron concentrations. A lower intracellular iron level was found in the 100 ng/mL folic acid as compared to 10 ng/mL folic acid exposure for all cell lines (Figure 3.4). The biggest decrease was found in HT29.

Figure 3.4 Intracellular iron levels



Values were determined using atomic absorption spectrometry and are normalized for protein content. Data are expressed as the mean ± SD (n=3) §: p-value < 0.02, stripped bars: 10 ng/mL folic acid, filled bars: 100 ng/mL folic acid, H: HT29, CC: Caco-2, CD: CCD841CoTr.

Table 3.2 Description of the differentially expressed genes

#	gene abbreviation	accession nr	name	function	associated with
1	SPINK1	NM_003122	secretory trypsin inhibitor	trypsin inhibitor	proliferation^{u}
2	CDH3	NM_001793	P cadherin	cell adhesion	differentiation^{d}, proliferation^{u}
3	SAT	NM_002970	spermidine/spermine N1-acetyltransferase	enzyme	proliferation^{u}
4	CARP	NM_014391	ankyrin repeat domain 1	transcription factor related protein	proliferation^{u}
5	DDK1	NM_012242	dickkopf homolog 1	wnt pathway	apoptosis^{u}
6	SOX4	NM_003107	sex determining region Y-box 4		apoptosis^{u}
7	AGR2	NM_006408	anterior gradient 2 homolog	tumor biology	tumour-related
8	FGA	NM_000508	fibrinogen A	matrix interaction	tumour-related
9	APOB	NM_000384	apolipoprotein B-100	lipid metabolism	
10	TI-227H	D50525	metastasis associated gene	metastasis	tumour-related
11	ANPEP	NM_001150	alanyl aminopeptidase	enzyme	
12	FOLR1	U20391	folate receptor 1	receptor	
13	GOT2	NM_002080	glutamic-oxaloacetic transaminase 2	enzyme	
14	CGI-85	NM_016028	CGI-85		
15	ANXA13	NM_004306	annexin A13	transporter	differentiation^{u}
16	FTL	NM_000146	ferritin light polypeptide	iron storage	differentiation^{u}, iron
17	ALDH1A1	NM_000689	aldehyde dehydrogenase 1	detoxification	differentiation^{u}
18	B2M	NM_004048	beta-2-microglobulin	cell matrix	
19	SLC40A1	NM_014585	iron-regulated transporter	transporter	iron
20	SLC7A7	NM_003982	cationic amino acid transporter	transporter	
21	LLID-57492	AB033061	KIAA1235 protein	hypothetical protein	
22	CHD17	NM_004063	LI cadherin	cell adhesion	

The association with cell turnover and iron metabolism is indicated. u : up regulation of this gene is associated with up regulation of this process, d : down regulation of this gene is associated with down regulation of this process

Discussion

The aim of this study was to determine effects of folic acid on the human colon epithelium. This was done by identification of shared responses of three different human colon epithelial cell lines, one immortalized cell line and two tumour cell lines. In all three cell lines the 100 ng/mL folic acid exposure, compared to the 10 ng/mL folic acid exposure, resulted in higher concentrations of intracellular 5'-methyltetrahydrofolate, of SAM and of SAH, in higher cell growth and lower intracellular iron levels. In addition to these similar effects, differences were seen. Overall HT29 seemed most responsive, while Caco-2 was least responsive. The responsiveness was not due to differences in intracellular 5'-methyltetrahydrofolate levels, which were largest in CCD841CoTr and smallest in HT29. Differences in responsiveness may result from differences in the levels or the balance of other folate metabolites. This may be caused by genetic differences between the cell lines, in particular polymorphisms in folate related genes (e.g. MTHFR, see material and methods).

Differences in responsiveness of the cell lines were clearly seen in the gene expression analysis, but in all cases only a small number of genes were affected. This is in agreement with three other papers that have investigated the effect of folic acid on gene expression. These papers all compared folate depletion and supraphysiological folate exposure. Although extreme differences in folate concentration were used, also under these conditions only a relative small number of genes (0.15-3.40%) responded. Remarkably among the differentially expressed genes very few were involved in folic acid metabolism. In agreement with this Crott et al. (55), using colonic rat mucosa, and Jhaveri et al. (56), using nasopharyngeal epidermoid carcinoma cells, found that folate depletion did not affect the expression of any folate related genes. We found only one gene related to folate metabolism in one cell line that showed to be differential expressed. This gene, folate receptor 1 was highest expressed at 100 ng/mL folic acid exposure in CCD841CoTr, the cell line that showed the highest intracellular 5'-methyltetrahydrofolate of all cell lines. None of the other genes involved in folate-metabolism (e.g. methylene tetrahydrofolate reductase, methionine synthase and serine hydroxymethyltransferase) or those involved in folate-transport (e.g. reduced folate carrier, SLC19A1 and folate receptor 2) were affected by the exposure conditions in any of the cell lines. This seems in contrast with Courtemanche et al. (57), who used non-epithelial cells. In primary human T lymphocytes these authors found that folate

deficiency resulted in down regulation of genes involved in folate metabolism, in particular the folate transporter. Besides the use of different cel types, a possible explanation for this difference could be the length of exposure. Courtemanche et al. (57) also reported higher expression of DNA repair and mitochondrial genes. The effect on mitochondrial genes points to an alteration of cellular energy metabolism. This agrees with our study, which showed an effect of folate on ATP content and MTT conversion. One carbon units in the mitochondria are required for the synthesis of formate, glycine and f-met-tRNA and mitochondria play an important role in carcinogenesis (104,105), but the precise role of folate on mitochondrial and cellular energy metabolism is not well investigated. This is an area that deserves further attention.

Cell proliferation and apoptosis are essential features in gut renewal, but excess cell proliferation is associated with carcinogenesis and generally considered as one of the early events in colon cancer (21,106). Recently, animal studies have indicated that timing and dose of folate intervention may be of critical importance in protection from colon carcinogenesis. It was observed that folate supplementation seems to enhance established neoplasms and deficiency has an inhibitory effect. This in contrast to normal epithelium, in which folate deficiency appears to increase the neoplasm risk, whereas moderate folate supplementation suppress the development of tumours (15,21,22,28,95,107). To understand the role of folate on tumour prone cells, we examined cell turnover parameters for the three cell lines that all have tumour characteristics. CCD841CoTr, is a SV40 immortalized cell line, while HT29 and Caco-2 are both colon tumor derived cell lines that can differentiate to form a mucous layer (HT29) or villi (Caco-2). All three cell lines showed a higher growth rate at higher folic acid concentrations. For HT29 and CCD841CoTr, the higher growth rate was accompanied with a higher rate of both proliferation and apoptosis, while for Caco-2 a lower proliferation and apoptosis rate was observed. This may imply that the rate of cell turnover, and therefore cell growth, is determined by a subtle balance between proliferation and apoptosis. Complete folate deficiency resulted in growth arrest, probably due to higher apoptosis and lower proliferation, as reported by others. A key feature in early and later stages in colon cancer is the inactivation of differentiation marker E-cadherin protein (15,108,109). E-cadherin protein expression levels are below detection in CCD841CoTr, but could be detected in HT29 and Caco-2. A higher concentration of folic acid showed a lower level of E-cadherin protein expression in HT29, indicative of risk

of cancer, but resulted in the opposite effect, i.e. a slightly higher expression in Caco-2, indicative of benefit. Together, our findings are supportive of the suggestion that the dosis of folic acid intervention may be critical in protection of carcinogenesis or otherwise enhancement of cancers (21,23,110). More insight will be obtained by analysis of the effect of a folic acid intervention on E-cadherin protein levels in the colon epithelium of healthy and at-risk individuals.

In all cell lines we found that intracellular iron levels are lower at 100 ng/mL folic acid exposure, which shows an effect of folate on iron metabolism. Previously, the converse effect of iron status on folate levels has been established, namely that iron deficiency results in folate deficiency in animals and humans (111-113). Furthermore a direct interaction between iron and folate metabolism has been shown; reduction of free iron by chelation of iron using either the chemical chelator mimosine (114) or the biological chelator ferritin heavy chain (but not FTL) (112) results in up regulation of translation rate of cytoplasmic serine hydroxyl methyl transferase (cSMHT) and increasing folate catabolism. We did not find an effect of folate levels on cSHMT gene expression (data not shown), but we did not investigate the SHMT protein levels. Our observation that folate affects total cellular iron and the expression of FTL, involved in binding of free iron and thus protecting the cell from its damaging oxidative properties, indicates a close interaction between both micronutrients, possibly to regulate cellular single carbon metabolism. This warrants further research, not only with respect to colon cancer risk, but also in view of the important role of iron and folate for the developing foetus (115).
In conclusion, the chronic exposure of cells to 10ng/mL compared to 100 ng/mL folate results in a lower cell growth, lower intracellular folate, SAM and SAH, and higher iron uptake, but cell turnover and gene expression response to folic acid seems to be cell line specific.
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Low intracellular folate levels inhibit glycolysis and growth of HT29 human colon cancer cells

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Submitted for publication

Abstract

Animal intervention trials suggest that folate deficiency can have an inhibitory effect on the progression of established colonic tumour cells. A characteristic of tumour cells is that they display an increased glycolysis and inhibition of glycolysis is a strategy to kill cancer cells. We investigated whether differences in folate status, obtained by exposure to physiological relevant folate concentrations, could affect the growth and the glycolytic rate of a model human colon cancer cell line. HT29 human colon cancer cells were grown in different types and concentrations of folate; synthetic folic acid (10 or 100 ng/mL pteroylglutamic acid; PGA) or natural folate (10 or 100 ng/mL 5-methyl-tetrahydrofolate; MTHF). Intracellular folate concentrations, growth, ATP content, lactate production, mitochondrial mass and oxygen consumption were assessed. Long-term culture of HT29 colon cancer cells in low levels of PGA (10 ng/mL) leads to low intracellular levels of tetrahydrofolate and 5-methyl-tetrahydrofolate and a diminished growth rate compared to long-term culture in either 100 ng/mL PGA or 10-100 ng/mL MTHF. Furthermore, HT29 cells with low intracellular folate levels and a reduced growth rate show a decrease in ATP content and lactate production. This decrease in glycolysis was accompanied with an increase in mitochondrial mass as well as mitochondrial oxygen consumption. Our results indicate that low intracellular folate levels (folate deficiency) can revert the phenotype of HT29 colon cancer cells towards a phenotype more characteristic for normal cells.

Introduction

Folate is a water-soluble B vitamin that naturally occurs in foods, such as green leafy vegetables, beans and peas and many other types of fruit and vegetables (116). Natural folates in foods are pteroylglutamate compounds that are all conjugated to a polyglutamyl chain containing different numbers of glutamic acids depending on the type of food. This polyglutamyl chain is removed by the intestinal folate conjugase enzyme before folate-monoglutamate is subsequently taken up by the intestine. If food folates are not in the 5-methyl-tetrahydrofolate (MTHF) form already, they will be converted to this form in intestinal mucosa cells (10).

Folic acid, the synthetic form of folate, is a monoglutamate (pteroylglutamic acid, PGA). This form does not require cleavage by the intestinal folate conjugase enzyme before uptake. Folic acid is used for fortification of grain products and is present in vitamin supplements because it is chemically more stable and is supposedly better bioavailable than natural folates (116). For physiological activity, folic acid needs to be reduced by the enzyme dihydrofolate reductase to dihydro- and tetrahydrofolate (DHF and THF). This occurs within intestinal mucosal cells and MTHF is released into the plasma (10).

Folates are thought to play a dual modularly role in colorectal carcinogenesis, depending on timing and dose of intervention (107). Animal intervention studies of Song et al. (117,118), using genetically predisposed murine models of intestinal tumourigenesis, support the dual modularly role of folate in carcinogenesis. These authors found that folic acid (PGA) supplementation suppressed intestinal tumor development in normal healthy epithelial tissues of Apc+/- and Apc+/-Msh-/- mice, whereas folate deficiency predisposed the animals to neoplastic transformation. In contrast, if intestinal neoplasms were already established, PGA supplementation had a promoting effect and deficiency inhibited progression into tumours. This inhibitory effect of folate deficiency in tissues with rapidly replicating cells could be explained by ineffective DNA synthesis. Folates are essential for purine and pyrimidine synthesis (119) and reduced levels of these building blocks may result in inhibition of tumour cell growth (22).

Rapidly growing cancer cells also have an impaired mitochondrial energy metabolism. Already in 1930, Otto Warburg described that cancer cells show a shift in energy production from mitochondrial oxidative phosphorylation (OXPHOS) to cytosolic glycolysis (120). Aerobic glycolysis, ATP production during the conversion of glucose to pyruvate and lactate, even in the presence of oxygen (121), is a major characteristic of tumour cells,

including colon tumours. Inhibition of glycolysis may have broad therapeutic implications in cancer treatment, as it is an effective strategy to kill cancer cells (121,122).

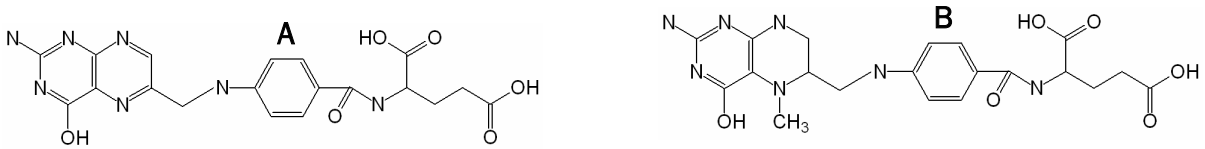
In the present study, we examined the effect of different physiological relevant concentrations and forms of folate on intracellular folate status, cell growth and energy metabolism of colon cancer cells. The highly glycolytic HT29 human colon cancer cell line (123) was chosen as an *in vitro* model to obtain more insight into the effect of folate status on colon cancer cell growth and to reveal whether inhibition of glycolysis could be involved in folate deficiency-induced growth inhibition of colonic tumour cells.

Material and Methods

Cell Culture

HT29 human colon cancer cells (ATCC number HTB-38, passage 141-150) were continuously (> 3 weeks) cultured in Dulbecco's modified Eagle's Medium (DMEM) without folic acid (Invitrogen, Breda, The Netherlands) supplemented with 3.7 g/l NaHCO3 (Sigma-Aldrich, Zwijndrecht, The Netherlands), 5% fetal calf serum (FCS) (Invitrogen), 1% non-essential amino acids (ICN, Zoetermeer, The Netherlands), 2% penicillin-streptomycin (5000 U - 5 mg/l, Sigma-Aldrich), 500 pg/ml methylcobalamin (vitamin B12; Sigma-Aldrich) and either 10 or 100 ng/mL of synthetic folic acid (pteroylglutamic acid; PGA) (Figure 4.1A) or 10 or 100 ng/mL of the natural form of folate, 5-methyl-tetrahydrofolic acid (MTHF) (Figure 4.1B) (Sigma-Aldrich). 10 ng/mL represents the normal human serum folate level and 100 ng/mL represents the human serum folate level that can be obtained upon dietary supplementation with folic acid (57,116). DMEM with folic acid normally contains 4000 ng/mL folic acid. The level of folate in FCS was 13.2 ng/mL and the level of vitamin B12 in FCS was 347 pg/ml.

Figure 4.1 Chemical structures of PGA and MTHF



Chemical structures of synthetic folic acid (pteroylglutamic acid, PGA) (A) and natural folate (5-methyl-tetrahydrofolate, MTHF) (B).

All cells were maintained in an atmosphere of 5% CO_2/95% air at 37 °C. Cells were sub-cultured at a ratio of 1-2:10 after they had reached 70-90% confluence in 75 cm^2 culture flasks (Corning, Schiphol-Rijk, The Netherlands). Cell culture medium was refreshed 3

times a week. Cell numbers were determined after trypsinisation using the Coulter Counter Z2 (Beckman Coulter, Mijdrecht, The Netherlands).

Intracellular THF and MTHF levels

Intracellular tetrahydrofolate (THF) and 5-methyl-tetrahydrofolate (MTHF) were measured in cell pellets of 75 cm^2 culture flasks using the HPLC fluorescent and diode array detection method of Konings (100). Each cell pellet was extracted with 200 µl of 50 mM CHES/HEPES buffer pH 7.0, mixed thoroughly and heated for 5 minutes in a boiling water bath. After cooling down, 50 µl of rat plasma conjugase (Rockland, Gilbertsville, USA) was added to each sample and the mixture was incubated for 4 hours at 37 °C. The extracts were then purified by affinity chromatography and analyzed by HPLC (Gynkotek M480 gradient system, Germering, Germany). Separation took place on a Symmetryshield RP18 5 µm, 4,6*150 mm column (Waters, Milford, MA, USA) protected by Brownlee C18 precolumn (Perkin Elmer, Shelton, CA, USA). Eluent A consisted of acetonitrile/phosphate 25 mM, pH 2.1 (94/6), eluent B consisted of acetonitrile/phosphate 25 mM, pH 2.1 (50/50). The following gradient was used: 0-20 min 0-19%B, 20-21 min 19%B, 21-22min 19-100%B, 22-24 min 100%B, 24-25 min 100-0%B, and the next injection at 31 min. Results are expressed in ng per mg protein. Total protein in cell pellets was measured according to Lowry (124) with use of the DC protein assay (Bio-Rad Laboratories, Veenendaal, The Netherlands).

Cell growth

HT29 cells were washed using HBSS, enzymatically harvested using 0.5% trypsin/5.3 mM EDTA and resuspended in fresh corresponding cell culture medium. Differences in cell growth were measured by re-plating 2.5 x 10^6 HT29 cells of the different culture regimens (10 or 100 ng/mL PGA or MTHF) in 75 cm^2 cell culture flasks after being in culture for > 3 weeks and determination of the amount of cells per flask after 1 and 3 days of culture, using the Coulter Counter.

ATP content

The CellTiter-Glo™ Luminescent cell viability assay of Promega (Leiden, The Netherlands) was used to measure the ATP content of HT29 cells. Cells were plated in 100 µl of cell culture medium at a concentration of 20000 cells per well, in 96-well flat clear bottom white polystyrene tissue culture plates (Corning). After 3 days, the ATP content was

measured according to the protocol of the manufacturer. Luminescence was recorded with the Luminoskan (Labsystems, Lifesciences International, Zellik, Belgium). Results are expressed in nmol per mg protein. Total protein in cell cultures was measured as stated above.

Lactate production

HT29 cells were plated in 500 µl of cell culture medium at a concentration of 100000 cells per well in 24-wells tissue culture plates (Corning). After 72 hours in culture, medium was collected from each well and lactate levels were measured with the ANALOX GM7 Analyser (Analox Instruments, Imlab BVBA, Boutersem, Belgium). The analyser measures the amount of oxygen uptake which is directly proportional to the concentration of lactate in the medium (lactate + O_2 → pyruvate + H_2O_2). The analyser is calibrated with the Analox Lactate standard (8 mmol/L). Results are expressed in µmol lactate produced per µg protein per 72 hours. Total protein in cell cultures was measured as stated above.

Mitochondrial oxygen consumption

HT29 cells were washed using Hanks Balanced Salts (HBSS) (Sigma-Aldrich), enzymatically harvested using 0.5% trypsin/5.3 mM EDTA (Invitrogen) and resuspended in their corresponding cell culture medium at a concentration of 1.0 x 10^6 cells/ml. Mitochondrial oxygen (O_2) consumption at 37 °C was analyzed by high-resolution respirometry using an Oxygraph (Oroboros Instruments, Innsbruck, Austria), with the chamber volumes set at 2 ml. DatLab software (Oroboros Instruments) was used for data recording (2 s time intervals) and analysis (125).

First of all routine respiration (R) was measured, which is defined as respiration in cell culture medium without additional substrates or inhibitors. After a steady-state respiratory flux was obtained, 2 µl of 2 mg/ml oligomycin (Sigma-Aldrich) was added to the chambers to inhibit ATP synthase (4O; oligomycin-inhibited state 4 respiration). Afterwards mitochondrial oxidative phosphorylation (OXPHOS) was uncoupled by stepwise titration of carbonyl cyanide p-trifluoromethoxyphenyl-hydrazone (FCCP) (Sigma-Aldrich) up to a maximum concentration of 3-6 µM (3U; uncoupled state 3 respiration). Finally, respiration was inhibited by addition of 5 µl of 0.5 mM rotenone (Sigma-Aldrich), to test for the effect of inhibiting OXPHOS complex I activity.

Besides information on absolute respiratory fluxes (expressed in pmol O_2 / sec / 1.0 x 10^6 cells) from the defined states of respiration (R;4O;3U), also important respiratory

control ratios can be derived from this protocol. These are the uncoupling control ratio (UCR = 3U/R), the respiratory control ratio (RCR = 3U/4O) and the phosphorylation respiratory control ratio (RCRp = (R-4O)/3U).

Mitochondrial mass

Mitochondrial mass of HT29 cells is determined using MitoTracker Green FM (MTGFM) (Molecular Probes, Invitrogen BV, Breda, The Netherlands). MTGFM is a green-fluorescent mitochondrial stain that localizes to mitochondria regardless of the mitochondrial membrane potential. HT29 cells were plated in 500 µl of cell culture medium at a concentration of 100000 cells per well in 24-wells tissue culture plates (Corning). After 3 days in culture, cells were washed using HBSS and 1 µM of MTGFM in cell culture medium was added for 30 minutes at 37 °C. After washing the cells for another 2 times with HBSS, fluorescence was recorded with the Cytofluor 4000 (Perceptive Biosystems; excitation 485/20 nm; emission 530/25 nm). Wells with culture medium but without cells were used for background correction. Mitochondrial mass is expressed in MTGFM fluorescence units (FU) per µg protein. Total protein in cell cultures was measured as stated above.

Statistical analyses

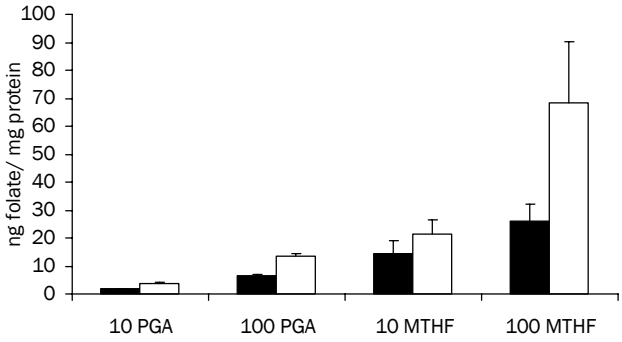
All results are expressed as means $\pm$ standard deviation (SD). Statistical analyses were performed using ANOVA with the Bonferroni test for post-hoc analysis. $p < 0.05$ was taken as the level of significance.

Results

Lower intracellular tetrahydrofolate (THF) and 5-methyl-tetrahydrofolate (MTHF) levels in HT29 human colon cancer cells as a result of synthetic folic acid (PGA) exposure compared to natural folate (MTHF) exposure

Intracellular THF and MTHF were measured in HT29 cells in order to verify uptake and bioavailability of different concentrations and forms of folate. As can be seen in Figure 4.2, intracellular levels of both THF and MTHF increase in the following order: 10 ng/mL PGA < 100 ng/mL PGA < 10 ng/mL MTHF < 100 ng/mL MTHF. Both the intracellular THF and MTHF levels of the 10 and 100 ng/mL PGA treatments were significantly lower compared to the 100 ng/mL MTHF treatment ($p < 0.05$).

Figure 4.2 Intracellular tetrahydrofolate (THF) and 5-methyl-tetrahydrofolate (MTHF) levels

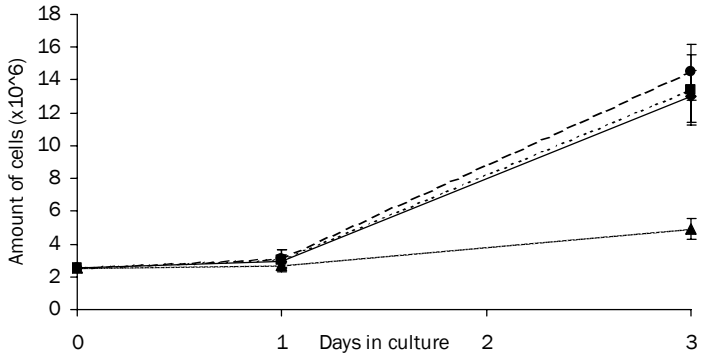


Intracellular tetrahydrofolate (THF, black bars) and 5-methyl-tetrahydrofolate (MTHF, white bars) levels (ng per mg protein) in HT29 human colon cancer cells cultured in 10 ng/mL PGA (10 PGA), 100 ng/mL PGA (100 PGA), 10 ng/mL MTHF (10 MTHF) or 100 ng/mL MTHF (100 MTHF). Results represent the means ± SD of duplicate determinations of 1 out of 2 independent experiments. Oneway ANOVA revealed a significant differences for both THF (p = 0.012) and MTHF (p = 0.015) between the groups. The Bonferroni post-hoc test revealed this difference for THF between 10 PGA – 100 MTHF (p = 0.02) and 100 PGA – 100 MTHF (p = 0.041). For MTHF, there were significant differences found between 10 PGA – 100 MTHF (p = 0.026) and 100 PGA – 100 MTHF (p = 0.046).

Low intracellular folate levels retard the growth rate of HT29 human colon cancer cells

HT29 cells are rapidly growing tumour cells. As illustrated in Figure 4.3, HT29 cells with low intracellular folate levels, cultured in 10 ng/mL PGA, grow significantly slower ($p < 0.02$), with a factor of approximately 2.7 fold, compared to HT29 cells cultured in 100 ng/mL PGA, 10 ng/mL MTHF or 100 ng/mL MTHF. These results clearly indicate that the

Figure 4.3 Growth rates



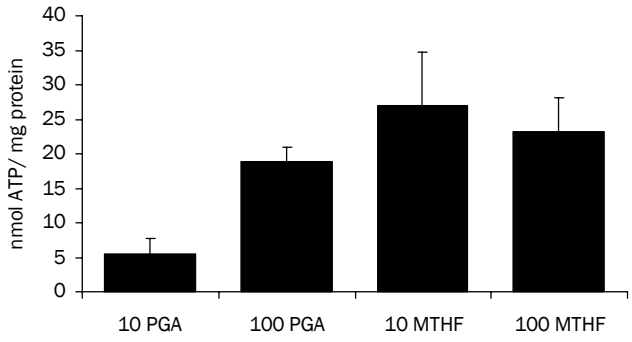
Growth curves of HT29 human colon cancer cells cultured in 10 ng/mL PGA (10 PGA, ▲), 100 ng/mL PGA (100 PGA, ●), 10 ng/mL MTHF (10 MTHF, ■) or 100 ng/mL MTHF (100 MTHF,♦). After > 3 weeks in culture, the cells were re-plated in 75 cm² cell culture flasks and the number of cells were counted after 1 and 3 days. Results represent the means ± SD of 3 independent replicate determinations. Oneway ANOVA revealed a significant difference in the number of cells between the groups at day 3 (p = 0.0001). The Bonferroni post-hoc test revealed this difference to be between 10 PGA and the other groups (100 PGA, p = 0.001; 10 MTHF, p = 0.001; 100 MTHF, p = 0.002).

growth rate of HT29 human colon cancer cells with low intracellular folate levels (~ folate deficiency) is inhibited. There was no significantly difference in growth rate between HT29 cells cultured in 100 ng/mL PGA, 10 ng/mL MTHF or 100 ng/mL MTHF. A clear difference is seen between exposure to 10 ng/mL of synthetic folic acid (PGA) versus 10 ng/mL of the natural folate (MTHF), which represents the human serum level.

A diminished energy status in HT29 human colon cancer cells with low intracellular folate levels

The ATP content of HT29 cells cultured in either 10 or 100 ng/mL synthetic folic acid (PGA) or natural folate (MTHF) was measured to identify differences in energy status between these different treatment regimens. Figure 4.4 illustrates the results of 6 replicate determinations of 1 out of 4 independent experiments. From the results, it is clear that the slow-growing HT29 cells with low intracellular folate levels, cultured in 10 ng/mL PGA, have a significantly ($p = 0.0001$) lower amount of intracellular ATP compared to the other treatments. There was no statistical difference in intracellular ATP level between 100 ng/mL PGA, 10 ng/mL MTHF and 100 ng/mL MTHF.

Figure 4.4 Energy status

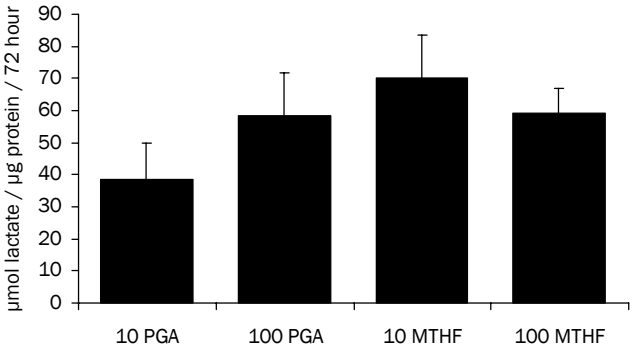


ATP content of HT29 human colon cancer cells cultured in 10 ng/mL PGA (10 PGA), 100 ng/mL PGA (100 PGA), 10 ng/mL MTHF (10 MTHF) or 100 ng/mL MTHF (100 MTHF). Results represent the means ± SD of 6 replicate determinations of 1 out of 4 independent experiments. Oneway ANOVA revealed a significant difference between the groups ($p = 0.0001$). The Bonferroni post-hoc test revealed this difference to be between 10 PGA and the other groups (100 PGA, $p = 0.0001$; 10 MTHF, $p = 0.0001$; 100 MTHF, $p = 0.0001$).

Lower rate of lactate production in HT29 human colon cancer cells with low intracellular folate levels

Cancer cells are highly glycolytic and we explored whether the observed the differences in ATP content were caused by differences in cytosolic glycolysis, as the source of ATP production. We investigated this by measuring lactate production. We indeed found a

Figure 4.5 Lactate production



Lactate production of HT29 human colon cancer cells cultured in 10 ng/mL PGA (10 PGA), 100 ng/mL PGA (100 PGA), 10 ng/mL MTHF (10 MTHF) or 100 ng/mL MTHF (100 MTHF). Lactate was measured in the media after 72 hours in culture. Results represent the means ± SD of 4 replicate determinations of 1 out of 3 independent experiments. Oneway ANOVA revealed a significant difference between the groups (p = 0.031). The Bonferroni post-hoc test revealed this difference to be between 10 PGA - 10 MTHF (p = 0.026).

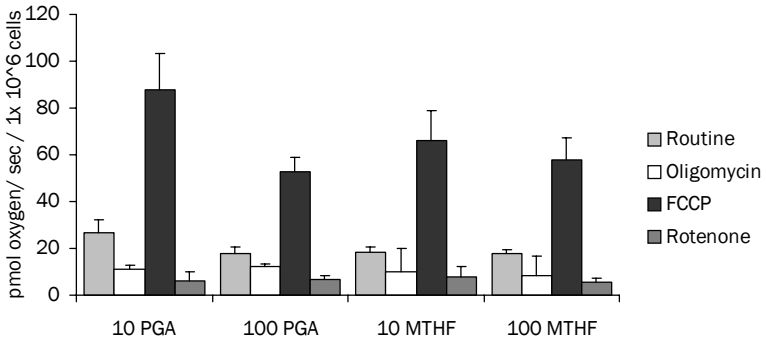
lower amount of lactate in the media of HT29 cells (corrected for the amount of protein) cultured in 10 ng/mL PGA compared to the other culture conditions. The lactate levels between 10 ng/mL PGA and 10 ng/mL MTHF were significantly different, with a p-value of 0.026 (Figure 4.5).

Upregulation of mitochondrial respiration in HT29 human colon cancer cells with low intracellular folate levels

In addition to glycolysis, ATP can also be generated via mitochondrial oxidative phosphorylation (OXPHOS). Mitochondrial respiration was measured in all culture conditions to verify whether the decrease in ATP content in HT29 cells cultured in 10 ng/mL PGA could also be explained by a decrease in mitochondrial OXPHOS. The mitochondrial respiratory capacity was analyzed in a standardized experimental regime (Figure 4.6; n=10). Strikingly, the routine respiration of HT29 cells with low intracellular folate levels (10 ng/mL PGA) was significantly higher compared to the other culture

conditions (100 ng/mL PGA; 10 ng/mL MTHF and 100 ng/mL MTHF) ($p < 0.004$). FCPP stimulated respiration, reflecting the respiratory capacity of uncoupled mitochondria in

Figure 4.6 Mitochondrial oxygen consumption



Mitochondrial oxygen consumption of HT29 human colon cancer cells cultured in 10 ng/mL PGA (10 PGA), 100 ng/mL PGA (100 PGA), 10 ng/mL MTHF (10 MTHF) or 100 ng/mL MTHF (100 MTHF). Routine respiration, oligomycin inhibited respiration, FCCP stimulated respiration and rotenone inhibited respiration are shown. Results represent the means ± SD of 10 independent replicate determinations. Oneway ANOVA revealed a significant difference between the groups for routine respiration ($p = 0.0001$), oligomycin inhibited respiration ($p = 0.005$) and FCCP stimulated respiration ($p = 0.0001$). The Bonferroni post-hoc test revealed this difference for the routine respiration between 10 PGA and the other groups (100 PGA, $p = 0.0001$; 10 MTHF, $p = 0.007$; 100 MTHF, $p = 0.004$). The differences for oligomycin were between 100 PGA – 100 MTHF ($p = 0.004$) and for FCCP also between 10 PGA and the other groups (100 PGA, $p = 0.0001$; 10 MTHF, $p = 0.028$; 100 MTHF, $p = 0.002$).

intact cells was also significantly higher in the HT29 cells cultured in 10 ng/mL PGA compared to the other treatments ($p < 0.028$). There was no statistical difference in routine respiration and FCCP stimulated respiration between 100 ng/mL PGA, 10 ng/mL MTHF and 100 ng/mL MTHF. Both oligomycin and rotenone inhibited respiration, with no difference in the final rates of oxygen consumption between the different culture conditions, only between the oligomycin inhibited respiration of 100 ng/mL PGA and 100 ng/mL MTHF ($p = 0.004$). The extent of respiration inhibition by both oligomycin and rotenone was higher in the HT29 cells cultured in 10 ng/mL PGA compared to the other treatments, however this was not statistically significant.

The results of the respiratory control ratios are shown in Table 4.1. RCR values greater than 10 are indicative of well-coupled mitochondria, lower values are indicative of "loose coupling" of the processes of substrate oxidation and ATP synthesis (126). The RCR are all lower than 10, but HT29 human colon cancer cells cultured in 10 ng/mL PGA have significantly ($p < 0.01$) the highest value. There was no difference in UCR. The RCRp values were also not significantly different between the different treatments.

Table 4.1 Respiratory control ratios

	10 PGA	100 PGA	10 MTHF	100 MTHF
UCR	3.3 ± 0.4	2.9 ± 0.5	3.6 ± 0.3	3.3 ± 0.5
RCR	8.1 ± 0.8[a]	4.3 ± 0.5[b]	6.8 ± 1.0[b]	6.9 ± 0.8[b]
RCRp	0.18 ± 0.04	0.12 ± 0.05	0.13 ± 0.04	0.16 ± 0.04

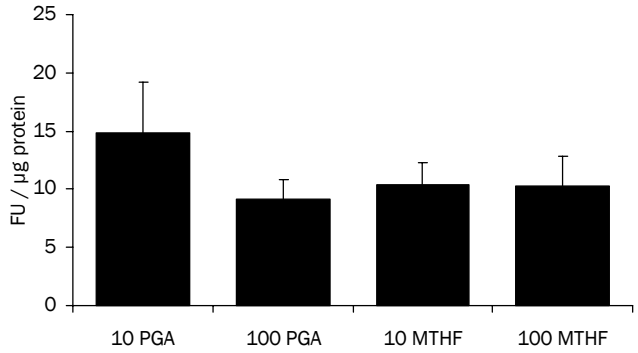
Respiratory control ratios of HT29 human colon cancer cells cultured in 10 ng/mL PGA (10 PGA), 100 ng/mL PGA (100 PGA), 10 ng/mL MTHF (10 MTHF) or 100 ng/mL MTHF (100 MTHF). *The uncoupling control ratio (UCR), respiratory control ratio (RCR) and phosphorylated respiratory control ratio (RCRp) for each culture condition are shown. Values are means ± SD.*

[a,b] Values with different letters are significantly different (p < 0.01).

Increased mitochondrial mass in HT29 human colon cancer cells with low intracellular folate levels

Mitotracker Green FM was used to measure mitochondrial mass in HT29 cells cultured in different forms and amounts of folate. Besides an upregulation of mitochondrial respiration in HT29 cells with low intracellular folate levels, we also found a significant increased Mitotracker Green FM fluorescence in HT29 cells cultured in 10 ng/mL PGA (Figure 4.7) ($p \leq 0.026$). This provides evidence of an increased mitochondrial mass (abundance) in these cells, compared to the other treatments (100 ng/mL PGA; 10 ng/mL MTHF; 100 ng/mL MTHF). There was no statistical difference in mitochondrial mass between 100 ng/mL PGA, 10 ng/mL MTHF and 100 ng/mL MTHF.

Figure 4.7 Mitochondrial mass



Mitochondrial mass of HT29 human colon cancer cells cultured in 10 ng/mL PGA (10 PGA), 100 ng/mL PGA (100 PGA), 10 ng/mL MTHF (10 MTHF) or 100 ng/mL MTHF (100 MTHF). Results represent the means ± SD of 8 replicate determinations of 1 out of 4 independent experiments. Oneway ANOVA revealed a significant difference between the groups (p = 0.002). The Bonferroni post-hoc test revealed this difference to be between 10 PGA and the other groups (100 PGA, p = 0.003; 10 MTHF, p = 0.026; 100 MTHF, p = 0.018).

Discussion

We found that low intracellular folate levels (~ folate deficiency) inhibit the growth of HT29 human colon cancer cells. Long-term culture of HT29 cells in 10 ng/mL PGA, that leads to low intracellular levels of THF and MTHF, also reduces the ATP content and lactate production of these cells compared to long-term culture in either 100 ng/mL PGA or 10-100 ng/mL MTHF. The decrease in glycolytic rate was accompanied with an increase in mitochondrial O_2 consumption as well as mitochondrial mass.

Intracellular levels of both THF and MTHF increase in HT29 human colon cancer cells in the following order: 10 ng/mL PGA < 100 ng/mL PGA < 10 ng/mL MTHF < 100 ng/mL MTHF. This is in contrast to the general idea that synthetic folic acid (PGA) is better bioavailable than natural folates. Two types of folate transport genes are involved in cellular absorption and transport of folates; folate receptors (FOLR1, 2 and 3 or folate binding proteins) and the reduced folate carrier (SLC19 A1 or RFC1). FOLR1, FOLR2, FOLR3 and SLC19A1 are all expressed in HT29 human colon cancer cells (unpublished microarray data). Verwei et al. (38) found in another human colon cancer cell line (CaCo-2) that cellular accumulation of synthetic folic acid (PGA) was lower compared to MTHF, despite a higher level of transport. Folate monoglutamates do not accumulate unless they are converted to folate polyglutamates by the enzyme folylpolyglutamate synthetase (FPGS) and these authors suggest that the higher degree of cellular accumulation of MTHF may be attributed to its higher affinity for FPGS. This explanation may also clarify our results in HT29 cells.

Our observation that low intracellular folate levels, obtained with culture in low levels of PGA, inhibit the growth of HT29 human colon cancer cells, parallels the results seen in the *in vivo* experiments of Song et al. (117,118). Inhibition of tumour cell growth could result from an interruption of folate metabolism causing ineffective DNA synthesis.

Besides folate, also cellular ATP is essential for cell growth and cell division. Cancer cells derive their ATP mainly via aerobic glycolysis, instead of mitochondrial OXPHOS. The factors causing tumour cells to increase aerobic glycolysis are unknown. It may be due to limitations in blood supply and consequently in the oxygen supply to fast growing cancer cells. Alternatively it may result from inactivation of normal mitochondrial function to prevent induction of apoptosis. Indeed, an altered regulation of apoptosis in cancer cells as a consequence of mitochondrial mutations has recently been established (127). The glycolytic shift of cancer cells has been shown to involve a diminished expression of the β-catalytic subunit of the mitochondrial H^+-ATP synthase (β-F1-ATPase) and an increased

expression of the glycolytic enzymes glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and inducible 6-phosphofructo-2 kinase (iPFK-2) (128,129). Levels of fructose-2,6-biphosphate, the glycolytic enzymatic endproduct of phosphofructokinase, have been found to be higher in HT29 cells as compared to normal tissue counterparts (128). Other experimental observations in literature that support the 'Warburg effect' are an increased expression of glucose transporter 1 (GLUT-1) in malignant tumours (130,131) that facilitates the entry of glucose in these cells. Also an increased activity of mitochondrial-bound hexokinase (132), the enzyme that converts glucose into glucose-6-phosphate, the first step in the glycolysis pathway, is observed.

Our results reveal that the slow-growing HT29 cells with low intracellular folate levels also have a lower amount of intracellular ATP, caused by a decrease in glycolysis, compared to the other treatments resulting in higher intracellular folate levels. This was accompanied with an increase in mitochondrial O_2 consumption and mitochondrial mass. Interestingly, the increase in mitochondrial O_2 consumption and mitochondrial mass did not lead to higher levels of cellular ATP, possibly due to mitochondrial defects in HT29 human colon cancer cells. Indeed, mitochondrial DNA mutations have been found in OXPHOS complex I, III and IV genes of human colorectal cancer cells (133).

The respiratory control ratios found in our study were comparable to other cancer cell line experiments (126,134). The increased respiratory control ratio (RCR; 3U/4O) in the slower-growing HT29 cells cultured in 10 ng/mL PGA indicates a tighter coupling between O_2 consumption and ATP production. However there was no significant difference in phosphorylation-related respiration. The phosphorylation respiratory control ratios (RCRp; (R-4O)/3U)) were approximately the same in all culture conditions, revealing no differences in mitochondrial ATP production. This result also supports the fact that the decrease in ATP content observed in HT29 cells cultured in 10 ng/mL PGA is not caused by a change in mitochondrial ATP production but is indeed caused by a decrease in the glycolytic rate. How folate modulates energy metabolism of HT29 cells is not known, but already in 1975 it was shown that intestinal glycolytic enzyme activities can be regulated by oral folic acid supplementation. Both pharmacologic and physiologic doses of folic acid were found to increase jejunal glycolytic enzyme activities in humans. When the folate was discontinued in the subjects, glycolytic enzyme activities returned to control values (135).

In summary, low intracellular folate levels can decrease the growth rate as well as the glycolytic rate of HT29 human colon tumour cells. Notwithstanding the limitations

associated with *in vitro* studies performed in only one cell line, data from the present study may suggest that deficient intracellular folate levels can revert the phenotype of colon cancer cells towards a phenotype more characteristic for normal cells.

5

5’-methyltetrahydrofolate and folic acid differently affect gene expression in HT29 human colorectal cancer cells

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Abstract

High dietary folate intake is associated with decreased colorectal cancer risk. However, recent animal studies indicate a potential risk of long term supplementation with folic acid (pteryol(mono)glutamic acid, PGA). Here, we investigated whether HT29 human colorectal cancer cells are differently affected by the natural folate 5'-methyltetrahydrofolate (MTHF) and the reduced, synthetic folate PGA. For this, HT29 cells were exposed for three weeks to serum levels (10 and 20 ng/mL) and physiological, supplemental serum levels (50, 100 and 200 ng/mL) of either MTHF or PGA. Using cDNA microarrays, genes involved in folate metabolism, cancer, protein synthesis and endoplasmatic reticulum (ER) were found to be differentially affected at 50-200 ng/mL. Dose-dependent gene expression (10-200 ng/mL) of selected genes related to folate metabolism, cancer and ER/Golgi were examined using real-time quantitative PCR. At 10 ng/mL, differences between MTHF and PGA were found in the folate metabolism related genes. At 50-200 ng/mL, ER/Golgi related genes, especially HERPUD1, STX3A and RAB1A, and cancer related genes, including CDH1, TACSTD1 and BRCA1, were differently expressed. The data suggest a more beneficial effect of MTHF as compared to PGA. The gene expression differences indicate a difference in response to supplemental MTHF and PGA, which warrants further examination *in vivo*.

Introduction

Folates are essential for maintenance of DNA integrity and stability (136). The diet consists of natural reduced folates, mainly 5-methyltetrahydrofolate (MTHF) and formyltetrahydrofolate (10,36). In dietary supplements the oxidised form of folate, folic acid (pteryol(mono)glutamic acid, PGA) is used (10,36). In the cell, PGA is reduced by dihydrofolate reductase to 7,8-dihydrofolate, which is then further reduced to the metabolically active form 5,6,7,8-tetrahydrofolate (10,137). This can be further converted to MTHF, but also to other metabolically active folates involved in different processes (9,10). MTHF is the primary form of folate entering human circulation from the intestinal cells (36,46,138). The requirement of folates in the synthesis of purines, pyrimidines and the universal methyldonor S-adenosylmethionine provides folates with an essential role in DNA synthesis and methylation. Low serum folate levels are associated with neural tube defects (139), cardiovascular disease risk (140) and colorectal cancer (95,141). Since most people consume less than the recommended daily intake of folates (300 µg per day for adults in The Netherlands (142)), supplementation with PGA at the early stage of pregnancy is advised. Furthermore in a number of countries, including the US, cereals are restored with PGA, primarily to reduce the incidence of neural tube defects (36,143). Although there are no known toxicities associated with elevated intakes of synthetic PGA, some concerns have been raised in the literature related to PGA supplementation. These include masking of vitamin B12 deficiency (110), reduced fetal growth (16) and accelerated growth of neoplasms (144). PGA supplementation in weanling animals before establishment of tumours seemed to decrease the tumour number, while after establishment of tumours supplementation increased the tumour number (21,117,118,145). Furthermore, animal and human studies suggest that not only the timing but also the dose of PGA intervention may be critical in protection of carcinogenesis or otherwise enhancement of cancers, especially in gastrointestinal tract (21,23). Since high dietary folate intakes are associated with reduced colon cancer risk, but supplementation with PGA was shown to pose some potential risks, we wondered whether this could be due to the different forms of folate that were involved (MTHF versus PGA). This is especially relevant for the intestinal tract since this is the only organ that is exposed to PGA.

There seemed to be no difference between PGA and MTHF on bioavailability, DNA stability and homocysteine lowering in case of short term and low dosage supplementation, but long term supplementation increases the bioavailability of PGA compared to MTHF

(9,35,40,41,43,46). Only three *in vitro* studies have used intestinal cell lines to study the difference between MTHF and PGA. These studies used 4000 ng/mL exposure conditions in the absence of vitamin B12 and focussed on epidermal growth factor receptor promoter activation, hyperproliferation and transport (38,66,146). 4000 ng/mL is supraphysiological, the normal serum concentration of folate in humans is 3-30 ng/mL, which can be increased to 200 ng/mL upon supplementation (36,147). In our own work (Dommels *et al.* submitted), we found differences in growth of human colon HT29 cells at low (10 ng/mL) concentrations of PGA and MTHF, but not at high (100 ng/mL) concentrations of MTHF and PGA, while increased intracellular folate levels were found in both MTHF conditions compared to PGA conditions. To examine whether supplementation with either MTHF or PGA can result in different steady state effects, we exposed the human colon cancer cell line HT29 to physiological relevant concentrations of 10-200 ng/mL of either PGA or MTHF. This was done in the presence of vitamins B2, B6, B12, which are all important cofactors in folate metabolism. We were especially interested to see whether folate metabolism and cancer related genes would be affected. This was accomplished by using real time quantitative PCR (qRT-PCR). Three genes encoding for proteins involved in folate metabolism were selected for analysis. 5,10'-methylene tetrahydrofolate reductase (MTHFR), which converts 5,10'-methylene tetrahydrofolate to MTHF (10), methionine synthase (MTR) which converts homocysteine to methionine and is necessary for the breakdown of homocysteine (10), and S-adenosylmethionine decarboxylase (AMD1), which catalyses the rate-limiting step in polyamine synthesis and is needed for normal cell growth (148). The four cancer related genes selected for examination were tumour protein p53 (TP53), E-cadherin (CDH1), breast cancer 1 early onset (BRCA1) and tumour- associated calcium signal transducer 1 (TACSTD1). TP53 and CDH1 are both tumour suppressors, known to be involved in colorectal carcinogenesis (106,108). Mutations in BRCA1 are linked to early breast cancer, but may have a role in colon cancer (149,150). Expression of TACSTD1 is assumed to be an early marker for malignancies, including colon adenomas. Activation of TACSTD1 protein causes cadherins to dissociate (151). In addition, we used cDNA microarrays to see whether other genes and processes could be identified that are differently affected by PGA and MTHF at supplemental, but still physiological, concentrations.

Material and methods

Experimental setup

The human colon epithelial cell line HT29 (ATCC, Manassas, VA, USA) was grown, in duplicate, on Dulbecco's modified Eagle's medium (DMEM) without folic acid (Invitrogen, Breda, The Netherlands), supplemented with pteroyl glutamic acid (PGA) or 5'-methyltetrahydrofolate (MTHF) for 3 passages. The concentration range of both folates (Sigma) was 10, 20, 50, 100 and 200 ng/mL (36,147). Cells were inoculated in 75cm^2 culture flasks at 80,000; 40,000; 40,000; 25,000 and 25,000 cells/cm^2 to obtain 80% confluence after one week for all cuture conditions. Cells were grown at 37°C in air with 5 % CO_2 and 100 % relative humidity. The growth medium was supplemented with $NaHCO_3$ (3.7 g/L, Sigma), non-essential amino acids (1x, ICN, Zoetermeer, The Netherlands), FCS (5%, Invitrogen), penicillin (5000 U, Sigma) and streptomycin (5 mg/L, Sigma) and methylcobalamin (vitamin B12, 500 pg/ml Sigma). Culture media were replaced every second day. Total RNA was extracted using TRIZOL according to the protocol of the supplier (Invitrogen), with addition of an extra phenol-chloroform-isoamylalcohol purification step. Concentrations were determined spectrophotometrically at A_{260nm} and all RNA samples were checked on 1% TAE/agarose gels. RNA samples were stored at -80°C for cDNA microarray analysis and quantitative real-time RT-PCR.

cDNA microarray analysis.

cDNA microarray analysis (array construction, RNA labeling and hybridization) was performed, as described in Pellis *et al.* (98) on the 50, 100 and 200 ng/mL folate exposure samples. Duplicate indirect RNA labelling was used, by incorporation of aminoallyl dUTP in the cDNA and subsequent chemical coupling to Cy5 monofunctional dye. A standard reference sample, consisting of a pool of mRNA from all exposed cell lines, was used and coupled to Cy3 monofunctional dye. Before hybridization, the Cy3- and Cy5-labeled samples were mixed 1:1 (v/v) and hybridised to in-house produced cDNA libraries. This in house constructed cDNA microarray contained 3000 unique cDNAs. These cDNAs are derived from Caco-2, HT29 and adipose cDNA libraries, all sequenced and annotated using BLAST searches in NCBI and also cDNAs encoding control sequences and known genes selected for functional relevance in folate metabolism, folate transport, DNA repair and carcinogenesis. After hybridization the microarrays were scanned using a confocal laser scanner (Scanarray ExpressHT, Perkin Elmer). Scans were made with a pixel resolution of 10 µm, a laser power of 60% and a photomultiplier tube

voltage of 55%. The software package ArrayVision (Version 7.0, Imaging Research, Ontario, Canada) was used for image analysis of the TIFF files, as generated by the scanner. Density values of each spot, multiplied by the area and the background (corners between spots) were collected and stored for further data processing in Microsoft Excel (Windows, Microsoft). Before data correction, background subtraction was performed on the raw data. From the quality check of the Cy3 plots, only spots with a CV smaller than 15% were used for further analysis. From duplicate slides spots belonging together were averaged. These values were used for discriminant analysis (GeneMath, Applied Maths, version NT), a form of principle component analysis that allows to identify differences in parameters between predefined groups, to identify differentially expressed genes.

Validation of microarray data with quantitative real-time PCR (qRT-PCR)

cDNA levels were quantified, in triplicate, using MyiQ real-time PCR detection system (Biorad, Veenendaal, The Netherlands). From one µg of total RNA, cDNA was synthesised using iScript cDNA Synthesis Kit (Biorad) according to the protocol of the supplier. qRT-PCR was performed for four housekeeping genes, calnexin (CANX), ß-actin (ACTB), annexin A5 (ANXA5) and chaperonin containing TCP1 subunit 5 (CCT5), and selected target genes (see Table 5.1). Each reaction contained 2.5 µl of each primer, 12.5 µl IQ SYBR Green Supermix (Biorad), 5.0 µl MilliQ-water and 2.5 µl of the cDNA template in a final volume of 25 µl. The two-step amplification procedure consisted of a pre-incubation step for activation of the Taq DNA polymerase (3 min at 90 °C), followed by 40 cycles consisting of a denaturation step (10s at 90 °C) followed by an annealing and extension step (45s at 60°C). For all measurements fluorescence was quantified during the extension step. A melting curve analysis was performed to evaluate efficiency and primer function. Target genes were corrected by the geometric mean of the four housekeeping genes (152).

Statistical analysis.

Statistical comparisons were made using an unpaired, two-tailed Student's t-test with a confidence level of 95% comparing the 50, 100 and 200 ng/mL PGA to 50, 100 and 200 ng/mL MTHF exposures.

Table 5.1 Primers used for qRT-PCR

Gene	Accession Number	Sense Primer	Anti-sense Primer	Efficiency (%)	S/AS (μM)
folate related					
AHCY	NM_000687	GCGACCTCACCAACCTCATCC	GACATTGATGGCAGGCACCTTG	94.3	800/400
AMD1	NM_001634	AAGAAGCAGCAACAACAGCAGAG	GCAACTACACAGTGGTGGTTATGG	94.8	800/800
MTHFD2	NM_006636	TGATCCTGGTTGGCGAGAATCC	CTGGAAGAGGCAACTGAACAAGG	107.9	800/800
MTHFR	NM_005957	AGAAGATGAGGCGGCGATTGG	CGGCGGTGCTGGCGATC	110.8	400/800
MTR	NM_000254	GTTAAGGCAACAGGCTGAGAAGG	TGCTGTAGTCGTCACCATCATCC	93.9	800/400
ODC1	NM_002539	ACTGTTGCTGCTGCCTCTACG	TGGCATCCTGTTCCTCTACTTCG	103.5	400/400
SHMT2	NM_005412	CCATCACTGCCAACAAGAACACC	CCTTCATCTATAAAGTCCACAACTCTCC	104.7	800/800
cancer related					
ARHA	NM_001664	TGTTGGTGATGGAGCCTGTGG	ATCAGTATAACATCGGTATCTGGGTAGG	105.3	800/800
BRCA1	NM_007306	AAGTCAGAGGAGATGTGGTCAATGG	TGTGGGCATGTTGGTGAAGGG	109.4	400/400
CDH1	NM_004360	CATTAACAGGAACACAGGAGTCATCAG	GATTGAAGATCGGAGGATTATCGTTGG	91.4	800/800
CEACAM6	NM_002483	GGACAGAGAAGACAGCAGAGACC	GCAGTGGTGGGTGGGTTCC	93.4	400/800
TACSTD1	NM_002354	ATAATCGTCAATGCCAGTGTACTTCAG	CCGCTCTCATCGCAGTCAGG	100.8	400/400
TDE1	NM_198941	CTTGTATTCTAGCATCCGCACTTCC	TCTTCATCACTGGCACCACTGG	105.6	400/400
TP53	NM_000546	CTATGAGCCGCCTGAGGTTGG	AGTGTGATGATGGTGAGGATGGG	107.5	800/800
endoplasmic reticulum and Golgi related					
HERPUD1	NM_014685	CTGCTCCTCAAGTGGTTGTTAATCC	TGTAGCTGCTGAATAGGTCCAATCC	104.3	400/400
MAL2	NM_052886	GTGGCTCAAATTGATGCTAACTGG	GGGATGTGGCTGCTGCTTC	99.3	400/400
RAB1A	NM_004161	AGTCTTGCCTTCTTCTTAGGTTTGC	TCTGTAATAACTGGAGGTGATTGTTCG	103.2	800/800
STX3A	NM_004177	TCATTGTGCTAGTAGTTGTGTTGC	CACTCGCTCTCAGGAAGGC	96.6	400/400
TRA1	NM_003299	TCTTAGGTTCCAGTCTTCTCATCATCC	AGGTTCTGTGAGGTAAATAACTTCATAGC	94.7	800/800
XBP1	NM_005080	GGATTCTGGCGGTATTGACTCTTC	AGGGAGGCTGGTAAGGAACTG	99.0	400/400
housekeeping					
ACTB	NM_001101	GCAAAGACCTGTACGCCAACAC	TCATACTCCTGCTTGCTGATCCAC	95.4	400/400
ANXA5	NM_001154	AGCATCCTGACTCTGTTGACATCC	GTTCATAAGCATCATAAAGCCGAGAGG	104.3	400/400
CANX	M94859	GAGAAACAGAAAAGTGATGCTGAAGAA	CAGGGGAGGAGGGAGAAGAAATC	92.7	400/400
CCT5	NM_012073	GATGGAGATGTGACTGTAACTAATGATGG	GGGTGAATGCCTCGGTCTAGC	107.8	800/400

The intron spanning primers of each gene, the accession number on which the primer was designed, the obtained PCR efficiency and used concentration for each primer. S means sense primer, AS means antisense primer.

Results

HT29 cells were grown at 50, 100 and 200 ng/mL MTHF and at 50, 100 and 200 ng/mL PGA, all in duplicate. After three weeks, RNA was isolated and all samples were individually hybridised to cDNA microarrays. The gene expression data from duplicates were averaged. Using discriminant analysis, the most differentially expressed genes between 50, 100 and 200 ng/mL MTHF on one side and PGA on the other side were identified. These genes are shown in Table 5.2, classified in subsets according to function. The MTHF/PGA expression ratios at 50, 100 and 200 ng/mL and the group wise p-values are given (cut off ratio > ±1.5 and p-value < 0.0125). One group consisted of folate related genes (all higher in MTHF) and a second group of cancer related genes. The third and largest subset consisted of genes involved in protein synthesis, all expressed

Table 5.2 Genes with a different steady state expression in HT29 in response to 50, 100 and 200 ng/mL 5'-methyltetrahydrofolate (MTHF) and folic acid (PGA).

gene	Unigene title	Unigene cluster	folate (ng/mL) 50	100	200
	folate related				
AHCY	S-adenosylhomocysteine hydrolase	Hs.388004	1.8	1.8	2.0
MTHFD2	methylene tetrahydrofolate dehydrogenase (NAD+ dependent), methenyltetrahydrofolate cyclohydrolase	Hs.154672	2.0	2.0	2.2
ODC1	ornithine decarboxylase 1	Hs.443409	1.9	2.0	1.6
SHMT2	serine hydroxymethyltransferase 2 (mitochondrial)	Hs.75069	1.7	1.7	1.4
	cancer related				
ARHA	ras homolog gene family, member A	Hs.77273	2.2	1.5	2.1
CEACAM6	carcinoembryonic antigen-related cell adhesion molecule 6	Hs.436718	-1.5	-1.4	-1.5
TDE1	tumor differentially expressed 1	Hs.272168	2.6	2.7	1.5
YWHAZ	tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide	Hs.386834	1.7	1.5	1.9
	protein synthesis				
CAPN13	calpain 13	Hs.325335	1.9	1.7	1.7
EEF1A1	eukaryotic translation elongation factor 1 alpha 1	Hs.439552	2.6	2.5	2.4
HNRPA1	heterogeneous nuclear ribonucleoprotein A1	Hs.356721	1.7	2.0	1.9
HSPA8	heat shock 70kDa protein 8	Hs.180414	2.1	2.3	2.3
RPL10A	ribosomal protein L10a	Hs.448396	2.1	1.9	2.4
RPL12	ribosomal protein L12	Hs.408054	2.3	2.0	2.2
RPL23A	ribosomal protein L23a	Hs.419463	2.6	2.5	2.4
RPL27A	ribosomal protein L27a	Hs.356342	2.2	2.5	2.0
RPL31	ribosomal protein L31	Hs.375921	2.0	2.3	2.2
RPL4	ribosomal protein L4	Hs.186350	2.1	2.3	2.2
RPS11	ribosomal protein S11	Hs.433529	2.3	2.8	2.4
SERPINA1	serine (or cysteine) proteinase inhibitor, clade A , member 1	Hs.297681	2.3	2.2	2.1
SUI1	putative translation initiation factor	Hs.150580	2.0	2.5	2.2
TRA1	tumor rejection antigen (gp96) 1	Hs.192374	1.8	2.0	1.8
	endoplasmic reticulum related				
HERPUD1	homocysteine-inducible, endoplasmic reticulum stress-inducible, ubiquitin-like domain member 1	Hs.146393	2.5	1.7	1.7
MAL2	mal, T-cell differentiation protein 2	Hs.76550	1.8	1.6	1.7
STX3A	syntaxin 3A	Hs.82240	1.7	1.6	1.7
TRA1	tumor rejection antigen (gp96) 1	Hs.192374	1.8	2.0	1.8
XBP1	X-box binding protein 1	Hs.437638	2.3	2.7	2.4
	cell cycle/growth				
DCN	decorin	Hs.156316	1.9	1.7	1.8
FTL	ferritin, light polypeptide	Hs.433670	1.9	1.9	2.3
TMSB10	thymosin, beta 10	Hs.446574	2.1	2.1	1.7
XPO1	exportin 1 (CRM1 homolog, yeast)	Hs.157367	1.7	1.8	1.5
	other				
AKR1C2	aldo-keto reductase family 1, member C2	Hs.201967	2.0	2.1	2.4
MTND4	NADH dehydrogenase subunit 4		2.0	1.9	1.7
ORF1-FL49	putative nuclear protein ORF1-FL49	Hs.323512	1.6	1.8	1.8
PAH	phenylalanine hydroxylase	Hs.325404	1.6	1.7	1.7
PRDX1	peroxiredoxin 1	Hs.180909	1.7	1.8	1.8

Genes were identified by discriminant analysis after cDNA microarray analysis. The expression level ratios at 50, 100 and 200 ng/mL MTHF compared to PGA are shown. The p-value was calculated between 50-200 ng/ML MTHF and PGA. Positive values represents genes higher expressed in MTHF exposure compared to PGA exposure.

higher with MTHF. The fourth subset consisted of endoplasmatic reticulum (ER) related genes (all higher in MTHF). A fifth subset consisted of a more diverse group of genes related to cell cycle and growth. The remaining genes could not functionally be grouped.

To verify the differences between MTHF and PGA exposure, we performed qRT-PCR and extended the analysis to cells exposed to 10 and 20 ng/mL folate. Folate metabolism and cancer related genes were found to be affected, therefore we added these genes to the pre-selection of these functional categories. The group of folate metabolism related genes analysed by qRT-PCR consisted of AMD1, MTHFR, MTR, as well as the array identified genes S-adenosylhomocysteine hydrolase (AHCY), mitochondrial methylene tetrahydrofolate dehydrogenase (NAD+ dependent) methenyltetrahydrofolate cyclohydrolase (MTHFD2), ornithine decarboxylase 1 (ODC1) and mitochondrial serine hydroxymethyltransferase 2 (SHMT2). The cancer related gene group consisted of BRCA1, CDH1, TACSTD1, TP53 as well as the array identified genes ras homolog gene family member A (ARHA), carcinoembryonic antigen-related cell adhesion molecule 6 (CEACAM6) and tumour differentially expressed 1 (TDE1). In addition, we selected all five genes that are related to endoplasmatic reticulum (ER). Homocysteine-inducible endoplasmic reticulum stress-inducible ubiquitin-like domain member 1 (HERPUD1), mal T-cell differentiation protein 2 (MAL2), syntaxin 3A (STX3A), tumor rejection antigen 1 (TRA1) and X-box binding protein 1 (XBP1) are ER proteins involved in protein folding. In addition, ras-associated protein 1 (RAB1A; not present on the array), a Golgi stress protein was selected based on literature (153,154). To accurately quantify the relative expression levels of the target genes, four housekeeping genes (ACTB, ANXA5, CANX, CCT5) were selected, based on a CV in the microarray analysis of less than 10 % in all six exposure conditions.

From the qRT-PCR data, normalised against the housekeeping genes, the ratio between the MTHF and PGA growth conditions was calculated for each concentration separately (Table 5.3). The biggest variation in geometric mean of the housekeeping genes between the different conditions was 1.4, therefore corrected target genes with ratio between –1.4 and 1.4 were considered not affected. The expression of most genes, in all three functional groups examined, was differently affected by MTHF and PGA at 10 ng/mL. In particular for genes involved in folate metabolism the biggest differences were seen at

this concentration, although also at 200 ng/mL ratios greater than 2 were seen for ODC1 and MTHFD2. Of the cancer related genes, in particular BCRA1, CDH1 and TACSTD1 were differently affected over the full concentration range. BCRA1 was always lower expressed

Table 5.3 Differences in mRNA expression levels of folate-, cancer- and ER/Golgi-related genes in HT29 cells after three week exposure to 5'-methyltetrahydrofolate (MTHF) or folic acid (PGA).

	folate (ng/mL)				
Gene	10	20	50	100	200
folate related					
AHCY	-3.0	-1.2	-1.7	-1.3	1.2
AMD1	-2.9	1.0	1.4	-1.4	1.1
MTHFD2	1.2	-1.3	-1.3	1.6	2.4
MTHFR	-2.0	-1.1	-1.7	-1.2	-1.4
MTR	1.0	1.1	-1.4	-1.5	-1.2
ODC1	1.8	-1.1	1.4	1.5	2.1
SHMT2	-2.4	1.9	1.2	-1.4	1.5
cancer related					
ARHA	-3.7	1.4	1.1	-1.5	1.1
BRCA1	-5.8	-6.4	-2.2	-2.7	-2.1
CDH1	-4.1	1.5	2.0	-1.7	3.8
CEACAM6	-5.3	-2.0	2.7	-1.7	-1.4
TACSTD1	15.5	2.0	2.5	1.1	-2.9
TDE1	8.3	8.5	1.6	1.7	-1.2
TP53	1.9	1.7	-1.1	1.0	-1.2
endoplasmic reticulum and Golgi related					
HERPUD1	-4.4	2.3	7.5	2.2	5.1
MAL2	-2.3	-1.5	-1.4	-1.4	-1.5
RAB1A	3.7	-2.2	-7.4	-4.4	-5.4
STX3A	2.1	1.7	1.1	2.3	2.4
TRA1	2.1	3.6	1.3	1.6	-1.1
XBP1	1.0	-1.3	1.0	1.1	1.9

mRNA levels were quantified by real time quantitative RT-PCR, normalised for four housekeeping genes. At each concentration the differences between MTHF and PGA were calculated. Positive values represent genes higher expressed in MTHF compared to PGA.

with MTHF. For TACSTD1 and TDE1, the difference in response to the two folates seemed to gradually disappear towards 200 ng/mL, in a concentration dependent pattern. Of the ER and Golgi related genes, the most striking differences were observed for HERPUD and RAB1A. HERPUD was lower expressed at 10 ng/mL MTHF, but higher at all other concentrations, while the reverse was true for RAB1A. STX3A showed a higher expression with MTHF as compared to PGA over the full concentration range. For TRA1, the difference seemed to gradually disappear towards 200 ng/mL

Discussion

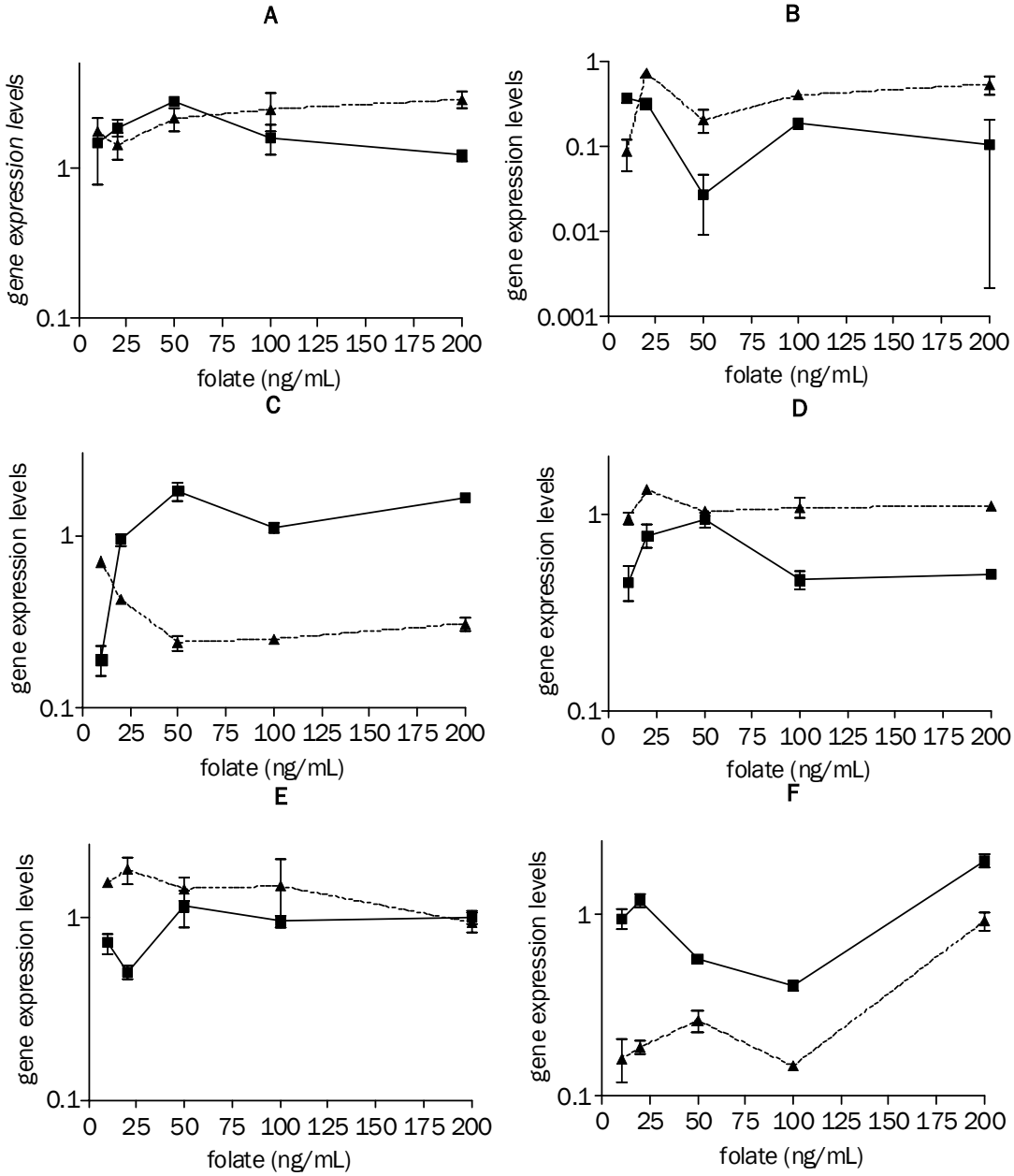
The objective of this study was to investigate if long term supplementation with 5'-methyltetrahydrofolate (MTHF) or folic acid (PGA) could result in physiological differences. Using HT29 human colorectal cancer cells folate metabolism, cancer, protein synthesis, endoplasmatic reticulum (ER) and cell cycle/growth related genes were found to be differentially affected by both folates. By means of qRT-PCR three of these categories (folate metabolism, cancer and ER/Golgi related genes) were further studied and the results were largely confirmed, especially for the cancer related and ER/Golgi related genes. These results show that the natural folate MTHF and the synthetic folate PGA, which is used in supplements, give differences in steady state gene expression at supplemental, but still physiological concentrations. This warrants further research in view of the possible negative side effects of folate supplementation on the colon.

The effects of genes involved in folate metabolism are most pronounced at 10 ng/mL exposure, where most genes involved in the folate metabolism were higher expressed after PGA exposure compared to MTHF exposure. This is in agreement with the observation that at this concentration the largest differences in intracellular concentrations of tetrahydrofolate and MTHF exist in HT29 cells (Dommels *et al.* submitted for publication). In Caco-2 cells, PGA was linked to lower intracellular folate concentrations compared to MTHF (38), which may explain the observed differences at low concentrations. Cancer related genes were also more favourable expressed in 10 ng/mL PGA compared to 10 ng/mL MTHF, which correlates well with the finding that low PGA levels can revert the phenotype of HT29 cells towards a phenotype more characteristic for normal cells (Dommels *et al.* submitted for publication). At high concentrations, differences were much less pronounced. The most pronounced difference in folate related genes was seen for methylene tetrahydrofolate dehydrogenase/cyclohydrolase (MTHFD2), which was higher expressed at 100 and 200 ng/mL MTHF (Figure 5.1). This mitochondrial protein converts 5,10'-methylene tetrahydrofolate to 10'-formyltetrahydrofolate and is needed for cytochrome c reduction (9). Since folate was shown to affect energy metabolism in HT29 cells (Dommels et al, submitted for publication) and here a number of mitochondrial related genes (MTHFD2, SHMT2 and MTND4) were found to be differentially expressed, it is of interest to further explore the relation between intracellular folate concentration and mitochondrial gene expression.

10'-formyltetrahydrofolate is also necessary for the formylation of the initiator methionine tRNA to start mitochondrial protein synthesis (155,156). A higher protein synthesis at supplemental MTHF concentrations as compared to PGA is clearly indicated in the microarray analysis, where the largest functional category consisted of genes involved in protein synthesis. Another group of related genes are involved in ER and Golgi, organelles which are involved in protein folding and transport (157,158). The ER can be expanded according to the demands placed upon the exocytic pathway (159). In Caco-2, syntaxin3A (STX3A) regulates exocytotic transport to the apical membrane (160-162). The higher expression of STX3A with MTHF (Figure 5.1) suggests increased exocytosis in the HT29 cells. RAB proteins serve as master regulators in uptake and intracellular transport of macromolecules (163,164). More specifically RAB1A coordinates the vesicle-mediated protein transport from ER to Golgi-apparatus and transport between Golgi compartments (153,154). RAB1A is higher expressed at 10 ng/mL MTHF, but significantly lower expressed ($p = 0.012$, average 5.7 fold) at higher MTHF concentrations (Figure 5.1), suggesting decreased endocytosis. HERPUD1 ($p = 0.044$, average 4.9 fold) and TRA1 play a crucial role in ER stress resistance by the unfolding protein response (159,165,166). When the protein folding capacity of the ER is challenged, the unfolded protein response maintains ER homeostasis by regulating protein synthesis and enhancing expression of resident ER proteins that facilitate protein maturation and degradation (159,167). The higher expression of HERPUD1 and TRA1 with MTHF (Figure 1) is in line, with the observed increased protein synthesis gene expression. Interestingly, both HERPUD and TRA1 can be induced by homocysteine. It has been shown that exposure to 100 µM homocysteine induces HERPUD1 expression and 5 mM homocysteine exposure induces TRA1 expression in neural cells (165,168,169). At the same time protein synthesis and cell viability is reduced (168,169). This suggests that the differential effect of MTHF and PGA may be mediated by homocysteine, a metabolite of folate metabolism.

HERPUD1 also has a role in stress resistance, calcium homeostasis and in regulating mitochondrial integrity (165,167,168). Sublethal ER stress levels increase HERPUD1 expression and maintain mitochondrial function, whereas higher stress levels decrease HERPUD1 expression and induce apoptosis (167,168). At 100 ng/mL PGA, HERPUD1 is lower expressed compared to 10ng/mL PGA, indicating more stress and apoptosis which

Figure 5.1 Expression of MTHFD2, HERPUD1, RAB1A, STX3A, TRA1 and BRCA1



Expression of MTHFD2 (A), HERPUD1 (B), RAB1A (C), STX3A (D), TRA1 (E) and BRCA1 (F) by folic acid (PGA) or 5'-methyltetrahydrofolate (MTHF) exposure in HT29 cells. The quantitative real time RT-PCR data were corrected using the geometric mean of four housekeeping genes. Straight line with squares represents the PGA exposure and dotted line with triangles represents the MTHF exposure.

correlates with our findings of increased apoptosis at 100 ng/mL PGA in a former study (Pellis *et al*. submitted for publication). These results imply that higher expression of HERPUD1 at 20-200 ng/mL MTHF compared to PGA may be linked to reduced apoptosis and increased differentiation. In colorectal cancer, loss of differentiation and increased invasiveness are associated with loss of expression and function of E-cadherin

(106,108,170). CDH1 is needed for the E-cadherin/catenin complex, while TACSTD1 disrupts these complexes (151). Disturbances in this adhesion complex is known to be involved in colon cancer (108). TACSTD1 is expressed in normal intestinal epithelium, but it is overexpressed in colorectal adenomas and carcinomas (151). Since CDH1 is higher expressed and TACSTD1 lower expressed at 200 ng/mL MTHF as compared to PGA, this may suggest increased differentiation. The potential positive effect of MTHF as compared to PGA is substantiated by lower expression of CEACAM6 and TDE1. CEACAM 6 is overexpressed in many cancers, has a function in adhesion and invasion, and is associated with decreased differentiation (171-173). TDE1 is expressed in colon cell lines and has been identified as a putative candidate gene in tumour progression in mice (174). However, this potential positive effect is contradicted by the lower expression of the breast cancer 1 early onset gene (BRCA1). Reduced expression of BRCA1 has been associated with colon carcinoma (149), while induced expression of BRCA1 has an antiproliferative effect (175). It should be noted that the higher expression of BCRA1 in colon cancer (150), has been contradicted in other studies (149). Finally, TP53 is not differentially affected by the higher concentrations of MTHF and PGA. TP53 is a tumour suppressor, with an important role in the balance between cellular proliferation and apoptosis and as the guardian of the integrity of the genome (106). Altogether, the cancer related gene expression data seem to suggest that high MTHF exposure increases the differentiation and adhesion of HT29 cells compared to high PGA exposure, both processes are decreased or lost in carcinomas (106,176). This may indicate a possible beneficial effect of supplemental MTHF levels, as compared to PGA, on HT29 colorectal cancer cells.

In conclusion, gene expression analysis of HT29 colorectal cancer cells shows differences in gene expression response to supplemental, but still physiological levels of MTHF as compared to PGA. In particular, the expression of genes related to protein synthesis, ER/Golgi and cancer were affected. In view of potential negative side effects of supplementation with PGA, further studies need to be conducted to investigate whether these gene expression differences will result in different physiological effects *in vivo*.

6

Human blood and colonic iron status in response to a 6 month folic acid and vitamin B12 intervention

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Abstract

Folate, vitamin B12, and iron are essential for normal cell physiology. Mechanistic studies have shown that iron increases folate catabolism. Reversely, increased folic acid (and vitamin B12) exposure decreased the total iron content of human colon cancer cell lines. It has also been shown that supplementation of adolescent girls with folic acid alone, decreased plasma ferritin levels. To examine the effect of folate, in the presence of vitamin B12, on the iron status of colon and blood, we used an existing randomised, placebo-controlled trial. 81 Subjects with a history of colorectal adenomas were randomly assigned to either the intervention or the placebo group. The intervention group received daily supplementation with 4.6 mg folic acid and 1.1 mg vitamin B12 for six months. Randomisation was stratified for MTHFR genotype. At baseline and after six months, the total iron concentration in rectal biopsies and haematocrit, haemoglobin, ferritin and iron concentrations in blood were measured. Folic acid supplementation did not significantly decrease the colonic iron status, but it decreased the serum ferritin concentrations significantly, especially among those with MTHFR 677 CC genotype and those with high serum folate or vitamin B12 levels at baseline. This finding emphasizes the importance of supplementation with iron, when a high dose of folic acid is used.

Introduction

Folate, vitamin B12 and iron are dietary components that are essential for our health. Folate, as a cofactor, is involved in the numerous intracellular reactions of one carbon metabolism (10). This includes the synthesis of purines and pyrimidines, building blocks of DNA, and a large number of methylation reactions, including DNA methylation. Furthermore, folate is essential for metabolism of methionine, serine and glycine and for mitochondrial protein synthesis (9). This provides folate with an essential role in normal development and growth. In humans, folate deficiency may result in neural tube defects (139). In addition, folate deficiency results in anaemia from ineffective erythrocyte production (177). Furthermore, a low folate intake is associated with increased risk for cardiovascular disease (9) and colorectal cancer (2).

Vitamin B12, cobalamin, is a cofactor of methionine synthase, which converts homocysteine to methionine and at the same time methyltetrahydrofolate to tetrahydrofolate and is thus involved in folate metabolism. Indeed, vitamin B12 deficiency can mask folate deficiency, and both deficiencies can result in anaemia (177,178). To examine effects of folate, it is essential that vitamin B12 is present in sufficient amounts. Furthermore, a decrease of ferritin synthesis in the spinal cord of rats under conditions of vitamin B12 deficiency indicates a relation between vitamin B12 and ferritin status.(179).

Iron is widespread in human cells and is involved particularly in metabolic processes involving oxygen (103,180,181). Iron in heme specifically binds oxygen and facilitates its transport by red blood cells (177). Iron is present in the multiple enzymes that have heme as part of their prosthetic groups, for example NO synthase and prostaglandin synthase, involved in signalling, and mitochondrial cytochromes, involved in oxidative phosphorylation and catalase, involved in defence (182-184). Iron is also present in iron-sulfur containing ferredoxins, which are involved in redox reaction (185). The role of iron as a cofactor provides iron also with a role in the regulation of normal as well as aberrant cellular proliferation (184). Iron deficiency, similar to folate and vitamin B12 deficiencies, can result in anaemia (177,186).

Iron and folic acid supplementation during pregnancy is commonly practiced to prevent maternal anaemia (187,188) Multiple studies have shown that iron-folate supplementation is more effective in increasing the iron status in anaemia than iron

supplementation alone (188-192). However, it has also been advised to use a high dose of folic acid supplementation (3-5 mg per day) alone to reduce anaemia (193,194). This seems remarkable, since it has been shown in Asian adolescent girls that 5 mg of folic acid supplementation per week (without vitamin B12) decreased plasma ferritin levels and thus decreasing iron status. While supplementation of 3.5 mg folic acid in combination with 60 or 120 mg iron per week increased plasma ferritin levels (190). In view of the different strategies for anaemia prevention and to confirm the link between folate and iron status in healthy, non deficient subjects, it is of importance to substantiate this finding in the presence of vitamin B12 to make sure that the effects are not attributable to vitamin B12.

Ferritin is the major iron binding protein in the body, and the body iron status is determined by measuring the plasma levels of ferritin, (103,195). Interestingly, ferritin has also been identified as folate catabolising enzyme (196,197), which provides a direct metabolic link between iron and folate. Studies employing the iron chelator mimosine provide evidence for a direct effect of iron on folate metabolism (114,197). In these studies, minosine was shown to act as a folate antagonist by decreasing cytosplasmic serine hydroxymethyltransferase expression (114). The reverse, an effect of folate on iron was observed in an *in vitro* study, where we found that folic acid and vitamin B12 supplementation resulted in a decreased expression of ferritin light chain and the iron transporter SLC40A1 in HT29 human colon cancer cells. In this study, the effect of folate on iron metabolism was confirmed by showing that folic acid supplementation resulted in a lower total iron content in three different human colon epithelial cell lines; HT29, Caco-2 and CCD841CoTr (Pellis *et al.* submitted). However, this result remains to be confirmed *in vivo*, in humans.

To establish whether folic acid supplementation in the presence of vitamin B12 lowers colonic and blood iron status in humans, we used an existing human intervention study. This study was originally conducted to examine the effects of folate supplementation on molecular events in the colon, in particular on uracil misincorporation and DNA methylation (69). In this randomized, placebo-controlled design, subjects were supplemented with a high dose of folic acid (4.6 mg/day) and vitamin B12 (1.1 mg/day) for six months. The subjects were healthy, but with a history of colorectal adenomas and had the either the 677 CC or TT polymorphism in the 5,10'-

methylenetetrahydrofolatereductase (MTHFR) gene. MTHFR is a key enyzme in folate-mediated one carbon metabolism, and converts 5,10-methylene tetrahydrofolate to 5-methyl tetrahydrofolate (11,198). It directs folate either to DNA synthesis and mitochondrial protein synthesis or to methylation reactions (11,199). Different functional MTHFR polymorphisms exist (199,200). The most studied polymorphism is MTHFR C677T with a prevalence of 10 % in the Dutch population (70,139). The homozygous TT genotype leads to 65% lowered activity of the MTHFR enzyme resulting in more 5,10'-methylenetetrahydrofolate and most likely directs the one carbon metabolism toward the DNA synthesis (198,201,202). The MTHFR genotype information allows us to see whether this influences the effect of folate on the colonic and blood iron status.

Material and methods

Study population

Participants were patients with a history of sporadic colorectal adenomas and had the MTHFR 677 CC or TT genotype. Eligibility criteria were: Dutch speaking; of European origin; aged 18 to 80 years; no hereditary colorectal cancer syndromes; no chronic inflammatory bowel disease; no history of colorectal cancer; no (partial) bowel resection; not pregnant or lactating; no significant liver or renal disease; not using anti-epileptic medication, antifolate medication or supplements containing B-vitamins. The participants were recruited between March 2002 and February 2003. The study was conducted in three hospitals in the Netherlands: the Radboud University Nijmegen Medical Centre in Nijmegen, the Gelderse Vallei Hospital in Ede, and the Slingeland Hospital in Doetinchem. The study protocol was approved by the Ethical Committees of all participating centres. Written informed consent was obtained from all participants.

Design

After stratification by MTHFR C677T genotype, participants were allocated to vitamin or placebo capsules at entry into the study. Capsules were produced by Dutch BioFarmaceutics (Helmond, The Netherlands). The study was designed for 5 mg folic acid and 1.25 mg vitamin B12. After analysis, vitamin capsules appeared to contain 4.6 mg folic acid (pteroylmonoglutamic acid) and 1.1 mg vitamin B12 (cyanocobalamin), whereas placebo capsules contained <0.04 µg folic acid and <0.002 µg vitamin B12. Within each genotype group, treatment was allocated using random permuted blocks with lengths of four and six. A random number table was used to determine block lengths and allocation

within each block. All participants and study personnel were blinded to treatment assignment for the duration of the study. Compliance was judged by pill-return counts and by analyzing plasma homocysteine and erythrocyte folate levels before and after the intervention period. Participants were advised not to alter their diet or lifestyle during the study.

Data collection

Dietary intake was assessed with a semi-quantitative food-frequency questionnaire that was originally developed for the Dutch cohort of the European Prospective Investigation into Cancer and Nutrition (EPIC) (203).Venous blood samples were obtained before and after the intervention period, to measure haematocrit and concentrations of haemoglobin, serum iron, serum ferritin, red blood cell (RBC) folate, serum folate, serum vitamin B12 and plasma homocysteine. Plasma samples for analysis of homocysteine concentration were immediately placed on ice and centrifuged within 30 min (4°C, 1200g, 10 min.). Serum samples were kept in the dark at room temperature for at least 30 min. before centrifugation (4°C, 1200g, 10 min.). Plasma and serum were stored at -80°C. Haematocrit (147) and haemoglobin concentrations were determined by the participating hospitals when blood and rectal biopsy were collected. Serum iron, serum ferritin, RBC folate, serum vitamin B12 and serum folate were measured by a chemiluminescent immunoassay analyser ((Immulite 2000, Diagnostic Products Company, Los Angeles, USA)) at the General Practitioner's Laboratory at Velp, the Netherlands. Plasma homocysteine was determined by HPLC as described by Voortman et al. (204). Rectal biopsies of normal appearing mucosa were obtained by flexible sigmoidoscopy without bowel preparation before and after intervention. Biopsies were frozen in liquid nitrogen and subsequently stored in liquid nitrogen. RBC folate, serum folate, and plasma homocysteine were assessed as measures of compliance.

Analysis of colonic iron levels

The intracellular iron concentration was analysed, in duplicate, using Atomic Absorption Spectrometry (Perkin Elmer SIMAA 6100, with graphite furnace and Zeeman background correction). Sample preparation was done as follows. Biopsies were digested after addition of 1.0 mL 70% HNO_3 (Baker) by 6 hours incubation at 65°C, followed by an overnight-incubation at room temperature and subsequent 6 hours incubation at 65°C. 1.5 mL Millipore filtered water was added and additionally incubated at 65°C overnight.

Mixtures were sonicated for 5 min and an 11-time dilution was prepared. Finally, the atomic absorption was measured at 248.3 nm. The coefficient of variation for multiple injections (n=10) of the same sample within a run was 4.1%.

Statistical analysis

Potential baseline differences between intervention and placebo group in intake and blood levels of relevant nutrients and other characteristics were verified at baseline using χ^2-tests and t-tests. We assessed differences in response between the intervention and placebo group with linear regression analysis to be able to adjust for MTHFR genotype. Sample size requirements were calculated by the use of an expected iron concentration difference of 11.8 ng/µg protein (with an estimated standard deviation of 2.4 ng/µg protein) between the supplemented and placebo group. To detect this difference with a power of 80% and a confidence level of 95%, each of the two groups consisted of sufficient participants.

Results

In total 81 eligible patients, 63 having the MTHFR 677 CC genotype and 18 the TT genotype, were randomly assigned to either the intervention or the placebo group. The proportion of women was somewhat lower in the intervention group (34%) compared with the placebo group (43%). Furthermore, the intervention group tended to have higher levels of haematocrit, haemoglobin, serum ferritine and RBC folate, and a lower level of plasma homocysteine at baseline (Table 1), compared with the placebo group. However, none of the differences were statistically significant. The mean daily iron intake did not differ between both groups (intervention: 11.8±2.6 mg, placebo: 11.6 ± 2.5 mg, p=0.69). When dividing the population in those below and above the median serum folate concentration at baseline, there were also no statistically significant differences between the intervention and the placebo groups (data not shown).

Both RBC folate and plasma homocysteine, as markers of compliance did not change in the placebo group, but significantly RBC folate increased and plasma homocysteine significantly decreased in the intervention group after supplementation. Haematocrit, the haemoglobin concentration, the serum total iron concentration and colonic total iron concentration decreased slightly in the intervention group, but the values were also decreased in the placebo group. Serum ferritin concentrations decreased in the

Table 6.1 Effect of folic acid and vitamin B12 intervention on blood iron status; haematocrit, haemoglobin, serum iron, serum ferritin, colonic iron, RBC folate, and plasma homocysteine concentrations

	Intervention			Placebo			Difference in response†
	Baseline*	End*	Change*	Baseline*	End*	Change*	
Haematocrit (%)	(n=42) 42.2±0.3	41.9±0.5	-0.4±0.4	(n=39) 41.0±0.4	40.7±0.4	-0.3±0.03	-0.1 (-0.009;0.007) p=0.80[a]
Haemoglobin (mmol/L)	(n=36) 9.03±0.09	8.94±0.09	-0.09±0.08	(n=35) 8.69±0.10	8.64±0.09	-0.06±0.03	-0.03 (-0.22;0.17) p=0.78[a]
Serum iron (µmol/L)	(n=41) 16.37±0.91	15.85±0.98	-0.51±0.96	(n=39) 16.39±0.82	15.64±0.73	-0.74±0.76	0.25 (-2.19;2.69) p=0.84[a]
Serum ferritin (µg/L)	(n=40) 133.25±15.16	123.24±15.35	-10.01±6.15	(n=39) 111.12±13.23	115.91±12.50	4.79±4.26	-14.94 (-29.37;-0.51) p=0.04[a]
Colonic iron (mg/kg)	(n=41) 38.50±3.07	29.48±1.66	-9.02±3.46	(n=38) 38.52±2.75	32.66±2.61	-5.85±4.06	-3.04 (-13.61;7.53) p=0.57[a]
RBC folate (nmol/L)	(n=42) 750±45	2556±95	1806±95 p<0.0001	(n=39) 641±40	601±31	-40±28 p=0.17	1849(1651;2048) p<0.0001[a]
Plasma homocysteine (µmol/L)	(n=42) 11.5±0.5	7.8±0.2	-3.7±0.4 p<0.0001	(n=39) 12.7±0.7	13.2±0.7	0.5±0.3 p=0.13	-4.2 (-5.2;-3.2) p<0.0001[a]

*[a] adjusted for MTHFR genotype, † Change intervention – change placebo (95%CI), * mean ± SEM*

Table 6.2 Effect of folic acid and vitamin B12 intervention on serum ferritin (µg/L)

	Intervention			Placebo			Difference in response†
	Baseline*	End*	Change*	Baseline*	End*	Change*	
All participants	(n=40) 133.25±15.16	123.24±15.35	-10.01±6.15	(n=39) 111.12±13.23	115.91±12.50	4.79±4.26	-14.94 (-29.37;-0.51) p=0.04[a]
TT genotype	(n=9) 128.28±32.58	105.34±29.30	-22.93±8.27	(n=9) 155.8±30.89	138.06±25.38	-17.74±10.32	-5.19 (-33.23;22.85) p=0.70
CC genotype	(n=31) 134.69±17.40	128.43±18.04	-6.26±7.50	(n=30) 97.71±13.87	109.27±14.37	11.56±3.91	-17.81 (-34.91;-0.72) p=0.04
Serum folate <14.3 nmol/L	(n=17) 125.25±21.79	122.55±19.99	-2.69±6.11	(n=23) 128.10±19.18	125.68±17.77	-2.42±6.03	-0.52 (-16.94;15.91) p=0.95[a]
Serum folate ≥ 14.3 nmol/L	(n=23) 139.16±21.20	123.74±22.61	-15.42±9.69	(n=16) 86.70±15.43	101.87±16.65	15.17±4.84	-29.58 (-54.47;-4.69) p=0.02[a]
Serum B12 <232 pmol/L	(n=17) 135.35±25.71	121.22±26.16	-14.14±12.03	(n=21) 110.38±17.40	110.66±14.91	0.28±5.65	-15.57 (-40.36;9.22) p=0.21[a]
Serum B12 ≥ 232 pmol/L	(n=23) 131.69±18.78	124.73±18.92	-6.96±6.18	(n=18) 111.98±20.80	122.04±21.16	10.07±6.41	-15.94 (-33.56;1.68) p=0.07[a]
Serum ferritine <104 µg/L	(n=17) 57.67±6.21	56.61±6.87	-1.05±2.98	(n=22) 51.29±5.06	61.91±6.84	10.63±3.83	-9.81 (-19.97;0.35) p=0.06[a]
Serum ferritine ≥ 104 µg/L	(n=23) 189.11±18.81	172.48±21.01	-16.63±10.37	(n=17) 188.55±15.70	185.79±15.21	-2.75±8.23	-19.78 (-47.53;7.96) p=0.16[a]
Serum iron <16 µmol/L	(n=18) 142.62±21.19	123.31±20.97	-19.31±11.67	(n=19) 109.35±18.28	109.6±14.75	0.25±6.35	-20.98 (-47.63;5.66) p=0.12[a]
Serum iron ≥16 µmol/L	(n=22) 125.57±21.73	123.17±22.45	-2.40±5.63	(n=20) 112.80±19.54	121.91±20.25	9.11±5.70	-9.02 (-24.08;6.04) p=0.23[a]

*[a] adjusted for MTHFR genotype, † Change intervention – change placebo (95%CI), * mean ± SEM*

intervention group, while it increased in the placebo group (p = 0.04). Table 2 shows the serum ferritin concentrations before and after the intervention period, stratified for MTHFR genotype. The decrease in serum ferritin by the intervention was more pronounced in the CC group (p =0.04). Also, the participants were divided in two groups, based on the serum concentrations at baseline of folate, vitamin B12, ferritin and iron, respectively that were either smaller or greater than their median value. A similar approach was taken for dietary intake of folate, vitamin B12 and iron. The decrease of the serum ferritin concentration was observed for those with the highest serum folate level (median 13.4 nmol/L) at baseline (p = 0.02), as well as for participants with the highest dietary folate intake (median 197 µg) (change intervention: -19.1 ± 9.9 µg/L, change placebo: 10.3 ± 4.9 µg/L, p=0.03). Also for vitamin B12 (median 232 pmol/L), the decrease of the serum ferritin concentration was observed for those with highest serum concentrations at baseline (p = 0.07), as well as for participants with the highest dietary vitamin B12 intake (median 4.2 µg) (change intervention: -13.1 ± 11.0 µg/L, change placebo: 13.8 ± 5.2 µg/L, p=0.10). The decrease of the serum ferritin concentration (p=0.06) was significant for participants with lowest serum ferritin concentrations at baseline (median 104 µg/L). A decrease was also observed for participants with highest serum ferritin concentrations, although not significant. For participants with lowest serum iron concentrations at baseline (median 16 µmol/L), a decrease of the serum ferritin levels was observed, although not statistically significant. In contrast to this, serum ferritine was statistically significantly (p=0.04) lower in participants with highest dietary intake of iron (median 11.7 mg). The decrease in intervention group was 16.7 ± 10.8 µg/L, while the placebo group had an increase of 12.3 ± 5.0 µg/L. Thus, folic acid and vitamin B12 supplementation decreased serum ferritin concentrations, especially in subjects with CC genotype and in subjects with higher folate levels or higher vitamin B12 levels at start of the intervention. When stratifying for MTHFR genotype it was observed that the CC group had a similar decrease in the colonic total iron concentration in the intervention and the placebo group. However, in the MTHFR 677 TT group, the colonic total iron concentration was decreased in the intervention group (-8.37 ± 5.2 µM, n=9), whereas in the placebo group an increase was observed (4.3± 6.6 µM, n=9), although this was not statistically significant (p=0.15).

Discussion

In vitro it was observed that increased folic acid exposure decreases the total iron content of human colon cancer cell lines. To examine whether these findings could be confirmed *in vivo* and in view of different intervention strategies to prevent anaemia, we evaluated the blood and colonic iron status before and after supplementation with folic acid and vitamin B12 in an existing randomized, placebo-controlled trial. We observed that supplementation with folic acid and vitamin B12 for six months decreased serum ferritin, especially in participants with high serum folate concentrations, or vitamin B12 concentrations, or low serum iron concentrations, or high iron dietary intake at baseline and among those with the MTHFR 677 CC genotype. No effect was seen on total colonic iron in the intervention group, as compared to the placebo group, although for the TT genotype a slight, but non-significant decrease was observed.

Serum ferritin, haemoglobin, and haematocrit were determined. These blood parameters and the daily intake of iron in our study were in the normal range (182,190,192,205), which allows to conclude that the population used was not anaemic. Whether iron status of the colon was in the healthy range was more difficult to establish, since not much is known about the usual concentration of iron in colon biopsies. To our knowledge only one study investigated the iron concentrations in intestinal cancer tissue and in colorectal polyps and found mean concentrations of 46.1 and 43.2 mg/kg, respectively (206). These values are somewhat higher than the levels we observed (mean: 34.8 mg/kg). However, our analysis was done in rectal biopsies of normal appearing mucosa and the discrepancy may be explained by pathology. In addition, it may be due to the differences in the methods of analysis that were used. We used Atomic Absorption Spectrometry in contrast to the total-reflection X-ray fluorescence method that was used in the other study.

In our previous *in vitro* study, we observed that the expression of the iron transporter SLC40A1 and of the ferritin light chain genes, as well as the total iron concentration was lowered in colonic cell lines by folic acid exposure. In the intervention study, no statistically significant lowering of the colonic iron concentration was observed as a consequence of folic acid supplementation. A discrepancy between results obtained in cell lines and in humans is also seen by many others (207). This may be explained by differences in cell or tissue physiology or in cellular composition. In the *in vitro* study,

immortalized and cancer derived cells were used, while *in vivo* healthy tissue was examined. Also, differences in physiology exist throughout the intestine. For example, uptake of dietary iron takes place in the duodenum (182), while we assessed the rectum. Furthermore, in contrast to the *in vitro* study, in which pure cultures of cell lines were used, rectal biopsies are comprised of multiple cell types. In addition to an epithelial cell layer, consisting of enterocytes, goblet and endocrine cells, also immune cells, gut-associated lymphatic tissue, blood and muscle are present and the different cells and cell types are spatially organised (208). It may well be that the power of analysis was too low to detect a decrease in total iron if this occurred only in the enterocytes. Alternatively, the down regulation of ferritin expression and of total iron by folate that was observed in the *in vitro* study may reflect the body iron store, rather than colonic iron status. Maybe *in vivo* the iron is not stored in the enterocytes. *In vivo*, iron is transported into the plasma from the intestinal mucosa, so plasma ferritin levels reflects the body iron store (205). To support this explanation, it would have been of interest to assess colonic ferritin levels in the intervention study, but this was not possible due to lack of additional biopsy material. Also, it will be of interest to examine the relation between folate, iron and ferritin *in vivo* specifically in enterocytes.

Our intervention study allowed us to examine the effect of folic acid and vitamin B12 supplementation on iron status and taken the MTHFR 677 genotype into account. We observed that participants with high serum folate concentrations at baseline had decreased serum ferritin levels after folic acid and vitamin B12 supplementation, in contrast to participants with low serum folate concentrations at baseline. The lowering of serum ferritin levels was also observed in participants having a high serum vitamin B12 concentration, compared to participants with low serum vitamin B12 concentration at baseline. This may indicate an additive effect of dietary folate and supplemental folic acid, and of dietary vitamin B12 and supplemental vitamin B12. Furthermore, it suggests that a direct interaction or physiological relation between folate-vitamin B12 and ferritin or iron exists. This is also strengthened by the observation that participants especially with the lowest serum ferritin concentration at baseline had a decreased serum ferritin concentration after supplementation. In agreement with the effect of folate on iron status, a decreased serum ferritin concentration was also observed for participants with the highest serum ferritin concentrations at baseline as well as for those with the lowest total iron concentrations, although not statistically significant. Stratifying for dietary iron

intake showed opposite results, which may be explained by the fact that the serum iron and ferritin concentrations are not directly dependent on the amount of iron consumed (182).

In addition, we observed effects of the MTHFR 677 genotype. Because the intervention was not designed for this analysis, we have to interpret the findings with caution. Nevertheless, it seemed that lowering of colonic iron, although not significant, could be observed in subjects with a MTHFR 677TT genotype, while this was not the case when all participants were analysed. It would be worthwhile to test this finding in larger number of subjects with the MTHFR677TT genotype. Furthermore, the lowering of serum ferritin levels could be observed in subjects with a MTHFR 677CC genotype (n=61), but not in those with the TT genotype. This can be the result of insufficient power to observe significant effects in TT genotypes (n=18). To further investigate these aspects, one could conduct a similar trial and include a larger number of TT genotypes. However, recruiting enough participants in such a trial will be difficult, as the prevalence of the TT genotype is only 10% in the Dutch population (70,139). If the observed differences could be confirmed, they can be explained by differences in metabolic functions caused by the MTHFR genotype. The TT genotype exhibits a lower MTHFR enzyme activity and thus provides more methyl groups for the DNA synthesis and a number of other functions including mitochondrial protein synthesis. It is thought that the TT genotype is favourable under conditions of folate sufficiency (11,199). It provides less methyl groups for (DNA) methylation (8.9), but this is limiting only under conditions of deficiency (201,202). Indeed a low folate status in TT subjects was shown to diminish genomic DNA methylation in blood, while this was not observed for either the CC genotype or for normal folate status (209). Thus the MTHFR genotype differences may affect cellular metabolism. This may result in differential effects on colon and plasma iron levels, either directly, by regulation of transcriptional activity, or indirectly, by altering homeostasis. Our results support the need for molecular dissection of the functions and interaction of folate and iron in cellular metabolism, to obtain an improved understanding of the importance of these dietary components in health and development.

In this human intervention study, a daily supplement of 4.6 mg folic acid and 1.1 mg vitamin B12 was given, which is relatively high. For comparison, the recommended daily intake in The Netherlands is 300 µg folate, 2.8 µg vitamin B12 and 9 mg iron (98). Iron-

folic acid supplementation during pregnancy consists of 400 µg folate and 15-30 mg iron per day, while during anaemia 5 mg folate and 200 mg iron is supplemented (189,191). However, in some cases of anaemia, in treatment of rheumatoid arthritis with methotrexate and in the prevention of neural tube defects, a high dose folic acid supplementation (3-5 mg) is used (193,194,210,211). Our finding that folic acid and vitamin B12 supplementation reduces serum ferritin levels, substantiates the finding that high folic acid supplementation (without vitamin B12) decreases plasma ferritin levels if no additional iron was supplied (190). Therefore, it is advisable, especially when high concentrations of folic acid and vitamin B12 are used, to include iron in supplementation, in particular in anaemic persons.

7

General discussion

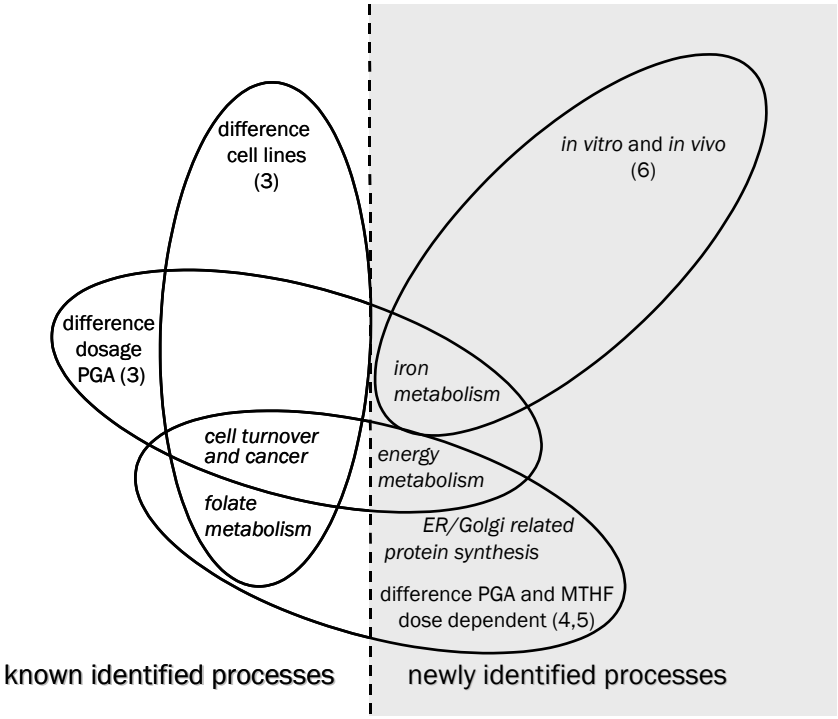
The main aim of this thesis was to identify processes, using *in vitro* studies, which are affected by folate in the colon, taking different folate metabolites and different dosages of folate into account. This was studied via a nutrigenomics approach using the transcriptomic technique, DNA microarrays. In this Chapter, we first summarise and discuss our main findings. Using these findings, we discuss the advantages and shortcomings of the studies and methods used. Next, we address the implications and directions for future research.

Main findings

A nutrigenomics approach using DNA microarrays was used to study the effect of folate on the human colon. Prior to this, in Chapter 2, the technical and biological variation of this method was investigated. The intraclass correlation coefficient (ICC), a novel approach in analysing data from DNA microarrays, was applied for analysing these variations. Using the ICC, we found that indirect labelling methods were more reproducible than direct methods. Therefore, this indirect labelling method was applied in the studies performed in Chapter 3 to 5. The optimal human rectal biopsy sampling appeared to be two biopsies per person. Furthermore, the analysis indicated that at least six persons per group would be needed using DNA microarrays to study gene expression in rectal biopsies. Chapter 3 to 5 describe all our studies on effects of folate on the human colon cells *in vitro*. In Figure 7.1 the main findings of these folate related studies are summarised, including the cellular processes involved. The *in vitro* study in Chapter 3 describes a difference in response to folate between the three human colon epithelial cell lines used. All cell lines showed an increased growth rate in the high PGA condition. This was coupled to a different proliferation and apoptosis rate for each cell line. This difference was also obtained for intracellular folate levels, the SAM concentration, the SAH concentration, the ATP concentration, the differentiation marker E-cadherin (CDH1), and the gene expression pattern. Gene expression data confirmed the difference found in the physiological assays. In addition, it revealed a difference in iron metabolism. For all cell lines a lower intracellular iron concentration was found with the high PGA exposure. Chapter 4 showed decreased glycolysis and increased mitochondrial mass and oxygen consumption in 10 ng/mL PGA compared to 100 ng/mL PGA, 10 ng/mL MTHF and 100 ng/mL MTHF. Chapter 5 revealed more details about the difference in mechanism of PGA and MTHF exposure, especially between the high exposure conditions (50, 100 and 200 ng/mL). High MTHF affected endoplasmatic reticulum (ER)/golgi related genes and

cancer related genes, which may suggest a more beneficial effect of MTHF as compared to PGA. Also in Chapter 5, a dose dependent effect of both PGA and MTHF was found on the expression of folate metabolism, cancer related and ER/golgi related genes. The *in vitro* findings on iron metabolism were confirmed *in vivo* by a human randomised intervention trial with a high dosage of folate (71) (Chapter 6).

Figure 7.1 Main folate related findings of the *in vitro* studies and the human intervention trial



In vitro *identified processes that are affected by folate in the colon are represented in italic, on the left side are known processes and on the right side (in grey) are newly identified processes. The relevant conditions are also indicated together with the relevant chapter number (between brackets).*

Summarising the outcome of the in vitro studies results in known and newly identified processes that are affected by folate in the colon. Known identified processes are cell turnover, folate metabolism and cancer related processes. Newly identified processes are iron metabolism, energy metabolism, protein synthesis and endoplasmatic reticulum/golgi related processes. Two other findings were obtained. Firstly, large differences in response to PGA exist between human colon epithelial cell lines and differences exist in response between PGA and MTHF exposure of HT29 cells. Secondly, identical levels of extracellular PGA and MTHF led to different intracellular folate and

folate metabolites concentrations. Also identical levels of extracellular PGA led to different intracellular folate and folate metabolites concentrations in different cell lines.

Methodological issues

In this paragraph we address the strength and limitations of the different studies described in this thesis, according to the introduction paragraph used in Chapter 1.

Folate related processes and metabolites

Using a nutrigenomics approach we identified several processes which where affected by folate in the colon. Cell turnover, cancer related processes (cell adhesion, viability and differentiation) and folate metabolism are described by others to be affected by folate using a nutrigenomics approach (55-57) or physiologicals assays (212-214). We identified other processes in the colon to be affected folate, namely iron metabolism, energy metabolism, ER/Golgi related processes and protein synthesis. Only a few studies have described the effect of folate on protein synthesis (8,9) and energy metabolism (3,9), all using large DNA microarrays. Iron metabolism and ER/Golgi related processes affected by folate are, to our knowledge, as yet undescribed. This could be due to the fact that we observed this in a study comparing PGA with MTHF using DNA microarrays, physiological folate concentrations and vitamin B12, and such study had not previously been performed.

To investigate the effect of folate on cell turnover, differentiation and energy metabolism we used established assays. For the effect on cell turnover, we investigated the proliferation, apoptosis and cell growth. This contrasts other studies, where only apoptosis is studied (46,49,212,214). This revealed that there were large differences in proliferation and apoptosis between three cell lines which all had a similar increased growth rate. Other studies state that increased apoptosis would lead to decreased cell turnover. We showed that it is important to investigate all parameters of cell turnover (apoptosis, proliferation and cell growth) to derive at this conclusion.

We expected the enzymes involved in the folate metabolism to differ in gene expression between PGA concentrations (Chapter 3), but these were stable. We also expected the folate metabolites to have a similar decrease or increase in all cell lines in identical PGA exposures. This was not the case. This can be explained by regulation of the enzymes on a translational, post-translational or protein level. For example, it is known that 5,10-

methylenetetrahydrofolatereductase (MTHFR) has a SAM/SAH regulatory domain (198,199). Different forms of serinehydroxymethyltransferase (SHMT) can arise by alternative splicing with different protein levels affecting folate metabolism (215). Therefore, it would be interesting to expand the transcriptomics studies to proteomic studies, or even with metabolomic studies, as we did not expect similar PGA and MTHF concentrations would give these differences in folate metabolite concentrations (Chapter 4). Due to limitations in sensitivity of the HLPC method and folate binding protein column that was used, we could only analyse the effect of folate exposure on intracellular THF and MTHF. It would have been interesting to know the concentrations of other key metabolites such as intracellular formylTHF and methyleneTHF.

Nutrigenomics

For Chapters 2, 3 and 5, we used an in-house produced microarrays that contained between 1100 and 3000 genes, which were obtained from subtracted cDNA libraries. At the time this study began, this was the state of the art technology. At present these arrays are considered to be small compared to Agilent and Affymetrix microarrays used in other studies, which contain 44,000 genes. Using these microarrays would allow us to study more known processes and identify new ones. Despite the limitations of our studies we have obtained insight in known and new processes that are affected by folate. Not only did we use the ICC to examine the reproducibility of our microarray data, but we also verified the microarray data with other techniques, such as physiological assays, including Western blot and qRT-PCR. Moreover, the microarray results of identical, but fully independent obtained exposure of Chapter 3 and 5 were compared, using different runs of in-house produced cDNA microarrays. Also, from all exposure conditions used in Chapter 4, microarray analysis was performed (not in this thesis), using MWG-oligo's (Dommels et al. submitted). The results obtained by these different microarrays were more or less similar. More specifically, the trend was the same (up or down regulation), only the fold-change differed, especially when comparing cDNA with oligo microarrays. It seemed that most differences were caused by differences in cell culture conditions between these studies, which were different in the exact exposure times and seeding density.

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In vitro studies

The use of *in vitro* models has several advantages over *in vivo* models. Many different conditions can be compared, exposure conditions can be to a high extend controlled and they are less time consuming than *in vivo* models (59,60). We wanted to confirm our *in vitro* findings *in vivo*. The best chance for this would be obtained if we would find common results for all cell lines. In Chapter 3 differences in response between the cell lines was found (e.g. proliferation, apoptosis and CDH1), but also common responses were observed (e.g. cell growth and iron metabolism). Lowering of CDH1 by folate supplementation could not be confirmed *in vivo*, in humans (data not shown in this thesis). However, an effect of folate on iron metabolism was observed *in vivo*, although this was not found as lowering of colonic iron levels, as observed *in vitro*, but in lowering of serum ferritin. Multiple articles are published concerning the difficulties of extrapolation of *in vitro* obtained data to humans (207,216-219). Large differences exist between cell culture systems and humans. Cell lines are placed outside their normal environment, and contact with other cell types and specific environmental conditions, such as colonic flora for intestinal cell lines is lacking. In addition, cell lines are often cultured for long periods, sometimes for many years, such as Caco-2 and HT29 cells, which may change their behaviour (59,61,220,221). Furthermore, due to absence of growth inhibition, the cell lines may not represent the physiology of normal cells. One could overcome this problem partially by using primary cell lines. However, the use of primary cell lines also has several disadvantages, one of these being the short life span (61). In addition, there are practical disadvantages such as how to obtain “fresh” human biopsies and how to isolate cells. To not only depend on cancer cell lines, we also used a SV40 transformed primary cell line (CCD841CoTr), although by this immortalisation cell physiology is altered (222). After the results from Chapter 3, we decided to further investigate the effect of folate in HT29 cells only. This was the most responsive cell line. Furthermore, culture time and passage number influences the morphological and physiological development of Caco-2 cells, while HT29 does not seem to have this problem (223).

There are three other important topics concerning the folate exposure to be considered, namely the luminal versus blood exposure, the PGA versus MTHF exposure and the dosage of folate exposure. In culture flasks, cells are only exposed on one side to the culture medium. However, colon epithelium is exposed to two sides, namely apical and

basolateral. Folate exposure from the apical or lumenal side consists of folates produced by the microflora, while basolateral or blood side consist of folate from the blood (39). Normally the diet consists of pteroylmonoglutamate and pteroylpolyglutamate forms and PGA, if supplements or fortified food are ingested, while the blood consists mostly of monoglutamate-MTHF (13). It is difficult to exactly predict the concentration of folate to which the lumenal side of the colon is exposed, due to the synthesis and breakdown of folate by the colonic microflora and their kinetics (32,208,224,225). Also, low dosages of oral PGA undergo extensive conversion to MTHF in the intestinal mucosa. This process however is saturable and it has been shown that high dosages (greater than 200 µg) give small amounts of unmetabolised PGA in plasma (32).

It would have been of great interest if it had been possible to analyse the human rectal biopsies from the intervention trial by DNA microarray. This could not be done due to breakdown of the RNA obtained from the biopsies, despite extensive precautions. The most likely reason for the degradation is that the time between sampling and degradation arrest by freezing was too long, possibly due to the large amount of specimens collected at one time and to the fact that these were not directly snap-frozen in liquid nitrogen before storage in the transport-container, where only the walls were filled with liquid nitrogen.

We investigated the effect of folate only in combination with vitamin B12. It would have been attractive to also study the effect of folate in combination with other B-vitamins, especially B2 and B6. These both serve as cofactors for folate metabolising enzymes (10) (see Chapter 1 Figure 1) and may influence the effect of folate on colorectal cancer risk (70). In normal culture media both vitamin B2 and vitamin B6 are present at supraphysiological concentrations (142), so a shortage of vitamin B2 and B6 could not have interfered with our results.

Overall the lessons learned from our *in vitro* studies are: use physiological concentrations of the dietary component of interest; use multiple cell lines and look for common responses, to investigate cell turnover; use the combination of apoptosis, proliferation and cell growth, and combine microarray data with physiological data.

Implications

In addition to the *in vitro* identified processes that were affected by folate, two other major findings were obtained in this thesis. Firstly, that large differences between human colon epithelial cell lines in response to PGA exposure exist and, secondly, that large differences in response exist between PGA and MTHF exposed HT29 cells. Furthermore, a discrepancy between extracellular folate exposure and intracellular folate metabolites was found in different cell lines and different folate exposures. The implications of these findings are discussed below, together with implications with regard to the use of a nutrigenomics approach.

Different response cell lines

Three different human colon epithelial cell lines were used and identically exposed to folate. We expected to find common responses, not only for the physiological assays that were used, but also for gene expression analysis. However, the responses of the cell lines varied substantially. Differences in response between different human colon epithelial cell lines were also found by others (49,68), when using another dietary component (curcumin) (226). The large differences between the cell lines reduces the predictive value of any (single) cell lines and makes it very difficult to faithfully extrapolate from *in vitro* to *in vivo*.

Different response to PGA and MTHF

A difference in response of HT29 cells to PGA and MTHF was observed in two separate studies (Chapter 4 and 5). A 10 ng/mL MTHF exposure resulted in a higher intracellular concentration of THF and MTHF and a higher growth rate as compared to a 10 ng/mL PGA exposure. Furthermore, the gene expression profile and mitochondrial activity differed between these conditions. Also high (50-200 ng/mL) MTHF and PGA exposure resulted in differences in gene expression, indicating functional differences between the two forms of folate. Differences in intracellular folate levels (4) and proliferation (5) were also found in Caco-2 cells. Not only molecular responses differ, but indications exist that also the physiological outcome is affected. Animal studies have shown adverse effects, supplementation with PGA after establishment of tumours increased intestinal tumour burden (21,23,117,118). With regard to other physiological parameters potential adverse effects have been observed. A study observed reduction of foetal growth in PGA supplementation (227). A human study showed that MTHF improved the endothelial

function of arteries compared to PGA supplementation (45). As MTHF and PGA give different molecular and physiological responses in our and other studies, and indications of potential adverse effects for PGA exist, it is of practical importance to know whether the same is true for MTHF and whether this form of folate may be better suited as supplement.

Intracellular folate metabolites

We found that the extracellular folate concentrations of different forms of folate result in different intracellular concentrations of folate. Furthermore, also identical extracellular PGA or MTHF concentrations of the different cell lines results in different intracellular concentrations of folate and folate metabolites. A discrepancy between exposure and intracellular concentrations was also seen by others in Caco-2 cells (38), confirming our observation. Also, in animal and human studies it is found that the folate intake does not correspond with colonic folate and folate metabolites, although it does correspond with plasma or serum folate (14,16,18-20,228). In our studies, the growth rate seems to correlate with intracellular folate metabolites concentrations and the SAM/SAH ratio, with strength in gene expression response. However, these parameters did not correlate with extracellular folate concentrations. It is of importance to know the intracellular concentrations of different folate metabolites in the tissue of interest in order to be able to correlate exposure to outcome.

Nutrigenomics approach

In our studies we have taken a hypothesis-generating approach using the transcriptomics technology and not a hypothesis-driven approach. The holistic hypothesis generating approach, which is also used in systems biology, requires highly robust technology and strongly developed bioinformatical data interpretation tools. Looking back it must be admitted that at the time of these studies the technology was not sufficiently robust. In fact, it was, and stil, is under constant development. Although the statistical tools to analyse data now exist, data interpretation tools especially are still relatively new and need to be further developed, and certainly with regard to nutrient relevant physiological processes (7). Another factor that makes interpretation difficult is that regulation not only takes place at transcription level but also at translation and post translation level. Using a multidisciplinary approach in which a combination of multiple “-omics” techniques, for instance transcriptomics, proteomics and metabolomics, describing the complete

biological processes can be used to circumvent some of these problems. However, this is only possible when combining the area of expertise of large scientific groups. In individual research projects, especially in view of the need for better data interpretation tools, it may therefore be advisable to concentrate on hypothesis-driven approaches to allow focused analysis of the data.

Future directions

In this paragraph the future directions and possible experiments are outlined following the four topics discussed in the implications.

Different response cell lines

Although there is a difference in response between the cell lines used, one should not completely rule out the use of cell lines. If, from *in vivo* studies, strong evidence is obtained for a certain gene or process, one can make use of cell lines to answer questions to a hypothesis-testing approach. A number of alterations can be performed compared to our studies, such as co-culturing of HT29 and Caco-2, transwells-grown cells, differentiated HT29 cells or primary cell cultures. The goal in establishing intestinal cell culture systems is to obtain cells that mimic as closely as possible their counter parts *in vivo*. Co-culturing has the advantage that a mixed population of cells mimics both the secretory and absorptive functions of the intestinal epithelium more closely, although until now it is only used in transport studies (229). Monolayers grown on transwells have polarity and tight junctions, making it possible to investigate the apical-basolateral transport (59,61). Differentiated HT29 cells become mucin-producing goblet cells, facilitating the ability to study a secreting cell (59,61). Primary cultures are most representative for the healthy intestine, although they can only be maintained for short-term experiments (61). Using RNAi-technique, *in vivo* identified genes can be silenced (230), so the same cell line, with and without expression, of a particular gene can be compared.

Different response to PGA and MTHF

It would be interesting to study the difference in response between PGA and MTHF *in vivo*. This can be accomplished in a human intervention trial, using different folate forms, with established biomarkers as endpoint, such as colorectal adenomas. As this is not ethical, in the case of PGA exposure and the length of time it takes to assess endpoints,

animal studies are an alternative option. In humans the diet always contains MTHF. Thus when investigating the effect of PGA supplementation, one is in fact studying the combination of both PGA and MTHF. In animal studies PGA and MTHF can be studied independently and in combination. Furthermore, it allows for analysis of effects before and after establishment of neoplastic foci. In *in vivo* studies, it is also attractive to study the effect of folate in combination with different B-vitamins, since there may be substantial interaction. Vogelstein (106) described the key genes involved in colorectal carcinogenesis. These are implemented in different knockout animal model. There are knockout models for sporadic colorectal cancer ($GSTM1^{min}$ model), as well as models for familial adenomatous polyposis (APC^{min} model) and hereditary non-polyposis colorectal cancer (mismatch repair genes knockout animal) (231-235). These, as well as models that use chemical induction, can be used to study the effect of folate on colorectal carcinogenesis. A disadvantage of the knockout models is that mutations are systemic and cause embryonic lethality or developmental compensation (236). The Cre-Lox technology is a new tissue specific inducible system (236,237). The advantage is that the immediate consequence of on, off, over- or under expression of a gene in otherwise normal tissue can be studied in any tissue at any life stage (236). This system can also be used to investigate newly identified genes, before and after folate supplementation.

Intracellular folate metabolites

As folate exposure and intracellular concentrations do not correlate and because a difference in metabolite balance may be essential to predict outcomes, a considerable effort should be made to measure folate metabolites in tissue. The microbial assay determines the total folate pool, while the HPLC method distinguishes between THF, MTHF, 5,10'-methyleneTHF and PGA (16,238). A combination of these two makes it possible to determine subnanomolar concentrations of PGA (239).

Recently a more sensitive method was developed to quantify simultaneously homocysteine, PGA and MTHF by stable isotope-dilution liquid chromatography/tandem mass spectrometry (LC/MS/MS) (240). Other folate forms, such as formylTHF, are less stable and cannot be detected with this method. Also the quantification methods of SAM and SAH are still under development using HPLC connected to tandem mass spectrometer (MS/MS) (241,242) or by capillary

electrophoresis (243). Most existing methods are not sensitive enough to measure folate metabolites in tissues. Furthermore, a number of folate metabolites are not stable. This

could interfere with interpretation, but an effort in this direction is essential to allow interpretation of the molecular effects resulting from folate exposure.

Nutrigenomics approach

"-Omics" technology is still under development. Current state-of-the-art transcriptomics technology is probably sufficiently robust and provides a full overview of possible effects. This is certainly not the case for proteomics and metabolomics. In view of this, and especially in view of the need for further development of data interpretation tools, it will currently be most effective for individual research projects to use nutrigensomics approaches in specific, hypothesis-driven research. Possibly, integrated research projects will allow to effectively combine transcriptomics, proteomics and metabolomics (4,5,7,8) and a number of different technological options that are becoming available can be incorporated. For example, making use of microarrays for screening of DNA methylation status of multiple genes simultanously (244) or the use of laser microdissection (245), so the epithelium only is investigated and not the surrounding tissue (4). However, for the large amounts of data obtained, first the data analysis and interpretations tools need to be further developed. This can then be used to further investigate the effect of PGA and MTHF on iron metabolism, cancer related processes or energy metabolism in humans. Hopefully this will lead to more insight in the effect of folate supplementation with regard to colorectal carcinogenesis.

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Summary

Folates are essential cofactors in single carbon transfer reactions and, consequently, are important for DNA synthesis and methylation. Several epidemiological studies have shown that low folate intake influences the risk of not only neural tube defects and vascular diseases but also colorectal tumours. A shortage of folate leads to increased uracil/thymidine ratio and altered DNA methylation. The increased uracil/thymidine ratio results in uracil misincorporation into DNA, leading to mutations in DNA and ultimately DNA strand breaks. Altered DNA methylation can increase gene expression of oncogenes and at the same time decrease the gene expression of tumour suppressor genes. All these processes are known to be involved in cancer. Little is know about other processes in the colon in which folate is involved. Folate is present as 5'-methyltetrahydrofolate (MTHF) in food and plasma, while in fortified foods and supplements the synthetic analogue folic acid (pteroyl glutamic acid, PGA) is used. Recent animal studies have shown that the dose and timing of folate intervention are critical, indicating a potential risk of long term supplementation with PGA. It is unknown whether PGA and MTHF may have different effects. The effects of folate(s) were investigated in traditional physiological assays, but we also combined these with gene expression analysis. Conventionally, gene expression analysis was restricted to one or a small number of genes. Recently, DNA microarray technology was developed. This transcriptomics technique allows scientists to investigate the expression of thousands of genes simultanously; when applied in nutrition research this is called nutrigenomics. We used this nutrigenomics approach to identify processes affected by folate in the colon. The following specific research questions were formulated (Chapter 1):

1. What is the effect of PGA supplementation on folate metabolites and cancer related processes, such as cell proliferation, apoptosis and differentiation?
2. Moreover, which unknown processes are affected by PGA supplementation?
3. Is this depending on which human colon cell line is used?
4. Can these similar effects be found with MTHF supplementation?
5. Can the *in vitro* results be verified *in vivo*, in humans?

To answer these questions we conducted *in vitro* studies using different cell lines and different dosages of folate and folate forms. We did not only investigate the effect of folate on gene expression, but combined this with physiological assays or other verification methods such as quantitative RT-PCR, ELISA or Western Blot.

Research question 1:

What is the effect of PGA supplementation on folate metabolites and cancer related processes, such as cell proliferation, apoptosis and differentiation?

We used three different human colon epithelial cell lines; Caco-2, HT29, CCD841CoTr, to investigate the response to folate (Chapter 3). All three cell lines showed an increased growth rate at 100ng/mL PGA, compared to 10 ng/mL PGA. Also, intracellular folate levels, SAM concentration and SAH concentration were increased in the high PGA exposure.

Research question 2:

Moreover, which unknown processes are affected by PGA supplementation?

Two unknown processes affected by PGA were identified; energy and iron metabolism. Physiological assays showed an effect of PGA on energy metabolism, which differed between cell lines. Gene expression analysis revealed a difference in iron metabolism in PGA exposed HT29 cells. However, for all cell lines a lower intracellular iron concentration was found in the high PGA exposure.

Research question 3:

Is this depending on which human colon cell line is used?

All cell lines showed an increased growth rate in the high PGA condition, although the rate of proliferation and apoptosis differed between cell lines. For all cell lines at 100 ng/mL PGA the intracellular folate levels, SAM concentration and SAH concentration were increased, but not in a similar manner. Energy metabolism (ATP concentration and MTT conversion), E-cadherin differentiation marker and gene expression were different between cell lines when exposed to 10 ng/mL and 100 ng/mL PGA.

Research question 4:

Can these similar effects be found with MTHF supplementation?

We performed two *in vitro* studies using HT29 cells to investigate the difference between PGA and MTHF supplementation. In Chapter 4 exposure to 10 ng/mL PGA compared to; 100 ng/mL PGA, 10 ng/mL MTHF and 100 ng/mL MTHF, showed decreased glycolysis and increased mitochondrial mass and oxygen consumption.

In Chapter 5 exposure to 10, 20, 50, 100 and 200 ng/mL PGA or MTHF showed a dose dependent effect of both folates on folate metabolism, protein synthesis, endoplasmatic

reticulum/Golgi related genes and cancer related genes. Expression of genes involved in folate metabolism were increased at 10 ng/mL PGA exposure, compared to 10 ng/mL MTHF. Supplemental levels of MTHF (50-200 ng/mL) seemed to increase protein synthesis, cell viability, differentiation and adhesion, compared to supplemental PGA levels.

Research question 5:

Can the in vitro results be verified in vivo, in humans?

A human folate and vitamin B12 intervention study (FOCO-trial) was used to confirm *in vitro* observed effects of folate on iron metabolism *in vivo* (Chapter 6). In the human study it was found that folate supplementation lowered the iron metabolism in blood (serum ferritin levels), but not in the colon.

The main findings from the work described in this thesis are summarised and discussed in Chapter 7. In this chapter, we reflect on the methodological issues of our studies. We further discuss difference in response between cell lines, and PGA and MTHF exposure, and the nutrigenomics approach, focusing on their implications and further directions.

In vitro, known processes were identified such as cell turnover, folate metabolism and cancer related processes. In addition, new processes were identified in which folate plays a role in the colon as iron metabolism, energy metabolism, protein synthesis and endoplasmatic reticulum/golgi related processes. The *in vitro* studies show a potential negative effect of PGA supplementation on cancer related processes, decrease of cell viability, differentiation and adhesion, compared to MTHF supplementation. Two other findings were also obtained. Firstly, large differences in response to PGA exist between human colon epithelial cell lines, and large differences exist in response between PGA and MTHF exposure of HT29 cells. Secondly, identical levels of extracellular PGA and MTHF led to different intracellular folate and folate metabolites concentrations. Also, identical levels of extracellular PGA led to different intracellular folate and folate metabolites concentrations in different cell lines. The difference between PGA and MTHF should be confirmed *in vivo* using an appropriate model and a clear hypothesis-driven nutrigenomics approach in combination with folate metabolite analysis and physiological parameters.

Samenvatting

Vitaminen zijn noodzakelijke bestanddelen van onze voeding en spelen een belangrijke rol bij onder andere de stofwisseling, botopbouw en spijsvertering. Eén van deze vitamines, folaat (vitamine B11) is belangrijk bij meerdere processen in de cel en het instandhouden van een 'gezonde' cel. Folaat is onder andere betrokken bij de synthese van DNA (erfelijk materiaal) en methyleringsreacties. Uit meerdere epidemiologische onderzoeken is gebleken dat een lage folaat inname de kans op neurale buis afwijkingen ('open ruggetje'), hart- en vaatziekten en dikke darmkanker vergroot. Een te kort aan folaat veroorzaakt een te veel aan uracil, in plaats van voldoende thymidine, en een verandering in de methylering van DNA. Het uracil kan nu in plaats van het thymidine in het DNA worden ingebouwd waardoor er mutaties in het DNA ontstaan. Dit kan er echter ook toe leiden dat het DNA gaat breken. Veranderingen in de methylering van DNA hebben tot resultaat dat de cel sneller gaat delen en groeien. Van al deze processen is bekend dat ze betrokken zijn bij het ontstaan van kanker. In voeding en bloed komt folaat voor als 5'-methyltetrahydrofolaat (MTHF). In producten verrijkt met folaat of voedingssupplementen zit de synthetische vorm van folaat (ook wel foliumzuur, pteroyl glutamaat zuur of PGA genoemd). Dierstudies hebben aangetoond dat de dosis en het tijdstip van de folaat interventie cruciaal zijn, deze geven een indicatie over mogelijk schadelijke effecten van PGA. Tot een paar jaar geleden kon men de expressie van één of slechts een paar genen (stukjes DNA) tegelijkertijd bekijken. Recentelijk is er een nieuwe techniek ontwikkeld, DNA microarrays. Op deze microarray staan duizenden genen. Dit maakt het mogelijk om al deze genen tegelijkertijd te bestuderen. In dit proefschrift is gebruik gemaakt van deze techniek om naar het effect van folaat op de dikke darm te kijken. Het toepassen van deze techniek in voedingsonderzoek noemt men nutrigenomics. Door gebruik te maken van de nutrigenomics aanpak willen we meer inzicht krijgen in de processen. Bovendien willen we nieuwe processen identificeren waarbij folaat betrokken is in de dikke darm. Hierbij kwamen we tot de volgende vijf onderzoeksvragen:

1. Wat is het effect van PGA blootstelling op de folaat metabolieten in de cel en welke kanker gerelateerde processen, zoals celgroei en differentiatie worden hierdoor beinvloed?
2. Welke onbekende processen worden beinvloed door PGA blootstelling?
3. Zijn deze onbekende processen afhankelijk van de cellijn die gebruikt is?
4. Verschillen deze processen ook in een PGA en MTHF blootstelling?
5. Kunnen we deze bevindingen bevestigen in de mens?

Om deze vragen te kunnen beantwoorden zijn er meerdere studies met cellijnen uitgevoerd, waarbij verschillende concentraties en vormen van folaat gebruikt zijn. Hierbij is een combinatie van DNA microarrays en fysiologische assays gebruikt .De verkregen gen expressie data is bevestigd met andere technieken, zoals kwantitatieve RT-PCR.

Onderzoeksvraag 1:

Wat is het effect van PGA blootstelling op de folaat metabolieten in de cel en welke kanker gerelateerde processen, zoals celgroei en differentiatie worden er beinvloed?

Er werden drie verschillende humane dikke darm cellijnen; Caco-2, HT29, CCD841CoTr, blootgesteld aan PGA. De folaat metabolieten zoals intracellulaire folaat, SAM, en SAH waren in alle cellijnen toegenomen in de hoge PGA blootstelling. Bij de hoge PGA concentratie groeiden alle drie de cellijnen sneller.

Onderzoeksvraag 2:

Welke onbekende processen worden beinvloed door PGA blootstelling?

Twee nieuwe processen bleken beinvloed te worden door de PGA blootstelling, namelijk het energie en ijzer metabolisme. Fysiologische assays lieten een effect van PGA op het energie metabolisme zien. Met behulp van de gen expressie vonden we dat genen die betrokken zijn bij het ijzermetabolisme minder tot expressie kwamen in de hoge PGA blootstelling. In alle drie de cellijnen werd een lagere concentratie ijzer in de cel gevonden in de hoge PGA blootstelling.

Onderzoeksvraag 3:

Welke onbekende processen worden beinvloed door PGA blootstelling?

Alle drie de cellijnen groeiden sneller bij een hoge PGA concentratie, terwijl de verhouding proliferatie (celdeling) en apoptosis (celdood) verschillend was tussen de cellijnen. De mate van toename van folaat, SAM en SAH in de cel verschilde per cellijn. Het energie metabolisme (ATP concentratie en MTT omzetting), het E-cadherine (differentiatie marker) en de gen expressie waren anders tussen de cellijnen.

Onderzoeksvraag 4:

Verschillen deze processen ook in een PGA en MTHF blootstelling?

Om verschil te vinden tussen PGA en MTHF zijn er twee studies uitgevoerd met de HT29 cellijn. In hoofdstuk 4 leidde de 10 ng/mL PGA blootstelling, vergeleken met 100 ng/mL

PGA, 10 ng/mL en 100 ng/mL MTHF, tot verminderde afbraak van glucose, toename van het aantal mitochondriën en toename van de zuurstof opname. In hoofdstuk 5 werd de HT29 cellijn blootgesteld aan 10, 20, 50, 100 en 200 ng/mL PGA of MTHF. Hierbij zagen we een dosis afhankelijk effect van beide folaten. Genen betrokken bij het folaat metabolisme vertoonden een hogere expressie bij lage PGA concentratie vergeleken met lage MTHF concentratie. Hogere folaat concentraties die door middel van suppletie verkregen kunnen worden verhoogden de eiwit synthese, conditie van de cellen, differentiatie en adhesie in MTHF vergeleken met PGA.

Onderzoeksvraag 5:

Kunnen we deze bevindingen bevestigen in de mens?

We hebben gebruik gemaakt van een humane interventie studie met folaat (PGA) en vitamine B12, om de verlaging van het ijzer onder invloed van PGA te bestuderen in de mens. In deze humane studie hebben we gevonden dat onder invloed van PGA het ijzer metabolisme in het bloed wordt verlaagd, maar dat er geen effect van PGA op het ijzer metabolisme in de dikke darm lijkt te zijn.

In de cellijnen hebben we meerdere reeds bekende processen geïdentificeerd waarbij folaat betrokken is, zoals cel groei, folaat metabolisme en kanker gerelateerde processen. Bovendien zijn er nieuwe processen geïdentificeerd, zoals ijzer metabolisme, energie metabolisme, eiwit synthese en endoplasmatisch recticulum/Golgi gerelateerde processen. Bovendien waren er nog twee opvallende dingen. Ten eerste dat het effect van PGA blootstelling verschillend is tussen de cellijnen en dat er grote verschillen tussen PGA en MTHF blootstelling zijn in de HT29 cellijn. Ten tweede dat identieke blootstellingen van PGA en MTHF verschillende concentraties van folaat en folaat metabolieten in de cel tot gevolg hebben tussen verschillende cellijnen. Identieke concentraties van PGA leiden tot verschillende concentraties van folaat en folaat metabolieten in de cel in verschillende cellijnen. De gevonden processen die verschillend waren tussen de PGA en MTHF blootstelling moeten in een daarvoor geschikte humane of dierstudie worden bevestigd. Gebruikmakend van gen expressie dient de vraagstelling echter duidelijk en hypothetisch geformuleerd te worden. De gen expressie dient echter gekoppeld te worden aan folaat metaboliet onderzoek en fysiologische parameters.

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Curriculum Vitae

About the author

Elisabeth Petronella Maria, Linette, Pellis was born on the 8th May 1974 in Etten-Leur, The Netherlands. She completed secondary school (HAVO) at "Katholieke Scholengemeenschap Etten-Leur" in 1991. In this year she started studying medical biochemistry at the Hogeschool West-Brabant in Etten-Leur. During this study she performed a traineeship of a year at Janssen Research Foundation in Beerse, Belgium. After obtaining her BSc degree in 1996, she started an MSc study in Molecular Biology and Genetics at Wageningen University. She ended this study with a traineeship at Genetics Department of Wageningen University. From 1998 until 2000 she worked as research technician in several companies; Tanox Biosystems, Organon and KeyGene. On the 15th of May 2000 she started her PhD project, the results are described in this thesis. She was appointed at the Division of Human Nutrition of Wageningen University and worked in the Food Bioactives Group at RIKILT – Institute of Food Safety in Wageningen. During her PhD she joined several congresses and international courses within the educational of the VLAG (Food Technology, Agrobiotechnology, Nutrition and Health Sciences) graduate school. She was part of the organisation of the PhD-study Tour 2001 to Italy, Switzerland and Germany.

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Educational Programme

	Organizing institute	Year(s)
Discipline specific activities		
ArrayNL platform	ArrayNL	2000-2001
meeting of Nutritional Science Community	NWO	2000-2003
symposium “nutrition and chronic disease”	NWO	2000-2003
Ecophysiology of the gastrointestinal tract	VLAG	2001
Nutrition and lifestyle epidemiology	VLAG	2001
Masterclass Nutrigenomics	VLAG	2001
Conference “homocysteine metabolism”, Italy		2001
Start symposium of Centre for Human Nutrigenomics	CHN	2001
Bioinformatics course	WUR	2001
GeneMath course	GeneMath	2002
Intestinal function	VLAG/CHN	2002
Conference “Folic acid, vitamin B12 and one carbon metabolism”, USA	FASEB	2002
Folate metabolism: new approaches and insights	WCFS	2003
Future Perspectives of Human Health: the Role of Nutrigenomics	VLAG/CHN	2003
Conference “folates, analysis, bioavailability and health”, Poland		2004
General courses		
PhD week	VLAG	2000
Scientific writing for PhD students	CENTA	2003
PhD week	VLAG	2004
Optional courses and activities		
PhD study Tour	WUR-HNE	2001
Talent day	NWO	2003
Meetings Homocysteine Club	WUR-HNE	2000-2003

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