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Modulation of One-Carbon Metabolism by B Vitamins: Implications for Transformation and Progression of Prostate Cancer

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1. Introduction

Extensive laboratory, epidemiological and clinical investigations suggest that prostate cancer might be affected by enhanced folate or B12 ingestion and or other perturbations of one-carbon (CH3−) metabolism. Over the last decade, largely due to government mandated dietary fortification with folic acid (FA), our clinic patients experienced a 4-6-fold increase in the median level of serum folate (5 ng/ml → 24 ng/ml). The National Health and Nutrition Examination Surveys (NHANES) confirm similar elevated levels Figure 1 (Dietrich et al, 2005; McDowell et al, 2008; Yang et al, 2010).
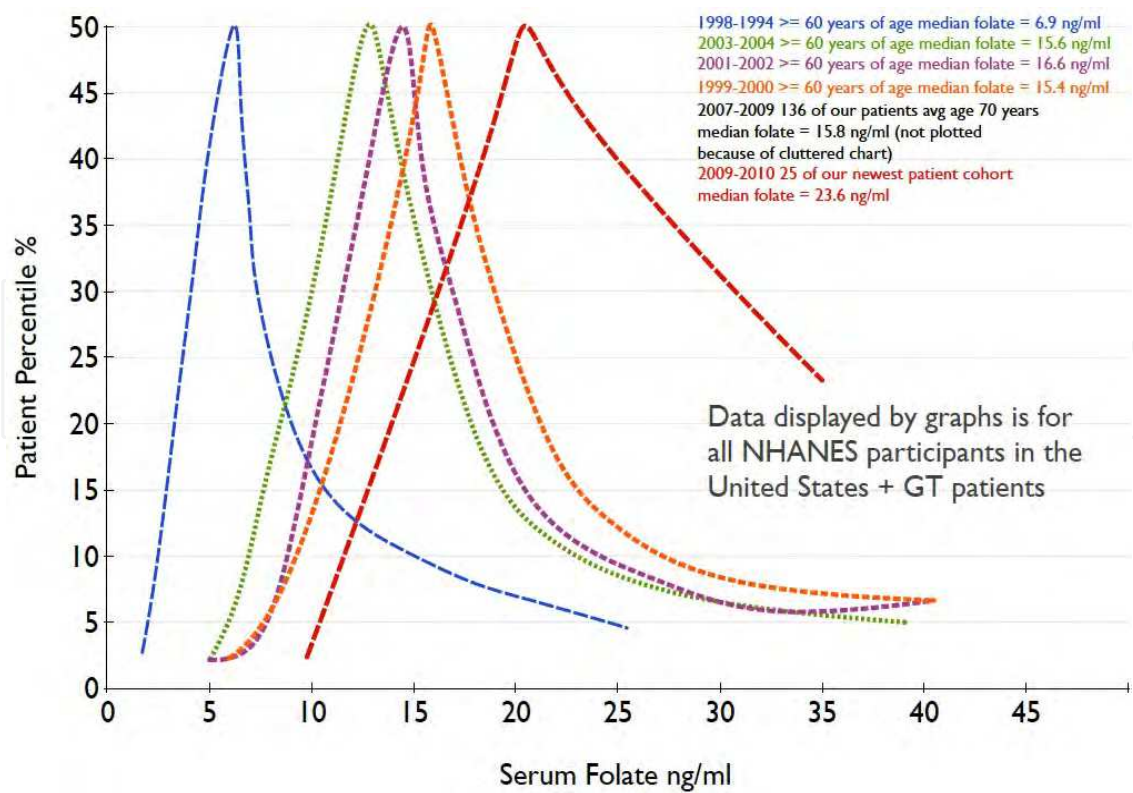

Fig. 1. Median serum/plasma blood folate levels NHANES and GT data.

Exposure to varying concentrations of FA and other B vitamins may transform benign cells to malignant and/or accelerate tumor growth. This review explores clinical observations and biochemistry of B vitamin mediated one-carbon metabolism with general emphasis on neoplasia and particular focus on prostate cancer.

2. Folic acid found to enhance tumor growth: Historical note

Sidney Farber, a pediatric pathologist practicing in Boston's Children Hospital in the mid 1940s, is generally given credit for establishing that FA stimulates human leukemia cell proliferation; however, a careful review of the literature does not confirm that assessment. In fact, Sidney Farber's clinical research group's seminal paper did not include treatment with FA (Farber et al, 1947). This manuscript, published in 1947 in the popular journal Science, reported on Farber's experience with the di- and triglutamate derivatives of oxidized folic acid (diopterin and teropterin, both supplied by Lederle Laboratories of Pearl River, NY under the direction of Yellapragada Subba Row, PhD). The fact is that Farber's landmark paper, routinely referenced in the literature, never stated FA could accelerate leukemia or any other malignancy.

Dr. Richard Lewisohn, famous for his excellence in surgery and his 1917 research on citrated blood as the preferred anticoagulant for transfusion was semiretired in the early 1940's and working in a trivial basement laboratory at Mount Sinai Hospital in NYC. At that time, he was given two folate compounds that were isolated at the Lederle Laboratories: liver L. casei factor (folic acid) and fermentation L. casei factor. The mislabeling of these compounds was the source of confusion for his initial paper mistakenly stating that folic acid was an inhibitor of spontaneous mouse mammary cancer (Leuchtenberger et al, 1945). The compound he thought was FA was in fact pteroyltriglutamate or teropterin.

Later, Hutchings and Stokstad of Lederle informed Lewisohn's group that the correct tentative designation of liver L. casei factor as used in Lewisohn's first report was actually fermentation L. casei factor (the triglutamate of pteroylglutamic acid or teropterin (Angier et al, 1946).

In Lewisohn's subsequent paper (Lewisohn et al, 1946) he confirmed that the mislabeled folic acid (liver L. casei factor) was actually fermentation L. casei factor or teropterin. Clarifying this confusing issue, the initial study revealed that teropterin injection inhibited spontaneous mouse mammary cancer while folic acid (liver L. casei factor), as confirmed in subsequent experiments, stimulated mouse mammary primary tumor growth and its pulmonary metastases while shortening overall survival (Lewisohn et al, 1946). To our knowledge, this is the first literature-documented study revealing FA stimulation of cancer cells (mouse mammary cancer) and their metastasis.

Next, in 1948, the hematologist Robert Heinle (Heinle & Welch, 1948) and later in 1950 the laboratory researcher Howard Skipper (Skipper et al, 1950) were first to document in the clinic and laboratory respectively that FA stimulated chronic myelogenous leukemia in man and acute leukemia in the rat.

The first mention that Farber was aware of what he termed an "acceleration phenomenon" appeared in his work published in 1948 in the NEJM (Farber et al, 1948). In that paper, he referred back to the Science 1947 manuscript stating that the reported children with leukemia displayed acceleration of the leukemic process within the marrow in response to diopterin or teropterin therapy. However, that finding had never been mentioned in the 1947 landmark Science report.

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The idea that B12 could accelerate chronic myelogenous leukemia in a patient with pernicious anemia and B12 deficiency was first demonstrated by Jose Corcino in 1971 while working in Dr. Victor Herbert's lab in the Bronx, NY (Corcino et al, 1971). In 1994, Dr. Ralph Green confirmed that observation in other patients with pernicious anemia (Green, 1994). Finally, in 2009, Tisman first demonstrated that B12 administration accelerated the growth of the epithelial prostate tumor in a patient with pernicious anemia and untreated prostate cancer, while at the same time correcting his anemia (Tisman et al, 2009).

3. Contemporary observations spur interest in folate, B12 and prostate neoplasia

In 1998 the US government mandated that the US diet be fortified with FA in an attempt to prevent birth defects such as spina bifida and anencephaly. Subsequent to government-mandated fortification of US, Canadian and Chilean diets with FA, numerous reports appeared documenting a higher incidence of certain cancers (colon, rectum, breast, prostate), reviewed by Young-In Kim and others (Hirsch et al, 2009; Kim, 2007; Kim, 2007; Kim, 2008; Smith et al, 2008).

The newest data relate elevated serum and prostate tissue folate to increased Gleason's grade and proliferation of prostate tumors compared to normal donor prostate tissue (Tomaszewski et al, 2011). FA supplementation was associated with a 2.6 fold increase in incidence (Figueiredo et al, 2009) and stage (Lawson et al, 2007) of prostate tumors. Collin noted serum folate-related increase in PSA velocity (Collin et al, 2010) enough to advance low-risk prostate cancer to higher risk with decreased survival (D'Amico et al, 2005) Figure 2.
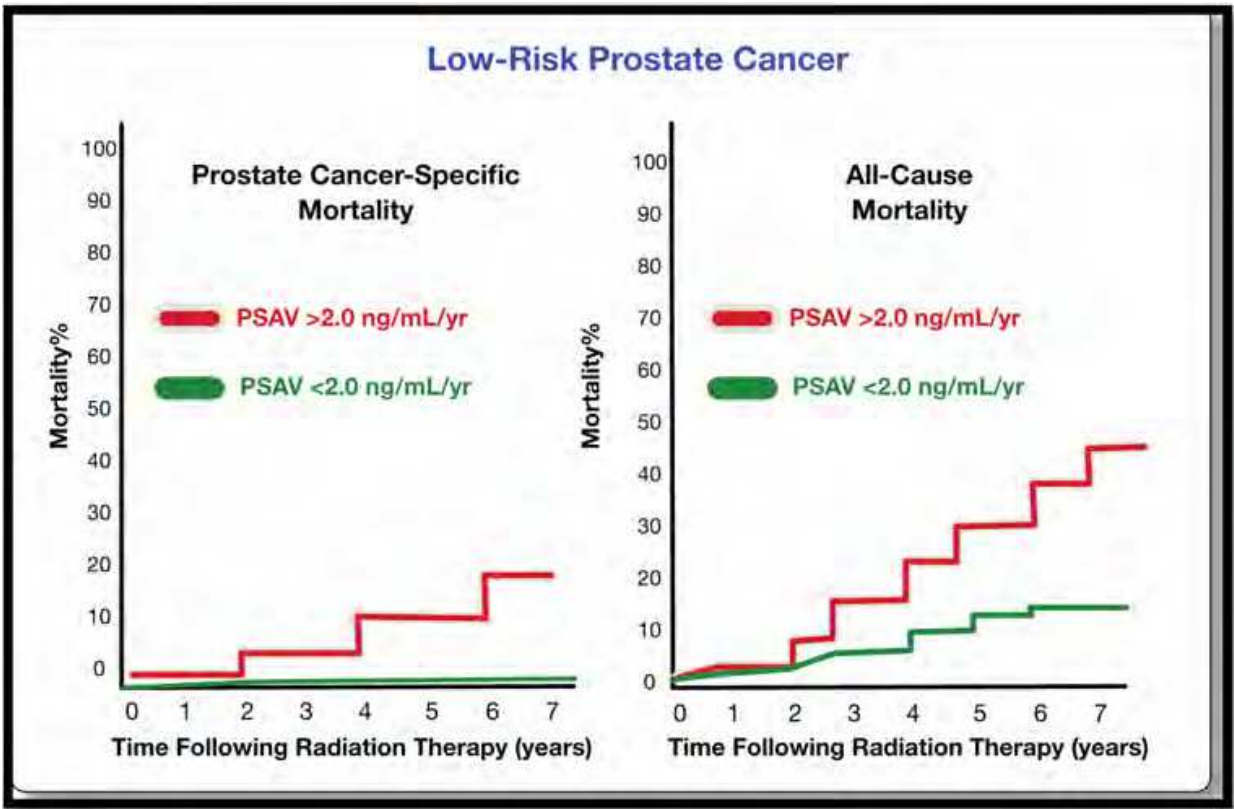

Fig. 2. Higher serum/plasma folate, associated with PSA velocity > 2.0 may increase mortality.

Others related high B12 levels to prostate cancer (Hultdin et al, 2005; Johansson et al, 2009; Weinstein et al, 2006; Vlajinac et al, 1997). We reported direct stimulation of prostate cancer by administration of B12 to a B12 deficient patient (Tisman et al, 2009) and by a supplement containing a combination of B12 and mixed folates Figures 3 and 4 (Tisman & Garcia, 2011). Patients with prostate cancer frequently ingest a variety of B vitamin-containing supplements (Velicer & Ulrich, 2008; Bailey et al, 2010) including FA and B12. We confirmed this almost universal finding in our clinic. Many are oblivious that their supplements contain larger than needed doses of vitamins. Others take comfort in supplement ingestion immediately after a cancer diagnosis while some use them in an attempt at prophylaxis (Holmes et al, 2010). Holmes' group noted folic acid supplement use before a colorectal cancer diagnosis was 35.4%. This statistic increased to 55.1% after receiving a diagnosis.

We start our review by briefly presenting two of our patients with prostate cancer whose clinical course was adversely impacted by the administration of B12 and mixed folates. This is to be followed by a rather in depth review of B vitamin metabolism as relates to biochemistry that could affect the prostate and its malignant transformation.

3.1 Patient 1

A 75 year-old man presented with prostate cancer and was later found to have pernicious anemia. After a period of 10 months of expectant surveillance it was noted that he was anemic; serum vitamin B_{12} level was 32 pg/ml (300-900 pg/ml) and holotranscobalamin 0 pg/ml (>70 pg/ml). There was an unexpected rapid progression of Gleason's score during watchful waiting. Therapeutic injection of vitamin B12 was accompanied by acceleration of PSA and prostatic acid phosphatase with shortening of prostate-specific antigen doubling time (Tisman et al, 2009) Figure 3.
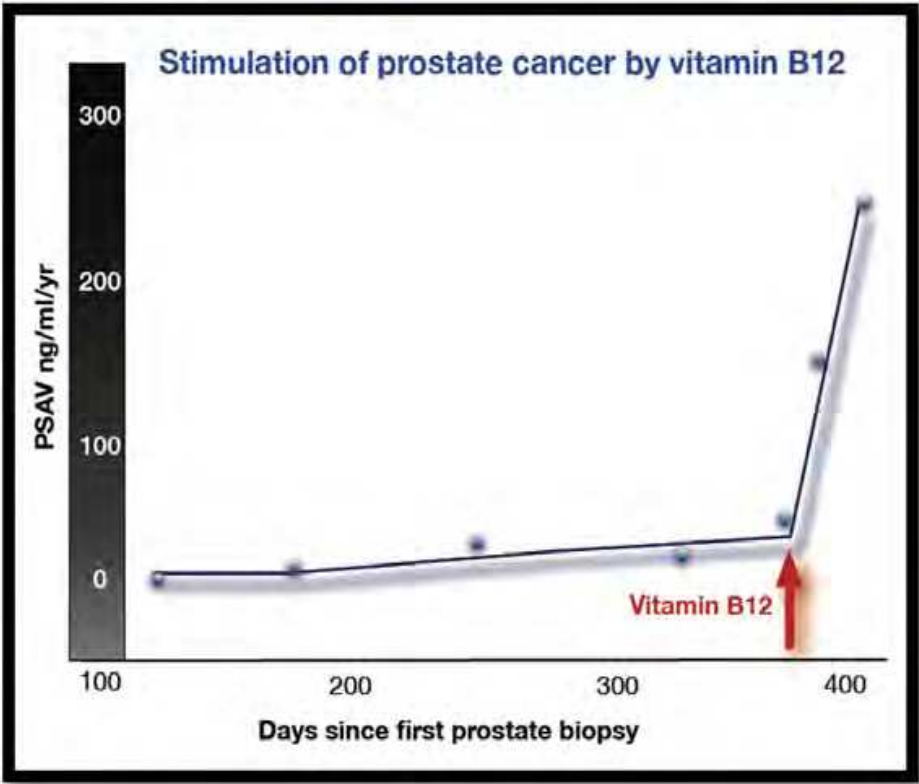

Fig. 3. Patient 1,PSA velocity in response to B12

3.2 Patient 2

A 71 year-old man was diagnosed in 1997 with Stage T1c prostate cancer, Gleason's score = 3+4 = 7. Primary therapy included intermittent androgen deprivation to resistance. While receiving docetaxel chemotherapy for 18 weeks with a continually increasing PSA, withdrawal of ingestion of 10 daily doses of a supplement composed of (500 mcg of vitamin B12 as cyanocobalamin, and 400 mcg each of folic acid as pteroylglutamic acid and 400 mcg of L-5-methyltetrahydrofolate = 800 mcg of mixed folates) was associated with a return to normal of serum prostate specific antigen Figure 4 (in press JMCR).
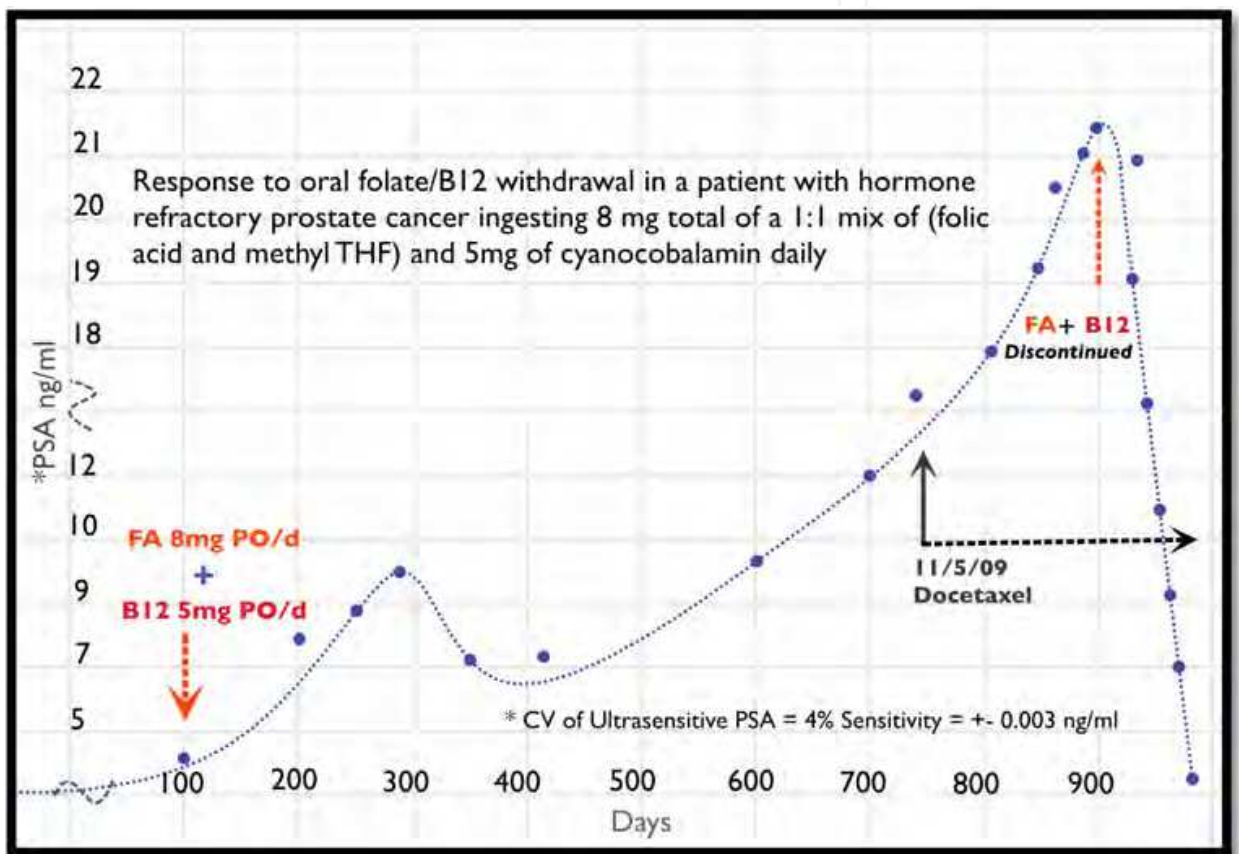

Fig. 4. Patient 2, PSA & withdrawal of B12 and folates

4. Mechanisms of modulation of one-carbon metabolism associated with B vitamin nutrition

Figures 5 and 6 help visualization of the nomenclature and structure of folate vitamers of the "one-carbon" pool. A normal diet supplies methyl groups through methionine and choline, however physiological needs exceed dietary intake. Man makes up the difference by *de novo* synthesis of methyl groups (CH3−). As illustrated in Figure 7, both dietary methionine and choline supply CH3− groups. Note the re-methylation of homocysteine generating methionine, later to be metabolized to S-adenosylmethionine (SAM).
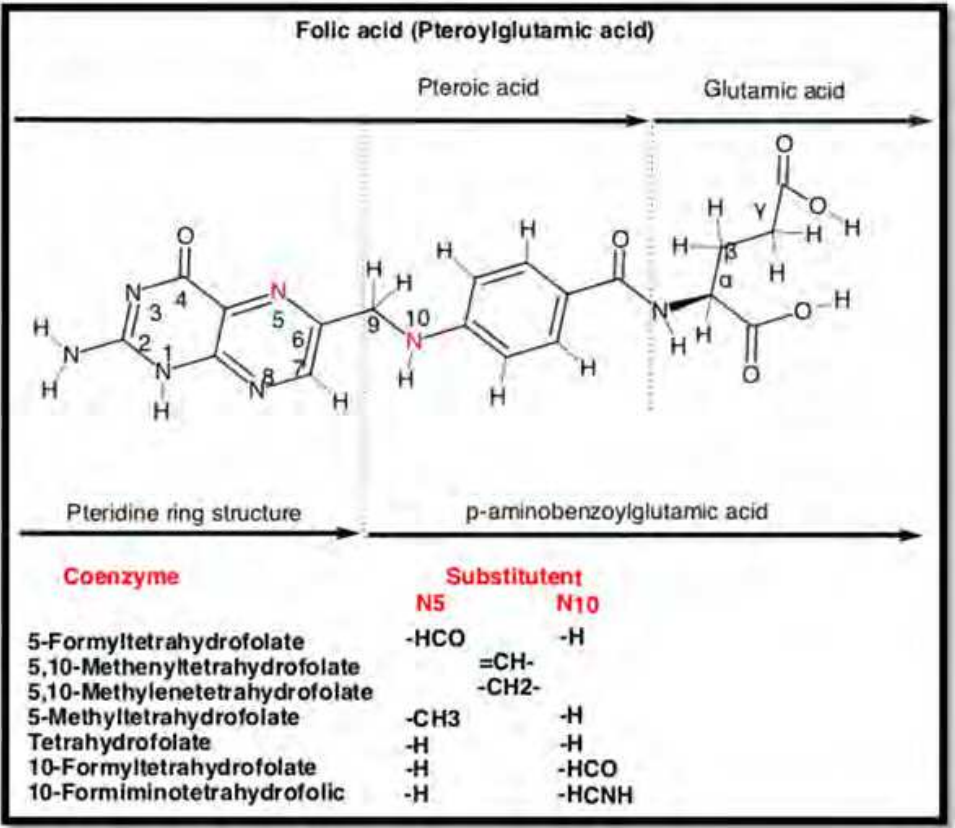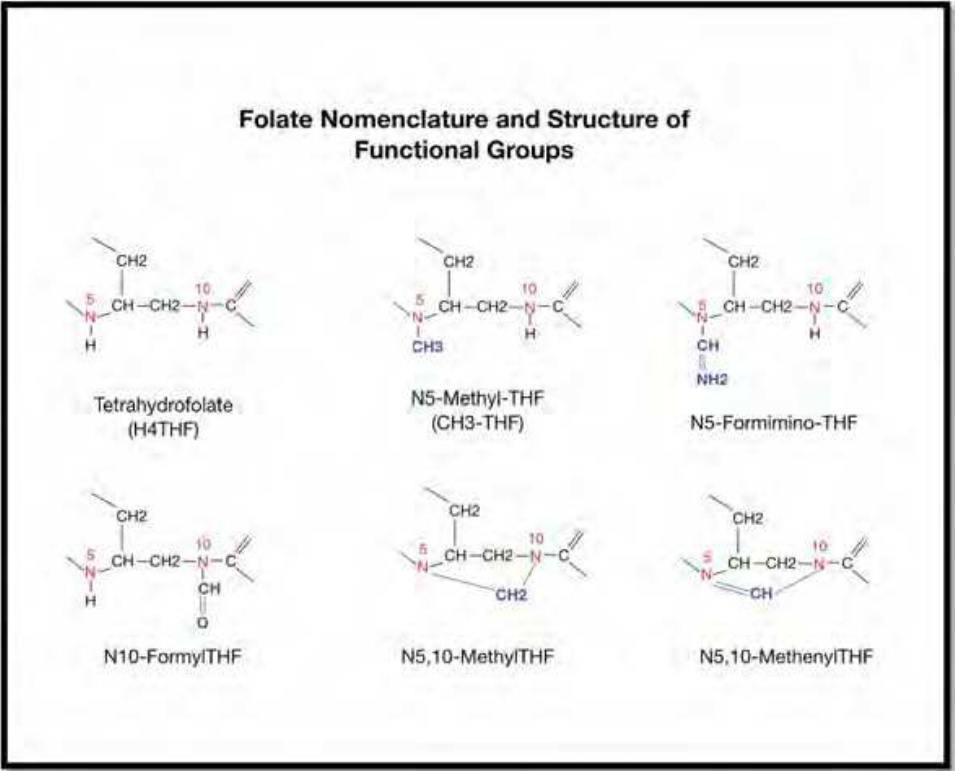
Fig. 5. Folate(s) nomenclature

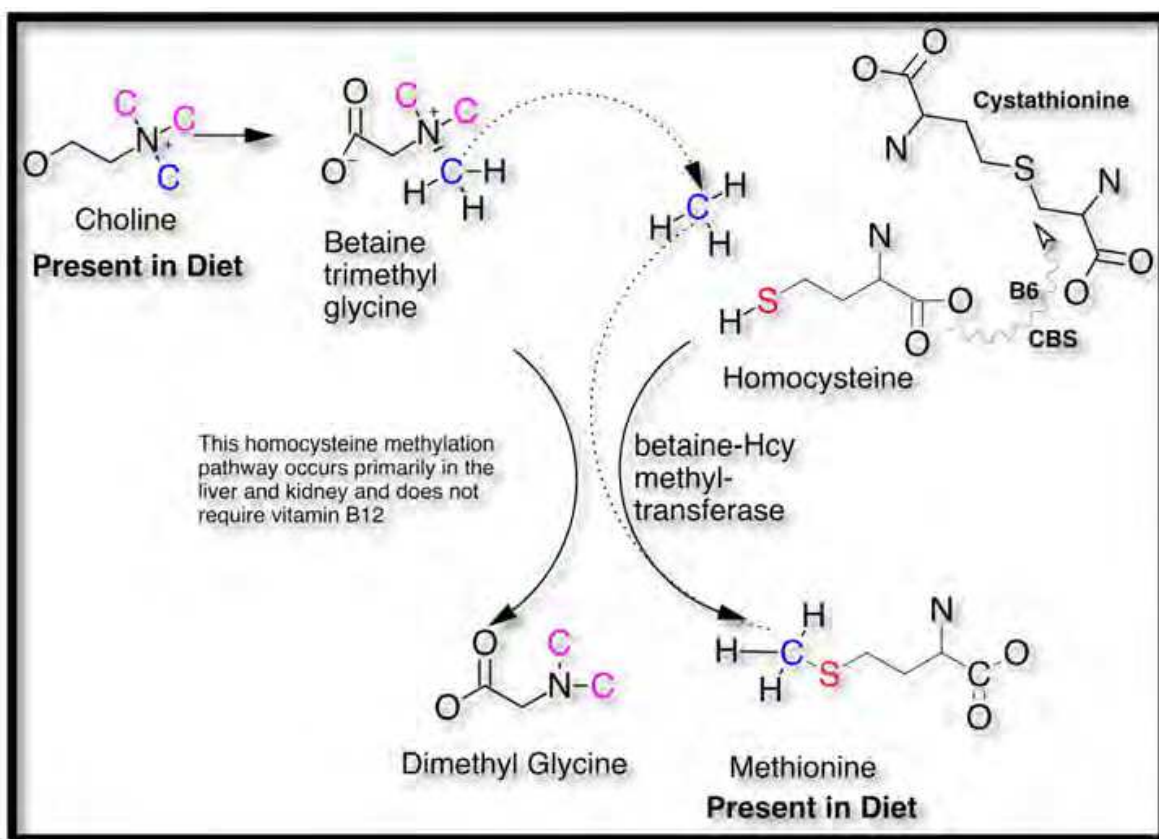Fig. 6. Folate nomenclature and structure

Fig. 7. Sources of dietary CH3— groups

Six fully reduced folate coenzymes, 5-formyl-THF, 5-formamino-THF, 10-formyl-THF, 5,10-methenyl-THF, 5,10-methylene-THF, and 5-methyl-THF (Figures) transfer methyl groups through folate-dependent enzymes. One-carbon fragments in various states of oxidation, when bound to reduced folate coenzymes, are referred to as the one-carbon pool Figures 5, 6 and 8 and are involved in multiple trans methylation reactions. These include the re-methylation of homocysteine leading to methionine and S-adenosylmethionine (SAM) (Figures 7 and 8). SAM is termed the universal methylator, and is involved in more than 100 methylation reactions either directly as in DNA epimethylation or indirectly in the synthesis of DNA-thymine from dUMP, purines (adenine and guanine) and for methylation of protein, RNA, histones etc.

5. Folate fortification, some adverse effects

For purposes of this review, B9 refers to folic acid (FA), the pharmaceutically synthesized, oxidized folic acid (FA) and folate(s) refers to the naturally occurring, usually reduced vitamers found in green leafy vegetables. Man is incapable of self-synthesis of folates and is fully dependent on folates provided by bowel flora, diet and supplements. FA is not normally found in nature, is inexpensive, water-soluble, and an artificial provitamin to reduced folates. Natural forms of the vitamin exist in nature with appended peptide tails of gamma linked glutamate amino acids (usually 5-8 in length). Polyglutamate forms are based mainly on 5-methytetrahydrofolate (CH3—THF) and account for approximately 90% of the folates in fresh foods, the majority of the remainder is based on 10-formyltetrahydrofolate (10-formylTHF) (Butterworth & Santini, 1963; Santini et al, 1964).
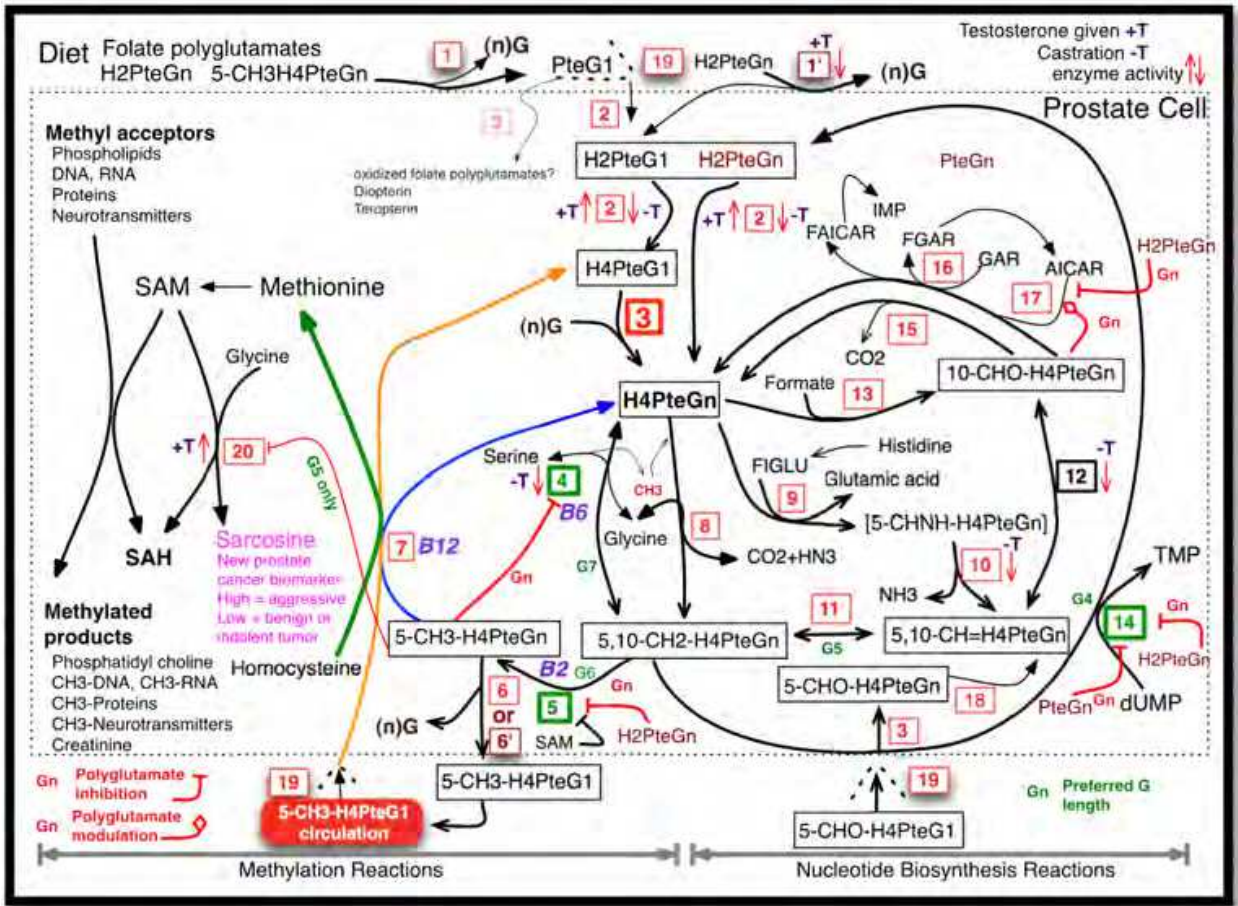
Fig. 8. Folate coenzyme forms, folate-requiring enzymes, important polyglutamates (Gn) and testosterone effects. 1) gamma-glutamyl hydrolase (enterocyte brush border) 1') PSMA prostate specific membrane antigen (2) dihydrofolate reductase 3) folylpolygamma-glutamate synthetase 4) Serinehydroxymethyl transferase 5) methylenetetrahydrofolate reductase 6) gammaglutamyl hydrolase (possibly lysosomal) 6') PSM' Prostate Membrane Antigen splice variant 7) cobalamin dependent methionine synthase 8) glycine cleavage enzyme system 9) glutamate ormimino-transferase 10) formiminotetrahydrofolate cyclodeaminase 11) methylenetetrahydrofolate dehydrogenase 12) methenyltetrahydrofolate cyclohydrolase* 13) formyl tetrahydrofolate synthetase 14) thymidylate synthase 15) formyltetrahydrofolate dehydrogenase 16) phosphoribosyl glycinamide (GAR) formyl transferase 17) phosphoribosylaminoimidazolecarboxamide (AICAR) formyl transferase 18) 5-formyl etrahydrofolate cycloligase 19) folate/methotrexate transport system 20)** glycinemethyl transferase. Dihydrofolate monoglutamate is a weak inhibitor with a Kt = 50 μM, while the pentaglutamate is a potent competitive inhibitor with a Ki of 3.8 μM (Bertrand et al, 1987). Only 5-Me-THFG5 inhibits this enzyme.

Prior to 1998, folate was primarily delivered by diet while FA was added to multivitamin products furnished by health food stores. After 1998, because neural tube defects were related to folate deficiency, US law mandated that FA be added to the food supply. Fortification has proven 19% effective (Honein et al, 2001) in rescuing approximately ~1500-2000 live births a year from neural tube anomalies (spina bifida and anencephaly). However, fortification was followed by reports of reversal of the declining incidence of colorectal cancer in the US, Canada and Chile (Mason et al, 2007; Hirsch et al, 2009). After FA

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supplementation in the US, there were approximately 15,000 additional cases of colorectal cancer per year (Mason et al, 2007). In the following years, larger amounts of FA were ingested to metabolically lower serum levels of the vasculotoxic amino acid, homocysteine. It was thought that lowering homocysteine would decrease the incidence of arteriosclerotic cardiovascular disease. However, studies thus far have been inconsistent and not confirmed this assumption save for a possible benefit in stroke prevention (Chen, J (J), Xu, X (X), et al., 2010; Wang et al, 2007; Robinson et al, 1998) and thrombophilia.

Alarmingly, there are now laboratory animal (Lindzon et al, 2009; Ly et al, 2011) and epidemiological reports pointing to excessive malignancy, including prostate (Figueiredo et al, 2009), breast ({Campbell 2002); Chen et al, 2005; Ericson et al, 2009; Stevens et al, 2010) colorectal and other cancers (Kim, 2005) associated with both hyper and hypo-sufficiency of folate and folate enzyme polymorphisms, reviewed by Young-In Kim and others (Kim, 2007; Kim, 2008; Kim, 2005; Smith et al, 2008) see Table 1.

Study	B2	B6	B12	Folate
Kasperzyk et al, 2009		Hi B6 less risk of death from CaP		
Key et al, 1997		Hi B6 Less risk of CaP		
Collin et al, 2010			Hi B12 Hi CaP	
Hultdin et al, 2005			Hi B12 Hi risk for CaP	Hi Fol Hi risk for CaP
Johansson et al, 2008			Hi B12 Hi risk for CaP	
Vlajinac et al, 1997			Hi B12 Hi risk for CaP	
Collin et al, 2010			Hi B12 Hi risk for CaP	Hi Fol Hi risk for CaP
Pelucchi et al, 2005				Hi Fol protects against CaP
Collin et al, 2010				Hi Fol Hi risk PSAV > 2
Figueiredo et al, 2009				Hi Fol Hi risk for CaP 2.7-fold higher
Johansson et al, 2009	Hi B2 Hiher risk for CaP			

Table 1. Significant epidemiological studies relating B vitamins with prostate cancer (CaP)

5.1 Folate fortification: Effects on chemotherapy

5-fluorouracil and capecitabine (a prodrug metabolized to fluoropyrimidines) are active in breast and colon cancer. In the presence of high serum levels of folate and or in the presence of certain folate-metabolizing enzyme polymorphisms (Kim, 2009; Maring et al, 2005; Sharma et al, 2008), their toxicity is greatly magnified (Midgley & Kerr, 2009). This requires lowering of the recommended dose of folate in the US (Hennessy et al, 2005). This appears to be due to strengthening of the ternary complex between 5,10 methyleneTHF, F-dUMP and thymidylate synthase with enhanced inhibition of DNA-thymine synthesis.

5.2 B vitamin metabolism, malignant transformation and effects of enzyme polymorphisms on neoplasia

Impaired *de novo* DNA-thymine synthesis associated with absolute, relative or functional deficiency of folate coenzymes or folate-metabolizing enzyme concentration shifts may result in uracil misincorporation into DNA, defective DNA base excision repair, double strand chromosome breaks, and DNA point mutations resulting in malignant transformation (Blount et al, 1997; Milic et al, 2010) Figure 9. The resulting chromosomal breaks are identical to specific breaks found in common cancers (Yunis & Soreng, 1984).
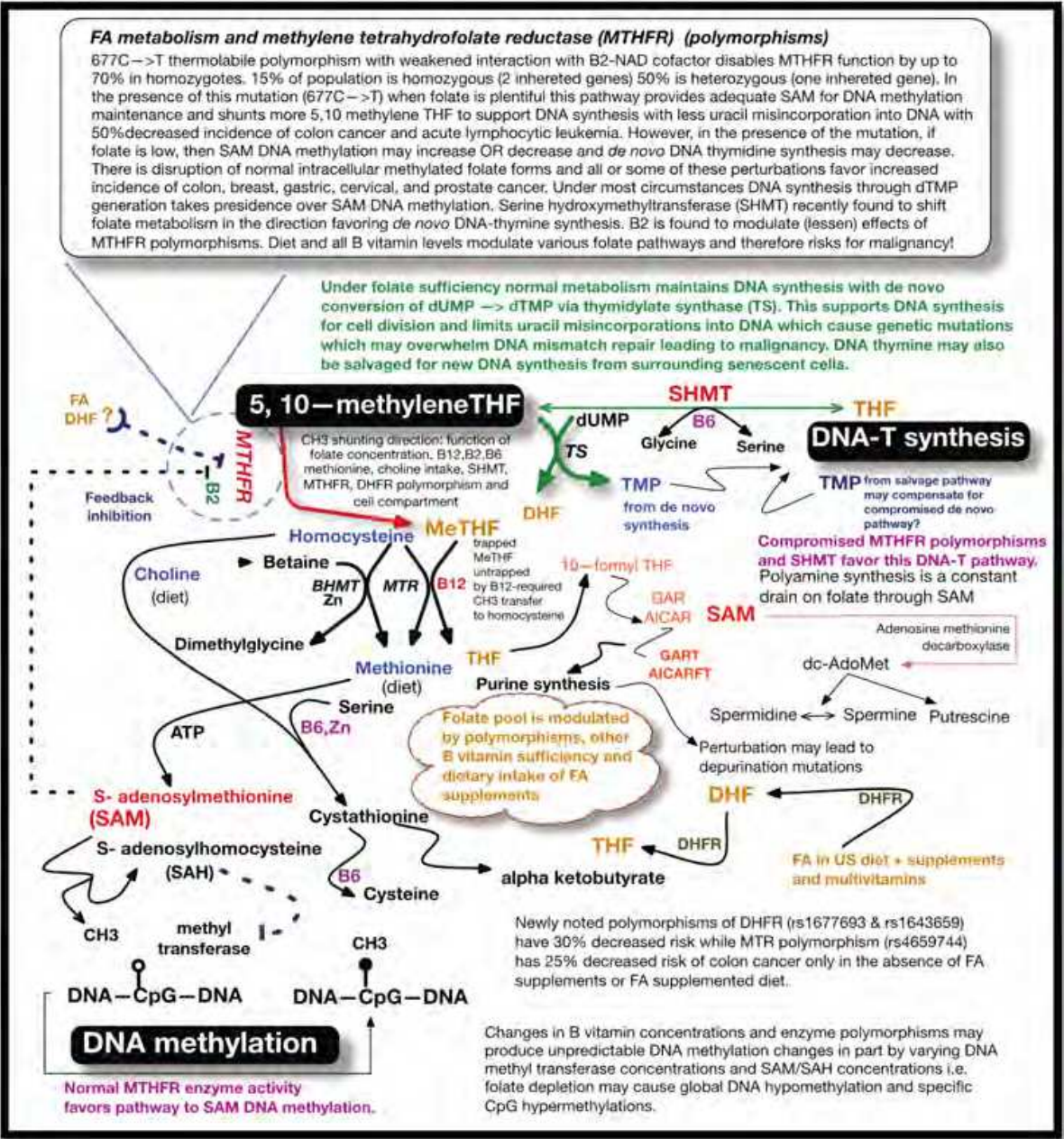

Fig. 9. *De novo* and salvage pathways to DNA-thymine synthesis, SAM methylation of DNA, polyamine synthesis and MTHFR polymorphisms

S-adenosylmethionine (SAM) is regulated by multiple factors including the enzyme glycine N-methyltransferase (GNMT) (Luka et al, 2009; Wang et al, 2011) and is governed in part by

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testosterone, vitamin A and specifically the potent inhibitory folatepentaglutamate, 5-CH3—THFG5 see Figure 10. The effects of altered SAM production may result in DNA hypo- and or hypermethylation thereby changing gene expression, which may result in malignant transformation (Davis & Uthus, 2004; De Cabo et al, 1995; Duthie, 1999; Duthie et al, 2002; Duthie, 2010; Esteller et al, 2002; Esteller, 2003; Esteller, 2007; Jones & Baylin, 2002; Ulrey et al, 2005; Wainfan et al, 1989) Compromised DNA-thymine synthesis (dUMP → TMP) results in uracil misincorporation into DNA and hastens malignant transformation, tumor proliferation and aggressiveness (Chango et al, 2009; Choi et al, 2004; James et al, 2003; Jang et al, 2005; Liu et al, 2006) Figures 9 and 10.

Additional studies show examples of change due to large doses of dietary FA. Rats fed 40 mg/kg compared to 2 mg/kg FA induced more hepatic SAM in Dams phenotypically resulting in fetuses of less weight and shortened vertex-coccyx length (Achón et al, 2000). High FA given to rats worsened experimentally induced liver fibrosis (Marsillach et al, 2008). Alternatively, folate insufficiency and/or B12 insufficiency slows cell metabolism and division. This is manifest in the clinic as macroovalocytic anemia with bone marrow megaloblastic change, and if severe, pancytopenia, as reviewed by Tisman (Tisman, 2005). Abnormal histones, targets for SAM-mediated epimethylation, are thought responsible for the clock-face
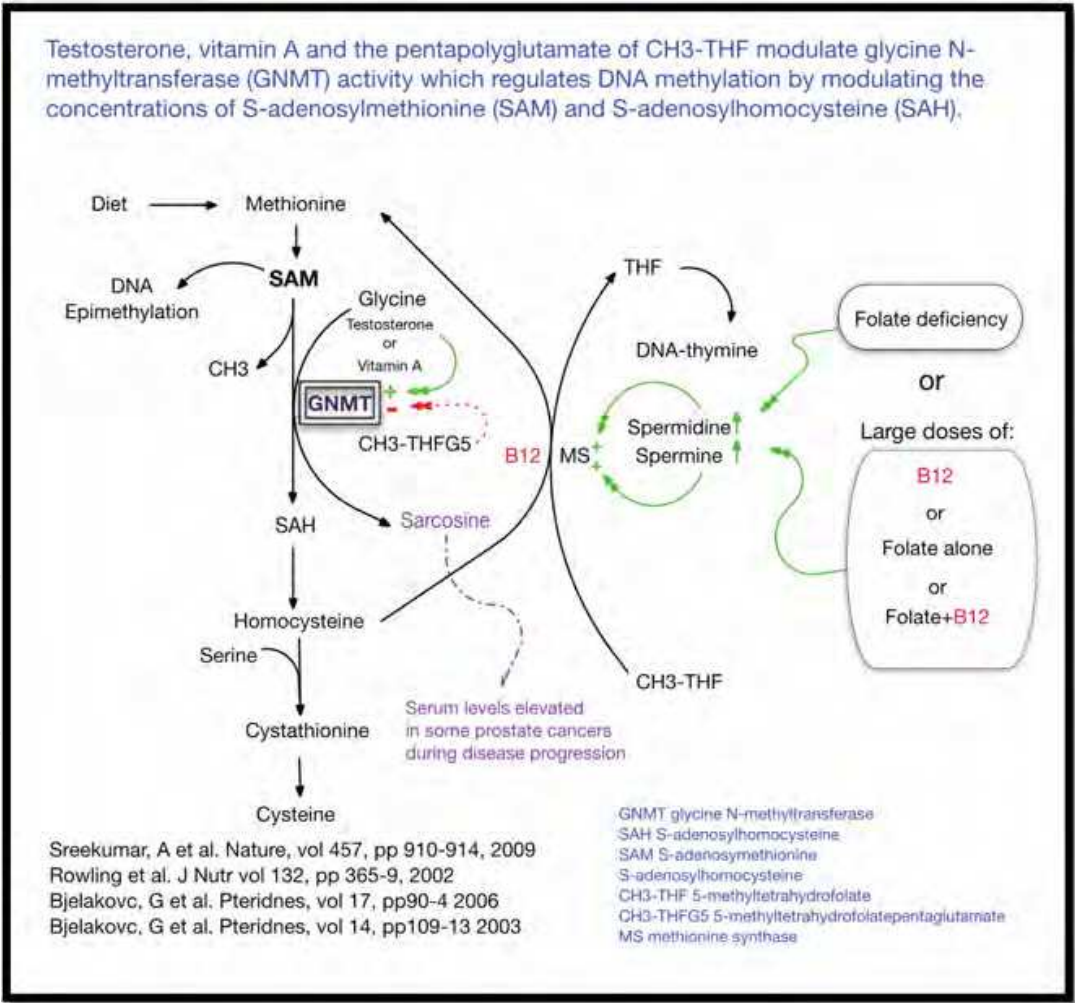

Fig. 10. GNMT modulation by Testosterone, CH3—THF**G5 polyglutamate** and vitamin A, effects of folates and B12 on polyamines and spermidine and spermine on methionine synthetase.

appearance of chromatin within basophilic megaloblasts (Das et al, 2005). Though megaloblastic change is characteristic of blood cells, similar morphology is noted in other cells undergoing cell division during B12 and or folate insufficiency. These include enterocytes, oral and glossal mucosa cells, bronchial mucosa and the uterine cervix (Herbert, 1959).

Exciting work in Young-In Kim's lab disclosed that high intrauterine and post-weaning dietary exposure to FA increased the risk of mammary tumors of offspring. They conjecture the tumor-promoting effect could have been mediated by altered DNA methylation and DNA methyl transferase activity during pregnancy (Ly et al, 2011). However, other experiments with rats by this group disclosed that maternal, but not post-weaning, FA supplementation reduced the odds of colorectal adenocarcinoma by 64% in carcinogen challenged offspring. It was proposed that the protective effect may have been due to increased global DNA methylation and decreased epithelial proliferation (Sie et al, 2011). They hint that there may be tissue-specific and divergent responses to both high and low FA exposure.

Historically, B12 and folate deficiency is associated with malignancy. B12 deficiency of pernicious anemia is accompanied by a 3-18 fold increase in gastric cancer (Kuster et al, 1972). Approximately 10% of those suffering from celiac disease, which is almost always associated with folate deficiency, were noted to develop malignancy (Chanarin, 1969; Dormandy et al, 1963;). Though B12 and folate may lead to pancytopenia in the short term, chronic insufficiency or gestational exposure to folate lack or hypersufficiency may lead to latent neoplastic change.

6. Newer concepts of nutrition: Compartmentalization, organ-specific nutrient deficiency, nutrient-nutrient interaction and cell kinetic modulation of nutrient adequacy

Epidemiological analyses measure nutrient consumption through questionnaires, serum levels of vitamins and metabolites and simultaneous analysis of clinical correlations with hypothesized relationships. Unfortunately, these studies frequently ignore micronutrient variation over long periods, presence of one or many interacting enzyme polymorphisms which vary with different populations, simultaneous variations of multiple interacting vitamers i.e. B12, B9, B2, B6, Figures 9 and 11 and the kinetic state of cellular metabolism at the time of the study.

As an example of simultaneous B vitamin variation, we present our data from our untreated cancer patient population as we studied serum levels of B vitamins, Figure 11. Forty-four percent of patients were found to have at least one of six measured parameters of B vitamin sufficiency in the abnormal range. Many were simultaneously deficient and or hypersufficient of several metabolically interacting B vitamins.

To understand how a specific organ such as the prostate may manifest vitamin deficiency while simultaneously others do not, we review relevant principles of nutrient distribution and utilization:

1. Clement Finch (Finch et al, 1950) taught that total body cellular iron deficiency appears gradually. There occurs an orderly decline in body iron starting with loss of plasma iron, followed by depletion of hepatic and marrow stores, decrease in red cell size finally culminating in anemia.

2. Victor Herbert expanded Finch's work demonstrating micronutrient distribution varied simultaneously from cell to cell and organ-to-organ. Nutrient sufficiency for folate and B12, he claimed, was compartmentalized and progressed in an orderly fashion (Herbert, 1987) (see Figures 12 and 13). One cell type could be nutritionally satisfied and in apparent equilibrium with hepatic, red cell and serum folate. Simultaneously, other more kinetically active cells could struggle with deficiency. Work in Herbert's lab by Das showed that the

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adequacy of a cell's folate and or B12 stores reflected the folate/B12 status at the time the cell was metabolically active (Das et al, 1980), which is the time that intracellular vitamin transport is active. Susan Duthie confirmed simultaneous discordance of folate stores between red blood and buccal mucosal cells (Basten et al, 2004).

3. Bruce Ames' theory of "micronutrient triage" (Ames, 2006) dovetails with that of Herbert. He teaches that through allocation and distribution, scarce micronutrients are triaged to the most metabolically acute process required for cell survival.

4. Finally, Heaney (Heaney, 2003) presented his view of "long-latency disease" whereby a minimal level of chronic nutrient deficiency would take years before tissue damage became apparent. As examples, he points to stroke due to folate-related hyperhomocysteinemia (Wang et al, 2007) and thrombophilia from folate and B6 lack (Remacha et al, 2002) resulting in thrombosis (Hron et al, 2007).
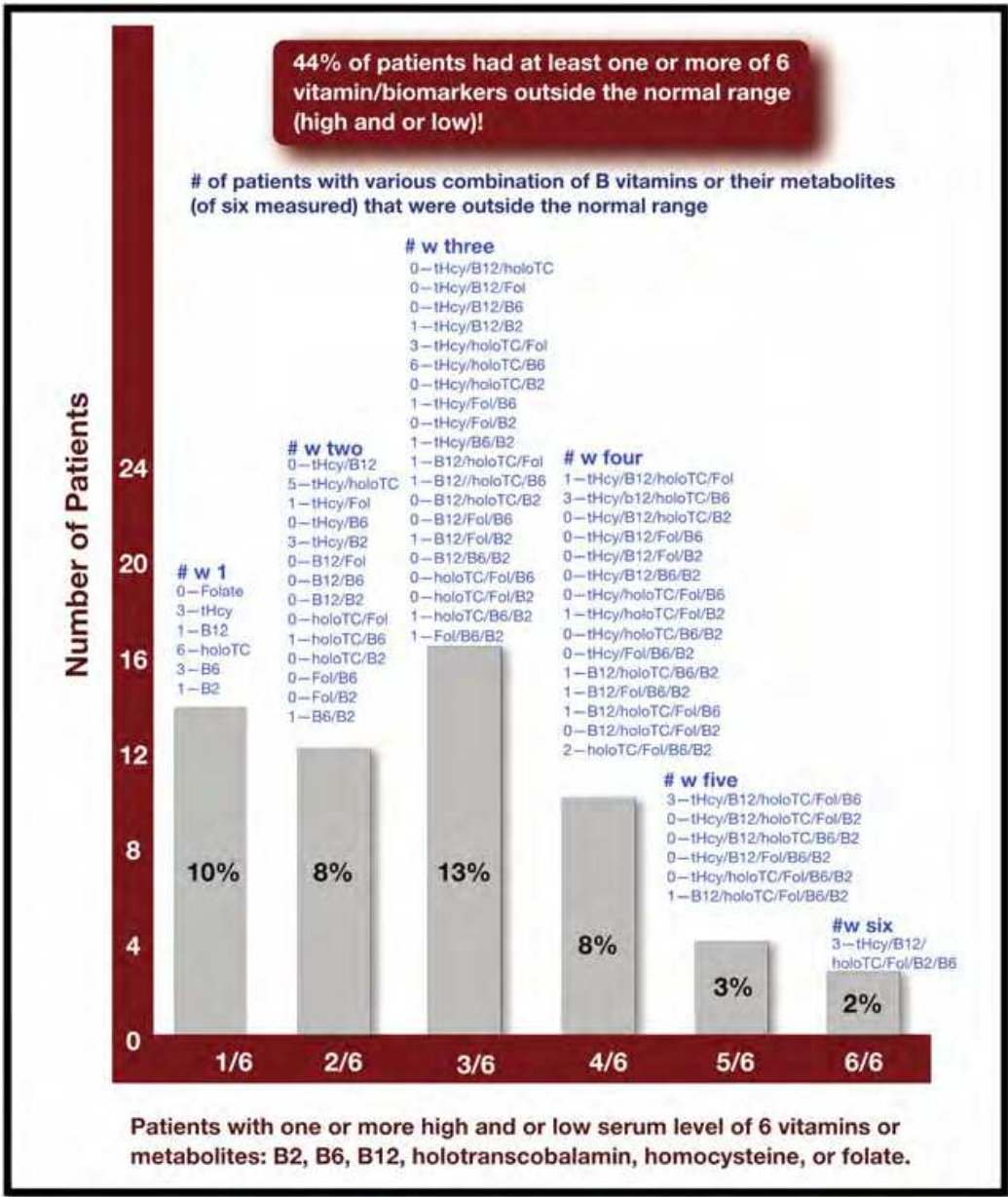

Fig. 11. Frequency of multiple simultaneous vitamin abnormalities among untreated cancer patients.

Awareness of these concepts helps in understanding how seemingly unrelated vitamin metabolism, cellular kinetics and nutrition might simultaneously starve select organs while others thrive.

7. Cell-specific metabolic preferences in one-carbon metabolism

Herbert demonstrated that patients' tumors and their own normal bone marrow cells more often than not differed in degree and preference for *de novo* and salvage pathways for DNA-thymine synthesis (Tisman et al, 1973) Figure 9. These inherent metabolic differences dictated sensitivity to vitamin deficiency and drugs such as fluorinated pyrimidines, methotrexate and other anti-fols.
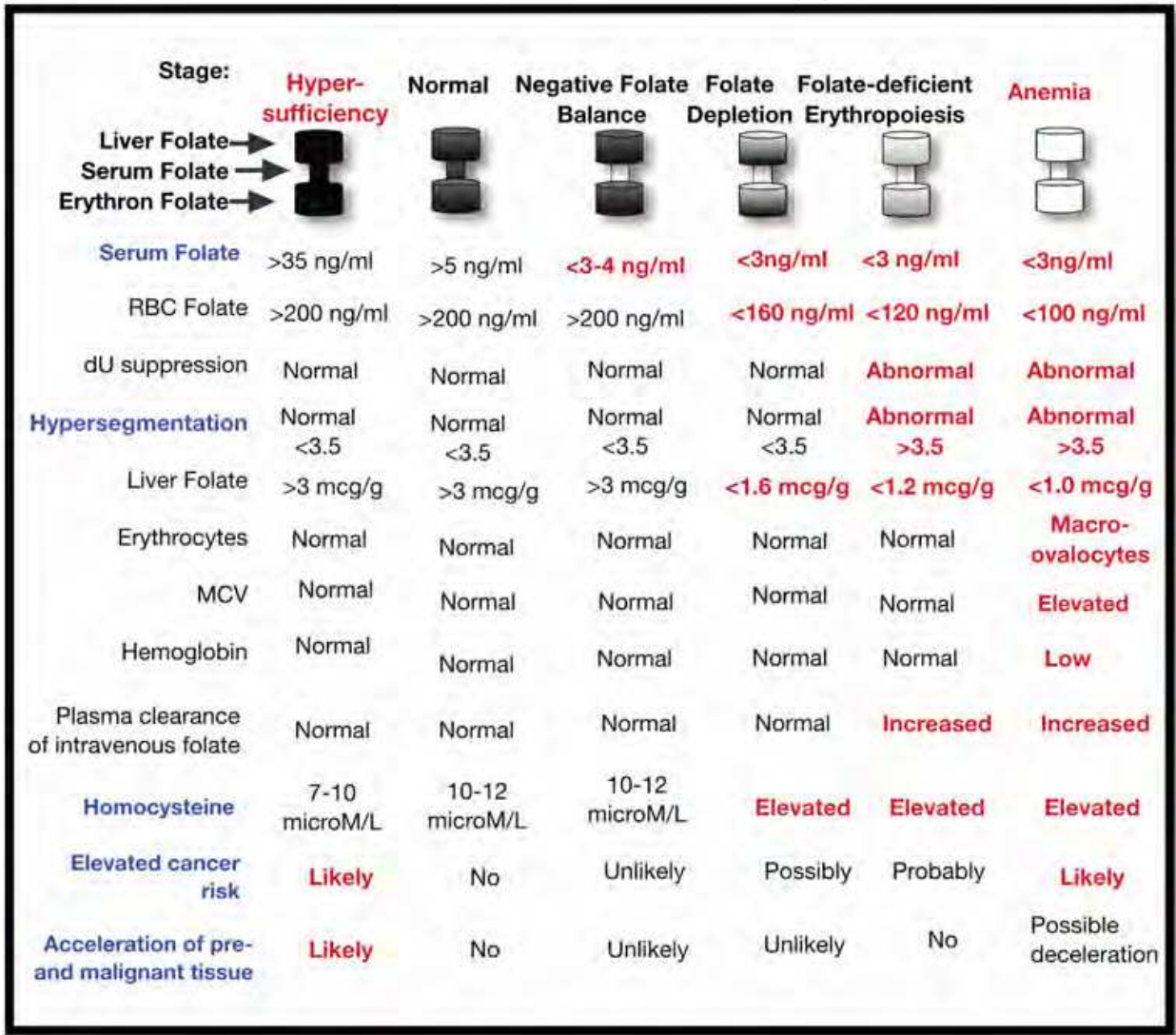
Stage:	Hyper-sufficiency	Normal	Negative Folate Balance	Folate Depletion	Folate-deficient Erythropoiesis	Anemia
Liver Folate → Serum Folate → Erythron Folate →						
Serum Folate	>35 ng/ml	>5 ng/ml	<3-4 ng/ml	<3ng/ml	<3 ng/ml	<3ng/ml
RBC Folate	>200 ng/ml	>200 ng/ml	>200 ng/ml	<160 ng/ml	<120 ng/ml	<100 ng/ml
dU suppression	Normal	Normal	Normal	Normal	Abnormal	Abnormal
Hypersegmentation	Normal <3.5	Normal <3.5	Normal <3.5	Normal <3.5	Abnormal >3.5	Abnormal >3.5
Liver Folate	>3 mcg/g	>3 mcg/g	>3 mcg/g	<1.6 mcg/g	<1.2 mcg/g	<1.0 mcg/g
Erythrocytes	Normal	Normal	Normal	Normal	Normal	Macro-ovalocytes
MCV	Normal	Normal	Normal	Normal	Normal	Elevated
Hemoglobin	Normal	Normal	Normal	Normal	Normal	Low
Plasma clearance of intravenous folate	Normal	Normal	Normal	Normal	Increased	Increased
Homocysteine	7-10 microM/L	10-12 microM/L	10-12 microM/L	Elevated	Elevated	Elevated
Elevated cancer risk	Likely	No	Unlikely	Possibly	Probably	Likely
Acceleration of pre- and malignant tissue	Likely	No	Unlikely	Unlikely	No	Possible deceleration

Fig. 12. Sequential stages of folate deficiency

Young-In Kim's group studied two colon cancer cell lines, HCT 116 and Caco2 (Hayashi et al, 2007), both grown in folate-limiting medium. They reported that the Caco2 line preserved *de novo* DNA-thymine synthesis while HCT 116 cells preserved SAM and DNA methylation pathways.

Bistulfi's lab (Bistulfi et al, 2009) studied an example of cell-specific nutrition in prostate cells. They presented evidence of high folate requirements by prostate cells to maintain SAM to support extraordinarily large amounts of polyamine synthesis (Figure 14). Folate insufficiency resulted in increased gene promoter CpG island and DNA-histone methylation Figure 14. The cell DNA methylome changed to produce the more aggressive phenotype *in vitro*; cells became anchorage-independent and showed reduced sensitivity to folate depletion.

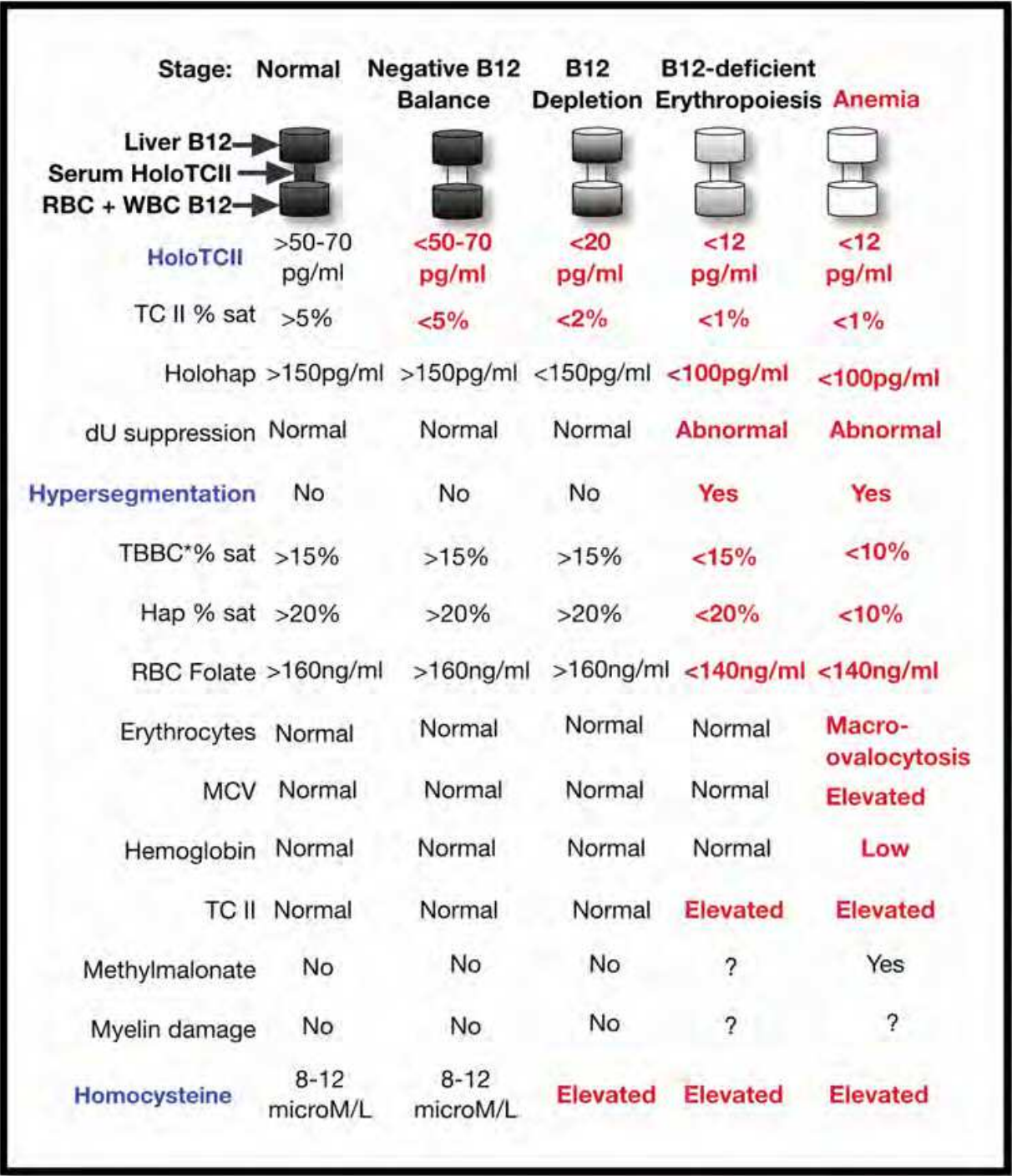| Stage:	Normal	Negative B12 Balance	B12 Depletion	B12-deficient Erythropoiesis	Anemia
HoloTCII	>50-70 pg/ml	<50-70 pg/ml	<20 pg/ml	<12 pg/ml	<12 pg/ml
TC II % sat	>5%	<5%	<2%	<1%	<1%
Holohap	>150pg/ml	>150pg/ml	<150pg/ml	<100pg/ml	<100pg/ml
dU suppression	Normal	Normal	Normal	Abnormal	Abnormal
Hypersegmentation	No	No	No	Yes	Yes
TBBC*% sat	>15%	>15%	>15%	<15%	<10%
Hap % sat	>20%	>20%	>20%	<20%	<10%
RBC Folate	>160ng/ml	>160ng/ml	>160ng/ml	<140ng/ml	<140ng/ml
Erythrocytes	Normal	Normal	Normal	Normal	Macro-ovalocytosis
MCV	Normal	Normal	Normal	Normal	Elevated
Hemoglobin	Normal	Normal	Normal	Normal	Low
TC II	Normal	Normal	Normal	Elevated	Elevated
Methylmalonate	No	No	No	?	Yes
Myelin damage	No	No	No	?	?
Homocysteine	8-12 microM/L	8-12 microM/L	Elevated	Elevated	Elevated

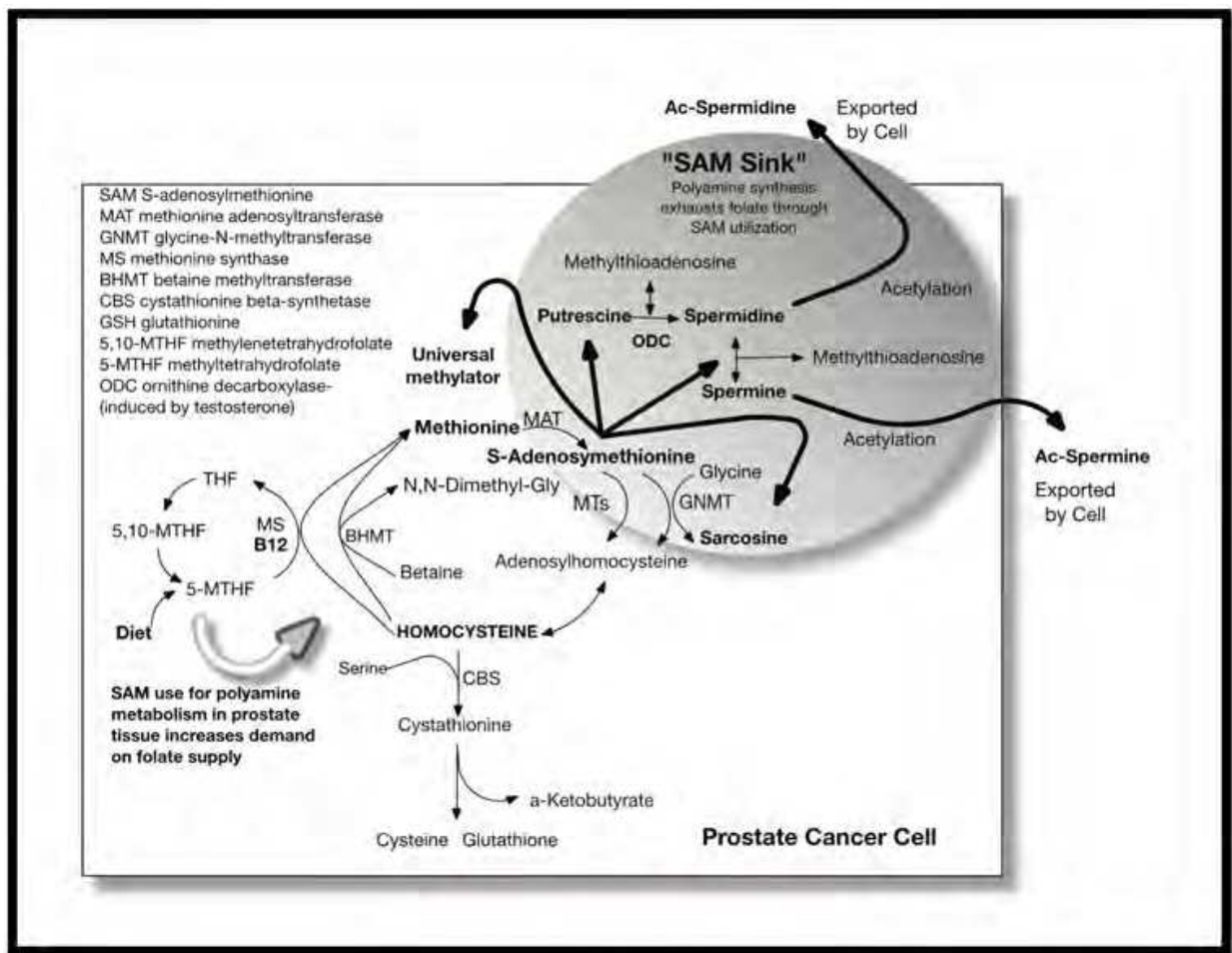Fig. 13. Sequential stages for B12 deficiency

Fig. 14. Prostate cells draw heavily of their SAM supply to maintain disproportionate polyamine synthesis

There is mounting biochemical and epidemiological support that cancers of the prostate may result in part from micronutrient imbalance leading to mutations and the aberrant epimethylome triggering abnormal gene expression (van Engeland et al, 2003; Cooper & Foster, 2009; Diaw et al, 2007; Ernst et al, 2002; Esteller, 2007; Esteller, 2008; Li et al, 2005; Li & Dahiya, 2007; Maruyama et al, 2002; Perry et al, 2006; Sasaki et al, 2002; Shukeir et al, 2006; Yegnasubramanian et al, 2008). Folate metabolizing enzyme polymorphisms complicate the overall picture (Cai et al, 2010) (Figure 9) through interaction with B vitamin metabolism causing results of epidemiological studies to be inconsistent; this has been extensively reviewed (Kim, 2007; Kim, 2004; Kim, 2005; Kim, 2009; Sanderson et al, 2007; Smith et al, 2008; Sohn et al, 2009).

8. Mechanisms for B vitamin control of one-carbon metabolism

8.1 Folate polyglutamate length modulation of one-carbon metabolism
Folylpolygammaglutamates are substrates for folate-dependent enzymes and generally have lower Km values (stronger enzyme binding) than corresponding monoglutamates. They result from reacting with folylpolygammaglutamyl synthetase (FPGS) (Cook et al,

1987; Lowe et al, 1993; Moran & Colman, 1984; Shane, 2010; Sun et al, 2001; Ward & Nixon, 1990). Polyglutamate tails increase folate cellular retention and coenzyme activity. Only folylmonoglutamates traverse cell membranes (Antony, 1992; Alemdaroglu, 2007; Perry & Chanarin, 1972; Shane, 2010). FPGS is up-regulated in cycling, dividing cells and in folate deficiency (Freemantle & Moran, 1997) Figure 15.
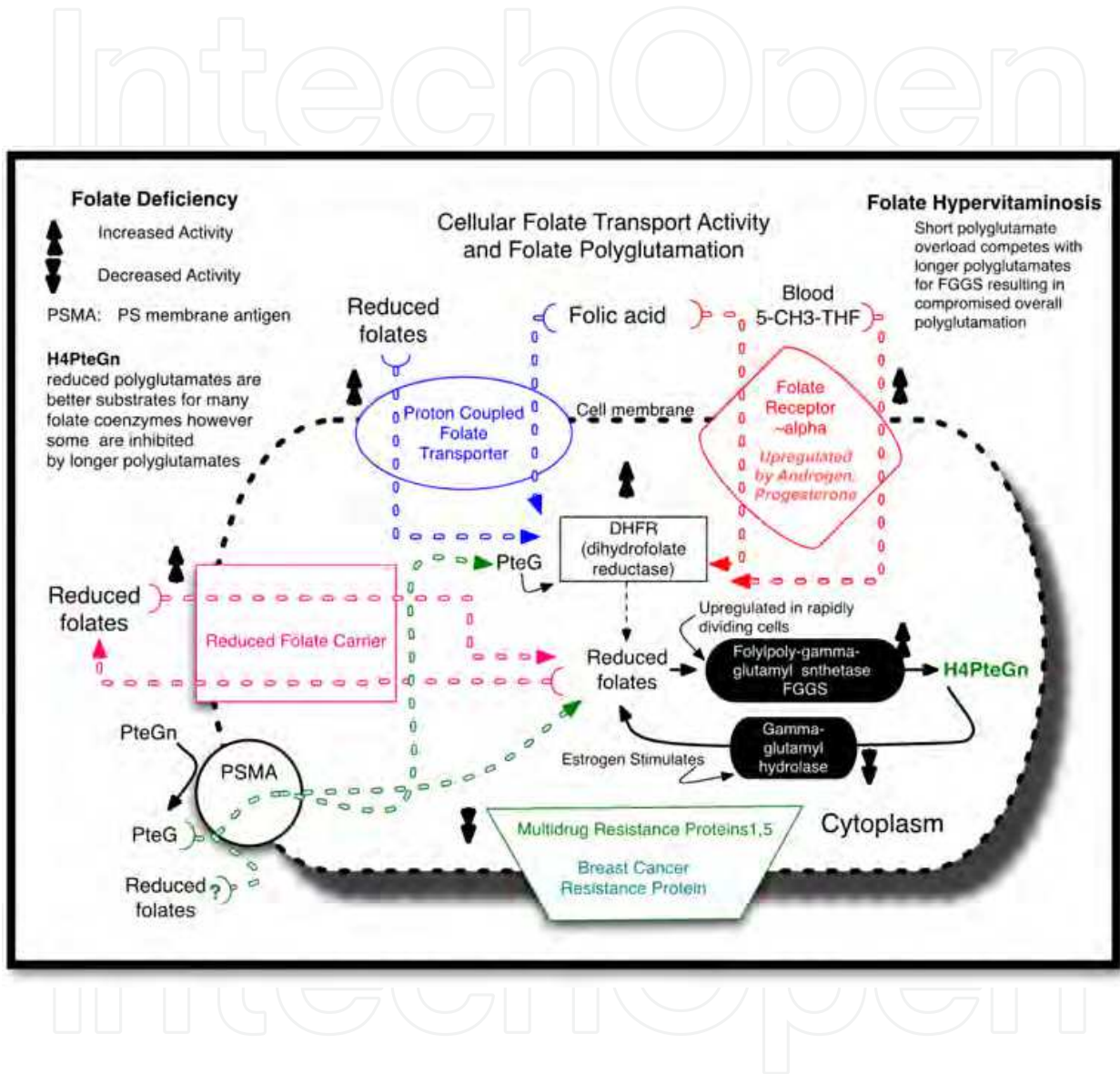

Fig. 15. Changes in folate metabolism in deficiency and sufficiency

Polyglutamation increases the affinity of folate forms (Krumdieck et al, 1992; Matthews et al, 1987) for most folate-dependent enzymes except for dihydrofolatereductase (DHFR) (Bailey & Ayling, 2009). DHFR is a crucial rate-limiting enzyme responsible for regenerating reduced tetrahydrofolates from spent dihydrofolates generated after reduced folates transfer their one-carbon moiety Figure 8. Polyglutamation of 5,10 methyleneTHF results in the potentiation of thymidylate synthase (TS) and thus the conversion of dUMP to thymidine monophosphate (TMP) by a factor 100-fold greater

than for the monoglutamate (Galivan et al, 1977; Kisliuk et al, 1974; Matthews et al, 1987; Moran, 1989).

The competing enzyme within intracellular lysosomes, folylpolygammaglutamalhydrolase (FPH) shortens polyglutamate length by splitting terminal glutamate residues (Krumdieck et al, 1976; Shane, 2010). FPGS and FPH are competing enzymes. FPH activity is duplicated in the prostate specific membrane antigen (PSMA) and its splice variants (PSM') implicated in prostate cancer carcinogenesis (Yao et al, 2010) Figure 19.

Methyltetrahydrofolate (CH3−THF) is a poor substrate for FPGS (Perry et al, 1983) and irreversible trapping of CH3−THF in B12 deficiency imposes both intracellular CH3−THF polyglutamate deficiency and loss of intracellular CH3−THF (Lowe et al, 1993). B12 deficiency was found to limit intracellular transport of CH3−THF (Tisman & Herbert, 1973), an example of B vitamin interaction compounding effects of B12 deficiency. Irreversible trapping of reduced folate as CH3−THF in B12 deficiency exhausts regeneration of reduced THF (Figure 9).

9. Sex steroid hormones and modulation of folate metabolizing enzymes and polyglutamate length

Relevant to the prostate, in rodents, estrogen and progesterone modulation of FPH affects intracellular polyglutamate length (Krumdieck et al, 1975) and thus folate polyglutamate concentrations and activity while testosterone impacts folate-metabolizing enzyme activity of the prostate and seminal vesicles (Bovina et al, 1972; Rovinetti et al, 1972). In women, estrogen administration to women lowers homocysteine and induces measurable changes of the epimethylome suggesting an effect on homocysteine remethylation and SAM methylation of DNA (Friso et al, 2007).

By shortening the folylpolyglutamate tails, increased activity of FPH can induce folate insufficiency. FPH levels of uteri of castrated rats increase in response to estrogen replacement (Krumdieck et al, 1975). FPH modulation by estrogen, a hormone active in prostate cancer, could involve regulation of folate metabolism. Estramustine (Emcyt™) and diethylstilbestrol might share similar effects on FPH. Orchiectomy and testosterone replacement induce intracellular folate-dependent enzyme concentration variations (Bovina et al, 1972; Rovinetti et al, 1972) Figures 8 and 16.

The effects of castration on folate metabolizing enzymes in rat prostate, seminal vesicle and liver tissue were reported by Rovinetti and Bovina (Bovina et al, 1972; Rovinetti et al, 1972). They found that for prostate tissue, castration caused reversible changes in content and tissue distribution of folate coenzymes Figures 8 and 16. Castration caused suppression of activity of dihydrofolate reductase (DHFR), 10- formyl THF synthase, and serine hydroxymethyl transferase (SHMT). Cytoplasmic cSHMT in concert with vitamin B6 acts as a metabolic switch with at least three functions: 1) preferentially supplies one-carbon units for DNA-thymidine synthesis, Figure 17 circle-number-1, 2) lowers methylene THF used for SAM synthesis Figure 17 circle-number-3, and 3) sequesters CH3−THF by adsorption to the enzyme thus limiting SAM synthesis Figure 17 circle-c. Administration of testosterone restored castration-related enzymatic activities to near normal and higher than normal values. The changes described by Rovinetti (Rovinetti et al, 1972) within prostate tissue could produce powerful metabolic and genetic changes regulated by the testosterone and folate status of the patient.
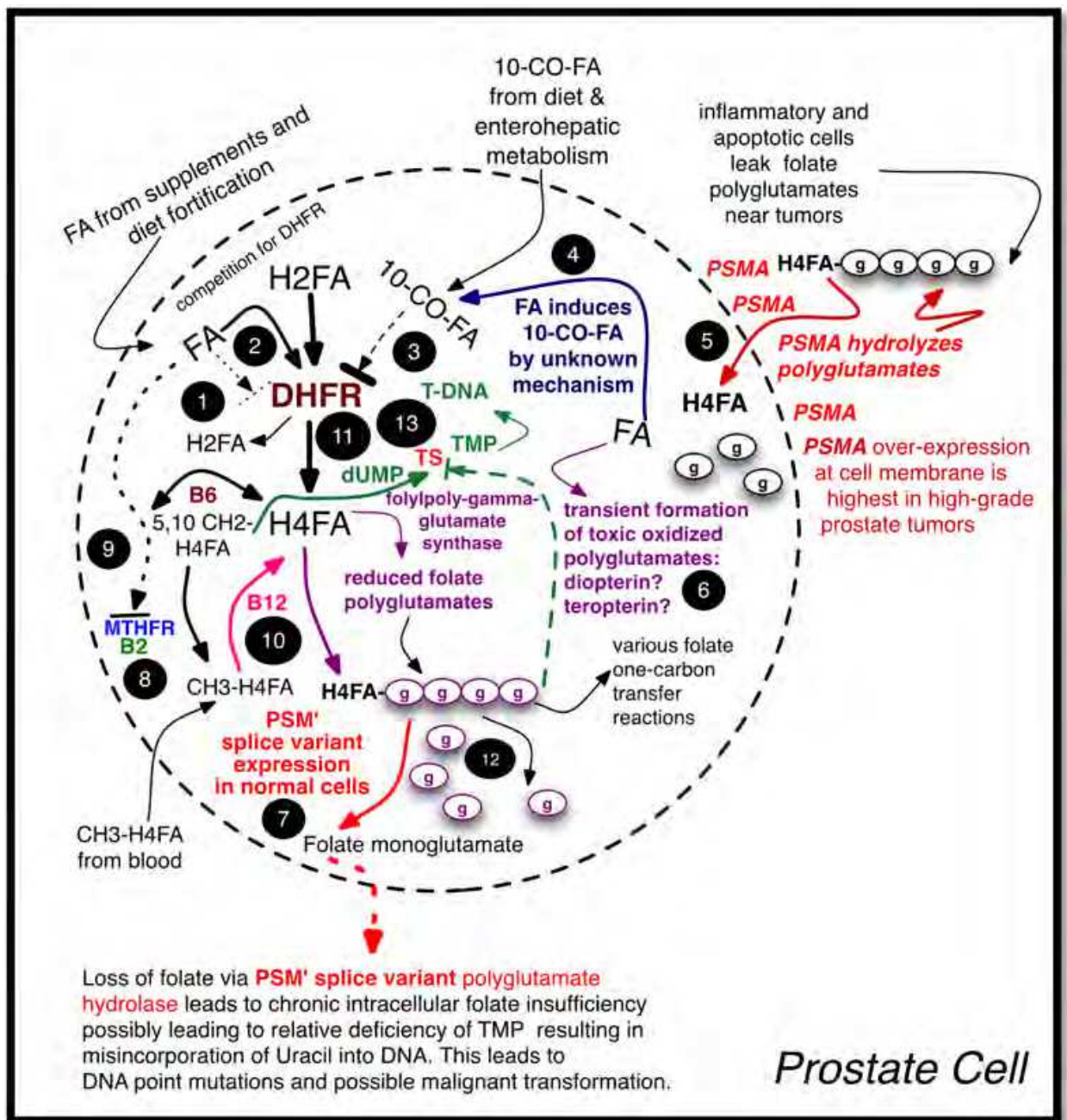
1) FA may directly inhibit DHFR (Kao et al, 2008) 2) FA competes with H2FA for reduction 3) 10-CO-FA inhibits DHFR 4) FA induces 10-CO-FA (D'Urso-Scott et al, 1974; Pratt & Cooper, 1971)? 5) PSMA of prostate cell membrane enhances folate monoglutamate of microenvironment 6) Possible transient production of toxic diopterin and teropterin and other polyglutamates by action of FPGS on FA (Hoffbrand et al, 1976; Shane, 2010) 7) PSM' splice variant FPH produces folate monoglutamates intracellularly 8) B2 modulates MTHFR (Bates & Fuller, 1986; Matthews & Baugh, 1980; Matthews & Haywood, 1979; McNulty et al, 2002) 9) DHFA SAM (Matthews & Daubner, 1982), and FA-(slightly) (Matthews & Haywood, 1979) inhibit MTHFR 10) CH3—THF trapped by B12 deficiency 11)Testosterone increases DHFR activity (Bovina et al, 1972; Rovinetti et al, 1972) 12) Estrogen increases FPH activity.

Fig. 16. Folate pathways affected by sex steroid concentrations and FA and other B vitamins.
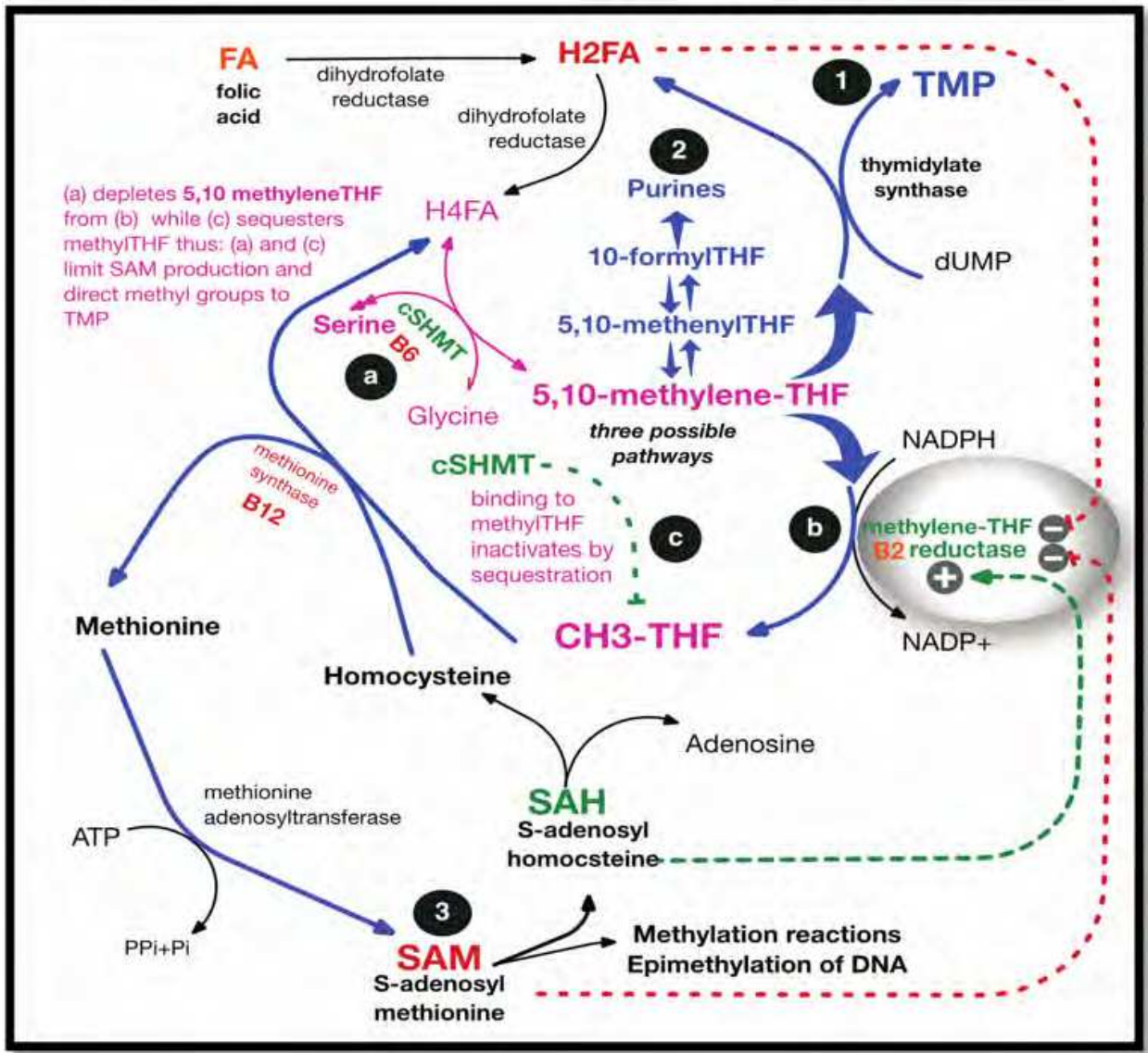
Fig. 17. DHFA, SAM, and S-adenosylhomocysteine (SAH) regulate 5, 10-methylene-THF reductase through inhibition and stimulation of the enzyme (see oval). Cytoplasmic serinehydroxymethylenetransferase (cSHMT) activity influences methylation of dUMP to DNA-thymine vs. homocysteine thru sequestration and inactivation of CH3−THF. Taken together, these compounds modulate methyl group flow to (1) dUMP for DNA-thymine (2) or purine synthesis (3) or for DNA epimethylation and other methylation reactions.

10. Modulation of folate polyglutamate tail length by folate hypersufficiency

Raising the folate concentration with FA competes for FPGS binding sites thereby lowering average polyglutamate length. This can reduce folate coenzyme substrate activity (Cook et al, 1987; Lowe et al, 1993; Shane, 2010), Figure 18.

As folate polyglutamate coenzymes increase in length, substrate-binding strength for FPGS decreases and polyglutamates are released from the enzyme limiting the maximum length to approximately 8 glutamate residues for mammalian cells. Polyglutamate tail length can change an active folate coenzyme to metabolically inert or to an anti-fol (Allegra et al, 1985; Kisliuk et al, 1974; Kisliuk & Gaumont, 1983; Kisliuk et al, 1981; Kisliuk, 1981; Matthews et al, 1987). As indicated in Figure 18, large doses of folate monoglutamates would compete for folate substrate binding, causing premature release of substrates resulting in shorter, less active folate coenzymes.
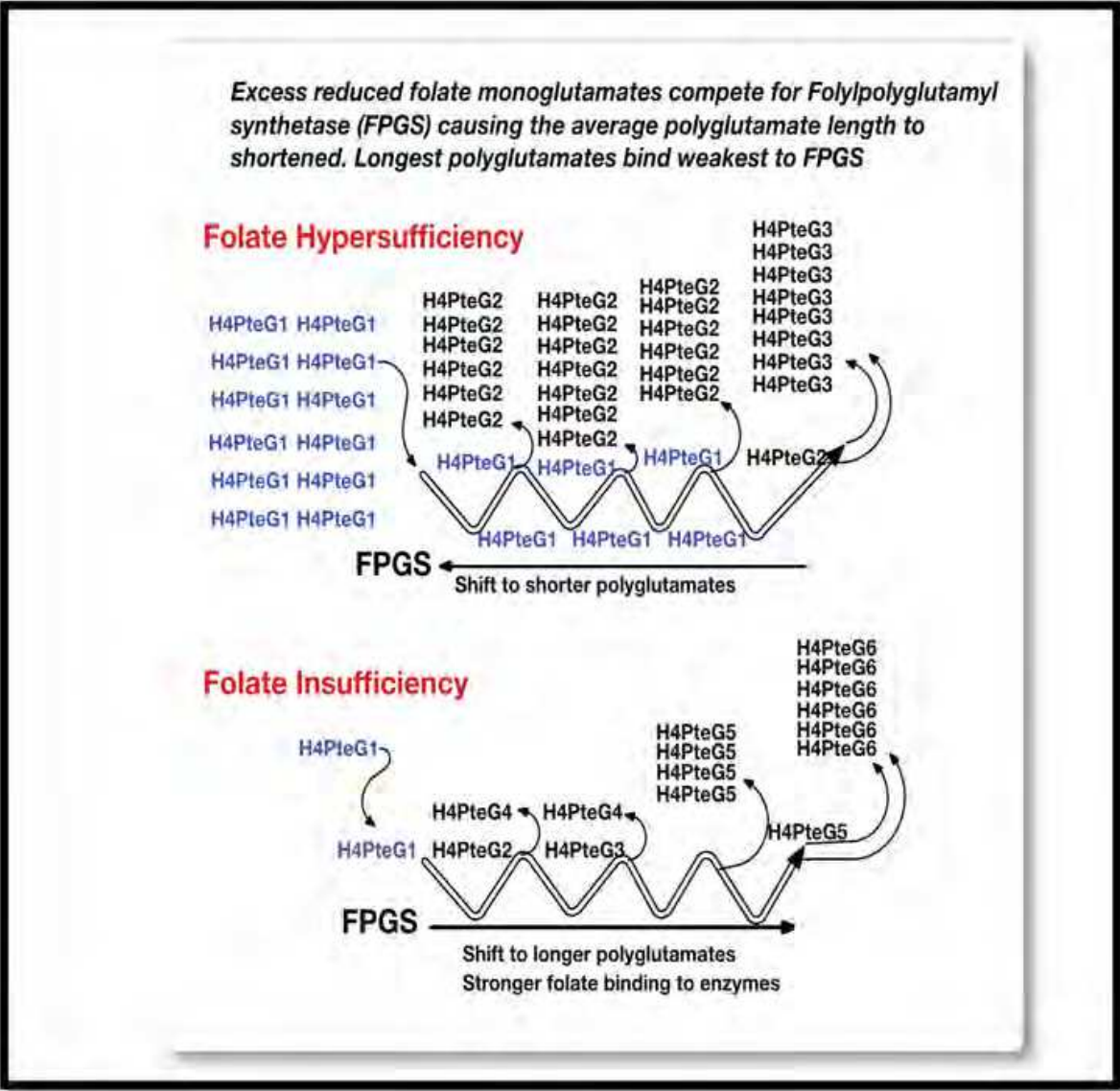

Fig. 18. Increasing reduced folatemonoglutamates competes for reduced folate substrate sites of FPGS tending to decrease overall lengths of folate polyglutamates (Lowe et al, 1993; Cook et al, 1987).

11. Modulation of folate activity by pteridine ring oxidation and DHFR polymorphisms

Matthews showed that several folate-dependent enzymes are inhibited by elevated concentrations of oxidized folate substrates. Dihydrofolate polyglutamates inhibit thymidylate synthase, AICAR transformylase and methylenetetrahydrofolate reductase (Krumdieck et al, 1992; Matthews et al, 1987; Matthews & Haywood, 1979; Ross et al, 1984). As discussed by Kisliuk, oxidation of the pteridine ring from the active tetrahydro to the dihydro state can change a stimulatory coenzyme to a powerful anti-fol (Kisliuk, 1981).

10-formylfolic acid is a potent inhibitor of dihydrofolate reductase of rat liver slices (Rauen et al, 1952; Bertino et al, 1965; D'Urso-Scott et al, 1974; Friedkin et al, 1975; Rauen et al, 1952; Silverman et al, 1954). However, the literature is inconsistent concerning the *in vivo* activity of 10-formylfolic acid in man. Whether this oxidized folate is active *in vivo*, a natural part of the diet (Butterworth & Santini, 1963; Konings et al, 2002; Konings et al, 2001) or an artifact of oxidation when purified is controversial. Pratt (Pratt & Cooper, 1971) and others (Ratanasthien et al, 1974) conjecture that this folate could be formed within the jejunum by bacteria and subject to entero-hepatic circulation and jejunal absorption. Long-term feeding of 10-formylfolic acid produces bioactive folates found to support the growth of chickens (Gregory III et al, 1984) and produces a relatively weak but measurable hematological response in humans with pernicious anemia (Spies & Garcia-Lopez, 1948). Needless to say, more work is needed here.

12. Prostate Specific Membrane Antigen (PSMA), PSMA splice variants (PSM'): Putative biology

PSMA, Figure 19, is a prostate cell membrane receptor with extracellular, transmembrane and intracellular components. The extracellular portion expresses both folylpolyglutamyl hydrolase and neurocarboxypeptidase activity. PSMA is expressed on epithelial cells of benign prostate, prostate hyperplasia, premalignant proliferative inflammatory atrophy (PIA), prostate intraepithelial neoplasia (PIN), and most intensely on high Gleason grade prostate carcinoma cells. PSMA was successfully targeted for radio-imaging vis-a-vis Prostascint scan (Taneja, 2004) and immunotherapy of prostate cancer (Bander et al, 2005; Harzstark & Small, 2009). Almost all Gleason grade 4 tumor cells express excessive amounts of surface PSMA. PSMA staining was positive for 49% of high-grade PIN and only 6% of normal prostate cells (Marchal et al, 2004). Surprisingly, PSMA, first thought to be specific for prostate cancer cells, is expressed on endothelial cells of neovessels involved in angiogenesis of most cancers and with normal wound healing (Chang et al, 1999; Gordon et al, 2008). PSMA expression on prostate epithelium is decreased by testosterone and increased in patients with prostate cancer refractory to testosterone withdrawal. As Gleason's grade and clinical stage increase, acceleration of cell proliferation requires an increased supply of nutrients and PSMA could help cells acquire folate by scavenging folatemonoglutamates from surrounding apoptotic inflammatory cells. Lighter molecular weight, so-called splice variants of PSMA, termed PSM', are present in large amounts within the cytoplasm of normal prostate cells. PSM' and its congeners are highly expressed in the normal prostate and in low-grade tumors retaining both FPH and neurocarboxypeptidase enzymatic activity (O'Keefe, Bacich, et al., 2001; Yao et al, 2008). As illustrated in Figure 18, and as suggested by Yao and O'Keefe, extensive expression of PSM' makes cells vulnerable to loss of folatemonoglutamates putatively causing intracellular folate deficiency (O'Keefe, Bacich, et al., 2001; Yao et al, 2008; Yao et al, 2010). Over

time, it is conceivable that such chronic, cell-specific deficiency could transform cells to a malignant phenotype (Yao et al, 2008).
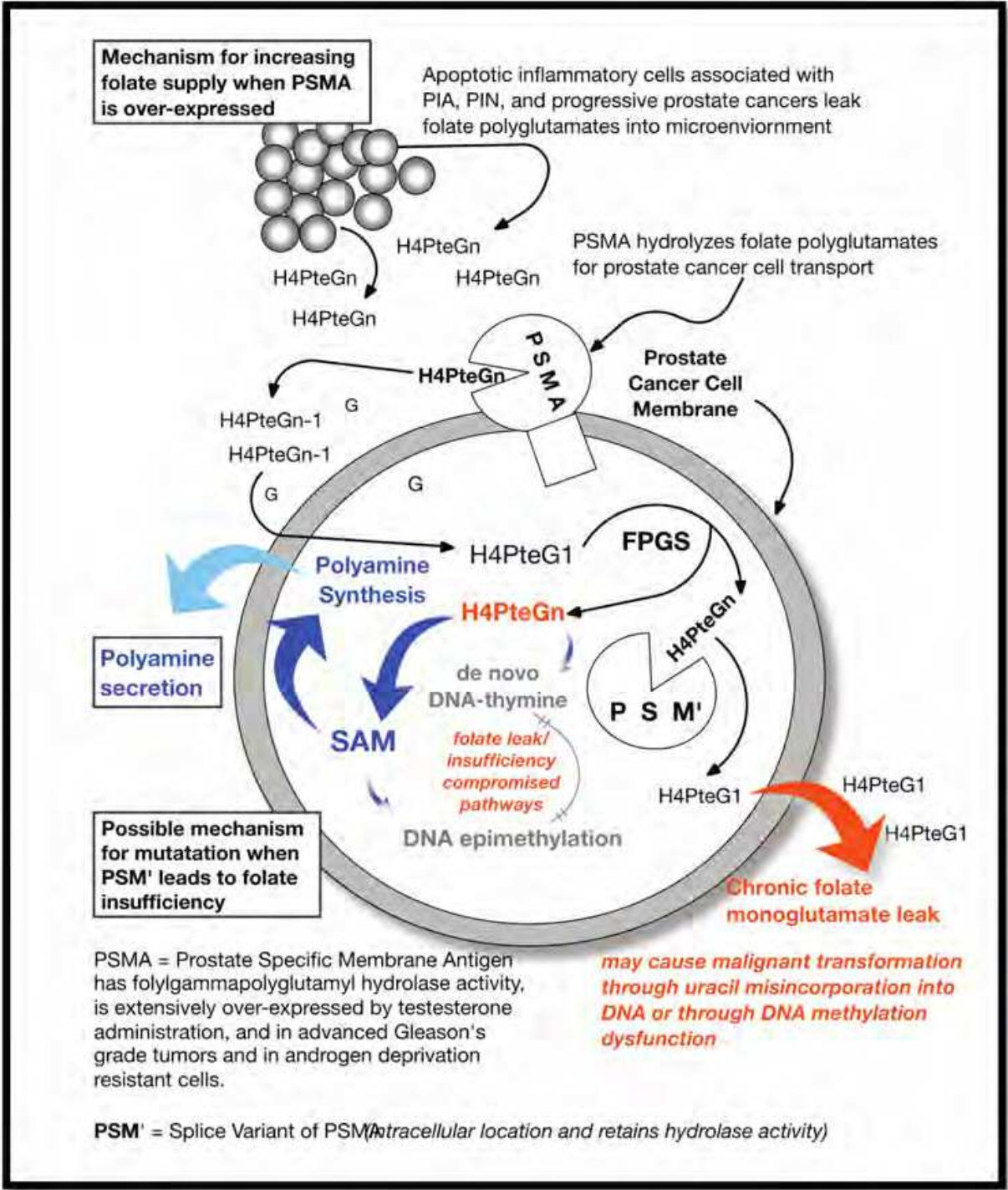

Fig. 19. Proposed balance between PSMA and PSM'. Prostate specific membrane antigen (PSMA) and its splice variant cogeners, PSM'. PSMA supplies intracellular folate monoglutamates from surrounding degenerating inflammatory cells that supply polyglutamates. PSM', present in high concentration in normal prostate cells, through its FPH activity generates folatemonoglutamates for extracellular transport leaving cells relatively deficient of folates.

13. Polyamines

These compounds contain two or more amine groups, such as spermidine and spermine and function as essential growth factors. The prostate is the major source of polyamine production and secretion. Metabolic demands for polyamine synthesis divert intracellular folate methyl groups from other metabolic processes including the synthesis of DNA-thymine and epimethylation of DNA (Bistulfi et al, 2009) Figure 14.
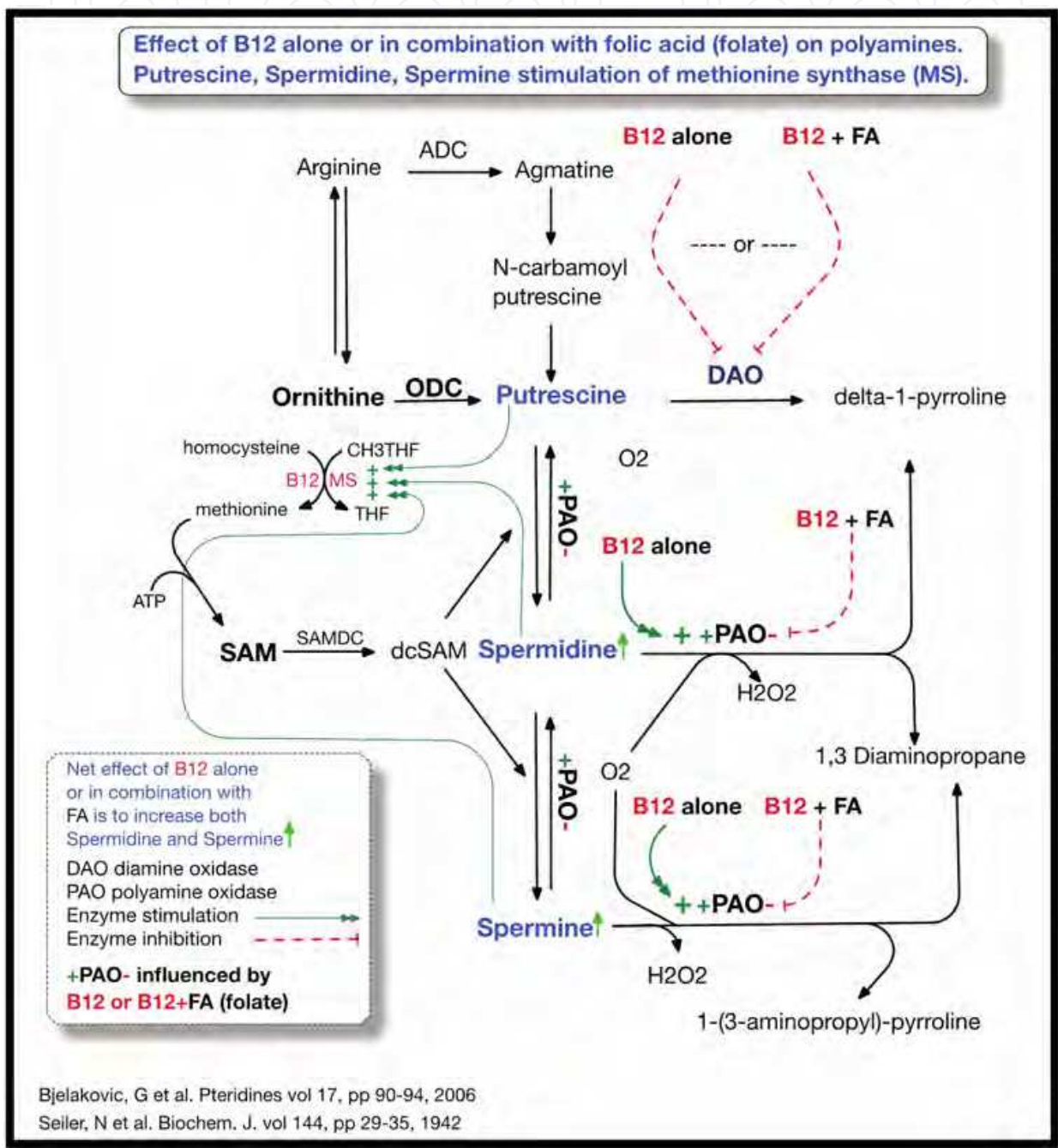

Fig. 20. Polyamine metabolism: B12 and FA influence polyamine metabolism and polyamines may modulate methionine synthase (MS)

Polyamines regulate cellular growth and participate in the evolution of prostate cancer. As noted in Figure 20 the enzyme ornithine decarboxylase (ODC) initiates polyamine synthesis. Testosterone increases the activity of ODC, S-adenosylmethionine decarboxylase and spermidine synthase of prostate epithelial cells. Figure 20 shows modulation by B12 and folate of the enzymes of polyamine oxidation, diamine oxidase (DAO) and polyamine oxidase (PAO). Spermine and spermidine modulate the key B12-requiring enzyme, methionine synthase, illustrating other areas of regulation between B vitamins, one-carbon transfer and polyamine metabolism critical for prostate cells.

In 1996 Kenyon's group reported that spermine increased methionine synthase (MS) activity by 400% and spermidine by 270% (Kenyon et al, 1996). In 2006 Bjelakovic (Bjelakovic et al, 2006) reported effects of mega doses of B12 alone and B12 plus folic acid on the activity of the polyamine metabolizing enzymes DAO and PAO, Figure 20. Dongmei Sun placed rats on a low folate diet inducing folate insufficiency and observed hepatic spermidine, spermine and putrescine increase by 58%, 67% and 27% respectively compared to controls fed a folate-replete diet (Sun et al, 2002). The polyamine concentrations of the jejunum, ileum, colon and brain remained stable. There was a tissue-specific polyamine response to folate deficiency. Low folate (Sun et al, 2002) as well large doses of folic acid alone, B12 alone, or B12 plus folic acid produced increased hepatic levels of spermidine and spermine (Bjelakovic et al, 2003; Bjelakovic et al, 2006). B12 and folate are thus modulators of polyamine metabolism and vice versa.

13.1 Other regulatory mediators of one-carbon metabolism: DHFR, SAM, GHMT and cSHMT

The pentaglutamate of CH3−THF modulates GNMT enzyme activity by non-substrate binding. CH3−THF, only as the pentaglutamate-G5, strongly inhibits GNMT activity (Wagner et al, 1985; Yeo et al, 1999). Likewise, cytoplasmic serinehydroxymethyltra-nsferase (cSHMT), by non-enzymatic sequestration of CH3−THF controls its metabolism to THF. These interactions add further diversity to mechanisms for control of one-carbon metabolism and surely wreak havoc on those trying to study them Figures 10 and 17.

When SAM levels are high, MTHFR is inhibited (Ubbink et al, 1996) and CH3−THF formation is reduced, resulting in active GNMT, which reduces excess SAM (Wang et al, 2011) Figures 10 and 17. SAM diversion to polyamine synthesis leads to active MTHFR increasing CH3−THF, which is polyglutamated to CH3−THFG5 leading to inhibition of GNMT (Williams & Schalinske, 2007; Yeo et al, 1999). GNMT catalyzes SAM mediated methylation of glycine to sarcosine (Rowling et al, 2002) Figures 8 and 10. Testosterone up-regulates GNMT, producing more sarcosine. Blood and urinary sarcosine has been the subject of intense debate in the literature. Sarcosine was initially identified as a valuable marker for aggressive prostate cancer (Sreekumar et al, 2009) but this has not yet been confirmed by others (Jentzmik et al, 2010; Jentzmik et al, 2011; Struys et al, 2010).

13.2 General genomic and chromosomal structure

Figures 21 and 22 summarize promoter and genomic architecture. The genome includes both the genes and the non-coding sequences of DNA. The DNA helix coiled around histones contains the genetic code. DNA coiled around a set of 4 histone dimers is referred to as a nucleosome (illustrated as a single bead). When the nucleosomes are

loosely packed in a relaxed state and spread apart they are collectively referred to as "euchromatin" and as such allow "epimethylation", the transfer of methyl groups delivered by S-adenosylmethionine to the cytosine bases of DNA (some arranged as CpG islands/groupings while others are more diffusely scattered about the global genome). CpG cytosine bases extend out from the DNA helix. Alternatively, tightly packed histones within chromosomes are termed "heterochromatin" and under this condition access to methylation of cytosine bases is prohibited. The packed beads of histones (loose or tight) are further folded into segments called chromosomes of which there are normally 46 in number.

14. Epigenetics, control of the epigenome, folic acid and malignant transformation

As a primer on the epigenome, methylation reactions and genetic control, we would recommend excellent comprehensive reviews (Choi & Friso, 2010; Dobosy et al, 2007; Foley et al, 2009; Jones & Takai, 2001;Perry et al, 2006). The definition of epigenetics given by Peter Jones at USC School of Medicine is "the study of heritable changes in gene expression that occur independent of changes in the primary DNA sequence or genetic code" (Jones & Laird, 1999; Sharma et al, 2010). The presence of a genetic code within DNA, though obviously necessary, is not sufficient for gene transcription. Only through gene expression do observable phenotypic changes become apparent. The prostate is under environmental controls mediated through DNA and histone methylation (Diaw et al, 2007; Esteller, 2008; Li & Dahiya, 2007; Li et al, 2005; Perry et al, 2006).

SAM, in concert with methyl transferases, mediates DNA methylation. Transfers of SAM CH3—groups occur at the carbon 5' position of cytosine within CpG dinucleotide rich islands through complex reactions involving cytosine bases that "poke out" of the double helix Figures 21 and 22. DNA CpG dinucleotide clusters/islands are extensive only at promoter regions of genes Figures 21 and 22 (Esteller, 2007). Unmethylated promoter status allows downstream portions of gene exons to be transcribed to m~RNA. The presence of methylated CpG islands at promoter sites changes regional chromatin geometry by affecting the binding properties of methylation-sensitive DNA-binding proteins. This ultimately leads to interference with gene transcription and down-regulation of gene expression.

DNA from normal tissue is globally methylated/epimethylated while their CpG promoter regions lack such methyl groups. These unmethylated promoter islands turn ON m~RNA transcription of regulatory genes, some of which are tumor suppressor genes. Global methylation of normal cells stabilizes genomes, thwarting global genetic expression of oncogenes. On the other hand, tumors are globally hypomethylated allowing for oncogene expression while at the same time promoter areas of tumors are hypermethylated turning OFF transcription (Ehrlich, 2002) of regulatory and tumor suppressor genes. Promoter or CpG island hypermethylation and global hypomethylation occur during malignant transformation Figures 21 and 22.

Each tumor subtype can be assigned a DNA hypermethylome profile, a CpG island hypermethylation pattern that closely defines a specific cancer. It is estimated that each tumor contains between 100-400 tumor-specific hypermethylated CpG islands (Esteller, 2007). These hypermethylated promoters appear before overt malignancy.
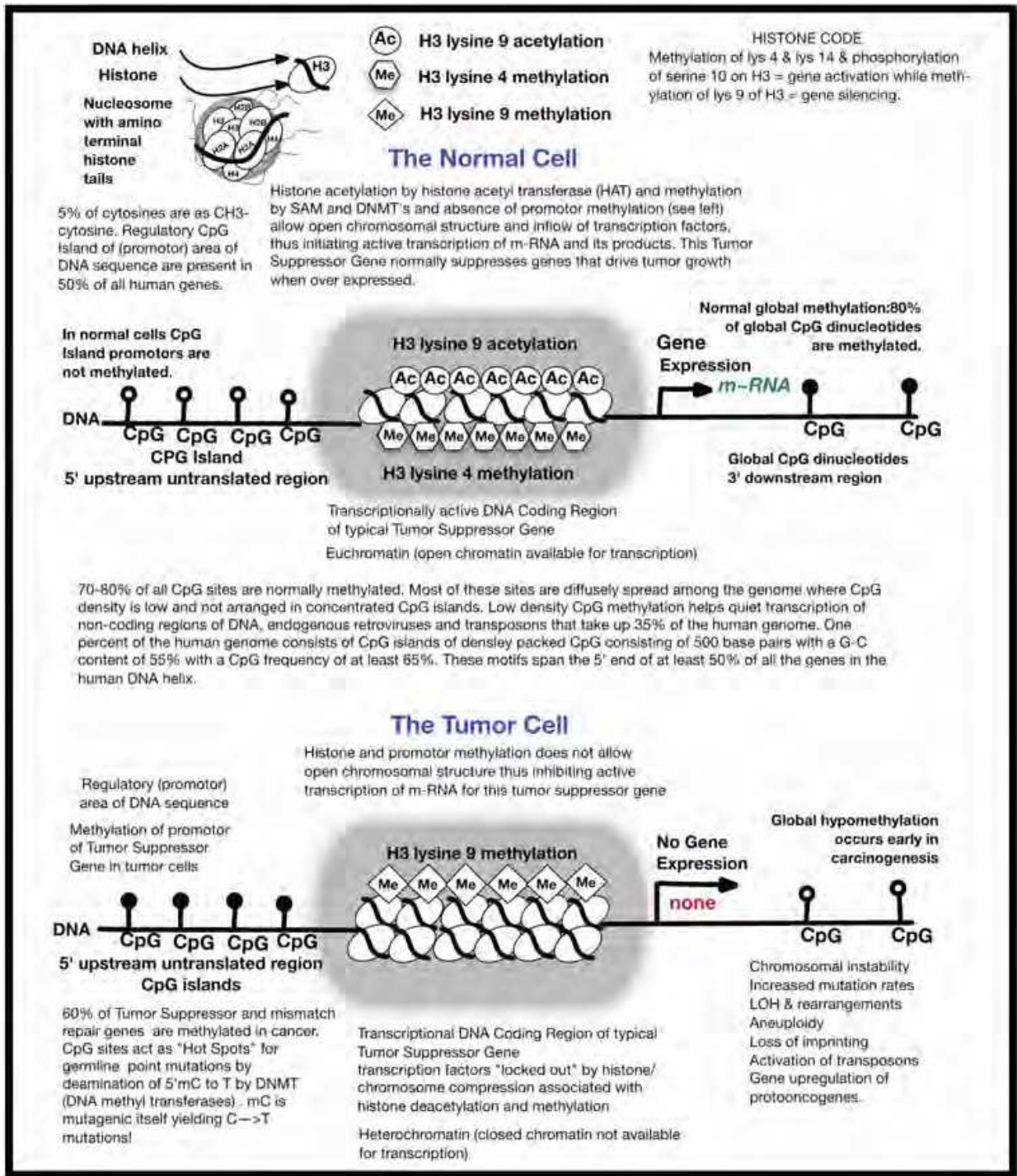
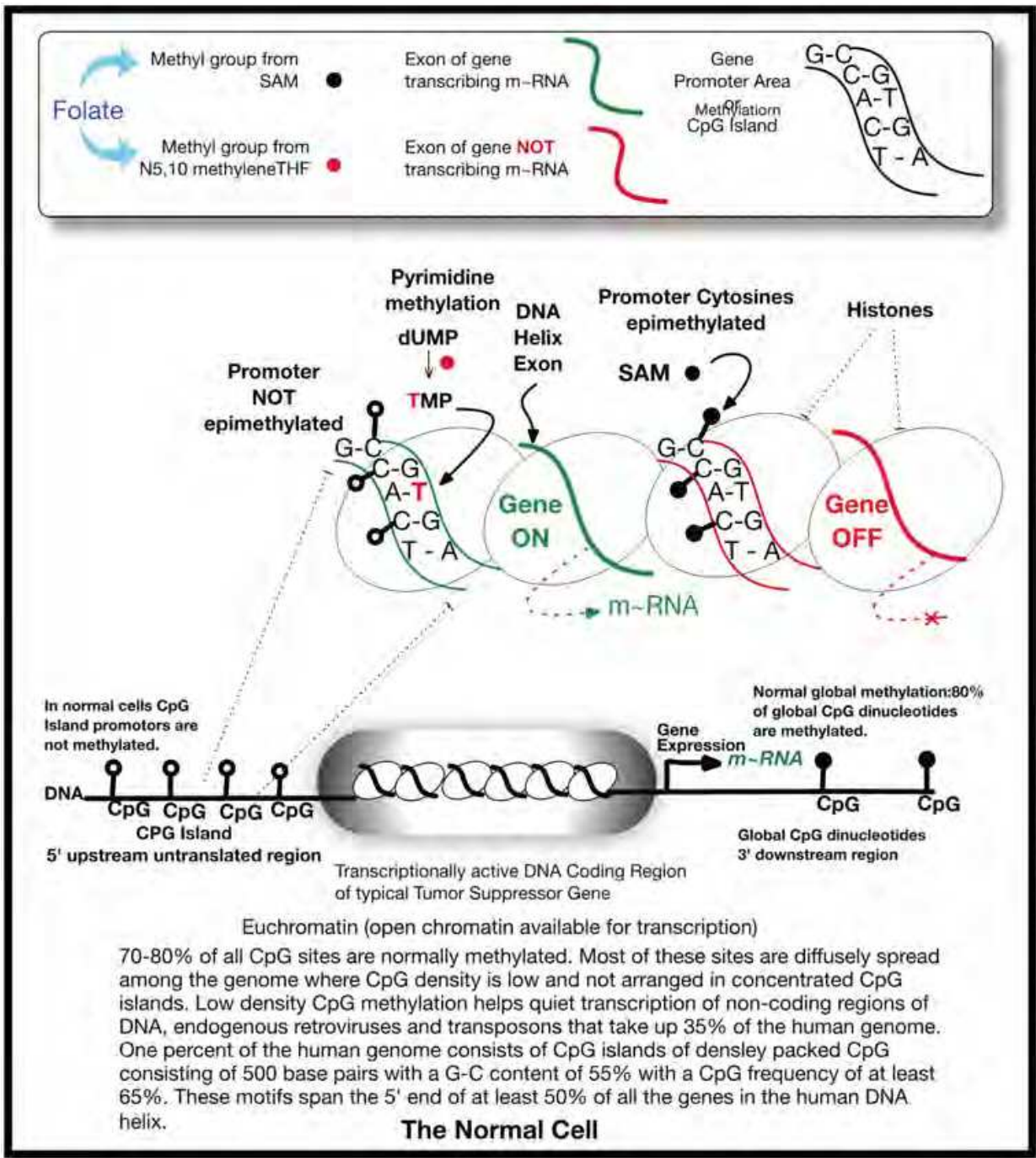Fig. 21. Epigenetic methylations in normal and tumor cells.

Fig. 22. Gene promoter ON/OFF control, a closer look.

15. Folate modifies the epigenome

In our opinion the US population suffers from dietary "iatrogenic hyperfolatemia." Study of the effects of both high and low concentrations of FA on the epigenome are important. Linette Pellis (Pellis et al, 2008) exposed colon cancer cells to elevated concentrations of FA, 100 ng/ml, levels routinely achieved by supplement users in the USA and compared growth to cells chronically exposed to 10 ng/ml. Exposure to FA 100 ng/ml induced greater proliferation and apoptosis compared to lower doses. Gene expression analysis of the 100 ng/ml cells revealed lower expression of E-cadherin. Rennie pointed to DNA hypermethylation as an important mechanism in prostate cancer for inactivation of key regulatory genes including E-cadherin (Rennie & Nelson, 1998). Reduction of E-cadherin expression in carcinomas correlates positively to the potential for invasion and metastasis. These changes occur in many tumors including hepatoma, advanced prostate, lobular breast and others (Hirohashi, 1998).

Studies by Wainfan (Wainfan & Poirier, 1992) disclosed that within seven days of initiating a methyl-deficient diet, depletion of S-adenosylmethionine pools resulted in DNA hypomethylation leading to expression of growth regulatory genes. There were decreases in overall levels of DNA methylation yielding patterns that closely resembled those reported to occur in livers of animals exposed to hepatoma-inducing carcinogens. Studies such as these indicate the importance of dietary FA as a regulator of gene expression. The rapidity of folate-induced changes of the epimethylome is alarming.

Man is not necessarily entirely hostage to fortune. As we learned, epigenetic changes dictated by diet can turn genes ON and OFF. The epimethylome is dictated in part by ingestion of nutrients and chemicals including B vitamins, medications, sun exposure (Vitamin D3), exercise induced decreases in insulin levels (Hsing et al, 2001) and methyl groups supplied by the diet such as methionine, choline, betaine, B12, folic acid and other supplements. We are in part a product of our environment, which can be changed (Hayashi et al, 2007; Kim, 2005; Kim, 2007).

16. DNA methylation and prostate cancer

The link between nutrition, epimethylation and cancer is now well established (Choi et al, 2004; Davis & Uthus, 2004, Esteller, 2007; Friso & Choi, 2005; Mason et al, 2008). In addition to gene-specific changes, studies of methylation of colonic mucosa indicate that age-specific, as well as organ site-specific (right or left colon) changes are demonstrable (Wallace et al, 2010). Genomic DNA aberrant methylation of tumor specific genes is almost always abnormal in malignant and transforming cells. These changes progress during carcinogenesis through clinical metastases and are more frequent than chromosomal mutations (Bastian et al, 2004; Diaw et al, 2007; Li & Dahiya, 2007; Perry et al, 2006; Sasaki et al, 2002; Song et al, 2002). Epimethylation's relevance to the genesis of prostate cancer is illustrated by sequential hypermethylation and hypomethylation of genes as prostate tumors dedifferentiate and clinically progress over time (Maruyama et al, 2002). Yegnasubramanian showed that CpG promoter hypermethylation occurs early in prostate tumors, before global genomic hypomethylation. Genomic hypomethylation occurs late in metastasis and varies at different metastatic sites (Yegnasubramanian et al, 2008) and is possibly responsible for heterogeneity of the therapeutic response. As

illustrated in Figure 23 the glutathione S-transferase (GSTP1) gene is methylated early in the evolution of prostate cancer. GSTP1 codes for proteins involved in processing carcinogenic metabolites and its dysfunction is understandably associated with malignant transformation.

Methylation of the GSTP1 promoter area is absent in normal prostate tissue and present in 6.4% of proliferative inflammatory atrophy, the precursor lesion of prostate cancer. GSTP1 hypermethylation is observed in 70% of high-grade PIN and in 90% of prostate cancer patients. As prostate tissue progresses from proliferative inflammatory atrophy (PIA) and PIN through hormone refractory metastatic disease a series of hypermethylated genes appear, see Figure 23 (Li & Dahiya, 2007). As tumors develop androgen independence, methylation of androgen and estrogen receptor genes becomes evident (Diaw et al, 2007; Sasaki et al, 2002).
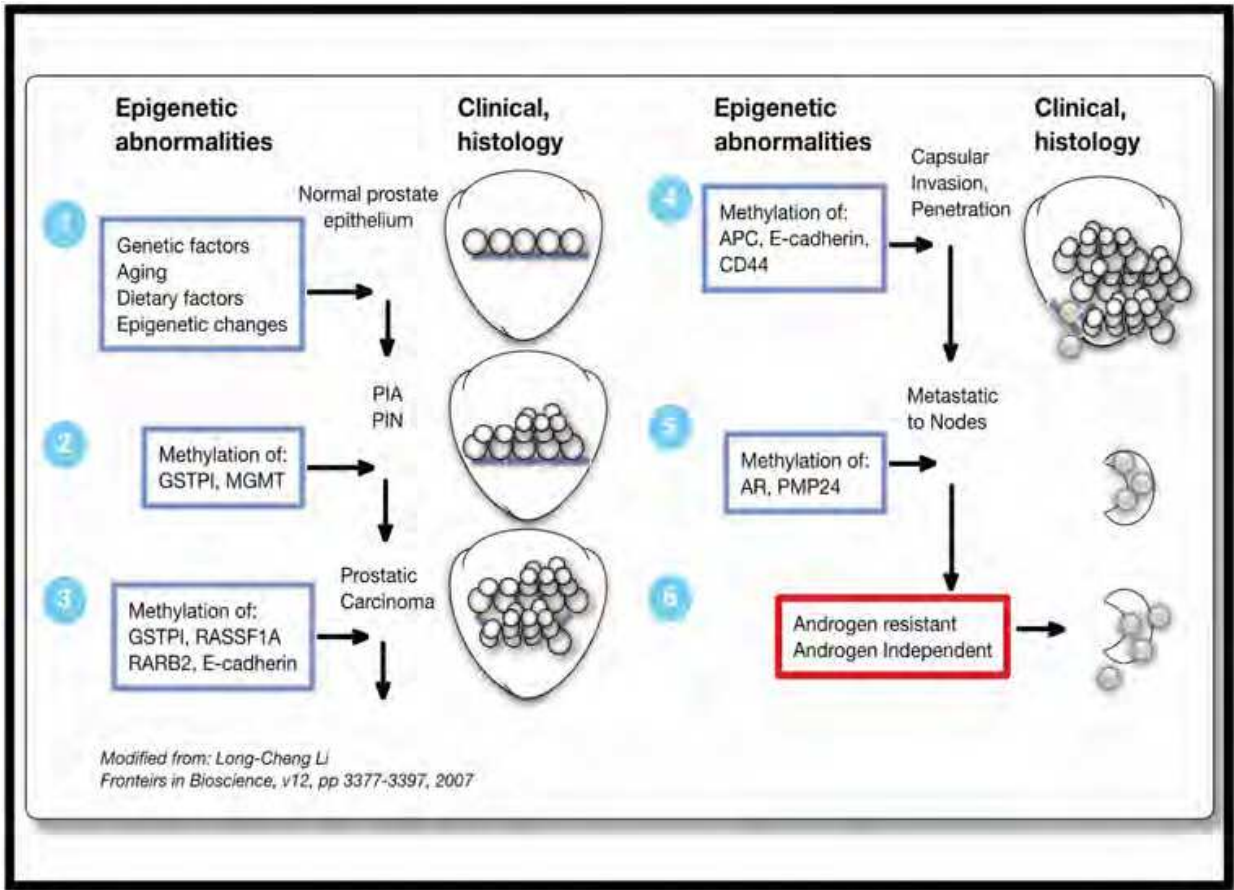

Fig. 23. Transformation of prostate tissue to malignancy involves sequential aberrant methylation of the genome.

17. Demethylating agents: Reversal of aberrant methylation in the clinic

Hypomethylation of oncogenes may activate cMYC , H-RAS (Das & Singal, 2004) and K-RAS (Feinberg & Vogelstein, 1983) contributing to oncogenesis while simultaneous promoter hypermethylation silences growth regulating tumor suppressor genes. Hypomethylation of prostate DNA is associated with BPH and metastatic prostate cancer but remarkably not with localized prostate cancer (Bedford & van Helden, 1987). Keep in

mind that either high or low (Duthie, 1999) concentrations of folate and its intermediates may induce simultaneous tissue-specific (Kawakami et al, 2003) and gene-specific aberrant hyper- and hypo-methylation (Duthie, 1999; Kawakami et al, 2003). Who said life would be easy?

As is now readily demonstrable in the clinic, 5-azacitidine (Vidaza™) therapy for patients with myelodysplastic syndromes variably reverses DNA hypermethylation after several weeks to months of therapy restoring phenotypic expression towards normal vis a' vis reversal of severe anemia and life-threatening thrombocytopenia to moderate transfusion-independent anemia and mild thrombocytopenia. Based on our clinical observations, responders may have complete reversal of disease for many years, or more often, clinical symptoms are walked back to a previous, tolerable stage no longer requiring aggressive blood product support.

5-azacitidine is a demethylating agent capable of activating genes repressed by promoter hypermethylation. In preclinical studies, demethylating agents reversed acquired androgen withdrawal-resistance of prostate cancer (Gravina et al,). Each steroid receptor gene active in prostate cancer that physicians have successfully targeted in the clinic (ER, PR, AR) may become inactive by CpG methylation both in prostate cancer tissue and cultured cell lines (Li et al, 2005; Sasaki et al, 2002).

A phase II clinical trial evaluated 5-azacitidine for men with hormone refractory prostate cancer that had progressed while on androgen deprivation therapy (Sonpavde et al, 2009). Patients with PSA doubling times (PSADT) ≤ 3 months were treated with 5-azacitidine 75 mg/m2 subcutaneously on days 1-5 of each 28-day cycle up to 12 cycles or until clinical progression or severe toxicity. A PSADT > 3 months was attained in 19 patients or 55.8%. Overall median PSADT was significantly prolonged compared to baseline 2.8 vs. 1.5 months. Fourteen patients had some PSA decline during therapy and 1 patient had a > 30% decline compared with baseline. It appeared the 5-azacitidine favorably modulated PSA kinetics and a correlation was made with decreasing plasma DNA methylation.

Braieth (Braiteh et al, 2008), using 5-azacitidine and valproic acid (Depakote™), a histone deacetylase inhibitor in combination treated a small group of patients with advanced, pretreated malignancies and observed stable disease lasting 4 to 12 months (median, 6 months) in 14 patients (25%), one of two patients with prostate cancer experienced stable disease. We caution that demethylating drugs lack gene specificity and may be associated with unexpected results. In our own clinic we observed decreases in PSA in occasional patients with myelodysplasia treated with 5-azacitidine without obvious prostate cancer, a possible lead that should be followed.

Global DNA hypermethylation associated with epigenetic reprogramming resulting in adriamycin chemotherapy resistance was reversed by the demethylator, hydralazine (Segura-Pacheco et al, 2006). Sensitivity to taxanes, the most active drugs in hormone resistant prostate cancer, was found to be a function of aberrant methylation of the CHFR (checkpoint with forkhead-associated and ring finger) gene in endometrial cancer (Yanokura et al, 2007). Thus, not only tumor transformation but chemotherapy resistance and sensitivity may be influenced by ones DNA epimethylome. Such may in part have been responsible for Patient 2's PSA response while on docetaxel (Taxotere™) after withdrawal of folate and B12. The therapeutic potential of demethylating agents and their pharmacology is reviewed by Szyf (Szyf, 2009) and Fenaux (Fenaux, 2005).

18. Review of epidemiological studies

18.1 Vitamin B12, folic acid, potential toxicity of dietary fortification

As is apparent in Figure 24, it doesn't take much extra FA to change serum levels to values demonstrated to impact DNA methylation (Basten et al, 2006; Jacob, 2000; Mokarram et al, 2008; Pufulete et al, 2005; Rampersaud et al, 2000; Smith et al, 2008; Tisman & Garcia, 2011; Zeisel, 2009). As previously noted, post 1998 serum levels have increased 4-6-fold in select populations. In the US, a daily bowl of FA-fortified cereal (400 μg FA) plus a routine multivitamin (400-800 μg FA) plus an afternoon "pick-me-upper" 5-Hour Energy™ drink (400μg FA+500μg B12+40mg B6), available at almost every gas station, liquor and convenience store across the US, plus a probiotic supplement supplying lactic acid bacteria-generated folates LeBlanc et al, 2007 could add up to an additional 40-200ng/ml increment to the usual basal serum level, which on average in the US is ~12 ng/ml. So, serum levels in our patient population are often greater than 30 ng/ml, median 24 ng/ml Figure 1. When reviewing epidemiological studies of folate and other vitamers often there are dose-response effects that may produce contrasting results, Figure 26.
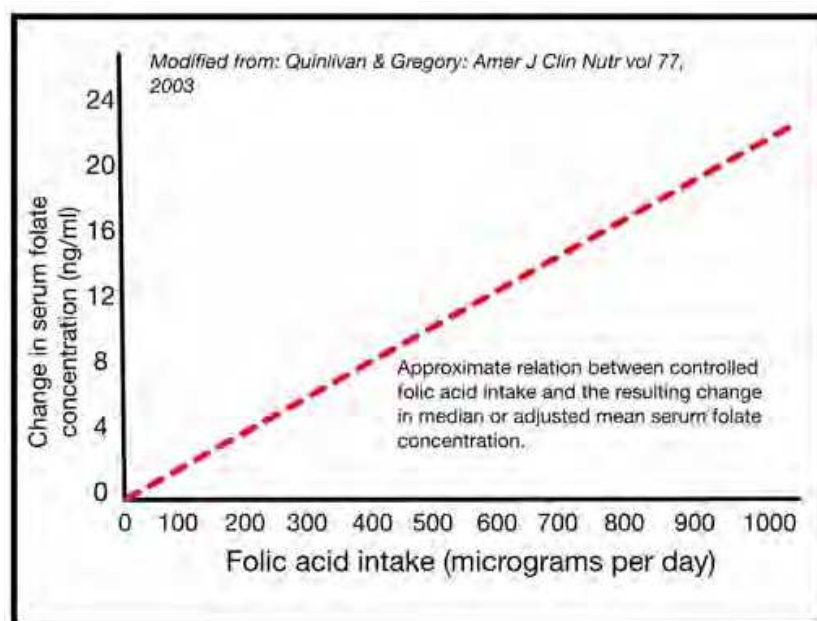

Fig. 24. Change in serum/plasma folate as a function of ingested folic acid.

There are epidemiological studies investigating prostate cancer risk based on dietary ingestion and serum folate. Most find no consistent relation (Hultdin et al, 2005; Johansson et al, 2008; Stevens et al, 2006, Weinstein et al, 2003) to prostate cancer. In one case control study by Pelucchi et al. of Italy (Pelucchi et al, 2005), folate ingestion was found to be protective against prostate cancer. The OR of prostate cancer was 0.66 for the highest versus the lowest quintile of folate intake.

A large study by Lawson (Lawson et al, 2007) of multivitamin and supplement use and risk of prostate cancer in the National Institutes of Health-AARP Diet and Health Study revealed absence of an association between multivitamin use and risk of localized prostate cancer. However, there was a statistically significant increased risk for advanced and fatal prostate cancers (RR = 1.32, and RR = 1.98, respectively) among men reporting excessive use of multivitamins (more than seven times per week) when compared with never users. The

incidence rates per 100,000 person-years for advanced and fatal prostate cancers for those who took a multivitamin more than seven times per week were 143.8 and 18.9, respectively, compared with 113.4 and 11.4 in never users. Also noted was a significant increase in risk of localized prostate cancer among heavy multivitamin users who consumed a folate supplement. Use of folate, as an individual supplement independent of a multivitamin, lacked an association with prostate cancer.

An important clinical study by Figueiredo (Figueiredo et al, 2009) reviewed prostate cancer occurrence in the Aspirin/Folate Polyp Prevention Study. This was a placebo-controlled randomized trial of aspirin and folic acid supplementation for the prevention of colorectal adenomas conducted between July 1994 and December 2006. The US government mandated folic acid fortification of the food supply by the end of 1998. Participants were followed for up to 10.8 (median = 7.0) years. Aspirin alone had no effect on prostate cancer incidence, but there were unexpected and marked differences according to FA treatment. Among the 643 men who were randomly assigned to placebo or daily supplementation with 1000 μg folic acid, the estimated probability of being diagnosed with prostate cancer over a 10-year period was 9.7% in the FA group and 3.3% for the placebo group. The age-adjusted hazard ratio = 2.63 and was found statistically significant with p=0.01.

A troublesome observation by Troen (Troen et al, 2006) among women with a diet low in folate (< 233 μ/d) found that those who used folic acid-containing supplements had significantly greater natural killer (NK) cytotoxicity (p = 0.01) while those who consumed a folate-rich diet and used folic acid supplements > 400 μg/d had reduced NK cytotoxicity, p = 0.02. Ingestion of > 400μg FA is associated with measurable serum FA in addition to usual CH_3—THF. Troen detected unmetabolized FA in 78% of plasma samples from fasting participants (Troen et al, 2006). There was an inverse relation between the presence of unmetabolized FA in plasma and NK cytotoxicity remembering that NK lymphocytes are important innate immune cells that target cancer cells and those infected with virus. NK cytotoxicity was 23% lower among women with detectable serum levels of FA (P = 0.04). Older women greater than 60 years were more susceptible to NK cell suppression. Complicating the picture, Young-In Kim found decreased NK-mediated cytotoxicity in rats made folate deficient (Kim et al, 2002).

Absorbed and circulating unmetabolized FA is potentially toxic (Lucock & Yates, 2005; Smith et al, 2008; Sweeney et al, 2009). Lucock studied three patients ingesting high doses of FA (5 mg/d) to lower serum homocysteine. Under these circumstances, serum folate levels may exceed 100-200 ng/ml and FA is frequently 50% of total serum folate. HPLC analysis of red cell folate coenzymes revealed an aberrant distribution from the usual. There was accumulation of methylene-, methenyl-, formyl- and unsubstituted THF at the expense of the MTHFR-downstream folate-coenzyme, CH_3—THF. He conjectured that *de novo* DNA-thymine synthesis would be preferred through high intracellular FA direct inhibition of MTHFR (Lucock & Yates, 2005) Figures 9 and 25. This would limit the production of SAM and possibly DNA methylation and relative to prostate metabolism, polyamine synthesis. Importantly, he points out that administration of the recommended daily dose of ~400 μg/d, the neural tube preventing dose (Daly et al, 1997), is not associated with significant blood levels of the oxidized provitamin, FA; however, as more FA is ingested, much is circulated as potentially dangerous FA. John Scott's group has studied this pharmacology (Sweeney et al, 2009) and reported no detectable serum FA after doses of 100 or 200 μg FA were ingested for 14 weeks, however, FA was measurable at the highest level (400 μg) tested (Sweeney et al, 2007). The review by Smith discusses potential metabolic interference (Kao et al, 2008) and toxicity (Smith et al, 2008) of the provitamin FA some of which is noted in Figure 25.
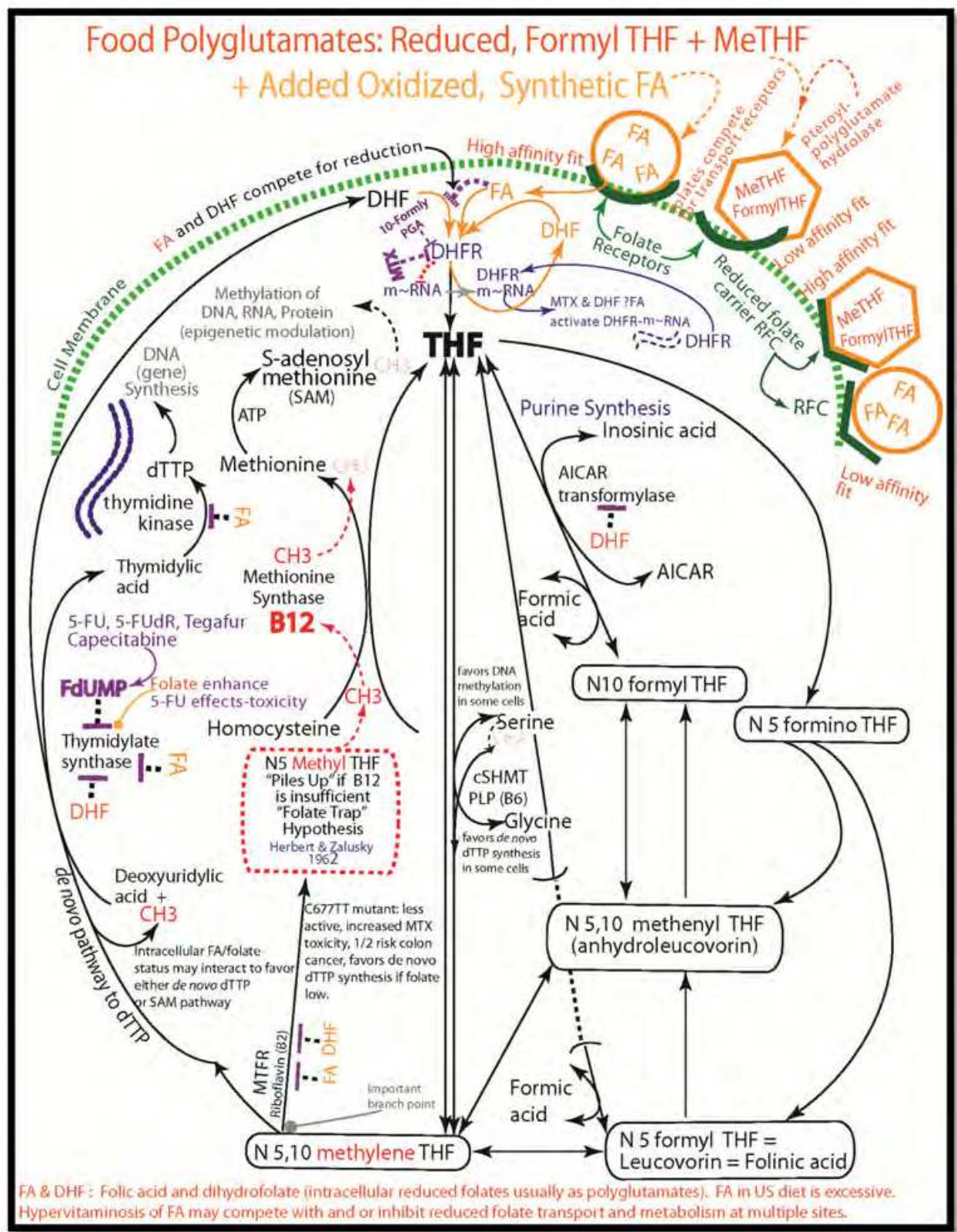
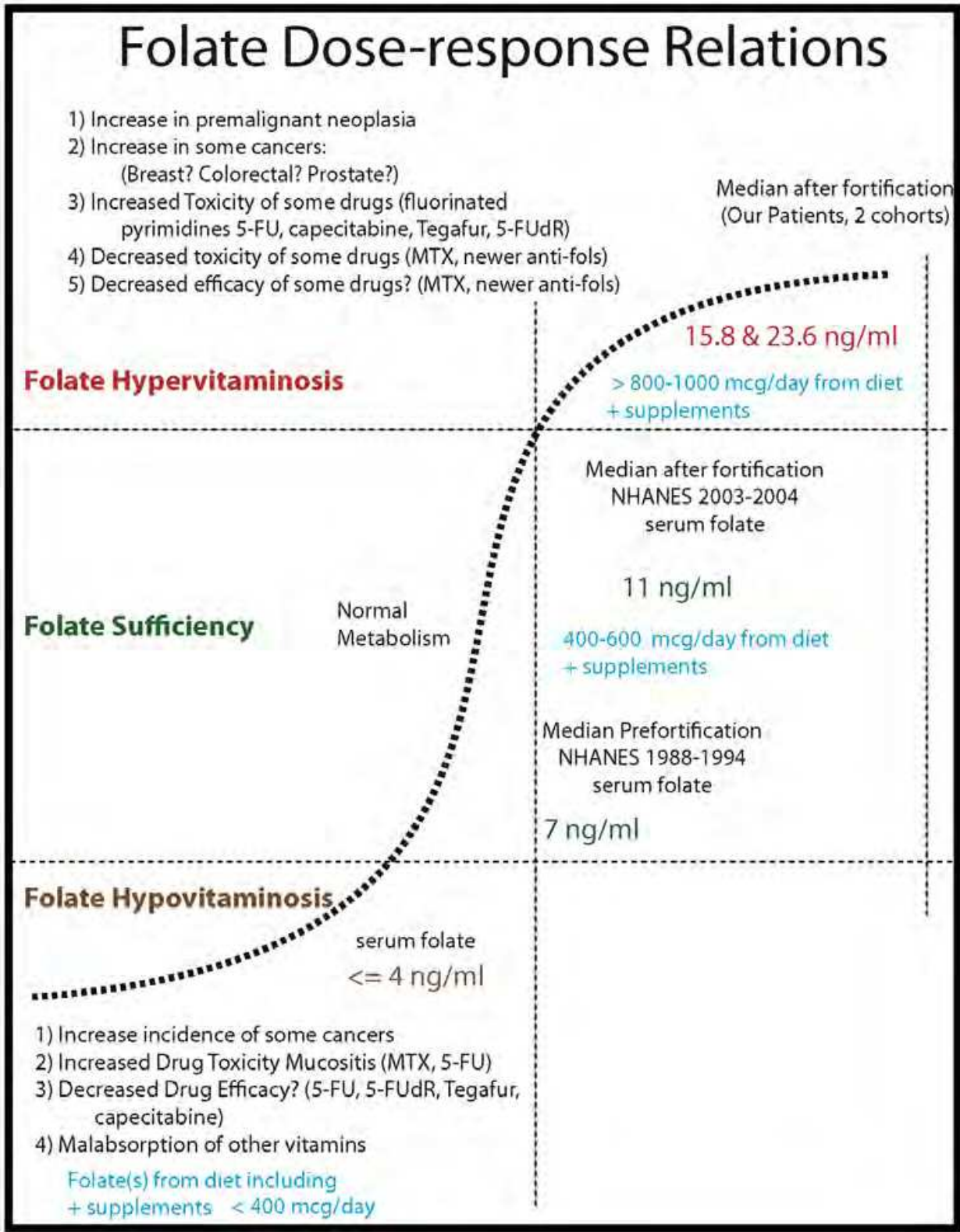Fig. 25. Folate and one-carbon metabolism.

Fig. 26. Dose-response curve for folate effects. Folate dose, stage (prenatal/postnatal) and duration of exposure are critical factors in determining tissue-specific outcome

Collin (Collin et al, 2010) measured plasma concentrations of folate, B12 (cobalamin), holohaptocorrin, holotranscobalamin ("active B12") total transcobalamin, and total homocysteine in 1,461 cases and 1,507 controls. They investigated whether both B12 and folate metabolism were associated with prostate-specific antigen (PSAV) as a proxy measure of prostate cancer progression in men with localized prostate cancer. Furthermore, thirteen folate pathway single nucleotide polymorphisms were genotyped for 311 participants. Post diagnosis PSAV was calculated. Median follow-up time was 2.5 years. Plasma folate was associated with an increased risk of PSAV > 2 ng/mL/yr, OR per unit increase in $\log_e$ concentration, OR = 1.57. This study provides some evidence that higher folate levels may be associated with faster prostate tumor progression. The MTRR 66A>G polymorphism was related to slower progression of localized prostate cancer. Note that the MTRR 66A>G polymorphism for methionine synthase reductase (MSR) catalyzes reductive methylation of the co-factor of [MS, B12, SAM], thus activating MS activity Figures 9 and 25. This polymorphism was associated with a reduced risk (OR, 0.33) for PSAV > 2 ng/ml/yr. The serinehydroxymethyltransferase (SHMT1) polymorphism (SHMT1 1420C>T) was associated with increased risk (per-allele OR, 1.49) for PSAV > 2 ng/ml/yr. Their work suggests that in addition to plasma levels of folate, folate-metabolizing enzyme polymorphisms are associated with the rate at which prostate tumors secrete PSA (PSAV), which relates to the rate of increase of prostate tumor volume (Berger et al, 2006). Elevated PSAV >2.0 ng/ml/yr as indicated by D'Amico increases the risk of death due to prostate cancer following radiation therapy despite having low-risk disease, Figure 2 (D'Amico et al, 2005). So it is conceivable that the combination of high plasma folate with or without associated folate enzyme polymorphisms may affect cure rates for some patients with prostate cancer.

Observations in our clinic of B12 alone and B12 plus folic acid stimulation of prostate cancer in two patients, and those of Tomaszewski's group (Tomaszewski et al, 2011) revealing direct correlation of Gleason's grade and Ki67 cellular proliferation index with both high serum and prostate tumor tissue folate, are compelling because they directly identify specific patients seen in the clinic that are adversely affected by what have largely been theoretical concepts developed from laboratory animal and large population studies.

19. Epidermal growth factor receptor (EGFR) and FA

Two colon cancer cell lines, Caco-2 and HCT-116, were maintained in medium containing 1μg/ml folic acid and supplemental folic acid was found to inhibit cell proliferation in a dose-dependent manner (Jaszewski et al, 1999). Pretreatment of the cell lines with supplemental FA (1.25 μg/ml) completely abrogated transforming growth factor-a (TGF-a)-induced proliferation in both. Tyrosine kinase activity and the relative concentration of EGFR were markedly diminished in both following a 24-h exposure to supplemental FA. FA-mediated reduced proliferation and EGFR tyrosine kinase pathway appeared to be involved.

To further establish the mechanism(s) by which FA affects EGFR, Nagothu (Nagothu et al, 2004) examined whether and to what extent supplemental FA or its metabolites modulate basal and serum-induced activation of the EGFR promoter in the HCT-116 colon cancer cell line. HCT-116 cells were pre-incubated with or without FA 10 μg/ml for 48 hours. Supplemental FA as well as its metabolites markedly inhibited EGFR promoter activity and its methylation status. Exposure of cells to 10% fetal bovine serum caused a marked

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stimulation of EGFR promoter activity and EGFR expression, both of which were greatly abrogated by supplemental FA or CH3—THF. Their work suggests that FA and CH3—THF inhibit EGFR promoter activity in colon cancer cells by enhancing promoter methylation. This could partly be responsible for FA-mediated inhibition of growth-related processes not only in colorectal but other neoplasia as well.

20. Effect of combined B vitamin insufficiency on the canonical Wnt kinase pathway

Inspection of Figures 8, 9 and 25 illustrates at least four different pathways where one-carbon vitamers of folate coenzymes depend on other B vitamins. Changes in the Wnt family of genes are prominent in prostate cancer (Yardy & Brewster, 2005; Zhu et al, 2004). In many tissues, activation of Wnt signaling is associated with neoplasia (Liu et al, 2007). Wnt-signaling was attenuated by combined B vitamin deficiency but not by singlet folate or doublet deficiencies. Increased levels of Wnt-11 found in prostate tumors contribute to tumor progression by promoting neuroendocrine dedifferentiation, tumor cell survival and cell migration/invasion (Uysal-Onganer et al, 2010). There is a positive correlation between Wnt-11 expression and PSA levels above 10 ng/ml. Beta-catenin protein, a critical molecular component of canonical Wnt signaling is capable of promoting androgen signaling through its ability to bind to the AR in a ligand-dependent (reminiscent of androgen) fashion thus allowing the beta catenin-AR complex to activate transcription of androgen-regulated genes. Based on Liu's study of colon cancer cells, one might expect that multiple deficiencies of B vitamins could modulate Wnt control of beta catenin in prostate cells. For excellent reviews of the importance of the Wnt gene and its effects in prostate metabolism, we refer Bisson and Prowse (Bisson & Prowse, 2009).

Various combinations of B vitamins and their metabolites are frequently elevated and/or low in untreated cancer patients as illustrated in Figure 11.

21. Conclusion

This review of B vitamin effects on one-carbon metabolism and neoplasia with focus on the prostate comes at a time when several countries are contemplating fortification of their nations' food supply with B12 and folic acid due to the known benefit in reducing neural tube defects in newborns. At the same time, "supplement mania" is rampant in the US and especially prominent in the prostate cancer patient population. FA-associated cancer acceleration as first reported by Lewisohn (Lewisohn et al, 1946) for mouse mammary cancer, Heinle (Heinle & Welch, 1948) for CGL, Tisman Tisman et al, 2009, Figueiredo (Figueiredo et al, 2009), Collin Collin et al, 2010 and Tomaszewski (Tomaszewski et al, 2011) for prostate cancer, is of utmost importance to those with potential or manifest occult prostate disease. Based on this review and our clinical experience we suggest a more targeted approach to vitamin supplementation, so that the benefit for one demographic does not come at the expense of another. Until more is learned, it may be best to adjust frequently abnormal serum levels of B vitamins into the normal range. It is clear that both vitamin deficiency and hypersufficiency have profound physiological effects that differentially affect select tissues. In the laboratory, the effects can be measured within days to weeks and have potential to cause life-threatening results that could have immediate effects on drug efficacy and toxicity or latent effects which may take years to manifest.

Intracellular one-carbon transfer reactions are essential for nucleotide (thymidylate from dUMP) and purine synthesis and diffuse methylation reactions that deliver methyl (CH3−) groups to DNA, RNA, proteins, and phospholipids. The one-carbon transfer reactions use intracellular polyglutamated reduced derivatives of FA. CH3-THF is the predominant form of folate in serum/plasma. The reduced folate carrier (RFC) delivers most CH3−THF into cells. RFC has a higher affinity for reduced folate and the chemotherapy folate inhibitor methotrexate than oxidized, pharmaceutical FA, and it accounts for the transport of most dietary, naturally occurring, reduced folate(s) (Brzezinska et al, 2000). Membrane-bound folate receptors, including folate receptor (FR) with very high affinity for pharmaceutical FA, are expressed in epithelial tissues and its expression is elevated in some malignant tumors. The predominant cytoplasmic folate, CH3−THF, donates its one-carbon moiety to methylate homocysteine to methionine, regenerating usable THF. THF is the much-preferred substrate to folypolygammaglutamyl synthetase (FPGS) that extends the glutamate chain enhancing intracellular retention and folate substrate binding strength to folate metabolizing enzymes. Methionine, from methylation of homocysteine and dietary ingestion, is converted to S-adenosylmethionine (SAM), a universal donor of CH3− groups to numerous methylation reactions through methyl transferase enzymes. SAM supplies CH3 − groups to cytosine bases positioned across from guanine as CpG islands of DNA and histones surrounding the DNA double helix. The methylation patterns are both heritable and subject to acute (drug) and chronic dietary effects restricting or enhancing methyl group precursors responsible for epigenetic control of both tumor suppressor and tumor promoter genes. The active coenzyme THF acquires a one-carbon moiety from the amino acid serine via serine hydroxy methyl transferase, a vitamin B6 requiring coenzyme. There are at least two forms of SHMT enzyme, one active in mitochondria, (mSHMT or SHMT2) and one active in cytoplasm (cSHMT or SHMT1). This metabolic catalysis of glycine with THF yields 5,10-methyleneTHF, a critical compound at the center of a switching point of CH3− delivery. The cSHMT form shuttles CH3−methylation reactions in the direction of the *de novo* synthesis of thymine from deoxyuridine. cSHMT activity is enhanced by the heavy chain of ferritin, a compound critical in iron metabolism. Many reactions depend on genetic coenzyme polymorphisms such as MTHFR C677TT weakening the enzyme's usual function by up to 70%. Regional B vitamin concentrations (B9, B12, B6, B2) affect delivery of CH3− from 5,10-methyleneTHF to either dUMP to form DNA thymine/thymidine (required in rapidly growing cells) or to methylation of homocysteine via B12-requiring transfer of CH3− from CH3−THF thus generating more SAM. Concentrations of SAM and DNA epimethylation enzymes control gene promoter and global gene epimethylation. This turns ON and OFF various tumor suppressor (TSG) and tumor associated (TAG) genes.

The robustness of the homocysteine methylation/SAM generation depends on the concentration and functional state of the methionine synthase-B12-enzyme complex. B12 deficiency from adsorptive, dietary, drug interference, N2O anesthesia inactivation (still common during routine surgeries) and anomalies of holotranscobalamin will eventually shut down global methylation through impaired synthesis of SAM and the ternary complex formed between thymidylate synthase, 5,10-methyleneTHF and dUMP which supplies TMP for DNA synthesis. This leads to uracil misincorporation into DNA resulting in DNA point mutations and finally to chromosomal breaks, and malignant transformation.

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22. Acknowledgements

23. References

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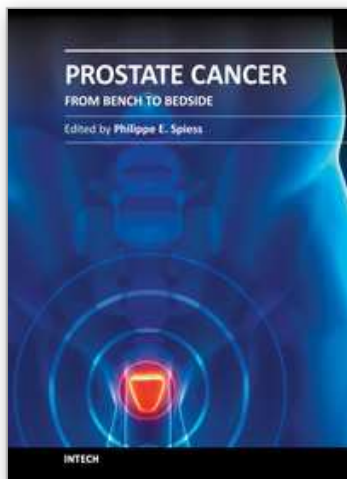
Prostate Cancer - From Bench to Bedside

Edited by Dr. Philippe E. Spiess

The present textbook highlights many of the exciting discoveries made in the diagnosis and treatment of prostate cancer over the past decade. International thought leaders have contributed to this effort providing a comprehensive and state-of-the art review of the signaling pathways and genetic alterations essential in prostate cancer. This work provides an essential resource for healthcare professionals and scientists dedicated to this field. This textbook is dedicated to the efforts and advances made by our scientific community, realizing we have much to learn in striving to some day in the not too distant future cure this disease particularly among those with an aggressive tumor biology.

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