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NUTRIENT-GENE INTERACTIONS WITHIN ONE-CARBON METABOLISM AND EFFECTS ON EPIGENETIC REGULATION THROUGH DNA METHYLATION IN PERIPHERAL BLOOD MONONUCLEAR CELLS

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ABSTRACT

Epigenetics is a field of molecular biology that copes with the study of gene function regulation without variations in DNA structure or nucleotide sequences.

Among the main epigenetic phenomema in eukaryotic cells there are DNA methylation and post-traslational mechanisms among which the major are histone methylation and acetylation.

Epigenetic changes are potentially reversible phenomena that are controlled also by nutritional factors as the methyl-donors involved in the folate cycle.

Plasma levels of B vitamins, among which "*in primis*" plasma folate concentrations, are implicated in epigenetic modulation so that it can be hypothesized that they may affect the modulation of gene expression through epigenetic mechanisms.

Epigenetic modifications represent one of the earliest events in the genesis of some complex pathologies, therefore the study of the interaction between epigenetics and nutritional status is of great interest either to define the physiopathological mechanisms of development of some illnesses, and for possible personalized strategies of prevention.

The present work has been articulated, at first, on the analysis of gene-nutritional interaction mechanisms within the folate cycle through the study of polymorphisms of enzymes involved in the metabolism of methyl-group donors; the aim was to study their possible role on the modulation of genomic DNA methylation in relationship to different plasma levels of idrosoluble B

vitamins. In this regard, the most important functional polymorfisms known on the genes of one-carbon metabolism and their relationship with methylation status of polymorphonuclear cells DNA have been analyzed from a cohort of around 800 subjects within a clinical study, underlining the role of the key folate-related enzymes in the modulation of DNA methylation.

Besides the function of genomic DNA methylation, the methylation status at specific sites has been also approached with the specific intent of considering a possible interrelationship between the role of promoter methylation and the co-presence of functional polymorphisms in the same genic site for a gene for which a precise functional effect is well-known. To address this issue the promoter region of coagulation factor VII gene was evaluated for both genetic and epigenetic modifications as a possible model of genetic-epigenetic interaction in the modulation of gene product regulation. The results showed the key importance of genetic-epigenetic interactions, so far unknowm, in modulating gene-expression at promoter gene sites.

The role of other vitamins involved in one-carbon metabolism in major chronic diseases, and specifically the emerging role of B6 vitamin, have been also studied.

Furthermore, a clinical study is now in progress to evaluate the function of gene-specific methylation in liver tissue where most of the folate cycle functions take place. The aim of this project is the evaluation of both genome-wide and gene-specific methylation status in the liver in comparison to that observed in peripheral blood mononuclear cells DNA to define

whether methylation status of peripheral blood DNA may be regarded as a good systemic biomarker for this epigenetic feature of DNA in relation to B vitamins nutritional status in cancer disease. Results from this study may help to define possible functional markers of gene-nutrients interactions with effects on epigenetic modulation for future preventive or therapeutic strategies. With that purpose, a novel high-throughput array-based technique for the detection of gene-specific methylation at promoter sites has been optimized in our laboratory.

INTRODUCTION

Epigenetics is defined by heritable changes in gene function that occur without changes in DNA sequence and it transmits non-coded information in DNA sequence from mother cell to daughter cell, from generation to generation, through mitosis. The main epigenetic phenomena are DNA methylation and histones modification, the DNA packaging proteins. When DNA methylation occurs within genes or gene regulatory elements, such as exons and promoters, generally suppresses the transcription of the gene, while when it takes place within gene deficient regions, as heterochromatin, assumes a crucial role in maintaining the conformation and integrity of the chromosome. Methylation of the mammalian genome undergoes major changes during early development and appears to be an integral mechanism for rapid differentiation and formation of various tissues and organs. When the differentiation is to be completed, the methylation pattern shows a tissue-specificity while the model remains essentially stable during later life. However, these models are not immutable and they can be influenced by nutritional status.

Nutrients involved in one-carbon metabolism, such as methionine, choline, betaine, folate, vitamin B12, vitamin B6, riboflavin, zinc and selenium, can alter the methylation status by interfering with the cellular levels of S-adenosylmethionine and S-adenosylhomocysteine. Those nutrients, in fact, play a crucial role in the epigenetic control of gene expression and genome

integrity. Therefore the study of the interaction between epigenetics and nutritional factors is critical both for a better understanding of the physiological mechanisms and for the assessment of possible underlying mechanisms of disease, especially those leading to carcinogenesis. Studies concerning the modulation of epigenetics by nutritional factors are needed to understand the molecular mechanisms underlying these interactions and, at the same time, to find new ways for disease prevention and treatment.

CHAPTER 1

ONE-CARBON METABOLISM BETWEEN GENETICS AND EPIGENETICS

FOLATE AND EPIGENETICS

One-carbon metabolism

One-carbon metabolism is a network of interconnected biochemical reactions in which a one-carbon unit is transferred from a donor to tetrahydrofolate (THF) for subsequent reduction or oxidation and/or transfer into biochemical pathways essential for DNA synthesis (thymidylate and purine synthesis) and biological methylation (methionine synthesis or serine-glycine interconversion) (Figure 1).

Folate coenzymes act as acceptors or donors of one-carbon units in a multiplicity of reactions involved in amino acid and nucleotide metabolism in mammalian tissue[1].

One-carbon metabolism is essential mainly for the methylation reaction and for the synthesis of nucleotides.

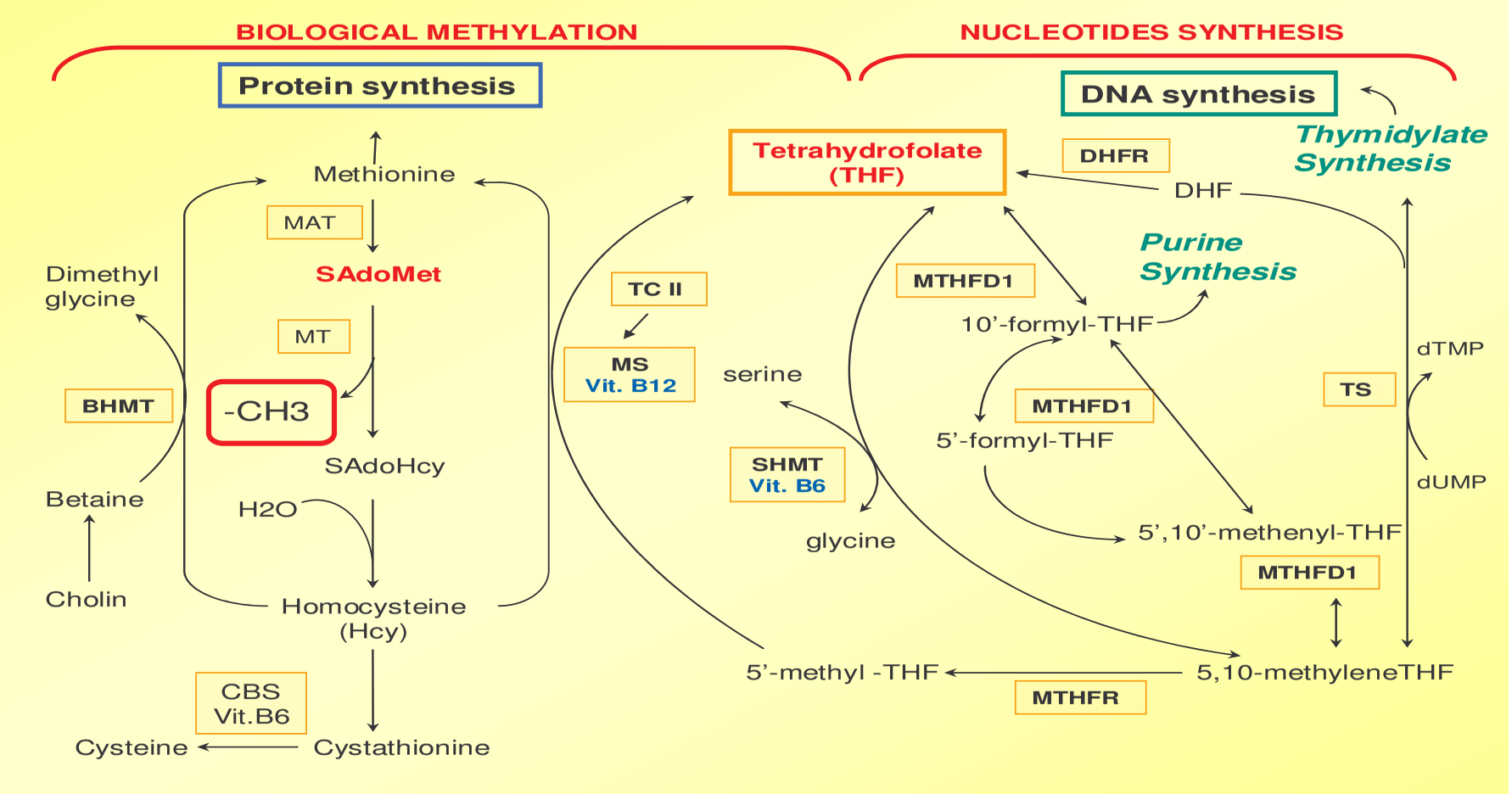

Figure 1. One carbon metabolism THF: tetrahydrofolate; DHF: dihydrofolate; SAdoMet: S-adenosylmethionine; SAdoHcy: S-adenosylhomocysteine; MTHFR: methylenetetrahydrofolate reductase; MS: methionine synthase; MT: methyltransferases; TS: thymidylate synthase; MAT: methionine-adenosyl transferase; SHMT: serine hydroxymethyltransferase; DHFR: didydrofolate reductase; CBS: cystathionine β-synthase; BHMT: betaine homocysteine methyltransferase; TC II: transcobalamin II; MTHFD1: methylene-tetrahydrofolate dehydrogenase/methenyl-tetrahydrofolate cyclohydrogenase/formyl-tetrahydrofolate syntetase.

Methylation reactions

One of the essential reactions in one carbon metabolism is the reversible transfer of formaldehyde from serine to tetrahydrofolate (THF) to generate glycine and 5,10 methylene-tetrahydrofolate (5,10-methyleneTHF): this reaction is catalyzed by Serinehydroxymethyl-transferase (cSHMT), an enzyme that utilizes pyridoxal-5'-phosphate (PLP or vitamin B6) as coenzyme. After that, another central enzyme, methylenetetrahydrofolate-reductase (MTHFR), irreversibly catalyzes the reaction from 5,10-methyleneTHF to 5-methyl-tetrahydrofolate (5-methyl-THF), the primary methyl donor for the remethylation of homocysteine to methionine by methionine-synthase (MS).

MS catalyses the remethylation of homocysteine to methionine, depending on cobalamin (vitamin B12) and folate, in a reaction in which 5-methyl-THF serves both as a cofactor and as a substrate. The reduced availability of 5-methyl-THF, the main circulating form of folate, decreases the biosynthesis of S-adenosylmethionine (SAdoMet or SAM), thus limiting the availability of methyl groups for methylation reactions.

Methionine is then converted to SAdoMet, the methyl group donor for over 80 biological methylation reactions, including those of DNA, RNA, proteins and lipoproteins.

After the transfer of a methyl group, SAdoMet is converted to S-Adenosylhomocysteine (SAdoHcy or SAH). Under normal conditions, SAdoHcy is hydrolyzed by SAdoHcy hydrolase to adenosine and

homocysteine. However, this reaction is rapidly reversible with equilibrium dynamics that strongly favor SAdoHcy synthesis rather than its hydrolysis. Thus, chronic elevation in plasma homocysteine levels may have an indirect and negative effect on cellular methylation reactions through a concomitant increase in intracellular SAdoHcy concentrations[2].

Homocysteine can be methylated to regenerate methionine by folate/vitamin B12 dependent MS reaction and additionally by the betaine-homocysteine methyltransferase (BHMT) reaction using choline and betaine pathway in the liver and kidney. Homocysteine also condenses with serine to form cystathionine in an irreversible reaction catalyzed by cystathionine-β-synthase (CBS), using vitamin B6 as a coenzyme.

Nucleotides synthesis

Thymidylate synthase catalyzes the transfer of methyl groups from folate to deoxyuridylate, producing thymidylate, *via* a rate-limiting reaction for DNA synthesis[1]. Since folate-derived one–carbon groups are essential for the *de novo* synthesis of thymidylate, inhibition of folate metabolism in mammalian cells results in uracil misincorporation into DNA due to an imbalance in the deoxyribonucleotide pool. It has also been shown that folate deficiency results in excess uracil incorporation into human DNA[3]; similarly, a moderate, chronic folate depletion in rats has been shown to result in excess uracil incorporation in colonic DNA[4]. One-carbon moieties from folate are also utilized in *de novo* purine biosynthesis.

Epigenetic mechanisms

Appropriate gene expression regulation is critical to maintain normal function and homeostasis in each cell type and aberrant gene expression can induce cell aberrations ultimately leading to disease development. At least seven potential control steps are involved in the regulation of gene expression: control of chromatin structure, initiation of transcription, processing of the transcript, transport to the cytoplasm, translation of mRNA, mRNA stability and protein activity stability [5]. Epigenetics is one of the main mechanisms to control gene expression. Epigenetics explains non-coding changes in the genome which are transmitted through mitosis and alter gene expression[6]. These epigenetic phenomena, which include DNA methylation and post-translational modifications of histones (acetylation, methylation, phosphorylation, ADP ribosylation, sumoylation and ubiquitination), are critical for the embryonic development, imprinting, aging, and the process of development of chronic diseases. In this regard, epigenetic phenomena have been mainly studied in cancer although several other pathological conditions have been proposed to be involved by underlying epigenetic phenomena. In contrast to mutations, in which DNA structure undergoes an irreversible change through induction of altered bases sequence, epigenetic phenomena are reversible and can be modulated by nutrients (figure 2).

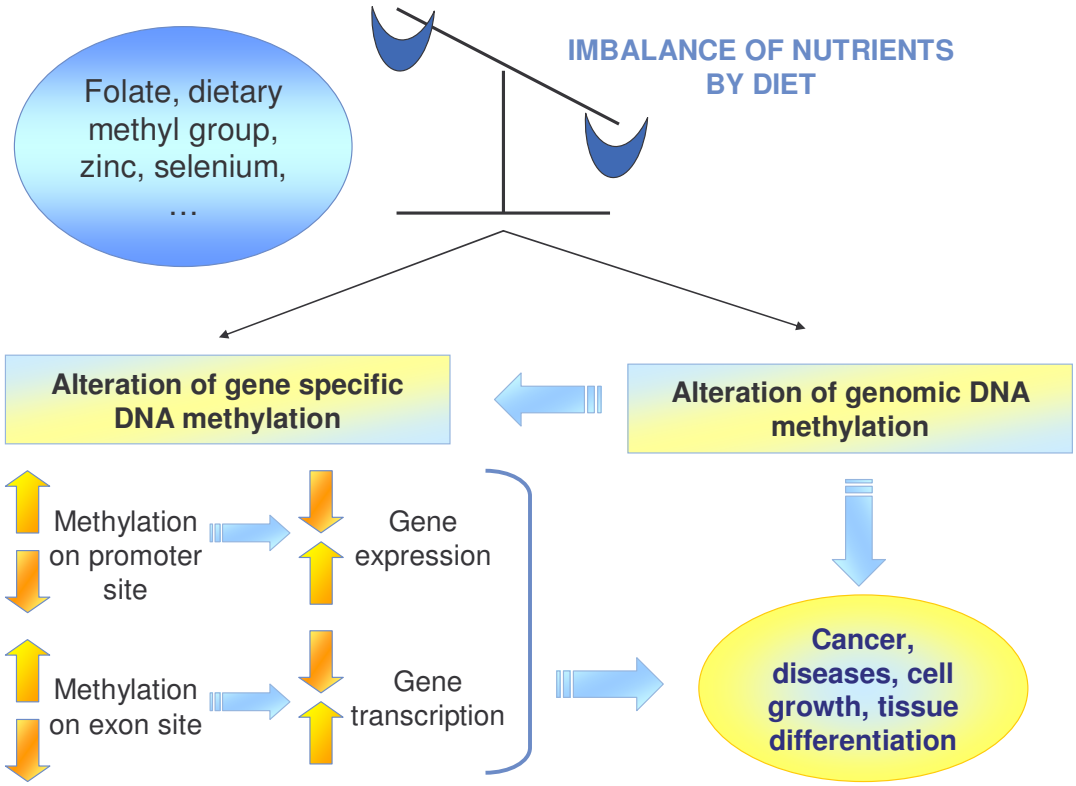

Figure 2: Gene expression and genome integrity may be modulated by DNA methylation through the nutrients in the diet. An imbalance of nutrients can determine aberrant gene expression and consequently chronic pathological conditions such as, for instance, cancer disease.

Methylation of cytosine is a unique endogenous modification of DNA occurring in mammalian cells in which DNA methyltransferases catalyze the transfer of a methyl group from SAdoMet to the carbon-5' position of cytosine in CpG dinucleotides[7].

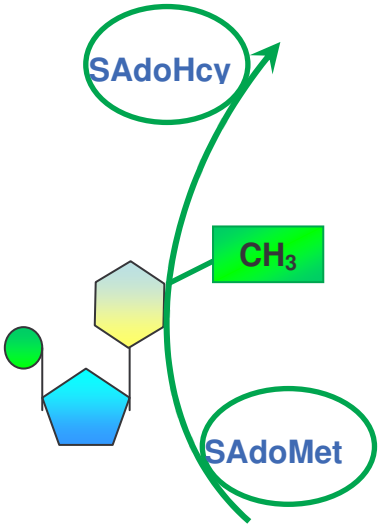

Figure 3: Representation of the DNA methylation process: the transfer of a methyl group from SAdoMet to carbon of a cytosine in the 5'-CpG-3' sequence.

Methylation within gene regulatory elements (promoters, enhancers and repressors) generally suppresses the function of the gene. Methylation within gene deficient regions (pericentromeric heterochromatin) is essential for maintaining the conformation and integrity of the chromosome.[8, 9] Methylation may be also a genome defense mechanism against movable genetic elements[10].

Genomic DNA methylation: effect of nutrients

Many micronutrients and vitamins are essential in DNA metabolic pathways[5,11] and, moreover, several nutrients are significant in maintaining genomic stability.

DNA methylation state, especially in genome-wide methylation, can be regulated through the availability of methyl groups, by aminoacids as methionine, choline, betaine and serine, contained in the diet, and also by bioactive nutrients, as zinc, selenium and retinoic acid in modifying the methyltransferases or the enzymes of one-carbon metabolism.[12, 13, 14, 15, 16,17] Nevertheless, folate and/or methyl group dietary supply provides the most convincing data for the interaction of nutrients and DNA methylation, because these dietary elements are directly involved in DNA methylation *via* one-carbon metabolism. The metabolic function of all coenzymatic forms of folate is to transfer one-carbon units for the synthesis of SAdoMet, the universal methyl donor for several biological methylation reactions, and the *de novo* deoxynucleoside triphosphate synthesis. Therefore, a dietary folate depletion may decrease genomic DNA methylation in humans[18, 19] and in animal models[20] and, on the other hand, a folate replete diet may restore the DNA methylation status[19].

Gene-specific DNA methylation at the promoter region

The 5'-promoter regions or the first exons of around one-half of human genes are areas very rich in CpG sequences called CpG islands[21]. At the promoter region, CpG-rich sequences usually are unmethylated[22]. The methylation of these CpG islands usually induces inhibition of their expression. Although the exact molecular mechanism by which DNA methylation represses the transcription is not yet clear, several data demonstrate an active role of

promoter methylation in gene silencing. For exemple, demethylation by 5-azadeoxycytidine, a DNA methyltransferase inhibitor, leads to re-expression of previously methylated genes[23].

The mechanism for the CpG island-associated gene silencing seems to involve the link of specific methylated DNA binding proteins, followed by the recruitment of a silencing complex that includes histone deacetylases[11, 24].

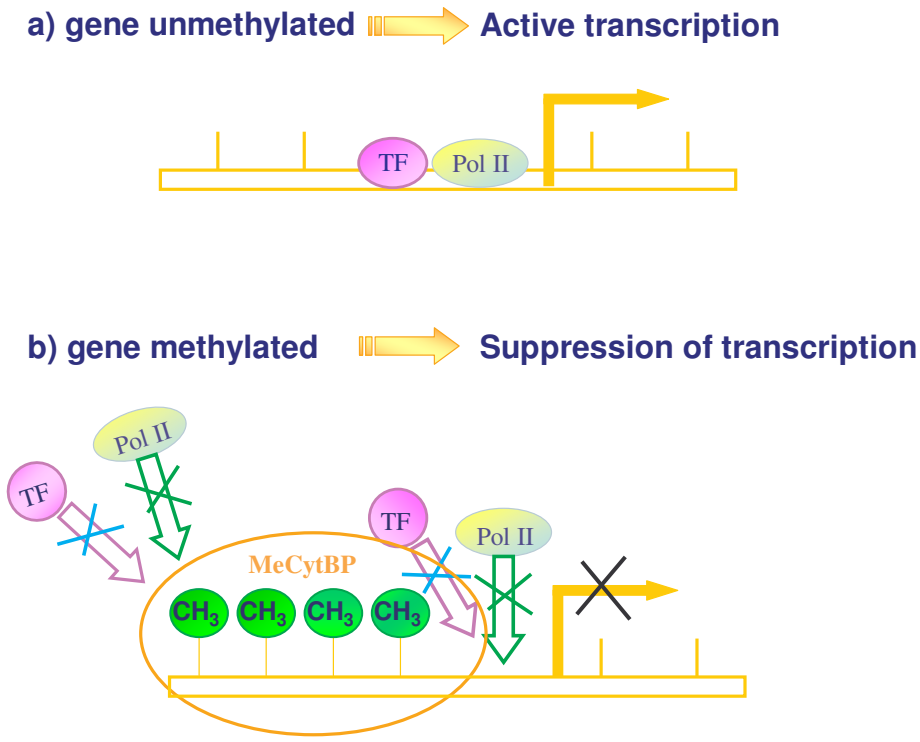

Figure 4: Gene-specific DNA methylation or unmethylation at the promoter region

Methylation, of DNA at promoter sites engages methyl-binding proteins, which also attracts specific protein complexes that contain histone deacetylases. Through the action of methyl-binding proteins and histone

deacetylases, the DNA structure changes to a compact, condensed chromatin configuration that results in permanent inhibition of messenger RNA and protein production[11]. In carcinogenesis, hypermethylation of CpG islands in promoter region is mainly associated with transcriptional silencing of gene expression, which has an important role as an alternative mechanism by which tumor suppressor genes are inactivated without mutation or allele deletion. On the other hand, hypomethylation of CpG islands is associated with the gene activation, which also is an important mechanism by which proto-oncogenes are activated.

Gene-specific DNA methylation at the coding region

Hypermethylation of the coding region can decrease the gene transcription since local cytosine methylation of a specific sequence can directly interfere with the binding of certain transcriptional factors[25]. On the other hand, the gene transcription of the coding region can be increased by hypomethylation, enhancing the binding of transcription factors.

Some observations propose that nutrients may affect gene transcription by gene-specific alteration of DNA methylation.

In hepatocarcinogenesis, where a chronic dietary methyl deficiency has been demonstrated, the progressive loss of methyl groups at most CpG sites on both coding and noncoding strands in hepatic DNA during the early phase of folate/methyl deficiency was observed[26]. After tumor formation, the majority

of cytosines became remethylated. In the preneoplastic nodules, the level of *p53 mRNA* was increased and associated with hypomethylation in the coding region, whereas in cancer tissue, *p53 mRNA* is decreased and associated with relative hypermethylation. This observation suggests that a folate/methyl-deficient diet induces liver cancer by affecting the methylation status of the *p53* gene coding region and by consequent alteration of *p53* gene transcription[26]. In other methyl-deficient animal studies, increased levels of mRNA for *c-fos*, *c-Ha-ras* and *c-myc* were correlated with hypomethylation at specific sites within these genes[27, 28].

Hypomethylation of the coding regions of critical genes can lead to genome instability either because this region becomes more susceptible to endogenous nucleases[29] or because the site of hypomethylation is likely to undergo enzymatic deamination to uracil[30, 31]. The second situation is more frequently observed in conditions where intracellular *S*AdoMet levels are low, such as in a folate depletion status[31].

Several animal studies and cell culture studies confirm that folate deficiency might induce DNA strand breaks and subsequent mutations through exon site hypomethylation[30, 32, 33, 34, 35].

At the other hand, in an animal model of chemical carcinogenesis, folate supplementation reduces gene disruption by reversing the site-specific DNA hypomethylation[33].

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GENE-NUTRIENT INTERACTIONS IN ONE-CARBON METABOLISM: ROLE OF CYCLE FOLATE POLYMORPHISMS

The *677C>T* polymorphism of *MTHFR* gene: a clear model of gene-nutrition interaction

Within the folate cycle, various enzymes have been studied in relationship to their role in the principal metabolic ways that interest the synthesis of nucleic acids and epigenetic modifications.

The enzymatic function mostly examined concerns the Methylenetetrahydrofolate reductase (MTHFR; EC 1.5.1.20).

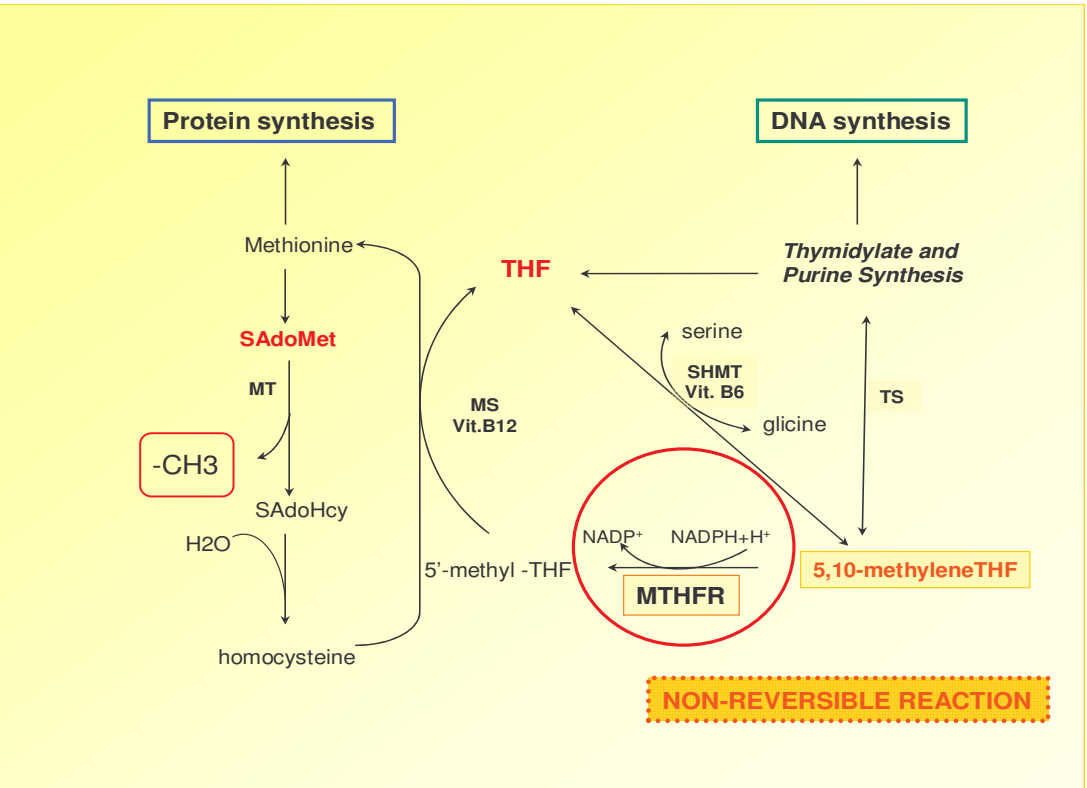


Figure 1: Role of *MTHFR* in one-carbon metabolism

MTHFR catalyzes the irreversible conversion of 5,10-methylene-THF to 5-methyl-THF, that is the donor of methyl groups for the synthesis "*de novo*" of methionine[1] and it represents the key enzyme in one-carbon metabolism because it is located to the intersection between the metabolic way of nucleic acids synthesis and that of methylation.

In 1988 a variant of the MTHFR enzyme that causes thermolability and reduced activity was identified[2]. The thermolabile variant of the *MTHFR* is due to a common missense gene mutation, a cytosine-to-thymine transition at base pair *677* (*677C>T*)[3] that results in an alanine-to-valine substitution in the MTHFR amino acid sequence. The prevalence of the valine-valine substitution is rather common, with a frequency of homozygous persons up to 20% in certain populations [3, 4, 5]. The mutant enzyme was associated with elevated plasma homocysteine levels, because the conversion of homocysteine to methionine is impaired[1]. It was demonstrated that only homozygous subjects for this polymorphism (*677TT* genotype) with inadequate blood folate levels, showed elevated plasma homocysteine concentrations[6]. An intermediate effect has also been observed in heterozygous subjects[5]. Following studies conducted also by our group[7] showed that the *MTHFR 677C>T* polymorphism, through an interaction with folate status, influences DNA methylation status: in fact genomic DNA methylation in peripheral blood mononuclear cells directly correlated with folate status and inversely with plasma homocysteine levels. *MTHFR 677TT* genotypes had a reduced level of DNA methylation compared with those with the *677CC* wild-type. When analyzed according to folate status, however,

only the *TT* subjects with low levels of folate accounted for the reduced DNA methylation.

An interesting model of gene-nutrient interaction in phenotypic expression is clearly shaped in this model[5, 6]. Moreover, the *MTHFR 677C>T* polymorphism is related to the reduced availability of methyl-THF and it has relevant implication in cardiovascular disease[8] and carcinogenesis [9, 10, 11, 12].

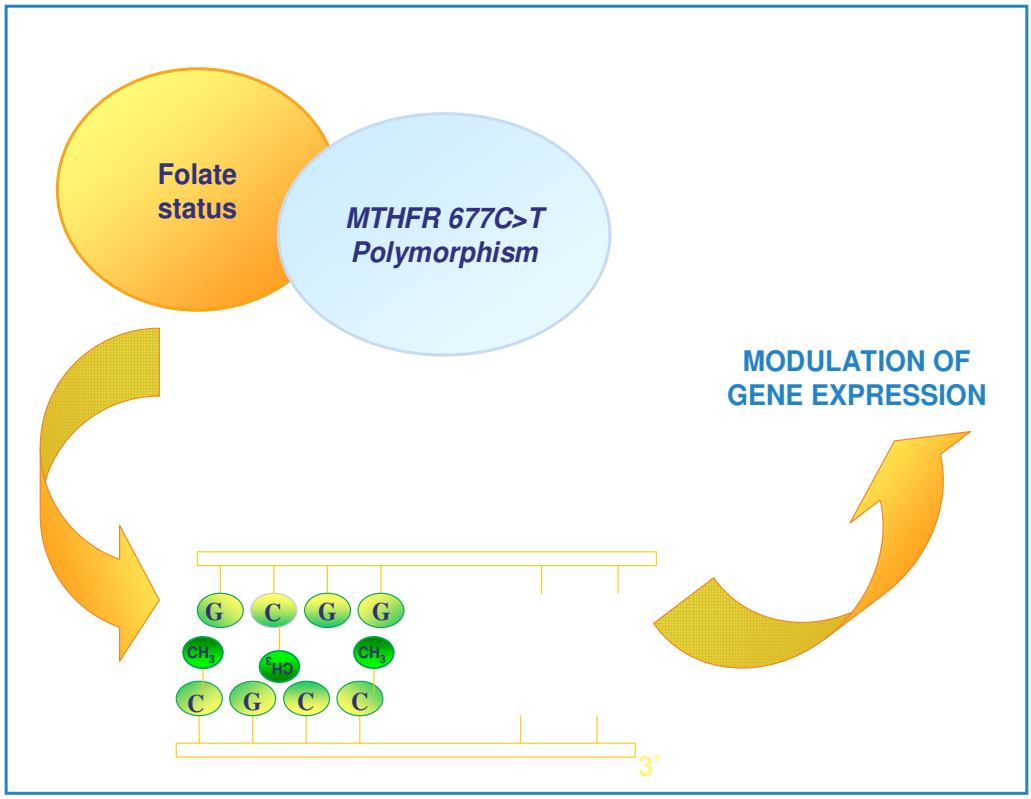


Figure 2: The *MTHFR* polymorphism: a clear model of gene-nutrition interaction

Aim

Starting from the observations on the role of MTHFR enzyme for gene-nutrition interaction models, we intended to appraise the principal polymorphisms of the other enzymes involved in folate metabolic cycle, to highlight possible similar examples of interactions among genetics, epigenetics and nutrition and therefore to study the possible effects of gene-nutrition interactions on the main epigenetic feature of DNA, DNA methylation.

Materials and methods

Around eight hundred subjects, sex and age-matched, have been recruited . They were for the greatest part native of the Veneto Region in Italy or born in neighbouring regions in Northern Italy. For each selected subject, biochemical data concerning one-carbon metabolism (plasma concentrations of folate, vitamin B12, vitamin B6 and homocysteine) and a complete clinical history were collected. Subjects with known conditions that could affect one-carbon metabolism were excluded from the study, such as subjects who used multivitamins supplements and/or with acute illnesses and/or making use of drugs interfering with folate metabolism (*i.e.* penicillinamine, anticonvulsants, methotrexate). The allelic frequencies and the genotypes were studied; moreover,the relationship between the presence or absence of each polymorphism and genomic DNA methylation status were analyzed.

DNA was extracted from peripheral blood mononuclear cells. Plasma homocysteine levels were measured by HPLC (High performance liquid chromatography), according to the method described by Araki and Sako[13]. Folate plasma levels and vitamin B12 were determined using an automatic method based on chemiluminescence technique (Chiron Diagnostics, East Walpole, MA).

The levels of vitamin B6 were measured as plasma pyridoxal phosphate (PLP), the active form of vitamin B6, by HPLC using a method described by Kimura et al[14].

Genotyping was performed using a standard PCR method followed by digestion with site-specific restriction enzyme or by allelic discrimination method through Real-Time PCR AB 7500.

Genomic DNA methylation was performed by a method that uses a combined technology with liquid chromatography and mass spectrometry (LC/MS)[15]. Briefly, DNA was denatured by heat at 100℃ for three minutes then rapidly cooled in ice and digested sequentially by three enzymes. Approximately 20 µL of solution containing DNA was hydrolyzed and instilled into the analytical column protected by a pre-column and a separation of four major DNA bases and the 5'-methyl-2'desossicytidine was obtained by an isocratic elution. The quantification of non-methylated and methylated cytosine bases were made through isotopic isomers as internal standards of known concentration (stable isotopes). DNA genomic methylation was defined as the amount of 5-methyl-

cytosine to total bases cytosine (methylated and non methylated) in the DNA analyzed.

Results and comments

The following polymorphisms were studied:

- *DHFR – Dihydrofolate-reductase (EC 1.5.1.3.): polymorphism 19 pb ins/del*
- *MS - methionine synthase (EC 2.1.1.13) polymorphism 2756 A>G*
- *TS - thymidylate synthase (EC 2.1.1.45) polymorphism 2rpt-3rpt*
- *cSHMT - Serine hydroxy-methyl-transferase (EC 2.1.2.1): polymorphism 1420 C>T*
- *MTHFD1 - Methylene-THF dehydrogenase (EC 1.5.1.5) / metenil-THF-cicloidrogenasi (EC 3.5.4.9) / formyl-THF synthetase (EC 6.3.4.3) polymorphism 1958 G> A*
- *TCII - transcobalamin II polymorphism 776 C> G*
- *BHMT - Betaine-homocysteine methyl-transferase: polymorphism 742 A>G*

Of each polymorphism we set out to assess the allele frequencies and genotypes in our population.

polymorphisms	Wild-type allele frequence	Mutant allele frequence	Wild type genotype	Eterozygous mutant genotype	Homozygous mutant genotype
MS 2756 A>G	0,81	0,19	66%	29%	5%
TS 2rpt/3rpt	0,46	0,54	21%	49%	30%
cSHMT 1420 C>T	0,75	0,25	55%	39%	6%
DHFR 19bp ins/del	0,61	0,39	38%	47%	15%
MTHFD1 1958 G>A	0,42	0,58	16%	52%	32%
TCII 776 C>G	0,58	0,42	33%	50%	17%

Table 1: allele frequences and genotypes for the most studied polymorphisms.

The analysis was particularly focused on *DHFR 19 bp ins/del* polymorphism. Among the other enzymes, the polymorphisms of *MS*, *TS* and *cSHMT* deserve a mention.

Methionine synthase and role of *MS polymorphism 2756 A>G* on epigenetic modulation

MS catalyzes the re-methylation of homocysteine to methionine with simultaneous conversion of 5-methyl-THF to THF. This reaction is dependent on vitamin B12 and folate which determine the availability of methyl groups as 5'-methyl-THF, because 5-methyl-THF, produced during the reduction catalyzed by MTHFR, forms the substrate.

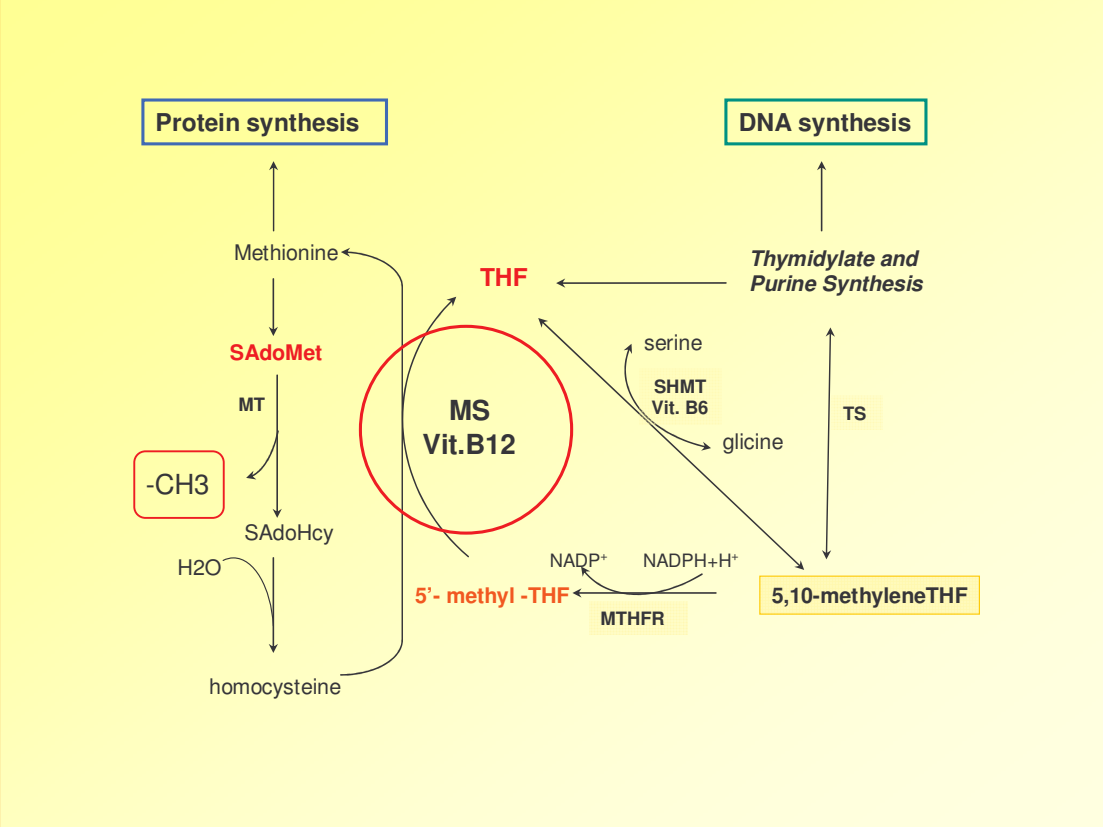


Figure 3: role of MS in the folate cycle

We studied the *polymorphism MS 2756 A> G* in the gene coding for the MS enzyme. This variant consists of replacement of one adenine (A) with guanine (G) at position 2756 on the gene, corresponding to the replacement of aspartate with glycine at codon 919 of the amino acid chain.

In some studies it was observed that the concentration of homocysteine would tend to decrease linearly in relation to genotype: the wild type (*2756AA*) would seem to be associated with a higher concentration of homocysteine[16].

Although our population had a homocysteine reduction in mutants compared to wild type, but this change was not statistically significant (15.96 µmol/L in *AA* vs 14.58 µmol/L in *GG*, *P*=NS).

The subjects carrying the polymorphism *2756 A>G* (genotype *2756 GG*-mutant) showed a significant reduction in genomic methylation compared to wild type (*2756 AA*).

This means that this polymorphism has effects on the epigenetic modulation.

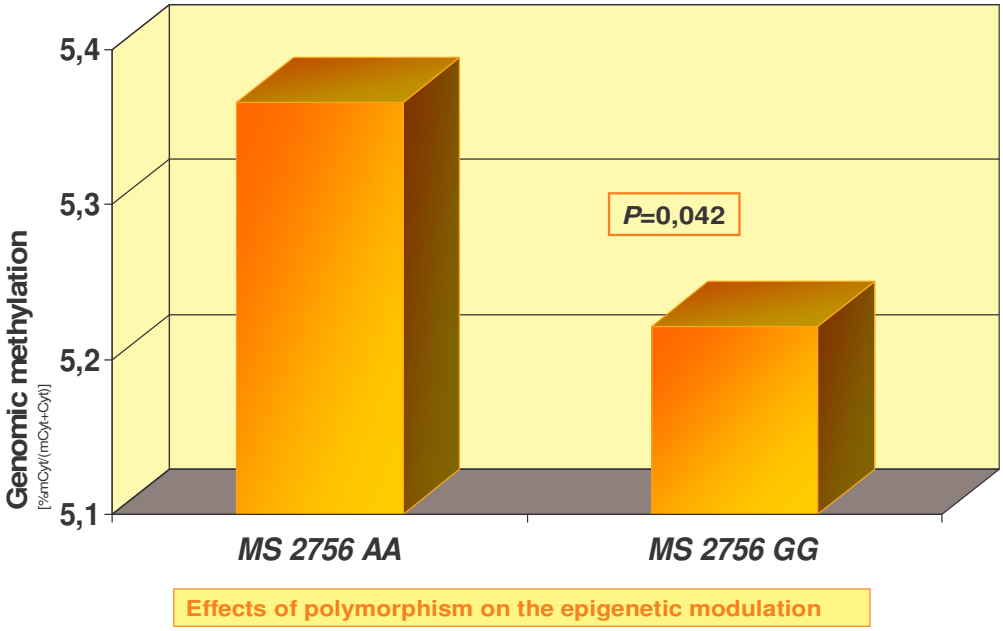


Figure 4: effects of *MS 2756 A>G* polymorphism on the epigenetic modulation

Thymidylate synthase and role of polymorphism *2rpt-3rpt* on plasma folate levels

One-carbon metabolism is also essential for the synthesis of deoxy-ribonucleotides.

The enzyme thymidylate synthase (*TS*) catalyzes the transfer of a methyl group (formaldehyde) from 5,10-methylene-THF to the position 5 'of the deoxy-uridyl (dUMP) with the production of deoxy-thymidylate (dTMP) and 7 ,8-dihydrofolate (DHF). Since the immediate deoxynucleotides are substrates for polymerases involved in DNA repair and replication, a correct DNA synthesis depends on the availability of deoxynucleotides.

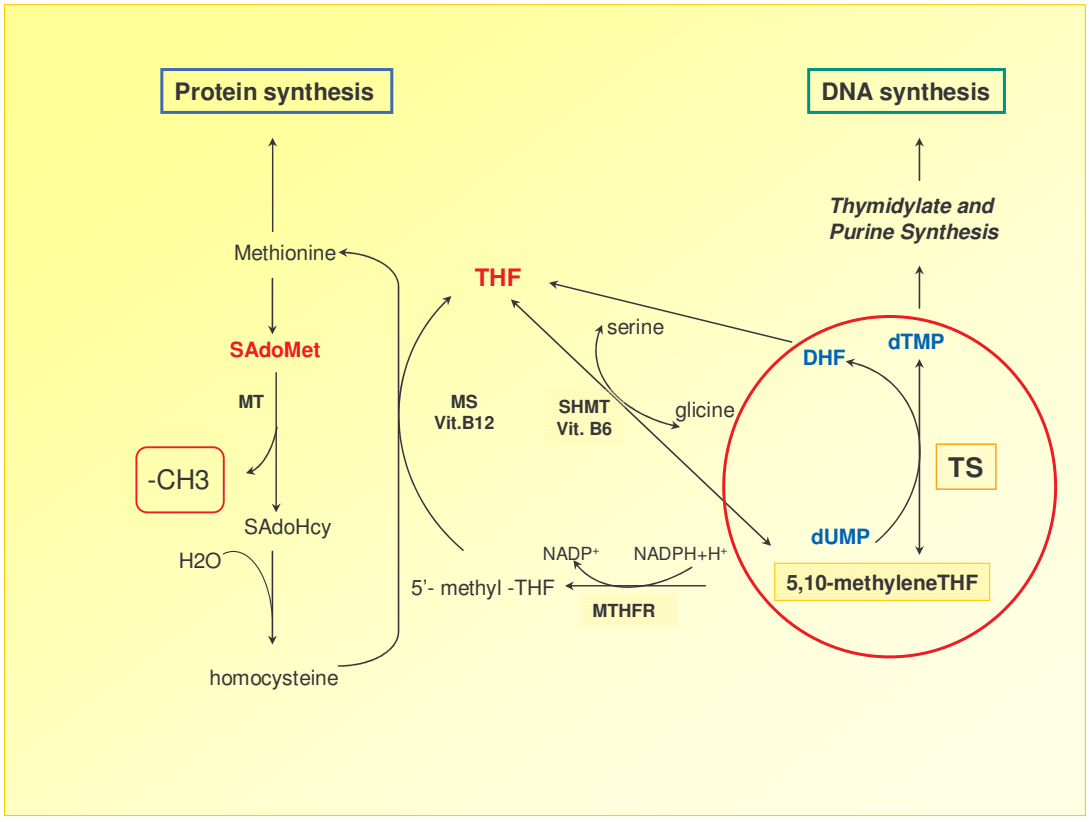


Figure 5: role of TS enzyme in the folate cycle.

We studied the *TS 2rpt/3rpt* polymorphism: in the promoter region, near the 5' position, the gene coding for the *TS* contains a variable number of "tandem repeats", consisting of *28 bp*, able of inducing transcription and thus increase gene expression[17, 18].

Wild type subjects have two sequence repeats (*2rpt/2rpt*), while the mutated are characterized by three sequence repeats (*3rpt/3rpt*) or, more rarely, by a higher number of *28 bp* repeats (4-9). In some studies[19], *3rpt/3rpt* genotype was associated with reduced folate plasma concentration and hyperhomocysteinemia (only in subjects with low folate levels). From the comparison between wild type subjects (*2rpt/2rpt*) and those who have the homozygous variant (*3rpt/3rpt*), there were no significant differences in the various endpoints.

	TS 2rpt/2rpt ***N=152***	***TS 3rpt/3rpt*** ***N=212***	***P***
tHcy (µmol/L)	14.84 (13.71-16.06)	16.33 (15.24-17.50)	N.S.
Folate (nmol/L)	12.12 (11.16-13.16)	11.54 (10.63-12.53)	**0,041**
Vitamin B_6 (nmol/L)	31.18 (28.23-34.44)	32.28 (29.27-35.59)	N.S.
Vitamin B_{12} (pmol/L)	383.56 (360.11-408.58)	395.16 (369.33-422.80)	N.S.
Genomic DNA methylation [%mCyt/(mCyt+Cyt)]	5.32 (5.32-5.32)	5.38 (5.34-5.41)	N.S.

Table 1: comparison between wild type and mutant homozygous for *TS 2rpt/3rpt* polymorphism.

There was, however, a significant reduction of folate plasma levels in the subjects with mutation compared to wild types (*P*=0,041), likely because the enzyme in its mutated form has higher enzyme activity and so it metabolized a higher amount of folate (figure 5).

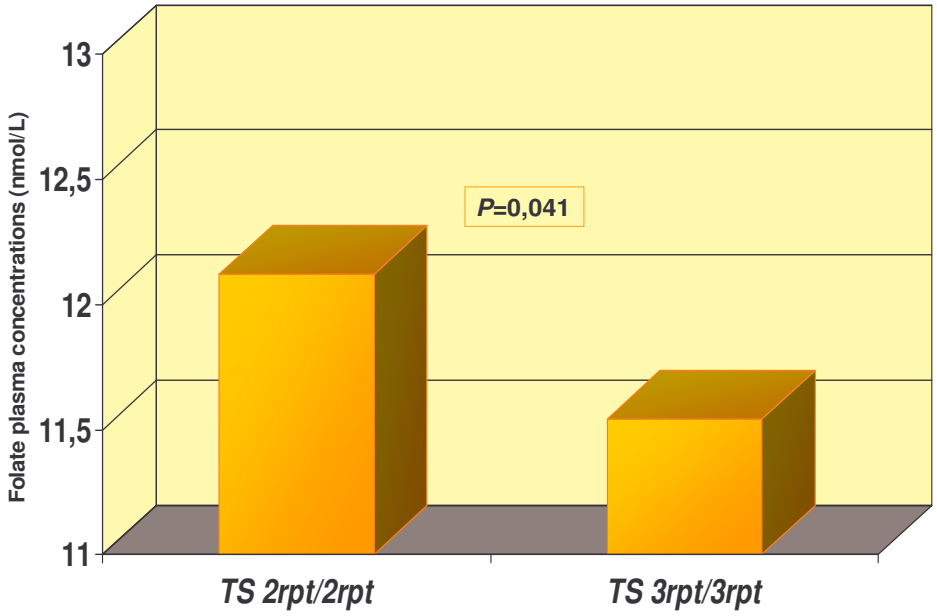


Figure 5: effects of *TS* polymorphism on folate plasma levels.

The subjects carrying genotype *2rpt/2rpt* did not show a significant reduction in genomic methylation compared to subjects carrying genotype *3rpt/3rpt* (5.32%, CI95% 5.32-5.32% in *2rpt/2rpt* genotype versus 5.38%, CI95% 5.34-5.41% in *3rpt/3rpt* genotype; *P*=NS) (figure 6).

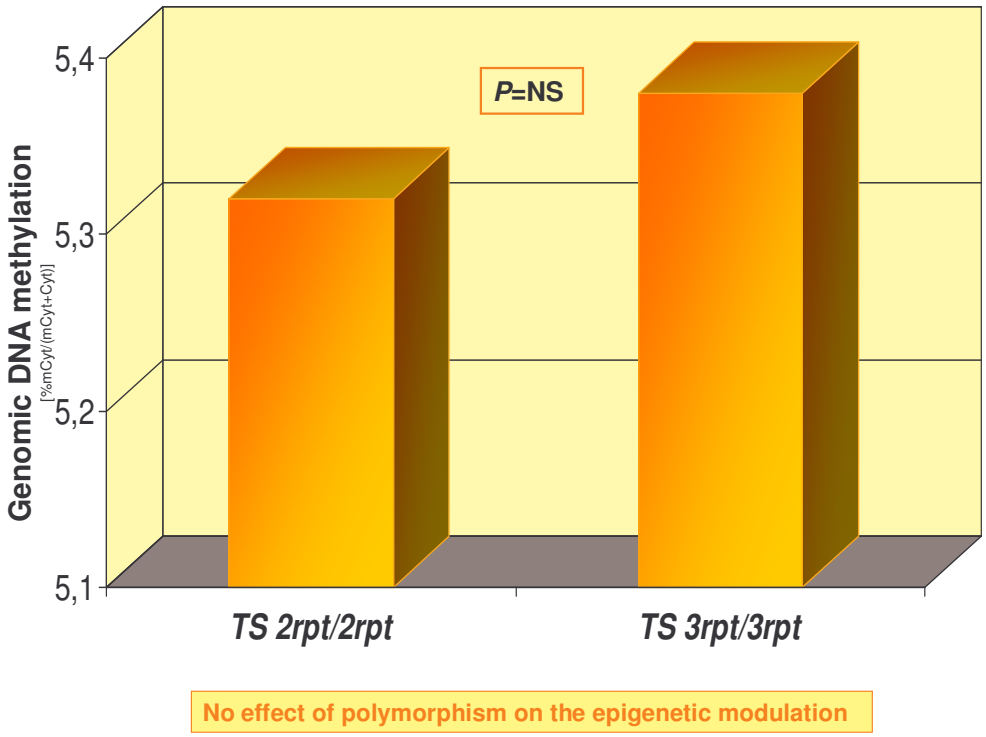


Figure 6: DNA genomic methylation and *TS 2rpt-3rpt* polymorphism

The genomic methylation is not modulated by the genotype of the *TS* polymorphism; and this is confirmation that the path on with TS acts is mainly the synthesis of nucleic acid and not the methylation.

Serine hydroxy-methyl-transferase and role of polymorphism *1420 C>T* on epigenetic modulation

Serine hydroxymethyltransferase (SHMT, EC 2.1.2.1), a pyridoxal-5'-phosphate (PLP)-dependent enzymes, is a major entry point for one-carbon units from serine into one-carbon metabolism and catalyzes the reversible transfer of the hydroxymethyl group of serine to tetrahydrofolate (THF) to form 5, 10 methyleneTHF and glycine[20], providing approximately 70% of the one-carbon units required for the biosynthesis of thymidine, purines, and methionine[21].

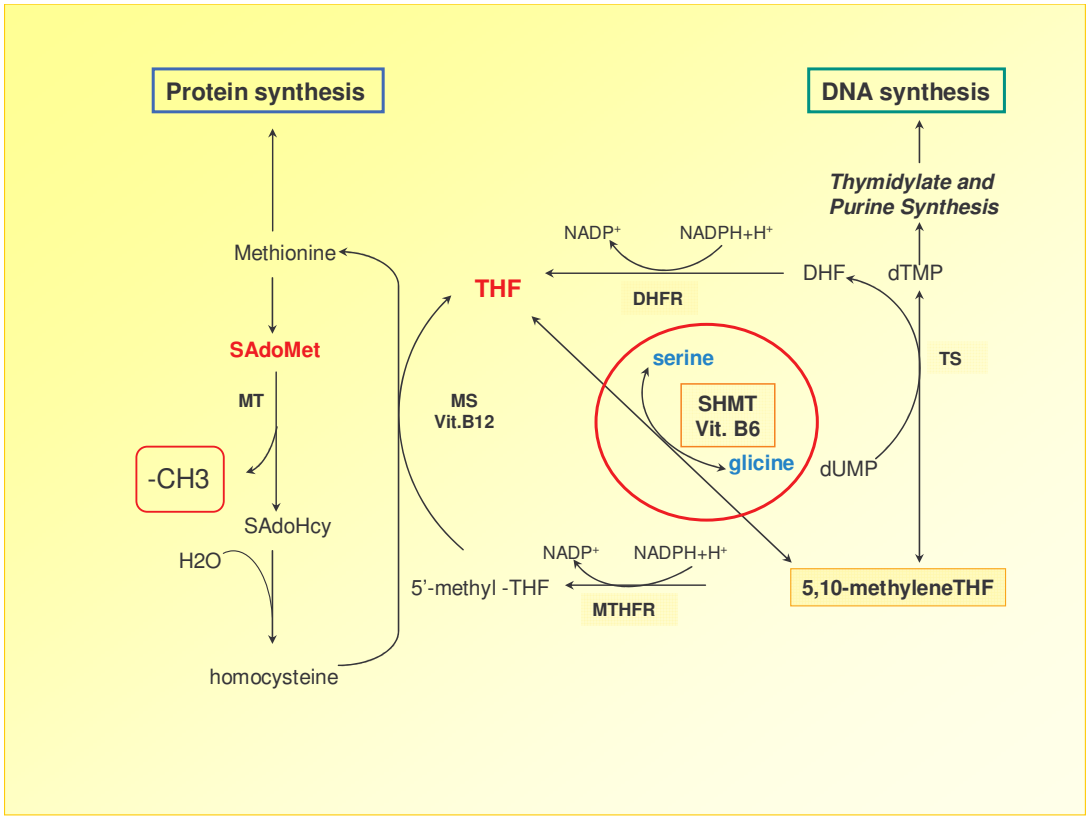
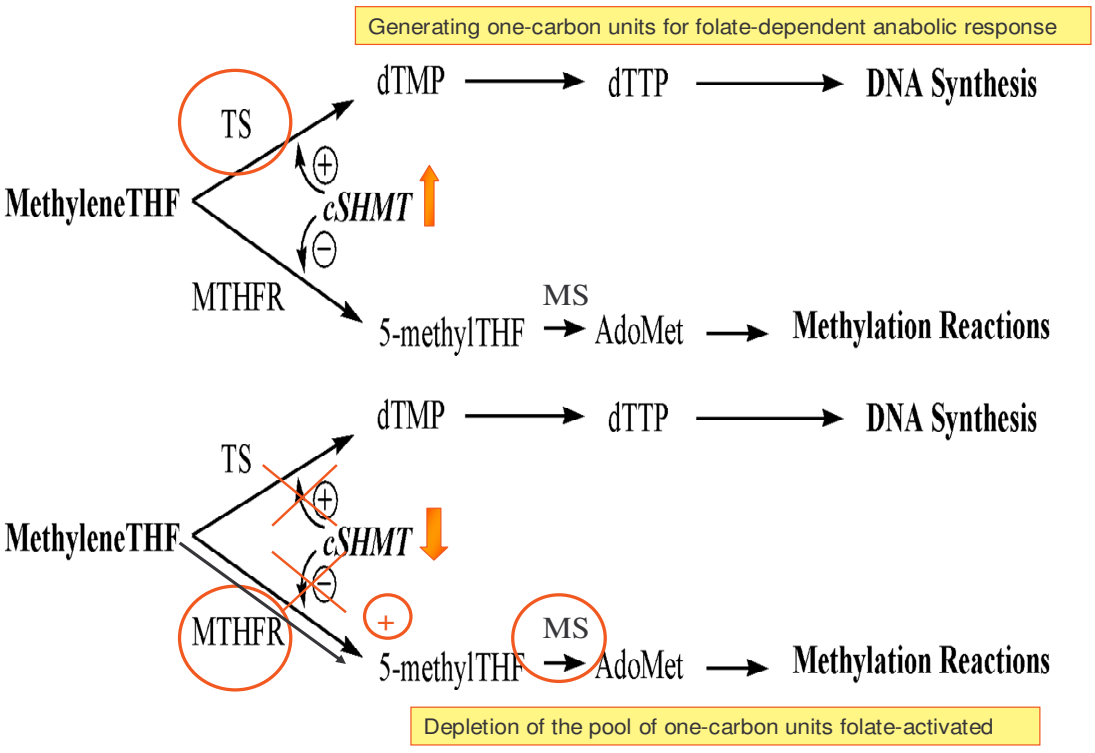


Figure 6: role of cSHMT enzyme in folate cycle

In humans, SHMT is present in isoforms, one located in the mitochondrion (mSHMT) and the other in the cytoplasm (cSHMT)[22]. Both SHMT isoezymes are sources of one-carbon units for cytoplasmic one-carbon metabolism. In the cytoplasm, folate-activated one-carbon units are required for the *de novo* synthesis of purines and thymidylate, and for the remethylation of homocysteine to methionine. The cSHMT enzyme is known to regulate the metabolic balance between thymidylate synthase (TS) and methylentetrahyfrofolate reductase (MTHFR).



SW Choi, Friso S, Editors "Nutrient-Gene Interactions in Cancer", 2006

Figure 7: cSHMT mediates the competition between TS and MTHFR for methylene-THF

Previous studies have indicated that a limited methyl group availability caused by folate depletion shifts the flux of one-carbon units to S-Adenosylmethionine (SAdoMet) synthesis and thereby suppresses DNA synthesis[23, 24]. However, recently Herbig et al. proposed that under certain conditions cSHMT acts as a switch to increase DNA synthesis at the expense of homocysteine remethylation[25]. Once this enzyme is activated, it preferentially supplies one carbon units for thymidylate synthesis, depletes MTHFR pools for SAdoMet synthesis by synthesizing serine and sequesters 5-methylTHF that is needed for SAdoMet synthesis. Collectively, these observations strongly suggest that alteration of cSHMT expression can influence DNA methylation status which needs SAdoMet as a one carbon donor.

In 2001 a genetic polymorphism of *cSHMT* was identified[22]. This variant consists in the substitution of a cytosine with a tymine in the nucleotide at position 1420 of the gene (chromosome 17p11.2) and consequently of a leucine (Leu) with a phenylalanine (Phe) at position 474 of the corresponding amino acid chain (Leu474Phe).

In our study, when we compare wild type (*1420CC*) and homozygous variants (*1420TT*), there was no significant difference at all endpoints.

However, homozygous mutant subjects (*1420TT*) subjects showed significantly decreased tendency in genomic DNA methylation compared with *1420CC* subjects. (*P*=0,005)

	cSHMT 1420 CC ***N=290***	*cSHMT 1420 TT* ***N=33***	***P***
tHcy (µmol/L)	15.67 (15.05-16.31)	16.28 (14.73-17.81)	N.S.
Folate (nmol/L)	12,03 (11.44-12.64)	11.10 (9.74-12.78)	N.S.
Vitamin B_6 (nmol/L)	32.13 (30.56-33.78)	30.87 (26.04-36.59)	N.S.
Vitamin B_{12} (pmol/L)	391.50 (376.15-407.48)	415.71 (376.15-454.86)	N.S.
Genomic DNA methylation (ng mCyt/µg DNA)	3.81 (3.63-3.97)	3.09 (2.45-3.85)	**0.005**

Table 2: comparison between wild type and mutant homozygous for *cSHMT 1420C>T* polymorphism.

Moreover, subjects homozygous carriers of the mutation *cSHMT 1420C>T* (*1420TT*) showed a reduced genomic methylation DNA both compared to wild-type *1420CC* and compared with subjects with the polymorphism in heterozygous (*1420CT*).

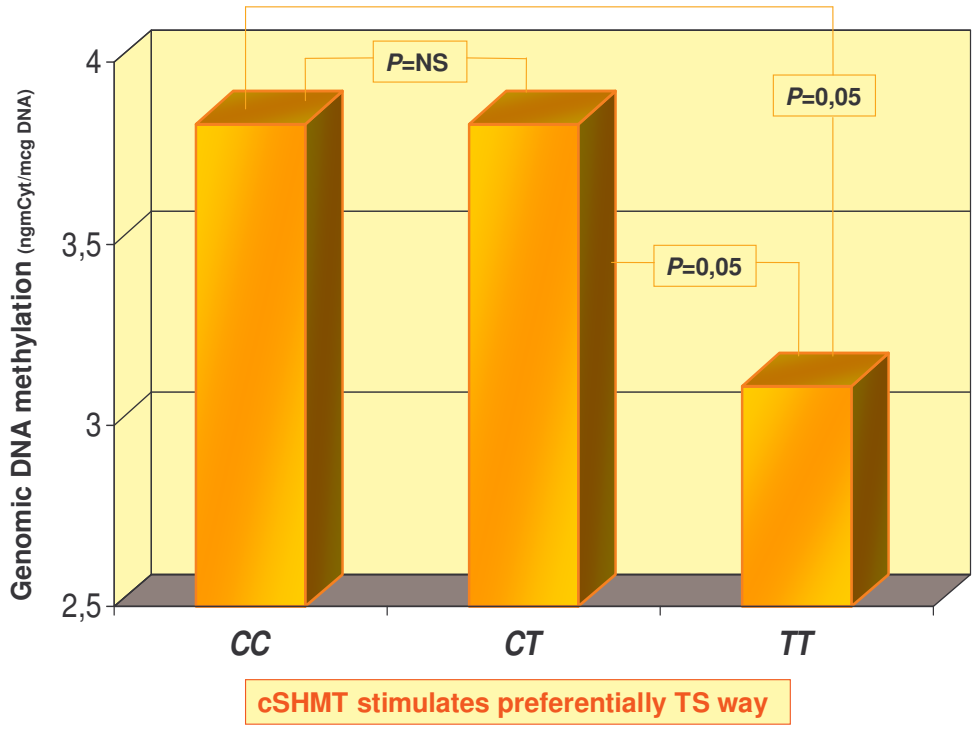


Figure 8: *cSHMT* and genomic methylation in relation to the three different genotypes

This observation confirms the role of cSHMT in stimulating preferentially TS pathway.

Dihydrofolate reductase (DHFR) and role of polymorphism *19bp-ins/del* on epigenetic modulation.

Enzyme dihydrofolate reductase (EC 1.5.1.3.) plays a central role in one-carbon metabolism because it catalyzes the conversion of dietary folic acid into dihydrofolate (DHF) and reduces DHF into tetrahydrofolate (THF), with NADPH as cofactor[26], making THF active and then available by the cell. In this way, it provides the essential cofactor in the reactions of biological methylation and purine and aminoacid synthesis.

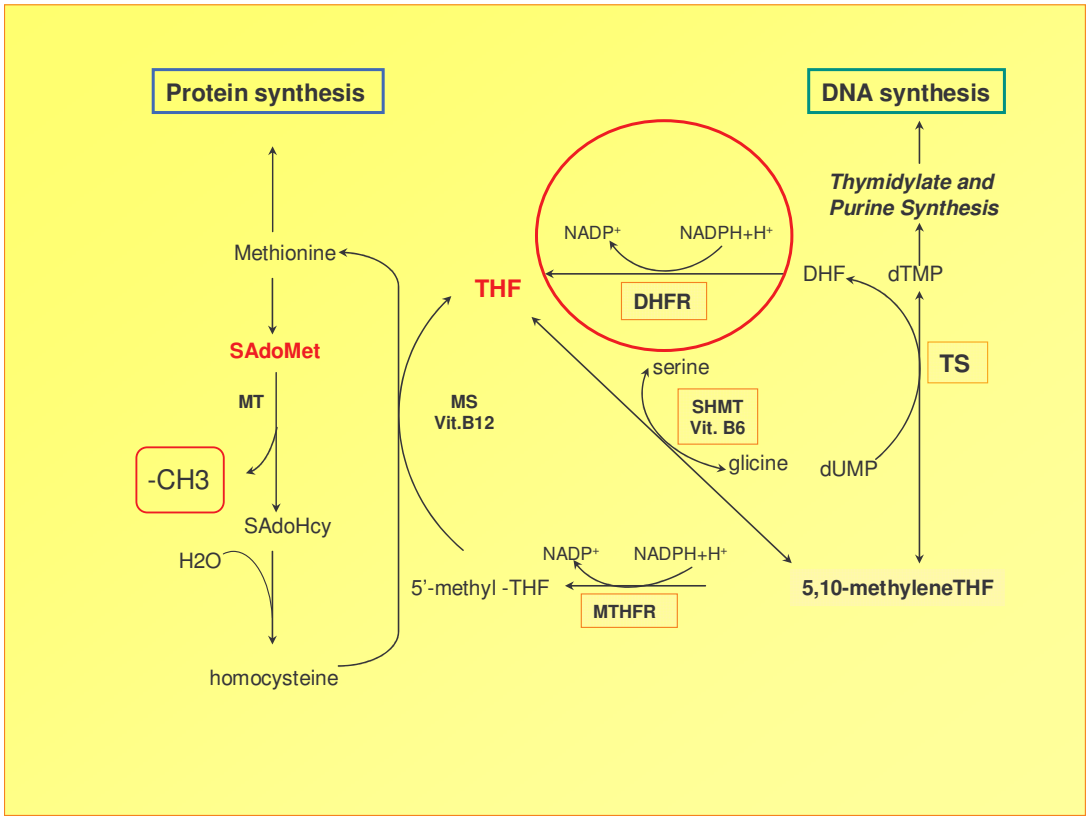


Figure 9: role of DHFR enzyme in folate cycle

Between the allelic variants identified on the *DHFR* gene, the polymorphism of interest from the point of view of the impact on enzyme activity is represented by a deletion of *19 bp* to the first intron. The mechanisms by which this deletion may cause a reduced activity of DHFR are manifold. First, it eliminates the binding site for a transcriptional factor (Sp1); second, the intron itself has a role in gene regulation, acting as a binding site for transcription factors or altering the splicing.

Some studies evaluated the role of *DHFR 19bp ins/del* in determining the onset of cancer. Gemmati and Collegues[27] examined the association between *DHFR 19bp ins/del* polymorphism and acute lymphoblastic leukemia (ALL) in adult subjects (245 patients and 245 control cases) and the possible interaction with the *MTHFR 677C>T* polymorphism. The deletion, removing a binding site for a transcription factor, induces an increase of enzyme expression, thus increasing the level of methylene-THF that favors the intracellular synthesis of DNA pathway. Polymorphism *MTHFR677C>T* has a synergistic effect by reducing enzymatic activity and the conversion of methylene-THF into methyl-THF leading to a build-up inside the cell. Both polymorphisms therefore play a protective role by reducing the risk of ALL[27]. The protection against the development of ALL appears to be attributable to a DNA synthesis more faithful and efficient thanks to a lot of THF reduced. Specifically, a better synthesis of thymidylate reduces the risk of DNA uracil misincorporation and prevents genomic instability. On the other hand, the reduction of methyl-THF, required for methylation, makes it less likely hyper-methylation and gene silencing of tumor suppressor genes.

In our study, we proposed not only to evaluate the possible role of *DHFR 19bp ins/del* polymorphism in modulating DNA genomic methylation but also to analyze the function of this polymorphism on gene-specific methylation in some genes involved in carcinogenesis, as *E-cadherin*, *RAR-beta*, *APC*, *RASSF1a*, *p15*.

Moreover, we evaluated the effect of co-presence of *DHFR 19bp ins/del* polymorphism and *MTHFR 677C>T* mutation in modulating DNA genomic methylation.

In our study, there was no statistically significant difference between wild type subjects and carrier of the mutant allele, for age, gender, folate concentration, plasma homocysteine, vitamins B6 and B12 levels. There were no significant differences also with regard to DNA genomic methylation. However, when the DNA genomic methylation was compared between subjects with or without the *DHFR 19bp del* mutant allele in agreement with plasma folate levels, there was a significant reduction of DNA methylation in the carriers of *DHFR 19bp del mutant allele del* and low folate concentrations. The subjects carrying the wild type *DHFR 19bp ins* allele showed no statistically significant differences in DNA methylation in relation to folate status (Figure 10).

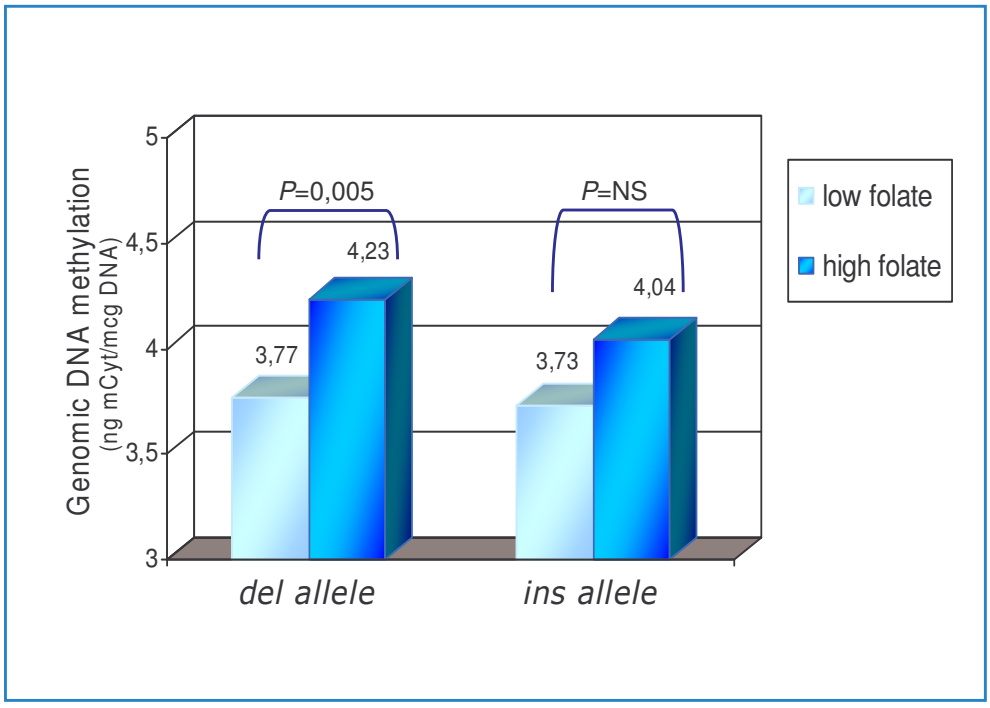


Figure 10: Genomic DNA methylation according to the presence of either *DHFR 19bp ins/del* allele according to folate plasma concentrations.

This observation costitutes another exemple of gene-nutrient modulation within folate cycle by an enzyme involved in the transport of methyl groups.

When DNA genomic methylation was considered according to both *DHFR 19bp ins/del* and *MTHFR 677C>T* polymorphisms, there was a statistically significant difference between combined subgroup *DHFR 19 bp ins/ins* + *MTHFR 677TT* and subgroup *DHFR 19 bp del/del* + *MTHFR 677CC* (3,45 *versus* 4,14 ng mCyt/µg DNA, respectively, *P*<0.0001). The co-presence of the mutated *MTHFR 677TT* genotype with the *DHFR 19bp ins/ins* wild type or the *DHFR 19bp del/del* mutant genotype was not associated to statistically

significant differences (3,45 *versus* 3,71 ng mCyt/µg DNA, *P*=0.09) (Figure 11).

These data show that carriership in homozygosity of the *677T* mutant allele (*MTHFR 677TT* genotypes) is apparently more important in determining DNA methylation status than the *19 ins/del* polymorphism of *DHFR* by itself, as reflected in the lack of difference in the methylation status between wild- type and mutant DHFR genotypes compared with the same carrier state of *MTHFR 677TT*. The observation, however, that both these mutations have a significant effect on DNA methylation appears as a novel and relevant finding, especially considering the rather common allele frequency of the two polymorphic sites in our population.

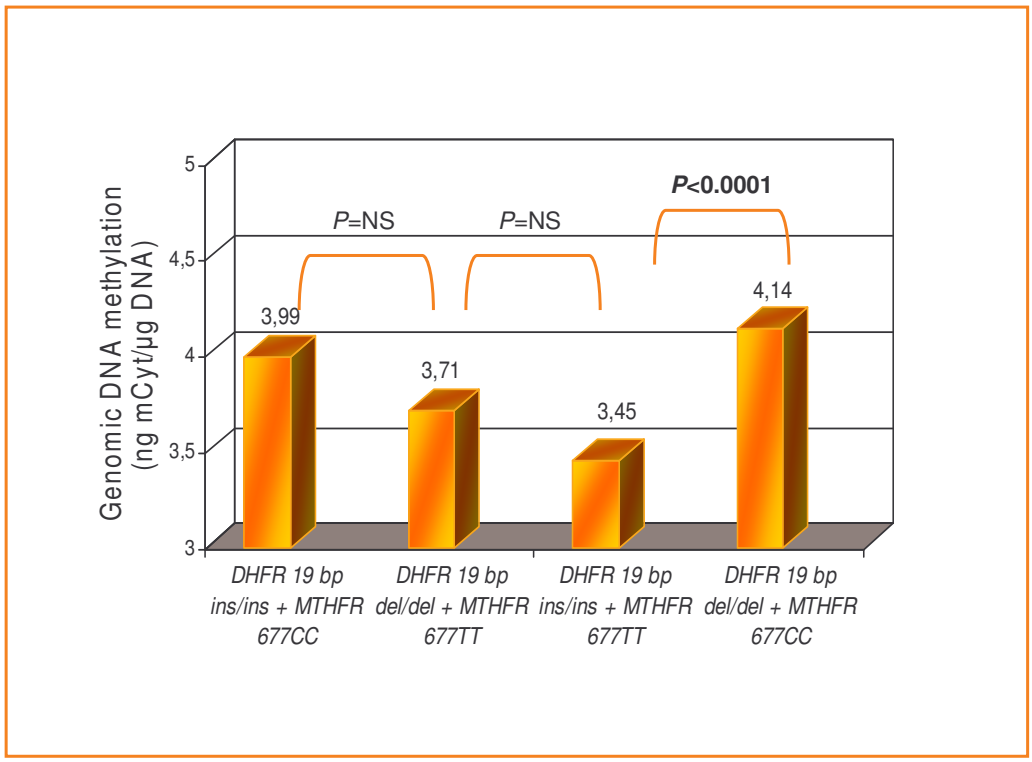


Figure 11: Genomic DNA methylation according to co-presence of two polymorphisms *MTHFR 677C>T* and *DHFR 19 bp ins/del*.

The study of DNA gene-specific methylation of *E-cadherin*, *RAR-beta*, *APC*, *RASSF1a*, *p15* promoter genes according to *DHFR 19bp ins/del* genotype, however, did not show any significant difference in the different genotypes. This finding can be due to different reasons being the most likely that in absence of a clear pathologic condition the methylation status at this gene sites in peripheral blood mononuclear cells may not be so striking to allow a significant difference. Another reason may be due to the relatively small number of subjects analyzed, therefore a larger study may be auscpicable in the future to consent to potentially identify differencies in genomic compared to gene-specific methylation in such cell model.

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Among the polymorphisms examined, the analysis was particularly focused on the latest polymorphism. DNA methylation has been mainly studied in cancer, but probably it is involved also in other pathological condition. Indeed we have observed, for example, that the *DHFR 19bp-ins/del* variant appears associated with coronary artery disease.

CHAPTER 2

A POLYMORPHIC VARIANT OF *DIHYDROFOLATE REDUCTASE* (*DHFR 19BP-INS/DEL*) IN CHRONIC DISEASE RATHER THAN CANCER.

Abstract

Folate metabolism disorders are associated with higher risk of cancer, neural congenital defects and cardiovascular diseases. The dihydrofolate reductase enzyme (DHFR) converts dietary folic acid into dihydrofolate (DHF) and reduces this latter into tetrahydrofolate (THF), essential cofactor in the reactions of biological methylation and purine and aminoacids synthesis. Recently a 19-bp deletion polymorphism has been described in intron 1, likely related with the risk of spina bifida, preterm delivery, autism, breast cancer and cardiovascular disease. We studied a group of 681 subjects enrolled in the Verona Heart project, subdivided into two groups according with the presence or not of coronary artery disease (CAD) and the prevalence of the polymorphism was determined by allele specific

polymerase chain reaction. A significantly higher frequency of the *DHFR 19bp del/del* genotype was found in the group of affected subjects as compared with healthy controls. Moreover, the presence of *del* allele was associated with higher risk of being affected by coronary artery disease in a fashion dependent on the number of allele (OD: 1.56 for *ins/del* and 1.85 for *del/del* respectively).

Introduction

Folic acid is a water soluble B-group vitamin and plays a crucial role in DNA synthesis and repair and in DNA methylation. The NADPH dependent enzyme dihydrofolate reductase (DHFR) catalyzes the hydrogenation of dihydrofolate (DHF) to tetrahydrofolate (THF). This latter molecule and its one carbon derivatives are essential cofactors for purine, thymidylate and aminoacids biosynthesis, through the so called "one-carbon transfer reactions"[1]. Moreover tetrahydrofolate is necessary for DNA methylation, since 5-methyltetrahydrofolate arising through the action of methionine sinthetase, is essential for synthesis of S-adenosylmethionine (SAdoMet), universal donor of methyl groups[2, 3].

In presence of low availability of THF, the synthesis of 5,10 methylene-THF and 5 methyl-THF is impaired, as well as the reconversion of homocysteine to methionine.

Plasma total homocysteine levels are a strong predictor of mortality in patients with angiographically confirmed coronary artery disease[4]. Moreover a meta-analysis study[5] demonstrated a causal association between homocysteine and cardiovascular disease and in a more recent work the same authors could show that the severity of angiographically detected coronary artery disease was positively associated with total homocysteine levels[6].

A close relationship exists between folate and homocysteine in circulating blood, with homocysteine levels being inversely related to folate both intake and plasma levels[7].

In 2004 Johnson and Colleagues[8] described for the first time a polymorphic variant of *DHFR* consisting in a 19bp deletion in the first intron of the gene, and reported an association between the presence of the polymorphism in a group of women and the increased risk of having a child affected by spina bifida.

More recently the presence of *DHFR 19bp-del* has been associated with low levels of plasma homocysteine[9] and the decrease correlated with both condition of heterozygosis (*ins*/*del*) and homozygosis (*del/del*); in the same year Xu and colleagues studied 2000 women and reported that the presence of the variant in homozygotic condition correlated with higher plasma and erythrocyte folate levels[10]. In 2008 the results of Framingham Offspring Study (Kalmbach 2008) suggested that, regardless of folate intake, the *del/del* polymorphism in *DHFR* was functional, since it was associated with

both higher levels of unmetabolized plasma folate and decreased red blood cell folate store.

The presence of allele *del* has been associated with increased *DHFR* gene expression: the raise was 2.4-fold in heterozygosis and 4.8-fold in homozygotic subjects, in comparison with wild type group[10]. This evidence is contrasting with a report where it was hypothesized that *DHFR 19-bp deletion* might decrease enzyme expression through the removal of a strong transcription factor binding site for Sp1[8].

Variants of DHFR that might affect plasma homocysteine and the status of folate in blood and erythrocytes were examined in a study[9] where the gene for *DHFR* was sequenced in twenty individuals and the presence of several SNPs and the 19-bp deletion were described. In 330 subjects the *19-bp del/del* genotype was found to be associated with lower plasma Hcy compared with the wild genotype. No association was found with plasma and red blood cell folate levels.

No data are available in literature about the possible association between *DHFR deletion* polymorphism and coronary artery disease; on the other hand the role of plasma homocysteine is well established as risk factor for coronary heart disease.

In this study we accomplished several targets: a. to determine the frequency of *DHFR 19-bp del* polymorphisms in a group of patients affected by coronary artery disease compared with control subjects; b. to ascertain the

possible association between *DHFR* genetic variants, B-vitamin status and homocysteine in coronary artery disease; c. to verify if the presence of the mutant polymorphism might represent an additional and independent risk factor for coronary artery disease.

Materials and methods

Study population. This study was performed within the framework of the Verona Heart Study, a regional survey aimed at searching for new risk factors for CAD in subjects with angiographic documentation of their coronary vessels. Details of the enrolment criteria were given elsewhere[11, 12]. A total of 681 subject were included in the present study. Two hundred forty-two subjects had completely normal coronary arteries, having undergone coronary angiography for reasons other than CAD, mainly valvular heart disease (CAD-free group), and they served as controls. These subjects were also required to have neither a positive history nor clinical or instrumental evidence of atherosclerosis in vascular districts beyond the coronary bed. Four hundred thirty-nine subjects had angiographically proven CAD (most of them were candidates for coronary artery bypass grafting).

All participants came from the same geographical area (Northern Italy) and were of similar socioeconomic status. At the time of blood sampling, a complete clinical history, including the assessment of cardiovascular risk factors such as obesity, smoking, hypertension, and diabetes, was collected.

Written informed consent was obtained from all participants after a full explanation of the study. The study was approved by our Institutional Ethical Committee.

Biochemical analysis. Samples of venous blood were drawn from each subject after an overnight fast. Plasma folate and vitamin B-12 levels were determined by a chemiluminescence technique (Chiron Diagnostics, East Walpole, MA, USA). Homocysteine was measured in plasma with HPLC following the method by Araki and Sako[13], with slight modifications. Levels of vitamin B-6 were determined as plasma concentrations of pyridoxal-5'-phosphate (PLP), the active metabolite of vitamin B-6 with a HPLC method[14,15]. Erythrocyte folate content was assayed by affinity method, followed by reversed-phase chromatography with electrical detection according to Bagley and Selhub[16].

Serum lipids were measured with an automatic analyzer (Technicon DAX96) and LDL cholesterol was calculated with Friedwald formula.

DNA extraction and DHFR 19bp ins/del determination. Genomic DNA was extracted from mononucleated blood cells with Wizard® Genomic DNA purification kit (PROMEGA Corporation, Madison, WI, USA) and the polymorphic variant 19-bp *ins/del* was analyzed by allele specific polymerase chain reaction, which was set up by modification of published methods[9], followed by agarose gel electrophoresis of amplification products. Briefly, the reaction was performed in 25 μl volume, containing 100 ng of genomic DNA, 0.4 μM each of the following primers: forward 1 (5'-

CCACGGTCGGGGTACCTGGG -3'), forward 2 (5'-*ACGGTCGGGGTGGCCGACTC*-3'), reverse (5'-*AAAAGGGGAATCCAGTCGG*-3'), 0.25 mM dNTPs, 1.5 mM $MgCl_2$, 0.75 U SuperTaq (AB-Analitica, Padova, Italy) and 8% DMSO. Thermocycler conditions were 4 min at 94° followed by 35 cycles of 94° 1 min, 62° 1 min, 72° 1 min, with the final extension step of 72° for 7 min.

PCR products were loaded on 4% agarose gel containing ethidium bromide, run at 100 V and analyzed on an image detector GelDoc (BioRad Laboratories). The insertion allele product was represented by a 113 bp band, whereas the deletion allele generated a 92 bp band.

Statistical methods. All statistical analyses were performed with SPSS 17.0 statistical package (SPSS Inc., Chicago, IL). Distribution of continuous variables in groups was expressed as means ± standard deviation. Logarithmic transformation was performed on all skewed variables, including homocysteine, folate, vitamin B12, vitamin B6. Therefore, the statistical differences concerning these parameters were also computed on the corresponding log-transformed values, although, for the sake of clearness, non-transformed data were reported in the Results. Quantitative data were assessed using the Student's t-test or by ANOVA and Tuckey's post-hoc test when indicated. Covariance analysis (ANCOVA) was performed to evaluate the possible confounding effects of classic risk factors for CAD, on the assessment of risk associated with each polymorphic variant.

Associations between qualitative variables were analyzed with the χ^2-test, and linear trend analysis when indicated. The median of plasma folate levels in the control group (12 nmol/L) was used as cut-off value for defining low and high folate concentrations. A value of $p < 0.05$ was considered significant.

To assess the extent to which genetic polymorphisms were associated with coronary artery disease, odds ratios with 95% CIs were estimated by univariate logistic regression analysis. Adjustment for classical cardiovascular risk factors (i.e. sex, age, smoke, diabetes, cholesterol, triglyceride) was performed by adding those covariates in a multiple logistic-regression model.

Results

The clinical and biochemical characteristics of the subjects are summarized in table 1. As expected the CAD group presented significant differences in most parameters acknowledged as risk factors for cardiovascular pathology: higher proportion of males and smokers and significantly elevated plasma levels of total cholesterol, LDL-cholesterol and triglycerides. Inversely HDL-cholesterol was lower in cases as compared with controls. Moreover plasma homocysteine concentrations were higher in CAD patients.

Variable	CAD N=439	CAD-free N=242	P-value
Age (years)	60.3±9.3	58±12.8	0.011
Sex (% males)	69.8	30.2	<0.0001
Smoke (%)	69.2	30.8	<0.0001
Total Cholesterol (mmol/L)	5.96±1.14	5.53±1.07	<0.0001
LDL Cholesterol (mmol/L)	4.06±0.99	3.58±0.93	<0.0001
HDL Cholesterol (mmol/L)	1.22±0.34	1.45±0.42	<0.0001
Triglycerides (mmol/L)	2.04±1.18	1.48±0.66	<0.0001
Creatinine (mmol/L)	96.55 (93.70-98.49)	91.84 (90.02-93.70)	0.04
Homocysteine (µmol/L)	16.45 (15.49-16.95)	14.88 (14.15-15.80)	0.008
BMI	26.6±3.3	25.3±3.4	<0.0001
Diabetes (%)	68.9	31.1	<0.001

Table 1 Clinical and biochemical characteristics of cases (CAD) and controls (CAD-free)

When Body Mass Index was calculated and compared in the two groups, it turned out to be higher in CAD group compared with CAD-free subjects (26.6±3.3 *vs* 25.3±3.4, p<0.0001) as expected. The prevalence of diabetes was significantly higher in CAD patients as compared to CAD-free controls.

When stratified for the genotype, the three groups did not differ for age, sex, BMI, plasma folate and homocysteine, red blood cell folate and vitamin B6 (Table 2). The levels of vitamin B12 were significantly lower according to the presence of one or two *del* alleles, as compared to the *ins/ins* genotype.

	DHFR genotype			
Variable	***ins/ins* (n=256)**	***ins/del* (n=320)**	***del/del* (n=105)**	*P*-value*
Age y (range)	58.9 (58-60)	59.8 (59-60)	60.9 (59-63)	0.26
Sex % males [1]	75.3	79.4	78.5	0.48
BMI kg/m^2 (range)	25.7 (25.1-26.2)	26.3 (25.7-26.6)	26.0 (24.726.5)	0.37
Plasma folate nmol/L (range)	11.8 (10.9-12.8)	12.0 (11.2-12.7)	12.0 (10.7-13.5)	0.91
Plasma Hcy µmol/L (range) [2]	15.0 (14.1-16.1)	14.9 (14.1-15.7)	15.5 (13.8-17.4)	0.81
RBC folate nmol/L	1038 (930-1146)	1052 (979-1124)	1178 (904-1452)	0.73
Vitamin B6 nmol/L (range)	29.5 (26.5-32.8)	29.6 (27.7-31.6)	31.7 (26.7-37.6)	0.37
Vitamin B12 pmol/L (range)	311 (292-331)	304 (286-322)	282 (255-312)	<0.01

Table 2 Characteristics of the subjects according to *DHFR* genotype

Values are expressed as geometric means (antilogarithms of the transformed means), and 95% CIs are reported in parentheses with 2-tailed P values.

* *P*<0.05 is considered statistically different.

[1] Adjusted for serum creatinine concentrations

[2] Data are expressed as percentage

No association was found between the presence of the allelic variant and plasma folate, homocysteine, B6 and B12 levels (data not shown).

The genotyping of DNA samples was performed on 439 CAD cases and 242 controls (CAD-free). The frequencies of the three polymorphic variants in the entire population were 37.6% homozygous wild (*ins/ins*), 47.1%

heterozygous (*ins/del*) and 15.3% homozygous mutant (*del/del*). The frequency of allele *del* was 0.39.

The prevalence of the polymorphic variants and the allele frequencies calculated in the two groups of subjects split according to the diagnosis are shown in Table 3.

	CAD (n.439)	CAD-free (n.242)	*P*
***DHFR* Genotype**			
ins/ins	33.5	45.1	ns
ins/del	49.5	42.5	<0.009
del/del	17.0	12.4	<0.010

Table 3 Frequencies of the DHFR polymorphic genotypes and alleles in the CAD samples and CAD-free controls

The association between *DHFR 19bp deletion* polymorphism and the relative risk for coronary artery disease was analysed with logistic regression, to determine the odds ratios (ORs) and the 95% confidence interval (Table 4). Compared with the *ins/ins* genotype subjects, individuals *ins/del* had an OR of 1.56 (95%CI: 1.11-2.20). The *del* allele for CAD vs. CAD-free was observed in 68.7 vs. 31.3% (*P*<0.001). The presence of the allele *del* in homozygosis increased the OR to 1.85 (95%CI: 1.14-3.03). Potentially confounding factors were evaluated in the model: age, sex, smoking, BMI,

total cholesterol, triglycerides, LDL cholesterol, HDL cholesterol, creatinine, homocysteine, diabetes, folate, vitamin B6 and B12. The addition of each covariate to the logistic regression model did not change significantly (10% or more) the effect.

	CAD	CAD-free	OR [a] (95% CI)
	n (%)		
DHFR ins/ins	147 (33.5)	109 (45.5)	1.00
DHFR ins/del	217 (49.5)	103 (42.5)	1.56 [b] (1.11-2.20)
DHFR del/del	75 (17)	30 (12.4)	1.85 [c] (1.14-3.03)

Table 4 Risk for coronary artery disease related with *DHFR genotype*

[a] *P* for trend among genotypes = 0.009
[b] *P* = 0.012, for the comparison between *ins/ins versus ins/del*
[c] *P* = 0.017, for the comparison between *ins/ins versus del/del*

Discussion

Our study demonstrates that the *19-bp deletion* allele has a significantly higher frequency in subjects affected by coronary artery disease than in controls, either in heterozygosis and homozygosis condition.

No association was found between the presence of the allelic variant and plasma folate, homocysteine, B6 and B12 levels, suggesting that the presence of *del* variant might represent an independent risk factor.

We analysed with logistic regression the association between *DHFR 19bp* deletion polymorphism and the relative risk for coronary artery disease, to determine the odds ratios (ORs) and the 95% confidence interval. Compared with the *ins/ins* genotype subjects, individuals *ins/del* had an OR of 1.56 and the presence of the allele *del* in homozygosis increased the OR to 1.85. Potentially confounding factors were evaluated in the model: age, sex, smoking, BMI, total cholesterol, triglycerides, LDL cholesterol, HDL cholesterol, creatinine, homocysteine, diabetes, folate, vitamin B6 and B12, but the addition of each covariate to the logistic regression model did not change significantly (10% or more) the effect.

Plasma total homocysteine level is a strong predictor of mortality in patients with angiographically confirmed coronary artery disease[4]. Moreover, a meta-analysis study[5] demonstrated a causal association between homocysteine and cardiovascular disease and in a more recent work the same group showed a positive association between the severity of angiographically detected coronary artery disease and total homocysteine levels[6].

A close relationship exists between folate and homocysteine in circulating blood, with homocysteine levels inversely related to both folate intake and plasma levels[7].

Very scarce are the data from scientific literature about the correlation between *DHFR* polymorphic variants and cardiovascular disease, while other enzymes involved in one-carbon metabolism have been investigated for their impact on homocysteine levels in blood. A recent review[17] presented an

overview of the association among metabolism of homocysteine, polymorphic variants of genes encoding for enzymes involved in the folate-homocysteine metabolic pathways and influence of nutrients on homocysteine levels and the risk for cardiovascular disease. The genes examined were *methylenetetrahydrofolate reductase (MTHFR), methionine synthase (MTR), methionine synthase reductase (MTRR) and cystathionine beta-synthase (CBS).* The authors suggested that in future studies other genes should be considered because genetic modifications of enzymes and changes of products depending on the B-group vitamins could alter Hcy status and modify disease risk . Our results lines up in this direction, demonstrating for the first time that the *DHFR 19-bp deletion allele* shows in subjects affected by coronary artery disease a significantly higher frequency than in controls, either in heterozygosis and homozygosis condition, in association with a higher risk of being affected by the disease.

The presence of *DHFR* 19bp-*del* has been associated with low levels of plasma homocysteine[9] and the decrease correlated with heterozygosis (*ins*/*del*) or homozygosis (*del/del*); in the same year Xu and colleagues studied 2000 women and reported that the presence of the variant in homozygotic condition correlated with higher plasma and erythrocyte folate levels[10]. Our results showed no association between *DHFR 19bp-del* variant and plasma homocysteine and B-group vitamins levels, moreover the addition of these covariate to the logistic regression model did not change significantly (10% or more) the relative risk for CAD.

Rather surprising is the finding that the levels of plasma B12 correlates with the presence of the polymorphic variant of *DHFR* (table 2). On the other hand the assay of plasma B12 is not a reliable biomarker of possible metabolism alterations, since the measurement of holotranscobalamin or methylmalonic acid are more sensible indicators of vitamin B12 status and subclinical deficit[18].

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CHAPTER 3

STUDY OF A FUNCTIONAL MODEL OF INTER-RELATIONSHIP BETWEEN GENETICS AND EPIGENETICS IN FACTOR VII GENE PROMOTER: EVALUATION OF TWO FUNCTIONAL POLYMORPHISMS ON *F7* PROMOTER GENE AND INTERACTION WITH METHYLATION INDEX IN THE *F7* PROMOTER REGION.

DNA methylation, the main epigenetic mechanism that controls gene expression, occurs in position 5 'only of cytosine dinucleotide sequences within 5'-CpG-3'. As known, the distribution of CpG dinucleotides in the genome is not homogeneous: they have high concentration especially in promoter regions and in the first exons of genes, where they are called "CpG islands", namely areas rich in these dinucleotide CpG sequences (70% of CpG sequences).

Based on the observations made on folic acid polymorphisms we chose to study the *F7* gene because in its promoter region, near the transcription start sites, polymorphisms with different functional effects, including *5'F7* and *-402G>A* were identified. Our aim was to assess the epigenetic control on the expression of a specific gene in presence/absence of functional polymorphisms.

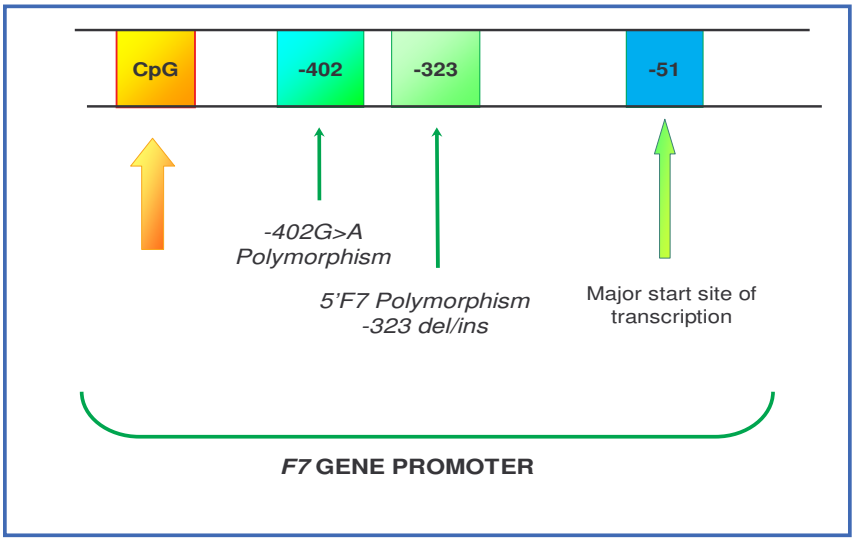

Figure 1: Two functional polymorphisms at *F7* promoter gene

PROMOTER METHYLATION IN COAGULATION *F7* GENE INFLUENCES PLASMA FVII CONCENTRATIONS AND RELATES TO CORONARY ARTERY DISEASE

Abstract

Plasma factor VII concentrations (FVIIa), an indicator of coronary artery disease (CAD) risk, are influenced by genetic markers at promoter site: the *A2* allele, due to a *10bp* insertion at position *-323*, is a determinant of lower FVIIa concentrations and reduced CAD risk, while the *-402A* allele, due to a *G>A* substitution, confers increased transcriptional activity *in vitro* resulting in higher FVIIa. Transcriptional regulation of *F7* by epigenetic features is, however, still unknown as is the inter-relationship of genetic and epigenetic modifications at promoter site. *F7* promoter methylation and its link with

promoter polymorphisms in modulating FVIIa and CAD risk was evaluated in 253 CAD and CAD-free subjects. Plasma FVIIa was inversely related to methylation in *A1A1* and *-402GG, i.e.* in the absence of the rare *A2* and *-402A* allele. The higher FVIIa paralleled with lower methylation in *A1A1* compared to *A2A2* (*P*=0.035) while no variation in methylation was associated with the different *-402G>A* genotypes. The methylation-induced FVIIa concentrations modulation was observed only in *A1A1* where the higher methylation resulting in lower FVIIa was prevalent within CAD-free compare to CAD subgroup (*P*=0.011).

These findings identify the role of *F7* promoter methylation in regulating plasma FVIIa and its association with CAD.

1. Introduction

Factor VII, for its key function in coagulation pathway [1], is related to atherothrombosis [2] and elevated concentrations of plasma activated factor VII (FVIIa) are recognized as a significant risk factor for coronary artery disease (CAD) and its main thrombotic complication, myocardial infarction [3, 4]. Several polymorphisms have been identified in *F7* gene with functional effects in modulating plasma concentrations of the gene product [5-8]. Among the major genetic determinants of FVII concentrations, an important role has been defined for a decamer insertion at position *–323* in the *5'* promoter region from the start of translation (0/10bp), where allele *A1* corresponds to the absence of the decamer and allele *A2* to its insertion [5, 9, 10]. We previously observed that the functional *-323 10bp del/ins*

polymorphism in the *5'* promoter region affects the risk of atherothrombosis [4]. The *F7* gene promoter activity is reduced by 33% in carriers of the *A2* allele [4, 11] so that subjects with the *A2A2* genotype have the lowest FVIIa concentrations and are more likely not to develop myocardial infarction compared to patients with the wild-type genotype [4]. Another polymorphism at *F7* gene promoter (*–402G>A*) has been also described having opposite effect *in vitro* [12]. The rare *-402A* allele confers increased transcriptional activity and is associated with higher plasma FVII concentrations [12], although with a gender specificity in a selected population subset [7].

While the characterization of *F7* genotype is indeed of help to predict FVIIa phenotype as well as the risk of CAD, genetic mechanisms alone may not fully explain the different FVIIa concentrations and the incidence of CAD.

Besides gene sequence variations, epigenetic modulation is also a major player in gene expression regulation and phenotype characterization [13]. DNA methylation is the main epigenetic feature of mammalian DNA [14, 15] with a critical role for gene expression regulation which has been extensively described mainly in cancer disease [16, 17]. The methylation-related gene-silencing phenomenon involves specific CpG–rich regions, the so-called CpG islands [18, 19] that preferentially reside in promoters of genes [18]. CpG islands hypermethylation within promoter sites is usually associated with gene transcriptional silencing [18] in cancer-related genes [19], therefore providing an important alternative path by which genes are modulated without structural changes.

Mechanisms of epigenetic control of genes related to atherothrombotic disease have not been extensively investigated, thus far. We recently described, however, the epigenetic regulation through repression *via* methylation at the 11 beta-hydroxysteroid dehydrogenase 2 gene promoter site, a hypertension-disease associated gene, therefore suggesting a novel link between regulation through epigenetic mechanism in diseases other than cancer [20].

As for atherothrombosis-linked genes, a distinctive high interest has been given to the role of *F7* gene [2, 3] for its key role in activating the extrinsic pathway by the exposure of tissue factor after plaque disruption [21] and for a strong genotype-phenotype correlation that defines a singular link between various genotypes at promoter site as predictor of plasma FVIIa concentrations and CAD risk [4, 7, 22].

While certain polymorphisms at *F7* promoter are well-recognized determinants of FVIIa concentrations and CAD risk [4], little is known about *F7* promoter DNA methylation as a determinant of plasma FVIIa concentrations by influencing gene product concentrations and possibly CAD risk, either in the presence or absence of known functional polymorphisms at promoter site.

The present study was therefore designed to evaluate: i) the role of DNA methylation at *F7* promoter site and its functional effects in phenotype characterization, *i.e.* FVIIa concentrations; ii) the significance of *F7* promoter

methylation relative to the presence of functional polymorphisms in affecting FVIIa concentrations; iii) the relationship between *F7* promoter methylation and risk of CAD, with regard to the presence/absence of *F7* promoter polymorphic site.

2. Materials and Methods

We studied 253 unrelated, age- and sex-matched subjects recruited from a single geographical area (Northern Italy) who were selected from consecutively recruited subjects among those referred to the Department of Clinical and Experimental Medicine and the Institute of Cardiovascular Surgery of the University of Verona in Italy and participating to the Verona Heart Project. Criteria for selection of the study population have been previously described in details.[4, 23]

All subjects underwent coronary artery angiography which was performed by two cardiologists unaware that the patients were participating to the study. According to coronary angiography documentation, the patients were classified as either being or not affected by severe coronary atherosclerotic disease (CAD and CAD-free, respectively).

Of these 253 patients, 165 CAD had angiographically documented, severe multivessel coronary atherosclerosis. Subjects with non significant coronary stenosis (*i.e.* <50%) were excluded from the study. As a control group, 88 CAD-free patients who were examined with coronary angiography for reasons other than possible coronary artery disease (mostly valvular heart

disease) were enrolled. They were required to have normal coronary arteries as documented by angiography and to have neither a history of atherosclerosis nor clinical or laboratory evidence of atherosclerosis in other vascular beds. This control group was included so that the atherosclerotic phenotype could be clearly defined and any association between methylation status of the *F7* gene promoter and coronary atherosclerosis itself could be identified.

A complete clinical history was collected from each subject. Patients who were taking an anticoagulant drug, vitamin supplements or other drugs that could potentially interact with one-carbon metabolism known to affect methylation status were excluded from this study.

The study was approved by our Institutional review boards. Written informed consent was obtained from all the participants after a full explanation of the study.

2.1. Biochemical analysis

After an overnight fast, samples of venous blood for FVII assay were drawn into Vacutainer® tubes containing 0.1 part of 0.129 M buffered sodium citrate per 10 parts blood. FVIIa was assayed as previously described[4] with a kit utilizing a soluble recombinant truncated tissue factor that is selectively deficient in promoting FVII activation but retains FVIIa cofactor function, thus allowing quantitative plasma FVIIa assessment (Staclot VIIa-rTF, Diagnostica Stago, Asnières-sur-Seine, France). The results were expressed in mU/ml, where 30 such units are equivalent to 1 ng of FVIIa. The within-run, and

between-run coefficients of variation were 7.8% and 6.4%, respectively. Routine biochemical parameters including those to assess major CAD risk factors were measured by standard established methods as previously described. [4, 23]

2.2. Genetic analysis: polymorphisms detection

DNA was extracted from peripheral-blood mononuclear cells (PBMC) using a phenol/chloroform protocol and subsequently stored at –80°C until analysis. The detection of the *5'F7 -323 del/ins* and the *-402G>A* polymorphisms was performed as previously described [10]. Polymorphism *5'F7* involves a decamer insertion at position *-323* in the 5'promoter region, where allele *A1* corresponds to the absence of the decamer and allele *A2* to its insertion. The *-402G>A* is due to the substitution of a guanine to an alanine at position *-402* at promoter site where the *-402G* represents the rare allele [12].

2.3. Epigenetic analysis: methylation at F7 gene promoter

In the *F7* gene promoter[24, 25] CpG-dinucleotide-rich regions were localized upstream to known functional polymorphisms including the 10bp insertion at *-323* and the *G>A* substitution at *-402* before setting for *F7* specific methylation primers design (Fig. 1).

A methylation-specific PCR assay [26] was set up to detect *F7* promoter methylation using bisulfite-treated DNA by designing *ad hoc* primers and after optimization of PCR conditions. Bisulfite treatment was performed using

the CpG Genome™ DNA modification kit (Chemicon International, Inc. Temecula, CA, USA).

Briefly, in the bisulfite reaction, all unmethylated cytosines are deaminated and sulphonated to be converted into uracil bases, while 5-methylcytosine remain unaltered.

Thus, the sequence of the treated DNA will differ depending on whether the DNA is originally methylated or unmethylated and, consequently, primer sets were designed to specifically amplify either a bisulfite-sensitive or a bisulfite-resistant sequence. In short, 1 μg genomic DNA was denatured by incubation for 10 minutes at 37°C with 7 μl 3M NaOH and subsequently bisulfite treated by overnight incubation at 55°C. Desalting procedure followed in sequential steps and desulphonation was then performed with 50 μl of 20 mM NaOH/90% ethanol before final elution. DNA was immediately used for PCR reaction or stored at -80°C until use. After sodium bisulfite treatment, enzymatic amplification of DNA was performed by PCR using hot start Taq polymerase (by AB Analitica, Padova, Italy) after detection of optimal conditions that allowed to perform the same protocol for the two sets of forward and reverse primers specifically designed to identify either methylated or unmethylated sites on the *F7* gene promoter region.

Primers set for unmethylated sites were TGTGGATTGTTGTTAGTTGGGTATAGT (forward) and CAAAAATAAAATTTCACCATATTAACCAAAC (reverse); for methylated sites were ACGTGGATTGTCGTTAGTCGG (forward) and AAATAAAATTTCACC

GTATTAACCAAACT (reverse). In order to verify the efficiency of the newly designed primers, both unmethylated and methylated primer sets were tested with bisulfite-treated fully unmethylated and fully methylated DNA (CpG Genome™ Universal Unmethylated or Universal Methylated DNA, respectively by Chemicon International, Inc. Temecula, CA, USA) which were both run during each PCR reaction simultaneously with the samples. Amplification products were resolved by gel electrophoresis and stained with ethidium bromide. The index of methylation is obtained by calculating the density (intensity/mm^2) of each band specific for methyl- and unmethyl-primers and expressed as the percent density of [methyl-band/(methyl-band + unmethyl-band)] measured under UV light by a Gel Doc XR 170-8170 (Bio-Rad, Laboratories, Hercules, CA, USA).

2.4. Statistical Analysis

All the calculations were performed using the SPSS 17.0 statistical software (SPSS Inc., Chicago, IL, U.S.A). Distributions of continuous variables in groups was expressed as means ± SD. Logarithmic transformation was performed on all the skewed variables, as is the case for FVIIa; therefore, geometric means with 95% confidence intervals are given for these variables. To investigate for significant associations between FVIIa concentrations and other continuous or categorical variables, multiple regression models estimating r2 and standardized β-coefficients were used. Categorical variables were analysed after transformation into binary variables (*i.e.* for *F7* gene polymorphisms, patients were considered for either being carriers or

non-carriers of the less frequent *A2* or *-402A* alleles). Differences in quantitative variables, including data on gene-specific methylation were assessed using the Student's t-test or by ANOVA with Tukey's post-hoc comparison of the means. Qualitative data were analyzed with the chi-square test. A two-tailed *P*-value of <0.05 was considered statistically significant in all instances.

3. Results

As shown in the graphic illustration in Fig. 1, CpG-dinucleotide-rich regions are located in the *F7* (GenBank accession no. U14580) upstream to the known functional polymorphisms [24, 25] including the 10bp insertion at *-323* and the *G>A* substitution at *-402* before set for *F7* specific methylation.

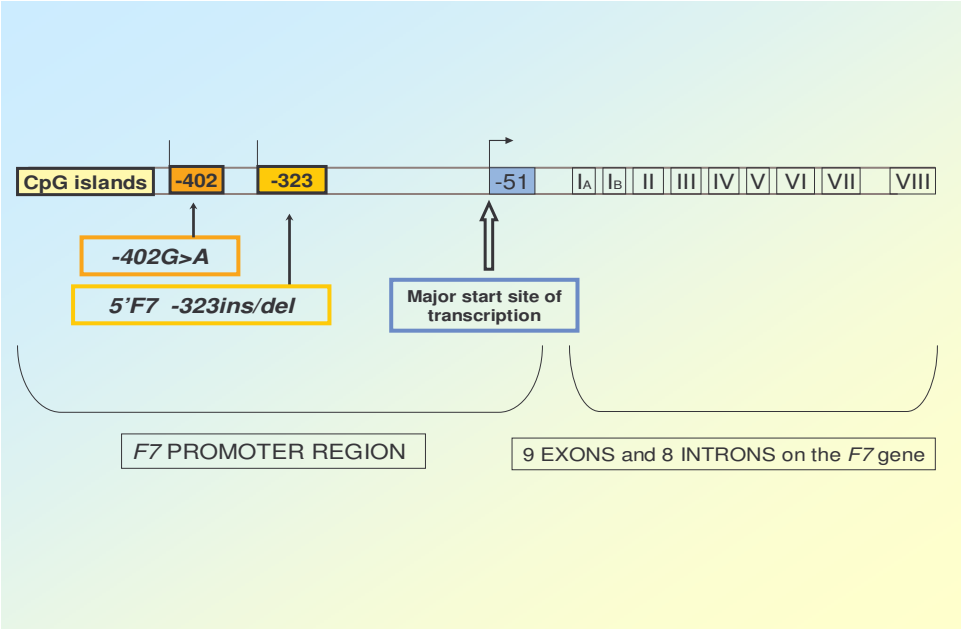

Figure 1. *F7* gene structure. Schematic drawing of *F7* gene 5'-region with a focus on promoter region where CpG-dinucleotide-rich sites are located upstream from the functional *-323del/ins* and *-402G>A* polymorphisms. Major start site of transcription is located at -51 with 9 exons and 8 introns downstream to that site.

Among the 253 selected patients, 211 were wild-type (*A1A1*) and 42 were homozygous mutant (*A2A2*) for the 5'*F7 -323del/ins* polymorphism. An inverse correlation was observed between *F7* promoter methylation and plasma FVIIa concentrations (Pearson's correlation coefficient –0.187; *P*=0.003).

Subjects carrying the *-323del/ins* functional polymorphism, the *A2A2* genotypes, had significantly lower FVIIa concentrations than did the *A1A1* wild-types, as expected (14.93, 95%CI 11.91-18.71 *vs.* 46.31, 95%CI 44.14-48.59 mUI/ml *P*<0.0001) (Fig. 2A).

The higher FVIIa concentrations, moreover, paralleled with the lower promoter methylation index in *A1A1* genotypes as compared to *A2A2* (30.31±9.45 *vs.* 33.69±9.31%, *P*=0.035) (Fig. 2B).

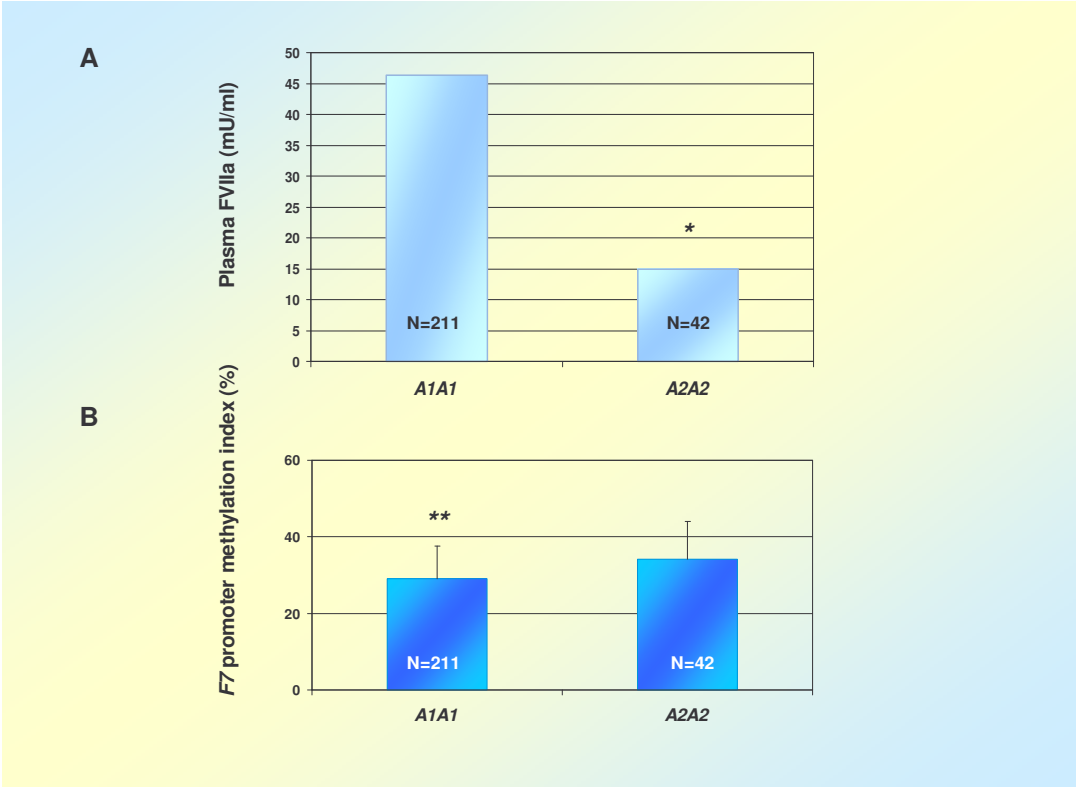

Figure 2. *F7* promoter methylation index according to *-323del/ins* polymorphism genotypes. The methylation index at *F7* gene promoter is shown accordingly with *-323del/ins* polymorphism genotypes at 5'*F7* site, *A2A2* mutants (n=42) and *A21A1* wild types (n=211), respectively. (A) Subjects carrying the *-323del/ins* functional polymorphism, the *A2A2*, show significantly lower concentrations of gene end-product, protein FVIIa than do the *A1A1* wild-types (14.93, 95%CI 11.91-18.71 *vs.* 46.31, 95%CI 44.14-48.59 mUI/ml *P*<0.0001). As shown in panel (B), the lower FVIIa concentrations in *A2A2* paralleled with the higher promoter methylation index as compared to *A1A1* (33.69±9.31 *vs.* 30.31±9.45 %, *P*=0.035). Results are expressed as mean plus or minus SD for continuous variables such as *F7* promoter methylation index. Error bars in panel (B) therefore indicate SD. Logarithmic transformation was performed on all the skewed variables, as in the case of FVIIa; therefore, geometric means with 95% confidence intervals are given for this variable. **P* <0.0001; ***P*=0.035.

The effect of methylation at promoter site was diverse if considering carriers or not carriers of the functional *-323del/ins* polymorphism so that only the *A1A1* wild-types accounted for the inverse correlation between methylation and plasma FVIIa concentrations (Pearson correlation coefficient –0.176; *P*=0.011); no such relationship was detected in *A2A2* genotypes (*P*=0.548). When considering carriers of the *-402G>A* variation at promoter site only the *-402GG* wild-types showed an inverse correlation between promoter methylation and plasma FVIIa concentrations (Pearson correlation coefficient –0.201; *P*=0.012) while there was no such relationship in *-402AA* homozygous mutants *(P*=0.30). No effect of the *-402G>A* polymorphism was detected in terms of FVIIa concentrations (47.01 *vs.* 50.6 mUI/ml, *P*=0.52) with no difference in promoter methylation index (30.74 *vs.* 23.71% for *-402GG vs. -402AA* genotypes, *P*=0.054).

As shown in Fig. 3, when analysis was conducted evaluating haplotypes, a higher *F7* promoter methylation index was detected in carriers of the *A2A2/-402GG vs.* both *A1A1/-402GG* and *A1A1/-402AA* (33.8 vs. 29.0%, *P*= 0.034 and 33.8 vs. 23.0%, *P*=0.016, respectively).

The higher methylation at *F7* promoter region paralleled with the lower concentration of FVIIa in the *A2A2/-402GG* compared to both *A1A1/-402GG* and *A1A1/-402AA* (*P*=0.0001). No carriers of the rare *A2A2/-402AA* were detected in the present population.

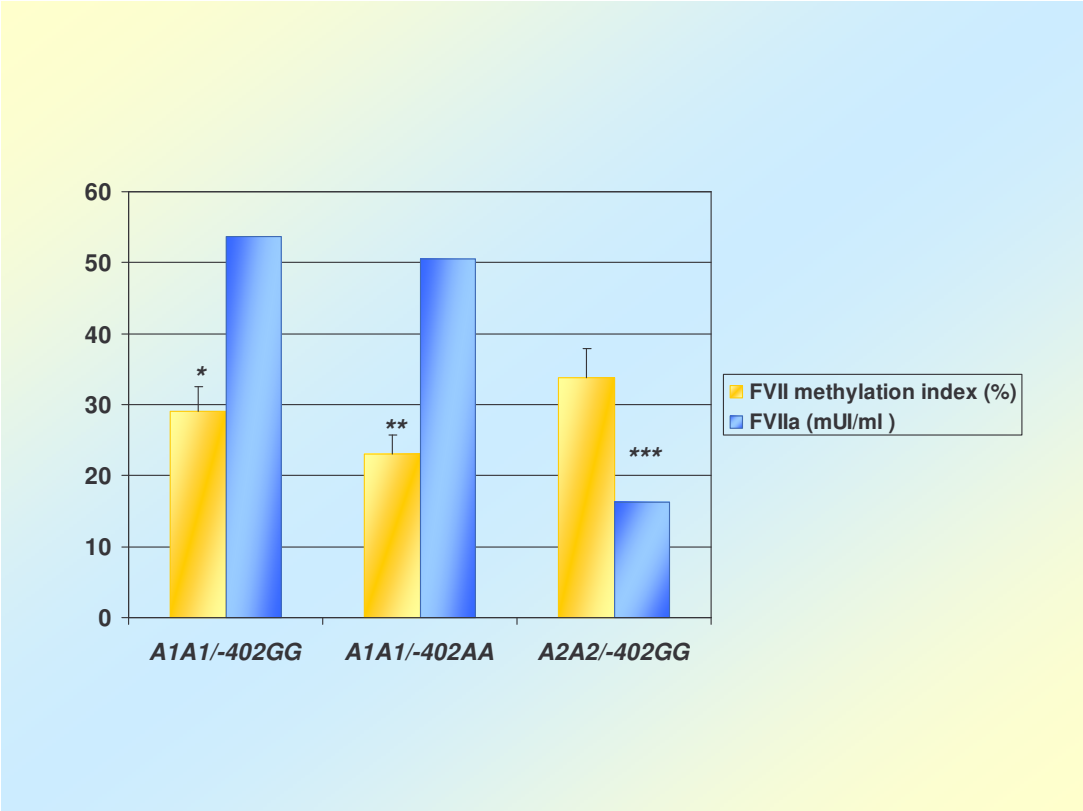

Figure 3. *F7* promoter methylation index and plasma FVIIa levels according to different haplotypes for the functional polymorphisms *-323del/ins* and *402G>A* at promoter site. Subjects carrying the *A2A2/-402GG* carriers had higher *F7* promoter methylation index compared to both *A1A1/-402GG* and *A1A1/-402AA* (33.8 *vs.* 29.0%, *P*= 0.034 and 33.8 *vs.* 23.0%, *P*=0.016, respectively) which paralleled with the lower concentration of FVIIa in the *A2A2/-402GG* compared to both *A1A1/-402GG* and *A1A1/-402AA* (*P*=0.0001) . Results are expressed as mean plus or minus SD for continuous variables such as *F7* promoter methylation index. Logarithmic transformation was performed on all the skewed variables, as in the case of FVIIa; therefore, geometric means with 95% confidence intervals are given for this variable.**P* <0.034; ***P*=0.016; ****P*=0.0001.

Moreover CAD-free subjects showed a higher *F7* methylation index compared to CAD patients (32.12±9.80 *vs.*30.22±9.23, *P*=0.012).

In a genotype-based analysis only the *A1A1* group accounted for such a difference in *F7* promoter methylation between CAD and CAD-free subjects, so that the CAD-free group showed higher methylation associated with lower FVIIa concentrations compared to CAD group, in a multiple logistic regression model analysis adjusted for the major risk factors for CAD (hypertension, diabetes, smoking habit, BMI, homocysteine) (31.65±10.29 *vs.* 29.60±8.93, *P*=0.011). The presence of the *-323del/ins* polymorphism was instead responsible for the lower plasma FVIIa concentrations and reduced CAD risk in *A2A2* genotypes where no difference was detected in *F7* promoter methylation (34.04±7.32 vs. 33.49±10.38, *P*=NS).

4. Discussion

The present study shows that epigenetic regulation by means of CpG sites methylation is a fundamental phenomenon at *F7* promoter region that significantly affects plasma FVIIa concentrations. An inverse correlation was identified between *F7* promoter methylation index and plasma FVIIa concentrations, therefore highlighting a transcriptional-silencing function of methylation at *F7* promoter site which affects the gene product.

The role of methylation appears significant in absence of known functional *F7* gene variations at promoter site and is associated to coronary artery disease.

To the best of our knowledge, this is the first report which demonstrates the function of methylation at *F7* promoter site in modulating plasma FVIIa concentrations and its association with coronary artery disease. The present data show also a relationship between *F7* methylation index with known functional polymorphisms at gene promoter region, emphasizing the role of this epigenetic feature of DNA as a main player for the gene product regulation specifically for *A1A1* genotypes.

Genotype variations at *F7* promoter are indeed well-assessed determinants of plasma FVIIa concentrations [5, 9, 10, 12]. The *-323del/ins* polymorphism is associated with lower FVIIa concentrations in homozygous mutants [5, 9, 10] and the *G>A* substitution at position *-402* confers instead a higher transcriptional activity associated with increased plasma FVIIa concentrations *in vitro* [12] and in selected populations [7].

As for epigenetic marks, not only is the role of methylation largely unexplored for atherothrombosis-related genes but the relationship between promoter methylation and the simultaneous presence of gene variations is also mostly unknown.

Coagulation *F7* promoter site polymorphisms have a definite functional effect for the protein end-product concentrations [5, 9, 10, 12]. Therefore, the study of this gene appears of peculiar interest to evaluate the role of methylation in the case of conserved gene sequence at the promoter site.

DNA methylation is the major epigenetic feature in mammalian DNA [14]. This epigenetic phenomenon has been mainly studied in cancer disease, thus far. Hypermethylation of CpG islands at promoter regions is generally associated with gene transcriptional silencing [18, 19, 27, 28] and represents, therefore, an unconventional mechanism through which tumor suppressor genes are inactivated without the presence of sequence anomalies such as mutations or allele deletions [19, 28]. Hypomethylation of CpG islands, instead, is usually associated with gene activation and represents an important mechanism by which proto-oncogenes are activated [28].

The epigenetic regulation of genes is still not entirely known for a large number of genes. Furthermore, the role of epigenetic modifications in DNA related to diseases other than cancer is yet mostly unexplored.

The present study shows that *F7* methylation index is inversely related to plasma FVIIa concentrations, therefore confirming earlier data referred to cancer-related genes on the prominent transcriptional repression function of methylation at promoter site [18, 19]. The silencing effect of methylation at *F7* promoter region appears certainly significant for this crucial atherothrombosis disease-related gene [2, 3].

Not only is the role of epigenetic mechanisms yet incompletely defined for many genes but the inter-relationship between regulation through methylation and the simultaneous presence of functional genetic variations at promoter site is yet unclear.

The present results highlight a substantial role for methylation in absence of the rare *A2* allele, along with the substantiation of previous results on the significant functional effect of the *F7 10bp del/ins* polymorphism at position *-323* in determining plasma FVIIa [4]. Subjects carrying the *A2* allele showed, as expected, lower FVIIa concentrations compared to *A1A1*. There was, however, no difference in FVIIa concentrations between carriers and wild types of the *-402G>A* polymorphism in the present setting of subjects, therefore being these findings partly different from previous studies in which a functional role of the rare *-402A* genotype was observed [7, 29]. However, an inverse correlation between promoter methylation and FVIIa concentrations was detected within the *-402GG* wild-type group, then indicating once more a possible role for methylation in the absence of this important functional polymorphic site at *F7* promoter. The limited number of subjects may be a reason for not reaching a statistically significant difference in the methylation index for the *-402GG versus* the*-402AA* genotype group (*P*=0.054).

The methylation score is appreciably lower in *A1A1* wild-types compared to the *A2A2* genotypes and corresponds to the higher plasma FVIIa concentrations in those not carriers of the rare functional *A2* allele. Furthermore, *F7* promoter methylation index is inversely related to plasma FVIIa concentrations only in *A1A1* genotypes, highlighting a link between an epigenetic marker and the biochemical phenotype in absence of the well-defined genetic variation, namely the *A2* allele. Modulation of FVIIa

concentrations by promoter methylation was apparently critical in carriers of the *A1A1* genotype.

Although, further studies are certainly needed to reveal the role of genetic-epigenetic relationships at promoter site, through these findings one can speculate that epigenetic regulation through methylation may exert a stronger effect in absence of functionally significant genetic variations at promoter site for the control of a gene product. Furthermore, for the unequivocal effect of the rare *A2* allele on regulating FVIIa levels, *i.e.* the gene end product, *F7* may in fact represent a paradigmatic example of different roles of genetic variations comparatively to epigenetic modifications in transcriptional regulation at promoter region. A correlation between methylation and plasma FVIIa concentrations was indeed observed only in *A1A1* wild type subjects, while DNA methylation was not associated with *A2* allele carriership which may be related to the localization of *-323del/ins* at the start of translation region which may also give reason for the minor effect of the genetic-epigenetic relationship observed according to the presence of the *-402G>A* genotype.

It is, in truth, interesting to point out that the higher F7 promoter methylation index corresponding to lower FVIIa concentrations, was higher in CAD-free compared to CAD subjects and related to lower risk in A1A1 subjects while no effect in terms of CAD risk was related to methylation in A2A2 genotypes. Our clear definitions of phenotypes should have reduced the chance of spurious results, a problem inherent in studies of allelic association [30].

This observation may allow, therefore, for considering F7 promoter methylation in PBMC DNA as a possible novel protective marker for coronary disease risk in a subgroup of patients not-carriers of the functional -323del/ins polymorphism. Because epigenetic mechanisms, differently from genetic aberrations, are potentially reversible, the observations of the present study are of possible high interest for opening up new insights for preventive strategies particularly in complex diseases such as CAD where several factors are into place.

A major strength of our study is its fairly large number of subjects for the evaluation of promoter methylation and its relationship with CAD disease in a case-control clinical study. The method used in this study allowed to detect significant differences and appeared the most suitable approach for the rather large number of cases and controls. The measurement of FVIIa in plasma is a highly accurate index of the gene end-product, as previously shown, for its correlation with genotype markers and CAD risk [4].

One possible limitation is to be seen in the use of PBMC DNA for methylation analysis, although the significant association between methylation index and FVIIa concentrations as well as CAD risk may emphasize a potential role of methylation at *F7* promoter site as a molecular biomarker related to CAD disease in an easily accessible tissue DNA such as that from peripheral blood mononuclear cells.

In conclusion, methylation at *F7* promoter region is a key mechanism through which the gene regulates FVII protein production *via* an epigenetic modality that acts differently from that related to the presence of polymorphic variants at the same site and is associated to modulation of coronary artery disease risk. Additional studies are definitely warranted to better understand the significance of the genetic-epigenetic cross-talks with effects on gene transcription, although the present findings certainly outlines the essential role of promoter methylation for gene transcriptional regulation of *F7* gene product, which appears to exert its prime function in the absence of functional polymorphisms in the promoter region.

Considering that, unlike sequence variants, epigenetic features of DNA are potentially reversible, this previously unknown mechanism of *F7* gene regulation at promoter site may represent a fundamental mechanism through which atherothrombotic disease risk may be modified, therefore opening up a fascinating prospective for future studies related to both pathogenesis and prevention of such a high impact disease for public health issues.

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CHAPTER 4

ONE-CARBON METABOLITES AND ROLE OF B VITAMINS BETWEEN PHYSIOLOGY AND PATHOLOGY AND ITS CORRELATION WITH EPIGENETIC MECHANISMS

Among the essential cofactors that interact with the enzymes of the folate cycle, there is vitamin B6. The relationship between vitamin B6, inflammation and cardiovascular disease still remains a debated topic and there are not recent works that allow to point out the scientific research in this field.

For this reason we have been concerned to review the available literature focusing on the relationship between nutrition and inflammation in cardiovascular disease.

PLASMA VITAMIN B6: A CHALLENGING LINK BETWEEN NUTRITION AND INFLAMMATION IN CARDIOVASCULAR DISEASE

Abstract

The objective of the present review is to highlight the relationship between low vitamin B_6 status and cardiovascular disease (CVD) through its link with inflammation.

While overt vitamin B_6 deficiency is uncommon in clinical practice, increasing evidence suggests that mild vitamin B_6 deficiency is rather frequent in a

consistent portion of the population and is related to an increased risk of inflammation-related diseases.

Ample evidence substantiates the theory of atherosclerosis as an inflammatory disease, and low plasma vitamin B_6 concentrations have been related to increased cardiovascular disease risk. Several studies have also shown that low vitamin B_6 status is associated with rheumatoid arthritis and chronic inflammatory bowel diseases, both of which hold an underlying chronic inflammatory condition. Furthermore, the inverse association observed between inflammation markers and vitamin B_6 supports the notion that inflammation may represent the common link between low vitamin B_6 status and CVD risk. In addition to the epidemiological evidence, there are a number of cell culture and animal studies that suggest several possible mechanisms relating impaired vitamin B_6 status with chronic inflammation.

A mild vitamin B_6 deficiency characterizes, in most cases, a subclinical at-risk condition in inflammatory-linked diseases which should be addressed for an appropriate individually-tailored nutritional preventive or therapeutic strategy.

Introduction

The two major derivates of vitamin B_6 are the coenzyme species pyridoxamine 5'-phosphate and pyridoxal 5'-phosphate (PLP)[1]. PLP is both the major coenzyme form of vitamin B_6 in plasma and the metabolically active coenzyme produced by the phosphorylation of the pyridoxal compound following the oxidation of other vitamin B_6 vitamers in the liver. Plasma PLP is

considered the most sensitive indicator available of tissue vitamin B_6 status because it reflects liver PLP concentrations and stores[2-6], although other measures of vitamin B_6 such as erythrocyte PLP, total plasma B_6, and urinary excretion of 4-pyridoxic acid are also useful marker of human vitamin B_6 status even if less commonly available in clinical practice [7,8]. Overt vitamin B_6 deficiency is a rare condition that is mainly defined by the appearance of specific clinical signs or symptoms whereas also a suboptimal vitamin B_6 status may influence the risk of development of several diseases. It has been previously suggested that vitamin B_6 deficiency corresponds to plasma PLP concentrations below 20 nmol/L while a borderline, marginal impairment of vitamin B_6 status may be observed for plasma PLP level < 30 nmol/L[7, 9], although a clear-cut definition for deficiency of this nutrient seems a notion to be considered in evolution for novel at-risk pathologic conditions due to a grade of impairment that fails to meet the magnitude of deficit classically defined as a deficiency state[8, 9].

Several authors have indicated a role for low plasma PLP concentrations in a number of pathological conditions. This suggests that, not only does overt vitamin B_6 deficiency increase the risk of certain chronic diseases, but even mild vitamin B_6 deficiency could be associated with increased risk of certain chronic diseases[10-12].

It has been reported that low plasma PLP concentrations are inversely related to C-reactive protein (CRP), a major marker of inflammation and a risk factor for atherosclerotic disease[13-14]. A number of studies have shown

that low plasma vitamin B_6 levels are associated with typical inflammatory chronic diseases, such as rheumatoid arthritis (RA)[15] and inflammatory bowel diseases (IBD)[16], and are inversely related to markers of inflammation[17,18]. These studies suggest that impaired vitamin B_6 status in RA patients is not solely caused by either lower intake, malabsorption or excessive catabolism of the vitamin, but is rather the result of metabolic mechanisms caused by the inflammatory process[18,19]. Among other possible hypotheses, it has also been suggested that the underlying inflammatory condition itself may reduce circulating and hepatic concentrations of PLP by mobilizing PLP from the liver and peripheral tissues to the sites of inflammation[18,19]. Chiang *et al.* observed that plasma PLP concentration in rats with adjuvant arthritis was about 53% of the controls at acme of inflammation and related to the content of PLP in the liver, thus suggesting that the lower circulating PLP levels observed in rheumatoid arthritis could be a sign of a decrease in hepatic PLP pools, and that plasma PLP is a valuable indicator of liver B_6 status during inflammation[18]. Further data showed that PLP in plasma seems a more relevant metabolic marker than PLP in the erythrocytes during inflammation[19] as also confirmed by the observation that plasma, but not erythrocyte PLP concentrations, inversely relates to both clinical and biochemical indices of disease activity and severity in patients affected by RA[19].

For decades, several studies have demonstrated that patients with RA or other inflammatory diseases have a higher risk of developing premature coronary artery disease (CAD)[20-21] and traditional CVD risk factors did not,

by themselves, clarify the increased prevalence of CAD in such patients[22]. Through studying the relationship between RA, inflammation, and CAD, some CVD prevention strategies in RA patients have been proposed, including anti-inflammatory therapy with cyclooxygenase-2-specific inhibitors and statins[23] as well as pyridoxine hydrochloride supplementation[24]. Several studies, moreover, confirmed the theory of atherosclerosis as an inflammatory disease, thus demonstrating that the link between atherosclerosis, RA and other inflammatory chronic diseases is through inflammatory processes. Inflammation may therefore be considered a major pathogenic mediator underlying atherosclerosis and its complications[25-26]. The chronic inflammation in atherosclerotic-related disease, such as CAD and stroke, and its major clinical complications, namely myocardial infarction (MI), have also been associated with low plasma vitamin B_6 concentrations[11,27-28, 29].

The relationship between atherosclerosis and vitamin B_6-linked inflammatory mechanisms is indeed of great interest. Does low plasma PLP indicate the sole effect of inflammation? Or could it be a cofactor that promotes the development of inflammation, potentially contributing to a sustained chronic inflammatory response? The answer to these questions may open way to novel and interesting approaches for dietary prevention and therapy.

Plasma vitamin B_6 and coronary artery disease, myocardial infarction and ischemic stroke

Vitamin B_6 and coronary artery disease

Table 1 summarizes the human studies which addressed the relationship between vitamin B_6 status and coronary artery disease. Low plasma vitamin B_6 concentrations are not only associated with an increased risk for atherosclerotic diseases[30] and more specifically with higher CAD incidence[27,31,32] but the higher risk also appears to be independent of other recognised risk factors for CAD, including homocysteine[27]. Moreover, adequate vitamin B_6 levels emerged as a protective factor for CAD[31]. In a prospective design study, Folsom *et al.*,[33] demonstrated the risk of developing coronary heart disease within five years significantly decreased with consensual increasing concentrations of the plasma PLP[33]. The association between low PLP and CAD was, however, not confirmed in all studies[34,35] (Table 1).

In addition to several epidemiological studies demonstrating a role for low PLP as an independent risk factor for CAD, some studies have shown that low PLP may be linked to CAD through inflammation. In an observational study conducted in an Italian population, a cohort of patients with angiography-defined, severe, multivessel CAD were compared to CAD-free subjects to evaluate the relationship between CAD risk and both plasma PLP concentrations and major markers of the acute-phase reaction[11]. An inverse

relationship between plasma PLP and both hs-CRP and fibrinogen was found, confirming previous findings of an inverse association between inflammatory markers and vitamin B_6[13]. The prevalence of low PLP (defined as PLP concentrations below 36.3 nmol/L, which was the median value in control group) was significantly higher among CAD patients compared to controls[11]. The association between low plasma PLP concentrations and increased CAD risk was also independent of the major classical risk factors for atherosclerosis, including total plasma homocysteine. This association continued to be significant even when hs-CRP and fibrinogen were included in the multiple logistic regression models. The strength of this independent relationship was confirmed even after adjustments for a number of other conditions known to be associated with low concentrations of plasma PLP, including aging, smoking status, and impaired renal function. Results from this study also showed that the combined presence of low PLP along with other major risk factors for CAD, such as higher hs-CRP and elevated LDL/HDL ratio, further increased the risk for CAD in a graded manner[11].

These plasma PLP levels could be described as a mild PLP impairment compared to previous studies that had defined PLP impairment by concentrations as low as 20 nmol/L[27], suggesting that even a moderate impairment of this vitamin is sufficient to confer a higher risk for CAD. In a case-control study performed by Lin and Colleagues[36], low PLP concentrations (defined as PLP below 30 nmol/L) were associated with a significantly increased risk of CAD for angiography-documented patients compared to healthy controls, even after adjustments for hs-CRP[36]. In a

case-control study evaluating whether plasma PLP exerts an independent or a synergic effect with inflammation in elevating the risk of CAD, Cheng and colleagues confirmed that low PLP levels (below 20 nmol/L) are independently associated with higher CAD risk. The inverse relationship between PLP and hs-CRP was observed only within the control group[37].

Vitamin B_6 and myocardial infarction

A specific association between low plasma PLP and incidence of MI, the main thrombotic complication of CAD, is supported by early reports[38-40-41] and by subsequent case-control and prospective studies[42,43,44,45], as shown in Table 2. Moreover, an association between lower dietary vitamin B_6 intake and higher risk of MI has also been suggested[42].

A number of studies observed an inverse association between plasma concentrations of vitamin B_6 and risk of MI, independent of known CAD risk factors[44,46], though not all studies confirmed this independent association[47]. In a study evaluating heart transplant recipient, it was observed that about 21% of the patients showed PLP lower concentrations and that only 9% of those with lower plasma PLP levels did not have CVD complications[48]. Subjects in the highest quintile of PLP had a significantly reduced risk of MI but adjustment for either low-grade inflammation or smoking diminished this association[47].

With regard to the relationship between vitamin B_6 and major markers of inflammation, results from early studies have shown that low plasma PLP

levels are related to acute phase reactants in patients with MI who are in the initial phase of the disease[41, 49].

Vitamin B_6 and stroke

In 1995, Selhub et al. described a relationship between lower vitamin B_6 concentrations and extracranial carotid artery stenosis through its role in homocysteine metabolism[28]. Moreover, recent case-control studies have described a possible relationship between low plasma vitamin B_6 concentrations and the onset of cerebrovascular disease, specifically ischemic stroke and transient ischemic attack[29,50]. A strong association between stroke/TIA and low PLP (defined as levels less than 20 nmol/L) was independent of other well-established vascular risk factors, including total plasma homocysteine concentrations. Furthermore, results from that study identified a possible protective effect for higher PLP concentrations[29].

In the context of the Health Professional Follow-up Study, He et al., evaluated dietary intakes of vitamin B_6 together with the intake of other B vitamins by a semiquantitative food frequency questionnaire[51] in relation to risk of ischemic and hemorrhagic stroke[52]. Unlike data related to other B vitamins, the intake of vitamin B_6 was not associated with risk of ischemic stroke after adjustments for lifestyle and dietary factors[52].

Data from the Spanish National Nutrition Survey, which was designed to assess the association between dietary vitamin B_6 intake and CVD mortality,

also showed no association between vitamin B_6 impairment and cardiovascular mortality[53].

Vitamin B_6 and peripheral artery disease

Reports on the relationship between vitamin B_6 and peripheral artery disease are few[53]. Wilmink et al., reported that daily vitamin B_6 intake is lower in patients with peripheral artery disease, as defined by an ankle-brachial pressure index below 0.9, and appears as an independent predictor of peripheral artery occlusive disease. An increase in daily vitamin B_6 intake by one standard deviation significantly decreased the risk of peripheral artery disease by 29%[53].

Plasma vitamin B_6 supplementation, inflammation and cardiovascular disease prevention

An early observation of patients given vitamin B_6 for inflammatory diseases or degenerative diseases found that subjects taking vitamin supplementation had a lower risk of developing MI compared with patients who had not taken vitamin B_6[55]. Table 3 summarizes the main studies in which B vitamin supplementation, including supplementation with B6 vitamin, have been performed. In the Nurses' Health Study, among women with no prior history

of CAD, users of multivitamins containing folate and vitamin B_6 had a reduced risk of CAD[56].

Very few human studies have been performed to evaluate the modifications of major markers of inflammation during supplementation with B vitamins, including vitamin B_6, despite the association with either folate and/or vitamin B_{12}[57,58]. Furthermore, results from those studies showed that CRP and pro-inflammatory interleukins levels are unchanged after vitamin supplementation[57,58]. Antioxidant activity of vitamin B_6 supplementation has been observed in a study performed on rats, but the exact mechanism is unclear[59].

Several large, prospective trials have been conducted in recent years with the principal aim of studying the effects of lowering serum homocysteine concentrations with the use of B vitamins, including vitamin B_6 on cardiovascular events[60-61].

Overall, the most compelling data from vitamin supplementation studies have demonstrated that vitamin B_6 is not effective for preventing the recurrence of cardiovascular events, including CAD, peripheral vascular disease and stroke[54,62-63].

Only a few small trials, performed with renal transplant patients that have hyperhomocysteinemia and subjects at risk for cerebral ischemia have demonstrated the effectiveness of vitamin B_6 supplementation with folate and vitamin B_{12} on carotid artery intima-media thickness progression[64,65]. This

marker for subclinical atherosclerosis was also evaluated in another recent double-blind, placebo-controlled, randomized clinical trial[66]. Vitamin supplementation significantly reduced subclinical atherosclerosis progression only in subjects at low risk for cardiovascular disease with total plasma homocysteine concentrations equal to or above 9.1 μmol/L[66]. Other trials evaluated CAD patients for the effects of B vitamin supplementation, though the interpretation of the results was ambiguous. Supplementation with vitamin $B_{6,}$ folate and vitamin B_{12} after coronary angioplasty decreased the rate of restenosis and the need for revascularization[67], while supplementation after coronary stenting increased the risk of in-stent restenosis and the need for target-vessel revascularization[68].

Despite the advantage of large randomized intervention studies, wisdom from studies of cancer chemoprevention with folate clearly suggest that two critical factors should be taken into account in the above mentioned trials: time and dose of B vitamin supplementation. Nutritional intervention is indeed considered to be a two-edged sword, where a beneficial effect may be observed with nutritional support in the prevention phase and a disease aggravating effect may be observed after the onset of illness, with nutritional support actually fueling the disease process. This observation may certainly apply to folate supplementation, which can effectively prevent the onset or progression of disease in the early phase, while it may accelerate the progression of disease in the late phase. It can therefore be speculated that

continuous supplementation with a single non-physiologic form of vitamin supplements might contribute to unexpected or even harmful outcomes, even in the at-risk condition of lower vitamin B_6 status. Furthermore, the major endpoints of these studies were those of evaluating the effect of lowering homocysteine on the recurrence of established disease, mainly by the simultaneous use of various B-vitamins that have many functions other than lowering total plasma homocysteine. Very little information is available, however, regarding the effects of B vitamin supplementation on inflammatory markers and more so for what pertain to the dose and timing issue as well as possible harmful effect for a possible primary or secondary preventive approach with vitamin B_6.

Even if there is consistently a lack of benefit in secondary prevention of cardiovascular disease with B vitamins supplementation, with or without vitamin B_6, (WENBIT[69], WAFACS[62], VISP[63], NORVIT[61], HOPE-2[70]), it should be also considered that all the above-mentioned studies are quite diverse from one another in terms of the time period of supplementation. In fact, one may raise the point that also the time period of supplementation is extremely critical to reach positive outcomes or avoid possible harmful effects in terms of the rate of CVD events[71]. However, despite the apparently clear outcomes[71], it could be argued that a potential benefit that modifies secondary prevention outcomes may not be observed over a period of moderate duration (between two to five years for the NORVIT, HOPE-2 and VISP studies or of about seven years for the WAFACS study).

Furthermore, the negative results of vitamin supplementation trials, including vitamin B_6 use, does not preclude the possibility of a protective effect in primary prevention. It could be difficult, however, to demonstrate that vitamin B supplementation is ineffective in patients who have had a clinical vascular event, while effective in those without a clinical event or with subclinical atherosclerosis. Clinical trials for primary prevention may require a longer duration and larger population studies in order to answer the key question on whether vitamin B_6 supplementation is effective in preventing CAD before the first vascular event or in younger life.

Studies are needed to find the specific time and optimal dose of vitamin B_6 in order to maximize efficacy, minimize adverse effects, and identify targets for vitamin B_6 interventions on the basis of genetic susceptibility and environmental factors. A better understanding of the mechanisms underlying the relationship between cardiovascular disease and vitamin B_6 may indeed be extremely helpful in designing the most accurate preventive strategies.

Inflammation and vitamin B_6-related atherogenesis

PLP functions as a coenzyme in more than one-hundred reactions that are involved in the metabolic pathways of neurotransmitters as well as the metabolism of amino acids, lipids, and carbohydrates[72]. PLP also takes part in other significant pathways related to immune function[73], thrombosis[74-75],

and inflammation[17], all of which are crucial mechanisms in every stage of the atherosclerotic process.

Furthermore, PLP is implicated in the synthesis and repair of both nucleic acids and proteins. Low vitamin B_6 concentrations could thus reflect an increased consumption of PLP in the accelerated synthesis of cytokines[76] and in the activation and proliferation of lymphocytes, both of which are key events in the inflammatory process[77,78].

Considering the epidemiological evidence of a role of vitamin B_6 in inflammatory-related diseases and the observed relationship with inflammatory markers[11,16,18,79], it is plausible that vitamin B_6 plays a role in CVD pathogenesis through mechanisms linked to inflammation.

Therefore, with the knowledge that systemic acute-phase markers are solid and independent risk factors for CAD[80, 81], that inflammation exerts an essential role in all stages of the atherosclerotic process[82], and the possibility that vitamin B_6 has a role in inflammatory processes, several mechanisms were proposed linking low vitamin B_6 and CVD using cell culture studies, animal studies[72], and clinical trials.

Animal studies reported that inflammation reduces circulating and hepatic concentrations of vitamin B_6. Plasma PLP is considered a sensitive indicator of tissue vitamin B_6 status[3-7, 15, 18]. As shown in Fig. 1, it is thus possible that, in patients in an inflammatory state, PLP is mobilized from the liver and peripheral tissues to the sites of inflammation[19]. Plasma PLP levels are

known to be inversely related to tumour necrosis factor alpha production and other inflammatory cytokines in rheumatoid arthritis [17]. It has been also reported that low plasma PLP concentrations are inversely related to both plasma fibrinogen[83] and CRP [11, 13] with a robust and independent association of other major biomarkers related to vitamin B_6 metabolism[11]. Moreover, inflammatory status increases oxidant stress, which results from an imbalance between oxidant production and antioxidant defenses (Fig.1). All these conditions characterized by the increase of inflammatory cytokines, fibrinogen, CRP and superoxide radicals may induce the consumption of vitamin B_6 with a consequent reduction of PLP plasma levels and its antioxidant effect and, at the same time, they may favour a thrombogenic effect thus triggering impairment of endothelial function a key event in the pathogenesis of atherosclerotic processes (Fig. 1)[84]. PLP has been also described to have an inhibitory effect on endothelial cell proliferation [85, 86]. The persistence of a chronic inflammatory condition may result in the depletion of vitamin B_6, which might then contribute to a sustained chronic inflammatory response (Fig. 1).

Most of the evidence demonstrates a potential role for vitamin B_6 in inflammatory processes where the low vitamin B_6 status in inflammation-related illnesses appears to result not from lower intake or excessive catabolism of PLP but from the inflammatory process underlying the disease itself[79].

Although vitamin B_6 represents an important coenzyme in the metabolism of homocysteine, a recognized risk factor for thrombosis, the role of PLP in atherosclerosis is only partly related to its function in the one-carbon pathway. Several studies thus supported a role for vitamin B_6 in the risk of CVD[27, 31, 42, 46] and stroke[29], independently of homocysteine and other risk factors. A hypothesis is that vitamin B_6 could be directly implicated as a cofactor in an anti-inflammatory mechanism where the utilization of the vitamin results in its consumption, and the consequent low vitamin B_6 concentrations could support and amplify the inflammatory process thereby leading to chronic progression of inflammatory disease. Indeed, a mild deficiency of vitamin B_6 may be associated with an increased risk, not only of atherosclerosis, but also of other chronic inflammatory diseases such as rheumatoid arthritis (RA)[18,87], and inflammatory bowel diseases (IBD)[16]. A number of studies highlight the relationship between PLP and inflammation, showing an inverse association between vitamin B_6 and major markers of inflammation, including plasma fibrinogen concentration[83], erythrocyte sedimentation rate (ESR), and CRP[13]. In the population-based Framingham Heart Study cohort, the association between PLP and CRP was strong and independent of other major biomarkers related to vitamin B_6 metabolism[13], supporting a possible role for plasma PLP in inflammatory processes.

Some reports did not support this observation[88], as the study by Folsom *et al.*, conducted among healthy middle-aged adults in the Atherosclerosis Risk in Communities (ARIC) Study, which did not find an inverse association between CRP and PLP. However, the authors did observe that lower plasma

PLP and dietary vitamin B_6 were associated with a higher white blood cell count, though this association was not found with the use of vitamin supplements[88]. It should be also taken into account that some diversity in the observations reported by the studies may be due to the different methods utilized to measure plasma PLP, although the methods used by most studies are considered highly reliable for the assessment of vitamin B_6 status[11, 15, 88-90]. The majority of studies, however, confirmed the inverse correlation between PLP and major markers of inflammation in various inflammation-related diseases. In subjects affected by RA[19,24], PLP was associated with ESR, CRP levels, and other markers of disease activity and severity, suggesting that impaired vitamin B_6 status is a result of inflammation. It also appeared that the relationship between lower vitamin B_6 and increased inflammation in these RA patients could be tissue-specific[24].

Plasma PLP concentrations are also altered in subjects who have acute diseases with an evident underlying inflammatory condition. Vasilaki et al, observed that, in patients admitted to the Intensive Therapy Unit with high concentrations of CRP, PLP and intracellular pyridoxal levels were significantly lower in those critically ill patients than in the group of subjects taken as controls[91].

Further support for the hypothesis of a link between vitamin B_6 and inflammation can be found in an analysis of data from a large population-based survey from participants in the 2003–2004 NHANES. Results showed

that higher vitamin B_6 intakes are protective against inflammation, as indicated by hs-CRP concentrations. Moreover, the level of vitamin B_6 intake that was associated with maximum protection against vitamin B_6 inadequacy was elevated in the presence of inflammation compared to its absence[79].

A strong inverse association between vitamin B_6 status, as measured by plasma PLP concentration, and the inflammatory marker CRP was also observed recently in a cohort of elderly Puerto Ricans living in Massachusetts[92]. In this study, chronic inflammatory conditions, such as metabolic syndrome, diabetes, and obesity, were significantly associated with lower plasma PLP. The patients affected by such diseases were also significantly more likely to demonstrate vitamin B_6 inadequacy. Furthermore, lower PLP plasma concentrations were associated with oxidative stress, as indicated by higher urinary concentrations of 8-hydroxydeoxyguanosine, a marker of DNA damage and oxidative stress[92]. Authors concluded that vitamin B_6 status may influence CAD risk through mechanisms that link vitamin B_6 to inflammatory processes rather than mechanisms related to the role of vitamin B_6 in homocysteine metabolism[92].

Conclusions

Several studies demonstrated an association between mild vitamin B_6 deficiency with inflammation-related diseases, including CVD[11,16,18,79], by highlighting an inverse relationship between vitamin B_6 and inflammatory markers[11,19,79]. In a consistent number of studies, this association between impaired vitamin B_6 status and higher risk of CVD is independent of other major traditional atherosclerosis risk factors[13,27,37,46,44] and is inversely related to major markers of inflammation[11]. This evidence suggests a link between impaired vitamin B_6 and CVD through inflammation. Because vitamin B_6 is involved in a large number of physiological reactions, it could be essential to design appropriate studies to define the exact mechanisms underlying the inter-relationships among suboptimal vitamin B_6 status, as defined by both plasma and tissue PLP concentrations, and biochemical-molecular alterations leading to development of inflammation-related diseases. A research priority may be that of investigating the kinetics and regulation of B6 vitamers and enzymes in different body compartments during inflammatory processes.

Mild vitamin B_6 deficiency is not a rare occurrence in population-based studies[12]. This issue, therefore, deserves further investigation, especially in terms of prevention strategies, for the purpose of promoting specific public health policies. Current clinical trials indicate that vitamin B_6 supplementation seems not to be effective for the prevention of recurrence of CVD, although remains still to be discussed the appropriate dosage and timing for possible

beneficial effects through the use of vitamin supplements. Other crucial issues pertain also to the evaluation of the actual effect of supplementation with vitamin B_6 in synthetic form, as well as to whether such approach may be as effective as an adequate dietary vitamin B_6 intake. The question of whether supplementation with vitamin B_6 may be useful for primary prevention of CVD is yet another key issue to be defined. The importance of considering vitamin B_6 status in relation to the risk for CVD may nevertheless open new insights for the potential identification of innovative preventive and therapeutic strategies. In order to help tailor an adequate nutritional approach on an individual basis, both dose and timing as well as possible harmful effect of vitamin B_6 supplementation should be carefully considered in the design of future *ad hoc* clinical trials that are aimed at identifying appropriate vitamin B_6 supplementation.

The notion of a definite deficient status for this nutrient is, as a consequence, a concept to be considered *in fieri* because the risk of certain diseases seems to result from degrees of vitamin B_6 reduction that fall short of the extent of depletion that has been classically defined as a deficiency state.

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Legend to Figure 1

Fig. 1. A simplified scheme of the proposed possible mechanisms for the relationship between low vitamin B_6 and cardiovascular disease.

This figure is a simplified representation of some of the possible mechanisms through which an impaired vitamin B_6 status has been hypothesized to exert its effect in atherosclerotic disease process. As shown, it is possible that vitamin B_6 is mobilized from the liver and peripheral tissues to the sites of inflammation which is characterized by an increase of C-reactive protein (CRP), fibrinogen, superoxide radicals (SOR), tumour necrosis factor alpha (TNFα) as well as other inflammatory cytokines. Plasma vitamin B_6 (pyridoxal-5'phosphate) concentrations are reported to be inversely related to TNFα and other inflammatory cytokines production as well as major markers of inflammation such as CRP and fibrinogen, in chronic inflammatory processes.

The inflammatory status, furthermore, increases oxidant stress, which results from an imbalance between oxidant production (SOR) and antioxidant defenses and may induce the consumption of vitamin B_6 with a consequent reduction of the active form of vitamin B_6 (pyridoxal-5'phosphate) plasma levels and its antioxidant effect and, at the same time, it may facilitate a thrombogenic effect which then triggers the impairment of endothelial function, a key event in the pathogenesis of atherosclerosis. Vitamin B_6 has been also described to have an inhibitory effect on endothelial cell proliferation, therefore the reduction of vitamin B_6 (pyridoxal-5'phosphate) plasma levels induced by inflammatory process may further favour the mechanisms leading to atherogenesis.

Figure 1. Mechanisms for the link between vitamin B_6 and inflammation

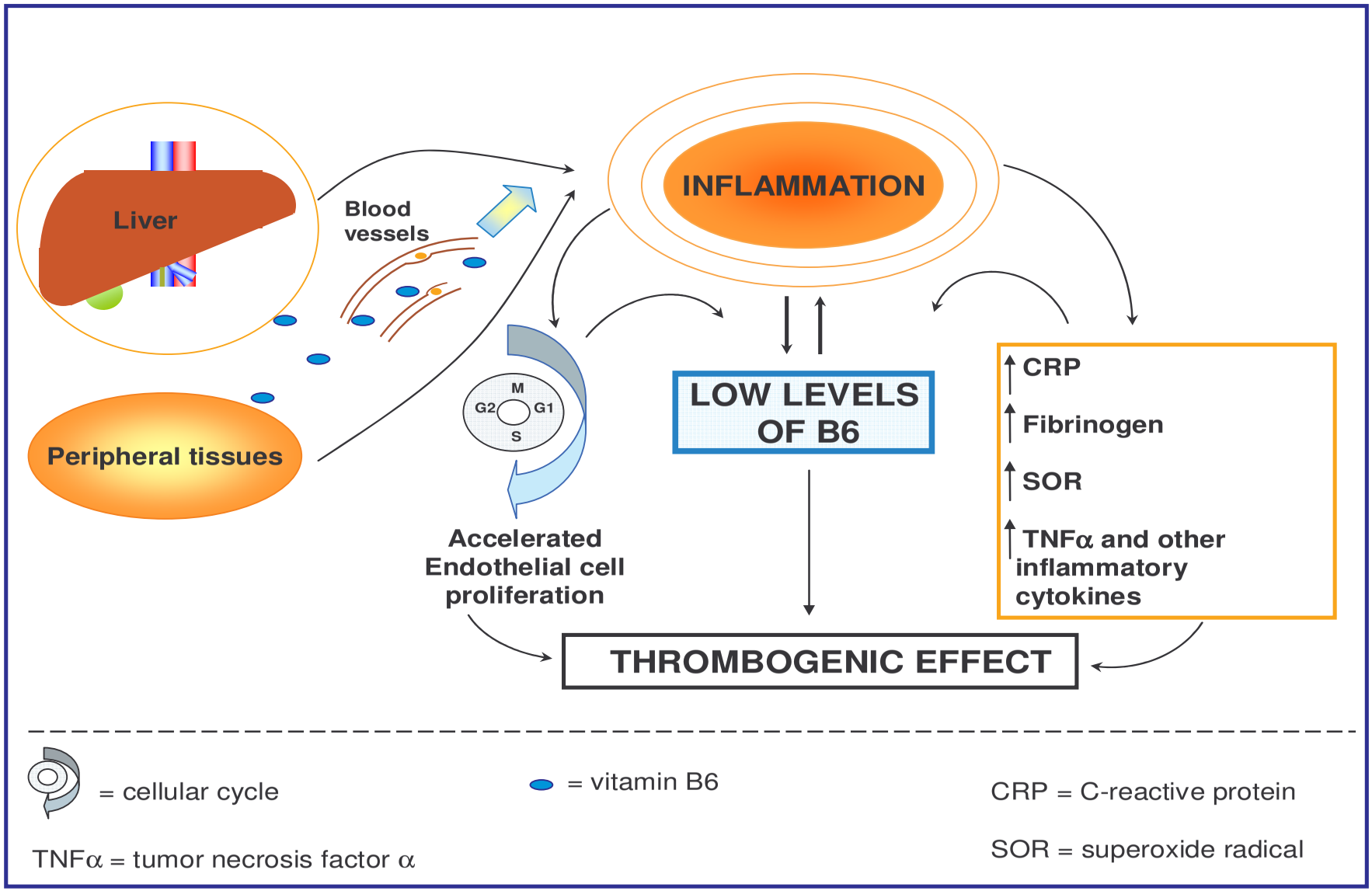

TABLE 1: Vitamin B_6 and risk of CAD

Authors and references	*Study design*	*Main findings*	*Low vit B_6 association with CVD risk*
Cheng et al. (2008) [36]	case-control	Risk of CAD was higher in subjects with plasma PLP < 20 nmol/L (OR, 2.39; 95% CI, 1.24-4.60) and those with hs-CRP > 0.6 mg/dL (OR, 3.37; 95% CI, 1.52-7.46) also after adjustment for potential confounders. The combined presence of low PLP and high hs-CRP levels increased CAD risk (OR, 4.62; 95% CI, 1.28-16.71). Plasma PLP concentrations were significantly and negatively associated with hs-CRP after adjusting for potential risk factors (β = -0.001, P = 0.03) in healthy controls.	yes
Folsom et al.. (2006)[87]	prospective	Risk of developing CHD within 5 years significantly decreased with increasing concentration of vitamin B_6. HR = 0.73 (P < 0.01) for an increase in vitamin B_6 by one SD increment (3.51 nmol/L).	yes
Lin et al. (2006)[35]	case-control	PLP < 30 nmol/L was associated with a significantly higher risk of CAD, as documented angiographically (OR, 1.85; 95% CI, 1.16-2.95), after adjusting for homocysteine and hs-CRP. The association between PLP and risk of CAD remained significant even after adjustment for lipid profile.	yes
Friso et al. (2004)[4]	case-control	There was a significant, inverse, graded relationship between PLP and both hs-CRP and fibrinogen (P < 0.001). PLP concentrations < 36.3 nmol/L were significantly higher for CAD patients (n = 475) than CAD-free subjects (n = 267) (P < 0.001). After adjustment for major classic CAD risk factors, including hs-CRP and fibrinogen, low PLP was still significantly associated with higher risk (OR, 1.89; 95% CI; 1.18-3.03; P = 0.008).	yes
Folsom et al. (1998)[27]	prospective case-cohort	In a population of middle-aged men and women, the incidence of CHD (definite or probable MI, silent MI, fatal CHD, and revascularization procedures) was recorded over an average of 3.3 years of follow-up. Risk of CHD was significantly lower in the highest quintile of PLP compared to the lowest quintile (RR, 0.28; 95% CI, 0.1-0.7, P = 0.001).	yes
Rimm et al. (1998)[55]	prospective case-cohort	Highest quintile of vitamin B_6 intake was associated with a reduced risk for CAD compared to lowest quintile, adjusted for CVD risk factors (RR, 0.67; 95% CI, 0.53-0.85, P = 0.002). Women who regularly used multiple vitamins also had a reduced CAD risk (RR, 0.76; 95% CI, 0.65-0.90).	yes
Robinson et al. (1998)[32]	case-control	Lowest quintile of PLP levels (< 23.3 nmol/L) was associated with an increased risk of atherosclerosis, independent of tHcy. Coronary artery disease, peripheral vascular disease and cerebrovascular disease were documented clinically and/or by angiography.	yes
Siri et al. (1998)[88]	case-control	Among patients with severe CAD, as documented by angiography, those in the lowest quartile ($\leq 25^{th}$ percentile) of vitamin B_6 did not have a significantly higher risk of coronary atherosclerosis (OR, 0.86; 95% CI, 0.33-2.22).	no
Verhoef et al. (1997)[34]	case-control	Plasma PLP was lower in CAD patients (as documented by angiography) than in controls, but the difference was not statistically significant.	no
Dalery et al.,(1995)[33]	case-control	PLP levels were significantly lower in CAD patients (as documented by angiography) than in controls (P < 0.005).	yes
Robinson et al. (1995)[26]	case-control	Patients with low PLP (< 20 nmol/L) had significantly higher risk of CAD adjusting for multiple risk factors, including tHcy (OR, 4.3; 95% CI, 1.1-16.9, P < 0.05).	yes

TABLE 2: Vitamin B_6 and risk of MI			
Author and References	***Study design***	***Main findings***	***Low vit B_6 independent association with MI risk***
Page (2009)[44]	Nested case-control	Fasting concentrations of PLP were significantly inversely associated with subsequent risk of MI: highest versus lowest quartile RR = 0.22 (95% CI, 0.09-0.55; *P* = 0.047). In a conditional logistic regression model, the effect of adding PLP with conventional MI risk factors was statistically significant (χ^2 = 15.8; *P* = 0.001). The relationship between PLP and risk of MI was stronger among women under 60 years old than among older women: highest versus lowest quartile RR = 0.05 (95% CI, 0.004-0.61).	yes
Dierkes (2007)[47]	Nested case-cohort	Subjects were recruited at random from the general population and excluded if they had a history of MI and stroke at baseline. The highest PLP quintile had a significantly reduced risk of MI (HR, 0.50; 95% CI, 0.29-0.83). Adjustment for either low-grade inflammation or smoking diminished this association. When adjusted for both low-grade inflammation and smoking, , the association between PLP and risk of MI was abolished. Adjustment for established risk factors also abolished the association.	no
Tavani (2004)[41]	case-control	Risk of acute MI was significantly lower for the highest tertile of vitamin B_6 intake compared to the lowest tertile of intake (OR, 0.34; 95% CI, 0.19-0.60).	yes
Nahlawi (2002)[89]	follow-up	21% of heart transplant recipients had PLP deficiency. Only 9% of the patients with low PLP did not have CVD complications/death (*P* = 0.05). Risk for CVD events, including death, was 2.7 times higher for patients with low vitamin B_6 (PLP $\leq$ 20 nmol/L) (95% CI, 1.2-5.9, *P* = 0.02).	yes
Folsom (1998)[27]	prospective case-cohort	In a population of middle-aged men and women, incidence of CHD (definite or probable MI, silent MI, fatal CHD, and revascularization procedures) was recorded over an average of 3.3 years of follow-up. Risk of CHD was lower in the highest quintile of PLP versus the lowest quintile (RR, 0.28; 95% CI, 0.1-0.7; *P* = 0.001).	yes
Chasan-Taber (1996)[29]	case-control	The lowest quintile of PLP (< 28.9 nmol/L) had a higher risk of MI when adjusting for multiple variables (RR, 1.5; 95% CI, 1.0-2.2). If adjusted for folate, RR was 1.3 (95% CI, 0.9-2.1).	yes
Verhoef (1996)[46]	case-control	Patients with first MI had lower dietary and plasma vitamin B_6 than controls. OR was 0.97 for the lowest quintile of plasma PLP ($\leq$ 29.7 nmol/L) when adjusted for age and sex and 0.97 (95% CI, 0.40-2.33) when adjusted for multiple variables,.OR was 0.32 for the highest quintile of plasma PLP (> 88.9 nmol/L) when adjusting for age and sex and 0.51 (95% CI, 0.19-1.36) when adjusting for multiple variables.	yes
Kok (1989)[28]	case-control	Patients with MI (n = 84) were compared to control subjects (n = 84). MI was more than 5 times more likely among subjects in the lowest quartile of plasma PLP (< 20 nmol/L) (OR, 5.2; 95% CI, 1.4-18.9).	yes

Legend: MI = myocardial infarction; CHD = coronary heart disease; CVD = cardiovascular disease; 95% CI = 95% confidence interval; RR = relative risk; OR = odds ratio; HR = hazard ratio

Table 3: B vitamin supplementation, including vitamin B6, and CVD				
Author and References	***Study design***	***Supplementation***	***Main findings***	***Benefit of vitamin supplementation***
Albert (2008)[60] WAFACS	Double-blind, placebo controlled randomized clinical trial	2.5 mg folic acid + 1 mg vit. B_{12} + 50 mg vit. B_6 Time: 7.3 years	Population of 5442 women with history of CVD or ≥ 3 coronary risk factors. Patients receiving vitamin supplementation had similar risk to patients receiving a placebo for the composite CVD primary end point (MI, stroke, coronary revascularization, CVD mortality) (RR, 1.03; 95% CI, 0.90-1.19; $P = 0.65$).	No benefit on CVD risk
Ebbing (2008)[61] WENBIT	Double-blind, controlled, randomized clinical trial Two-by-two factorial design	4 groups: 1) 0.8 mg folic acid + 0.4 mg vit. B_{12} + 40 mg vit. B_6 2) 0.8 mg folic acid + 0.4 mg vit. B_{12} 3) 40 mg vit. B_6 4) placebo Time: 7 years (but terminated early, after 38 months)	Population of 3090 patients undergoing coronary angiography with double or triple vessel disease, stable angina pectoris, or acute coronary syndromes. Patients receiving vitamin supplementation had a similar risk to subjects receiving a placebo for the composite end-point (all-cause death, nonfatal AMI, acute hospitalization for unstable angina pectoris, nonfatal thromboembolic stroke). Comparing group 2 vs. 4: HR, 1.09; 95% CI, 0.90-1.32; $P = 0.36$. Comparing group 3 vs. 4: HR, 0.90; 95% CI 0.74-1.09; $P = 0.28$.	No benefit on CVD risk
Bønaa (2006)[63] NORVIT	Double-blind, controlled, randomized clinical trial Two-by-two factorial design	4 groups: 1) 0.8 mg folic acid + 0.4 mg vit. B_{12} + 40 mg vit. B_6 2) 0.8 mg folic acid + 0.4 mg vit. B_{12} 3) 40 mg vit. B_6 4) placebo	Population of 3749 subjects with an acute myocardial infarction within seven days before randomization. Supplementation with vitamin B_6 was not associated with any significant benefit on recurrent myocardial infarction, stroke, or death attributed to coronary artery disease (RR, 1.14; 95% CI, 0.98-1.32; $P = 0.09$). In the group given folic acid, vitamin B_{12}, and vitamin B_6, there was a trend toward an increased risk (RR, 1.22; 95% CI, 1.00-1.50; $P = 0.05$).	No benefit on CVD risk
Lonn (2006)[62] HOPE-2	Double-blind, placebo controlled randomized clinical trial	2.5 mg folic acid + 1 mg vit. B_{12} + 50 mg vit. B_6 or placebo Time: 5 years	Population of 5522 patients with vascular disease or diabetes. Compared with placebo, active treatment did not significantly decrease the risk of death from cardiovascular causes (RR, 0.96; 95% CI, 0.81-1.13) or myocardial infarction (RR, 0.98; 95% CI, 0.85-1.14).	No benefit on CVD risk
Toole (2004)[59] VISP	Multicenter, double-blind, controlled, randomized clinical trial	2 groups: 1) High-dose multivitamin formulation (n = 1827): 2.5 mg folic acid + 0.4 mg vit. B_{12} + 25 mg vit. B_6 2) Low-dose multivitamin formulation (n = 1853): 20 µg folic acid + 6 µg vit. B_{12} + 200 µg vit. B_6 Time: September 1996 – May 2003	Population of 3680 adults with non-disabling cerebral infarction. There was a difference between two groups in mean reduction of tHcy, but there was no treatment effect on any end point. Mean reduction of tHcy was 2 µmol/L greater in the high-dose group than in the low-dose group. The unadjusted RR for stroke, CAD or death was 1 (95% CI, 0.8-1.1).	No benefit on recurrent cerebral infarction or CVD risk
Hodis (2009)[67]	Double-blind, placebo controlled randomized clinical trial	5 mg folic acid + 0.4 mg vit. B_{12}+ 50 mg vit. B_6 or placebo Time: 3.1 years	Population of 506 subjects with tHcy > 8.5 µm/L without diabetes and CVD. Among subjects with tHcy ≥ 9.1 µm/L, those randomized to supplementation had a statistically significant lower average Rate of cIMT progression, as assessed using high-resolution B-mode US, compared with placebo ($P = 0.02$). Among subjects with tHcy < 9.1 µm/L, there was no significant effect of vitamin supplementation on subclinical progression of atherosclerosis.	Limited benefit on subclinical atherosclerosis progression
Till (2005)[66]	Double-blind, placebo controlled randomized clinical trial	2.5 mg folic acid + 0.5 mg vit. B_{12} + 25 mg vit. B_6 Time: 1 year	Population of 50 patients with cIMT ≥1 mm. In the treatment group, cIMT significantly decreased after supplementation ($P = 0.034$). The mean changes in cIMT differed significantly ($P = 0.019$) between vitamin supplementation and placebo groups. Multiple regression analysis revealed that the observed effect on cIMT depended only on medication. This effect was independent of tHcy concentration.	Benefit on cIMT in patients at risk
Marcucci (2003)[65]	Double-blind, placebo controlled randomized clinical trial	5 mg folic acid + 0.4 mg vit. B_{12} + 50 mg vit. B_6 or placebo Time: 6 months	Population of 56 stable hyperhomocysteinemic RTRs. cIMT of common carotid arteries, an early sign of atherosclerosis, was measured with high-resolution B-mode US. In the treatment group, cIMT significantly decreased after supplementation ($P < 0.0001$). In hyperhomocysteinemic patients without vitamin supplementation, there was a significant increase in cIMT after 6 months ($P < 0.05$).	Benefit on cIMT in group of RTRs

Legend: CAD = coronary artery disease; CVD = cardiovascular disease; MI = myocardial infarction; tHcy = total homocysteine; RR = relative risk; OR = odds ratio; HR = hazard ratio; 95% CI = 95% confidence interval; RTRs = renal-transplant recipients; cIMT = carotid intima-media thickness;

Table 4:Vitamin B_6 and inflammation			
Author and References	***Study design and participants***	***Main findings***	***Conclusions***
Morris (2010)[78]	Observational study on a portion of the participants in 2003-2004 NHANES (general U.S. population); 2686 adults were eligible.	Higher vitamin B_6 intakes were protective against inflammation (as measured by CRP concentrations). After multivariate-adjusted analysis, the prevalence of vitamin B_6 inadequacy was <10% when serum CRP concentrations were ≤ 3 mg/L and about 50% if serum CRP concentrations were > 10 mg/L ($P < 0.001$).	Low vitamin B_6 status in inflammation-related illnesses appears to be caused, not by lower intake or excessive catabolism of PLP, but from the inflammatory process underlying the disease itself.
Shen (2010)[6]	Cross-sectional study on 1205 Puerto Rican adults living in Massachusetts.	Plasma PLP was related to plasma CRP in a clear dose-response relationship. Plasma CRP significantly decreased with increasing quartiles of plasma PLP ($P < 0.001$).	Low PLP concentrations are associated with markers of inflammation and may influence CAD risk through different mechanisms than homocysteine.
Cheng (2008)[36]	Case-control study with 184 CAD patients and 516 CAD-free control subjects.	In the controls but not the cases, PLP was negatively associated with hs-CRP ($P = 0.03$). Risk of CAD was 2.39 times higher for low PLP and 3.37 times higher for high hs-CRP. The co-occurrence of low PLP and high hs-CRP was associated with 4.35 times higher risk of CAD.	PLP and hs-CRP are independently associated with CAD risk. Low PLP and high hs-CRP occurring together increased risk of CAD.
Vasilaki (2008)[85]	Case-control study on 96 critically ill patients and 126 control subjects.	PLP and PL were significantly lower in critically ill patients than in control subjects ($P < 0.001$ and $P < 0.01$, respectively). Among patients, the PLP:PL ratio was significantly lower in plasma than in red blood cells ($P= 0.001$).	The inflammatory condition may induce an increased utilization of vitamin B_6.
Chiang (2005)[20]	Double-blind study on subjects with RA and low vit. B_6 levels randomized to receive either active vit. B_6 (50 mg of pyridoxine, n = 14) or placebo (n = 14) tablets for 30 days.	Subjects taking methotrexate or prednisone treatment were stratified in two subgroups, and the subjects in each group were randomized to receive either active vitamin B_6 or placebo treatment. There were significant improvements in vitamin B_6 parameters only in active treatment group. In patients with RA, PLP and net increase in plasma homocysteine (post-methionine load test) were related to CRP.	Vit. B_6 supplementation should be considered in RA patients to reduce the potential for adverse consequences of B_6 vit. B_6 deficiency. CRP could be a potential target for vit. B_6 supplementation.
Friso (2004)[4]	Case-control study on 742 participants that either had severe multivessel CAD (n = 475) or were CAD-free (n = 267).	A significant, inverse relationship was observed between PLP and both hs-CRP and fibrinogen ($P < 0.001$). The prevalence of PLP < 36.3 nmol/L was significantly higher in CAD patients than in CAD-free subjects ($P < 0.001$). The OR for CAD risk related to low PLP concentrations after adjusting for the major classic CAD risk factors, including hs-CRP and fibrinogen, was 1.89 (95% CI, 1.18-3.03; $P = 0.008$).	Low plasma PLP is inversely related to markers of inflammation and independently associated with increased risk of CAD.
Chiang (2003)[12]	Cross-sectional study on 37 patients with RA.	PLP levels were inversely correlated with VES (r = -0.37, $P = 0.02$), CRP (r = -0.52, $P = 0.002$) and with other markers of disease activity and severity (disability score, morning stiffness and degree of pain).	In patients with RA, there is a consistent association between PLP and several indicators of inflammation. Impaired vitamin B_6 status may be a result of inflammation.
Folsom (2003)[84]	Cross-sectional study on 519 healthy middle-aged adults in the ARIC Study.	Plasma PLP was not associated with CRP concentrations, but it was significantly inversely associated with both factor VIIIc and WBC count.	B-vitamin status does not correlate strongly with circulating levels of inflammatory markers.
Saibeni (2003)[9]	Case-control study on 61 patients with IBD.	Median vitamin B_6 levels were significantly lower in IBD patients than in controls ($P < 0.01$). Low vitamin B_6 levels were significantly more frequent in patients with active disease than in patients with quiescent disease ($P < 0.001$). Low PLP levels were significantly correlated with CRP ($P < 0.01$).	Low PLP is frequent in patients with IBD, especially those with active disease.
Friso (2001)[5]	Observational study on 891 participants from the population-based Framingham Heart Study cohort.	Mean plasma PLP levels were lower in subjects with CRP ≥ 6 than in subjects with CRP < 6 mg/L (mean values were 36.5 nmol/L and 55.8 nmol/L, respectively; $P < 0.001$). After multiple logistic regression, including adjustment for tHcy, the association between PLP and CRP remained highly significant ($P = 0.003$).	Low plasma PLP is associated with higher CRP levels independent of tHcy. Vitamin B_6 may be decreased due to its increased utilization in the site of inflammation

Legend: CAD = coronary artery disease; RA = rheumatoid arthritis; IBD = Inflammatory Bowel Disease; ARIC = Atherosclerosis Risk in Communities; PL = pyridoxal; PLP = pyridoxal phosphate; VES = erythrocyte sedimentation rate; CRP = C-reactive protein; hs-CRP = high-sensitivity C-reactive protein; WBC = white blood cell; OR = odds ratio; 95% CI = 95% confidence interval US = ultrasound

ONE-CARBON METABOLISM, ADMA PLASMA CONCENTRATIONS AND CARDIOVASCULAR RISK

With the aim to identify other risk markers of disease, detectable in the plasma and correlated to one-carbon metabolism, we set out to study, asymmetric dimethylarginine, a metabolite of the folate, to assess its role on the risk of developing cardiovascular disease in our population.

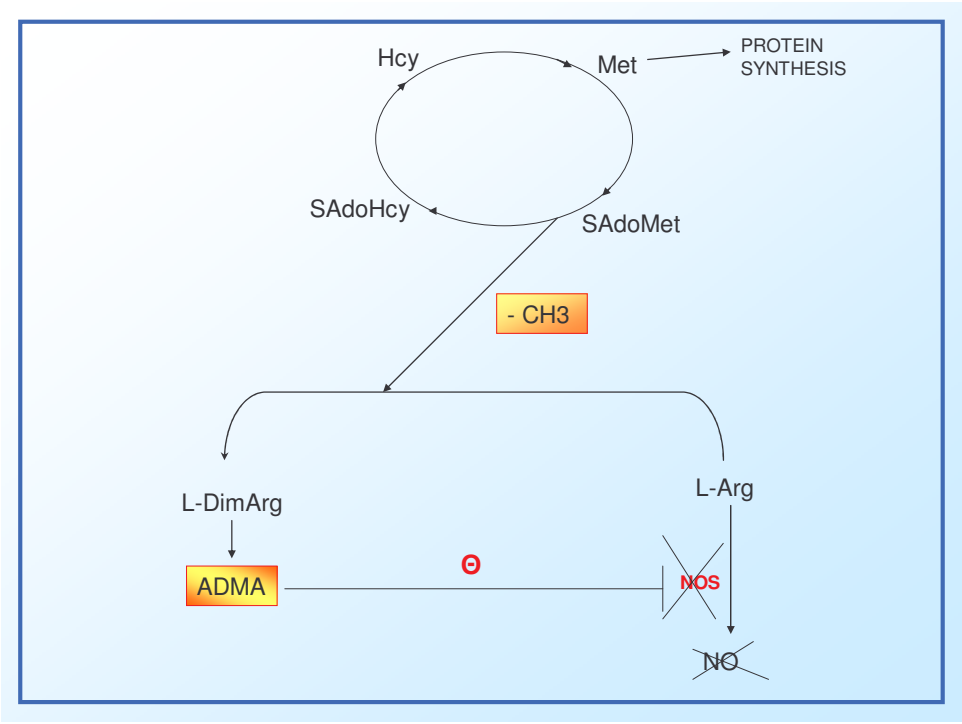

Figure 1: schematic representation of inter-relationship between ADMA and one-carbon metabolism. Hcy: homocysteine; Met: methionine; SAdoMet: S-adenosylmethionine; SAdoHcy: S-adenosylhomocysteine; -CH3: methyl group; L-DimArg. L-Dimethyl-arginine; L-Arg: L-Arginine; ADMA: Asymmetric dimethylarginine; NO: nitric oxide; NOS: nitric oxide synthase

Abstract

Asymmetric dimethylarginine (ADMA), is an endogenous competitive inhibitor of nitric oxide synthase (NOS), enzyme responsible for the production of nitric oxide (NO) by endothelial cells. NO is able both to induce vascular dilation and to influence monocytes adhesion, platelets aggregation and smooth muscle cells proliferation.

In 300 subjects of Verona Heart Project, the association between ADMA and coronary artery disease (CAD) was evaluated. ADMA plasma concentrations higher than 0.69 µmol/L (the highest tertile) defined a significant increase risk of myocardial infarction (MI), the main thrombotic complication of CAD (OR=2.14, 1.17-3.92, IC 95%, *P*=0.012). This association concerned only the female subgroup (OR=2.79, 1.07-7.27, IC 95%, *P*=0.032), where the increase of MI risk maintained a statistically significant difference in a multivariate model analysis adjusted for the major risk factors for CAD (OR=2.04, 1.09-3.82, IC 95%, *P*=0.026). This finding could allow to highlight a novel markers of CAD risk more specific for female patients.

Introduction

The endothelium plays a crucial role to maintain the vascular tone and structure. One of the major endothelium-derived vasoactive mediators is nitric oxide (NO). Asymmetric DiMethylArginine (ADMA) is a natural component of human blood plasma, produced by protein turnover. It is an endogenous competitive inhibitor of all three isoforms of nitric oxide synthase

(NOS) and elevated ADMA plasma levels have been reported in relation with diseases connected with an impaired endothelial L-arginine-NO pathway and endothelial dysfunction[1, 2]. ADMA is relased when methylated proteins are degraded into their amino acid components during hydrolytic protein turnover. The majority of ADMA is eliminated by degradation to dimethylamine and citrulline by the enzyme dimethylarginine dimethylaminohydrolase (DDAH); the other is eliminated by renal excretion. DDAH dysfunction may be a crucial unifying element of increased ADMA levels as well as cardiovascular risk[1, 3, 4]. Moreover, DDAH is currently investigated as a promising target for therapeutic interventions: transgenic and knock-out mouse with altered DDAH activity have been developed to study drug effects[5].

When ADMA levels are elevated, NO production by NOS is decreased since ADMA inhibits NOS by competitive displacement of the physiological substrate (L-arginine), from the enzyme. This inhibition leads to a decreased NO production in the endothelium of vessel walls with consequent increased resting vascular tone (vasoconstriction) and enhanced pro-atherogenic mechanisms including proliferation of vascular smooth muscle cell, platelet aggregation and adherence of monocytes, extracellular matrix formation[6] (Figure 2).

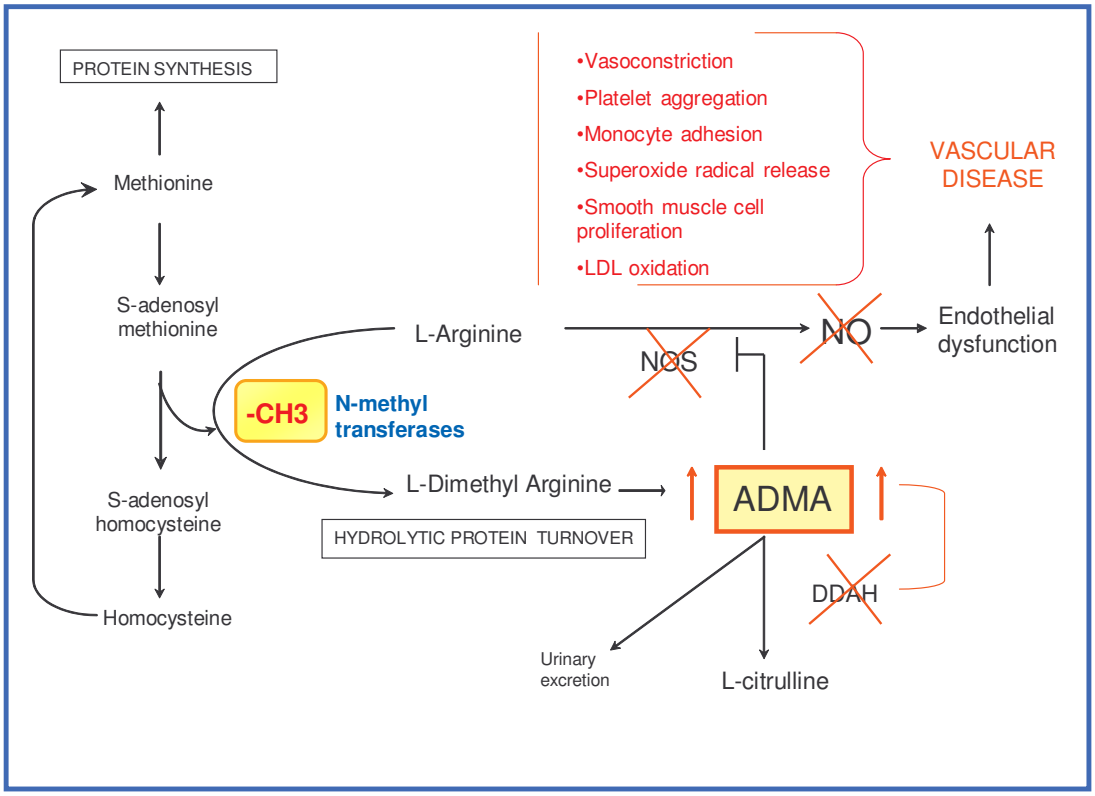

Figure 2: Effects of hight ADMA plasma concentrations on the endothelial dysfunction.

In 1992, P. Vallance and co-workers described for the first time, a molecule with structural omology to L-arginine, but containing one o two methyl groups, acting as inhibitors of NO synthesis[7]. They showed that asymmetric dimethylarginine belonged to this group of substances and was in position to inhibit NO synthesis[7]. By contrast, symmetric dimethylarginine (SDMA), the structural isomer of ADMA, had no effect on NO production. Subsequently, many researchers tried to elucidate the pathophysiological role of ADMA, and now they are agree that ADMA play a prominent role in the pathogenesis and in the progression of cardiovascular disease, specifically atherosclerosis[6, 8].

Many clinical studies demonstrated a significant and independent relationship between ADMA levels and the incidence of major adverse cardiovascular events[9,10], death [8,11], and higher risk of disease relapse[6].

Elevated ADMA concentration may play an important pathophysiological role also in many other clinical condition, such as hypertension[12], hypercholesterolemia[13], chronic renal failure[14, 15], chronic heart failure[16], diabetes mellitus[17, 18]. Moreover, other situations have been reported to be associated with elevated ADMA concentration as smoking, erectile dysfunction and preeclampsia[19].

The main aim of this project was to evaluate a possible association between plasma concentration of ADMA and coronary artery disease, including its main thrombotic complication, myocardial infarction,. The second aim was to analyse the role of ADMA in male and female patients to evaluate a possible risk variation with regard to gender.

Methods

Study Population

The Verona Heart Study (VHS) is a case-control study ongoing since May 1996 and designed to identify novel risk factors for CAD and MI in a population of subjects with documented coronary angiography data. Study design and details about enrolment/exclusion criteria have been previously described[20]. In the present study, we examined a total of 300 subjects for whom complete biochemical and clinical data were available. Of these, 200

had angiographically documented severe coronary atherosclerosis (CAD) with or without previous history of MI (MI group), documented by combining data from clinical history with a thorough review of medical records including diagnostic electrocardiogram and biochemical data on myocardial enzymes, and/or the typical sequelae of MI on ventricular angiography. One hundred subjects, that underwent coronary angiography for reasons other than CAD (mainly valvular heart disease) and showed normal coronary arteries, were considered as controls (CAD-free group). Controls were also required to have neither history nor clinical or instrumental evidence of atherosclerosis in vascular districts beyond the coronary bed. At the time of blood sampling, a complete clinical history was collected, including the assessment of cardiovascular risk factors such as obesity, smoking, hypertension and diabetes, as well as medical therapy.

The study was approved by our Institutional Ethical Committee. Informed consent was obtained from all the subjects after a full explanation of the study.

Biochemical analyses and ADMA assay

Samples of venous blood were drawn from each subject after an overnight fast. Laboratory assessment of several biomarkers of CAD were determined as previously described[20, 21]. Total plasma homocysteine levels were determined by high-performance liquid chromatography (HPLC) with fluorescent detection, according to Araki and Sako[22]. Plasma folate and

vitamin B12 concentrations were measured by an automated chemiluminescence method (Chiron Diagnostics, East Walpole, MA).

Asymmetric dimethylarginine (ADMA) levels were determined in EDTA plasma samples stored at –80°C before use and without freeze-thaw cycles by an enzyme-linked immunosorbent assay (DLD Diagnostika, Hamburg, Germany) according to the manufacturer's instructions.

Statistical analysis

All statistical analyses were performed with SPSS 17.0 statistical package (SPSS Inc., Chicago, IL). Distributions of continuous variables in groups were expressed as means±standard deviation. Logarithmic transformation was performed on all skewed variables, including tHcy, folate, vitamin B12 and ADMA. Therefore, the statistical differences concerning these parameters were also computed on the corresponding log-transformed values. Quantitative data were assessed using the Student's t-test or by ANOVA. Associations between qualitative variables were analyzed with the χ^2-test. A value of $P < 0.05$ was considered significant.

To assess the extent to which ADMA was associated with CAD and MI, odds ratios with 95% CIs were estimated by univariate logistic regression analysis. Adjustment for classical cardiovascular risk factors (i.e. gender, age, hypertension, smoke, diabetes, cholesterol and triglyceride) was performed by adding those covariates in a multiple logistic-regression model.

Results

Table I shows the biochemical features about one-carbon metabolism and plasma ADMA concentration according to CAD or CAD-free history. There was no difference in plasma concentrations of ADMA, homocysteine, folate, vitamins B6 and B12 between CAD and CAD-free subjects (respectively 0,64 µmol/L vs 0,62 µmol/L; 13,44 mmol/L vs 12,45; 9,51 nmol/L vs 10,79; 28.79 nmol/L vs 35,64; 413,23 pmol/L vs 407,48. All P=NS).

	CAD patients N=200	CAD-free subjects N=100	*P*-value
Homocysteine (mmol/L)	13,44 (12,14-14,88)	12,45 (12,52-14,45)	NS
Folate (nmol/L)	9.51 (7.91-12.40)	10.79 (14.42-8.15)	NS
Vitamin B6 (nmol/L)	28.79 (25.03-35.16)	35.64 (29.08-42.95)	NS
Vitamin B12 (pmol/L)	413.23 (391.51-450.34)	407.48 (304.91-685.40)	NS
ADMA plasma concentrations (µmol/L)	0,64 (0,62-0,66)	0,62 (0,59-0,64)	NS

Table I: Biochemical features concerning one-carbon metabolism and ADMA concentrations of the study population according to CAD or CAD-free diagnosis.

Table II shows the plasma concentrations both of ADMA and of homocysteine within the CAD group according to history of MI. ADMA levels were higher among those with a positive MI history (n=100) compared with those who are affected by CAD but without MI history (n=100) (0,66, 95% CI 0,63-0,69 vs. 0,61, 95%CI 0,60-0,63 µmol/L, P=0.011).

	MI patients (N=100)	MI-free patients (N=199)	*P*-value
ADMA plasma concentrations (µmol/L)	0,66 (0,63-0,69)	0,61 (0,60-0,63)	0,011
Homocysteine (µmol/L)	14,87 (13,41-16,49)	12,79 (11,92-13,72)	0,017

Table II. ADMA and homocysteine plasma concentrations in patients with positive (MI) or negative (MI-free) history for myocardial infarction.

Table III shows the biochemical features of the study population concerning one-carbon metabolism and ADMA concentrations according to subjects'gender (162 male versus 138 female patients).

	males (n=162)	females (n=138)	*P*
ADMA (µmol/L)	0,60 (0,59-0,62)	0,66 (0,64-0,69)	0,001
Folate (nmol/L)	10.58 (9,38-11,91)	14,23 (12,16-16,70)	0,03
Vitamin B12 (pmol/L)	329,11 (287,69-376,49)	319,64 (268,46-380,62)	NS
Homocysteine (µmol/L)	13,89 (12,73-15,14)	13,26 (11,94-14,72)	NS

Table III. Biochemical features of the study cohort concerning one-carbon metabolism and ADMA concentrations according to sex.

As illustrated in Figure 3, ADMA plasma concentrations in women were higher than those in men and the difference between two groups was statistically significant (0.60 µmol/L, IC 95% 0.59-0.62 µmol/L *versus* 0.66

µmol/L, IC 95% 0.64-0.69 µmol/L; *P*=0.001). Moreover, this relationship remained statistically significant even after adjustment for age (*P*= 0,003).

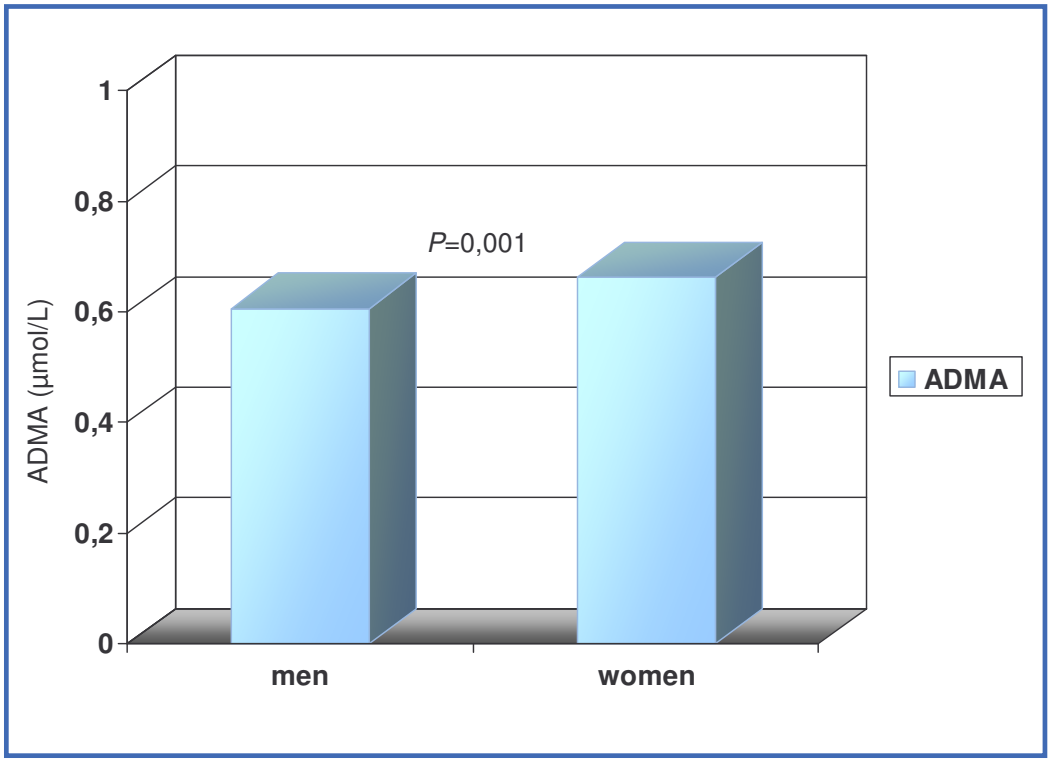

Figure 3: ADMA plasma concentration according to sex

The same difference between male and female appeared also for folate plasma concentrations, as exemplified in figure 4 (10.58 nmol/L, IC 95% 9.38-11.91 nmol/L *versus* 14.23 nmol/L, IC 95% 12.16-16.70 nmol/L).

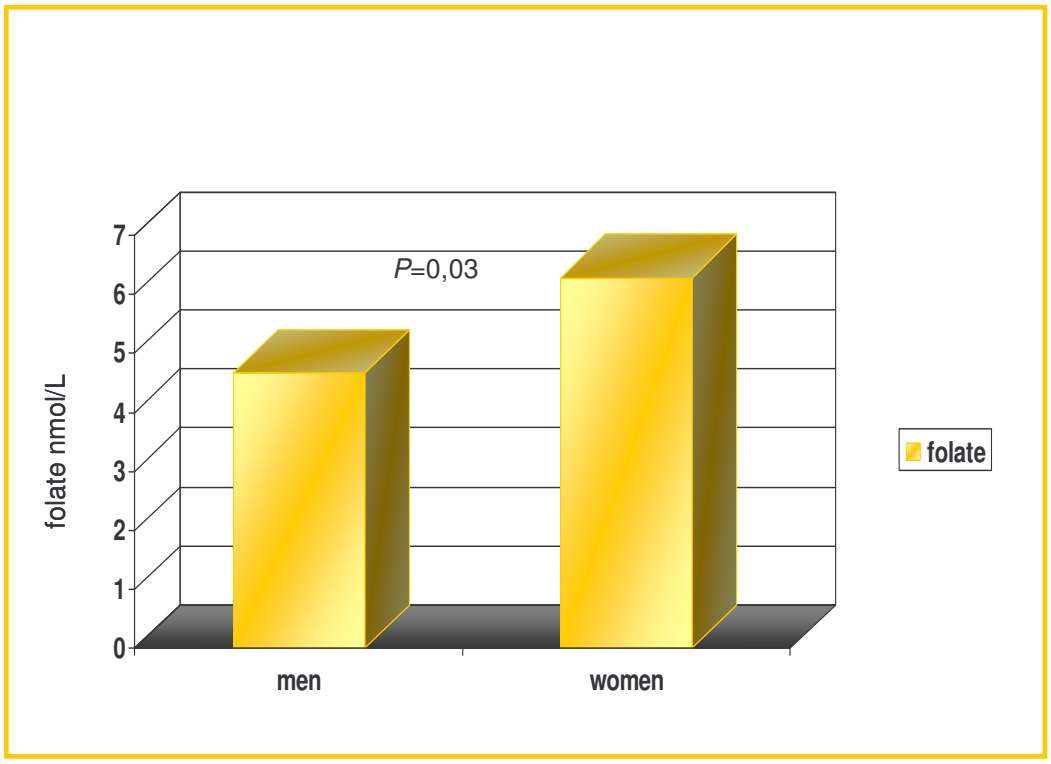

Figure 4: Folate plasma concentrations according to sex

There were not statistically significant differences between male e female groups with regard to B12 vitamin levels (329.11 pmol/L, IC 95% 287.69-376.49 pmol/L *versus* 319.64 pmol/L, IC 95% 268.46-380.62 pmol/L; *P*=NS) and homocysteine concentrations (13.89 µmol/L, IC 95% 12.73-15.14 µmol/L *versus* 13.26 µmol/L IC 95% 11.94-14.72 µmol/L; *P*=NS).

Table IV illustrates plasma ADMA concentrations in males and females, according to history for myocardial infarction (MI). The women had ADMA plasma concentrations significantly higher than those in men, both in group with MI history (0.70 µmol/L, IC 95% 0.65-0.76 µmol/L *versus* 0.63 µmol/L, IC 95% 0.59-0.66 µmol/L, *P*=0.015) and in MI-free group (0.64 µmol/L, IC 95% 0.61-0.67 µmol/L *versus* 0.59 µmol/L, IC 95% 0.57-0.62 µmol/L, P=0.010).

Table IV. ADMA plasma concentrations (µmol/L), in relations to history for MI, according to sex.

	MI	MI-free	*P-value*
men	0,63 (0,59-0,66)	0,59 (0,57-0,62)	NS
women	0,70 (0,65-0,76)	0,64 (0,61-0,67)	0.038
P-value	0.015	0.010	

There was no significant difference in ADMA plasma concentrations between men with or without history for MI. On the contrary, in female group, ADMA plasma concentrations were statistically different (*P*=0.038) between women with or without history for MI (Fig 5).

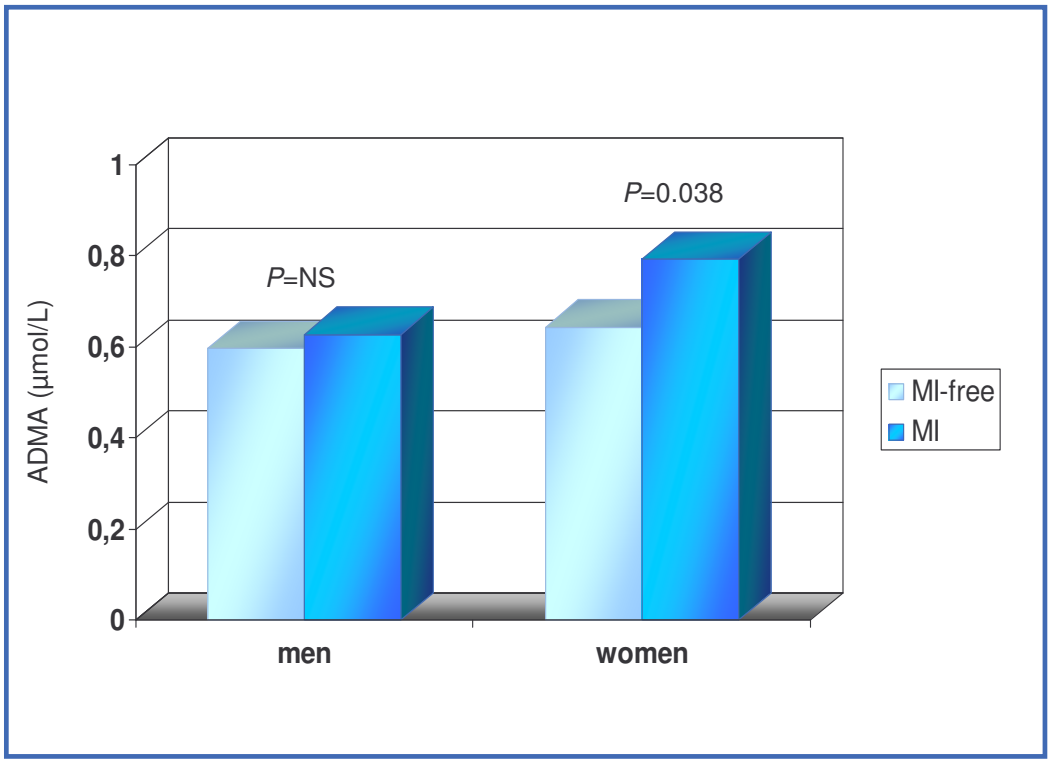

Figure 5: ADMA plasma concentrations in male and female according to history of MI

Further analysis determined whether there was a difference in the estimate of risk of CAD according to increasing ADMA quartiles, the study participants were stratified in four groups according to ADMA concentrations of 0.54 e a 0.73 µmol/L respectively for 25th and 75th percentile. The univariate logistic regression analysis did not show any difference among groups. The same analysis to estimate the risk of CAD performed on ADMA concentrations subdivided among tertiles (ADMA concentration of 0.56 e a 0.69 µmol/L respectively for the lowest and the highest tertile), also did not shown any statistically significant difference. When the univariate logistic regression analysis was performed to define the risk of MI, a more elevated risk for MI

according to ADMA concentrations higher than the upper tertile was observed (OR=2.14, 1.17-3.92, IC 95%, *P*=0.012).

The univariate logistic regression analysis performed to define the risk of MI according to sex, highlighted a more elevated risk depending on ADMA concentrations higher than the superior tertile only for the female subgroup (OR=2.79, 1.07-7.27, IC 95%, *P*=0.032). There was no such associations in male subgroup of subjects (OR=1.71, 0.77-3.85, IC 95%, *P*=0.19). Moreover, in the female subgroup, the higher risk for MI for ADMA concentrations higher than 3rd tertile was maintained in a multiple logistic regression model adjusted for menopause (OR=2.75, 1.04-7.28, IC 95%, *P*= 0.041) and also for other factors such as age, family history for CAD, hypertension and BMI (OR=2.23, 1.09-4.54, IC 95%, *P*=0.027).

In the multinomial regression analysis, higher risk for MI remained statistically significant also after adjustment for smoking (OR=2.04, 1.09-3.82, IC 95%, *P*=0.026) and diabetes mellitus (OR=1.86, 0.99-3.47, IC 95%, *P*=0.050). When the risk of MI associated to ADMA tertiles was adjusted for homocysteine plasma levels, the statistical analysis lost the statistically significant value (*P*=0.19), since there was a correlation between the two variables. In the multinomial regression analysis, however, also homocysteine lost the statistically significant value (*P*=0.21).

Discussion

The cardiovascular disease has a composite genesis based on many different factors. In the recent years, several studies attempted to identify some new risk factors to support classic factor as diabetes, cholesterol, obesity, smoking, age and familiarity.

Among new parameters that have been studied, ADMA may be considered a cardiovascular risk marker in numerous diseases related to endothelial dysfunction increased cardiovascular mortality and morbidity.

In the multicenter case-control CARDIAC study (Coronary Artery Risk Determination investigating the Influence of ADMA Concentration) Schulze and colleagues[23] analysing 131 CAD patients and 131 controls from the general population, found that cases had higher ADMA plasma levels than controls. This relationship remained statistically significant (p=0,001) also after multivariare logistic regression model including laboratory parameters and traditional risk factors (homocysteine was not take into consideration), so they concluded that ADMA is an independent risk factors for coronary heart disease[23]. Some Researchers report that ADMA is significantly increased in patients with coronary artery disease, especially in presence of chronic renal failure[24].

The present study suggests the hypothesis that a marker within one-carbon metabolism, ADMA plasma concentration, may be a link a risk factor for MI to be considered more carefully in female gender.

One of the main peculiarities of this study is the angiographic method to enlist the population in case-group and control-group for coronary atherosclerosis.

These data highlighted that ADMA plasma concentrations did not affect the risk of CAD, but its main thrombotic complication, myocardial infarction. That result may be of particular interest because, to date, the factor influencing the evolution of CAD in MI is yet not completely known. Moreover, ADMA plasma concentrations higher than 0.69 µmol/L, that corresponding to 3th tertile in studied population, may be considered an index for elevated MI risk only in female subgroup (OR=2.79, *P*=0.032), on the contrary in male subgroup a higher risk of MI associated to elevated ADMA levels did not appear (OR=1.71, *P*=0.19). Higher risk of MI in women remains statistically significant also after adjustment for smoking and diabetes; furthermore after adjustment for age, familiarity, coronary artery disease, hypertension and BMI. The degree of CAD risk increase for high plasma ADMA concentrations is little but this cardiovascular disease has a composite genesis with a lot of genetic and biochemical-enviromental factors.

Other Authors identified an association between ADMA concentrations and increase of CAD risk[2], hypothesizing that high baseline ADMA levels may have prognostic value and independently predict future cardiovascular risk[10].Therefore, our study lines up with previous studies. A Moreover, the population studied presented the same social, economical and environmental conditions and for each subject there were much knowledge about its history,

its clinical and biochemical data and the other main classical risk factors, to define more precisely the risk of cardiovascular disease.

In 2003, Lu e Coll.[9] observed that high ADMA levels in patients undergoing percutaneous coronary intervention (PCI) predicted an increased risk of restenosis. In 2005 Schnabel and Coll. related that, in 1874 patients with CAD, high concentrations of baseline ADMA were independently correlated with future death from cardiovascular causes or non-fatal myocardial infarction[10].

The risk of future cardiovascular event was also associated with increasing thirds of baseline ADMA (*p* for trend <0,001) and this relationship remained nearly unchanged after adjustment for traditional risk factors (homocysteine didn't take into consideration).

Other observations pointed out that ADMA plasma concentrations were positively correlated with age[25] in 1126 non-smoking healthy individuals from the Framingham Offspring Study; in that same study, ADMA levels were higher both in women than in men and in post-menopausal women than in pre-menopausal women.

In our study there were not statistically significant differences between male and female for age.

Bae and colleagues[26] observed that in patients with acute coronary syndrome ADMA is significantly higher than in controls and it rapidly decreases after short-term medical therapy. Analogously, other studies have demonstrated that ADMA in patients with angina instable is higher than in

patients with stable angina and there is a significant reduction of ADMA levels after percutaneous coronary intervention in patients with unstable angina[6]., High ADMA levels may independently predict major adverse cardiovascular events also in subjects with advanced peripheral artery disease (PAD)[27].

Korandji and Colleagues[28] investigated the relationship between increased levels of ADMA and plasma homocysteinemia (tHcy), an independent risk factors for atherosclerotic vascular disease, in patients with acute myocardial infarction. In the 138 patients with MI studied, ADMA was positively associated with SDMA (p<0,001) and tHcy (p=0,03). By multiple linear regression, SDMA but not ADMA was independently associated with tHcy (p=0,005; vs 0,74). They concluded that in AMI patients, ADMA levels are independent of traditional cardiovascular risk factors and high levels of tHcy are indirectly related to ADMA.

With regards to the relationship among ADMA levels and homocysteinemia, Antoniades and colleagues[29] observed that ADMA levels increased rapidly in acute homocysteinemia. Increased oxidative stress is the main probable mechanism underlying endothelial dysfunction homocysteinemia-induced, with increased concentration of pro-inflammatory cytokines.[30,13,31]

In our study, ADMA correlates with homocysteine and the latter correlates with folate. So ADMA may have significance in risk definition and prevention in relation to biochemical-nutritional data. Other studies are needed to confirm this hypothesis.

In conclusion, ADMA may represent an important prognostic factor to developing thrombotic complications in CAD patients. According to previous results reported in other studies, ADMA may be considered a risk factor for myocardial infarction, although the homocysteine have a predominant role among these.

Many studies dealt with defining generic risk, though knowing the different risk for atherosclerosis in woman and in man. Considering the significance to define markers of atherosclerotic disease according to gender, the results of this study highlights some relevant observations that contribute to identify new factors of MI risk, the main complication of CAD, in female population.

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After we attended both to study the main polymorphisms of the enzymes involved in one-carbon metabolism and to evaluate its impact on genomic methylation, investigating the possible existence of nutrient-gene interaction examples, we proposed to initiate a clinical study to identify possible functional markers of nutrient-gene interaction with effects on epigenetic modulation.

To achieve this goal, we set out not only to explore genomic methylation but also to compare gene-specific methylation on blood tissue with gene-specific methylation on liver tissue, healthy and cancerous changes affected.

Our aim was to study also the co-interaction with alcohol, another dietary factor that may determine some changes in folate metabolism with effects on both hematopoietic tissue and the liver tissue.

The liver, in fact, is the tissue which occurs in almost all the enzymatic functions relating to the folate cycle and it is known that epigenetic mechanisms and alcohol play an important role in carcinogenesis and tumor progression both in hepatocellular carcinoma and in cholangiocarcinoma intrahepatic. However, in humans liver tissue is obviously not easily accessible to be studied in terms of biochemical and molecular level.

Consequently, in the literature there are no demonstrations of the correlation between epigenetic mechanisms, nutrition and liver carcinogenesis. The search by a gene-nutrition approach of functional markers, documented both in the liver and in the blood, we believe that it would open new frontiers in terms of prevention and therapeutic approach of these tumors.

CHAPTER 5

FUTURE PERSPECTIVES

GENETICS AND EPIGENETICS RELATIONSHIP IN CANCER TYPE WITH A LINK TO ONE-CARBON METABOLISM: A POSSIBLE MODEL FOR THE ROLE OF EPIGENETIC MARKERS IN PERIPHERAL BLOOD MONONUCLEAR CELLS

Abstract

Folate-dependent one-carbon pathway is related to carcinogenesis for its function in nucleic acid synthesis and biological methylation. Alcohol consumption is one of the most important dietary factors implicated in cancer development and is metabolically related to folate metabolism. Specific polymorphic genes of folate-related enzymes in association with different folate and other B-vitamins status influence methyl transfer capacity and DNA methylation. Such models of gene-nutrient interactions may, therefore, modulate epigenetic mechanisms strictly linked to carcinogenesis. A causal relationship between one-carbon metabolism and cancer. in particular colorectal and liver cancer, has been previously demonstrated in a rodent model. The effect of alcohol consumption in association with different genetic patterns of folate-related enzymes and B-vitamins status has not been studied in humans for their possible role in modifying epigenetic phenomena. Our hypothesis is that different nutritional pattern alters genomic DNA

methylation status and histone epigenetic modifications *via* one-carbon metabolism, therefore inducing molecular aberrations that ultimately lead to neoplastic degeneration and can be detected in the hematopoietic tissue as a systemic marker of cancer disease. We also hypothesize an interrelationship between alcohol, one of the main nutrients known to affect both carcinogenesis and one-carbon metabolism, folate-related polymorphic genes and B-vitamins status that may alter epigenetic mechanisms and the expression of critical cancer pathway genes in a gene-nutrient interaction fashion. To address these issues we proposed a human study to determine either in peripheral blood mononuclear cells and in liver tissue whether different degrees of chronic alcohol consumption induce altered genomic DNA methylation as well as promoter site DNA methylation profiling by an array-based platform for single-site CpG resolution and to evaluate the expression of genes by an array-based technology.

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Summary and specific aims

Different B vitamins status either due to dietary factors or altered absorption are hypothesized to be related to epigenetic phenomena. Among the most important dietary factors for hepatocellular carcinoma and colorectal cancer is alcohol consumption[1] which is metabolically related to folate in the complex biochemical scheme of one-carbon metabolism[2]. Alcohol has not,

however, been studied extensively in an experimental fashion in regard to how it interacts with one-carbon metabolism in modulating the epigenetic phenomena which are related to cancer risk and particularly that of hepatocellular carcinoma and colorectal cancer and how this interaction can be ameliorated by dietary intervention. In an animal study we previously observed that chronic alcohol consumption induces hyperhomocysteinemia and genomic DNA hypomethylation in the colon but folate supplementation alone cannot prevent this phenomenon, therefore suggesting other possible mechanisms involved in carcinogenesis which are potentially reversible.

Evidence from both human and animal studies has repeatedly demonstrated that alcohol impedes one-carbon metabolism. In 1993 Hultberg et al.[3] found that chronic alcoholics were hyperhomocysteinemic. In human alcoholics Cravo et al.[4] also observed hyperhomocysteinemia which was associated with low blood folate levels and a significant decrease in serum pyridoxal-5'-phosphate (the active form of vitamin B6). Alcohol stimulates catabolism of methionine to generate cysteine and replenish glutathione, but at the same time, the cell attempts to conserve methionine through the choline and betaine pathway. This results in a drastic wastage of methyl groups and B6. Studies in a number of different mammals indicate that chronic alcohol ingestion diminishes methionine synthase activity in the liver, reducing the availability of methionine and SAdoMet, the universal methyl donor. In a recent rodent study we observed an impairment in one-carbon metabolism in the colonic mucosa of the alcoholic rat, leading to diminished methylation of DNA[5]. It is also not surprising that chronic alcohol ingestion would cause a

substantial shift in the distribution of the various forms of intracellular folate. Alcohol ingestion also causes an increase in the proportion of intracellular folate appearing as methylated THF and THF and a decrease in the proportion of formylated THF. Alcohol diverts formylated THF towards serine synthesis, thus folate-mediated thymidine synthesis is also interrupted. These alcohol-induced changes in folate form distribution are similar to what occurs in vitamin B12 deficiency (*i.e.* a reduction in the proportion of those co-enzymatic forms of folate which are used for nucleotide synthesis), a condition which increases uracil misincorporation as well as decreased DNA methylation status in the colon[6]. Vitamin B12 deficiency is thought to be less common in chronic alcoholics. Nonetheless, tissue deficiencies of this vitamin may occur despite subnormal, normal, or even higher circulating concentrations, suggesting that chronic alcohol consumption may impair the availability of B12 in tissues[7]. In a four-months long animal study Barak et al. reported that chronic alcohol feeding inhibited the activity of MS throughout the study, but increased the activity of BHMT and decreased betaine levels to maintain vital tissue levels of SAdoMet [8].

Our long-term goal is to highlight possible markers of genetic and epigenetic disruption that are related to one-carbon metabolism associated to alcohol consumption and, ultimately, to find an effective strategy for possible chemoprevention. Such models of gene-nutrient interactions may, therefore, modulate epigenetic mechanisms strictly linked to carcinogenesis. Considering that: a) metabolism of folate and other water-soluble B vitamins such as vitamin B12, B6, thiamine and betaine are altered in diseases of the

hepatic tissue; b) such B vitamins alterations are mainly observed in hepatic diseases due to alcohol ingestion; c) despite similar amounts of alcohol ingestion not all people develop alcohol liver disease including hepatocellular carcinoma, therefore suggesting the role of possible genetic factors; d) both alcohol and B vitamins impairment alter one-carbon metabolic pathway and may lead to epigenetic aberrations that potentially underlie neoplastic transformation of liver tissue; e) both alcohol and B vitamins impairment are also reflected in hematopoietic tissue aberrations. We, therefore, hypothesize that B vitamins impairment whether or not associated with alcohol ingestion may induce molecular aberrations in a gene-nutrient interaction fashion that are similar in peripheral blood mononuclear cells and in hepatocellular carcinoma secondary to alcohol-induced cirrhosis tissue and liver metastatic tissue due to colon adenocarcinoma.

For the easier availability of hematopoietic tissue in humans, the identification of biochemical and molecular markers in such tissue may represent an important finding for risk stratification study and for chemopreventive strategies. In particular, since epigenetic phenomena are potentially reversible, their modulation may be done by dietary habits modifications in which nutritional intervention may reduce the pro-carcinogenic effects of alcohol consumption and prevent carcinogenesis.

The present proposal will constitute the first study to investigate the mechanism(s) by which altered one carbon metabolism induced by alcohol consumption alters epigenetic phenomena in hematopoietic tissue and liver as well as liver metastatic tissue from colorectal cancer. To address these

issues, we propose to conduct an observational and prospective study in human subjects. In brief, our specific aims are as follows: a) to determine gene-nutrient interactions between B vitamins (folate, vitamin B12, B2, B6, betaine, choline, thiamine) and polymorphic genes within one-carbon metabolism that may affect epigenetic phenomena such as DNA genomic and gene-specific methylation as well as histone modifications in human peripheral blood mononuclear cells and liver tissue in a different age and dietary setting; b) to determine B vitamins status, one-carbon pathway biochemical markers such as homocysteine and genetic polymorphisms within one-carbon metabolism genes in subjects either assuming or not alcohol drinks and of different age (adults or elderly); c) to determine whether B vitamins abnormalities either alone or in association to alcohol consumption also induces altered expression of gene pathways related to carcinogenesis in human peripheral blood mononuclear cells and liver tissue.

The main goal of the study is therefore to determine the 'molecular signature' (genetic and epigenetic markers) in peripheral blood mononuclear cells as wells as hepatocarcinoma tissue or liver metastasis tissue from colonic cancer compared to normal tumour-surrounding tissue, in order to focus on those genes whose expressions most likely mediate folate metabolism-related epigenetic phenomena.

We expect that the study of mononuclear cells from peripheral blood show similar molecular signatures to those in primary or secondary tumour of the liver tissue and therefore can be used as a valid biochemical marker of folate-related carcinogenesis

Our main hypothesis is that of the existence of a gene-nutrient interaction between folate-related B vitamins, polymorphisms within one-carbon metabolism genes that can affect epigenetic phenomena, other endpoints will be biochemical markers related to folate pathway such as plasma folate, vitamin B12 and holotranscobalamin levels[9, 10], pyridoxal phosphate as the active form of vitamin B6[11] as well as homocysteine[12], SAdoMet, SAdoHcy.

We, therefore, proposed to test biochemical parameters such as plasma folate, vitamin B6 and B12 concentrations, homocysteine and the major biochemical parameters defining the stage of disease and as well as to determine the genotype of polymorphisms in folate-related genes.

We are planning to explore mechanistic pathway with a high-throughput novel array technology for DNA promoter methylation, chromatin remodelling and gene expression in a limited subset of subjects in order to define specific pathways or genes of interest related to cell cycle control. Genomic DNA methylation will be also determined by a precise quantitative method[13].

Based on the data including new genes will be found from microarray, we would like to pursue to find the exact mechanism(s) by which alcohol enhances hepatocellular carcinogenesis and to find whether molecular markers expressed in peripheral blood mononuclear cells of healthy subjects consuming alcohol may show a biochemical-molecular signature related to aberrant folate-related pathway that may offer novel chemopreventive strategy including optimal dose, time and combination of B vitamins such as mainly folic acid, vitamin B6 or betaine. We will also extend our observation to genes involving retinoic acid metabolism and JunN-terminal kinase-

dependent signaling pathway, alteration of which has been demonstrated in the liver of alcohol fed animals.

These future studies will provide us with a better mechanistic understanding of how one-carbon nutrients modulate cancer risk and will provide much greater insight into how to effectively prevent cancer, and which individuals we should target for nutritional modulation.

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CONCLUSIONS

In recent years, the scientific research in nutritional field has pointed out that many vitamins and micronutrients play an important role in genomic regulation[1]: they are, in fact, crucial substrates and cofactors in metabolic pathways for the DNA synthesis/repair and for the maintenance of DNA methylation patterns and thus, ultimately, for gene expression regulation.

It is also known that nutrient deficiencies can lead to impairment of genomic stability by modifying the normal process of methylation. DNA methylation represents, therefore, a fundamental mechanism for the control of gene expression and maintainance of genomic integrity[2, 3], with important consequences for the regulation of cell growth, tissue-specific differentiation[4-6] and carcinogenesis[3].

It is clear, therefore, the strong link between nutritional status and modulation of gene expression.

Folate is one of nutrients most widely studied for its role as a methyl group carrier for nucleotide synthesis and biological methylation. The discovery of polymorphic variants of enzymes involved in folate-related metabolic pathway largely contributed to new insights to better define the balance between genetics-epigenetics and nutrition in phenotypic expression.

The answer to a particular nutritional status in many cases appears to be specific for each genotype, and alterations of specific nutrients leads to a different gene expression, in relation to each genotype.

A clear example of interaction between nutrition (folate status) and genotype is represented by a common polymorphic variant of the key enzyme in one-carbon metabolism, the MTHFR, that is responsible for the availability of methyl groups for biological methylation reactions[7, 8]. In presence of this polymorphism, changes in DNA methylation, appears only in the case of impaired folate status.

Based on these premises, the first part of our research was performed, in order to identify new patterns of gene-nutrient interaction regarding other enzymes involved in one-carbon metabolism. Particular attention has been directed to interactions between genes and nutrients in the modulation of DNA methylation.

The field of nutrient-gene interactions appears a fascinating model to potentially explain different phenotypic expression which may occur in response to the environment and diet. It is moreover to be pointed out that the interaction between nutrients and DNA methylation has been recently emphasized since epigenetic mechanisms are potentially reversible phenomena, dependent on the methyl groups availability for metabolic processes.

Thus, the hypothesis of a possible modification of phenotypic expression, by affecting epigenetic mechanisms through nutritional status, seems to open new frontiers in the field of disease prevention and new therapeutic approaches.

For this reason, in the second part of our study we evaluated some possible

pathological conditions due to B vitamins deficiency and to identify potential risk markers for disease. We therefore started a novel study project focused on carcinogenicity, aimed at evaluating either genomic DNA methylation and gene-specific methylation, in blood mononuclear cells compared with liver tissue, organ, in which almost all the enzymatic functions of the folate cycle occur.

In conclusion, our work contributed to a better understanding of the mechanisms of nutrition and DNA methylation interrelationship and allowed to deepen our understanding on gene-nutrient interactions to propose novel pathways involved in the development of certain chronic diseases as well as to propose possible innovative prevention strategies by modifying epigenetic modulation through a nutritional approach.

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