INSTITUTO SUPERIOR DE CIÊNCIAS DA SAÚDE EGAS MONIZ

MESTRADO INTEGRADO EM CIÊNCIAS FARMACÊUTICAS

FOLIC ACID, ONE-CARBON METABOLISM, MTHFR POLYMORPHISMS AND PATHOLOGIES

Trabalho submetido por
Patrícia Muíla Bragança Sambo
para a obtenção do grau de Mestre em Ciências Farmacêuticas

Outubro de 2014

INSTITUTO SUPERIOR DE CIÊNCIAS DA SAÚDE EGAS MONIZ

MESTRADO INTEGRADO EM CIÊNCIAS FARMACÊUTICAS

FOLIC ACID, ONE-CARBON METABOLISM, MTHFR POLYMORPHISMS AND PATHOLOGIES

Trabalho submetido por
Patrícia Muíla Bragança Sambo
para a obtenção do grau de Mestre em Ciências Farmacêuticas

Trabalho orientado por
Doutora Alexandra Maia e Silva

Outubro de 2014

DEDICATION

I dedicate this thesis to my dearest husband, Anders, my partner in life, for his remarkable patience, support and encouragement during this challenging phase.

Additionally, I dedicate this thesis to my parents, Rosário and Eduardo, for teaching me the value of hard work and for always love me unconditionally, and to my sister, Indira, who always stood by me when I felt hopeless.

ACKNOWLEDGEMENTS

My thanks and appreciation to Prof. Alexandra Maia e Silva, for her leadership, guidance, attention and for persevering with me as my supervisor during this period of scientific research.

Special thanks to my friends, colleagues and librarians who supported and guided my research and writing.

Finally, I must acknowledge as well Instituto Superior de Ciências da Saúde Egas Moniz, my second home, for providing the knowledge that made this thesis possible.

ABSTRACT

The interaction between folate and methylenetetrahydrofolate reductase (*MTHFR*) gene is an example of a strong gene-nutrient interaction. *MTHFR* 677C→T polymorphism may be associated with homocysteine in the modulation of the cardiovascular disease (CVD). Additionally, the interaction between the polymorphism and folate has been linked to a protective effect in individuals with colorectal cancer (CRC). The aim of this work is to assess the possible association between *MTHFR* 677C→T polymorphism, CVD and CRC, modified by folate and homocysteine. The predictive response of CRC patients carrying *MTHFR* 677C→T polymorphism, treated with 5-fluorouracil, is also briefly examined, along with the current strategies to inhibit tumours, involving *MTHFR* gene as a target. Studies were found by searches of electronic literature for papers up to October 2014 using the terms "*MTHFR* 677C→T polymorphism" "folate," "cardiovascular disease," "homocysteine," "colorectal cancer" and "chemotherapy". Collected studies were mainly observational, randomised controlled trials, meta-analyses and systematic reviews, approximately from the last 15 years. The association between *MTHFR* 677C→T polymorphism and CVD was found, although results from folate supplementation trials demonstrated no benefit in CVD. High supply of folate and TT genotype carriers may have a lower risk to develop colon cancer in some populations. The type and amount of folate, along with its supplementation when carcinogenesis is already stablished may increase the risk for CRC. *MTHFR* 677C→T polymorphism seems to be associated with better prognosis and less toxicity in 5-fluorouracil monotherapy. *MTHFR* inhibition technique shows promising results as an anti-cancer therapy. Findings are inconsistent to recommend folate supplementation in TT genotype carriers with CVD and CRC. A genetic and environmental risk assessment for CRC risk in primary care, regarding folate and *MTHFR* 677C→T polymorphism is worth considering. Further research on combinatory *MTHFR* polymorphisms and riboflavin effect, could help clarify the association between *MTHFR* 677C→T polymorphism, folate and disease.

Keywords:

MTHFR 677C→T polymorphism; folate; cardiovascular disease; colorectal cancer

RESUMO

Pensa-se que a variante do enzima codificado pelo polimorfismo *MTHFR* 677C→T possa estar associado à homocisteína e consequentemente, associado ao risco de doenças cardiovasculares (CVD); assim como a associação entre o folato e a mesma variante polimórfica possa proteger contra o cancro colorretal (CRC). O objetivo deste trabalho é correlacionar o polimorfismo *MTHFR* 677C→T com CVD e CRC, associado ao folato e à homocisteína. A resposta terapêutica ao 5-fluorouracil em pacientes com CRC e com o polimorfismo é também, brevemente analisada, juntamente com as atuais estratégias de inibição de tumores, envolvendo *MTHFR*. Os estudos foram identificados por pesquisa eletrónica de referências bibliográficas publicadas até outubro de 2014, usando as terminologias: "polimorfismo *MTHFR* 677C→T", "folato", "doença cardiovascular", "homocisteína", "cancro colorretal" e "quimioterapia". Compreenderam principalmente estudos observacionais, clínicos randomizados, meta-análises e revisões sistemáticas dos últimos 15 anos. A associação entre o polimorfismo e CVD foi encontrada, embora os resultados de estudos com suplementação de folato não demonstrem benefício, em CVD. O elevado consumo de folato e o genótipo TT parecem estar associados a menor risco de cancro do cólon. O tipo e quantidade de folato, juntamente com a fase da carcinogénese na qual se inicia a suplementação do mesmo podem estar relacionados com o aumento do risco de CRC. O polimorfismo parece estar associado a um melhor prognóstico e menor toxicidade em tratamentos com 5-fluorouracil. A técnica de inibição de *MTHFR* mostra resultados promissores como terapia anticancerígena. Os resultados são inconsistentes para recomendar suplementos de folato em indivíduos TT. Testes de avaliação da interação gene-ambiente, em relação ao folato e ao polimorfismo referido podem ser justificáveis, em indivíduos em risco de CRC. Mais estudos que combinem os polimorfismos do *MTHFR* e que analisem o efeito da riboflavina, ajudariam a compreender a associação entre o polimorfismo *MTHFR* 677C→T, o folato e a doença.

Palavras-chave:

Polimorfismo *MTHFR* 677C→T; folato; doenças cardiovasculares; cancro colorretal

RESUMÉ

Interaktionen mellem methylentetrahydrofolat reduktase (*MTHFR*) genet og folat er et eksempel på en stærk gen-næringsstof interaktion. *MTHFR* 677C → T polymorfi kan være associeret med homocystein i modulering af kardiovaskulær sygdom (KVS). Derudover er interaktionen mellem polymorfi og folat blevet associeret med en beskyttende effekt hos personer med kolorektal cancer (KRC). Målet med dette speciale er at vurdere den mulige sammenhæng mellem *MTHFR* 677C → T polymorfi, KVS og KRC modificeret af folat og homocystein. Den prædiktive reaktion hos KRC- patienter, som bærer *MTHFR* 677C → T polymorfi behandlet med 5-fluorouracil-baseret kemoterapi er også kortvarigt undersøgt sammen med de nuværende strategier til at hæmme tumorer, der involverer MTHFR som et mål. Studier blev fundet ved søgninger i elektronisk litteratur for artikler frem til oktober 2014 ved brug af søgetermerne "*MTHFR* 677C → T polymorfi", "folat", "hjertekarsygdom", "homocystein", "kolorektal cancer" og "kemoterapi". Indsamlede studier var hovedsageligt observationsstudier, randomiserede kontrollerede studier, metaanalyser og systematiske reviews, alle hovedsageligt fra de sidste 15 år. Associationen mellem *MTHFR* 677C → T polymorfi og KVS blev fundet, selvom resultater fra folat-supplement studier ikke viste forbedring for KVS. Hypertension i TT-genotype bærere synes justerbare med riboflavin-tilskud. Høj forsyning af folat og TT-genotype bærere kan have en lavere risiko for at udvikle tyktarmskræft i visse populationer. Type og mængde af folater kan sammen med karcinogenes fase øge risikoen for KRC. *MTHFR* 677C → T polymorfi synes at være associeret med bedre prognose og mindre toksicitet i 5-fluorouracil monokemoterapi. *MTHFR*-hæmning-teknik viser lovende resultater som en anticancer-terapi. Resultaterne er inkonsistente i forhold til at anbefale folat-supplement i TT-genotype bærere med KVS og KRC. For visse populationer er gen-miljø vurderingstests vedrørende folat og *MTHFR* 677C → T polymorfi værd at overveje for KRC risikoindivider. Yderligere forskning på kombinatoriske *MTHFR*-polymorfier og riboflavin kunne bidrage til at afklare associationen mellem *MTHFR* 677C → T polymorfi, folat og sygdom.

Nøgleord:

MTHFR 677C → T polymorfi; folat; hjertekarsygdom; kolorektal cancer

CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

5-FU – 5-Fluorouracil

AdoHcy – S-Adenosyl-L-Homocysteine

AdoMet – S-Adenosylmethionine

AHCY – Adenosylhomocysteinase

ALL – Acute Lymphocytic Leukaemia

APC - Adenomatous Polyposis Coli

ASOs – Antisense Oligonucleotides

BHMT – Betaine-Homocysteine-Methyltransferase

CBS – Cystathionine-β-Synthase

CHD – Coronary Heart Disease

CI - Confidence Interval

CRC – Colorectal Cancer

CSE – Cystathionine-γ-Lyase

CVD – Cardiovascular Disease

DHF – Dihydrofolate

DHFR – Dihydrofolate Reductase

DNA – Deoxyribonucleic Acid

dNTP – Deoxynucleotide

dTMP – Deoxythymidine Monophosphate

dUMP – Deoxyuridine Monophosphate

dUTP – Deoxyuridine Triphosphate

FAD – Flavin Adenine Dinucleotide

FdUMP – Fluorodeoxyuridine Monophosphate

FOLFIRI – Folinic Acid, Fluorouracil, Irinotecan

FOLFOX – Folinic Acid, Fluorouracil, Oxaliplatin

HCC – Hepatocellular Carcinoma

Hcy – Homocysteine

HHcy – Hyperhomocysteinemia

HS – Haemorrhagic Stroke

ICH – Intracerebral Haemorrhage

IS – Ischemic Stroke

KRAS **-** Kirsten Rat Sarcoma Viral Oncogene Homolog

LDL – Low-Density Lipoproteins

MAT – S-Adenosylmethionine Synthetase

MI – Myocardial Infarction

MMIF – Macrophage Migration Inhibitory Factor

miRNA – Micro Ribonucleic Acid

mRNA – Messenger RNA

MTHFD – Methylenetetrahydrofolate Dehydrogenase

MTHFR – Methylenetetrahydrofolate Reductase

NADPH – Nicotinamide Adenine Dinucleotide Phosphate Hydrogen

NO – Nitric Oxide

OR – Odds Ratio

PCFT – Proton-Coupled Folate Transporter

PLP – Pyridoxal 5'-Phosphate

RNA – Ribonucleic Acid

SAM – S-Adenosylmethionine

tHcy – Plasma Homocysteine

THF – Tetrahydrofolate

TYMS – Thymidylate Synthase

VTE – Venous Thromboembolism

INTRODUCTION

Multifactorial diseases, such as cancer and cardiovascular disease are expanding globally in prevalence and mortality. Most of these conditions have a multifactorial inheritance due to genetics, but lifestyle and environmental factors, such as diet can prompt their start. Therefore, understand the interaction between genetics and diet is a major step in controlling multifactorial diseases (Lobo, 2008).

Extensive investigation upon the past 20 years has disclosed important aspects about one-carbon metabolism and its regulation. One-carbon metabolism plays a critical role in both DNA methylation and DNA synthesis, through the association of folate and methionine metabolism, along with homocysteine catabolism, being folate the central cofactor, ensuring the correct flow of carbon moieties (Ikeda *et al.*, 2012).

The interconnection between folate and methionine cycle happens with the irreversibly reaction catalysed by methylenetetrahydrofolate reductase (MTHFR) that produces 5-methyltetrahydrofolate, a methyl donor in homocysteine remethylation to methionine, directing the folate pool towards methylation reactions, in detriment of DNA synthesis (Bailey & Gregory, 1999).

However, some polymorphisms from the *MTHFR* gene have been linked to the impairment of the enzyme function. *MTHFR* 677C→T polymorphism is the most significant, and accumulating evidences have shown that this variant of the *MTHFR* gene is associated with many disease outcomes, particularly in cardiovascular disease and colorectal cancer (Kennedy *et al.,* 2012; Klerk *et al.,* 2002). Individuals with the 677C→T homozygous variant (TT genotype) have lower MTHFR activity than heterozygotes (CT genotype) or homozygotes wild-type (CC genotype), and under conditions of low dietary folate, people with the TT genotype can have elevated plasma homocysteine concentrations because not enough folate is metabolized to 5-methyltetrahydrofolate (Trabetti, 2008).

A number of molecular mechanisms of homocysteine have been suggested, and the underlying common pathogenic mechanisms are predominantly associated with

vascular injury (Brustolin, Giugliani, & Felix, 2010). Due to its impact on vascular tissues, homocysteine has been hypothesised as an independent risk factor for cardiovascular disease. However, homocysteine concentrations are positively correlated with many other cardiovascular risk factors, such as dyslipidemia, hypertension, sedentary lifestyle and unhealthy diet that can be potential confounders in epidemiological studies (Ueland & Loscalzo, 2012). Thus, many evidences suggest that homocysteine is more an epiphenomenon of vascular disease, rather than a casual factor (Brattström & Wilcken, 2000).

By contrast, several findings indicate that high intake of folate and the TT genotype are associated with reduced risk of colorectal cancer, compared to low intake of folate, heterozygous and wild-type genotypes (Kennedy *et al.,* 2012). However, a dual effect of folate in colorectal cancer has been recently reported, suggesting a possible enhancement of carcinogenesis (Castillo-Lancellotti, Marí & Dagach, 2012). Consequently, folate supplementation effect has been vastly evaluated on lowering homocysteine concentrations in cardiovascular disease, through several large-scale interventional trials and in colorectal cancer patients, mainly through observational studies (Clarke *et al.,* 2010; Hubner & Houlston, 2008).

The fact that MTHFR enzyme influences the concentrations of substrates involved in some antimetabolites chemotherapy supports the idea that *MTHFR* 677C→T polymorphism might have a pharmacogenetic role in predicting the efficacy and the toxicity of some cancer drugs (Chua *et al.,* 2009). Additionally, emerging studies point to an alternative anti-cancer therapy targeting MTHFR enzyme (Stankova, Shang & Rozen, 2005).

Overall, in the present thesis, the possible association between *MTHFR* 677C→T polymorphism, cardiovascular disease and colorectal cancer modified by the level of folate intake and homocysteine is reviewed, with regard to the observational studies, randomised controlled trials, meta-analyses and systematic reviews from the last 15 years. The predictive clinical outcomes from *MTHFR* 677C→T polymorphism of colorectal cancer patients, treated with 5-fluorouracil chemotherapy is also briefly examined, as the current strategies to inhibit the growth of tumours, involving *MTHFR* gene as possible target.

CHAPTER I – ESSENTIAL BIOCHEMISTRY OF ONE-CARBON METABOLISM

1.1. OVERVIEW

One-carbon metabolism can be understood as a biochemical network that regulates nutrient physiologic status, centred on folate and methionine. This process involves three major pathways: folate metabolism, cycle of methionine and transsulfuration pathway of homocysteine (Hcy) [Fig. 1]. In addition to folate, methionine and Hcy, other nutrients are required to maintain the flow of carbon units, such as pyridoxal 5'-phosphate (PLP, vitamin B_6), riboflavin (vitamin B_2), cobalamin (vitamin B_{12}), serine and betaine (Ikeda *et al.*, 2012).

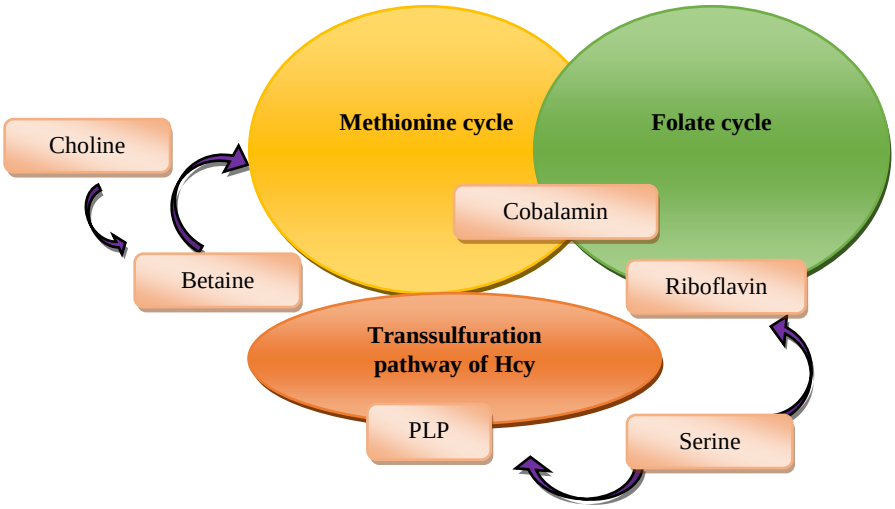


Figure 1. Inputs in one-carbon metabolism *(adapted from: Locasale, 2013)*.
PLP – pyridoxal 5'-phosphate

The terminology "one-carbon" refers to carbon moieties, such as methyl, methylene, methenyl and formyl units carried by folate coenzymes, essential for purine and thymidylate synthesis and, consequently, crucial for the preservation of genomic stability. Besides that, another function of one-carbon metabolism includes remethylation of Hcy in order to form methionine, the precursor of S-adenosylmethionine (SAM). SAM is a methyl donor for a wide range of methylation reactions involving lipids, hormones, DNA, proteins, etc. (Hazra *et al.*, 2009). Therefore, one-carbon metabolism nutrients and metabolites participate in multiple biological processes, such as epigenetics (through methylation), correct DNA synthesis (via regulation of nucleotide pools), vitamin and amino acid metabolism, and lipid

biosynthesis. In addition to biosynthesis and methylation, one-carbon metabolism plays a role in redox homeostasis (Locasale, 2013).

The activity of enzymes involved in one-carbon metabolism is greater in the liver, followed by the pancreas and kidney. Moreover, one-carbon metabolism is highly compartmentalised in eukaryotic cells, with some studies suggesting that the compartmentalisation of folate coenzymes and one-carbon units are mechanisms of regulation for the entire process (Tibbetts & Appling, 2010).

1.2. FOLATE CYCLE AND METABOLIC PATHWAY

Folate is found mainly in leafy vegetables, justifying its name origin from the Latin word "folium", which means "leaf". This nutrient is included in the vitamin B complex and cannot be produced by humans. However, folate differs from other B vitamins because it serves as a catalytic substrate for the transfer of one-carbon units, playing a major role ensuring the equilibrium between redox and methylation status in the body, in addition to the synthesis of purine and thymidylates (Czeizel, Dudás, Vereczkey & Bánhidy, 2013).

Using accurate terminology, folate refers to the family of substances containing a pteridine ring conjugate to both ρ-aminobenzoic acid and (poly)-glutamate(s) found in dietary food. They can be found in a reduced form linked to a polyglutamyl chain containing different numbers of glutamic acids depending on the type of diet (Ikeda *et al.*, 2012).

Polyglutamate residues are critical for the retention of folates in eukaryotic cells leading to efficient substrates for many folate-dependent enzymes in the one-carbon metabolism. Formyltetrahydrofolate synthetase is an example of that, the affinity of the enzyme for tetrahydrofolate (THF) with four or five glutamate residues is 150 times greater than for the monoglutamate form of THF (Fowler, 2001).

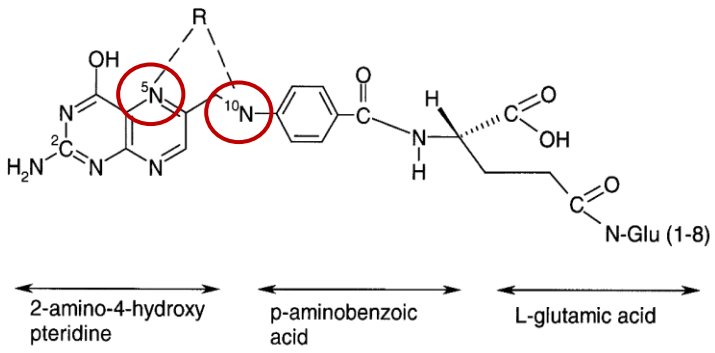
The structure of folate derivatives varies from unsubstituted polyglutamyl THFs to various substituted one carbon forms of THF (e.g. 10-formyl-, 5, 10-methylene-, and predominantly 5-methylTHF). Nevertheless, the unsubstituted forms are poorly stable

due to the fragile bond between the C-9 and N-10 leading to a substituted pteridine, and ρ-aminobenzoylglutamate that carries no biologic activity. Even though the substitution of a carbon group at N-5 or N-10 lowers the tendency of breakage, the molecule is still vulnerable to oxidation, and, therefore, predisposed to inactivation (Brody & Shane, 2001).

Within the group of folate derivatives stands out folic acid, a synthetic compound not found in nature in significant quantities. Folic acid is fully oxidised, and used industrially as nutritional supplements in fortified foods, or for pharmaceutical purposes. In this form, the pteridine ring is not reduced, making the molecule more resistant to oxidative rearrangements (Hoffbrand & Weir, 2001).

Summing up, folate from food differs from folic acid, mostly in three aspects: in additional glutamate residues, in reduction to di- or tetrahydroforms, and in additional single carbon units attached to N-5 or N-10 nitrogen atoms [Fig. 2] (Brody & Shane, 2001; Ikeda *et al.*, 2012).

a)



b)

Folic acid species	Substituent at N-5	Substituent at N-10
Tetrahydrofolic acid	- H	- H
5-methyltetrahydrofolic acid	- CH_3	- H
5,10- methylenetetrahydrofolic acid	- CH_2-	- CH_2-
5,10- methenyltetrahydrofolic acid	- $CH{=}N^+$-	- $CH{=}N^+$-
5-formyltetrahydrofolic acid	- CHO	- H
10-formyltetrahydrofolic acid	- H	- CHO
10-formiminotetrahydrofolic acid	- H	- HCNH

Figure 2. Structure of folic acid (a) and related compounds (b) *(adapted from: Fowler, 2001).*

After ingestion, folic acid is rapidly absorbed, mainly in jejunum. Absorption occurs by active transport via the proton-coupled folate transporter (PCFT), and folic acid suffers a two-step reduction to dihydrofolate (DHF). Subsequently, DHF is converted in

THF by the enzyme dihydrofolate reductase (DHFR) in the presence of ascorbic acid. Folic acid appears to have a higher bioavailability than folate in food, about 80% or greater (Winkels *et al.*, 2007).

In contrast, the polyglutamyl chain from natural folates is removed in the brush border of the mucosal cells by folate conjugase, reducing the bioavailability of folate as much as 25–50% because the reaction is not complete. After, folate monoglutamate is absorbed by both passive diffusion and active transport via PCFT. Both folate monoglutamates and folic acid, in the form of THF, are methylated to 5-methylTHF by one-carbon metabolism enzymes and release to circulation, being 5-methylTHF the principal circulating form of folate (Ikeda *et al.*, 2012).

Peripheral cells take up 5-methylTHF via reduced folate carrier or folate receptor, and incorporate it into one-carbon metabolism, which then must be polyglutamated for cellular retention and one-carbon metabolism coenzyme function. Thus, the central folate acceptor molecule in the one-carbon cycle is a polyglutamyl form of THF (Bailey & Gregory, 1999). Therefore, 5-methylTHF must be converted to THF via methionine synthase, explained in more detail in the next section.

The beginning of one-carbon metabolism via folate cycle starts with the conversion of THF into 5, 10-methyleneTHF. This reaction is a decisive step in the cycle, using the 3-carbon of serine as a main carbon source. Serine can be originated from 3-phosphoglycerate involved in glycolysis, or can also be directly imported from the extracellular environment by facilitated transport through amino acid transporters. The one-carbon unit is transferred from serine side-chain to THF via PLP-dependent serine hydroxymethyltransferase to form 5, 10-methylene-THF and glycine, in a reversible reaction (Locasale, 2013).

5, 10-methyleneTHF can follow two different pathways depending on the cell needs: can enter methionine cycle via reduction to 5-methylTHF by the riboflavin-dependent enzyme MTHFR, or be metabolised in several one-carbon transfer reactions leading to the synthesis of thymidylates and purines [Fig. 3] (Blom & Smulders, 2011; Forges *et al.*, 2007).

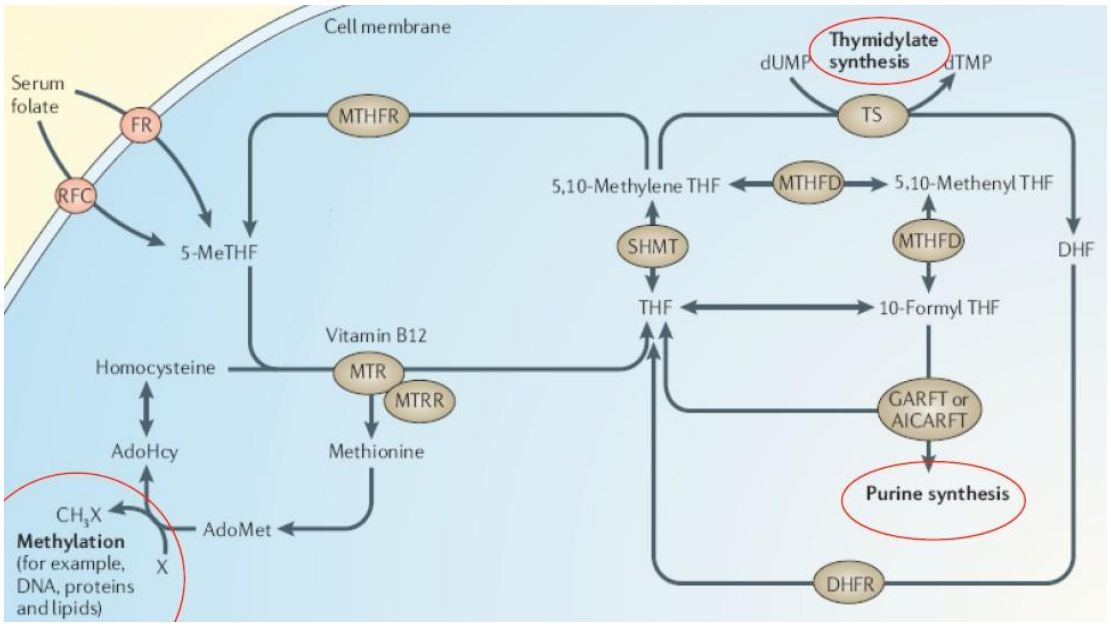


Figure 3. Simplified representation of the one-carbon metabolism pathway *(Humpath.com - Human pathology, 2003).* FR – folate receptor; RFC – reduced folate carrier; MTHFR – methylenetetrahydrofolate reductase; MTR – methionine synthase; MTRR – methyltetrahydrofolate-homocysteine methyltransferase reductase; SHMT – serine hydroxymethyltransferase; MTHFD - methylenetetrahydrofolate dehydrogenase; TS – thymidylate synthase; GARFT – glycinamide ribonucleotide formyltransferase; AICARFT – 5-aminoimidazole-4-carboxamide ribonucleotide formyltransferase; DHFR – dihydrofolate reductase; THF – tetrahydrofolate; AdoHcy – S-(5′-adenosyl)-L-homocysteine; AdoMet – S-adenosylmethionine.

During the synthesis of thymidylates, the one-carbon group of 5, 10-methyleneTHF is donated to deoxyuridine monophosphate (dUMP), resulting in the formation of deoxythymidine monophosphate (dTMP) and dihydrofolate (DHF), reaction catalysed by the thymidylate synthase (TYMS). TYMS reaction is considered a limiting step in DNA synthesis, and, more importantly, reduces dUMP levels. Finally, DHFR catalyses the reduction of DHF back into THF (Forges *et al.*, 2007).

In the purines synthesis, 5, 10-methyleneTHF is catalysed by the trifunctional enzyme methyleneTHF dehydrogenase (MTHFD) that has formyltetrahydrofolate synthetase, methenylTHF cyclohydrolase and methyleneTHF dehydrogenase activities. This reaction originates 5, 10-methenylTHF, and, subsequently 10-formylTHF that can donate a formyl groups for purines biosynthesis (Blom & Smulders, 2011).

1.3. METHIONINE AND S-ADENOSYLMETHIONINE CYCLE

Methionine is an essential sulfur-containing proteinogenic amino acid in humans and serves as the initiating amino acid in eukaryotic protein synthesis. It is an overly consumed amino acid in developed countries at about 60% over that is required to the organism. Methionine cycle serves to regulate cellular methylation through adequate supply of SAM, and is a source of glutathione, the cell principle antioxidant, enabling the redox buffering. Methionine cycle could not accomplish its function without being interconnected with folate cycle in a network implying more than twenty enzymes (Joint, 1998).

The interconnection between folate and methionine cycle happens with the irreversibly conversion of 5, 10-methyleneTHF to 5-methylTHF, reaction catalysed by MTHFR, using NADPH or FAD, as reducing agents. MTHFR has a key role in one-carbon metabolism by irreversibly directing one-carbon moieties to Hcy remethylation, rather than DNA synthesis [Fig. 4] (Crider, Yang, Berry & Bailey, 2012). The enzyme is a dimer with each monomer constituted by a catalytic domain that binds the FAD cofactor and folate, and a regulatory domain that binds SAM (Ulvik *et al.*, 2007).

After 5-methylTHF production, remethylation of Hcy to form methionine occurs via cobalamin–dependent methionine synthase, a ubiquitously expressed enzyme. Additionally, remethylation can happen as well via betaine-homocysteine-methyltransferase (BHMT), mainly expressed in the liver and kidneys, the main organs storing large amounts of betaine, required as a methyl group donor (Obeid, 2013).

Methionine synthase reaction involves the generation of a complex, cobalamin(I)methionine synthase, between cobalamin coenzyme, intermediate carrier of the methyl group, and methionine synthase, that binds to the methyl group of 5-methyTHF to form methylcobalamin(III)methionine synthase. After transferring the methyl group to Hcy, and originating methionine, cobalamin(I)methionine synthase is reformed, being able to accept another methyl group from 5-methylTHF, restarting the cycle (Blom & Smulders, 2011).

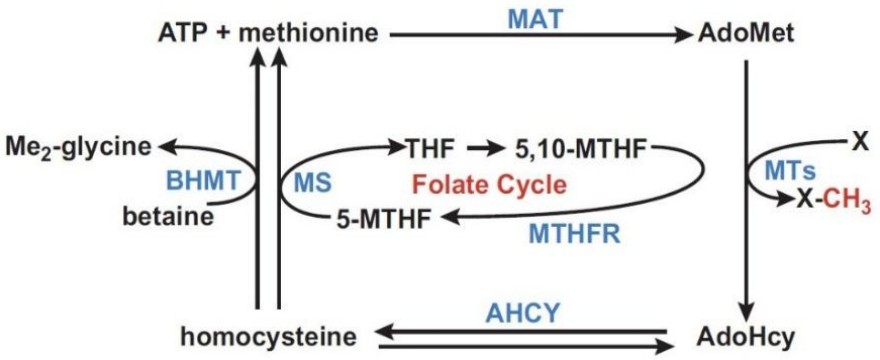


Figure 4. Methionine metabolism *(Lu & Mato, 2012).* MAT – S-adenosylmethionine synthetase; BHMT – betaine-homocysteine-methyltransferase; MS – methionine synthase; AHCY – S-adenosylhomocysteine hydrolase; MTHFR – methylenetetrahydrofolate reductase; MTs – methyltransferases; Me_2-glycine – dimethylglycine; AdoMet – S-adenosylmethionine; AdoHcy – S-adenosyl-L-homocysteine; 5-MTHFR – 5-methyltetrahydrofolate;
5, 10-MTHFR – 5,10-methylenetetrahydrofolate.

Thus, 5-methylTHF is converted to THF closing the cycle of folate (Blom & Smulders, 2011). BHMT is an alternative pathway for the remethylation of Hcy, connecting the oxidative catabolism of choline to methionine metabolism. In this reaction, the methyl group is donated by betaine that is converted into dimethylglycine (Forges *et al.*, 2007). Once remethylation of Hcy occurs, the resulting methionine is, either incorporated into proteins, or converted into SAM by S-adenosylmethionine synthetase (MAT) (Ikeda *et al.*, 2012).

SAM is the major methyl donor for most methyltransferases and is involved in numerous cellular reactions, including methylation of DNA, histone, proteins, synthesis of polyamines, binding to mRNA, methylation and synthesis of lipids, and antioxidants reactions (such as glutathione and taurine) [Fig. 5] (Mato & Lu, 2007). Thus, SAM is determinant for a wide range of biological processes, from membrane fluidity, gene expression, cell growth, differentiation, to suppression of cell division and apoptosis.

In methylation reactions, SAM has a methyl group removed, resulting in S-adenosylhomocysteine (SAH) a potent competitive inhibitor of many methyltransferases. Then, SAH can be hydrolysed to Hcy and adenosine [Fig. 4]. Physiologically, the biosynthesis of SAH is favoured, rather than its hydrolysis. The

reverse situation can happen if adenosine and Hcy are being rapidly eliminated, which is crucial to avoid accumulation of SAH (Lu & Mato, 2012).

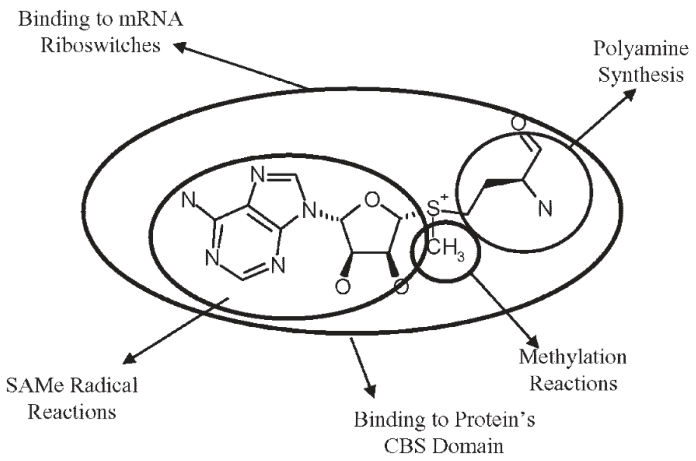


Figure 5. Structure of S-adenosylmethionine *(Mato & Lu, 2007).*

CBS – Cystathionine-β-Synthase

The effectiveness of the cycle depends on the replenishment of methyl groups in order to keep a proper concentration of SAM. Therefore, the remethylation of Hcy back to methionine and thus to SAM is essential for the cell survival (Joint, 1998).

1.4. HOMOCYSTEINE METABOLISM AND TRANSSULFURATION PATHWAY

Hcy is a thiol-containing non-proteinogenic amino acid exclusively originated from the demethylation of methionine. In most mammalian cells, Hcy can suffer different metabolic fates: *1)* remethylation, which is the conversion to methionine by methionine synthase or BHMT, as described before in last section; *2)* catabolism via transsulfuration pathway, where it is irreversibly converted to cysteine [Fig. 6]; *3)* release into the extracellular medium (Brustolin, Giugliani, & Felix, 2010). However, when the remethylation pathway is saturated, or when cysteine is required, Hcy follows the transsulfuration pathway (Blom & Smulders, 2011).

The transsulfuration pathway is an alternative mechanism to Hcy remethylation, and is the real mechanism by which methionine is catabolised, occurring in most tissues except in muscle and endothelium. Furthermore, transsulfuration pathway plays a role in the maintenance of the redox homeostasis, providing an endogenous route where Hcy

can be used for the generation of redox-controlling molecules, such as glutathione and taurine (Zhang *et al.*, 2013).

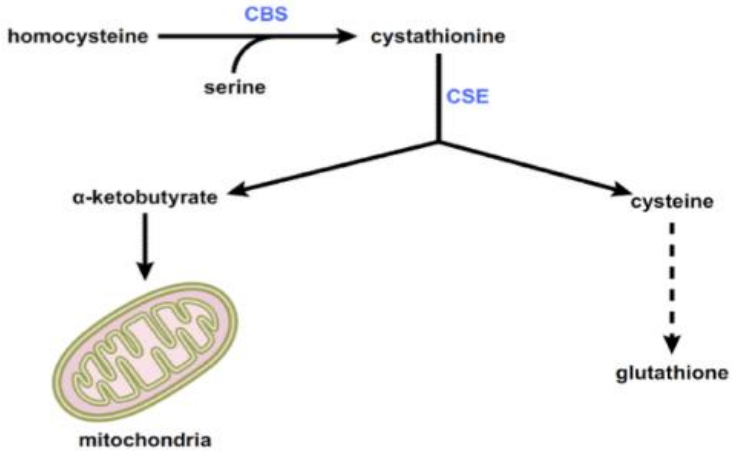


Figure 6. Transsulfuration pathway for homocysteine degradation and cysteine synthesis *(Lu & Mato, 2012).* CBS – cystathionine-β-synthase; CSE - cystathionine-γ-lyase.

In the transsulfuration pathway, Hcy is condensed with serine in a β-replacement irreversible reaction of the hydroxyl group of serine by cystathionine-β-synthase (CBS) forming cystathionine, with release of a water molecule. CBS is a heme-containing enzyme that is subject to regulatory control, as it is the first and rate-limiting enzyme for the transsulfuration pathway (McBean, 2012). On the second step of the transsulfuration pathway, the resulting cystathionine, from CBS reaction, is then hydrolysed to cysteine, alpha-ketobutyrate (from the Hcy carbon chain), and ammonia (from the amino group of Hcy) by cystathionine-γ-lyase (CSE) [Fig. 6]. Both enzymes, CBS and CSE are dependent on PLP, the active form of vitamin B_6. Alpha-ketobutyrate can suffer oxidative decarboxylation catalysed by pyruvate dehydogenase complex producing propionylcoenzyme A that can enter the tricarboxylic acid cycle. Cysteine can be used for the glutathione biosynthesis, and originate metabolites in mammals such as taurine, pyruvate, and hydrogen sulfide (Stipanuk & Ueki, 2011).

The balance of transsulfuration depends on the quantity of prooxidants and antioxidants. The prooxidants increase the transsulfuration flux, while antioxidants decrease it. Some studies agree that redox regulation of the transsulfuration pathway occurs at the level of CBS, due to the heme portion that serves as a sensor for the oxidative medium (Brosnan & Brosnan, 2006).

CHAPTER II – *MTHFR* 677C→T POLYMORPHISM: GENERAL ASPECTS

2.1. CYTOGENETIC LOCATION

MTHFR gene is located on the short (p) arm of chromosome 1 at position 36.3. (Trabetti, 2008). The majority of polymorphisms reported for *MTHFR* gene have a minor impact on enzymatic activity, with the exception of 677C→T and 1298A→C. *MTHFR* 677C→T and 1298A→C polymorphisms seem to be in linkage disequilibrium, with findings pointing to the appearance of 677 variant later than the 1298C variant on a chromosome harbouring 1298A. However, only *MTHFR* 677C→T polymorphism has been established to lower considerably serum folate and higher plasma homocysteine (Ulvik *et al.*, 2007).

MTHFR 677C→T polymorphism is transmitted in an autosomal recessive way and is characterized by a point mutation at base pair 677 (exon 4) that converts a cytosine (C) into a thymine (T) leading to an amino acid substitution (alanine to valine) at codon 222 in the *MTHFR* gene [Fig. 7] (Trabetti, 2008).

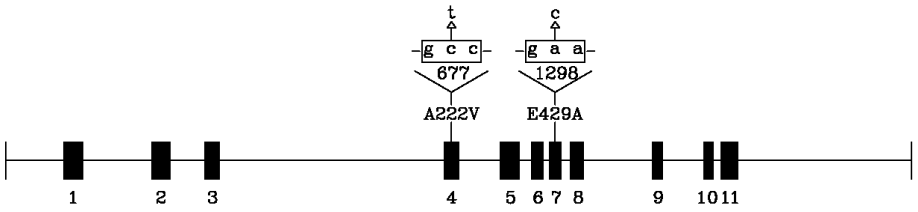


Figure 7. Schematic representation of the *MTHFR* gene, located on chromosome 1p36.3. Exons are shown in black boxes. The two most investigated polymorphisms, 677C→T and 1298A→C, corresponding to the amino acid substitution A222V and E429A, respectively, are indicated above their corresponding exons *(Trabetti, 2008).*

The enzyme becomes thermolabile in homozygotes for the variant (TT genotype), with lower activity (50-60% at 37°C and nearly 65% at 46°C), leading to increased Hcy levels in individuals with low folate status. In heterozygotes (CT genotype), enzyme activity is in an intermediate range, and functions normally in wild-type homozygotes (CC genotype) (Leclerc, Sibani & Rozen, 2005).

Many conditions have been associated with *MTHFR* 677C→T polymorphism, such as birth defects, psychiatric disorders [Table 1], vascular disease and cancer (both discussed in the following chapters); supporting the idea, that folate supplementation could have a role in the treatment and prevention of those diseases (Gilbody Lewis & Lightfoot, 2007). The relationship between the *MTHFR* 677C→T polymorphism and disease involves several mechanisms, such as plasma Hcy (tHcy), DNA methylation and DNA synthesis (Ueland *et al.*, 2001).

Table 1. Results from studies relating diseases and *MTHFR* 677C→T polymorphism.

Study / Authors	Study type	Results / Conclusions
Psychiatric disorders (unipolar depression, anxiety disorders, bipolar disorder, and schizophrenia) **Gilbody, Lewis & Lightfoot, 2007**	Meta-analysis	• For unipolar depression and the fixed-effects odds ratio (OR) for homozygote variants (TT) versus the wild type (CC) was 1.36 (95% confidence interval (CI): 1.11, 1.67), on 1,280 cases and 10,429 controls. • For schizophrenia the fixed-effects OR for TT versus CC was 1.44 (95% CI: 1.21, 1.70), on 2,762 cases and 3,363 controls. • For bipolar disorder the fixed-effects OR for TT versus CC was 1.82 (95% CI: 1.22, 2.70), on 550 cases and 1,098 controls. ➔ This meta-analysis demonstrates an association between the *MTHFR* 677C→T polymorphism and depression, schizophrenia, and bipolar disorder, raising the possibility of the use of folate in treatment and prevention.
Neural tube defects (spina bifida aperta or encephalocele) **Kirke *et al.*, 2004**	Case-control Cases: 397 individuals with spina bifida aperta (380) or encephalocele (17) Controls: 1000 newborn	• The heterozygous genotype is associated with an increased risk of neural tube defects (OR 1.52; p= 0.0015). • Risk is also raised for the homozygous TT genotype (OR 2.56; p< 0.0001). • CT genotype is responsible for at least as many neural tube defects in the population as the TT genotype (14.9% vs 11.3%), because a much greater proportion of the population are heterozygous for this allele. ➔ CT genotype needs to be considered as a risk factor for other conditions where homozygosity has been shown to be associated with increased risk.

2.2. POPULATION GENETICS

The worldwide distribution of *MTHFR* 677C→T polymorphism varies extensively among regions and populations. The frequency of the T allele ranges from 1% or less in Blacks from sub-Saharan, but increases substantially, up to 20%, among Caucasians and Chinese (Botto & Yang, 2000). In Europe, the prevalence of the TT genotype increase towards south direction, from low values in the north (4-7% in Finland, Helsinki,

Northern Netherlands, and Russia), to intermediate values (8-10%) in France and Hungary, to higher values in Southern Europe (12-15% in Spain and Northern Italy), peaking in southern Italy (20-26% in Campania and Sicily). In North America, the frequency of TT homozygotes increase from Western Canada to Southeastern United States, reaching a peak in Mexico [Fig. 8] (Wilcken *et al.*, 2003).

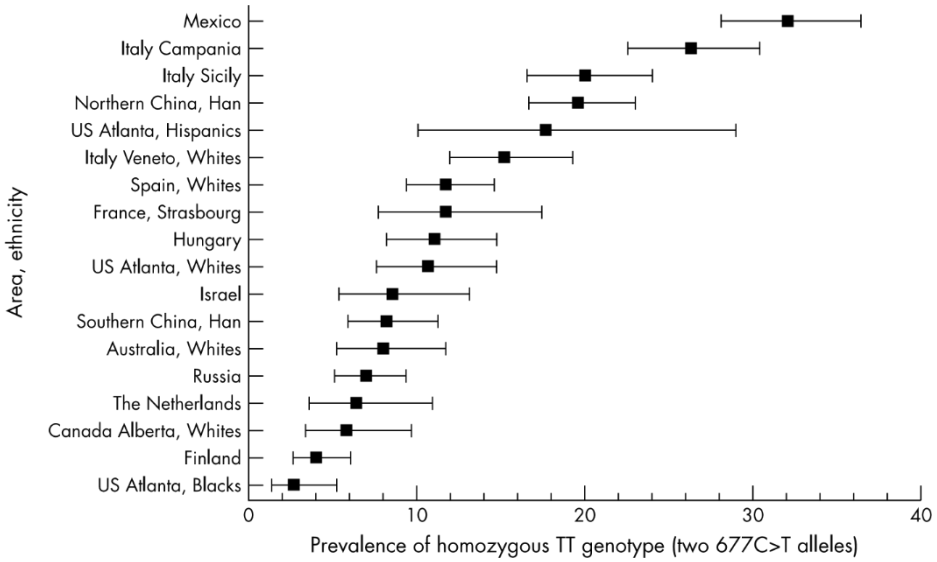


Figure 8. Prevalence of homozygous TT genotype among newborns by area and ethnic background *(Wilcken et al., 2003).*

The prevalence of *MTHFR* 677C→T polymorphism varies considerably in China through a geographic gradient. For *MTHFR* 677C→T, the frequencies of the T allele and the TT genotype are notably higher in the north (63.1% and 40.8%, respectively) compared to the south (24.0% and 6.4%, respectively) (Yang *et al.*, 2013).

The explanation for the differences found geographically and ethnically is thought to involve, in part, gene-nutrient interactions, between *MTHFR* 677C→T gene polymorphism and folate as suggested by Guéant-Rodriguez *et al* (2006). The authors reported Mexico City and Sicily as having the highest concentration of folate, the lowest rate of folate deficiency and the areas where influence of the TT genotype on tHcy was the lowest. Additionally, these areas exhibit the highest T allele frequency. In fact, the main resemblance concerning those two populations is their characteristic diet, richer in fresh fruit and vegetables, which are wide sources of folate. In another hand, they found a high prevalence of folate deficiency in West Africa, where the lowest

frequency of the T allele was registered. Additionally, in West Africa, the genotype influence on tHcy was the most evident; the majority of individuals with the T allele had elevated tHcy. These data indicate a possible interaction between the environment and genetic pressure and suggest that TT genotype may confer a survival advantage in populations with adequate dietary folate consumption (Guéant-Rodriguez *et al*, 2006).

In fact, other studies indicate that heterozygous or homozygous mutant genotypes with adequate folic acid consumption may have, in certain circumstances, a selective advantage over wild-type genotype, which may explain the current observed variability in its frequency in different populations. Further facts that support this hypothesis are the beneficial effect to heterozygotes during times of starvation and the decreased risk of 677C→T homozygotes for colon cancer (Schneider, Rees, Liu & Clegg, 1998), which is going be examined in the last chapter.

2.3. RELATIONSHIP WITH PLASMA HOMOCYSTEINE

Hcy concentrations are regulated by many factors such as the cofactors cobalamin, PLP, folate and enzymes implicated in methionine metabolism. When discrepancies between Hcy production and catabolism take place, tHcy can increase. Impairments in Hcy metabolism can derive from various nutritional and/or congenital disorders, such as *MTHFR* 677C→T polymorphism (Medina, Urdiales, & Amores-Sánchez, 2001).

Most of the tHcy is found in the oxidised form. Only 1–2% are present in its reduced form. Approximately 20% occurs as acid-soluble free Hcy in a form of homocysteine-cysteine mixed disulfide and homocystine (a dimer of Hcy). Approximately 70% to 80% circulate bounded to plasma proteins; the great majority travels linked to the cysteine of albumin, its main carrier in plasma. However, current data suggest that approximately 10%–30% of protein bound Hcy and cysteine are linked to globulins. The total tHcy is the sum of all protein-bound and free forms containing a thiol group (Amorim *et al.*, 2011).

In healthy individuals, concentrations of Hcy oscillate between 5 and 15 μmol/L. Hyperhomocysteinemia (HHcy) is the presence of an abnormally elevated concentration of plasma or serum, and can be distinguished by category, including cause, prevalence

and severity [Table 2]. HHcy is dependent on genetic, lifestyle, gender, being higher in men than in women and age, increasing from 10.8 µmol/L at age 40-42 up to 12.4 µmol/L between 65-67 years. Moreover, HHcy is observed in approximately 5% of the general population, and it is implicated in several morphological and physiological changes that are thought to increase the risk for many disorders, including vascular and neurodegenerative diseases, autoimmune disorders, birth defects, diabetes, renal disease, osteoporosis, neuropsychiatric disorders, and cancer (Brustolin, Giugliani, & Felix, 2010).

Table 2. Classification of hyperhomocysteinemia *(Brustolin, Giugliani & Felix, 2010).*

Severe hyperhomocysteinemia
tHcy levels at all times (31 to >100 µmol/L) caused, for example, by deficiencies in CBS, MTHFR, or in enzymes of cobalamin metabolism.
Mild hyperhomocysteinemia
Moderately high tHcy levels (15-30 µmol/L) under fasting conditions; reflects impaired Hcy methylation (folate, cobalamin or moderate enzyme defects, e.g., thermolabile MTHFR)
Post-methionine load
Abnormal increase in tHcy (>15 µmol/L) after a methionine load (100 mg/kg); reflects impaired Hcy transsulfuration (heterozygous CBS defects, PLP deficiency)

Apart from that, some findings reveal that elevated tHcy is associated with administration of certain medications, including antiepileptic drugs, methotrexate, antihypertensive, lipid-lowering and antidiabetic drugs (Malinow, Bostom & Krauss, 1999).

Ham *et al* (2014) conducted a study with 2,912 participants to assess the link between medication use, tHcy levels, and the potential mediation by serum vitamin B_{12} and folate [Table 3]. The only cases with higher mean Hcy levels were observed in users vs. non-users for diuretics, high-ceiling sulphonamide diuretics, medication acting via the renin-angiotensin system and metformin. Non-selective β-blocker use was associated with lower mean Hcy levels and only this association was mediated by an underlying association with vitamin B_{12} and folate levels. Nonetheless, the associations between tHcy levels and medication use appears to be unassertive, suggesting that medication use is unlikely to contribute to clinically relevant changes in tHcy levels.

Table 3. Associations between medication use and homocysteine levels in an older population, and potential mediation by vitamin B_{12} and folate *(Ham et al., 2014)*.

Medication group	Results and possible mechanisms
• **Diuretics in general** • **High-ceiling sulphonamide diuretics** • **Agents acting via the renin-angiotensin system**	• Small but significant positive association with tHcy. • Largely independent of vitamin B_{12} and folate levels. • Diuretic use has previously been shown to be associated with higher tHcy, possibly through decreasing folate levels. • High-ceiling sulphonamides are known as potent inhibitors of the reabsorption of electrolytes in the kidneys, resulting in reduced water reabsorption into the blood and thus increased water excretion. This alteration in fluid status, or mild dehydration, might cause the observed relative increase of the tHcy.
• **Non-selective β-blocker**	• Inverse significant association with tHcy was observed. • Characterised by an underlying association with vitamin B_{12} and folate levels. • How non-selective b-blockers may affect vitamin B_{12} and folate levels is still unknown.
• **Thiazides** • **Selective β-blockers** • **Statins** • **Sulphonylurea derivatives**	• No association was observed with tHcy, especially for metoprolol.
• **Metformin**	• Higher tHcy. • Relationship between metformin and tHcy was independent of vitamin B_{12} and which became slightly stronger after including folate levels. • Metformin increases insulin sensitivity, and potentially insulin levels are involved. Nevertheless, the literature reports contradictory findings, as both insulin sensitivity as well as insulin resistance were associated with higher tHcy.
• **Proton pump inhibitor** • **Histamine H_2 receptor antagonist** • **Methotrexate** • **Acetylsalicylic acid** • **Theophylline** • **L-dopa**	• Lack of association with tHcy.
• **Anticonvulsants**	• Lack of association with tHcy. • The association has been consistently reported in the literature. • Anticonvulsant drugs may reduce folate levels and particularly in combination with the *MTHFR* TT genotype, this may result in elevated tHcy. • The discrepancy between these findings and other studies may be explained by the non-significant interaction with the *MTHFR* polymorphism and the low frequency of folate deficiency in the population studied.
• **Combination of medication groups significantly associated with higher homocysteine level**	• No additive effect of the association was observed. • There is no literature regarding a potential additive effect of a combination of medication use on tHcy. • Nevertheless, because of multiple drug use, older individuals are more prone to unintended drug effects of which increased tHcy might have been one.

2.3.1. MOLECULAR MECHANISMS OF HOMOCYSTEINE

Endothelial cells participate in the regulation and preservation of vascular system and are extremely susceptible even to a mild increase in Hcy concentration. This sensitivity may be justified by the fact, that human endothelial cells do not express active form of CBS, and consequently, cannot initiate Hcy catabolism through transsulfuration pathway [Fig. 9]. Thus, a number of molecular mechanisms of Hcy have been suggested, and the underlying common pathogenic mechanisms are predominantly associated with vascular injury [Fig. 10] (Perła-Kaján, Twardowski & Jakubowski, 2007).

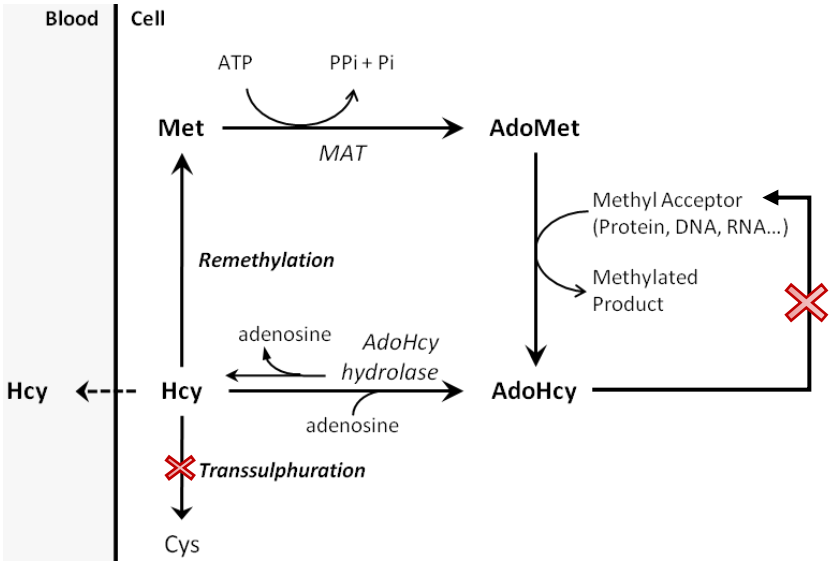


Figure 9. Simplified homocysteine metabolism (*adapted from: Esse et al., 2012).* AdoMet – S-adenosylmethionine; AdoHcy – S-adenosyl-L-homocysteine; MAT – S-adenosylmethionine synthetase; Met – methionine; Cys – cysteine; Hcy – homocysteine.

One of the pathogenic mechanisms proposed includes oxidative damage of the endothelium through nitric oxide (NO) suppression. The excess of Hcy leads to inhibition of NO synthase, by reducing the catabolism of its inhibitor, and, consequently, decreasing NO bioavailability. Under normal conditions, NO detoxify plasma from Hcy through formation of S-nitro-Hcy. However, in presence of large amounts of Hcy, and subsequent inhibition of NOS, NO cannot neutralize the excess, leading to the auto-oxidation of Hcy to homocystine with release of toxic free radicals to the vascular endothelium (Stühlinger *et al.*, 2001).

Additionally, Hcy may affect glutathione peroxidase activity, changing the dissemination of reactive oxygen species. Endothelial glutathione peroxidase catalyses the reduction of hydrogen and lipid peroxides to the equivalent alcohol, avoiding the oxidative inactivation of NO. The mechanism by which Hcy may act is thought to involve the downregulation of glutathione peroxidase expression (Yilmaz, 2012).

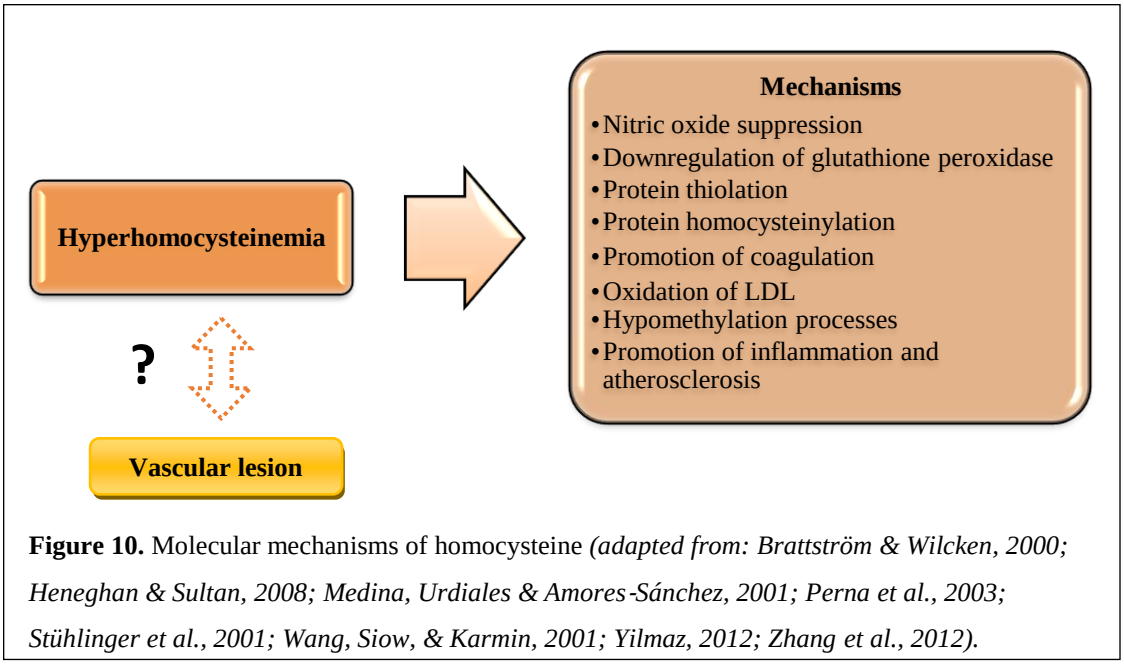


Figure 10. Molecular mechanisms of homocysteine *(adapted from: Brattström & Wilcken, 2000; Heneghan & Sultan, 2008; Medina, Urdiales & Amores-Sánchez, 2001; Perna et al., 2003; Stühlinger et al., 2001; Wang, Siow, & Karmin, 2001; Yilmaz, 2012; Zhang et al., 2012).*

Hcy is also known as thrombogenic agent, boosting platelet aggregation, acting on the coagulation cascade, directly inducing a synergistic pathway with other risk factors. It activates coagulation factor V, X, and XII, inhibits protein C and cell-surface thrombomodulin, and modulates tissue plasminogen activator, through annexin II (an endothelial receptor) (Perna *et al.*, 2003).

HHcy can have a direct impact on DNA methylation, leading to modifications in the gene expression, affecting both endothelial cells and smooth muscle cells. An increase in tHcy generates a growth in the intracellular concentration of SAH that can bind to methyltransferases with higher affinity than SAM. However, SAH is a potent inhibitor of DNA methyltransferases, affecting cell homeostasis (Castro *et al.*, 2003). Several studies suggest that hypomethylation processes, due to of HHcy, induce proliferation of vascular smooth muscle cells, resulting in a decrease of the vessel calibre (Zhang *et al.*, 2012).

Another molecular pathogenic mechanism of Hcy is believed to involve the formation of adducts with disulfide linkages with sulphydryl residues of proteins, in a reaction known as thiolation [Fig. 11]. Moreover, the detrimental effect of Hcy in proteins may be caused by the formation of Hcy thiolactone, leading to protein homocysteinylation, when transsulfuration or remethylation pathways are compromised. Both occurrences can lead to an irreversible damage of the enzymatic activity and denaturation of proteins, such as haemoglobin, low-density lipoproteins (LDL) and plasma proteins (Medina, Urdiales & Amores-Sánchez, 2001).

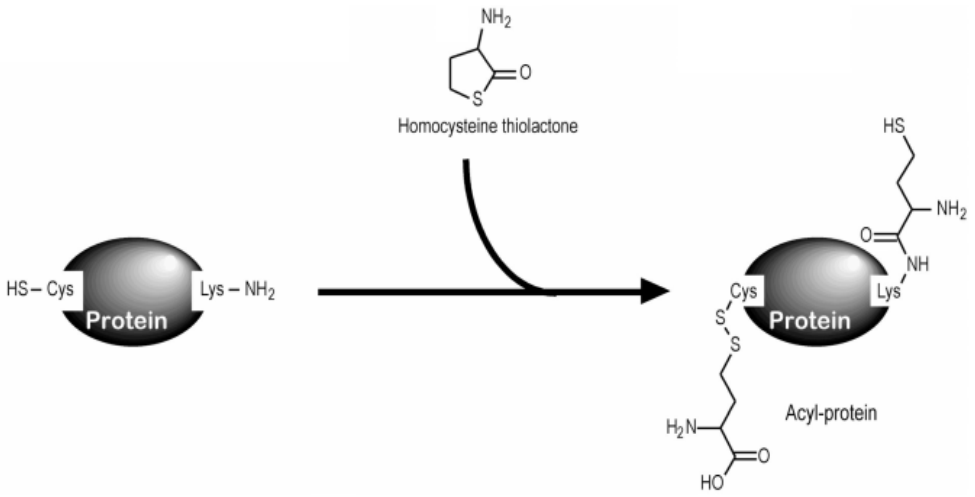


Figure 11. Generation of reactive homocysteine thiolactone *(adapted from: Medina, Urdiales & Amores-Sánchez, 2001).*

As a result of the vascular injury effect, Hcy has been frequently referred as "the cholesterol of the 21st century". HHcy can produce vascular injury by increasing the oxidation of LDL within vascular cells and tissues. LDL aggregates of Hcy in the bloodstream can be taken up by macrophages, originating foam cells, the precursors of atherosclerotic plaques. Within these plaques, Hcy modifies the oxidative and synthetic processes of artery wall cells, propagating plaque formation (Heneghan & Sultan, 2008).

Hcy has been associated with the promotion of inflammation and atherosclerosis, activating NF-κB responsible for the induction of chemokines and interleukins expression. Yet, Hcy can activate monocytes proliferation, inducing cytokines expression and inhibiting the expression of MMIF (Wang, Siow, & Karmin, 2001).

However, the origin HHcy is yet to be understood. Brattström and Wilcken (2000) suggest that, in reality, HHcy is more an epiphenomenon of vascular disease, having a minor role in the vascular damage. They hypothesised that the decline in renal function can be the actual cause of HHcy in vascular disease patients. Risk factors for vascular disease, such as atherogenesis and hypertension, frequently progress in a discrete way over the time before the appearance of clinically evident vascular episodes. Therefore, atherogenesis and hypertension can conduct to renal impairment, affecting the clearance of Hcy. As matter of fact, the existence of vascular illnesses would promote the raise of Hcy in plasma through the impairment of renal function.

CHAPTER III – *MTHFR* 677C→T POLYMORPHISM AND CARDIOVASCULAR RISK

3.1. *MTHFR* 677C→T POLYMORPHISM, HOMOCYSTEINE AND CARDIOVASCULAR RISK

Cardiovascular disease (CVD) or heart disease is a group of illnesses that involves the heart and/or the blood vessels, leading mostly to cardiac disease, vascular diseases of the brain and kidney, and peripheral arterial disease (Kelly, 2010). The concerns about CVD are reasonable due to its considerable high mortality rate, being the primarily worldwide cause of death. Additionally, the overall CVD is estimated to cost the EU economy almost €196 billion a year (Nichols *et al.*, 2012). The causes underlying CVD are diverse and multifactorial, but atherosclerosis and hypertension are the most common causes, defined by genetic and environmental aspects, such as gene-gene and gene-environment interactions (Sabetisoofyani, Larson & Watson, 2010).

As discussed in the last chapter, HHcy is cogitated by many researchers to grant a mild vascular risk alone, and to increase the risk of vascular lesions in association with other factors. Therefore, the attention given to cardiovascular risk and mild increase of circulating Hcy has been motivating investigators to establish a connection between *MTHFR* 677C→T polymorphism and CVD risk (Clarke *et al.*, 2012; Xuan *et al.*, 2011).

The metabolic phenotype of low concentrations of folate and tHcy elevated over 25% in individuals with the TT genotype compared with those with the CC genotype has been vastly assessed in Mendelian randomisation studies (Brattström, Wilcken, Öhrvik & Brudin, 1998). If a gene variant is responsible for higher tHcy levels, random allocation of this gene during conception produces a randomised trial of low versus high Hcy from birth on [Fig. 12]. Many studies revealed increased cardiovascular risk in individuals with the TT genotype compared with those carrying the wild-type (Smulders & Blom, 2011).

MTHFR 677C→T polymorphism was initially pointed as a possible risk factor for CVD in Canadians and afterwards it was also described in Japanese, in Italians, in Chinese, and in English population (Sazci *et al.*, 2006). If proved such association, the high frequency of the T allele within a certain population could serve to emphasize

recommendations concerning nutrient consumption, and avoidance of other cardiovascular risk factors.

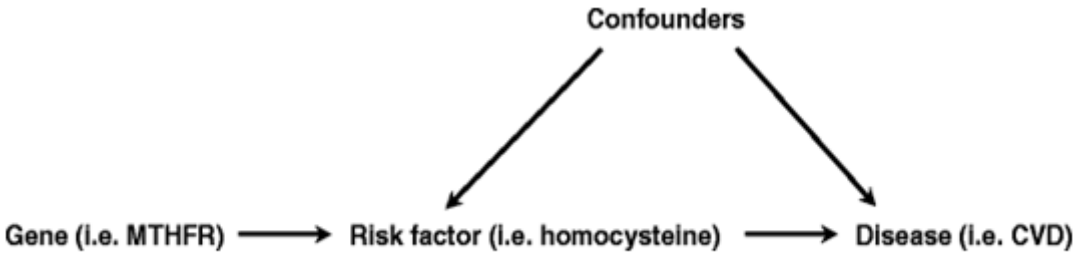


Figure 12. Principle of Mendelian randomisation as applied to hyperhomocysteinemia.
Essential for the validity of the model is the absence of arrows between the gene and confounders, as well as the absence of an arrow (a direct association not dependent on the risk factor) between the gene and the disease *(Smulders & Blom, 2011).*

Several *MTHFR* 677C→T polymorphism studies, including retrospective and prospective studies, suggest that the association between the polymorphism and CVD is causal, stating HHcy as the underlying mechanism (Wald, Law & Morris, 2002). However, the debate about this topic has intensified in the beginning of the 21st century with several studies showing discordant results. Many studies advocate that mild HHcy associated with *MTHFR* 677C→T polymorphism is not high enough to be an independent risk factor for the development of CVD. Thus, although the association between tHcy concentrations and CVD has been already demonstrated, the causality is not yet established (Brattström, Wilcken, Öhrvik & Brudin, 1998).

3.2. CORONARY HEART DISEASE

Several meta-analyses, using all data published concerning the association between the risk of coronary heart disease (CHD) and *MTHFR* 677C→T polymorphism, have been conducted to aid the assessment of causality [Table 4]. In 2002, a meta-analysis, including single participant data from all case-control studies on the polymorphism and risk of CHD, involved data from *MTHFR* 677C→T genotype, levels of tHcy, folate, and other cardiovascular risk factors. The results pointed to a relation between TT genotype and increased CHD risk, when folate status were low, suggesting an interaction between the *MTHFR* 677C→T polymorphism and folate status, holding the hypothesis that HHcy could be the triggering mechanism (Klerk *et al.*, 2002).

However, results of CVD studies do not seem to be concordant for the overall population. In 2011, another meta-analysis on published studies, studied the association between the polymorphism and risk of myocardial infarction (MI), and found association only for Caucasians (Xuan *et al.*, 2011).

Even though collected evidences have shown an association between HHcy and CHD, there is a strong divergence in results, with studies supporting the idea that HHcy might be just a consequence of CHD. Additionally, the divergence is magnified when the causality between *MTHFR* 677C→T polymorphism and risk of CHD is hypothesised. An updated meta-analysis of unpublished studies on *MTHFR* 677C→T polymorphism and CHD performed in 2012 found that the additional CHD risk in TT compared to CC genotype carriers was only 2%, a negligible increase, certainly coincidental. The stratification of the study populations by folate status showed, as well, no evidence that T allele had an extra CHD risk compared to the wild-type [Table 4] (Clarke *et al.*, 2012).

Table 4. Results from studies relating coronary heart disease and *MTHFR* 677C→T polymorphism.

Study / Authors	Study type	Sample	Results
Coronary heart disease **Klerk *et al.*, 2002**	Meta-analysis of 40 published and unpublished studies	11,162 cases 12,758 controls	• Individuals with the TT genotype had a 16% (OR, 1.16; 95% CI, 1.05-1.28) higher odds of CHD than those with the CC genotype. • Significant heterogeneity between the results obtained in European populations (OR, 1.14; 95% CI, 1.01-1.28) compared with North American populations (OR, 0.87; 95% CI, 0.73-1.05).
Myocardial Infarction **Xuan *et al.*, 2011**	Meta-analysis of 30 published studies	8,140 cases 10,522 controls	• Significant association was found between *MTHFR* 677C→T polymorphism and risk of MI (OR, 1.183; 95% CI, 1.076–1.300). • The same association was found in overall Caucasians (OR, 1.139; 95% CI: 1.007–1.288) and young/middle-aged (<50 years) Caucasians (OR, 1.275; 95% CI, 1.077–1.509). • No associations were detected between *MTHFR* 677C→T polymorphism and the risk of MI in elderly male or female Caucasians, East Asians, South Asians, and African-Americans.
Coronary heart disease **Clarke *et al.*, 2012**	Meta-analysis of 19 unpublished studies	48,175 cases 67,961 controls	• The case-control CHD OR and 95% CI comparing TT vs CC homozygotes was 1.02, and 0.98–1.07, respectively (p= 0.28).

3.3. VENOUS THROMBOSIS

The specific mechanism of susceptibility for venous thrombosis remains unclear. Interest in the genetic basis of venous thrombosis was enhanced by discover of genes with a possible effect in thrombophilia, such Leiden V factor, prothrombin G20210A and *MTHFR* 677C→T polymorphism (Yin *et al.*, 2012).

Due to HHcy association with venous thrombosis, *MTHFR* 677C→T polymorphism has been proposed as a genetic risk factor candidate for venous thrombosis. Consequently, several studies have suggested that the effect of *MTHFR* 677C→T polymorphism on venous thrombosis is only perceptible in particular minor groups with other predisposing genetic or environmental factors or, in contrast, in minor groups in which conventional risk factors for venous thrombosis are missing. Based on this premise, one could suggest that in clinical practice, measurement of Hcy levels (and the assessment of folate levels) would be reasonable for individuals with unsolved idiopathic, persistent venous thrombosis, or venous thrombosis occurring at early age or at an uncommon site (Den Heijer, Lewington & Clarke, 2005).

In 2012, a study examined the possible relation between *MTHFR* 677C→T polymorphism and venous thromboembolism (VTE) [Table 5]. The results showed that the frequency of T alleles and TT carriers was significantly higher in patients compared with that of the healthy controls. In addition, the plasma levels of Hcy in the VTE group were higher compared with those in the control group. The results of the study imply that HHcy and *MTHFR* 677C→T polymorphism could be considered risk factors for VTE [Fig. 13] (Yin *et al.*, 2012).

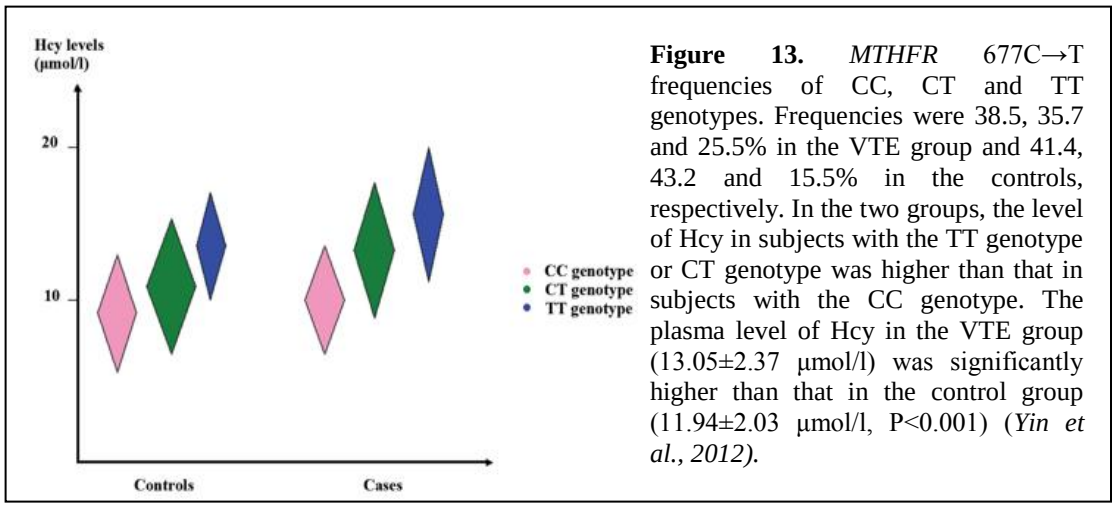


Figure 13. *MTHFR* 677C→T frequencies of CC, CT and TT genotypes. Frequencies were 38.5, 35.7 and 25.5% in the VTE group and 41.4, 43.2 and 15.5% in the controls, respectively. In the two groups, the level of Hcy in subjects with the TT genotype or CT genotype was higher than that in subjects with the CC genotype. The plasma level of Hcy in the VTE group (13.05±2.37 μmol/l) was significantly higher than that in the control group (11.94±2.03 μmol/l, P<0.001) (*Yin et al., 2012).*

Joachim and colleagues (2013) conducted a study to assess the prevalence of HHcy and *MTHFR* variant in a pediatric population with VTE, and the association with thrombus outcome. Subjects were enlisted in an institution-based prospective cohort of children with VTE. The prevalence of HHcy or *MTHFR* variant was not amplified in comparison with the control group. Plasma Hcy did not change between those with CC genotype versus *MTHFR* 677C→T heterozygotes or homozygotes, and adverse thrombus outcomes were not associated either.

In concordance with the previous study, a meta-analysis evaluating risk factors for venous thrombosis showed the same lack of association. The study used the Multiple Environmental and Genetic Assessment including subjects with first venous thrombotic event, deep vein thrombosis of either the leg or pulmonary embolism [Table 5]. In this study, *MTHFR* 677C→T polymorphism was not associated with the risk of venous thrombosis. Stratification by risk factors did not provide evidence of an association in specific groups. Since genotype distributions did not differ between case and control groups, there was no excess risk of venous thrombosis associated with *MTHFR* 677C→T polymorphism. Consequently, based on this study, routine testing of *MTHFR* 677C→T genotype as part of a thrombophilia evaluation with venous thrombosis would not be justified (Bezemer *et al.*, 2007).

Table 5. Results from studies relating venous thrombosis and *MTHFR* 677C→T polymorphism.

Authors	Study type	Sample	Results
Yin *et al.*, 2012	Population -based case-control	440 cases 440 controls	• The plasma levels of Hcy in the VTE group (13.05±2.37 µmol/l) were significantly higher compared with those in the control group (11.94±2.03 µmol/l, P<0.001). • Compared with the CC genotype, the TT genotype was significantly correlated with an increased risk of VTE (OR, 1.753; 95% CI, 1.215–2.529; P=0.003). • In the VTE group (F=106.051; P<0.001), the level of Hcy in subjects with the CT+TT genotype (14.49±2.51 µmol/l) was significantly higher than that in subjects with the CC genotype (11.35±1.57 µmol/l, P<0.001).
Bezemerl *et al.*, 2007	Population -based case-control	4375 cases 4856 controls	• The TT genotype was present in 440 patients (10%) and 517 control subjects (11%), and the CT genotype in 1891 patients (43%) and in 2094 control subjects (43%). • Stratification by known risk factors for venous thrombosis, such as V Leiden, prothrombin 20210G→A, family history, age, and presence of predisposing factors provided no evidence of an association in specific groups.

3.4. ISCHEMIC AND HAEMORRHAGIC STROKE

The *MTHFR* 677C→T polymorphism has been associated, as well, to ischemic and haemorrhagic stroke in many studies but not in others [Table 6]. In 2010, a study evaluated the *MTHFR* 677C→T polymorphism in ischemic and haemorrhagic stroke patients in a Northern Indian population. Despite the elevated tHcy found in TT genotype ischemic stroke patients, *MTHFR* 677C→T gene polymorphism was associated with neither haemorrhagic nor ischemic stroke (Somarajan, Kalita, Mittal & Misra, 2011). However, recent meta-analyses suggest that the T allele of the *MTHFR* 677C→T polymorphism is associated with increased risk of haemorrhagic stroke (HS) and ischemic stroke (IS) [Table 6]. These results, if confirmed, are significant for the genetic epidemiology, diagnosis, treatment and prevention of HS and ARE (Kang *et al.*, 2013; Li & Qin, 2014).

Table 6. Results from studies relating stroke and *MTHFR* 677C→T polymorphism.

Study / Authors	Study type	Sample	Results
Ischemic stroke and Intracerebral haemorrhage (ICH) **Somarajan *et al.*, 2007**	Case–control	Cases: 207 IS 215 ICH 188 controls	• The frequency of the CC genotype in controls was 68.6%, CT in 28.7% and TT in 2.7%. It was 75.3%, 20.5% and 4.2% in ICH and 66.2%, 39.4% and 2.4% respectively in IS. • The frequency of these genotypes as well as allele frequency was not different in IS, ICH as compared to controls • Allele was more frequent in IS compared to ICH. • Hcy level was higher in IS patients with variant genotype.
Haemorrhagic stroke **Kang *et al.*, 2013**	Meta-analysis of 15 case-control studies	2034 cases 4485 controls	• Significant associations between the *MTHFR* 677C→T polymorphism and the risk of HS were observed in dominant (OR, 1.611, 95% CI, 1.336–1.942), codominant (OR, 1.500, 95% CI, 1.330–1.692), and recessive (OR, 1.695, 95% CI, 1.409–2.038) models.
Ischemic stroke **Li & Qin, 2014**	Meta-analysis of 19 case-control studies	2223 cases 2936 controls	• Statistically significant association with IS was identified for allele T (OR = 1.28; 95% CI, 1.17–1.40, P < 0.00001). • Marginally significant association was detected with genotype CT (OR = 1.13; 95% CI: 1.01–127; P = 0.04) and genotype (OR = 1.43; 95% CI: 1.20–1.70; P < 0.001).

A possible mechanism linking *MTHFR* 677C→T polymorphism and stroke events would be through elevated tHcy inducing endothelial dysfunction, mostly through coagulative effect in IS and rupture of microaneurysms in HS (Li *et al.*, 2003). It was also postulated that elevated tHcy could cause, initially, IS and later, HS (Sazci *et al.*, 2006).

3.5. CAN LOWERING HOMOCYSTEINE LEVELS REDUCE CARDIOVASCULAR RISK?

An approach that could disclose the uncertainty around TT genotype, HHcy and CVD triangle would be to carry out several trials that evaluate the decrease of cardiovascular risk or the protective effect in TT genotype when tHcy level is reduced by supplementation of folate. If folate supplementation were demonstrated to reduce cardiovascular risk, this would be of considerable importance for public health and clinical practice. However, the majority of follow-up studies on the assessment of therapeutic intervention with folic acid-based vitamin supplements has indicating no beneficial effect in CVD prevention (Schwammenthal & Tanne, 2004).

A daily dose of folic acid ranging 0.5 and 5 mg was associated with a 25% decrease in tHcy levels addressed in a meta-analysis of the short-term trials. This decrease in tHcy, accomplished with folic acid supplements, is similar to the variation in tHcy levels between TT and CC genotypes. Despite the positive results in some observational studies, a great part of interventional trials firmly contests any satisfactory result of lowering Hcy concentrations with folic acid treatment on CVD (Den Heijer, Lewington & Clarke, 2005).

Recent meta-analyses of randomised trials on the impact of lowering tHcy level by vitamin B supplementation on CVD showed that even with the decrease of 25% in tHcy no beneficial effect was found on the progression of the disease [Table 7] (Clarke *et al.*, 2010).

Table 7. Results from studies relating folate supplementation and *MTHFR* 677C→T polymorphism.

Authors	Study type	Sample	Results
Clarke *et al.*, 2010	Meta-analysis of 8 large, randomised, placebo-controlled trials	37 485 individuals at increased risk of cardiovascular disease	• Folate supplementation led to an average of 25% reduction in Hcy levels • During a median follow-up of 5 years, folate supplementation had no significant effects on vascular outcomes, with an OR of 1.01 (95% CI, 0.97-1.05) for major vascular events; OR, 1.03 (95% CI, 0.97-1.10) for major coronary events, and 0.96 (95% CI, 0.87-1.06) for stroke.
Clarke *et al.*, 2012	Meta-analysis of 10 large-scale placebo-controlled trials	50,378 individuals	• Little or no effect on the 5 years incidence of CHD (OR, folate *vs* placebo, 1.02; 95% CI, 0.96–1.08).

3.6. POSSIBLE EXPLANATIONS FOR DISCREPANCIES AMONG STUDIES

Sample dimension, linkage disequilibrium between polymorphisms, nutrition status, setting, and study design could be responsible for the discrepancy [Fig. 14]. Population specificity might play a role, because different groups of patients may have various sets of genetic factors that predispose to the disease, stressing the importance of ethnicity and genetic background (Yin *et al.*, 2012). Specific alleles of candidate genes can be deeply correlated with the disease in one population, while in another this connection can be low because of other genetic factors or particular interactions between genetic and non-genetic factors (Trabetti, 2008).

The geographical inconsistency of results may be as well attributed to the higher folate intake in certain countries. The impact of the polymorphism on tHcy levels depends largely on the folate intake. Consequently, if dietary folate is high, the expression of the TT phenotype is diminished, Hcy is less increased and so the risk of CVD is improbable (Wald, Law & Morris, 2002). For instance, the venous thrombosis risk estimated for TT genotype, obtained from studies carried out in North America, differed from those carried out in Europe and elsewhere, which may be explained by the higher dietary intake of folate and riboflavin in North America compared to Europe (Den Heijer, Lewington & Clarke, 2005).

Another reason might be due to discrepancies in study design. The clustering of additional risk factors among cases in case-control studies may introduce bias toward a higher risk estimate than in population-based studies (Yin *et al.*, 2012). Thus, several well-established standard risk factors, such as, sex, age, smoking, blood pressure, cholesterol level, and sedentary habits might be associated with tHcy and may confound the relation between tHcy and CVD (Brattström *et al.*, 1998). Relatively small case-control studies may not have the statistical power to adjust and fully eliminate the effects of those risk factors on tHcy concentration. In addition, study heterogeneity reflected by the different selection of patients and control subjects, and occasional incomplete information on Hcy levels and folate status can be a source of discrepancies (Trabetti, 1998).

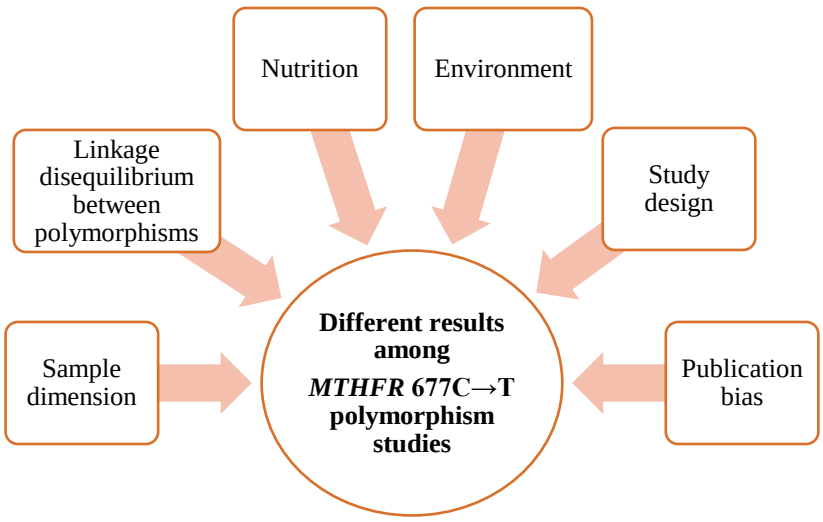


Figure 14. Possible explanations for positive and negative association between *MTHFR* 677C→T polymorphism, homocysteine and cardiovascular disease among studies *(adapted from: Brattström et al., 1998; Clarke et al., 2012; Den Heijer, Lewington & Clarke, 2005; Haynes & Clarke, 2012; Trabetti, 1998; Wald, Law & Morris, 2002; Yin et al., 2012).*

Authors suggest that apart from the common methodological problems, such as sampling, stratification, and analysis of cases and controls, the different outcomes of *MTHFR* 677C→T polymorphism studies might reveal the impact of publication bias [Fig. 14] (Haynes & Clarke, 2012). Mendelian randomisation meta-analyses of unpublished genetic epidemiology datasets are a helpful solution to assess the impact of publication bias on controversial topics, since it is not materially affected by publication bias. Majority of meta-analysis of unpublished datasets about *MTHFR* 677C→T

polymorphism suggested no association between lowering tHcy and decrease risk in CVD (Clarke *et al.*, 2012).

Although the majority of randomised controlled trials suggest no benefit from lowering tHcy with folate supplementation, in the prevention of CVD, some other hypotheses where given to explain these results [Fig. 15] (Clarke *et al.*, 2012). The absence of benefit from lowering tHcy, in the prevention of CVD registered in some randomized trials might be due to the fact that they are carried out in regions with established policies of population folate supplementation, which minimizes the effect expected from of the interventional trials (Holmes *et al.*, 2011).

Another reason might be based on the aspirin reducing or negating the anti-platelet effect of lowering tHcy in the trials. Based on this assumption, folate would have a part in the primary prevention of CVD, when aspirin is not administrated routinely, but not in secondary prevention, when it is routine. Thus, the negative trial evidence on CVD should not weaken the positive evidence from the *MTHFR* 677C→T polymorphism studies and erroneously lead to a conclusion that there is no role for folate in preventing CVD (Wald, Morris & Wald, 2011).

Moreover, the power of some of arguments against can be questioned because HHcy and TT genotype could also have opposite effects on processes related to CVD. The TT genotype may autonomously modulate CVD risk, being independent from Hcy, through the distribution of folates in the direction of purine and pyrimidine biosynthesis. If this assumption is confirmed, the application of Mendelian randomisation studies into the association of *MTHFR* 677C→T polymorphism, Hcy and CVD [Fig. 12] has to be subject of further analysis to assess its real validity (Ueland *et al.*, 2000).

By contrast, some of the studies have suggested that B vitamins treatment might promote CVD [Fig. 15]. Trials of lowering Hcy concentrations are based, mainly, on prescription of folic acid and one of the main functions of folate metabolism is to provide one-carbon building blocks for the synthesis of purines and pyrimidine, increasing the DNA synthesis. Consequently, although high amounts of folic acid may be beneficial through the Hcy lowering, a secondary effect of folic acid use may prompt

atherosclerotic plaque progression, through vascular proliferation and inflammation (Blom & Smulders, 2011).

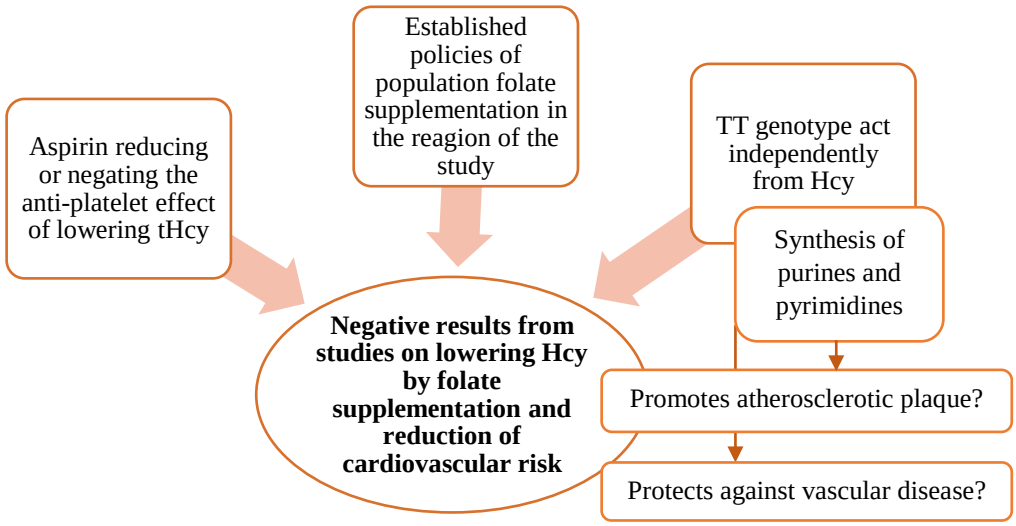


Figure 15. Possible explanations for negative results from studies of folate supplementation, *MTHFR* 677C→T polymorphism and reduction of cardiovascular risk *(adapted from: Blom & Smulders, 2011; Holmes et al., 2011; Ueland et al., 2000; Wald, Morris & Wald, 2011).*

Current guidelines recommend that patients with the TT genotype, with normal tHcy should be reassured that there is no current evidence of increased risk for venous thrombosis related to their *MTHFR* status, common reasons for which clinical testing is performed. A patient, who is homozygous for the *MTHFR* 677C→T polymorphism but also with elevated Hcy, may be at mildly elevated risk for venous thrombosis. The patient can also be reassured that there is no evidence of any association with homozygosity and mortality, from CVD. Furthermore, when an individual is homozygous for the polymorphism, it is suitable to assess some of the recognised associations, possible risks, highlighting that the effects have been small, the absolute risks are probable low, and it may even be established in the future that there is no increase above population risk (Hickey, Curry & Toriello, 2013).

3.7. NEW PERSPECTIVES: HYPERTENSION AND RIBOFLAVIN

Hypertension, considered one of the major risk factors of CVD, is a condition where systolic and diastolic blood pressure reach 140/90 mmHg or higher. Reducing blood pressure values to normal addresses great improvements to the incidence and outcome of CV events. Apart from the traditional risk factors, understanding the role of genetic factors that might prompt hypertension has been a major challenge for researchers (McNulty, Strain & Ward, 2014).

In recent years, many studies have found an association between the *MTHFR* 677C→T polymorphism and hypertension, suggesting that CVD patients with TT genotype have significantly higher blood pressure. In 2014, a meta-analysis found a significant association between the *MTHFR* 677C→T polymorphism and hypertension, supporting the evidence that individuals with the TT genotype are susceptible to hypertension, compared with those with other genotypes [Table 8] (Yang *et al.*, 2014).

Additionally, was observed that 63% of patients with the TT genotype, being treated with one or more antihypertensive agents, failed to achieve the goal blood pressure (<140/90mmHg), indicating that the therapeutic effect of some specific antihypertensive drugs is unlikely to be accomplished in patients with the TT genotype [Table 8] (Horigan *et al.*, 2010).

The mechanism through which the *MTHFR* 677C→T polymorphism affects blood pressure is not yet completely defined, although reduced levels of riboflavin have been already suggested as possible cause. Riboflavin is the precursor of the coenzyme FAD, a cofactor for MTHFR enzyme. The variant enzyme becomes less active due to its tendency to separate from FAD (McNulty, Strain & Ward, 2014).

Low levels of riboflavin in association with the TT genotype would conduct to a decrease in enzymatic function in cells including those of the arterial system, resulting in impaired vascular function leading to hypertension. Vascular concentrations of 5-methylTHF (the active form of folate), an important regulator of NO, were found to be reduced in patients with the TT genotype (Wilson *et al.*, 2012).

Blood pressure was found to be highly responsive to riboflavin, especially in patients with the *MTHFR* 677C→T polymorphism. A randomised trial investigated the sensitivity of blood pressure to riboflavin supplementation in hypertensive individuals with the TT genotype, without overt CVD. The results showed that riboflavin supplementation could decrease blood pressure more efficiently than current antihypertensive drugs, predominantly in individuals with the TT genotype. Consequently, the achievement of goal blood pressure (≤140/90 mm Hg) in this genetically susceptible group may be significantly improved with the supplementation of riboflavin in addition to antihypertensive medication (Wilson *et al.*, 2013).

The mechanism underlying riboflavin effect is based on its corrective function on MTHFR enzyme in cells. Some researchers believe that riboflavin may interact with MTHFR to influence blood pressure independent from tHcy, and elevated tHcy may only be a marker of decreased MTHFR activity in cells rather than being the cause associated with hypertension (Horigan *et al.*, 2010).

Table 8. Results from studies relating hypertension, riboflavin supplementation and *MTHFR* 677C→T polymorphism.

Authors	Study type	Sample	Results
Yang *et al.*, 2014	Meta-analysis of 27 studies	5,418 cases 4,997 controls	• A significant association between the *MTHFR* 677C→T polymorphism and hypertension was found under the allelic (OR, 1.32; 95% CI, 1.20‑1.45), dominant (OR, 1.39; 95% CI, 1.25‑1.55), recessive (OR, 1.38; 95% CI, 1.18‑1.62), homozygote (OR, 1.59; 95% CI, 1.32‑1.92), and heterozygote (OR, 1.32; 95% CI, 1.20‑1.45) genetic models. • A strong association was also revealed in subgroups, including Asian, Caucasian and Chinese.
Horigan *et al.*, 2010	Randomised control trial	181 premature cardiovascular disease patients	• Among patients taking one or more antihypertensive drugs at recruitment (82%), a target blood pressure (<140/90 mmHg) was achieved in only 37% patients with the TT genotype compared with 59% with the CT and 64% with the CC genotype ($P < 0.001$). • Riboflavin intervention reduced mean blood pressure specifically in those with the TT genotype (from 144/87 to 131/80 mmHg; $P<0.05$ systolic; $P<0.05$ diastolic), with no response observed in the other genotype groups.

Although the decrease of tHcy concentration was already demonstrated in large-scale trials with folate supplementation, no blood pressure response was registered, supporting the idea that the direct involvement of Hcy is unlikely. On top of that, repairing 5-methylTHF levels, through riboflavin re-establishment of MTHFR function, may improve NO activity, lowering blood pressure. The decline of methylation status observed in patients with the TT genotype may also respond to riboflavin and could have an impact in blood pressure control. Thus, CVD risk associated with TT genotype could be explained with the relation between *MTHFR* 677C→T polymorphism and hypertension, leading to the invalidation of the application of Mendelian randomisation to the *MTHFR* 677C→T polymorphism, Hcy and CVD risk triangle (Wilson *et al.*, 2012).

Current findings reinforce the benefits of personalised medicine, a topic vastly debated among the scientific community and clinical practitioners. Professionals sharing this view believe that the adjustment of medical interventions to specific individuals can markedly increase health. The 10% of the general population, and the higher proportions of some ethnic groups, who carry the TT genotype may profit from targeted treatment with riboflavin (Wilcken *et al.*, 2003).

As a final remark, studies seem to be more concordant about the impact of TT genotype on hypertension. Moreover, this genotype group seems to be responsive to riboflavin, especially in cases where conventional antihypertensive therapy is ineffective. Consequently, riboflavin could offer an affordable approach associated with antihypertensive drugs in hypertensive TT genotype patients. However, further studies need to be conducted to assure this association and the riboflavin benefit in hypertension treatment (Horigan *et al.*, 2010).

CHAPTER IV – *MTHFR* 677C→T POLYMORPHISM, FOLATE AND CANCER

4.1. GENE-NUTRITION INTERACTION AND EPIGENETICS ON CANCER

Carcinogenesis is a molecular multistep process initiated by modifications in normal cellular mechanisms, such as DNA repair, cell cycle, apoptosis, differentiation, proliferation, hormonal regulation, inflammation and immunity [Fig. 16]. Failures in any of these mechanisms can conduct to genome rearrangements and instability, apoptosis inhibition, angiogenesis and biological active chemical induction. Changes in the expression of multiple genes happen not only due to genetic alterations, but also as a result of epigenetic changes, leading to activation of protooncogenes and prometastatic genes, or inactivation of tumour suppressor genes and anti-metastasis genes (Stefanska, 2012).

Figure 16. Shared acquired capabilities that enable malignant growth in human cancers *(Hanahan & Weinberg, 2011).*

Epigenetics can be described as an inherited and/or self-propagated phenomenon affecting gene expression without altering the primary genomic sequence. It comprises mainly, DNA methylation, histone acetylation and methylation and non-coding RNA-related mechanisms, caused by metabolic, nutritional and other environmental factors (Kim, Friso, & Choi, 2009).

DNA methylation is probably the best-understood epigenetic mechanism. The reaction is reversible and catalysed by a DNA methyltransferases, which transfer SAM methyl groups to the fifth position of a cytosine pyrimidine ring (Yoo & Jones, 2006). Global DNA hypomethylation together with hypermethylation of certain promoters, especially in the cytosine rich areas (known as CpG islands), have been discovered to take place both independently and in combination with each other. When acting simultaneously, global hypomethylation (translated in an increase of gene expression) has been suggested to occur as an initial stage in tumourigenesis, whereas promoter hypermethylation (translated in a reduction of gene expression) is a subsequent phenomenon, associated with the silencing of tumour suppressor and DNA repair genes (Jung, 2014).

The interaction between epigenetics and nutrition, specially "low-methyl" diet, in affecting cancer in humans is an emerging area of research and DNA methylation has been confirmed to be affected by nutrition in neoplastic alterations (Lee, 2009). Folate and one-carbon metabolism are linked to cancer risk through their fundamental role in DNA synthesis and methylation. An interesting model of gene-nutrient interaction in phenotypic expression is patent in *MTHFR* 677C→T polymorphism and has been associated with risk of several cancers in epidemiological studies. Dietary folate may differentially alter the effect of *MTHFR* 677C→T polymorphism, increasing or decreasing cancer risk (Hubner & Houlston, 2008).

Two mechanisms have been suggested by which folate deficiency could have an impact on carcinogenesis [Fig. 17]. On one hand, folate deficiency involves a decrease in available 5-methylTHF, which originates a reduction in the conversion of Hcy to methionine, reducing the availability of SAM and consequently, leading to global hypomethylation, gene-specific hypomethylation and possibly activation of proto-oncogenes (Stefanska, 2012). On the other hand, insufficient folate availability during mitosis can lead to a lower production of thymidine, inducing uracil misincorporation into DNA. Failures in the attempt to repair DNA can increase the frequency DNA strand breakage and chromosome damage. Even though evidences in support of these mechanisms are limited, it has been shown that low folate originates micronuclei (suggestive of chromosome breakage) and that the TT genotype leads to increased micronuclei formation under low folate conditions (Crider *et al.*, 2012).

The carcinogenesis of breast cancer in premenopausal women, gastric cancer, bladder cancer, esophageal cancer may be more associated to genome hypomethylation, while the carcinogenesis of prostate cancer and colorectal cancer may be more correlated to DNA synthesis (Jin, Qu & Shen, 2009).

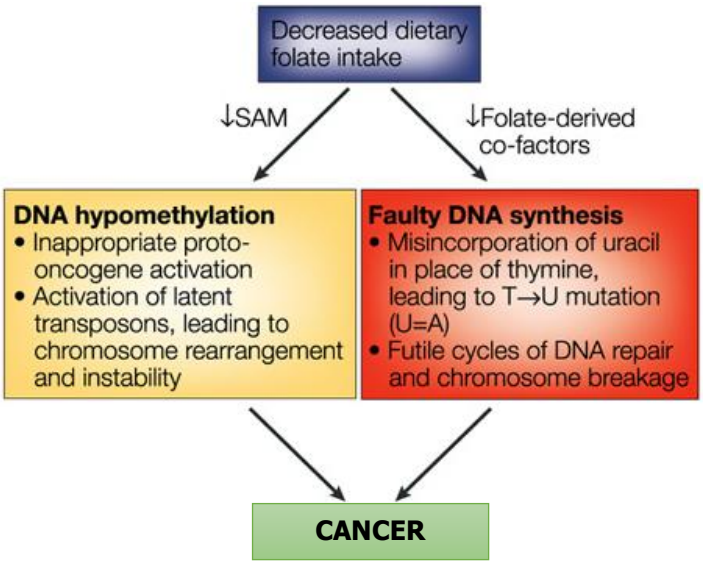


Figure 17. Decreased dietary folate intake markedly perturbs both DNA methylation and biosynthesis (*adapted from: Lamprecht & Lipkin, 2003).*

Several observational studies have suggested a causal role of *MTHFR* 677C→T polymorphism, low folate intake and impaired folate metabolism in cancer progression. Numerous evidences indicate that this gene-diet interaction might be involved in the development of many types of cancer [Table 9] (Bai *et al.,* 2009; Larsson *et al.,* 2006).

So far, results from recent meta-analyses are not unanimous showing different degrees within the association between *MTHFR* 677C→T polymorphism and cancer risk. On one hand, results demonstrate a statistically significant association. On the other hand, some suggest that the polymorphism has low-penetrance susceptibility or may even be protective against cancer risk in TT genotype individuals. This situation can be related to the role of folate in modulating carcinogenesis, and reinforce the need to incorporate data on folate intake when interpreting results of *MTHFR* 677C→T polymorphism in relation to cancer risk (Bai *et al.*, 2009; Zhang *et al.*, 2010).

Table 9. Results from studies relating cancer and *MTHFR* 677C→T polymorphism.

Cancer / Authors	Results / Conclusions
Prostate cancer **Bai *et al.*, 2009**	<u>Meta-analysis of 3511 cases and 2762 controls</u> • T allele was more likely to exert a protective effect on prostate cancer risk (OR = 0.81, 95% CI: 0.68–0.98) with a recessive genetic model. • No association was found for the CT genotype and the TT homozygote with prostate cancer risk compared with CC, with OR = 1.13 (95% CI: 0.88–1.45) and OR = 0.85 (95% CI: 0.71–1.03), respectively. ➔ ***MTHFR* 677C→T polymorphism is a low-penetrance susceptibility gene for prostate cancer, and might provide protective effects against prostate cancer risk.**
Hepatocellular carcinoma (HCC) **Fabris *et al.*, 2009**	<u>Cohort of patients transplanted for end stage liver disease</u> • Among the 65 patients with HCC, 22 had the CC genotype, 30 the CT, and 13 the TT genotype. • Only in patients with alcoholic liver disease was a significant association detected between the TT genotype and the presence of liver cancer. • At stepwise logistic regression analysis, the independent selected predictors of HCC were found: age at transplantation >55 years ($p < 0.001$) and the association among male gender, alcoholic liver disease, and TT genotype ($p = 0.002$). ➔ **Male TT carriers with alcoholic cirrhosis bear an increased risk of developing HCC.**
Esophageal, Gastric, and Pancreatic Cancer **Larsson *et al.*, 2006**	<u>Systematic review with meta-analysis of epidemiologic studies</u> • The summary relative risks for the highest versus the lowest category of dietary folate intake were 0.66 (95% CI, 0.53–0.83) for esophageal squamous cell carcinoma (4 case-control), 0.50 (95% CI, 0.39–0.65) for esophageal adenocarcinoma (3 case-control), and 0.49 (95% CI, 0.35–0.67) for pancreatic cancer (1 case-control, 4 cohort). • In most studies, the **TT genotype was associated with an increased risk** of esophageal squamous cell carcinoma, gastric cardia adenocarcinoma, noncardia gastric cancer, gastric cancer, and pancreatic cancer; all but one of 22 OR were >1, of which 13 estimates were statistically significant. ➔ **Folate may play a role in carcinogenesis of the esophagus, stomach, and pancreas**.
Breast cancer **Zhang *et al.*, 2010**	<u>Meta-analysis involving 15,260 cases and 20,411 controls</u> • Significantly elevated breast cancer risk was associated with TT genotype in homozygote comparison and dominant genetic model (TT vs. CC: OR = 1.11, 95% CI = 1.01–1.23; dominant model: OR = 1.04, 95% CI = 1.00–1.09). • In the subgroup analysis by ethnicity, significantly increased risks were found for TT allele carriers among Asians (TT vs. CC: OR = 1.18, 95% CI = 1.04–1.35; recessive model: OR = 1.15, 95% CI = 1.03–1.29). • When stratified by study design, statistically significantly elevated risk was found in hospital-based studies (TT vs. CC: OR = 1.18, 95% CI = 1.02–1.38; recessive model: OR = 1.17, 95% CI = 1.05–1.29). • In the subgroup analysis by menopausal status, statistically significantly increased risk was found among postmenopausal women (CT vs. CC: OR = 1.12, 95% CI = 1.02–1.23; dominant model: OR = 1.11, 95% CI = 1.01–1.22). ➔ **MTHFR T allele is a low-penetrant risk factor for developing breast cancer.**
Childhood acute lymphocytic leukaemia (ALL) **Franco *et al.*, 2001**	<u>Case-control</u> • *MTHFR* 677C→T polymorphism was detected in 34 patients (48%) and in 49 control subjects (69%), yielding an overall OR for ALL of 0,4 (95% CI 0,2–0,8). OR for heterozygotes was 0,5 (95% CI 0,2–0,9) and for homozygotes the OR was 0,3 (95% CI 0,09–0,8). ➔ **The polymorphism was linked to a significant 2,4-fold decreased risk of developing childhood ALL.**

An important characteristic of these studies that assure reliable results is stratification. Some meta-analyses that have stratified studies by design showed significantly elevated risk in hospital-based studies (Zhang *et al.*, 2010). Hospital patients are likely to have different features than the population, for instance, they may have higher levels of alcohol ingestion, tend to be smokers, or be nutritional imbalanced, features that are related to cancer risk, which may result in biased results (Morabia, Stellman & Wynder, 1996).

Although randomised controlled trials are more applicable to disclose association between folate and cancer risk, observational studies turn out to be used more frequently, due to ethical issues. Thus, observational nutritional studies are more difficulty to get reliable results from, because the evaluation of the actual amounts of folate consumed by individuals is very inaccurate, and is almost impossible to control other nutrients in the diet that may have an impact on the association between folate and cancer risk (Byers, 1999).

When stratification was made regarding ethnicity, the great majority of studies have revealed significantly increased cancer risk for TT allele carriers among Asian, while in Caucasians this association was, in most cases, not very statistically significant (Zintzaras, 2006).

Surprisingly, the great amount of studies regarding cancer risk and *MTHFR* 677C→T polymorphism were conducted in China and there is a pronounced unanimity among results when compared to studies conducted in Western countries. The high frequency of the T allele (Yang *et al.*, 2013), the high incidence of cancer and the fact that 20% of the population is considered to be folate deficient in China, might be the causes for the rise of studies about the relation between *MTHFR* 677C→T polymorphism and cancer risk (Song *et al.*, 2001). Since the major pool of studies has been conducted in China, it is imperative to stratify by ethnicity, while conducting a meta-analysis, to avoid worldwide erroneous extrapolations of results (Szumilas, 2010).

4.2. FOLATE AND COLORECTAL CANCER

Colorectal cancer (CRC) is the third most commonly diagnosed cancer in men and the second in women and is strongly characterised by biochemical and genetic pathways associated with diet and lifestyles. The process by which CRC progresses comprises an accumulation of genetic mutations in tumour suppressor and proto-oncogenes genes, mainly APC, K-Ras and p53, leading to an adenoma that can reach a carcinoma stage [Fig. 18]. Moreover, several epigenetic modifications are associated with the development of CRC, such as genomic global hypomethylation, gene promoter region hypermethylation, histone modifications and alteration of miRNA patterns (Fearon, 2011).

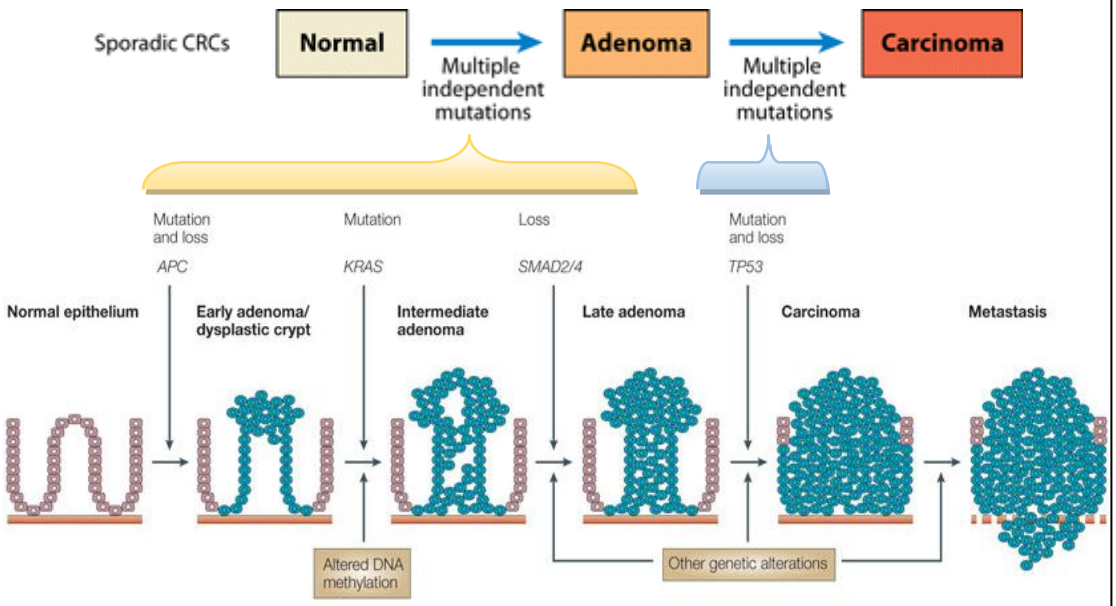


Figure 18. Steps in the development of sporadically occurring cancer in a normal colon epithelium. Not all colorectal tumours exhibit all the mutations presented above *(adapted from: Davies, Miller & Coleman, 2005; Fearon, 2011).*

Chemoprevention with bioactive food components has been supported by several studies as a result of risk reduction in CRC. Due to evidences from epidemiological studies pointing to a modest protection against CRC, folate has been contemplated as a chemopreventive candidate and the relation with CRC risk has been vastly scrutinised (Guerreiro *et al.*, 2008).

Although some short-term trials do not confirm this association, others suggest that a long latency period (15 years or more) of folate supplementation is required for a

significant 75% risk reduction in CRC. This association has been found mostly in colon rather than rectum cancer (Hubner & Houlston, 2008). The mechanism is thought to be related with decrease misincorporation of uracil into DNA, as described in the previous section. In contrast to cardiovascular risk, HHcy has not been linked to CRC (Van Guelpen *et al.*, 2006).

However, a dual effect of folate in CRC has been reported. This duality involves timing, the amount and the type of folate consumed. If the supplementation is implemented before the establishment of the neoplasia, a protective effect is noticed. Conversely, if an increased intake of folate starts once CRC is already present folate can have a detrimental effect. Some evidences suggest that nutritionally adequate baseline folate status has a positive effect in reducing CRC risk, whereas excessive intake or fortification and supplements of synthetic folic acid can fuel the growth of neoplastic cells. In agreement with this idea, mice models genetically predisposed to develop tumours showed increased microscopic lesions when fed with folic acid supplements [Fig. 19] (Castillo-Lancellotti, Marí & Dagach, 2012).

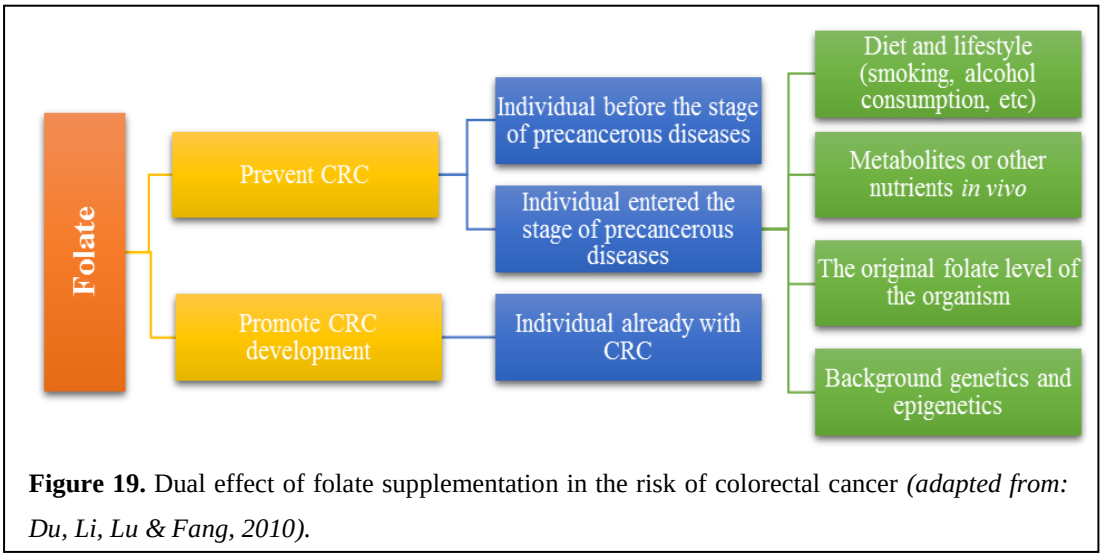


Figure 19. Dual effect of folate supplementation in the risk of colorectal cancer *(adapted from: Du, Li, Lu & Fang, 2010).*

Several mechanisms regarding this duality have been proposed. folic acid differently from folate in diet has to be reduced before it can be metabolised. In cases of increased intake of folic acid the enzyme responsible for the reduction can easily saturate leading to elevated plasma levels of unmetabolised folic acid. Thus, the circulating folic acid can be channelized directly to nucleotides synthesis increasing proliferation of precancerous cells and promoting tumourigenic processes (Williams, 2012).

Another suggestion is that the beneficial effect reported mainly for folate, present in the diet, can be due to the interaction with other food compounds. Furthermore, there is a possibility that folate may interact with other risk factors, such as smoking, alcohol consumption, diet, physical activity, and hormone-related factors in a "folate-independent pathway" influencing the carcinogenesis process (Chuang *et al.*, 2013). Finally, a recent study revealed that moderate dietary folate depletion decreases the expression of genes involved in pro-inflammatory and immune-related pathways, whereas repletion or supplementation have the opposite effect in most cases (Protiva *et al.*, 2011).

4.3. *MTHFR* 677C→T POLYMORPHISM AND COLORECTAL CANCER

The intricacy between folate and CRC risk is enhanced by the presence of functional polymorphisms. Studies point to a selective advantage for TT genotype carriers, suggesting a 12% reduced risk of CRC compared to heterozygous genotype and wild-type. However, under circumstances of high total folate intake, the associated risk of CRC is equally reduced for both CC and TT genotypes. The association has been found mainly in colon, rather than in rectum cancer, particularly at a later stage (Kennedy *et al.*, 2012).

Low intake diets of methyl groups, such as high alcohol consumption (folate antagonist interfering with folate absorption), low in folate, choline and methionine (important dietary sources of methyl groups) have been associated with increased risk of CRC. The risk of CRC through the combination of high alcohol-low folate and the TT genotype is thought to be related to epigenetics [Fig. 20]. In cases of low folate-high alcohol status, the *MTHFR* 677C→T polymorphism may increase the risk of elevated DNA hypomethylation. Conversely, TT genotype, in combination with balanced folate status and low alcohol, could allow a larger pool of 5, 10-methyleneTHF for thymidylate synthesis and therefore decrease uracil misincorporation and consequent chromosome breakage, reducing the risk of CRC (Giovannucci, 2004).

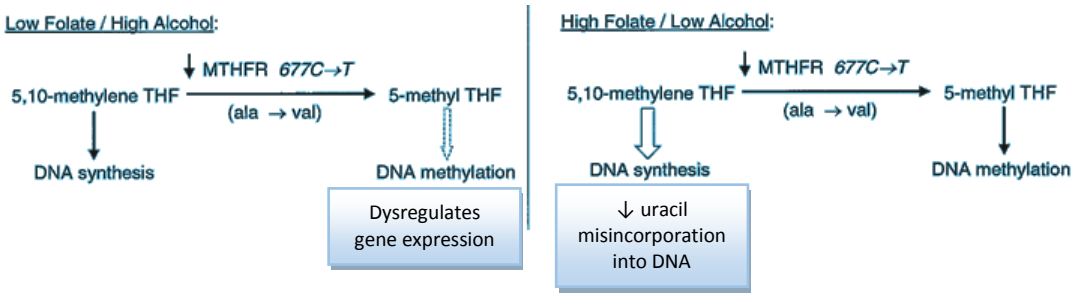


Figure 20. Interactions between high alcohol-low folate, TT genotype and risk of colorectal cancer *(adapted from: Giovannucci, 2004).*

Nonetheless, recent publications still reveal conflicting results [Table 10]. One meta-analysis conducted in 2012 showed that *MTHFR* 677C→T polymorphism contributes to the decreased CRC risk in overall population (Kennedy *et al.*, 2012). Another meta-analysis, performed in 2013, found that *MTHFR* 677C→T polymorphism is a potential risk factor of CRC in overall populations, except among African descent population. However, only four studies in African populations were included in this meta-analysis (Teng *et al.*, 2013).

Comparison of high versus low folate intake within the genotype can help shed light on inconsistent results about this subject. In fact, a study conducted in the Portuguese population indicates that conditions of low folate intake, can lead to an increased risk of CRC in the TT genotype. Authors suggested that the lack of folic acid fortification and the low use of vitamin supplements, which is characteristic in many countries, might lead to a relatively low folate intake (Guerreiro *et al.*, 2008). This fact could explain the association between TT genotype and increased risk of CRC.

Although meta-analyses have been employed to assess the link between *MTHFR* 677C→T polymorphism and CRC risk, the quality of single studies incorporated may differ considerably. For example, the studies that are conducted in different countries can represent different sources of folate and different food choice combinations, increasing the heterogeneity of the results. Then, publication bias may occur because only published studies are included in the majority of meta-analysis. In addition, the selection of individuals in control group in some studies is based only on the lack of symptoms, not excluding latent cancer cases. Apart from that, in some studies, interactions between *MTHFR* 677C→T polymorphism and some environmental risk

factors are not entirely explored, along with the risk of gene-gene or linkage disequilibrium between other polymorphisms (Teng *et al.*, 2013).

Table 10. Results from studies relating colorectal cancer risk, folate and *MTHFR* 677C→T polymorphism.

Authors	Study design	Results
Guerreiro *et al.*, 2008	Case-control 196 patients with CRC and 200 healthy controls matched for age and sex were evaluated for intake of methyl-donor nutrients and the polymorphism.	• Except for folate intake, which was significantly lower in patients (P = 0.02), no differences were observed in the dietary intake of other methyl-donor nutrients between groups. • High intake of folate (>406.7 µg/d) was associated with a significantly lower risk of CRC (odds ratio: 0.67; 95% CI: 0.45, 0.99). • In contrast, TT genotype presented a 3,0-fold increased risk of CRC (95% CI: 1.3, 6.7). • Low intake of all methyl-donor nutrients was associated with an increased risk of CRC in TT genotype, but a statistically significant interaction was only observed for folate (odds ratio: 14.0; 95% CI: 1.8, 108.5).
Kennedy *et al.*, 2012	Systematic Review and Meta-Analysis 10 observational studies were included which assessed the risk of CRC for the polymorphism and/or had defined levels of folate intake for the polymorphism and assessed the risk of CRC.	• The summary risk estimate comparing the CT versus CC genotype was 1.02 (95% CI 0.95–1.10) and for TT versus CC was 0.88 (95% CI 0.80–0.96) both with heterogeneity. • The summary risk estimate for high versus low total folate for the CC genotype was 0.70 (95% CI 0.56–0.89) and the TT genotype 0.63 (95% CI 0.41–0.97). • TT genotype is associated with a reduced risk of developing CRC, under conditions of high total folate intake, and this associated risk remains reduced for both CC and TT genotypes.
Teng *et al.*, 2013	Meta-Analysis 71 publications including 31,572 cases and 44,066 controls	• *MTHFR* 677C→T polymorphism is significantly associated with increased risk of colorectal cancer. • In the stratified analysis by ethnicity, significantly increased risks were also found among: → Caucasians for CC vs TT (OR = 1.076; 95%CI = 1.008–1.150; I^2 = 52.3%), CT vs TT (OR = 1.102; 95%CI = 1.032–1.177; I^2 = 51.4%) and dominant model (OR = 1.086; 95%CI = 1.021–1.156; I^2 = 53.6%). → Asians for *CC vs TT* (OR = 1.226; 95%CI = 1.116–1.346; I^2 = 55.3%), *CT vs TT* (OR = 1.180; 95%CI = 1.079–1.291; I^2 = 36.2%), recessive (OR = 1.069; 95%CI = 1.003-1.140; I^2 = 30.9%) and dominant model (OR = 1.198; 95%CI = 1.101-1.303; I^2 = 52.4%). → Mixed populations for *CT vs TT* (OR = 1.142; 95%CI = 1.005-1.296; I^2 = 0.0%). - No associations were found in Africans for all genetic models.

In 2012, a comparison of approaches for association studies of polymorphisms and CRC risk was conducted. From this analysis, authors assumed that there is variation in calculated relative risk and changes in tests for publication bias that were dependent on the inclusion criteria used for association studies of polymorphisms and CRC. Thus, normalising study inclusion conditions may decrease the variation in findings for meta-analyses of gene-association studies of CRC (Ramsey *et al.*, 2012).

Recently, the role of riboflavin has been also explored. Some studies suggest that high intake of riboflavin may enhance folate protective effect in colorectal adenomas, which seems to be more significant in TT genotype individuals [Fig. 21a]. In an in vitro study, the decrease of nuclear buds (a marker of gene amplification), through the increase of folic acid concentration was greater in the presence of high levels of riboflavin. Furthermore, a randomised, placebo-controlled trial, stratified by the genotype was performed in individuals with normal mucosa and in those with adenomatous polyps. Subjects were randomly given placebo, low folic acid, low folic acid plus riboflavin, or high folic acid. Results showed that riboflavin administration boosted the systemic response to low-dose folic acid in polyp patients, particularly in TT and CT genotypes [Fig. 21b] (Powers, 2005).

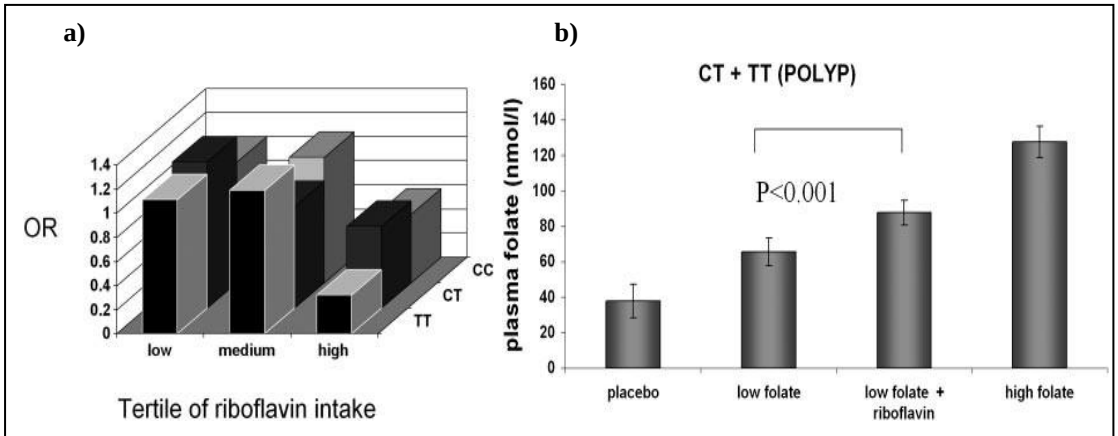


Figure 21. Interaction among folate, riboflavin, genotype, and colorectal cancer. a) Interaction between *MTHFR* 677C→T polymorphism (CC, CT, TT, genotypes) and riboflavin intake in risk of CRC; b) effect of riboflavin administered with low-dose folic acid in determining plasma folate concentration in patients (CT, TT, genotypes) with adenomatous polyps *(Powers, 2005).*

One can hypothesize that, as cofactor of MTHFR, low riboflavin intake along with folate deficiency could increase uracil misincorporation and decrease 5-methylTHF availability for Hcy remethylation, leading to dysregulation of gene expression through epigenetic mechanisms. By contrast, high concentrations of riboflavin could moderately balance enzyme activity in heterozygotes and homozygotes when folate concentrations are low. Thus, not only folate, but also riboflavin could protect against MTHFR thermolability and, therefore, help reduce the risk of CRC.

4.4. *MTHFR* 677C→T POLYMORPHISM AND CHEMOTHERAPY IN COLORECTAL CANCER

In conjunction with chemoprevention, the attention drawn to folate metabolism is due to its possible interference in pharmacodynamics of anticancer agents. One of the most common chemotherapeutic agents used in CRC is 5-fluorouracil (5-FU). Nonetheless, the standard chemotherapy used in adjuvant and advanced CRC comprises several regimens along with the simple 5-FU based treatment, such as 5-FU-folinic modulation, FU combination with oxaliplatin (FOLFOX) or irinotecan (FOLFIRI), and the associations of FU-containing chemotherapies with biological targeted therapies (Etienne-Grimaldi *et al.*, 2010).

To optimize response rate, prognosis and to minimize the side effects associated to chemotherapy treatments, some predictive factors have been investigated. 5-FU, an antimetabolite acting as a TYMS inhibitor, requires concentrations of 5, 10-methyleneTHF. The 5-FU active metabolite fluorodeoxyuridine monophosphate (FdUMP) binds to the nucleotide-binding site of TYMS and forms a stable ternary complex with TYMS and 5, 10-methyleneTHF, blocking access of dUMP to the nucleotide-binding site and inhibiting dTMP synthesis. This results in deoxynucleotide (dNTP) pool imbalances and increased levels of deoxyuridine triphosphate (dUTP), causing DNA damage [Fig. 22] (Longley, Harkin & Johnston, 2003).

Elevated concentrations of 5, 10-methyleneTHF are dependent on MTHFR enzyme activity, opening the possibility for a correlation between the *MTHFR* 677C→T polymorphism and cancer response to FU-based chemotherapy. Thus, increase intracellular folate concentrations in conjunction with *MTHFR* 677C→T polymorphism

would increase the availability of 5, 10-methyleneTHF and therefore would improve sensitivity to fluoropyrimidines (Sharma *et al.*, 2008).

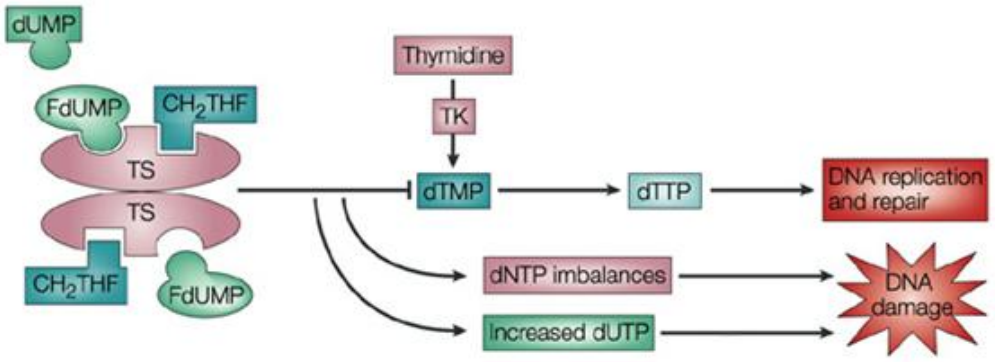


Figure 22. Mechanism of thymidylate synthase inhibition by 5-fluorouracil *(adapted from: Longley, Harkin & Johnston, 2003).*

The research carried out in this field is relatively recent and the results found are inconsistent. Some findings suggest that the type of chemotherapy treatment used can have an impact on the predictive effect of *MTHFR* 677C→T polymorphism, with TT genotype patients having a better response to 5-FU monotherapies, in comparison with combined therapy such as, FOLFOX or FOLFIRI. Furthermore, *MTHFR* 677C→T polymorphism was found to be considerably associated with toxicity, particularly with grade 3 or 4 diarrhoea after FOLFOX administration (Chua *et al.*, 2009).

Based on the current knowledge, it would be very premature to agree with an established association between *MTHFR* 677C→T polymorphism and CRC treatment, centred only in the small and conflicting evidences. Nonetheless, pharmacogenomics can be a useful tool to optimize the selection of the chemotherapy regimen according to the genomic profile of the patient, highlighting the need for prospective and larger cohort studies to reach consistent results about *MTHFR* 677C→T polymorphism and the response to 5-FU in CRC treatment.

4.5. *MTHFR*: AN ALTERNATIVE IN ANTI-TUMOUR THERAPY.

MTHFR has been investigated as a potential new strategy for cancer chemotherapy. Due to the need for methionine by transformed cells, disruption of methionine cycle through *MTHFR* inhibition could be useful for cancer therapy. Some *in vitro* and *in vivo* studies demonstrated that antisense-mediated inhibition of *MTHFR* can lead to cytotoxicity, and, consequently, reduced progression of human cancer cells. Antisense-mediated inhibition is a transfection technic of antisense oligonucleotides (ASOs), such as EX5 or 677T, commonly used to downregulate the expression of genes in tumour cells. ASOs form RNA-DNA duplexes, causing a decrease in the gene expression. *MTHFR* antisense was showed to lead to a decrease in cell survival by 70% (EX5) or 80% (677T) in the colon and breast carcinoma lines and in neuroblastoma (Sekhon *et al.*, 2002).

Another *in vivo* study demonstrated that the combination of *MTHFR* antisense-mediated inhibition with cytotoxic drugs, such as 5-FU and cisplatin has a potentiated effect on inhibiting cell growth. EX5 inhibited the growth of human colon carcinoma xenografts, by 90%, in combination with 5-FU, 40% more when compared with EX5 alone. In combination with cisplatin, EX5 decreased the growth of lung tumours xenografts by 85%, an additional 40% compared with cisplatin alone (Stankova, Shang & Rozen, 2005).

The mechanism underlying growth inhibition by EX5 can be linked to low concentrations of MTHFR enzyme and to apoptosis. *MTHFR* inhibition can cause DNA hypomethylation, contributing to gene expression changes that might affect cell growth. Moreover, *MTHFR* inhibition can lead to elevated tHcy, which promotes apoptosis through oxidative stress enhancement, as demonstrated in some cell cultures (Stankova, Shang & Rozen, 2005). Thus, *MTHFR* downregulation approach seems to be a promising alternative route in cancer treatment.

CONCLUSIONS

In summary, the function of folate in one-carbon metabolism efficiency has been demonstrated, which is extremely depend on the total tetrahydrofolate cofactors pool, and tightly regulated by different feedback loops. Additionally, *MTHFR* 677C→T polymorphism seems to be a significant co-determinant involved in plasma homocysteine levels, DNA synthesis and methylation, particularly when folate levels are low, and should be integrated into the multifaceted role of folate in the prevention of disease. Thus, the interaction between the *MTHFR* gene and folate is a robust example of a gene-nutrient interaction (Ueland *et al.*, 2001).

The explanation behind the geographical and ethnic distribution of the *MTHFR* 677C→T polymorphism remains hypothetical. Additional assessment of gene-nutrition, medical, and nutritional factors involving *MTHFR* 677C→T polymorphism, along with improved understanding of human demographic history is necessary to elucidate its high frequency and extensive distribution. A better comprehension would encourage specific preventive measures that could be conducted in specific ethnicities, such as public-health education, emphasizing the impact of balanced folic and B-vitamin consumption (Guéant-Rodriguez *et al*, 2006).

In addition, the association between *MTHFR* 677C→T polymorphism and cardiovascular disease, through plasma homocysteine has been found in many observational studies (Xuan *et al.*, 2011). Nevertheless, recent evidences seem to point towards the hypothesis that increased plasma homocysteine is indeed an epiphenomenon (Clarke *et al.*, 2012), although the topic remains an open question, without scathing and unanimous results, with the possible exception of stroke (Li & Qin, 2014).

One should contemplate that hyperhomocysteinemia could result from other diverse genetic and biological conditions, and that cardiovascular disease is directly linked to this particular conditions leading to the elevation of plasma homocysteine, confounding the results (Ueland & Loscalzo, 2012). Furthermore, an alternative mechanism of *MTHFR* 677C→T polymorphism, independent from homocysteine cannot be totally

dismissed, jeopardizing the foundation of Mendelian randomisation when reviewing this association (Ueland *et al.*, 2000). Further research is need to understand the mechanisms underlying *MTHFR* 677C→T polymorphism and cardiovascular health.

Even though, hyperhomocysteinemia do not seem to be a casual factor for cardiovascular disease, we could still consider plasma homocysteine elevation as biomarker, predictor of cardiovascular disease, and *MTHFR* 677C→T polymorphism could be equally determined as a measure in public health (Den Heijer, Lewington & Clarke, 2005). However, according to evidences from several meta-analysis concerning the implication of interactions between *MTHFR* 677C→T polymorphism, tHcy levels and cardiovascular disease it would be precipitate to propose such procedure. Moreover, the negative results in secondary-intervention studies have demonstrated no benefit from supplementation with folic acid in patients with established cardiovascular disease (Clarke *et al.*, 2012). An intervention that decreases the biomarker without modifying the disease incidence is worthless; therefore, folic acid supplementation for cardiovascular patients without evident vitamin deficiency cannot be encouraged.

Another fact that cannot be rejected is the discrepancy observed between meta-analyses of published Mendelian randomisation studies that could be attributed to several methodological problems, but publication bias might seems to be the large source of discrepancies. Some studies may have been chosen for publication due to their positive results in favour of others that reveal negative results (Haynes & Clarke, 2012).

Regarding hypertension, although the exact mechanism linking *MTHFR* 677C→T polymorphism to hypertension is yet to be fully understood, it seems that higher blood pressure in individuals with the TT genotype is adjustable by correcting the variant of the MTHFR enzyme by riboflavin status improvement. Consequently, supplementation of riboflavin in this genetic group, could present a personalized approach to the treatment of hypertension (Horigan *et al.*, 2010). Nevertheless, more prospective, double-blind, randomized studies, including riboflavin intervention, hypertension and *MTHFR* 677C→T polymorphism should be conducted.

Another important remark is the role of genetic factors, such as *MTHFR* 677C→T polymorphism in carcinogenesis, along with explicit evidences that environmental

factors, such folate, are crucial in defining cancer risk. Therefore, several studies have shown that the TT genotype is related to biomarkers, such as global DNA methylation particularly in a low folate diet, confirming that the reduce availability of folate can lead to exacerbation of genetic variations in MTHFR enzyme function (Crider *et al.*, 2012). Therefore, different dietary habits among populations could be the source inconsistencies in findings from molecular epidemiologic studies relating cancer risk and *MTHFR* 677C→T polymorphism (Guerreiro *et al.*, 2008).

Taking together, the great majority of studies suggest that high supply of folate and TT genotype carriers may have a lower risk to develop colorectal cancer, particularly colon cancer, since adequate intake reduces the imbalance in the synthesis of nucleotides, assuring the correct DNA replication (Kennedy *et al.*, 2012). However, this may not hold true for all populations, due to the different degrees of folate consumption (Teng *et al.*, 2014). A genetic and environmental risk assessment for colorectal cancer risk in primary care, regarding folate and *MTHFR* 677C→T polymorphism is worth considering (Hall *et al.*, 2012).

Currently, evidences indicate that differences in the type and amount of folates, along with colorectal cancer onset may increase the risk for developing adenomas and colorectal cancer, forcing the re-evaluation of criteria and levels of folic acid supplementation in some populations. Thus, scientific evidences are not solid enough to recommend folate supplementation in populations at risk for colorectal cancer. Further research on gene-environment interactions is compulsory to define the association between increased intake of folate and colorectal cancer (Castillo-Lancellotti, Marí & Dagach, 2012).

Further studies should focus on less well studied populations, such as Africans. Other combinations of polymorphisms in *MTHFR* gene and its prevalence should also be considered in persons with different levels of intake of dietary factors, along with the research of possible explanatory mechanisms (Lee, 2009). Additionally, folate and riboflavin interaction on the *MTHFR* 677C→T polymorphism should be further investigated, due to recent findings that support the beneficial effect of riboflavin in modulating folate levels (Powers, 2005).

Furthermore, *MTHFR* 677C→T polymorphism seems to have a pharmacogenetic role in the response estimation of some chemotherapy protocols, allowing another step in the direction of personalized therapy. Clinicians could use genetic tests to assess which patient carries the variant of *MTHFR* gene, which can be associated, in theory, with better prognosis, and also less side effects to the 5-fluorouracil monotherapy regimen. If proved, these findings could offer an adjustment in the concentrations of 5-fluorouracil chemotherapy regimens in patients without a beneficial *MTHFR* genotype. However, the overall of recent studies do not seem to be consistent regarding this issue (Chua *et al.,* 2009). Thus, more large-scale human studies of combined polymorphisms from *MTHFR* gene may result in a more efficient method than single polymorphism studies.

Finally, the comprehension of *MTHFR* polymorphism mechanisms was essential for the development of new alternative anti-cancer drugs (Sekhon *et al.,* 2002). Recent research on *MTHFR* inhibition technique is showing promising results as a potential anti-cancer therapy, through the increased response of cancer cells to some cytotoxic drugs. The confirmation of this finding, apart from the additive or synergistic effect with the standard anti-cancer drugs, could allow the reduction of their concentrations, minimizing toxicity and drug resistance (Stankova, Shang & Rozen, 2005).

The findings from this review could serve as background information of *MTHFR* 677C→T polymorphism for future epidemiologic studies on cardiovascular disease, colorectal cancer, and on the involvement of *MTHFR* gene in cancer treatment.

REFERENCES

• Amelio, I., Cutruzzolá, F., Antonov, A., Agostini, M., & Melino, G. (2014). Serine and glycine metabolism in cancer. *Trends in biochemical sciences*, *39*(4), 191-198.

• Amorim, F. G., Rezende, L. C. D. D., Coitinho, L. B., Freitas, J. V. D., Scherr, J. A., & Dettogni, R. S. (2011). Bioquímica clínica da aterosclerose provocada por hiperhomocisteinemia. *Revista Eletrônica de Farmácia*, 8(1), 24.

• Bai, J. L., Zheng, M. H., Xia, X., Ter-Minassian, M., Chen, Y. P., & Chen, F. (2009). *MTHFR* C677T polymorphism contributes to prostate cancer risk among Caucasians: A meta-analysis of 3511 cases and 2762 controls. *European Journal of Cancer*, *45*(8), 1443-1449.

• Bailey, L. B. (Ed.). (2010). Folate status: effect on carcinogenesis. In: Bailey LB, ed. *Folate in health and disease*. New York, NY, Marcel Dekker, 1995:361–378.

• Bailey, L. B., & Gregory, J. F. (1999). Folate metabolism and requirements. *The Journal of nutrition*, *129*(4), 779-782.

• Bailey, L. B., & Gregory, J. F. (1999). Polymorphisms of methylenetetrahydrofolate reductase and other enzymes: metabolic significance, risks and impact on folate requirement. *The Journal of nutrition*, *129*(5), 919-922.

• Bezemer, I. D., Doggen, C. J., Vos, H. L., & Rosendaal, F. R. (2007). No association between the common MTHFR 677C→ T polymorphism and venous thrombosis: results from the MEGA study. *Archives of internal medicine*, *167*(5), 497-501.

• Blom, H. J., & Smulders, Y. (2011). Overview of homocysteine and folate metabolism. With special references to cardiovascular disease and neural tube defects. *Journal of inherited metabolic disease*, *34*(1), 75-81.

• Botto, L. D., & Yang, Q. (2000). 5, 10-Methylenetetrahydrofolate reductase gene variants and congenital anomalies: a HuGE review. *American journal of epidemiology*, *151*(9), 862-877.

• Boushey, C. J., Beresford, S. A., Omenn, G. S., & Motulsky, A. G. (1995). A quantitative assessment of plasma homocysteine as a risk factor for vascular disease: probable benefits of increasing folic acid intakes. *Jama*, 274(13), 1049-1057.

• Brattström, L., & Wilcken, D. E. (2000). Homocysteine and cardiovascular disease: cause or effect?. *The American journal of clinical nutrition*, *72*(2), 315-323.

• Brattström, L., Wilcken, D. E., Öhrvik, J., & Brudin, L. (1998). Common methylenetetrahydrofolate reductase gene mutation leads to hyperhomocysteinemia but not to vascular disease the result of a meta-analysis. *Circulation*, *98*(23), 2520-2526.

• Brody, T., & Shane, B. (2001). Folic acid. Handbook of the vitamins, 3rd ed. New York: Marcel Dekker, 427-62.

• Brosnan, J. T., & Brosnan, M. E. (2006). The sulfur-containing amino acids: an overview. *The Journal of nutrition*, *136*(6), 1636S-1640S.

• Brouwer, I. A., van Dusseldorp, M., West, C. E., & Steegers-Theunissen, R. P. (2001). Bioavailability and bioefficacy of folate and folic acid in man. *Nutrition research reviews*, *14*(02), 267-294.

• Brustolin, S., Giugliani, R., & Félix, T. M. (2010). Genetics of homocysteine metabolism and associated disorders. *Brazilian Journal of Medical and Biological Research*, *43*(1), 1-7.

• Byers, T. (1999). What can randomized controlled trials tell us about nutrition and cancer prevention?. *CA: a cancer journal for clinicians*, *49*(6), 353-361.

• Castillo-Lancellotti, C., Marí, J. T., & Dagach, R. U. (2012). Suplementación con ácido fólico y prevención de recurrencia de adenomas colorrectales; revisión sistemática. *Nutrición Hospitalaria*, *27*(n01).

• Castro, R., Rivera, I., Struys, E. A., Jansen, E. E., Ravasco, P., Camilo, M. E., ... & de Almeida, I. T. (2003). Increased homocysteine and S-adenosylhomocysteine concentrations and DNA hypomethylation in vascular disease. *Clinical Chemistry*, *49*(8), 1292-1296.

• Chua, W., Goldstein, D., Lee, C. K., Dhillon, H., Michael, M., Mitchell, P., ... & Iacopetta, B. (2009). Molecular markers of response and toxicity to FOLFOX chemotherapy in metastatic colorectal cancer. *British journal of cancer*, *101*(6), 998-1004.

• Chuang, S. C., Rota, M., Gunter, M. J., Zeleniuch-Jacquotte, A., Eussen, S. J., Vollset, S. E., ... & Vineis, P. (2013). Quantifying the dose-response relationship between circulating folate concentrations and colorectal cancer in cohort studies: a meta-analysis based on a flexible meta-regression model. *American journal of epidemiology*, kwt083.

• Clarke, R., Bennett, D. A., Parish, S., Verhoef, P., Dötsch-Klerk, M., Lathrop, M., ... & MTHFR Studies Collaborative Group. (2012). Homocysteine and coronary heart disease: meta-analysis of MTHFR case-control studies, avoiding publication bias. *PLoS medicine*, *9*(2), e1001177.

• Clarke, R., Halsey, J., Lewington, S., Lonn, E., Armitage, J., Manson, J. E., ... & Collins, R. (2010). Effects of lowering homocysteine levels with B vitamins on cardiovascular disease, cancer, and cause-specific mortality: meta-analysis of 8 randomised trials involving 37 485 individuals. *Archives of Internal Medicine*, *170*(18), 1622-1631.

• Crider, K. S., Yang, T. P., Berry, R. J., & Bailey, L. B. (2012). Folate and DNA methylation: a review of molecular mechanisms and the evidence for folate's role. *Advances in Nutrition: An International Review Journal*, *3*(1), 21-38.

• Cuskelly CJ, McNulty H, Scott JM. Effect of increasing dietary folate on red-cell folate: implications for prevention of neural tube defects. *Lancet*, 1996, 347:657–659.

• Czeizel, A. E., Dudás, I., Vereczkey, A., & Bánhidy, F. (2013). Folate deficiency and folic acid supplementation: the prevention of neural-tube defects and congenital heart defects. *Nutrients*, *5*(11), 4760-4775.

• Davies, R. J., Miller, R., & Coleman, N. (2005). Colorectal cancer screening: prospects for molecular stool analysis. *Nature Reviews Cancer*, *5*(3), 199-209.

• Den Heijer, M., Lewington, S., & Clarke, R. (2005). Homocysteine, MTHFR and risk of venous thrombosis: a meta-analysis of published epidemiological studies. *Journal of Thrombosis and Haemostasis*, *3*(2), 292-299.

• Du, W., Li, W. Y., Lu, R., & Fang, J. Y. (2010). Folate and fiber in the prevention of colorectal cancer: between shadows and the light. *World journal of gastroenterology: WJG*, *16*(8), 921.

• Eftychiou, C., Antoniades, L., Makri, L., Koumas, L., Costeas, P. A., KYRIAKOU, E., ... & Papadogiannis, D. (2012). Homocysteine levels and MTHFR polymorphisms in young patients with acute myocardial infarction: A case control study. *Hellenic J Cardiol*, *53*(3), 189-194.

• Esse, R., Leandro, P., Rivera, I., de Almeida, I. T., Blom, H. J., & Castro, R. (2012). Deciphering protein arginine methylation in mammals.

• Etienne-Grimaldi, M. C., Milano, G., Maindrault-Gœbel, F., Chibaudel, B., Formento, J. L., Francoual, M., ... & De Gramont, A. (2010). Methylenetetrahydrofolate reductase (MTHFR) gene polymorphisms and FOLFOX response in colorectal cancer patients. *British journal of clinical pharmacology*, *69*(1), 58-66.

• Fabris, C., Toniutto, P., Falleti, E., Fontanini, E., Cussigh, A., Bitetto, D., ... & Pirisi, M. (2009). MTHFR C677T polymorphism and risk of HCC in patients with liver cirrhosis: role of male gender and alcohol consumption. *Alcoholism: Clinical and Experimental Research*, *33*(1), 102-107.

• Fearon, E. R. (2011). Molecular genetics of colorectal cancer. *Annual Review of Pathology: Mechanisms of Disease*, *6*, 479-507.

• Forges, T., Monnier-Barbarino, P., Alberto, J. M., Gueant-Rodriguez, R. M., Daval, J. L., & Gueant, J. L. (2007). Impact of folate and homocysteine metabolism on human reproductive health. *Human reproduction update*, *13*(3), 225-238.

• Franco, R. F., Simoes, B. P., Tone, L. G., Gabellini, S. M., Zago, M. A., & Falcão, R. P. (2001). The methylenetetrahydrofolate reductase C677T gene

polymorphism decreases the risk of childhood acute lymphocytic leukaemia. *British journal of haematology*, 115(3), 616-618.

• Gilbody, S., Lewis, S., & Lightfoot, T. (2007). Methylenetetrahydrofolate reductase (MTHFR) genetic polymorphisms and psychiatric disorders: a HuGE review. *American journal of epidemiology*, *165*(1), 1-13.

• Giovannucci, E. (2004). Alcohol, one-carbon metabolism, and colorectal cancer: recent insights from molecular studies. *The Journal of nutrition*, *134*(9), 2475S-2481S.

• Gregory JF. Bioavailability of folate. *European Journal of Clinical Nutrition*, 1997, 51:554–559.

• Guéant-Rodriguez, R. M., Guéant, J. L., Debard, R., Thirion, S., Hong, L. X., Bronowicki, J. P., ... & Mutchinick, O. M. (2006). Prevalence of methylenetetrahydrofolate reductase 677T and 1298C alleles and folate status: a comparative study in Mexican, West African, and European populations. *The American journal of clinical nutrition*, *83*(3), 701-707.

• Guerreiro, C. S., Carmona, B., Gonçalves, S., Carolino, E., Fidalgo, P., Brito, M., ... & Cravo, M. (2008). Risk of colorectal cancer associated with the C677T polymorphism in 5, 10-methylenetetrahydrofolate reductase in Portuguese patients depends on the intake of methyl-donor nutrients. *The American journal of clinical nutrition*, *88*(5), 1413-1418.

• Hall, M. J., Manne, S. L., Myers, R. E., Keenan, E. M., Balshem, A. M., & Weinberg, D. S. (2012). Predictors of patient uptake of colorectal cancer gene environment risk assessment. *Genome medicine*, *4*(11), 92.

• Ham, A. C., Enneman, A. W., van Dijk, S. C., Araghi, S. O., Swart, K. M., Sohl, E., ... & van der Velde, N. (2014). Associations Between Medication Use and Homocysteine Levels in an Older Population, and Potential Mediation by Vitamin B12 and Folate: Data from the B-PROOF Study. *Drugs & aging*, *31*(8), 611-621.

• Hanahan, D., & Weinberg, R. A. (2011). Hallmarks of cancer: the next generation. *Cell*, *144*(5), 646-674.

• Haynes, R., & Clarke, R. (2012). Homocysteine, the kidney, and vascular disease. *BMJ-British Medical Journal*, *344*(7863), 11.

• Hazra, A., Kraft, P., Lazarus, R., Chen, C., Chanock, S. J., Jacques, P., ... & Hunter, D. J. (2009). Genome-wide significant predictors of metabolites in the one-carbon metabolism pathway. *Human molecular genetics*, *18*(23), 4677-4687.

• Heneghan, H. M., & Sultan, S. (2008). Homocysteine, the cholesterol of the 21st century. Impact of hyperhomocysteinemia on patency and amputation-free survival after intervention for critical limb ischemia. *Journal of Endovascular Therapy*, *15*(4), 399-407.

• Hickey, S. E., Curry, C. J., & Toriello, H. V. (2013). ACMG Practice Guideline: lack of evidence for MTHFR polymorphism testing. *Genetics in Medicine*, *15*(2), 153-156.

• Hoffbrand, A. V., & Weir, D. G. (2001). The history of folic acid. British journal of haematology, 113(3), 579-589.

• Holmes, M. V., Newcombe, P., Hubacek, J. A., Sofat, R., Ricketts, S. L., Cooper, J., ... & Casas, J. P. (2011). Effect modification by population dietary folate on the association between *MTHFR* genotype, homocysteine, and stroke risk: a meta-analysis of genetic studies and randomised trials. *The Lancet*, *378*(9791), 584-594.

• Horigan, G., McNulty, H., Ward, M., Strain, J. J., Purvis, J., & Scott, J. M. (2010). Riboflavin lowers blood pressure in cardiovascular disease patients homozygous for the 677C→ T polymorphism in MTHFR. *Journal of hypertension*, *28*(3), 478-486.

• Hubner, R. A., & Houlston, R. S. (2008). Folate and colorectal cancer prevention. *British journal of cancer*, *100*(2), 233-239.

• Humpath.com - Human pathology. (2003, October 15). Retrieved October 20, 2014, from http://www.humpath.com/spip.php?article1229

• Ikeda, S., Koyama, H., Sugimoto, M., & Kume, S. (2012). Roles of One-carbon Metabolism in Preimplantation Period. *Journal of Reproduction and Development*, *58*(1), 38-43.

• Jacques, P. F., Bostom, A. G., Williams, R. R., Ellison, R. C., Eckfeldt, J. H., Rosenberg, I. H., ... & Rozen, R. (1996). Relation between folate status, a common mutation in methylenetetrahydrofolate reductase, and plasma homocysteine concentrations. *Circulation*, *93*(1), 7-9.

• Jin, F., Qu, L. S., & Shen, X. Z. (2009). Association between the methylenetetrahydrofolate reductase C677T polymorphism and hepatocellular carcinoma risk: a meta-analysis. *Diagn Pathol*, *4*(1), 39-46.

• Joachim, E., Goldenberg, N. A., Bernard, T. J., Armstrong-Wells, J., Stabler, S., & Manco-Johnson, M. J. (2013). The Methylenetetrahydrofolate reductase polymorphism (MTHFR c. 677C> T) and elevated tHcys in a US pediatric population with incident thromboembolism. *Thrombosis research*, *132*(2), 170-174.

• Joint, F. A. O. (1998). WHO Expert Consultation on Human vitamin and mineral requirements. Vitamin and mineral requirements in human nutrition: report of a joint FAO/WHO expert consultation.

• Jung, A. Y. (2014). B vitamins and DNA methylation in colorectal carcinogenesis: across a continuum of differential risk for colorectal cancer.

• Kang, S., Zhao, X., Liu, L., Wu, W., & Zhang, D. (2013). Association of the C677T Polymorphism in the MTHFR Gene with Hemorrhagic Stroke: A Meta-Analysis. Genetic testing and molecular biomarkers, 17(5), 412-417.

• Kelly, B. B. Institute of Medicine; Fuster, Valentin (2010). Promoting Cardiovascular Health in the Developing World: A Critical Challenge to Achieve Global Health.

• Kennedy, D. A., Stern, S. J., Matok, I., Moretti, M. E., Sarkar, M., Adams-Webber, T., & Koren, G. (2012). Folate intake, MTHFR polymorphisms, and the risk of colorectal cancer: a systematic review and meta-analysis. *Journal of cancer epidemiology*, *2012*.

• Kim, K. C., Friso, S., & Choi, S. W. (2009). DNA methylation, an epigenetic mechanism connecting folate to healthy embryonic development and aging. *The Journal of nutritional biochemistry*, 20(12), 917-926.

• Kirke, P. N., Mills, J. L., Molloy, A. M., Brody, L. C., O'Leary, V. B., Daly, L., ... & Scott, J. M. (2004). Impact of the MTHFR C677T polymorphism on risk of neural tube defects: case-control study. *Bmj*, *328*(7455), 1535-1536.

• Klerk, M., Verhoef, P., Clarke, R., Blom, H. J., Kok, F. J., & Schouten, E. G. (2002). MTHFR 677C→ T polymorphism and risk of coronary heart disease: a meta-analysis. *Jama*, *288*(16), 2023-2031.

• Lamprecht, S. A., & Lipkin, M. (2003). Chemoprevention of colon cancer by calcium, vitamin D and folate: molecular mechanisms. *Nature Reviews Cancer*, *3*(8), 601-614.

• Larsson, S. C., Giovannucci, E., & Wolk, A. (2006). Folate Intake,< i> MTHFR</i> Polymorphisms, and Risk of Esophageal, Gastric, and Pancreatic Cancer: A Meta-analysis. *Gastroenterology*, *131*(4), 1271-1283.

• Leclerc, D., Sibani, S., & Rozen, R. (2005). Molecular biology of methylenetetrahydrofolate reductase (MTHFR) and overview of mutations/polymorphisms. *MTHFR Polymorphisms and Disease. Georgetown, TX: Landes Bioscience/Eurekah. com*, 1-20.

• Lee, S. A. (2009). Gene-diet interaction on cancer risk in epidemiological studies. *Journal of Preventive Medicine and Public Health*, *42*(6), 360-370.

• Li, P., & Qin, C. (2014). Methylenetetrahydrofolate reductase *MTHFR* gene polymorphisms and susceptibility to ischemic stroke: A meta-analysis. Gene, 535(2), 359-364.

• Li, Z., Sun, L., Zhang, H., Liao, Y., Wang, D., Zhao, B., ... & Hui, R. (2003). Elevated Plasma Homocysteine Was Associated With Hemorrhagic and Ischemic Stroke, but Methylenetetrahydrofolate Reductase Gene C677T Polymorphism Was a Risk Factor for Thrombotic Stroke A Multicenter Case-Control Study in China. Stroke, 34(9), 2085-2090.

• Lobo, I. (2008). Multifactorial inheritance and genetic disease. *Nat Educ*, *1*(1).

• Locasale, J. W. (2013). Serine, glycine and one-carbon units: cancer metabolism in full circle. *Nature Reviews Cancer*, *13*(8), 572-583.

• Longley, D. B., Harkin, D. P., & Johnston, P. G. (2003). 5-fluorouracil: mechanisms of action and clinical strategies. *Nature Reviews Cancer*, *3*(5), 330-338.

• Lu, S. C., & Mato, J. M. (2012). S-adenosylmethionine in liver health, injury, and cancer. *Physiological reviews*, *92*(4), 1515-1542.

• Malinow, M. R., Bostom, A. G., & Krauss, R. M. (1999). Homocyst (e) ine, diet, and cardiovascular diseases a statement for healthcare professionals from the Nutrition Committee, American Heart Association. *Circulation*, *99*(1), 178-182.

• Mato, J. M., & Lu, S. C. (2007). Role of S-adenosyl-L-methionine in liver health and injury. *Hepatology*, *45*(5), 1306-1312.

• McBean, G. J. (2012). The transsulfuration pathway: a source of cysteine for glutathione in astrocytes. *Amino acids*, *42*(1), 199-205.

• McNulty, H., Strain, J. J., & Ward, M. (2014). Riboflavin lowers blood pressure in hypertensive people with the MTHFR 677TT genotype. *Archives of Public Health*, *72*(Suppl 1), K2.

• Medina, M. A., Urdiales, J. L., & Amores-Sánchez, M. I. (2001). Roles of homocysteine in cell metabolism. *European Journal of Biochemistry*, *268*(14), 3871-3882.

• Medina, M. Á., Urdiales, J. L., & Amores-Sánchez, M. I. (2001). Roles of homocysteine in cell metabolism. *European Journal of Biochemistry*, *268*(14), 3871-3882.

• Morabia, A., Stellman, S. D., & Wynder, E. L. (1996). Smoking prevalence in neighborhood and hospital controls: implications for hospital-based case-control studies. *Journal of clinical epidemiology*, *49*(8), 885-889.

• Nichols, M., Townsend, N., Luengo-Fernandez, R., Leal, J., Gray, A., Scarborough, P., & Rayner, M. (2012). European cardiovascular disease statistics 2012. *European Heart Network, Brussels, European Society of Cardiology, Sophia Antipolis*, P104.

• Obeid, R. (2013). The metabolic burden of methyl donor deficiency with focus on the betaine homocysteine methyltransferase pathway. *Nutrients*, *5*(9), 3481-3495.

• Perła-Kaján, J., Twardowski, T., & Jakubowski, H. (2007). Mechanisms of homocysteine toxicity in humans. *Amino acids*, *32*(4), 561-572.

• Perna, A. F., Ingrosso, D., Lombardi, C., Acanfora, F., Satta, E., Cesare, C. M., ... & De Santo, N. G. (2003). Possible mechanisms of homocysteine toxicity. *Kidney International*, *63*, S137-S140.

• Podda, G., Faioni, E. M., Zighetti, M. L., & Cattaneo, M. (2003). No effect of fasting plasma total homocysteine on protein C activity in vitro. *Blood*, *101*(6), 2446-2446.

• Powers, H. J. (2005). Interaction among folate, riboflavin, genotype, and cancer, with reference to colorectal and cervical cancer. *The Journal of nutrition*, *135*(12), 2960S-2966S.

• Protiva, P., Mason, J. B., Liu, Z., Hopkins, M. E., Nelson, C., Marshall, J. R., ... & Holt, P. R. (2011). Altered folate availability modifies the molecular environment of the human colorectum: implications for colorectal carcinogenesis. *Cancer Prevention Research*, *4*(4), 530-543.

• Ramsey, S. D., Holmes, R. S., McDermott, C. L., Blough, D. K., Petrin, K. L., Poole, E. M., & Ulrich, C. M. (2012). A comparison of approaches for association studies of polymorphisms and colorectal cancer risk. *Colorectal Disease*, *14*(9), e573-e586.

• Rozen, R. (1997). Genetic predisposition to hyperhomocysteinemia: deficiency of methylenetetrahydrofolate reductase (MTHFR). *Thrombosis and haemostasis*, *78*(1), 523-526.

• Sabetisoofyani, A., Larson, D. F., & Watson, R. R. (2010). Homocysteine: Role in Cardiovascular Disease. In *Modern Dietary Fat Intakes in Disease Promotion* (pp. 405-415). Humana Press.

• Sazci, A., Ergul, E., Tuncer, N., Akpinar, G., & Kara, I. (2006). Methylenetetrahydrofolate reductase gene polymorphisms are associated with ischemic and hemorrhagic stroke: Dual effect of MTHFR polymorphisms C677T and A1298C. *Brain research bulletin*, *71*(1), 45-50.

• Schneider, J. A., Rees, D. C., Liu, Y. T., & Clegg, J. B. (1998). Worldwide distribution of a common methylenetetrahydrofolate reductase mutation. *American journal of human genetics*, *62*(5), 1258.

• Schwammenthal, Y., & Tanne, D. (2004). Homocysteine, B-vitamin supplementation, and stroke prevention: from observational to interventional trials. *The Lancet Neurology*, *3*(8), 493-495.

• Sekhon, J., Pereira, P., Sabbaghian, N., Schievella, A. R., & Rozen, R. (2002). Antisense inhibition of methylenetetrahydrofolate reductase reduces survival of methionine-dependent tumour lines. *British journal of cancer*, *87*(2), 225-230.

• Sharma, R., Hoskins, J. M., Rivory, L. P., Zucknick, M., London, R., Liddle, C., & Clarke, S. J. (2008). Thymidylate synthase and methylenetetrahydrofolate reductase

gene polymorphisms and toxicity to capecitabine in advanced colorectal cancer patients. *Clinical Cancer Research*, *14*(3), 817-825.

• Smulders, Y. M., & Blom, H. J. (2011). The homocysteine controversy. *Journal of inherited metabolic disease*, *34*(1), 93-99.

• Somarajan, B. I., Kalita, J., Mittal, B., & Misra, U. K. (2011). Evaluation of MTHFR C677T polymorphism in ischemic and hemorrhagic stroke patients. A case–control study in a Northern Indian population. Journal of the neurological sciences, 304(1), 67-70.

• Song, C., Xing, D., Tan, W., Wei, Q., & Lin, D. (2001). Methylenetetrahydrofolate reductase polymorphisms increase risk of esophageal squamous cell carcinoma in a Chinese population. *Cancer research*, *61*(8), 3272-3275.

• Stankova, J., Shang, J., & Rozen, R. (2005). Antisense inhibition of methylenetetrahydrofolate reductase reduces cancer cell survival in vitro and tumour growth in vivo. *Clinical cancer research*, *11*(5), 2047-2052.

• Stefanska, B., Karlic, H., Varga, F., Fabianowska-Majewska, K., & Haslberger, A. G. (2012). Epigenetic mechanisms in anti-cancer actions of bioactive food components–the implications in cancer prevention. *British journal of pharmacology*, *167*(2), 279-297.

• Stipanuk, M. H., & Ueki, I. (2011). Dealing with methionine/homocysteine sulfur: cysteine metabolism to taurine and inorganic sulfur. *Journal of inherited metabolic disease*, *34*(1), 17-32.

• Stühlinger, M. C., Tsao, P. S., Her, J. H., Kimoto, M., Balint, R. F., & Cooke, J. P. (2001). Homocysteine impairs the nitric oxide synthase pathway role of asymmetric dimethylarginine. *Circulation*, *104*(21), 2569-2575.

• Szumilas, M. (2010). Explaining odds ratios. *Journal of the Canadian Academy of Child and Adolescent Psychiatry*, *19*(3), 227.

• Teng, Z., Wang, L., Cai, S., Yu, P., Wang, J., Gong, J., & Liu, Y. (2013). The 677C> T (rs1801133) polymorphism in the MTHFR gene contributes to colorectal cancer risk: a meta-analysis based on 71 research studies. *PloS one*, *8*(2), e55332.

• Tibbetts, A. S., & Appling, D. R. (2010). Compartmentalization of mammalian folate-mediated one-carbon metabolism. *Annual review of nutrition*, *30*, 57-81.

• Trabetti, E. (2008). Homocysteine, MTHFR gene polymorphisms, and cardio-cerebrovascular risk. *Journal of applied genetics*, *49*(3), 267-282.

• Ueland, P. M., Hustad, S., Schneede, J., Refsum, H., & Vollset, S. E. (2001). Biological and clinical implications of the MTHFR C677T polymorphism. *Trends in pharmacological sciences*, *22*(4), 195-201.

• Ueland, P. M., & Loscalzo, J. (2012). Homocysteine and cardiovascular risk: The perils of reductionism in a complex system. *Clinical chemistry*, *58*(12), 1623-1625.

• Ueland, P. M., Refsum, H., Beresford, S. A., & Vollset, S. E. (2000). The controversy over homocysteine and cardiovascular risk. *The American journal of clinical nutrition*, *72*(2), 324-332.

• Ulvik, A., Ueland, P. M., Fredriksen, Å., Meyer, K., Vollset, S. E., Hoff, G., & Schneede, J. (2007). Functional inference of the methylenetetrahydrofolate reductase 677 C> T and 1298A> C polymorphisms from a large-scale epidemiological study. *Human genetics*, *121*(1), 57-64.

• Van Guelpen, B., Hultdin, J., Johansson, I., Hallmans, G., Stenling, R., Riboli, E., ... & Palmqvist, R. (2006). Low folate levels may protect against colorectal cancer. *Gut*, *55*(10), 1461-1466.

• Wald, D. S., Law, M., & Morris, J. K. (2002). Homocysteine and cardiovascular disease: evidence on causality from a meta-analysis. *Bmj*, *325*(7374), 1202.

• Wald, D. S., Morris, J. K., & Wald, N. J. (2011). Reconciling the evidence on serum homocysteine and ischaemic heart disease: a meta-analysis. *PloS one*, *6*(2), e16473.

• Wang, G., Siow, Y. L., & Karmin, O. (2001). Homocysteine induces monocyte chemoattractant protein-1 expression by activating NF-κB in THP-1 macrophages. *American Journal of Physiology-Heart and Circulatory Physiology*, *280*(6), H2840-H2847.

• Wilcken, B., Bamforth, F., Li, Z., Zhu, H., Ritvanen, A., Redlund, M., ... & Botto, L. D. (2003). Geographical and ethnic variation of the 677C> T allele of 5, 10 methylenetetrahydrofolate reductase (MTHFR): findings from over 7000 newborns from 16 areas world wide. *Journal of medical genetics*, *40*(8), 619-625.

• Williams, E. A. (2012). Folate, colorectal cancer and the involvement of DNA methylation. *Proceedings of the Nutrition Society*, *71*(04), 592-597.

• Wilson, C. P., McNulty, H., Ward, M., Strain, J. J., Trouton, T. G., Hoeft, B. A., ... & Scott, J. M. (2013). Blood Pressure in Treated Hypertensive Individuals With the MTHFR 677TT Genotype Is Responsive to Intervention With Riboflavin Findings of a Targeted Randomised Trial. *Hypertension*, *61*(6), 1302-1308.

• Wilson, C. P., Ward, M., McNulty, H., Strain, J. J., Trouton, T. G., Horigan, G., ... & Scott, J. M. (2012). Riboflavin offers a targeted strategy for managing hypertension in patients with the MTHFR 677TT genotype: a 4-y follow-up. *The American journal of clinical nutrition*, *95*(3), 766-772.

• Winkels, R. M., Brouwer, I. A., Siebelink, E., Katan, M. B., & Verhoef, P. (2007). Bioavailability of food folates is 80% of that of folic acid. *The American journal of clinical nutrition*, *85*(2), 465-473.

• Xuan, C., Bai, X. Y., Gao, G., Yang, Q., & He, G. W. (2011). Association Between Polymorphism of Methylenetetrahydrofolate Reductase (*MTHFR*) C677T and Risk of Myocardial Infarction: A Meta-analysis for 8,140 Cases and 10,522 Controls. *Archives of medical research*, *42*(8), 677-685.

• Yang, B., Liu, Y., Li, Y., Fan, S., Zhi, X., Lu, X., ... & Sun, G. (2013). Geographical distribution of MTHFR C677T, A1298C and MTRR A66G gene polymorphisms in China: findings from 15357 adults of Han nationality. *PloS one*, *8*(3), e57917.

• Yang, K. M., Jia, J., Mao, L. N., Men, C., Tang, K. T., Li, Y. Y., ... & Zhan, Y. Y. (2014). Methylenetetrahydrofolate reductase C677T gene polymorphism and essential hypertension: A meta-analysis of 10,415 subjects. *Biomedical reports*, *2*(5), 699-708.

• Yilmaz, N. (2012). Relationship between paraoxonase and homocysteine: crossroads of oxidative diseases. *Arch Med Sci*, *8*(1), 138-153.

• Yin, G., Yan, L., Zhang, Z., Chen, K., & Jin, X. (2012). C677T methylenetetrahydrofolate reductase gene polymorphism as a risk factor involved in venous thromboembolism: A population-based case-control study. *Molecular medicine reports*, *6*(6), 1271-1275.

• Yoo, C. B., & Jones, P. A. (2006). Epigenetic therapy of cancer: past, present and future. *Nature reviews Drug discovery*, *5*(1), 37-50.

• Zarychanski, R., & Houston, D. S. (2004). Plasma homocysteine concentration is not associated with activated protein C resistance in patients investigated for hypercoagulability. *Thrombosis and haemostasis-stuttgart-*, *91*, 1115-1122.

• Zhang, D., Chen, Y., Xie, X., Liu, J., Wang, Q., Kong, W., & Zhu, Y. (2012). Homocysteine activates vascular smooth muscle cells by DNA demethylation of platelet-derived growth factor in endothelial cells. *Journal of molecular and cellular cardiology*, *53*(4), 487-496.

• Zhang, J., Qiu, L. X., Wang, Z. H., Wu, X. H., Liu, X. J., Wang, B. Y., & Hu, X. C. (2010). MTHFR C677T polymorphism associated with breast cancer susceptibility: a meta-analysis involving 15,260 cases and 20,411 controls. *Breast cancer research and treatment*, *123*(2), 549-555.

• Zhang, W., He, H., Wang, H., Wang, S., Li, X., Liu, Y., ... & Liu, X. (2013). Activation of transsulfuration pathway by salvianolic acid a treatment: a homocysteine-lowering approach with beneficial effects on redox homeostasis in high-fat diet-induced hyperlipidemic rats. *Nutrition & metabolism*, *10*(1), 68.

• Zintzaras, E. (2006). Association of methylenetetrahydrofolate reductase (MTHFR) polymorphisms with genetic susceptibility to gastric cancer: a meta-analysis. *Journal of human genetics*, *51*(7), 618-624.