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GENETIC POLYMORPHISMS OF THE FOLATE METABOLIC PATHWAY IN CHILDHOOD  
ACUTE LYMPHOBLASTIC LEUKEMIA. A MOLECULAR STUDY AND A PROPOSAL FOR AN  
INTERPRETATIVE MODEL.

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**1**

# **INTRODUCTION**

## 1.1 ACUTE LYMPHOBLASTIC LEUKAEMIA

Acute lymphoblastic leukemia (ALL) is defined as a clonal proliferative disorder originating from lymphoid precursors in the hematopoietic bone marrow. As a result of this transformation, a single B or T clone will stop the cell maturation process and will reveal an enhanced proliferation activity and as a consequence there will be a large infiltration of malignant lymphoblasts in the hematopoietic bone marrow and in the peripheral blood.

Although ALL is considered a rare disease, it is the most frequent neoplasia in the pediatric population accounting for 80% of leukemia with a peak incidence in those aged 2-5 years (Barry et al., 2008). About 80% of ALL are malignant proliferations of precursors-B cells while less common (about 20%) are ALL that involve precursors-T cells. The causes of ALL remains still unknown in the majority of cases nevertheless, during the last 20 years survival rates have strongly improved thanks to current treatment regimens. There are several conditions strongly linked to the pathology and able to upgrade the risk of developing ALL: ionizing radiations and some congenital disorders such as Fanconi's anemia, Bloom's Syndrome and Down's Syndrome (Kaatsch et al., 2010; Belson et al., 2007; Mathew et al., 2006; Zwaan et al., 2010). In 1976, thanks to Franco-American-Britannic Classification (FAB) it was possible to classify ALL in 3 main groups: L1, L2, L3, according to the nucleocitoplasmatic characteristics of leukemic elements.

L1 includes small blast cells with scant cytoplasm. L1 is the most common form of ALL in young under 15 years old.

L2 includes greater blast cells, with variable dimensions and irregular nuclei containing evident nucleoli.

L3 includes big dimensions blast cells with strongly basophilic cytoplasm.

In 2008, the World Health Organization (WHO) revised the FAB classification of ALL and classified ALL as follows:

Acute Lymphoid B Leukemia divided into:

Acute Lymphoid B Leukemia Not Otherwise Specified

Acute Lymphoid B Leukemia with recurrent genetic abnormalities

ALL with t(9;22)/BCR-ABL

ALL with abnormalities of MLL gene

ALL with t(12;21)/ETV6-RUNX1

ALL with hyperdiploida

ALL with hypodiploida

ALL with t(1;19)/TCF3-PBX1

ALL with t(5;14)/IL3-IGH

Acute Lymphoid T Leukemia divided into 3 categories according to signs and symptoms:

Signs and Symptoms arising from hematopoiesis suppression;

Signs and Symptoms arising from the presence of leukemic elements in peripheral blood and releasing of inflammation mediators;

Signs and Symptoms arising from tissue infiltration.

Of particular relevance is the t(9;22) that brings to a disruption in the bcr region of BCR gene (breakpoint cluster region) quite similar to Chronic Myeloid Leukemia (CML) in about 50% of cases. In the remaining 50% of cases the breaking point is located more proximal (5') than in CML. The fusion gene bcr-abl leads to the constitution of a protein of 190kD, different from the classical fusion protein of 210kD produced in CML. This 190kD protein allows a differentiation on molecular basis of ALL Ph<sup>+</sup> from classical CML.

In the prognosis of ALL different factors assume a relevant importance: age, gender, blasts morphology, leukocytosis, immuno-phenotype and cytogenetic factor. The complete remissions are around 95% in children but considering adult patients (50-60 years old) the complete remissions diminish to less than 60%. Furthermore, male patients have a lower tendency to the complete remission than female patients (McCredie et al., 1999). Considering the blast morphology, the remission percentage and the length of the disease are lower and statistically significant in L3 patients than L1 and L2 patients. Prognosis could be strongly influenced by particular immunophenotypes. In the therapeutic phase three principal and consecutive phases there exist: the induction therapy, the consolidation therapy and the maintenance therapy.

The remission induction therapy consists of a combination of pharmacological treatments with vincristine, daunorubicin, prednisone and asparaginase that have distinct mechanisms for their antileukemic effects and act synergistically (Kawedia et al., 2012).

The consolidation therapy allows the control of the minimal residual disease (MRD) and the prevention of relapses.

During the maintenance therapy, that is essential for all ALL patients, the treatment is prolonged for 2-3 years using pharmacological treatment such as 6-mercaptopurin and Methotrexate (MTX) even though there is no consensus on the best regimens and their duration (Pui et al., 2012). The marrow transplantation is today the therapeutic strategy which aims to cut out MRD once the complete remission is obtained.

## 1.2 MOLECULAR MECHANISMS OF ACUTE LYMPHOBLASTIC LEUKAEMIA

The molecular analysis of common genetic alterations in lymphoblastic cells has strongly contributed to the comprehension of ALL pathogenesis. There are different genetic sub-type frequencies between adult and young ALL patients but the general molecular mechanisms that lead to ALL are quite similar. They include: anomalous expression of pro-oncogenes, chromosomal translocations that produce fusion genes coding for active kinases and altered transcriptional factors, hyperploidy that includes more than 50 chromosomes (Gale et al., 1997; Wiemels et al., 2002; Mori et al., 2002; Greaves et al., 2006). All the above alterations contribute to the leukemic transformation of hematopoietic stem cells modifying their cellular function. The cells regulatory processes are therefore altered leading to an unlimited capacity of self-renewal. Accordingly, this mechanism removes the normal control of the cell proliferation, blocking the differentiation and promoting the resistance to apoptosis (Pui et al., 2004). Some genetic lesions influence only one pathway whereas other alterations can interfere with more pathways. The ABL proto-oncogene codes for a tyrosin-kinase with a closely controlled activity. The fusion protein BCR-ABL (the “Philadelphia chromosome”) is a kinase constitutively activate, instead. It could modify the signal pathway that controls the cell proliferation, the survival and the self-renewal capacity of hematopoietic stem cells (Pane et al., 2002). However, the oncogenic events that lead to chromosomal rearrangements are probably insufficient to singly cause ALL: probably, a combination of several factors such as gene-gene and gene-environment interactions appears to be necessary. Recently, Eden summarized the major putative factors for causation of childhood leukemia, principally treating the genetic predisposition, the genetic susceptibility and the environmental factors (Eden et al., 2010). Childhood leukemia like other cancers derived from two or more molecular changes in stem-like cells that can divide and at the same time, maintain an immature state. Several common genetic mutations in leukemia such as TEL-AML1 and AML1-ETO have been evaluated for their formation during the foetal development. Studies revealed that these translocations occur at a rate of 1% or more in the normal population so mutations associated with leukemia cannot cause by themselves the disease (Zuna et al., 2011; Mori et al., 2002). Some studies in the early 20<sup>th</sup> century proposed that infection was the cause of leukemia, now hypothesized as a reaction to infection (McCarthy et al., 1992). Greaves and Kinlen proposed a link with infection through population mixing and a key role of the infection in the promotion of a pre-leukemic clone’s transformation into leukemia (Greaves et al., 1988; Kinlen et al., 1990). Graves

met the hypothesis that infection through an immune response would lead to the postnatal genetic errors seen in childhood ALL especially, strongly close to the “hygiene” hypothesis. The absence of an early immune challenge and priming, with delayed exposure to infections may predispose the individual to pathological responses to infection that would create the second ‘genetic’ hit through proliferation or apoptotic stress considering that the first hit that is pre-leukemic clone, was present from fetal life (Wiemels et al., 2002; Greaves et al., 2003). The infection hypothesis is supported by several studies that emphasize the importance also of the maternal infection and influenza during pregnancy concluding that a link there exists between maternal infection and increased risk of leukemia (Lehtinen et al., 2003; Naumburg et al., 2002). Considering the environmental factor, it is well known a remarkably high rate of cellular kinetics in fetal hematopoiesis and a high degree of cellular proliferation that could set up a situation of perturbation via environmental insults, including chemicals, inducing mutations (Wiemels et al., 2012). However, the only confirmed chemical cause is ionizing radiation caused by atomic bomb exposure or diagnostic imaging during pregnancy (Little et al., 2008). Other potential contributors to leukemogenesis are diet of the mother and child, parental smoking, pesticides and traffic fumes (Metayer et al., 2008; Chang et al., 2009). Several candidate gene studies have also implicated DNA repair, carcinogen detoxification and folic acid metabolism pathways (Vijayakrishnan et al., 2010). It has been estimated that in industrialized countries at least one third of cancer death can be attributed to inappropriate diet, malnutrition and lifestyle (World Cancer Research Found, American Institute of Cancer Research 2007).



## 1.3 FOLATE

Nutrients and vitamins may influence cell metabolism, immune and inflammatory processes, hormone pathways, response to carcinogens and stimulate detoxification and antioxidant mechanisms, altering gene transcription and translation, DNA repair and apoptosis, modifying in turn cell growth, differentiation, proliferation and death. All these stages are considered noteworthy in tumorigenesis-related processes (Barta et al., 2006; Lampe et al., 2003; Mutch et al., 2005). The gene-nutrient interactions are other important factors that could influence the establishment of tumors: the genes encoding for metabolic enzymes contain a lot of polymorphic variations and several of these have functional consequences on the expressed protein under a particular nutritional condition (Mutch et al., 2005; Afman et al., 2006).

Folate, essential nutrition component in the human diet, not synthesized by mammalian cells is involved in several metabolic pathways such as carbon transfer reactions in purine, pyrimidine biosynthesis and amino acid interconversion (Iyer et al., 2009). The daily recommended intake (DRI) is 400 µg/d for adults and 600 µg/d for pregnant women and the principal sources of folate are green leafy vegetables, crucifers, lettuce, legumes, tomatoes, asparagus, potatoes, whole-grain cereals, beans, citruses, strawberries, kiwi, fermented dairy products and milk which is well-known as a source of both free and bound folate (Lin et al., 2000). Folate is a generic term for naturally occurring food vitamin while the synthetic folic acid form is used in food fortification and supplements (Iyer et al., 2009). The proton-coupled folate transporter allows the absorption of folic acid across the intestinal epithelium and then reduces the synthetic molecule to Tetrahydrofolate (THF). Serum folate circulates as monoglutamate derivatives and to be absorbed by the intestine, needs an enzymatic hydrolysis by the glutamate carboxypeptidase II (GCPII), an intestinal brush-border enzyme (McNulty et al., 2004) which catalyzes the substitution of C1 at the N-5 or N-10 positions (Zhao et al., 2009). This reaction occurs at pH 6-7, therefore the acidification due to ingested foods could change folate absorption by the hindrance of GCPII activity. GCPII is a Zn-dependent exopeptidase, thus, zinc deficiency can reduce the efficiency of folate utilization (Wei et al., 1998). Significant folate losses (>50%) can result from a variety of thermal and refining processes. High time of boiling of green vegetables proving that folate bioavailability can be affected by the method, the time of cooking and the type of food (McKillop et al., 2002). There are many counteracting studies about the folate bioavailability, some of which indicate a higher bioavailability of the synthetic folic acid than the natural folates and some others, indicate nearly

equivalent bioavailability of monoglutamyl and polyglutamyl vitamins forms (Hannon-Fletcher et al., 2004; Melse-Boonstra et al., 2004; McKillop et al., 2006). It has been observed that the folic acid absorption on an empty stomach is doubled as available as food folate while the folic acid absorption on a full stomach is 1.7 folds as available as food folate (Hendler et al., 2001).

Folate is a water-soluble B vitamin essential for cell growth and development, involved as a cofactor in several single-carbon transfer reactions. Different isoforms of folate (Figure 1) are distinguishable by their oxidation-state but the term “folate” refers to a family of substances composed of Para Amino Benzoic Acid (PABA), a pterin residue and a  $\gamma$ -linked glutamate moiety (Iyer et al., 2009). The most oxidised and stable form of folate is folic acid (Figure 2), also called pteroylmonoglutamic acid (PGA). The most abundant form of folate in serum and red cells is 5-methyl-Tetrahydrofolate (Bailey et al., 1999) but each form of folate has specific roles in the metabolic pathway, thus, from a medical point of view the two main one-carbon isoforms are 5,10-methylene-Tetrahydrofolate (5,10-methylene-THF) and 5-methyl-Tetrahydrofolate (5-methyl-THF), involved in amino acid metabolism, methylation processes and in DNA and RNA biosynthesis (Shane et al., 2010). Folic acid is necessary for the synthesis of thymidylate (dTMP) from deoxyuridine monophosphate (dUMP). Generally, when a situation of folate deficiency is established, dUMP accumulates and uracil is incorporated into DNA, instead of thymine (Eto et al., 1986). The clinical manifestation of folate deficiency is called Megaloblastic Anaemia, characterised by red cell precursors (megaloblasts) larger than normal cells in the bone marrow, presence of macrocytes in the peripheral blood and giantism of the proliferating cells (Chanarin et al., 1985). Furthermore, from the time it is known that an imbalance of substrates and cofactors involved in one-carbon metabolism and an altered enzymatic activity can lead to a backlog of Homocysteine (Hcy) and S-adenosyl-homocysteine (SAH) responsible for specific pathological conditions such as Hyperhomocysteinemia (Engbersen et al., 1995). The Homocysteine accumulates in the cell and subsequently is exported into the blood causing toxic effects against endothelial cells and circulating proteins (Abahji et al., 2007, Perla-Kajan et al., 2007). Additionally, several studies linked low folate status to heterogeneous types of pathological conditions, like anemia, Neural Tube Defects (NTDs) in offspring, depression, cardiovascular disease (CVD) and cancer. Red blood cells have a very high turnover rate and low folate availability can cause a reduction in the de-novo nucleic acid biosynthesis leading to prolongation of the cell division phase. Without enough folate, the body cannot maintain new blood cells proliferation, causing in turn, anemia. Even a vitamin increased requirement can create a secondary deficitary status, like during pregnancy when folate needing doubles and the acquired low maternal folate status can associate with premature birth and increased risk for NTDs (van der Put et al.,

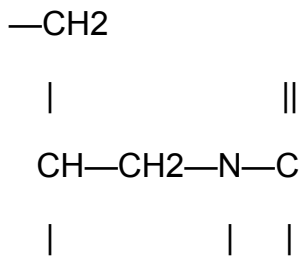
2001). A chronic severe folate deficiency is instead associated with several mental problems such as Alzheimer's disease, schizophrenia, depression, behavioural disorders and cognitive decline (Luchsinger et al., 2007). Furthermore, many studies highlight a correlation between low folate intake and elevated plasma Hcy levels which is a strong risk factor for venous and arterial thrombotic disease, suggesting the hypothesis that lowering Hcy levels in the blood with an appropriate consumption of folate could be a potential method to prevent CVD (Verhaar et al., 2002; Klerk et al., 2002). Last but not least is the correlation between low folate level and elevated cancer risk. Several studies underline the possibility to reduce the link thanks to optimization of folate intake (Divisi et al., 2006, Aune et al., 2011) without forget that folate has been recognized a potential risk factor for cancer as well as other supplementations do to other complex disease (Mok et al., 2011).

Before the absorption in the small intestine, natural food folates are hydrolysed to pteroylmonoglutamate forms and the bioavailability of ingested monoglutamates is significantly higher than that of folate polyglutamates likely because of the requirement for hydrolysis of the latter (Fitzpatrick et al., 2003). The polyglutamates folates have greater affinity for folate-dependant enzymes (Table 1) than polyglutamate forms and the tetrahydrofolate that is produced together with its derivatives are involved in a series of reactions as acceptor or donor of one carbon units. The two main biochemical reactions within this metabolic pathway are:

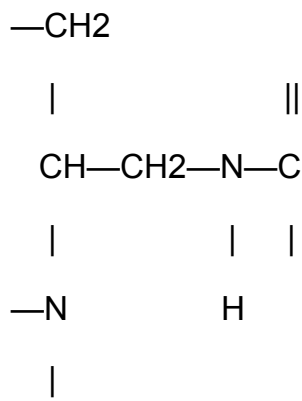
- i) Nucleotide synthesis, that is DNA synthesis
- ii) DNA methylation

The **5,10-methenyl-THF**, produced by 5,10-Methenyl-THF-cyclohydrolase enzyme (MTHFCH) can be hydrolyzed to **10-formyl-THF**. The latter is used as donor of one-carbon units by 5'ribosil-5-aminoimidazole-4-N-Succin-carboxamide-formyl-transferase (AICAR-FT) during purine and formyl-methionine synthesis, with a consequent production of **Tetrahydrofolate** (THF). The starting point of this metabolic pathway is represented by THF which can be used as acceptor of one-carbon units in many reactions. THF can be linked to formimino, generating **5-formimino-THF** for the conversion to glutamate and to glycine. Furthermore, it can intervene in the splitting of glycine into CO<sub>2</sub> and NH<sub>4</sub><sup>+</sup> with creation of **5,10-methylene-THF**. The methylation of THF to 5,10-methylene-THF, as methyl donor, can be catalysed also by Serine-Hydroxymethyl-Transferase (SHMT) enzyme activity. 5,10-methylene-THF can be transformed into THF also by other two pathways: as cofactor of Thymidylate Synthase (TS) enzyme during the conversion of dUMP into dTMP or by Dihydrofolate Reductase (DHFR) enzyme activity that generates THF by methylation

reaction of Dihydrofolate (DHF). MTHFR enzyme than catalyzes the reduction of 5,10-methylene-THF to **5-methyl-THF**, allowing the transfer of a methyl unit to cobalamin-I, cofactor of Methionine Synthase (MS). Cobalamin-I is activated as methylcobalamine providing the methyl unit for the remethylation of Hcy to methionine. Another enzyme implicated in the methyl unit transfer reaction to Hcy methylation is the Bethaine-Hcy-Methyltransferase (BHMT). The reduced activity of BHMT could lead theoretically, to an enhancement of Hcy level (Heil et al., 2000). Moreover, folate and B12 vitamin as methyl-cobalamin are respectively substrate and cofactor for the remethylation of Hcy to methionine. The latter is an essential amino acid and after their conversion to S-adenosylmethionine (SAM) is the most important donor of methyl groups within the cell. Therefore, a methionine shortage can bring to hypomethylation of lipids, proteins and DNA. Also a low availability of 5-methyl-THF can diminish methionine synthesis and consequently, SAM levels that is necessary to the cytosine methylation process (Friso et al., 2002). Thus, it is clear that anomalous levels of folate can have significant consequences for several pathway primarily involved in the synthesis and methylation processes perturbing the biochemical status within the cell. DNA methylation is directly dependant on the reduced folate pool finely tuned MTHFR enzyme (Friso et al., 2002). Generally, the gene methylation degree correlates with the transcription activity and an altered methylation status, referring to oncogenes and onco-suppressor genes has been identified as fundamental for the pathogenesis of tumours and haematological malignancies (Kuppers et al., 1999, Vanesse et al., 1999).

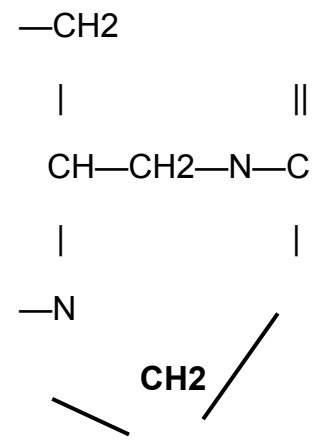


**Tetrahydrofolate**  
**[H4-folate]**

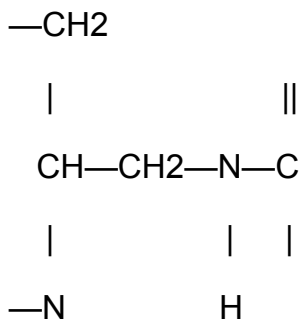


**CH3**

**5-methyl-H4-folate**



**5,10-methylene-H4-folate**

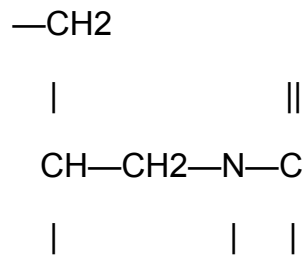


**CH**

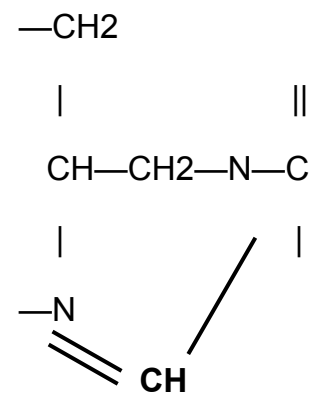
**||**

**NH2**

**5-formimino-H4-folate**



**10-formyl-H4-folate**



**5,10-methenyl-H4-folate**

Fig.1 Principal folate isoforms involved in the one-carbon metabolism.

**Table 1** Main enzymes involved in the folate pathway

<b>Enzima/Recettore</b>	<b>Acronimo</b>
Human Folate Receptor	hFR
Reduced Folate Carrier	RFC
Dihydrofolate Reductase	DHFR
Methylenetetrahydrofolate Reductase	MTHFR
Methionine Synthase	MS
Methionine synthase reductase	MTRR
Serine hydroxymethyltransferase	SHMT
Methylenetetrahydrofolate dehydrogenase	MTHFD
Thymidylate synthetase	TS
Cystathionine- $\beta$ -synthase	CBS

### **1.3.1. Folate distribution**

Folate, essential component in the human diet cannot be synthesized by mammalian cells so it must be integrated by the diet in order to prevent nutritional deficiency that can occur by malnutrition or by malabsorption. The latest is determined by several conditions such as Celiac disease, Chron's disease, liver failure, tropical sprue and gluten sensitive enteropathy (Bemeur et al., 2010). Serum and red blood cell folate concentrations are finely dependent on folate status changing with a faster response of serum folate (Herbert et al., 1987). The plasmatic physiological concentrations vary in the range 3 ng/ml-30 ng/ml. The circulating form 5-methyl-THF is used by the liver that finely modulates this omeostasis as it allows the activation of specific biochemical reactions. There is in fact a balance between THF isoforms used by tissues and isoforms synthesized and hydrolysed by the cell in order to maintain an optimal bioavailability of 5-methyl-THF isoform. In case of high consumption, malabsorption, underutilization or poor introduction by the liver, the standard level of folate can be temporarily maintained by the vitaminic pool stored within the cell.

Red blood cells achieve all their amount of intracellular folate during erythropoiesis in the bone marrow. The progenitor cells keep folate through membrane-associated folate receptors that in any case are not significantly present on mature red blood cells (Antony et al., 1992). As a consequence, mature red blood cells gain a neglectable amount of folate. This is the reason why the intracellular

folate will shift relatively slowly after an altered intake and may not reach steady-state levels until after 35 weeks. Conversely, serum folate is used to provide information about the recent dietary intake: studies based on reduction of folate intake to inadequate levels showed that serum folate decreased over 7 weeks and stabilized after 8 weeks (Gregory et al., 2010). Generally, serum and intracellular folate results may provide different information about the patient's folate status mostly because the different isoforms of folate within the cell can be easily interconverted. In recent years, studies have considered different approaches evaluating serum and intracellular folate as markers of deficiency, but at the moment, is not present a gold standard to diagnose a folate deficiency. Several studies consider an elevated Hcy levels as a sensitive marker of folate deficiency: under condition of low dietary folate, the remethylation and the transsulfuration pathways, responsible for maintaining adequate level of Hcy in blood and cells, are altered and as a consequence it increases, being a potential cytotoxic sulphur amino acid (Shinawi et al., 2007). It is evident that, the assessment of a patient's folate status is of clinical importance. From previous studies it seems that serum folate is a superior marker in patients with deficit of B12 vitamin and that serum folate appears to better discriminate between different levels of folate intake (Flynn et al., 2003). It is important to consider also the genetic implication in the distribution of folate forms. From the time it is known that the C677T polymorphism of MTHFR gene may alter folate concentrations in populations with low folate status. This polymorphism also alters the distribution of folate isoforms in red cells causing analytical variation in the measurement of folate concentrations. At the same time the individual folate status appears to mediate the interaction between MTHFR genotype and circulating folate concentrations. In the study of Shelnutz et al. after a restriction folate diet for 7 weeks, those subjects with homozygote polymorphic genotype (677TT) had significantly lower concentrations in serum folate, while red cell folate was unable to respond as rapidly to the changes in folate intake (Shelnutt et al., 2004). However, some other studies have shown no difference (Esfahani et al., 2003, Caudill et al., 1997). It is noteworthy that the difference in serum and red blood cell folate results in the presence of specific genotypes seems to reflect a real change in folate metabolism. To the diminished levels of folate in 677TT genotype is associated an increment of plasma Hcy (Bowron et al., 2005, Crider et al., 2011). Other observations suggest that the Hcy variation between different genotypes exhibits interaction with folate status. Subjects 677TT present higher Hcy levels when folate intake is reduced but there is no difference when folate status is high (Kluijtmans et al., 2003).

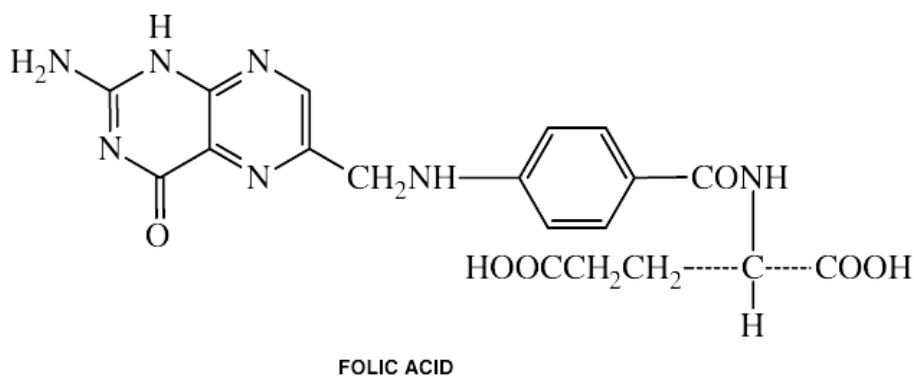


Fig.2 Acid folic molecule

### 1.3.2. Folate and cancer

Different nutrients have a role in genomic stability and integrity maintenance. Folate is an important molecule used by the cell both for DNA synthesis and methylation, being the main source of methyl groups (Oommen et al., 2005, Crider et al., 2012, Friso et al., 2002) (Fig. 3). Not only inadequate intake/absorption, but also nutritional interactions (Navarro-Peran et al., 2005, Navarro Peran et al., 2007) or genetic polymorphisms in the one-carbon transfer enzymes (Gemmati et al., 2009, Han et al., 2012, Lee et al., 2011) can limit folate and the other related isoforms bioavailability. Recent reviews on folic acid food fortification effectiveness suggest that high vitamin levels might either prevent or promote some cancer types (Sauer et al., 2009; Kim et al., 2004; Crider et al., 2011) hypothesizing that an early exposure to folic acid may prevent tumour supplying enough methyl groups to properly maintain the methylation pattern but, after tumour growth, a higher intake of folic acid may even promote its expansion. Till now, direct evidence that folate deficiency produces hypermethylation of tumour suppressor gene promoters is lacking. Suboptimal folate level instead, could be considered an accelerator of carcinogenesis by altering both DNA repair process and the balance of purine and pyrimidine DNA precursors. Uracil is not normally present in DNA and when misincorporated due to thymidine paucity, it activates risky removal processes, responsible for DNA strand breakage, chromosomal damage and possible malignant transformation (Beetstra et al., 2005, Narayanan et al., 2004). Genetic polymorphisms can reverse the situation, rebalancing the cell folate isoforms modifying the cancer risk in carrier subjects (Gemmati et al., 2009, Han et al., 2012; Lee et al., 2011). Nowadays, there is a growing body of interest about the linkage between the key enzyme MTHFR and childhood leukemia (Yan et al., 2012) where MTHFR polymorphisms



play a crucial role in the intracellular folate balancing. Among the most investigated folate genes, MTHFR is indeed, of particular interest because of its implication in the conversion of 5,10-methylene-THF to 5-methyl-THF, the main form of circulating folate and the primary methyl donor for the remethylation of Hcy to methionine. The most common identified and widely investigated gene variant associated to MTHFR gene is the C677T. It is mainly studied in cardiovascular disease, venous thrombosis and neoplasia (Gemmati et al., 1999; Klerk et al., 2002; Bolufer et al., 2006). The SNP is associated with a reduced enzyme activity leading in the case of 677TT-homozygous condition, to a reduction of about 65% (Frosst et al., 1995). Several studies found the SNP protective against cancer, reducing in turn cancer susceptibility (Chen et al., 1996, Franco et al., 2001, Gemmati et al., 2004). However, at the same time, recent papers ascribed to the same 677T-allele increased negative side effects, such as increased drug toxicity and low survival rates in cancer patients (Gemmati et al., 2007). This dual-faced behaviour has been defined as the *Judas-allele* characteristic (Gemmati et al., 2008) now better called *false-friend* allele (Gemmati et al., 2009). Common gene variants within genes of enzymes considered pivotal for cancerous cell cycle, acting as modifying the susceptibility, survival and response to treatment of patients, may influence global disease outcome, though the results obtained are still controversial (Wu et al., 2007, Habuchi et al., 2006, Sinnet et al., 2006; Bolufer et al., 2006; Loktionov et al., 2003). Several polymorphic genes involved in folate metabolism are recognized to work in concert, in order to generate the different substrates necessary for the optimal folate cell cycling. A paradox there exists when such polymorphisms are investigated among cancer populations: cancer patients carrying such gene variants may complain the polymorphism's effect in dual and opposite fashion. They may have firstly reduced cancer susceptibility but a contextual increased drug-related toxicity, particularly when they are treated with folate antagonists such as Methotrexate (MTX) (Gemmati et al., 2008, Ongaro et al., 2009, Gemmati et al., 2007). Also a reduced survival rate is associated to the presence of this polymorphisms, or combination of them, in carrier patients. The same allele could be considered on one hand a protective factor against the establishment of the disease and on the other hand, a risk factor for poor prognosis. No clear and definite mechanisms have been suggested to explain this opposite behaviour but folate balancing and other acquired or transient conditions together with a complex genetic background may contribute to the clinical phenotype in patients. This could in part explain why such gene variants should be considered *false-friend* alleles acting friendly in healthy subjects but unfriendly when the same individuals get sick.

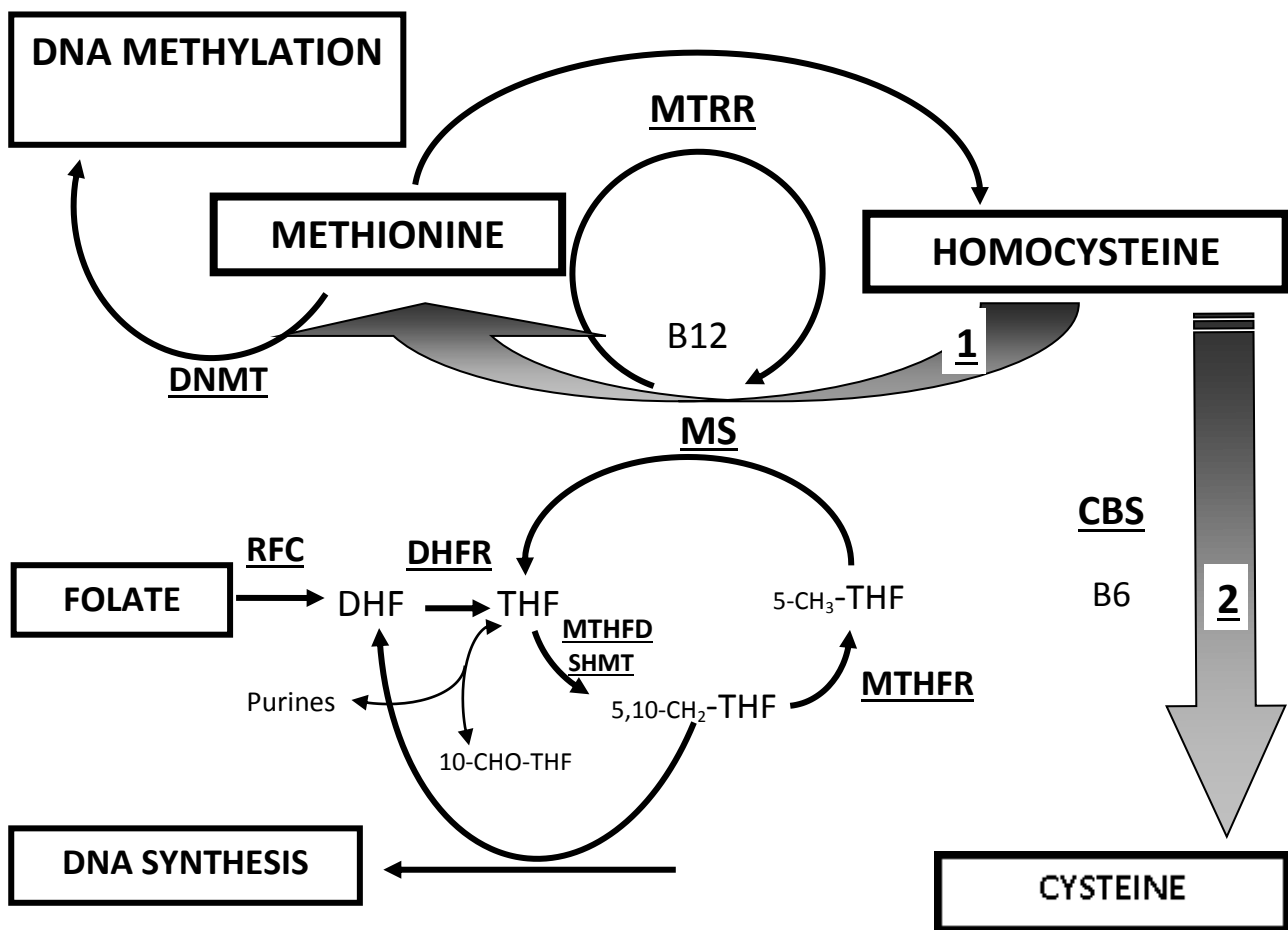


Fig.3 Folate metabolism [Gemmati et al.,2008]

### 1.3.3 Folate deficiency: DNA damage and repair

Folate deficiency has been implicated in the development of several cancer types such as breast, ovary, pancreas, colon and cervix but an entirely protective role for folate against carcinogenesis has been questioned. In effect, recent papers suggest that an excessive intake of folate or synthetic folic acid may enhance cancer risk by accelerating growth of precancerous lesions (Kim et al., 2007). Folate plays a crucial role in maintaining genomic stability through DNA biosynthesis, repair and methylation processes and its deficiency has been associated to the perturbation of each of these pathways. If folate is limited, the balance of DNA precursor molecules is altered and normal DNA repair is inhibited: low 5,10-methylene-THF levels hampers conversion of dUMP to

dTMP leading to thymidine deficiency and high uracil concentration. Uracil is thus misincorporated into DNA but is readily removed by DNA repair enzymes. When folate is steadily limited, this breakage-repair cycle continues, leading to double-strand breaks, chromosomal abnormalities and malignant transformation (Blount et al., 1997). Several cell culture models have been performed including human lymphocytes, human colonocytes and Chinese hamster ovary cells (CHO) in response to folate deficiency (Duthie et al., 2000, Beetstra et al., 2005, Duthie et al., 2008). These experiments showed that human lymphocytes grown under folate restriction conditions, increased gene-specific DNA strand breaks in the p53 tumor suppressor gene. Branda et al demonstrated that folate deficiency could increase the susceptibility of mammalian cells to mutagens and irradiation (Branda et al., 2007), whereas Melnyk et al. in 1999 showed that folate deficiency could promote malignant transformation in CHO cells inoculated in mice (Melnyk et al., 1999). Several studies have been performed not only in animal models but also in humans. Bone marrow from megaloblastic anaemia patients showed high uracil levels and low thymidine levels (Wickramasinghe et al., 1994). Furthermore, Blount et al demonstrated that uracil misincorporation and chromosomal abnormalities in severely deficient patients, could be reversed by high dose synthetic folic acid (5mg/d for 8 weeks) (Blount et al., 1997). Basten et al., showed that uracil is also present in DNA from healthy individuals and it is responsive to folate supplementation (Basten et al., 2006). Besides DNA damage processes, also defective DNA repair is linked to the development of human cancer: polymorphisms in DNA repair genes such as XRCC1 and PARP1 have been shown to correlate with altered cancer risk and disease progression in colorectal carcinoma (Bigler et al., 2006). Base excision Repair (BER) pathway is obstructed in folate-deficient cultured cells. Duthie et al demonstrated that excision of alkylation and oxidative damages are delayed in human lymphocytes and colonocytes grown in folate deplete medium (Duthie et al., 2008, Duthie et al., 1998). Animal models such as folate-deficient rats have also been studied demonstrating that intracellular nicotinamide adenine dinucleotide (NAD) is high, suggesting upregulation of DNA repair enzymes, possibly because of increased DNA strand breakage (James et al., 1992). Duthie et al in 2010 showed that the deficit of folate can significantly increase by 25% the activity of two incision repair proteins: methylguanine-DNA methyltransferase (MGMT) and oxoguanine-DNA glycosylase (OGG1) in rat liver, whereas the same group in 2008, determining global protein expression in human colon cells in vitro, shown that folate deficiency upregulates specific DNA incision repair enzymes, including MSH2 and XRCC5, involved in mismatch repair and double-strand break repair. These studies probably confirm the hypothesis that the induction of DNA repair's proteins is linked to increased DNA damage caused by a deficit of folate (Duthie et al., 2010, Duthie et al., 2008).

### **1.3.4 Folate status and DNA methylation**

DNA methylation is the most widely studied mechanism of epigenetic gene regulation. Epigenetic modifications are dynamic and can be strongly influenced by external factors, thus, these factors may alter gene expression, potentially inducing disease formation (Li et al., 2002, Reik et al., 2001). Evidences suggest DNA methylation is fleeting towards nutritional and environmental influences. Methionine is converted into S-adenosylmethionine (SAM), the primary methyl donor for the DNA methylation process and it derives from a cyclical cellular pathway called one-carbon metabolism. During this cycle, DNA methyltransferases (DNMTs) covalently link methyl unit from SAM to cytosine bases, composing 5-methylcytosine, thus methylating DNA. In mammals, DNA methylation process is dynamic and this regulatory event can occur in a global or gene specific manner (Maunakea et al., 2010). MTHFR is a key enzyme of the one-carbon metabolism and it is involved in the conversion of Hcy to methionine in order to generate 5-methyl-THF. This is the reason why MTHFR activity has been widely investigated in DNA methylation process. Shelnutt et al in 2004 studied the relationship between the MTHFR C677T polymorphism in young women under controlled folate intake and DNA methylation, showing that reduced dietary folate intake was associated with decreased global methylation of leukocyte DNA. Furthermore, after a re-supplementation with folate, there was a DNA re-methylation on the global scale (Shelnutt et al., 2004). Folate is reduced to DHF and then to THF by DHFR enzyme. THF is necessary as one carbon donor to form 5-methyl-THF that donates its methyl group to Hcy in order to convert it to methionine. Under low dietary folate intake, SAM is reduced and SAH is elevated: folate depleting is thus, generally associated with decreased DNA methylation and, potentially, increased proto-oncogene expression (Kim et al., 2007). Many studies on animal models, have evaluated the relationship between global methylation and maternal folate status. In a study conducted by McKay et al., murine offspring under low folate diet during pregnancy showed a reduction of global methylation in small intestinal tissue (McKay et al., 2011). Conversely, Ly et al., showed an association between the measurement of global methylation following in utero folate supplementation and the CpG hypomethylation of mammary tissue among rat offspring of 28 weeks (Ly et al., 2011). Another study conducted on murine colorectal tissue, demonstrated that post weaning folate supplementation equivalent to recommended intake levels for women of child-bearing age linked to a decrease of global methylation levels (Sie et al., 2011). Several human epidemiological studies have also taken into account the global DNA methylation, looking at the link between folate intake and DNA methylation in colorectal cancer (CRC) patients and in postmenopausal women. One study observed, in patients CRC negative, an association between

colonic mucosa and lymphocyte DNA methylation and serum and dietary folate concentrations, whilst, in patients CRC positive the group observed a correlation between folate supplementation and increasing in global DNA methylation (Pufulete et al., 2005). Another group observed that among postmenopausal women, dietary and serum folate decrease, were associated with decreased DNA methylation in leukocytes, in a dose-dependent manner (Rampersaud et al., 2000). However, the positive impact of the folate supplementation is strongly dependent on dose and timing of intervention and on health status (Pufulete et al., 2005).

### **1.3.5 Genetic variants and possible effects on cancer**

The folate pathway seems to play a crucial role in the development of childhood ALL because of its involvement in the DNA synthesis and repair processes and in the DNA methylation pathway. Recently, especially in childhood acute lymphoblastic leukemia the influence of polymorphisms in genes of the folate pathway has been studied, indeed. Malignant cells are rapidly dividing cells and require a high amount of folate. Thus, these cells are vulnerable to folate deficiency. There are strong evidences that suggest that maternal folate supplementation during pregnancy can reduce the risk of childhood ALL (Milne et al., 2010). Furthermore, several studies suggest that some functional polymorphisms of the MTHFR gene could alter cancer susceptibility by the regulation of folate metabolism (Alcasabas et al., 2008, Damnjanovic et al., 2010). For this reason it possible to speculate that individuals with reduced MTHFR enzyme activity may have reduced risk of developing ALL because of an enhancement of folate bioavailability. From recent meta-analysis it was found that MTHFR polymorphisms have a significant impact on childhood ALL risk particularly when considering the variant T allele of MTHFR C677T that was found protective against ALL risk (Yan et al., 2012). Nevertheless, in the past decade, studies on the implication of MTHFR polymorphisms in the development of ALL reported conflicting results, frequently showing no associations of the two common variants MTHFR C677T and A1298C with childhood ALL risk (Chan et al., 2011, Kamel et al., 2007). A study conducted by Semsei et al. showed high protection in boys carrying 677CT and 1298AA genotypes and less protection against ALL in girls with 677CC and 1298AC genotypes (Semsei et al., 2010). The results are still inconclusive and some authors suggest the hypothesis that C677T SNP could be in linkage with some unknown functional SNPs that can modulate ALL risk. Another important factor to consider is ethnicity that may influence SNP-cancer risk association through the different allele frequencies, the different genetic backgrounds and the possible gene-environment interactions, due to different lifestyles (Yan

et al., 2012). Many authors considered also the association between MTHFR polymorphisms and toxicity during chemotherapy, especially with methotrexate: some studies did not find significant associations between the 677T allele and toxicity (Imanishi et al., 2007, Pakakasama et al., 2007), whilst some others showed that individuals with 677T allele had to stop the treatment frequently, suggesting the 677T allele as a toxicity predictor during the chemotherapy maintenance (Shimasaki et al., 2008, Huang et al., 2008). All the studies conducted till now are not sufficient but necessary at the same time, in order to get an appropriate treatment for individuals with unfavourable polymorphisms towards leukemia treatment.

#### The MTHFR gene:

Methylenetetrahydrofolate reductase (MTHFR) catalyzes the conversion of 5, 10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, necessary as a co-substrate for the remethylation of Homocysteine to methionine. MTHFR gene lies on chromosome 1, locus 1p36.3 and contains 11 exons, coding for a protein of 656 amino acid with a predicted molecular weight of 74.5 kD (Goyette et al., 1995)

In the MTHFR gene are present two well-described and widely studied common polymorphisms: C677T and A1298C. Other less common polymorphisms have been reported at 1059 bp, 1289 bp, 1317 bp and 1793 bp (Trembath et al., 1999, Weisberg et al., 1998, Rady et al., 2002). The MTHFR C677T polymorphism occurs in exon 4 and consists of a C-to-T nucleotide transition at nucleotide 677 leading to an alanine to valine change at codon 223 (A223V) in the catalytic domain of the protein (Frosst et al., 1995). This substitution renders the enzyme thermolabile, affecting the associated activity *in vitro* (Goyette et al., 2000, Yamada et al., 2001). Subjects carrying the CT heterozygous condition have been shown to have 65 percent of their *in vitro* enzyme activity, compared with homozygotes for the common variant CC whereas those with the homozygous variant condition TT have been found to have 30 percent compared with the wild type (Frosst et al., 1995). Moreover, Molloy et al. reported that CT heterozygous carriers have 10 percent lower red cell folate levels, when compared with the wild type CC, whilst the TT homozygotes have 18 percent lower red cell folate (Molloy et al., 1997). Other observations showed that subjects carrying the TT variant also present lowered circulating folate, B12 vitamin levels and increased plasma Hcy levels (Ma et al., 1999, Jacques et al., 1996). Gemmati et al. in 1999 conducted a study to evaluate the different correlation degree between plasma Hcy and folate levels among the three genotype conditions of MTHFR C677T polymorphism (Gemmati et al., 1999). Homozygotes TT showed a

significant increasing of Hcy as the plasma folate level decreased. This correlation decreased among heterozygotes CT and disappeared among wild-type CC. This observation is in line with the study of Malinow who reported that depending on the number of polymorphic 677T allele in MTHFR gene, the response to folate supplementation is different (Malinow et al., 1997). The same paper reported a significant overrepresentation of TT-carriers among subjects with hyperhomocysteinemia. In 1998 Bagley and Selhub hypothesized that MTHFR C677T polymorphism could be associated with an altered distribution of folate within the red blood cells: the polyglutamated 5-methyl-THF isoforms were the unique folate isoforms rediscovered within red blood cells of subjects with 677CC genotype. The exclusive presence of formil folate isoforms within red blood cells of subjects with 677TT genotype is in line with the hypothesis that there exists, *in vivo*, a displacement of the activity of MTHFR thermolabile variant and that this displacement will lead to an altered folate distribution within erythrocytes. The thermolabile variant of the enzyme present in 677TT subjects catalyzes less efficiently the synthesis of 5-methyl-THF respect to 677CC subjects (Bagley et al., 1998). Skibola et al in 1999 hypothesized a protection against leukaemia explicable with an increment of methylene-THF cytosolic levels available for thymidylate synthesis, an essential precursor for the DNA de-novo synthesis. Regarding MTHFR polymorphisms and DNA methylation, it is generally known that DNA from subjects with the thermolabile variant of MTHFR (TT) is hypomethylated respect to DNA from subjects with the wild-type variant (CC). Nevertheless, this finding is not confirmed in a study of Narayanan (Narayanan et al., 2004). Recent data, suggest the hypothesis that DNA methylation status could be influenced by genotype only among those subjects presenting low plasma folate levels. Friso et al thus, have demonstrated that subjects with MTHFR 677TT genotype and lower plasma folate concentration had the lower methylation level compared to all the other groups (Friso et al., 2005).

A second MTHFR polymorphism lies in the S-adenosyl-methionine-regulatory domain of the enzyme and it consists of an A-to-C transversion at nucleotide 1298, causing a glutamate to alanine amino acid change at codon 429 (A1298C/E429A) (Matthews et al., 1984, Wiemels et al., 2001). The binding of SAM causes conformational changes that inhibit MTHFR's activity (Matthews et al., 1984). Studies performed on lymphocytes from individuals in homozygous variant 1298CC demonstrate to have approximately 60 percent specific wild-type *in vitro* MTHFR activity, whereas individuals heterozygotes 677CT and heterozygotes 1298AC were found to have 50-60 percent wild-type MTHFR activity (Weisberg et al., 1998; van der Put et al., 1998). The C677T polymorphism has been most extensively studied than A1298C and researches on the relationship between plasma folate and Hcy levels and MTHFR A1298C are less consistent than those performed on C677T variant, probably because of the lower effect of A1298C variant on the

enzyme properties. Some in vitro studies such as site-directed mutagenesis and in vitro expression experiments have been performed to create a recombinant protein containing both gene variations (Goyette et al., 2000, Yamada et al., 2001). Mutant enzymes with the two separate variants 1298 or 677 showed respectively about 68 percent and 45 percent of the control activity whereas the recombinant enzyme containing both variations had 41 percent of the control activity. The two polymorphisms sites are 2,1 kb apart and several groups have found a strong linkage disequilibrium between 677 and 1298 gene variants (Stegmann et al., 1999, Chen et al., 2002). The two polymorphisms very rarely exist on the same allele and double homozygous carriers 677TT/1298CC are extremely uncommon in general population. Furthermore, Zetterberg et al showed in a study performed in 2002 an increased frequency of combined polymorphic alleles C677T and A1298C in spontaneously aborted embryos (Zetterberg et al., 2002).

Generally, it was demonstrated that there are significant associations between specific polymorphisms in folate-related genes and susceptibility to develop childhood ALL. MTHFR is the most widely investigated gene linked to one-carbon metabolism and C677T and A1298C are the two most common polymorphisms considered. Recent reviews have summarized the results of several studies that focussed on the link between MTHFR polymorphisms and risk of ALL. Interesting observations indicate the possibility of a different impact of polymorphisms on susceptibility to ALL, depending on the type of populations (Koppen et al., 2010). Some studies found an increased risk for ALL with the MTHFR A1298C in Filipino and non-white Brazilian populations (Zanrosso et al., 2006, Alcasabas et al., 2008) whilst some others found a decreased risk with the same polymorphisms within British and Egyptian populations (Skibola et al., 1999, Kamel et al., 2007). Robien and Ulrich in 2003 showed that for MTHFR A1298C, the polymorphic allele C frequency ranges from 0.27-0.36 in Western Europe to 0.17-0.19 among Asian populations (Robien et al., 2003) and this finding could support the theory described above.

#### The DHFR gene:

Dihydrofolate reductase (DHFR) is ubiquitously expressed in all organisms and it is controlled by a TATA-less promoter that, in turn is governed by several transcription factors, including Sp1 and E2F (Chen et al., 1984, Jensen et al., 1997). The DHFR gene family includes the functional DHFR gene that lies on chromosome 5q11.2 and three intronless pseudogenes: DHFRP1 on chromosome 18, DHFRP2 on chromosome 6, DHFRP4 on chromosome 3 (Anagnou et al., 1984). The functional gene is expressed in three mRNA isoforms with alternatively spliced 3' UTR ends (Morandi et al.,



1982). DHFR catalyzes the reduction of dihydrofolate (DHF) to tetrahydrofolate (THF) with the presence of NADPH as cofactor. DHFR is also necessary for the intracellular conversion of synthetic folic acid into the THF forms needed for the folate/Hcy metabolism. Since THF is the principal one-carbon donor within the folate cycle in order to synthesize purines and thymidylate, absence of DHFR activity leads to fast breakdown of THF and consequent stop of de-novo DNA synthesis and cell proliferation (Skibola et al., 1999). This is the reason why DHFR is a common drug target in several pathologies (McGuire et al., 2003) and functional polymorphisms within DHFR can potentially lead to changes in its expression or activity, thus influencing the risk of folate-dependent diseases or therapeutic responses to antifolates, leading to higher adverse drug event frequency. In 1989 Detera-Wadleigh firstly reported a polymorphism within the first intron of DHFR as a restriction fragment length polymorphism (RFLP) and in 2004 Johnson et al determined the location and size of the polymorphism identifying a 19bp insertion to deletion and suggesting its functional role, since Sp1 transcription factor binding-site is located within the deleted sequence and the deletion allele could affect gene expression (Detera-Wadleigh et al., 1989; Johnson et al., 2004). In fact, intron-1 has important roles in the transcription of DHFR and the amount of protein produced by translation (Noè et al., 2003). In the past years has been observed a dose-dependent relation between DHFR expression and the deletion. Subjects 19bp DD (deleted allele) homozygotes had higher mRNA levels than subjects carrying the counterpart genotype WW (wild-type allele): the DD genotype has been associated with up to 4.8-fold increase in mRNA levels compared with the WW genotype (Xu et al., 2007). This could be responsible for a filling of THF and other isoforms within the cell, supporting as a consequence DNA synthesis as reported for MTHFR C677T (Frosst et al., 1995, Bagley et al., 1998, Kluijtmans et al., 1996). During the last years several studies have been performed on DHFR but results are still inconclusive and often conflicting: some studies provided evidences that the risk of having a child with spina bifida and the risk of pre-term delivery is higher for women with the DHFR DD genotype (Johnson et al., 2004, Johnson et al., 2005). Parle-McDermott otherwise, reported a lower risk of having a child with Neural Tube Disease (NTD) in women with the deleted allele (Parle-McDermott et al., 2007). A study reported a non-significant increase in mRNA levels for homozygous deleted individuals whereas another study observed a significant increase in DHFR expression with the number of deleted allele, suggesting the possible maternal protective role of 19 bp D allele in NTD by raising the quantity of DHFR available for the reduction of DHF (Parle-McDermott et al., 2007; Xu et al., 2007). Other authors have analysed the relation between the DHFR 19 bp ins/del polymorphism and the risk of breast cancer reporting non-significant association with overall breast cancer risk (Cam et al., 2009). However, an important consideration should be done when stratification according to

vitamin use is performed: patients that used the multivitamin supplements with the DHFR-DD genotype had a 50 percent increase in breast cancer risk compared with women with DHFR-WW genotype (Xu et al., 2007). It is possible that polymorphisms associated with a higher expression of DHFR may protect against cancer because of the high amount of 5,10-methylene-THF available for thymidilate synthesis, but at the same time a variation in the pool of 5-methyl-THF may influence methylation processes and consequently, affect the cancer risk. Gemmati et al, reported a protective role of DHFR 19 bp D allele in adult acute lymphoblastic leukemia particularly when the D-allele was associated with MTHFR 677TT genotype (Gemmati et al., 2009). It has also been reported a correlation between lower Hcy levels, increased serum and red blood cell folate level and DHFR-DD genotype. These studies suggest the hypothesis that the 19 bp deletion could increment DHFR expression facilitating the remethylation of Hcy (GelleKink et al., 2007, Stanislawska-Sachadin et al., 2008). Nevertheless, the effects of DHFR 19 bp ins/del polymorphism have not yet been clarified in detail in the context of solid tumours and data about adult or childhood haematological neoplasia are not yet present, considering the single polymorphism or its combination with other metabolic pathways.

DHFR is one of the key-enzyme of the one-carbon metabolic pathway and it is considered a target molecule for clinically important drugs such as chemotherapeutic agents. Methotrexate (MTX) is a potent inhibitor of DHFR, resulting in the depletion of reduced folates, cofactors for thymidylate synthetase. The same gene variations that define THF accumulation within the cell may protect against cancer risk as shown in the study of Gemmati et al. but contextually, they may affect antifolate treatment as shown in the paper of Ongaro et al. In the latest, authors showed that MTX-related hepatic toxicity increased 2.07-4.57-fold in adult acute lymphoblastic leukaemia patients with the 19 bp deletion allele (Ongaro et al., 2009). This dual and opposite paradoxical effect can be summarized as follow: cancer patients carrying such gene variants may have firstly reduced cancer susceptibility but a contextual increased drug-related toxicity, particularly when they are treated with folate antagonists such as Methotrexate (MTX) (Gemmati et al., 2008).

#### B cell lymphoma gene-2 (Bcl-2):

Bcl-2 is an inner mitochondrial membrane protein codified by the bcl-2 gene that lies on chromosome 18q21.3 and consists of 3 exons and 2 promoters with different functional properties (Hockenbery et al., 1990). P1 promoter drives the transcription activity while P2 promoter is located 1400 bp upstream of the translation initiation site and diminishes P1 promoter's activity,

acting as a negative regulatory element (Young et al., 1993, Seto et al., 1988). Preliminary studies have been effectuated in 1985 as a result of the involvement of the *bcl-2* gene in the most common t(14;18) translocation found in follicular B lymphomas (Bakhshi et al., 1985; Tsujimoto et al., 1985, Bakhshi et al., 1987). The t(14;18) translocation leads to a chimeric transcript because of the fusion of the 3' nontranslated region of *bcl-2* with the joining (JH) segment of Ig heavy chain gene, causing a deregulation of *bcl-2* transcription (Clearly et al., 1986). The *bcl-2* gene encodes a major nonglycosylated protein of 26 Kd that resides in mitochondria and that promotes the survival of several hematolymphoid cells (Hockenberry et al., 1990, Vaux et al., 1988, Nunez et al., 1990), inhibiting apoptosis (programmed cell death). An increased survival of a cell population defines the accumulation of genomic instability within the cells thus inducing leukemogenesis. Apoptosis is a natural process regulated by several pathways that comprise genes that both promote or inhibit apoptosis. Bcl-2 possesses anti-apoptotic activity and several studies have showed that *bcl-2* gene was upregulated in high proportion of acute myeloid leukemia (AML) patients (Karakas et al., 1998, Bensi et al., 1995). Bcl-2 thus, may play a key role in the survival of hematolymphoid cells, inhibiting apoptosis through the prevention of the release of cytochrome c from mitochondria, inducing the block of caspase activation (Reed et al., 1994). It can be hypothesized that polymorphisms in the *bcl-2* gene, which modify protein function and/or expression could play an important role in the balancing of apoptosis regulation mechanisms. A single-nucleotide polymorphism (SNP) in the promoter region locates at nucleotide position -938 and consists of a C-to-A change. The SNP -938C>A has been reported to decrease the prostate cancer risk but the study considered a relative small sample size (Kidd et al., 2006). Other papers did not reveal any significant association between the functional SNP -938 C>A and the risk of different cancer types such as squamous cell carcinoma of the head and neck (SCCHN) or B-cell chronic lymphocytic leukemia (B-CLL) (Chen et al., 2007, Nuckel et al., 2007). Nevertheless, the same authors showed that the -938 C>A SNP had a statistically significant impact on transcriptional activity of a region of the *bcl-2* gene that inhibits promoter activity. The functional -938 C>A promoter polymorphism is located in the inhibitory P2 promoter region and an increased activity of the inhibitory P2 promoter seems to be associated with the C allele (Nuckel et al., 2007), suggesting a potentially different binding of transcription factors to the C and A alleles. Several others studies performed on breast cancer, oropharyngeal squamous cell carcinoma, chronic lymphocytic leukemia, glioblastoma multiforme and prostate cancer have showed an association between the A allele and an higher Bcl-2 expression level. Furthermore, the -938 C>A genotype has been linked to survival of patients suffering from those cancers (Bachmann et al., 2007; Lehnerdt et al., 2009, Nuckel et al., 2007). Recently, Kunkele et al., have evaluated the possible correlation between -938 C>A

functional polymorphism and the risk group classification in childhood ALL, considering that this SNP is known to influence the balance of apoptosis and survival in hematolymphoid cells. Data suggest that genotyping ALL patients for -938 C>A polymorphism could be of benefit for therapy response prediction: authors indeed, have showed that childhood ALL patients with bcl-2 -938CC genotype have about 3-fold higher risk of belonging to a high-risk group (Kunkele et al., 2013). Chauhan et al., suggested that bcl-2 could be used as possible marker for therapy response during induction chemotherapy treatment of adult ALL patients (Chauhan et al., 2012), whilst Singh et al. showed that Bcl-2 inhibition with concomitant use of methadone increased the induction of apoptosis in childhood ALL cells (Singh et al., 2011). Until now, there are few reports on the association between bcl-2 polymorphism and cancer risk or disease progression or therapy response and survival. More intensive treatment regimens can leads to severe side effects and/or long-term adverse effect so additional markers to ameliorate the specificity of treatment could be considered a great goal, reducing short- and long-term adverse effects.

## 1.4 METHOTREXATE

During the last 20 years advances in specific chemotherapy allowed an improving of the survival rates for ALL, with expected cure rates higher than 80 percent (Pui et al., 2008). Methotrexate (MTX) (Fig. 4) was the first drug considered curative for patients with a solid tumour, choriocarcinoma and it is an important component of the therapy for childhood ALL. Often, the treatment with MTX causes severe and different toxicities that require the cessation or the reduction of the therapy then, an appropriate predictor for the adverse outcome of MTX treatment would be strong useful (Imanishi et al., 2007). MTX enters the cell by two mechanisms: with the first mechanism the uptake of MTX occurs by passive diffusion, dependent on the amount of extracellular concentrations of the drug; with the second mechanism MTX enters via active transport mediated by the reduced folate carrier (RFC1) (Gorlick et al., 1996). Once inside the cell, MTX is polyglutamylated by the enzyme folylpolyglutamate synthetase and it acts by inhibiting two key enzyme of the folate metabolic pathway: DHFR and thymidilate synthase (TS). The block of these enzymes affects DNA synthesis and cell replication. DHFR is necessary for the conversion of DHF to THF and its inhibition therefore gets reduced THF isoforms necessary for folate homeostasis out. TS catalyses the methylation of dUMP to dUTP, a precursor for DNA synthesis: the inhibition of TS leads therefore to the depletion of thymidine and DNA damages (Salazar et al., 2012). High-dose MTX is frequently used during the consolidation phase of ALL treatment. Hypothesizing that MTX toxicity can be related to polymorphic variants in genes of the folate/MTX metabolic pathway, the identification of these genetic markers could be considered a high contribute to the individualization of chemotherapy in order to reduce related toxicities (Cheok et al., 2006). MTHFR is a key enzyme of the folate metabolic pathway. It catalyzes the conversion of 5,10-methylen-THF to 5-methyl-THF which is required for protein synthesis and nucleic acid methylation. Alterations of folate isoforms as a consequence of impaired MTHFR activity may have an important effect on the responsiveness of malignant and non-malignant cells to MTX. Several data stress the pharmacogenetic link of MTHFR genotype in chemotherapeutic treatments including MTX: Salazar et al found that ALL children with favorable MTHFR 677CC genotype treated with high doses MTX ( $5\text{g m}^{-2}$ ) had significantly better event-free survival (EFS) at 48 months than patients with MTHFR 677TT genotype treated with the classical MTX dose  $3\text{g m}^{-2}$  in a 24-h infusion (Salazar et al., 2012). Other studies showed that patients with MTHFR 677T/1298A haplotype had a poorer EFS, nevertheless different ALL protocols were used (Krajinovic et al., 2004); Aplenc et al showed that the MTHFR C677T polymorphism was associated with relapse

whilst, MTHFR A1298C polymorphism was not (Aplenc et al., 2005). One of the major adverse effects of MTX is nephrotoxicity. MTX and its metabolite 7-hydroxy-MTX precipitates in the renal tubules causing renal dysfunction (Yarlagadda et al., 2008). As a consequence, MTX excretion may be delayed, maintaining high plasma MTX levels, increasing in turn, toxicity. Haematological toxicities such as thrombocytopenia may also occur, due to high serum MTX level. Kantar et al in a recent study showed that childhood ALL patients with MTHFR A1298C polymorphism treated with high dose MTX, had significantly higher MTX levels at 48 h and haematological toxicities such as thrombocytopenia (Kantar et al., 2009). Recently, a systematic review by Lopez-Lopez et al concluded that today there is no evidence to support the use of either MTHFR C677T or the A1298C polymorphisms as MTX toxicity markers (Lopez-Lopez et al., 2013). It is clear that other polymorphisms involved in the folate pathway and other genetic and epigenetic mechanisms may contribute to the variability in treatment responses. DHFR plays a crucial role in the conversion of DHF to THF necessary for nucleic acid synthesis and methylation processes. It is considered a major MTX target and plays an important role in the development of MTX resistance in ALL. Several experimental and clinical settings were made, founding altered levels of DHFR and/or DHFR gene amplification in relapsed leukemia patients and in leukemia and colon cell lines with MTX-resistant phenotypes (Morales et al., 2009, Matherly et al., 1995, Serra et al., 2004, Goker et al., 1995). Moreover, Bertino et al showed that DHFR levels increase within hours in both normal leucocytes and acute leukemia cells after MTX treatment. MTX slowly dissociates from DHFR and survival cells continue to synthesize THF and proliferate (Bertino et al., 2009). Chu et al have demonstrated that DHFR translation is inhibited by binding of DHFR to its mRNA and this inhibition is relieved by MTX which binds to DHFR and leads to a conformational change in the protein (Chu et al., 1993). This mechanism thus, limits MTX effectiveness (Guo et al., 1999). Several years ago, a polymorphism in the 3' untranslated region (UTR) of DHFR was found. This SNP, a cytosine to thymine substitution at position 829 was associated with higher expression of DHFR transcript (Goto et al., 2001). Also Mishra et al showed that the presence of the SNP increased levels of DHFR mRNA, the amount of protein and resistance to MTX (Mishra et al., 2007). Alterations in DHFR expression levels and thus, in sensitivity to MTX, can be also caused by genetic polymorphisms in the promoter (Dulucq et al., 2008, Al-Shakfa et al., 2009). Dulucq et al, showed an association of individual polymorphisms and resulting haplotypes with ALL outcome, revealing a reduction in relapse-free survival with the presence of A and C alleles located at position 317 and 1610 upstream from a minor transcription initiation site, respectively (Dulucq et al., 2008). Thus, results are still scarce and inconclusive. However, the application of a tailored therapy would represent a huge advance in the treatment of childhood ALL and the identification of

pharmacogenetic markers in treatments will provide an improvement in risk classifications allowing an optimal and personalized therapy selection.

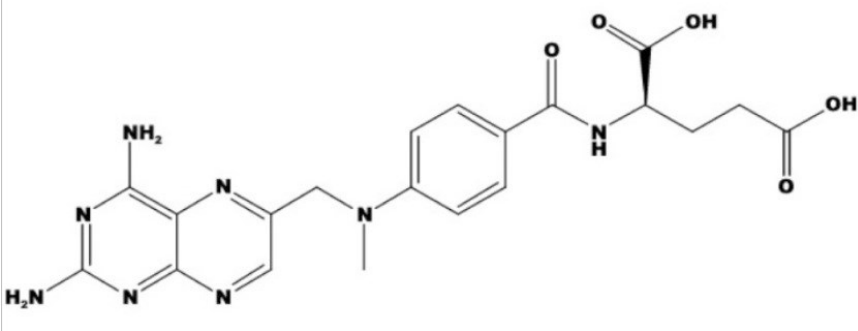


Fig.4 Methotrexate molecule.

## 1.5 AIM AND SCOPE

Acute lymphoblastic leukemia (ALL) is the most common pediatric cancer accounting for 80% of childhood leukemia. Different gene polymorphisms (most of them SNPs) play an important role in the susceptibility to childhood ALL which probably derives from a combination and relation of genetic and environmental factors. Folates are bioactive nutrients involved in several processes including DNA synthesis and methylation and purines synthesis. Polymorphisms in genes coding for enzymes of the folate metabolic pathway can alter the intracellular folate status or distribution. Several studies have investigated on the role of the genetic variants of the one carbon pathway and on the susceptibility to develop childhood ALL. It is to note that current treatment regimens achieve levels about 80% in overall survival (OS). Unfortunately, the side effects derived from the chemotherapeutic agents used can be severe, especially for high-risk patients. Therefore, the identification of additional markers which can improve risk stratification and individually tailored therapy regimens would be a great goal, in order to avoid over-treatment which can increase long-term adverse side effects.

The aim of the present study was, therefore, to investigate whether common polymorphisms (i.e. MTHFR C677T and A1298C in addition to DHFR 19 bp INS/DEL and Bcl-2 -938 C>A) might influence the risk of childhood ALL. For this purpose we genotyped by PCR 197 childhood ALL patients and 201 matched healthy controls for the DHFR and MTHFR gene polymorphisms hypothesizing their single/combined effect on cancer risk by means of intracellular folate isoforms unbalancing. Similarly, we genotyped the same samples for the -938 C>A polymorphism in the anti-apoptotic Bcl-2 gene, hypothesizing its effects on cancer risk by itself or in combination with the previous ones polymorphisms. Finally, other parameters such as disease onset, therapy duration and risk scoring, were stratified by the polymorphisms investigated.

In an exploratory way and to validate the proposed model we evaluated possible differences in cellular viability under MTX treatment, between healthy PBL cells harvested from subjects with opposite genotype condition considering DHFR and MTHFR genes. One of the most interesting facets of the relationship between folate status and cancer risk is the modulation of the one-carbon metabolism by key folate-metabolizing enzymes. The amount of intracellular THF is due to the relative balancing between DHFR and MTHFR activities: the first by production and filling activity, the second one by consumption and depletion activity. Changes in their activity resulting



from polymorphisms in DHFR and MTHFR genes could modify the susceptibility to cancer and cancer treatment. MTX is indeed an antifolate agent necessary in the maintenance therapy of ALL. It interferes with folate metabolism leading to depletion of reduced forms of THF (including 5,10-methylene-THF) and to inhibition of nucleic acid synthesis, favouring cell death. Therefore a paradox there exists when some polymorphisms are investigated among cancer populations: patients carrying specific gene variants may have reduced cancer risk but increased drug-related toxicity when they are treated with folate antagonists. The same allele could be considered, therefore a protective factor against cancer establishment and a risk factor for prognosis of the same disease. Genotyping of folate and apoptotic pathways' polymorphisms might be useful to assess the effects on childhood ALL susceptibility and to assess the effects of anti folate drug therapy on toxicity. Considering several parameters such as onset, therapy duration and risk stratification, the latter one could provide important information to clinicians to optimize MTX therapy, reducing the associated toxicity with possible effects on survival.

The proposed *in vitro* model could be considered a starting point for further investigations that could in part explain the reason why some gene conditions result protective towards the onset of cancer and at the same time risk factors for increased toxicity towards anti-folate therapy.

**2**

## **MATERIALS AND METHODS**

## 2.1 SELECTION OF CASES AND CONTROLS

Recruitment of childhood ALL cases consisted of 197 Italian and Egyptian individuals with newly diagnosed ALL according to the French-American-British classification. They were from the files of patients who received hematological diagnosis by the Unit of Pediatrics of University-Hospital of Ferrara (n= 3), by the Unit of Pediatrics of University-Hospital of Tanta, Egypt (n=20) and by the Unit of Pediatrics of Bari-Policlinico (n=174) in the period between January 2011 and January 2014. The whole group of ALL cases consisted of patients with an age range of 0.5 – 17.33 years with a mean age of  $10.76 \pm 5.68$  years. 38.6% were male. Among ALL cases, 86% were ALL-B and 14% were ALL-T. The control group (n= 201) consisted of Italian healthy individuals, who were not relatives of the cases and were randomly selected from the local blood donor lists in the period between January 2011 and January 2014. The age range was 1 – 21.5 years with a mean age of  $15.83 \pm 6.41$  and 42% were male. Peripheral blood samples for all cases were collected at the date of diagnosis before any pharmacological treatment and for controls at the moment of blood donation by venipuncture. Informed consent was obtained from donors and patients' parents.

Within the control group genotyped for MTHFR C677T and DHFR -19bp INS/DEL (W/D), 4 volunteer homozygotes 677TT/-19bp DD and 4 volunteer homozygotes 677CC/-19bp WW were selected for the cell culture protocol described in detail in the next chapter.

## 2.2 GENOTYPIC ANALYSIS

### 2.2.1 Extraction Protocol

Three mL peripheral blood samples, anticoagulated by EDTA were obtained. DNA was isolated and extracted by automated procedure with the instrument Bio Robot EZ1 (Qiagen, Milan, Italy). The DNA extraction kit (EZ1 DNA Blood mini kit 350 µl) is based on the magnetic microparticles technology (Fig.5). The steps include:

- i) Whole blood sample lysis
- ii) Link between nucleic acid (DNA) and magnetic nanoparticles
- iii) Nucleic acid wash and final elution

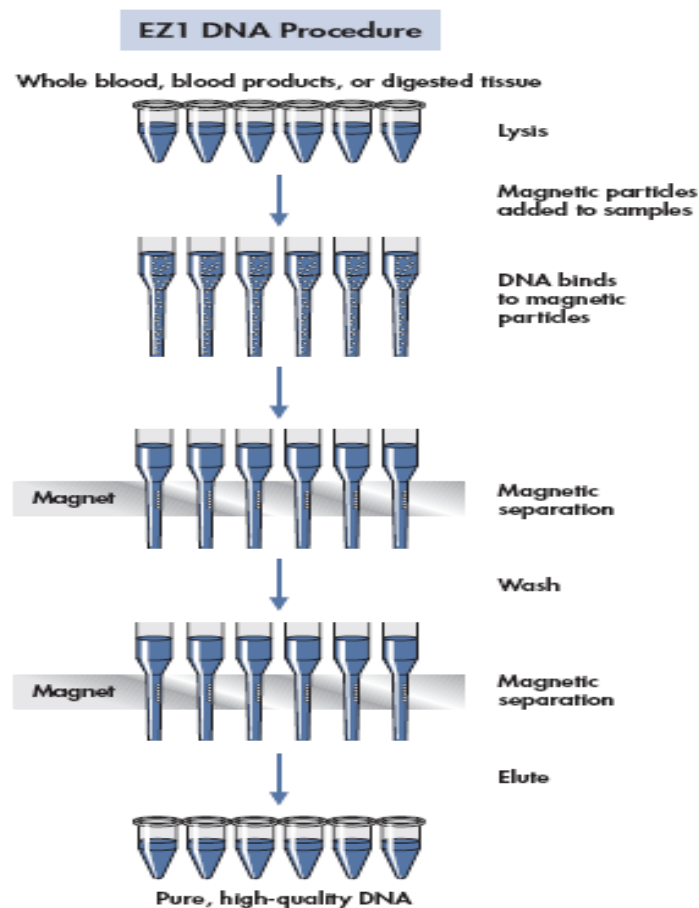


Fig.5 Steps of the DNA extraction protocol

## 2.2.2 Polymerase Chain Reaction (PCR)

PCR technique is a useful tool for the exponential amplification of a DNA fragment, and its principle is based on the mechanism of DNA replication in vivo: the double strand dsDNA is denatured to a single strand ssDNA, duplicated, and this process is repeated along the reaction according to the following formula:  $C = C_0(1+E)^n$  where,

C: final amount of DNA

C<sub>0</sub>: initial amount of DNA

E: efficiency

n: number of cycles

s: slope of the exponential phase.

During the denaturation step, the dsDNA melts opening up to ssDNA, and all enzymatic reactions stop. To DNA denaturation, the temperature is usually raised up to 93-96 °C, breaking the H-bonds and thus increasing the number of non-paired bases. The temperature at which half of the dsDNA is single-stranded is known as the melting temperature, T<sub>m</sub>. The second phase, i.e. annealing of primers to ssDNA, takes place at temperatures closer to their T<sub>m</sub> (usually 55-65 °C) and is named as temperature of annealing (T<sub>a</sub>). The oligonucleotides used as primers typically consist of relatively short sequences (15-25 nt) complementary to recognition sites, flanking the segment of target DNA to be amplified. Once the temperature is reduced, the two complementary ssDNA chains tend to rehybridise into a dsDNA molecule. If primers adequately anneal to the template, the ionic bond is strong enough between the template and the primer to stabilize the nascent double stranded structure and allow the polymerase to attach and begin copying the template. The extension phase is carried out across the target sequence by using a heat-stable DNA polymerase in the presence of dNTPs and MgCl<sub>2</sub>, resulting in a duplication of the starting target material. This enzyme has 5' → 3' DNA polymerase activity, i.e. it adds dNTPs from 5' to 3', reading the template from 3' to 5'. After each cycle, the newly synthesized DNA strands can serve as template in the next cycle. Potentially, after 30 PCR cycles there will be around 230-fold amplification, assuming 100 % efficiency during each cycle. The efficiency of a PCR will vary from template to template and according to the degree of optimization that has been carried out.

### **2.2.3 Genotype protocols**

#### **MTHFR C677T**

PCR amplification was performed using specific Forward and Reverse primers (Table??) and the PCR conditions were as follows: initial denaturation at 95°C for 5 minutes, followed by 30 cycles of 45 seconds at 94°C, 60 seconds at 65°C, 90 seconds at 72°C and a last elongation step for 10 minutes at 72°C, adapted from Frosst et al (Frosst et al., 1995).

The amplicon was digested over night at a temperature of 65°C with restriction enzyme Taq I (New England BioLabs, Beverly, MA), according to the manufacturer's instructions.

The amplified, digested product was separated on 10% acrylamide gel at 120V for 2 hours and half and stained with Ethidium Bromide. The obtained band sizes are shown in Table 2.

#### **MTHFR A1298C**

PCR amplification was performed using specific Forward and Reverse primers (Table??) and the PCR conditions were as follows: initial denaturation at 95°C for 5 minutes, followed by 30 cycles of 30 seconds at 94°C, 13 seconds at 59°C, 17 seconds at 72°C and a last elongation step for 10 minutes at 72°C, adapted from Weisberg et al (Weisberg et al., 1998).

The amplicon was digested over night at a temperature of 37°C with restriction enzyme Fnu4HI (New England BioLabs, Beverly, MA), according to the manufacturer's instructions.

The amplified, digested product was separated on 10% acrylamide gel at 120V for 2 hours and half and stained with Ethidium Bromide. The obtained band sizes are shown in Table 2.

### **DHFR 19 bp INS/DEL**

The genotyping protocol to detect the DHFR 19 bp deletion polymorphism was adapted from Johnson et al. (Johnson et al., 2004). The deleted and non-deleted alleles were genotyped by using a system of three specific primers: two different allele-specific forward primers, respectively for the non-deleted and deleted polymorphic alleles and one common reverse primer. The PCR conditions were as follows: : initial denaturation at 95°C for 5 minutes, followed by 30 cycles of 50 seconds at 94°C, 50 seconds at 62°C, 50 seconds at 72°C and a last elongation step for 10 minutes at 72°C.

PCR products were analyzed by 11% PAGE Ethidium Bromide stained, to distinguish the two different alleles (non-deleted allele, 113 bp and deleted allele 92 bp) (Table 2).

### **Bcl-2 -938 C/A**

PCR amplification was performed using specific Forward and Reverse primers (Table 2) and the PCR conditions were as follows: initial denaturation at 96°C for 5 minutes, followed by 35 cycles of 45 seconds at 96°C, 40 seconds at 56°C, 30 seconds at 72°C and a last elongation step for 10 minutes at 72°C, adapted from Chen et al (Chen et al., 2007).

The amplicon was digested over night at a temperature of 37°C with restriction enzyme Bcc I (New England BioLabs, Beverly, MA), according to the manufacturer's instructions.

The amplified, digested product was separated on 10% acrylamide gel at 120V for 2 hours and half and stained with Ethidium Bromide. The obtained band sizes are shown in Table 2.

All PCR cycles were performed in an Agilent Thermal Cycler apparatus (Sure Cycler 8800, Agilent Technologies). Confirmation of genotypes was carried out by randomly re-genotyping cases and controls for all the polymorphisms investigated and DNA samples known for all the genotype conditions were used as internal controls. There were no discrepancies between genotypes determined in duplicate and compared with controls.

**Table 2** Primer Sequences

PRIMER	SEQUENZA	AMPLICON SIZE	BAND SIZE
MTHFR C677T F MTHFR C677T R	5'-TGA AGG AGA AGG TGT CTG CGG GA-3' 5'-AGG ACG GTG CGG TGA GAG TG-3'	233 bp (C)	175bp (T) 58bp (T)
MTHFR A1298C F MTHFR A1298C R	5'-CTT TGG GGA GCT GAA GGA CTA CTA C-3' 5'-CAC TTT GTG ACC ATT CCG GTT TG-3'	138 bp(A)	119bp (C) 19bp (C)
DHFR-19bp INS/DEL F1 DHFR-19bp INS/DEL F2 DHFR-19bp INS/DEL R	5'-CCA CGG TCG GGG TAC CTG GG-3' 5'-ACG GTC GGG GTG GCC GAC TC-3' 5'-AAA AGG GGA ATC CAG TCG G-3'	113bp(W)	92bp (D)
Bcl-2 -938 F Bcl-2 -938 R	5'- CTGCCTTCATTTATCCAGCA-3' 5'-GGCGGCAGATGAATTACAA -3'	300bp(C)	189,111 (A) 300 (C)

**Table 3** Components for MTHFR C677T PCR

Genomic DNA	100ng
10X Buffer PCR	2,5 µl
MgCl <sub>2</sub>	1,5 mM
Primer 5'	0,25 mM
Primer 3'	0,25 mM
DNTPS	1,66 mM
Taq Roche [5u/µl]	2U/A
H <sub>2</sub> O	-



**Table 4** Components for MTHFR A1298C PCR

Genomic DNA	100 ng
10X Buffer PCR	2,5 µl
MgCl <sub>2</sub>	1,5 mM
Primer 5'	0,25 mM
Primer 3'	0,25 mM
DNTPS	1,66 mM
Taq Roche [5u/µl]	2U/A
H <sub>2</sub> O	-

**Table 5** Components for DHFR 19 bp INS/DEL

Genomic DNA	100 ng
10X Buffer PCR	2,5 µl
MgCl <sub>2</sub>	1,5 mM
Primer Forward1	0,25 mM
Primer Forward2	0,25 mM
Primer Reverse	0,25 mM
DNTPS	1,66 mM
DMSO	2%
Taq Roche [5u/µl]	2U/A
H <sub>2</sub> O	-

**Table 6** Components for Bcl-2 -938 C>A

Genomic DNA	100 ng
10X Buffer PCR	2,5 µl
MgCl <sub>2</sub>	1,5 mM
Primer 5'	0,25 mM
Primer 3'	0,25 mM
DNTPS	1,66 mM
Taq Roche [5u/µl]	2U/A
H <sub>2</sub> O	-

## 2.2.4 Cell preparation and culture

Within the control group genotyped for MTHFR C677T and DHFR -19bp INS/DEL (W/D), 4 volunteers homozygotes 677TT/-19bp DD and 4 volunteers homozygotes 677CC/-19bp WW were selected. Peripheral Blood was collected from healthy donors in the presence of sodium citrate by venipuncture. Peripheral Blood Lymphocytes (PBL) were isolated by centrifugation on a layer of Histopaque® (Sigma Chemical Co.). PBL were resuspended in Rosewell Park Memorial Institute culture medium (RPMI 1640; Sigma Chemical Co.), supplemented with 10% FCS, 2 mM L-glutamine and antibiotics (penicillin 100 U/ml, streptomycin 100 mg/ml). Cultures were maintained at 37°C in a humid atmosphere containing 5% CO<sub>2</sub> and PBL were activated by PHA (5µg/ml) for 48 hours and then dead cells were removed by centrifugation on a layer of Histopaque® (Sigma Chemical Co.), whilst PHA-activated PBL were harvested, washed in PBS, and then resuspended in Rosewell Park Memorial Institute culture medium (RPMI 1640; Sigma Chemical Co.) and incubated in 96-well micro-plates (7,5x10<sup>5</sup>/ml) (Costar, Cambridge, MA) with MTX 100 µM together with folic acid (1mg/L) for 5/6 days. Cells were cultured in duplicate and every 24 hours, cell count and MTT viability assay were performed.

### **MTT Viability Assay (Roche Diagnostic GmbH, Germany):**

The assay is based on the cleavage of the yellow tetrazolium salt MTT to purple formazan crystals by metabolic active cells (Vistica et al., 1991). The formazan crystals formed are solubilized and the resulting colored solution is quantified using a scanning multiwell spectrophotometer (ELISA reader) with a wavelength to measure absorbance of the formazan product of 550nm.

Stage 1: cells grown in a 96 well culture plate are incubated with the yellow MTT solution for approximately 4 hours;

Stage 2: after the incubation period, purple formazan salt is formed and it can be solubilized by adding the solubilization solution (10% SDS, 0.01 M HCl) and incubating the plate overnight in humidified atmosphere;

Stage 3: the solubilized formazan product is spectrophotometrically quantified using an ELISA reader. An increase in number of living cells results in an increase in the total metabolic activity in the sample.

## 2.3 STATISTICAL ANALYSIS

Statistical differences between case and control populations were tested using  $\chi^2$  and Student's tests. Where appropriate, Yates' correction or Fisher's exact test were applied.  $P \leq 0.05$  was considered statistically significant. Odds ratios (OR) and 95% confidence intervals (95% CI) were used to estimate the risk of developing childhood ALL. Adjusted ORs for single or combined comparisons were calculated with logistic regression models, using the homozygous wild-type genotypes as the reference group. The expected frequency of control genotypes were checked by the Hardy-Weinberg equilibrium test.

In a subgroup of cases coming from the Unit of Pediatrics of Bari University (n=141) Kaplan-Meier survival analyses and the log-rank test were used to evaluate the relationship between onset, duration therapy and MTHFR A1298C and Bcl-2 genotypes corroborated also by Hazard Risk (HR) calculation. The duration therapy was calculated from the date of first diagnosis until the date off therapy. All analyses were performed by Systat V.5.0 (Systat Inc., Evanston, IL) and SPSS Statistical Package (SPSS Inc., Chicago, IL).

**3**

## **RESULTS**

### 3.1 GENOTYPIC ANALYSIS OF MTHFR, DHFR, BCL-2 POLYMORPHISMS

#### 3.1.1 Clinical-pathological characteristics of cases

**Table 7**

<b>BARI ALL CASES</b>	
<b>Total number</b>	141
<b>Mean age (yy) ± SD</b>	10.76yy ± 5.68
<b>Range age (yy)</b>	(0.5 – 17.33)
<b>Gender (male/female)</b>	76/68
<b>Mean Onset (yy) ± SD</b>	6.55yy ± 4.3
<b>Range Onset (yy)</b>	(0.5 – 17.33)
<b>ALL Phenotype (%)</b>	
B	121 (83.5%)
T	16 (11.4%)
<b>Translocation (%)</b>	
YES	30 (21%)
NO	95 (66%)
NP	19 (13%)
<b>Risk Therapy (%)</b>	
HR	19 (13%)
MR	88 (62%)
SR	36 (25%)
<b>Mean Therapy Duration (dd)± SD</b>	634.836 dd ± 263.16
<b>Range Therapy Duration (dd)</b>	(180 – 1855)

Table 7 presents the patient's characteristics of the principal subgroup of samples from the Unit of Pediatrics of Bari-Policlinico. HR refers to High Risk group due to poor response to therapy; MR refers to Median Risk group due to uncertain response; SR refers to Standard Risk group due to better response to therapy. NP referred to analysis Not Performed; (yy) referred to age and age onset expressed as years; (dd) referred to mean therapy duration expressed as days.

### **3.1.2 Evaluation of childhood ALL risk: single analysis**

Table 8 shows the genotype frequencies of childhood ALL cases and healthy controls and the risk evaluation for the four polymorphisms investigated. Considering the MTHFR C677T polymorphism, it was noted an under-representation of the polymorphic allele yielding a significant risk reduction, computing T-allele carrier cases versus controls (OR 0.66; 95%CI 0.44-0.99; P=0.046). Considering the MTHFR A1298C polymorphism, although it did not reach statistical significance, it is important to note an over-representation of the polymorphic C allele, that yielded an increased risk of ALL combining together CC homozygotes and AC heterozygotes versus AA wild-type genotype. As regards the Bcl-2 -938 C>A polymorphism, it is to note a significant over-representation of the polymorphic A allele, that yielded an increased risk of childhood ALL combining together AA homozygotes and CA heterozygotes versus CC wild-type genotype (OR 1.5; 95%CI 1-2.26; P=0.049). Similarly, analyzing the frequency of the allele distribution, a significant border-line result was observed for MTHFR 677-T allele, according to the under-representation of the polymorphism among cases (OR 0.77; 95%CI 0.57-1.02; P=0.07). No significant results were obtained regarding the genotypic distribution.

	<b>DHFR -19bp INS/DEL</b> (tot <sub>cases</sub> =195)(tot <sub>Contr</sub> =201)			<b>MTHFR C677T</b> (tot <sub>cases</sub> =195)(tot <sub>Contr</sub> =201)			<b>MTHFR A1298C</b> (tot <sub>cases</sub> =197)(tot <sub>Contr</sub> =201)			<b>Bcl-2 -938 C&gt;A</b> (tot <sub>cases</sub> =197)(tot <sub>Contr</sub> =197)		
	WW(%)	WD (%)	DD (%)	CC (%)	CT (%)	TT (%)	AA (%)	AC (%)	CC (%)	CC (%)	CA (%)	AA(%)
<b>CASES</b>	86(44%)	84(43%)	25(13%)	86(44%)	81(41%)	28(14%)	84(42%)	82(41%)	31(15%)	66(33%)	87(44%)	44(22%)
<b>CONTROLS</b>	84(42%)	84(42%)	33(16%)	69(34%)	98(48%)	34(16%)	101(50%)	70(34%)	30(15%)	85(43%)	72(36%)	40 (20%)
<b><i>P-value</i></b> <b><i>(genotypic distribution)</i></b>	NS			NS			NS			NS		
OR <sub>(++/+ vs --)</sub>	0.9 (0.6-1.35)			0.66 (0.44-0.99)			1.35 (0.91-2.01)			1.5 (1-2.26)		
<b><i>P-value</i></b>	NS			<b>0.046</b>			NS			<b>0.049</b>		
OR <sub>(++ vs other genotypes)</sub>	0.7 (0.42-1.31)			0.8 (0.47-1.41)			1.06 (0.61-1.836)			1.12 (0.69-1.82)		
<b><i>P-value</i></b>	NS			NS			NS			NS		
<b>ALLELS</b>	<b>W</b>		<b>D</b>	<b>C</b>		<b>T</b>	<b>A</b>		<b>C</b>	<b>C</b>		<b>A</b>
<b>CASES</b>	256 (65%)		134 (33%)	253 (64%)		137 (35%)	250 (63%)		144 (36%)	219 (55%)		175 (44%)
<b>CONTROLS</b>	252 (62%)		150 (37%)	236 (58%)		166 (41%)	272 (67%)		230 (32%)	242 (61%)		152 (38%)
OR <sub>(+vs-)</sub>	0.87 (0.65-1.17)			0.77 (0.57-1.02)			1.2 (0.89-1.61)			1.270.96-1.69)		
<b><i>P-value</i></b>	NS			<b>0.07</b>			NS			NS		

**Table 8**

### 3.1.3 Evaluation of childhood ALL risk (MTHFR C677T, DHFR): combined analysis

Table 9 shows the combined results for DHFR and MTHFR C677T polymorphisms. All the possible genotype combinations were considered: only the appreciable results were showed. Interestingly, the DHFR-WW/MTHFR-TT genotype combination yielded a significant 3.3-fold risk reduction (OR 0.3; 95%CI 0.11-0.8; P=0.01). Every combination of alleles was computed considering subjects with DHFR WW- and MTHFR CC-genotypes as the reference. The evaluation of the combined risk for double homozygotes (i.e. DHFR DD/MTHFR TT), however, did not show statistical significance, probably for the lower number of subjects computed in the analysis.

Genotype		Cases n = 195 (%)	Controls n = 201 (%)	OR (95% CI)	P
DHFR	MTHFR				
WW	CC	47 (24)	34 (17)	Reference	
WD	CT	39 (20)	48 (24)	0.58 (0.3-1.08)	0.08
DD	CT	10 (5.1)	17 (8.5)	0.42 (0.17-1.04)	0.06
WW	TT	7 (3.6)	17 (8.5)	0.3 (0.11-0.8)	<b>0.01</b>

**Table 9**



### 3.1.4 Disease onset and different polymorphisms.

Tables 10-13 show the stratification of the mean age disease onset by the three different genotype conditions, among the four polymorphisms examined. For all the tables 10-13, the mean age disease onset is expressed as years (yy).

We found significant results only for MTHFR A1298C polymorphism (Table12). Homozygotes CC patients had a significant higher age disease onset when compared to homozygotes AA and heterozygotes AC. An appreciable result, even though not significant, was obtained comparing homozygotes CC patients towards homozygotes AA patients (P=0.07). Interestingly, no homozygotes CC patients developed disease before 37 months, respect to the earlier age disease onset observed among the remaining genotypes (respectively, 6 months for homozygotes AA and 12.6 months for heterozygotes AC).

Genotype DHFR -19bp INS/DEL (W/D)			
	<b>WW (n=55)</b>	<b>WD (n=66)</b>	<b>DD (n=20)</b>
Mean Onset ± SD (yy)	7.12 ± 4.58	5.57 ± 3.37	7.38 ± 4.5
Range (yy)	(1.05 – 16.58)	(1.41 – 17.16)	(0.5 – 17.33)
$P_{DDvsWW}$		0.4	
$P_{DDvsWD+WW}$		0.17	
$P_{WWvsWD+DD}$		0.19	

**Table 10**

Genotype MTHFR C677T			
	CC (n=58)	CT (n=60)	TT (n=23)
Mean Onset ± SD	6.51 ± 4.37	6.46 ± 4.4	6.9 ± 3.9
Range (yy)	(0.5 – 17.16)	(1.05 – 17.33)	(2 – 15.66)
P <sub>CCvsTT</sub>		0.33	
P <sub>CCvsCT+TT</sub>		0.45	
P <sub>TTvsCT+CC</sub>		0.32	

**Table 11**

Genotype MTHFR A1298C			
	AA (n=58)	AC (n=63)	CC (n=20)
Mean Onset ± SD (yy)	6.31 ± 4.4	6.3 ± 3.9	8.0 ± 4.9
Range (yy)	(0.5 – 17.33)	(1.05 – 17.16)	(2.25 – 15.75)
P <sub>AAvsCC</sub>		<b>0.07</b>	
P <sub>CCvsAC+AA</sub>		<b>0.05</b>	
P <sub>AAvsAC+CC</sub>		0.28	

**Table 12**

Genotype Bcl-2 -938 C>A			
	CC (n=46)	CA (n=59)	AA (n=36)
Mean Onset ± SD (yy)	6.9 ± 4.5	6.6 ± 3.9	5.9 ± 4.6
Range (yy)	(1.41 – 16.91)	(2 – 17.16)	(0.5 – 17.33)
P <sub>CCvsAA</sub>		0.16	
P <sub>CCvsCA+AA</sub>		0.24	
P <sub>AAvsCA+CC</sub>		0.15	

**Table 13**

### 3.1.5 Therapy duration and different polymorphisms

Tables 14-17 show the stratification of the mean therapy duration by the three different genotypes conditions, among the four polymorphisms examined. For all tables 14-17, the mean therapy duration is expressed as days (dd).

We found a significant association for MTHFR A1298C polymorphism (Table 16) and Bcl-2 -938 C>A polymorphism (Table 17). As regard to the MTHFR A1298C polymorphism, CC homozygotes showed a slight higher mean therapy duration (651.75dd ± 204.16dd) when compared with the mean therapy duration of the heterozygotes AC computed together with homozygotes AA (P=0.05, respectively 589.5dd ± 255.07dd and 651.75dd ± 204.16dd).

As regard to Bcl-2 -938 C>A polymorphism, interesting results were obtained comparing the two homozygous conditions: -938-CC versus -938-AA (P=0.03). Comparing the AA homozygotes with heterozygotes AC computed together with homozygotes CC we noted interesting results (P=0.056), whereas a borderline result was obtained from the comparison of CC homozygotes with AC heterozygotes computed together with AA homozygotes (P=0.061) (Table 17).

Genotype DHFR -19bp INS/DEL (W/D)			
	<b>WW (n=55)</b>	<b>WD (n=66)</b>	<b>DD (n=20)</b>
Mean Therapy Duration $\pm$ SD (dd)	621.2 $\pm$ 269.94	643 $\pm$ 223.9	657 $\pm$ 342.2
Range (dd)	(210 – 1155)	(180 – 1520)	(210 – 1855)
$P_{WWvsDD}$		0.3	
$P_{DDvsWD+WW}$		0.34	
$P_{WWvsWD+DD}$		0.36	

**Table 14**

Genotype MTHFR C677T			
	<b>CC (n=58)</b>	<b>CT (n=60)</b>	<b>TT (n=23)</b>
Mean Therapy Duration $\pm$ SD (dd)	663.53 $\pm$ 279.08	615.23 $\pm$ 264.18	613.6 $\pm$ 219.2
Range (dd)	(180 – 1855)	(180 – 1520)	(180 – 760)
$P_{CCvsTT}$		0.19	
$P_{CCvsCT+TT}$		0.14	
$P_{TTvsCT+CC}$		0.13	

**Table 15**

Genotype MTHFR A1298C			
	AA (n=58)	AC (n=63)	CC (n=20)
Mean Therapy Duration $\pm$ SD (dd)	678.24 $\pm$ 284.86	589.5 $\pm$ 255.07	651.75 $\pm$ 204.16
Range (dd)	(180 – 1855)	(180 – 1305)	(210 – 850)
$P_{AAvsCC}$		0.32	
$P_{CCvsAC+AA}$		0.35	
$P_{AAvsAC+CC}$		<b>&lt; 0.05</b>	

**Table 16**

Genotype Bcl-2 -938 C>A			
	CC (n=46)	CA (n=59)	AA (n=36)
Mean Therapy Duration $\pm$ SD (dd)	588 $\pm$ 229.07	634.3 $\pm$ 268.35	694.72 $\pm$ 289.34
Range (dd)	(210 – 760)	(180 – 1520)	(180 – 1855)
% Genotype <760 dd	46 (100%)	55 (93.2%)	32 (88.8%)
% Genotype <730 dd	45 (97%)	51 (86%)	31 (86%)
$P_{AAvsCC}$		<b>0.033</b>	
$P_{CCvsCA+AA}$		<b>0.061</b>	
$P_{AAvsCA+CC}$		<b>0.056</b>	

**Table 17**

### 3.1.6 Kaplan-Meier analysis. Correlation with MTHFR A1298C polymorphism

Figure 6 shows the stratification of the age disease onset of single patients among the three genotype conditions of MTHFR A1298C polymorphism. For all figures 6-8, the X-axis expresses the onset age as years (yy); the Y-axis expresses the survival probability as percentage (%). It is to note that the CC homozygotes had an appreciable different behavior in the establishment of the disease when compared with the survival curves of AC heterozygotes and AA homozygotes, which completely overlapped (HR 1.2868; P=0.5175). This is the reason why we combined together AA homozygotes and AC heterozygotes and compared them with CC homozygotes patients (Fig.7), showing an appreciable improvement in the significance (HR 1.3; P=0.2).

In addition, we divided the whole onset period (0.5yy – 17.33yy) by tertiles: the first tertile in the range 0.5yy – 7.25yy; the second tertile in the range 7.26yy – 12.83yy; the third tertile >12.83yy. Interestingly, the sub-analysis by tertiles, ascribed to MTHFR 1298CC homozygotes a significant later onset compared with AA homozygotes together with AC heterozygotes, when the third tertile is considered (HR 1.6; 95%CI 1.0206-2.5395) (Fig.8), which correspond to a contextual risk reduction.

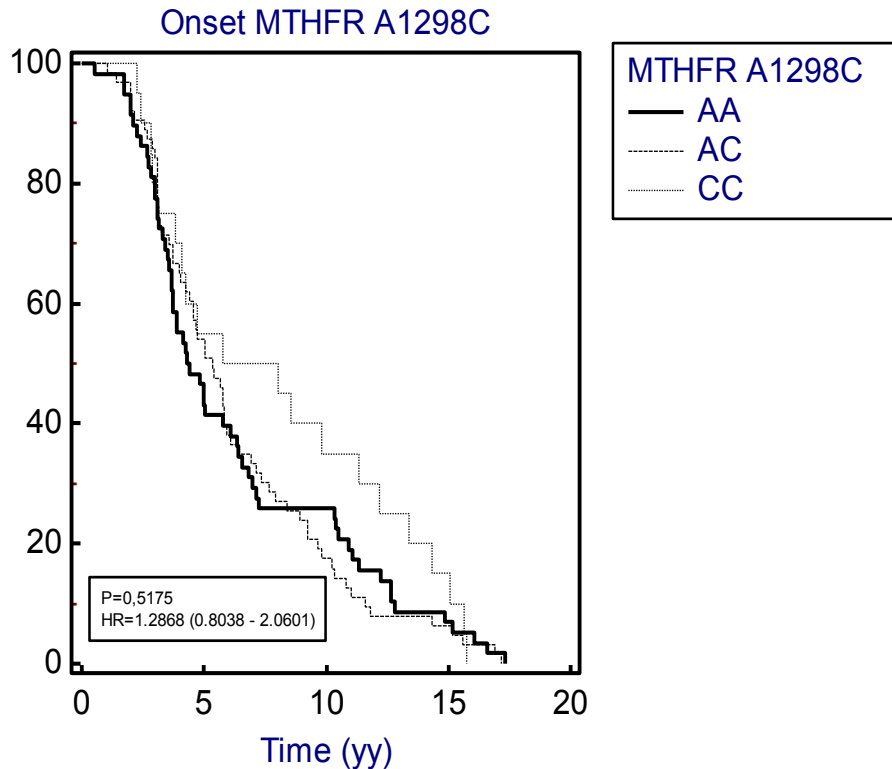


Fig.6

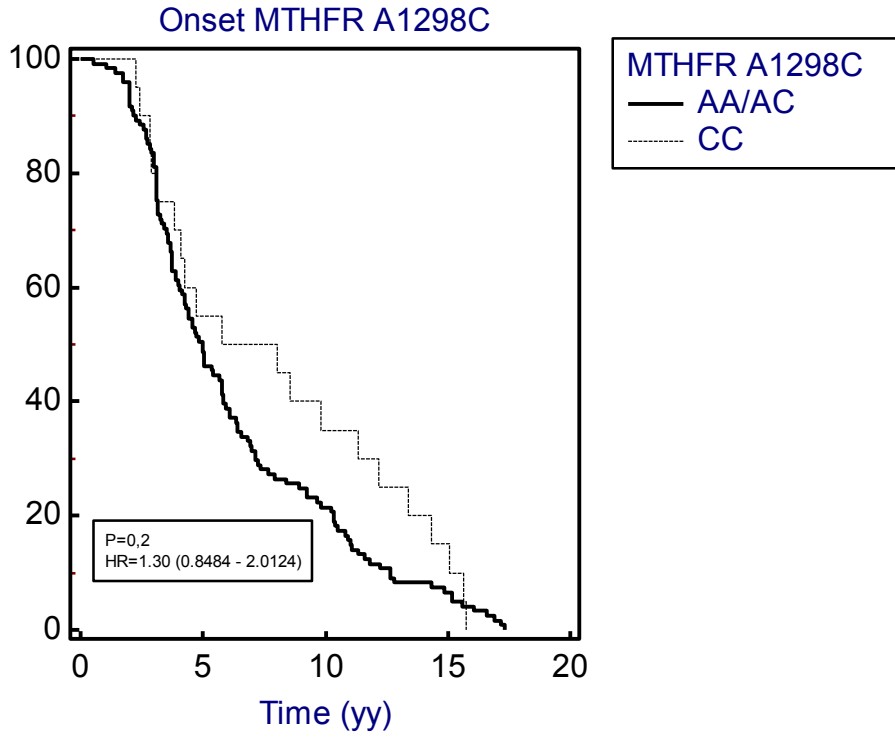


Fig.7

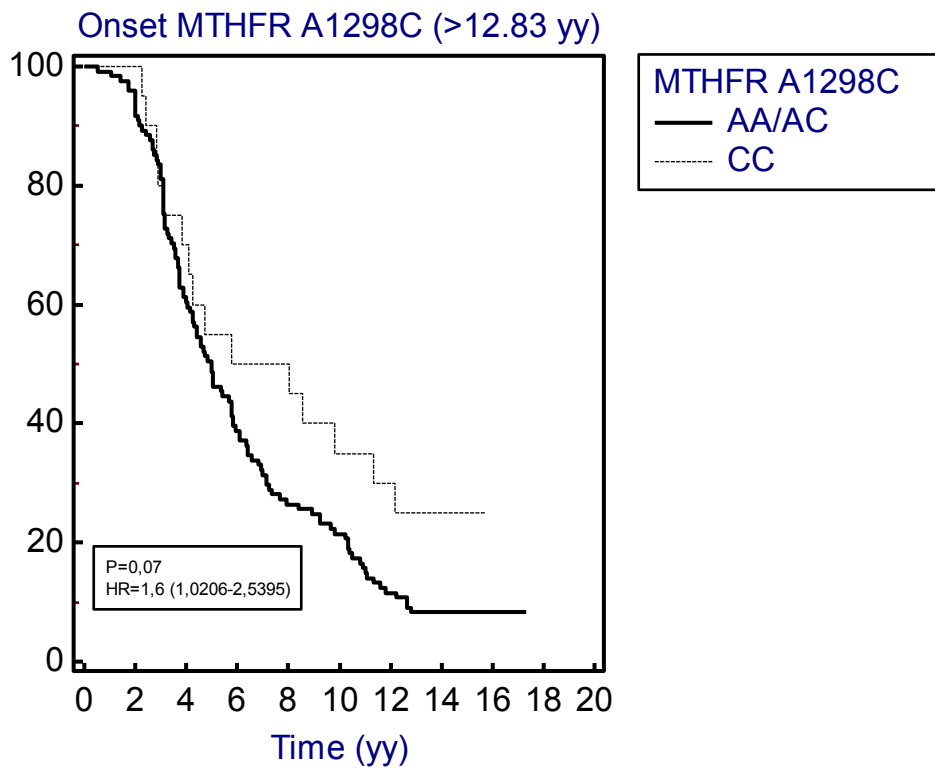


Fig.8

### 3.1.7 Kaplan-Meier analysis. Correlation with Bcl-2 -938 C>A polymorphism

Figure 9 shows the stratification of the therapy duration time of single patients among the three genotype conditions of Bcl-2 -938 C>A polymorphism. Figure 10 shows an allargement of follow-up period less than 2 years.

Interestingly, in a sub-analysis of the follow-up period less than 2 years, we noted an overlapping of the CC homozygotes and CA heterozygotes curves, whereas the AA homozygotes curve strongly diverged. This is the reason why we merged together the CC homozygotes and the CA heterozygotes (Fig. 11). Considering those patients who stopped therapy within two years, the AA patients were over-represented among those with longer therapy (HR 1.3; P=0.07) (Fig. 12). Finally, comparing the opposite homozygotes Bcl-2 -938CC versus -938AA, the risk of longer therapy duration ascribed to the -938AA genotype further increases, becoming significant (HR 1.4; P=0.03) (Fig.13-14, respectively full follow-up period and 2-years follow-up period).

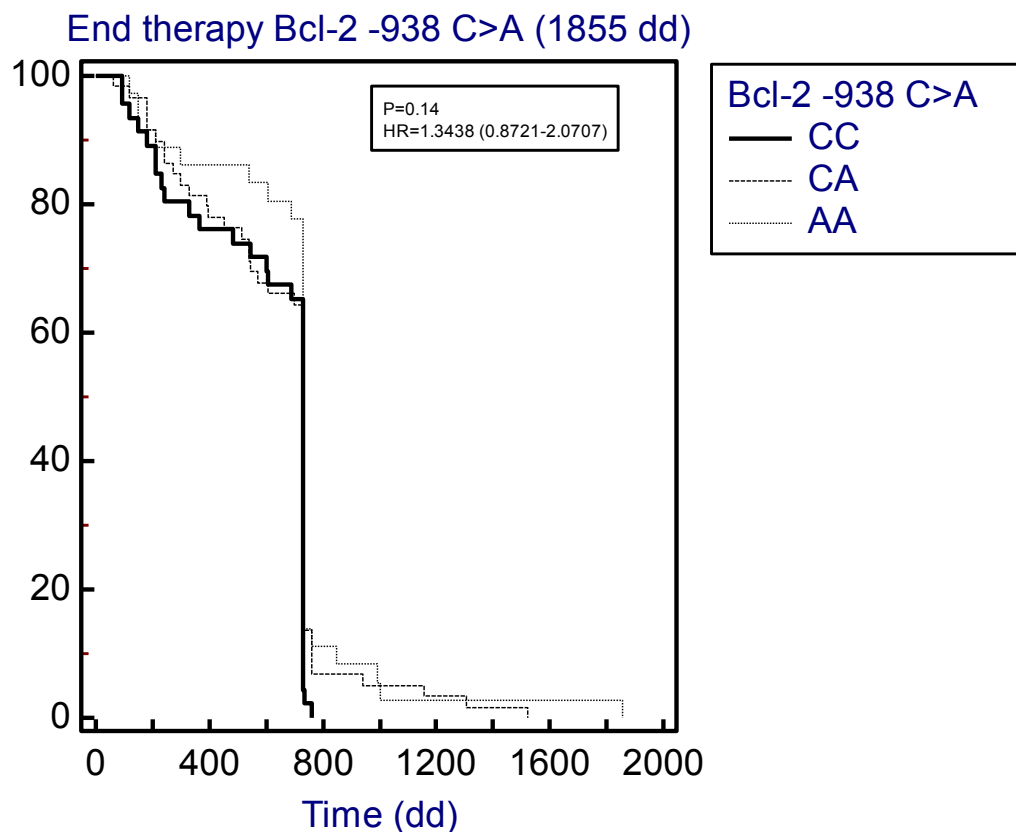


Fig.9



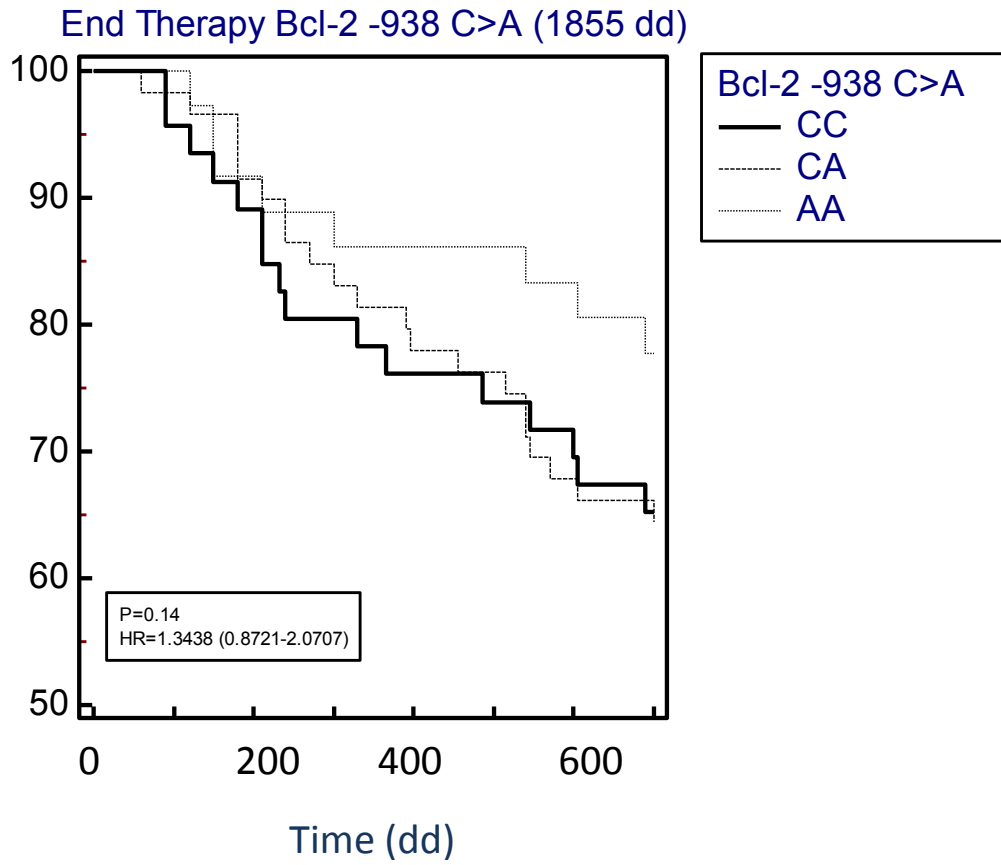


Fig.10

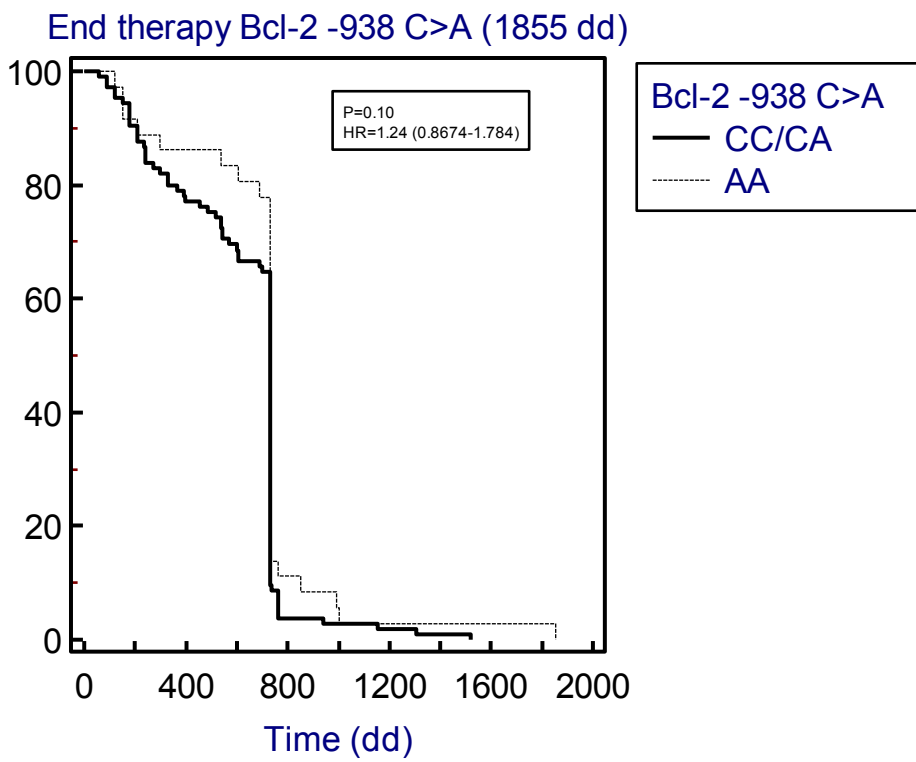


Fig.11

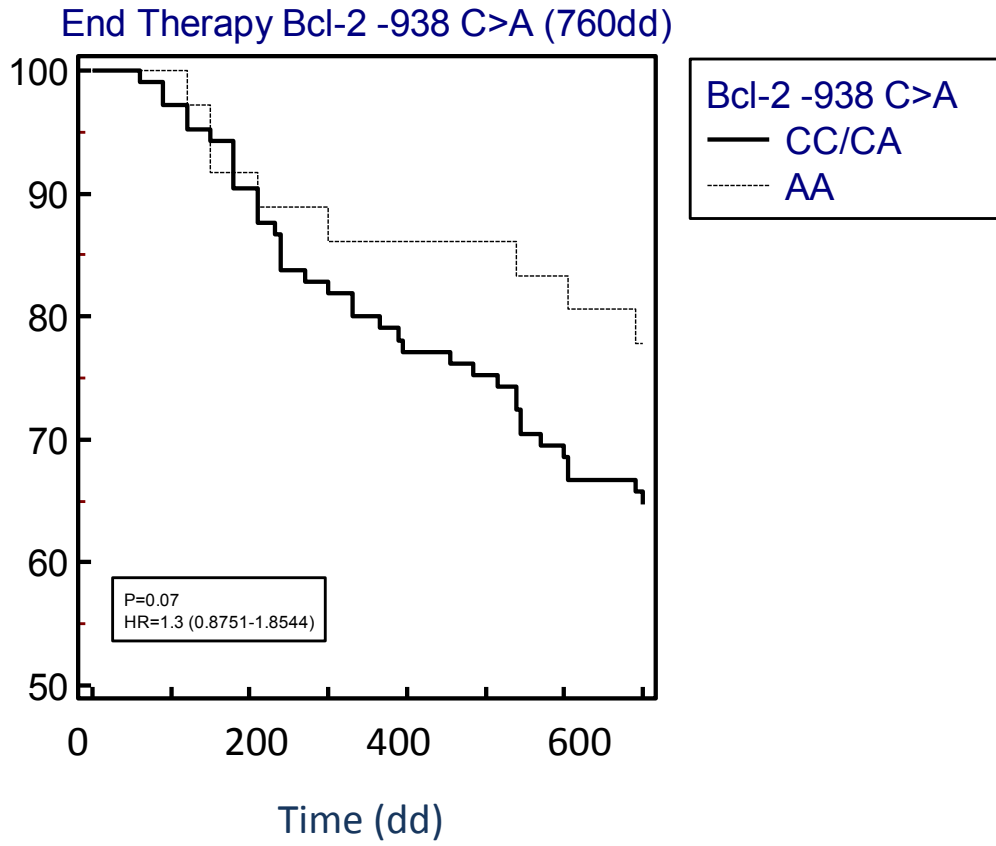


Fig.12

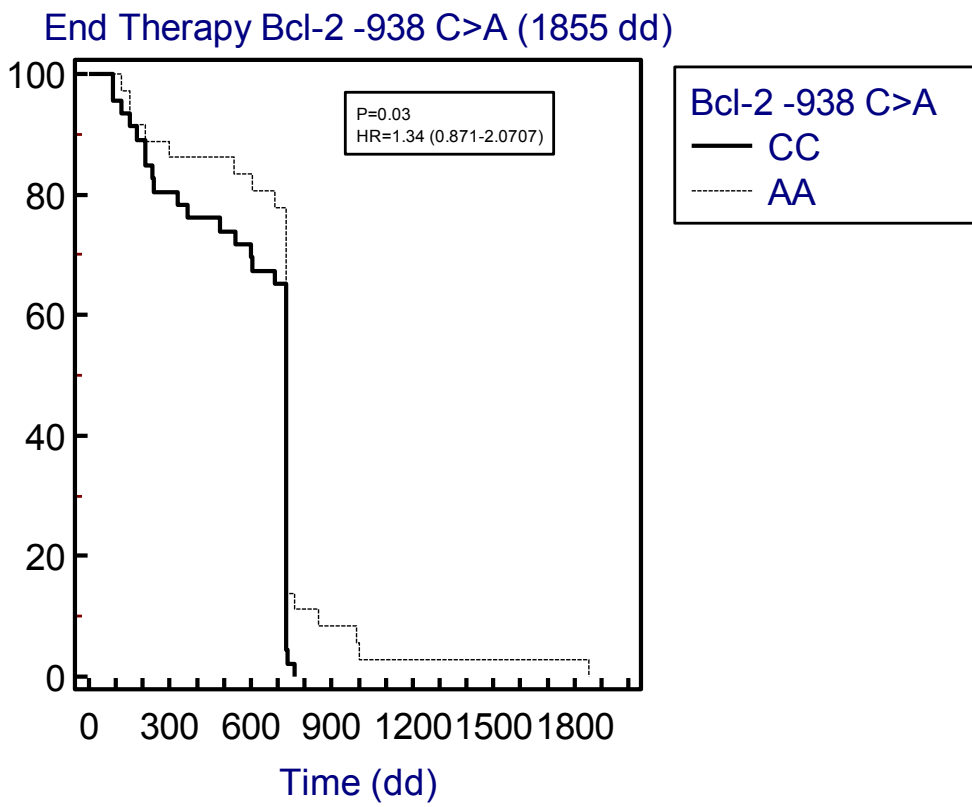
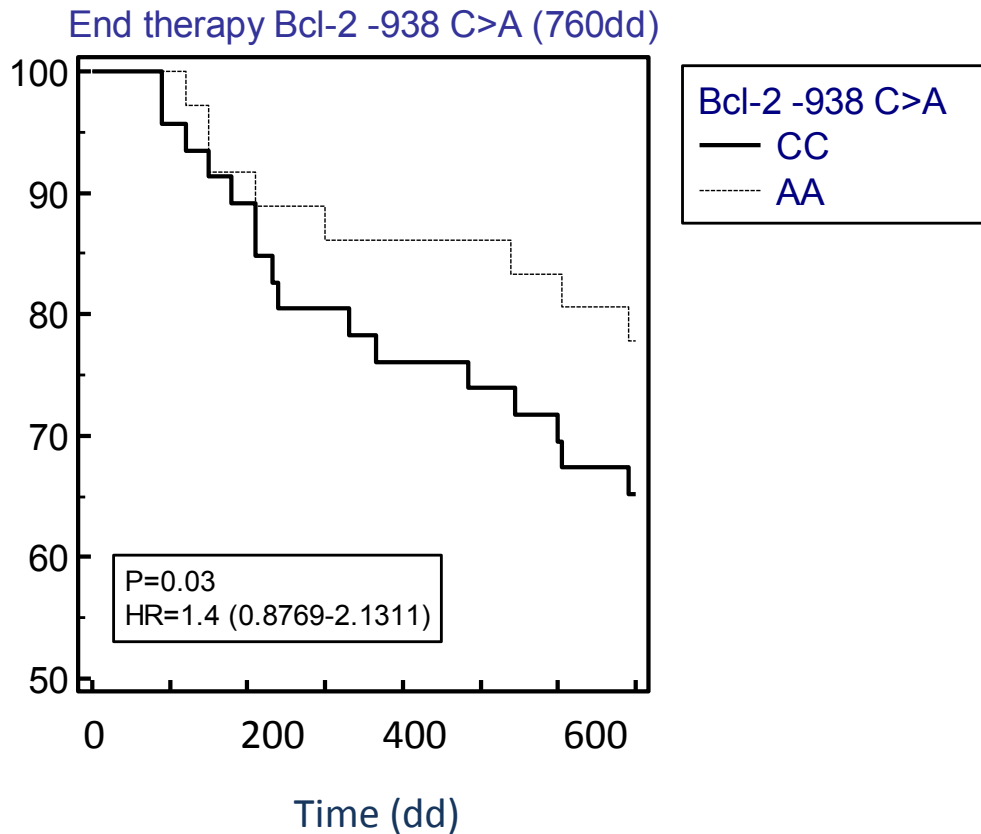


Fig.13



**Fig.14**

### 3.1.8 Folate and anti-folate: the in vitro cellular model

Figures 15 and 16 show the kinetics of cellular viability in the two opposite conditions (wild-type homozygotes DHFR WW/MTHFR CC and polymorphic homozygotes DHFR DD/MTHFR TT) when the respective PBL are treated or not with 100 $\mu$ M MTX. After an activation with PHA for 48 h, having the same basic culture conditions, the same pharmacological treatment and the same starting number of cells (1.5 x10<sup>5</sup>cells/ml) for both genotypic conditions, the number of cells monitored for 5 days, showed that WW/CC were mainly higher (see Tables 18 and 19). It is to note that this gap increased over the five days since to reach a 1.4-fold higher at t5, ascribable to WW/CC genotype. Considering the cellular viability after the MTX treatment we can observe that the absolute number of residual viability was higher among WW/CC respect to viable DD/TT cells for each day considered (Fig.17). This result was confirmed also considering the global treated

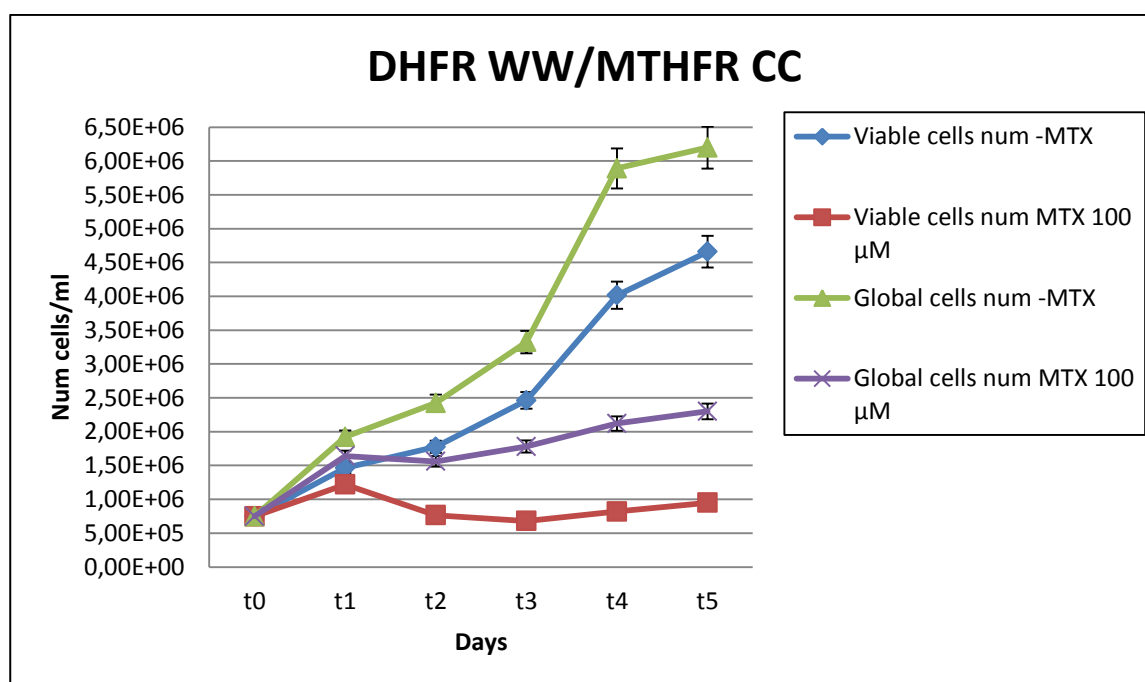
cells. The observation that MTX treated cells (global and viable) diverged between the two genotypes leads us to analyze in detail cells number and viability respectively by cell counting and MTT test. A sub-analysis revealed that the two different genotypes significantly diverged at t1 (1.22E+06 vs 8.45E+05; P=0.07) and t5 (9.5E+05 vs 4.61E+05; P=0.04) in favour of the WW/CC genotype (Fig. 17). MTT test confirmed the results obtained (Tables 20, 21).

WW/CC	t0	t1	t2	t3	t4	t5
Viable cells num/ml -MTX	7,50E+05	1,46E+06 ±2.4E+05	1,78E+06 ±5.88E+05	2,46E+06 ±8.16E+05	4,02E+06 ±1.38E06	4,67E+06 ±1.5E+05
Viable cells num/ml MTX100 µM	7,50E+05	1,22E+06 ±4.62E+05	7,65E+05 ±3.78E+05	6,81E+05 ±3.12E+05	8,20E+05 ±3.74E+05	9,50E+05 ±0.9E+05
Global cells num/ml -MTX	7,50E+05	1,92E+06 ±1.28E+05	2,42E+06 ±6.48E+05	3,32E+06 ±1.04E+06	5,0E+06 ±1.76E+06	6,20E+06 ±3.5E+05
Global cells num/ml MTX100 µM	7,50E+05	1,64E+06 ±5.64E+05	1,56E+06 ±4.04E+05	1,78E+06 ±6.43E+05	2,12E+06 ±7.49E+05	2,30E+06 ±1.5E+05

**Table 18.** t0-t5 indicate the days of cell culture with counting and MTT test every 24 h for WW/CC genotype; Viable cells num/ml -MTX and Viable cells num/ml MTX100 µM indicate the count of the viable cells as control and treated with 100 µM MTX, respectively; Global cells num/ml -MTX and Global cells num/ml MTX 100 µM indicate the count of the total cells as control and treated with 100 µM MTX.

DD/TT	t0	t1	t2	t3	t4	t5
Viable cells num/ml -MTX	7,50E+05	1,21E+06 ±9.19E+05	1,93E+06 ±1.02E+06	2,56E+06 ±1.67E+06	3,00E+06 ±1.42E+06	3,28E+06 ±0.9E+06
Viable cells num/ml MTX 100 µM	7,50E+05	8,45E+05 ±2.34E+05	6,41E+05 ±2.18E+05	6,33E+05 ±3.1E+05	5,64E+05 ±2.39E+05	4,61E+05 ±2.74E+05
Global cells num/ml -MTX	7,50E+05	1,60E+06 ±1.1E+06	2,36E+06 ±1.19E+06	3,41E+06 ±1.99E+06	4,40E+06 ±1.15E+06	4,51E+06 ±0.95E+06
Global cells num/ml MTX 100 µM	7,50E+05	1,26E+06 ±3.04E+05	1,40E+06 ±3.61E+05	1,60E+06 ±2.45E+05	1,72E+06 ±4.17E+05	1,65E+06 ±8.1E05

**Table 19** t0-t5 indicate the days of cell culture with counting and MTT test every 24 h for DD/TT genotype; Viable cells num/ml -MTX and Viable cells num/ml MTX100 µM indicate the count of the viable cells as control and treated with 100 µM MTX, respectively; Global cells num/ml -MTX and Global cells num/ml MTX 100 µM indicate the count of the total cells as control and treated with 100 µM MTX.



**Fig.15**

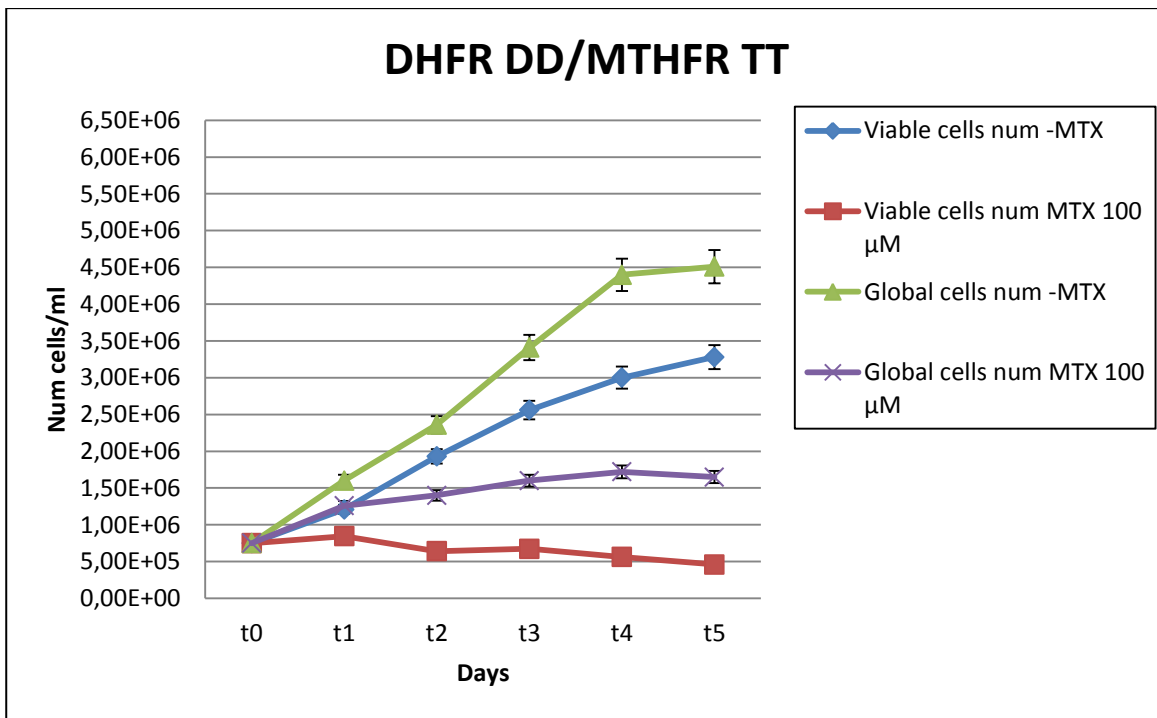


Fig. 16

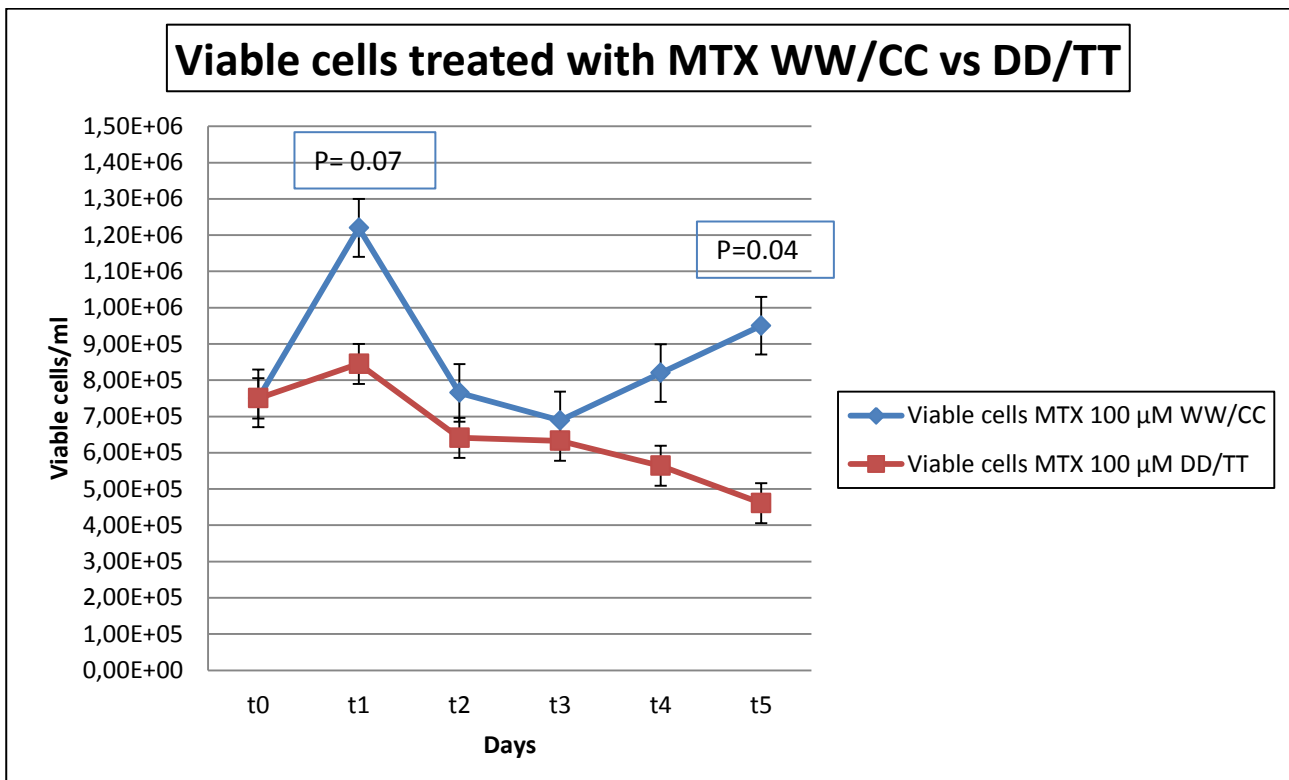


Fig.17

**DHFR DD/MTHFR TT  
MTT test**

	DAYS					
OD (A550nm)	t0	t1	t2	t3	t4	t5
Viable cells -MTX	0,355	0,563	0,645	0,700	0,735	0,782
Viable cells MTX 100 µM	0,350	0,435	0,388	0,216	0,133	0,145
Blank	0.051	0.046	0.048	0.047	0.047	0.048

**Table 20**

**DHFR WW/MTHFR CC  
MTT test**

	DAYS					
OD (A550nm)	t0	t1	t2	t3	t4	t5
Viable cells - MTX	0,415	0,581	0,660	0,691	0,681	0,682
Viable cells MTX 100 µM	0,414	0,548	0,412	0,341	0,330	0,31
Blank	0.051	0.046	0.048	0.047	0.047	0.048

**Table 21**

**4**

## **DISCUSSION**



## 4.1 DISCUSSION

Several molecular rearrangements are involved in the etio-pathogenesis of cancer disease and similarly, numerous polymorphisms affect its susceptibility. Folate balancing has a critical role in normal and malignant cell cycle by influencing fundamental processes such as DNA synthesis and methylation (Blount et al., 1997). Sub-optimal/anomalous folate levels/distributions are correlated to an elevated risk of developing several forms of neoplasia including ALL. From another point of view, one of the most important processes related to the development of cancer is the balancing of apoptosis regulation mechanisms: an increased survival of a cell population, indeed, defines the accumulation of genomic instability within the cells (Karakas et al., 1998). Apoptosis is a natural process regulated by several pathways that comprise genes that both promote or inhibit it. It can be hypothesized that polymorphisms of genes involved in the regulation of apoptosis, which modify protein function and/or expression could play an important role in the balancing of this cell regulation mechanism (Kidd et al., 2006).

With the present thesis, we aimed to investigate the effect of three polymorphisms within two genes of the folate metabolic pathway (MTHFR and DHFR) on the risk of developing childhood ALL, and similarly, we investigated the effect of one polymorphism within the anti-apoptotic Bcl-2 regulatory gene on childhood ALL susceptibility. From this study we can observe that the homozygous DHFR-DD genotype and the homozygous MTHFR 677-TT genotype are under-represented among childhoods ALL cases (Tab.8). Nevertheless, the analysis of DHFR polymorphism did not show significant results. Considering the MTHFR C677T polymorphism, it was noted an under-representation of the polymorphic T-allele yielding a significant ALL risk reduction of 1.5-fold ( $P=0.046$ ). At the same time, considering the MTHFR A1298C polymorphism, although it did not reach statistical significance, it is important to note an over-representation of the polymorphic C allele, that yielded an increased risk of ALL combining together CC homozygotes and AC heterozygotes versus AA wild-type genotype. The two enzymes considered, are involved in cyclization of folate isoforms. DHFR is responsible for the conversion of dihydrofolate to tetrahydrofolate, favouring the synthesis of 5,10-methylene-THF (Skibola et al., 1999). The elevated expression levels associated to the D-allele could therefore facilitate the filling of the cell with 5,10-methylene-THF, supporting consequently the DNA synthesis process (Xu et al., 2007). MTHFR catalyzes the reduction of 5,10-methylene-THF to 5-methyl-THF (Goyette et al., 1995). A lower enzyme activity associated to the polymorphic T-allele leads to an

underutilization of methylene-THF within the cell, with consequent accumulation of partially reduced folate isoforms. This statement is in agreement with studies demonstrating an accumulation of THF formylated isoforms within red blood cells (Bagley et al., 1998). As a consequence, a balance within the cell there exists between 5,10-methylene-THF isoforms and formylated THF isoforms so the synthesis of purine and pyrimidine is positively influenced. If the rate of intracellular THF isoforms is determined by the relative balancing between DHFR (by production/filling) and MTHFR (by use/emptying) activities, we aimed to investigate if the coexistence in the same individual, of the two polymorphic conditions could ascribe a synergistic or additive effect on the risk of developing childhood ALL. Interestingly, from the combined analysis of the two polymorphisms (DHFR -19bp INS/DEL and MTHFR C677T) the DHFR-WW/MTHFR-TT genotype combination yielded a significant 3.3-fold childhood ALL risk reduction ( $P=0.01$ ), whilst border-line results are obtained from the combination of double heterozygotes DHFR-WD/MTHFR-CT and from the combination of homozygotes DHFR-DD/homozygotes MTHFR-CT (Tab.9). The evaluation of the combined risk for double homozygotes (i.e. DHFR DD/MTHFR TT), in which a maximal protective effect is hypothesized however, did not show statistical significance, probably for the lower number of subjects computed in the analysis. As regards the Bcl-2 -938 C>A polymorphism, it is to note a significant over-representation of the polymorphic A allele among childhood ALL cases, that yielded an increased risk of 1.5-fold of childhood ALL combining together AA homozygotes and CA heterozygotes versus CC wild-type genotype ( $P=0.049$ ). Bcl-2 seems to play a crucial role in the survival of hematolymphoid cells, by inhibiting apoptosis through the prevention of cytochrome c release from mitochondria, blocking caspase activation (Reed et al., 1994). Our results are in line with the data that ascribe to the A-allele a higher Bcl-2 transcription, responsible for an increased Bcl-2 level (Nuckel et al., 2007). Since high Bcl-2 expression correlates with unfavorable prognosis and disease stage this could lead to poor prognosis among A-carrier patients. The polymorphic -938 A-allele could therefore represents a risk factor for the susceptibility to childhood ALL.

These are the reasons why, the second phase of this study focusses on the relationship between polymorphisms, disease onset and therapy duration. From the results obtained considering the relationship between MTHFR A1298C and disease onset, it is to note that the CC homozygotes had an appreciable different mean age onset when compared with the remaining genotypes (età e  $P=0.05$ ). We can ascribe to the MTHFR 1298CC homozygotes a significant later onset also confirmed by survival analysis ( $P=0.05$ ). Other significant results were obtained analyzing therapy duration. MTHFR 1298CC had a significant higher mean therapy duration compared with the

remaining genotypes ( $P=0.05$ ). Conversely, similar results were not obtained considering the C677T polymorphism, although 677T-allele affects MTHFR enzyme activity at higher extent than MTHFR A1298C polymorphism, maybe due to the stronger partial link diseq between the two gene variants. The same analytical approach computing Bcl-2 -938 C>A polymorphism ascribed to the -938 AA genotype a significant longer therapy duration ( $P=0.03$ ). Also -938 C-carriers had appreciable longer therapy duration and this result completely strengthened the above mentioned hypothesis that ascribes to the A-allele a higher Bcl-2 transcription, responsible for an increased Bcl-2 level (Nuckel et al., 2007), correlating with unfavorable prognosis and disease stage.

In order to verify effective actions of the folate cycle polymorphisms investigated on base-line cells viability and on residual viability of anti-folate treated cells, we selected two groups of opposite genotypes for DHFR and MTHFR to improve possible differences. Accordingly, under the same in vitro culture condition we observed appreciable effects in cell number, in favor of the WW/CC genotype. The difference became even higher when the same two groups were treated with anti-folate drug. On the contrary, the initial rate in cells growth was completely comparable between DD/TT and WW/CC genotypes, being about + 60% for DD/TT genotype and about +58.3% for WW/CC (data were referred to the first 48 hours of culture for the viable not treated cells).

The pharmacological induced restriction in folate availability (MTX) yields to different responses in the two opposite genotypes, in favor of WW/CC, though they have the same initial growth rate. This is in line with the hypothesis that the combination of DD/TT genotype helps the storage of folate isoforms (useful for the cell viability) by production and filling activity, compared to the WW/CC responsible for a lower filling and consuming activity. As for the hypothesized different genotype-dependent intracellular folate distribution, also MTX being itself a synthetic analogue of folate should follow the same handling process. Now, a higher MTX level inside the cell should result in an elevated toxicity level. In fact, both global and residual viable treated cells significantly increase among the WW/CC genotype.

## **Conclusion**

It has been well established since the last 15 years that the role of some folate pathway gene polymorphisms have effects on risk establishment, prognosis, survival and toxicity in several hematological malignancies. The dual and opposite role often ascribed to this kind of polymorphisms could be in part explained by the fact that the same mechanism (e.g. folate storage/consuming) could be responsible for different outputs when an healthy cell or a cancer cell is considered.

Theoretically, a cell genetically prone to accumulate folate isoforms (useful for the cell viability) carries an advantage in proliferation. However, when a toxic folate analogue is furnished together with folate, the cell is not able to discriminate the right from wrong molecule and the positive mechanism above mentioned became lethal.

**5**

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