

# Folates and Anti-folates – Chemical Perspectives, *in vitro* and *in ovo* Assessments

BOGDAN SOROP<sup>1#</sup>, VLAD LAURENTIU DAVID<sup>1#</sup>, ALINA HEGHES<sup>2\*</sup>,  
DELIA BERCEANU-VADUVA<sup>1\*</sup>, LAVINIA BALAN<sup>1</sup>, MARIA SOROP FLOREA<sup>3</sup>,  
DORU ANASTASIU<sup>1</sup>

<sup>1</sup>Victor Babes University of Medicine and Pharmacy Timisoara, Faculty of Medicine, Faculty of Medicine, 2 Eftimie Murgu Sq., 300041, Timisoara, Romania

<sup>2</sup>Victor Babes University of Medicine and Pharmacy Timisoara, Faculty of Medicine, Faculty of Pharmacy, 2 Eftimie Murgu Sq., 300041, Timisoara, Romania

<sup>3</sup>University of Medicine and Pharmacy of Craiova, Faculty of Medicine, 2 Petru Rares Str., 200349, Craiova, Romania

**Abstract:** *Folates participate in DNA replication reactions, act as a substrate in enzymatic reactions related to amino acid synthesis and vitamin metabolism while antifolates participate in reactions that inhibit the formation of tetrahydrofolate with consequences on protein and nucleic acid synthesis and implicitly on growth and development both types of cells, healthy and diseased. In the present study, the viability of healthy cells, keratinocytes and human fibroblasts was evaluated in the presence of three folates (folic, dihydrofolic and tetrahydrofolic acids), one antifolate (methotrexate) and combinations between them by Alamar blue assay. The antiangiogenic potential was also evaluated by in ovo technique, CAM assay. Cell viability was influenced in a cell-dependent and dose-dependent manner, fibroblasts being more sensitive to the action of the test compounds, especially the combination of methotrexate and dihydrofolate. Data related to CAM assay showed that methotrexate revealed a slightly higher vessel density, but without inducing toxicity on vascular architecture and functionality. The data obtained highlight the greater sensitivity of the viability of fibroblasts in the presence of methotrexate and its combinations with folates used in the study.*

**Keywords:** *folates, methotrexate, reactivity, viability, irritation*

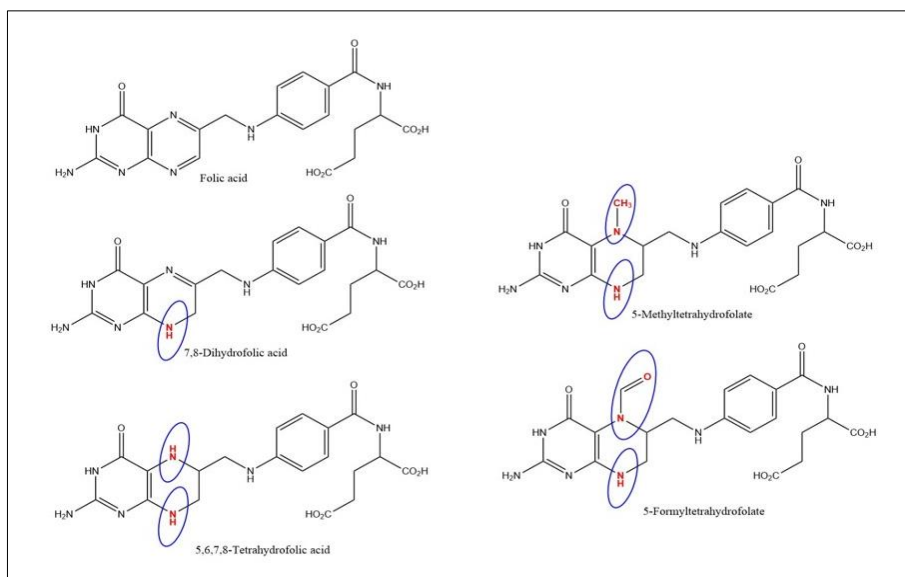
## 1. Introduction

The term *folates* describes the class of water-soluble vitamins, consisting of a pterin structure, a *p*-aminobenzoic acid and a residue of one or more molecules of glutamic acid. Folates can come in two forms: (i) oxidized and synthetic, found in food supplements (*eg* folic acid) and (ii) reduced, found in food and the human body (*eg* 5-methyltetrahydrofolate). The reduced form has a higher importance than the oxidized one due to the fact that it plays a crucial role in the metabolic activity, probably to the glutamate residues and to the units with a single carbon [1]. The main natural forms are partially reduced (dihydrofolate compounds) or completely reduced (tetrahydrofolate compounds), as can be seen in Figure 1.

The importance of folate in growth and development has been studied for decades, but there are mechanisms and processes that have not yet been elucidated or fully understood. One of these is related to the amount of unmetabolized folic acid (FA) that reaches from mother to fetus with adverse consequences due to the resulting poor methylation processes. Genetics and epigenetics have gained attention in recent years due to the premise that if genetics cannot be modified, epigenetic factors are flexible and can be adjusted to regulate, correct or change certain chemical processes of a physiological nature. At the same time, genetics and epigenetics are the basis of differences between individuals and populations, being the key in understanding the different symptoms and clinical responses between them. MTHFR (Methylene Tetra Hydro Folate Reductase) mutations are a key factor especially in the pre-conception period and to prevent genetic abnormalities the administration of methylfolate to the detriment of folic acid is indicated [2]. MTHFR mutations (both gene and

\*email: [heghes.alina@umft.ro](mailto:heghes.alina@umft.ro), [berceanu.delia@umft.ro](mailto:berceanu.delia@umft.ro)

# Authors with equal contribution



**Figure 1.** Chemical structures of folic acid, dihydrofolic acid, tetrahydrofolic acid and its methyl and formyl derivatives

protein) are closely related to the following: pre-eclampsia, recurrent miscarriages, spina bifida, anencephaly, Down syndrome [3]. One of the main risk factors for embryonic genetic defects is poor embryonic methylation due to lack of erythrocyte folate. Erythrocyte folate levels are directly influenced by the administration of drugs in the following classes: oral contraceptives, anti-epileptics, non-steroidal anti-inflammatory drugs and drugs such as methotrexate and cholestyramine [4]. Considering the multiple reactions that are involved in the folate cycle and the normal growth and development from the conception stage, the mechanisms that need clarification are of real interest. The human body undergoes a series of changes in the presence of various factors, which have multiplied significantly in the context of global development (pollutants, changes in food structure, stress, etc.). Pregnant women are prone to several diseases, require the administration of folates and in the context of the existence of certain pathologies even the administration of antifolates, which leads to normal or aberrant reactions. Methotrexate (MTH) is a synthetic compound, a structural analogue of folate, which acts by inhibiting key catalytic enzymes of purine and pyrimidine, which leads to depletion of intracellular folates downgrading cytotoxic effects, especially manifested by impairing DNA synthesis, methylation and repair [5].

In recent years there have been a number of controversies related to folate action, selective effects of methotrexate and cellular behavior, especially due to differences in bioavailability and metabolism of synthetic and natural folates but also different characteristics of subjects and evaluation methods [6-8]. Cells such as keratinocytes and fibroblasts are found in organs and tissues and have specific roles in the body. Although non-myocytes (e.g. fibroblast) occupy a relatively small volume fraction, they are essential for normal heart homeostasis, providing the extracellular matrix, intercellular communication, and vascular supply needed for efficient cardiomyocyte contraction and long-term survival [9].

The current study was purported to analyze the viability of human keratinocytes and fibroblasts (due to their complex role in the processes of physiological and pathological stress) in the presence of folate (recommended for fortification especially for women who want to become pregnant and pregnant), methotrexate (an anti-folate commonly prescribed in cancers, inflammatory and autoimmune diseases) and combinations between them in order to obtain preliminary data related to the dual role of these compounds in the body cells. The anti-angiogenic potential of the compounds was also evaluated *in ovo* on embryonated eggs by the means of CAM assay.

## 2. Materials and methods

### 2.1. Reagents

Folic acid (FA, code F7876), 7,8-Dihydropteroyl-L-glutamic acid, (DHF, code D7006); 5,6,7,8-Tetrahydropteroyl-L-glutamic acid (THF, code T3125) and methotrexate (MTH, code M8407) were purchased from Merck (Germany).

### 2.2. Cell cultures and testing

In the current research study were chosen human immortalized keratinocytes (HaCaT, code CLS300493) and human skin fibroblast (1BR3, code 90011801) cell lines, received as frozen items from CLS Cell Lines Service GmbH (Germany) and ECACC General Collection (Salisbury, UK). The specific media, reagents and kits necessary for cell growth and testing were acquired from Sigma Aldrich and American Type Culture Collection. The cells were treated according to the manufacturer's specifications and as specified in the literature [10,11]. In brief, human immortalized keratinocytes were cultured DMEM (Dulbecco's modified Eagle Medium) high glucose (a specific culture medium) supplemented with 10% FCS (fetal calf serum), human skin fibroblast in EMEM (Eagle's Minimum Essential Medium) supplemented with 15% FCS, while 1% antibiotic mixture (penicillin/streptomycin) solution was added to the culture of both cell types [12]. Alamar blue assay was selected in order to verify the behavior of the compounds on the cells viability. The protocol applied is described in literature and includes the following steps: the cells (10,000/200  $\mu$ L medium/well) were seeded in a 96-well plate, allowed to adhere to the plate, and subsequently incubated for 24 h with the different compounds studied (at four different concentrations) and finally the absorbance was measured at two wavelengths (570 and 600 nm) on a dedicated spectrophotometer (xMark <sup>TM</sup> Microplate, BioRad) [12].

### 2.3. *In ovo* evaluation - CAM assay

A simple and easily applied *in vivo* technique is the CAM assay based on which the effects exerted on the angiogenesis process can be investigated, and not only taking into account the fact that it is also used to evaluate the tumor process. The method involves the use of fertilized chicken eggs and is carried out in several stages: cleaning the eggs with 70% ethanol before incubation (37°C, 50% controlled humidity), in EDD 3 (day 3 of embryonic development) is removed 2-3 mL of albumen, in EDD 4 (day 4 of embryonic development) a hole is made in the upper part which is covered at the reintroduction in the incubator and in EDD 9 (day 9 of embryonic development) the solutions of the analyzed compounds are tested. By stereomicroscopy, eggs are evaluated daily. The positive control used in the experiments is represented by SDS (1% in PBS) and the negative control used is PBS; the concentration of the compounds is determined after performing *in vitro* tests and is the median result in these tests [12].

The particular reactions induced by the compounds are: hemorrhage (H), vascular lysis (L), coagulation (C) and the time required for their production has been set at 300 s. An irritation score is established, which can have values in the range 0-21 and is calculated based on the formulas described in the literature [13]. In addition to the irritation score, the severity score (values in the range 0-3) is also calculated to determine the severity of the irritation [12].

### 2.4. Statistical data

The programs and software utilized in this research study were GraphPad Prism 7 (GraphPad Software, CA, USA), AxioVision SE64. Rel. 4.9.1, Gimp v2.8, ImageJ v1.50e (<https://www.gimp.org/>) and results were processed with one-way ANOVA (coupled with Tukey's post-tests) to establish the statistical difference between controls and compounds (\*, \*\*, \*\*\* and \*\*\*\* denote  $p < 0.05$ ,  $p < 0.01$ ,  $p < 0.001$  and  $p < 0.0001$ , respectively).



[18,19].

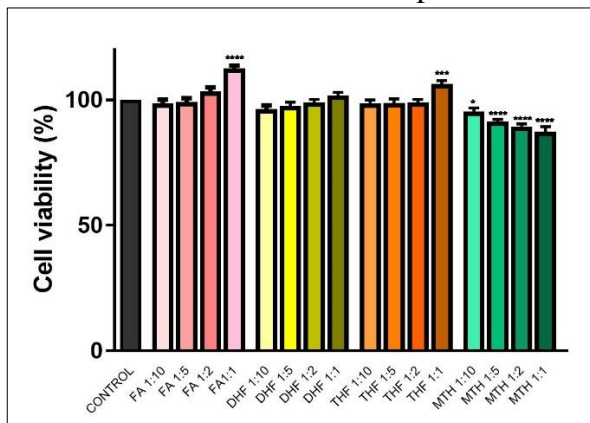
In order to establish the optimal recommendations regarding the administration of folate, the possible negative effects produced must be taken into account, namely: masking vitamin B12 deficiency, deficient metabolism with the increase of unmetabolized compound that can lead to aberrant and deficient proliferation with serious pathological consequences. Folic acid is a molecule that can interact with various chemical and molecular formations. Compounds known generically as antioxidants (flavonoids, cinnamic acids, stilbene, etc.) can influence the breakdown of folic acid, proving to be useful protectors [20]. Folates found in plasma have low affinity for proteins, instead folic acid could react with several proteins: through both hydrophobic and hydrophilic interactions it can interact with human and bovine serum albumin (due to tryptophan in their structure), with lactoferrin forming saturated reactive complexes, with  $\beta$ -casein forming stable complexes due to certain hydrophobic parts that are found in both structures [21-23].

In the case of the class of anti-folate compounds there are a number of gaps in terms of their selectivity and the reactions in which they are involved. The elucidation of the reactions that imply them, the establishment and validation of the mechanisms that lead to sensitivity or resistance would contribute decisively to certain therapeutic schemes depending on the selective pathologies, especially for pregnant women.

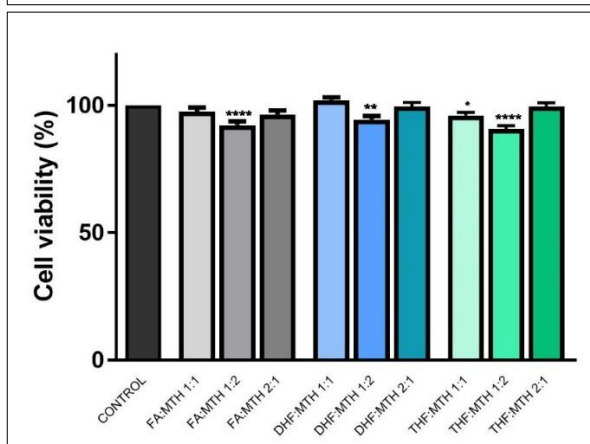
### 3.2. *In vitro* activities of folates and anti-folates

In order to evaluate the effects induced by FA, DHF, THF, MTH and some of their combinations on human keratinocytes, different concentrations were tested in a 24 h exposure assay.

The data revealed that MTH induced a slight decrease of HaCaT cells at the highest concentrations tested, the activity was dose-dependent, and none of the compounds exerted significant toxicity as can be seen in Figure 3. Even in the case of the combination of the two types of compounds, folate with anti-folate, there was no significant decrease in the number of viable cells (Figure 4) analyzed by the means of Alamar blue test in a 24 h post-stimulation assay.



**Figure 3.** Cell viability of human keratinocytes after stimulation with folates (folic, dihydrofolic and tetrahydrofolic acids) and anti-folate (methotrexat) at 24h post-stimulation



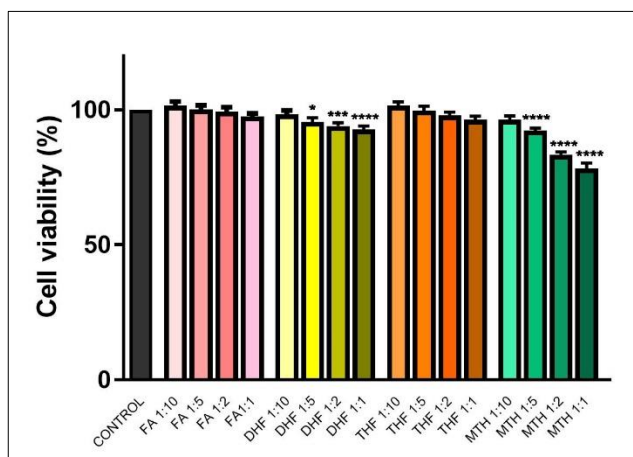
**Figure 4.** Cell viability of human keratinocytes after stimulation with the combinations between folates (folic, dihydrofolic and tetrahydrofolic acids) and anti-folate (methotrexat) at 24h post-stimulation



Tissue homeostasis and the epidermal barrier are decisively influenced by the following processes: the movement of mitotic active cells from the basal layer to the skin surface by terminal differentiation; transcriptional and morphological modification of keratinocytes simultaneously with the previous process; differentiation and stratification of immature ectoderm (during embryogenesis) resulting in a single layer of undifferentiated epithelial cells that is subsequently covered by the periderm (barrier layer existing only before birth, with the role of preventing pathological epithelial adhesions) [24]. Any disturbance to the described processes, centered on keartinocytes, can lead to abnormalities of the lips / palate, therefore the behavior of keratinocytes in the presence of biologically active compounds is a first step in elucidating the mechanisms involved in the body's response.

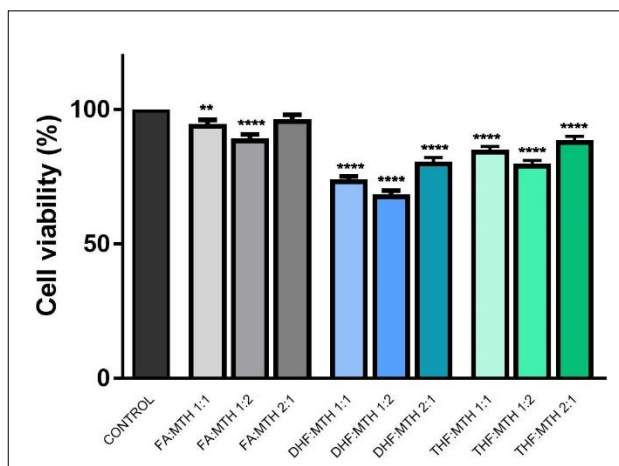
In the study conducted by Pagano *et al*, immortalized human keratinocytes and human primary fibroblasts were treated with different volumes of diluted FA with specific culture medium (DMEM), viability was assessed by MTT method at 24 h, and cells showed a viability > 77 % at all dilutions tested [25]. In another study, HaCaT cells were treated with different concentrations of MTH solution (between 1  $\mu$ M and 18.6  $\mu$ M) and cell viability (assessed by the means of MTT assay) was around 60% for all concentrations tested [26]. In the case of keratinocyte, folate depletion leads to arrest of S-phase proliferation and increased levels of inherent DNA damage, being sensitized to apoptosis induced by various factors (eg ultraviolet radiation) and, show a low capacity for repair DNA destroyed by photochemical and oxidative means. These effects can be counteracted by supplementing folic acid which has been shown to increase the resistance of HaCaT cells to cellular degradation processes [27]. After stimulation of HaCaT cells with methotrexate it was observed a decrease of cells viability (Figure 3), data that are in line with the results of Panonnummal and Sabitha, which showed a significant toxicity (61% viable cells) at a higher concentration – 0.18 mg/mL after 24 h [28].

Fibroblasts are another type of cell that is involved in managing physiological and pathological stress. Human fibroblast in the presence of folates did not show significant changes in viability, dihydrofolic acid had a more significant influence on viability compared to the other two folates (folic and tetrahydrofolic acids) tested, as can be seen in Figure 5. The viability of fibroblasts in the presence of methotrexate, established by the Alamar blue test, in a 24h exposure, decreased to 78% at the highest concentration used.



**Figure 5.** Cell viability of human fibroblasts after stimulation with folates and antifolate methotrexat at 24h post-stimulation

In the case of testing the combinations between metrotrexate and folic, dihydrofolic and tetrahydrofolic acids, the viability percentages varied as follows: the combination FA:MTH (1:2) decreased the viability of fibroblasts to around 89%, THF: MTH (1:2) to around 79% and the most pronounced effect was observed in the combination DHF: MTH (1:2) where there was a decrease of up to 68% (Figure 6).



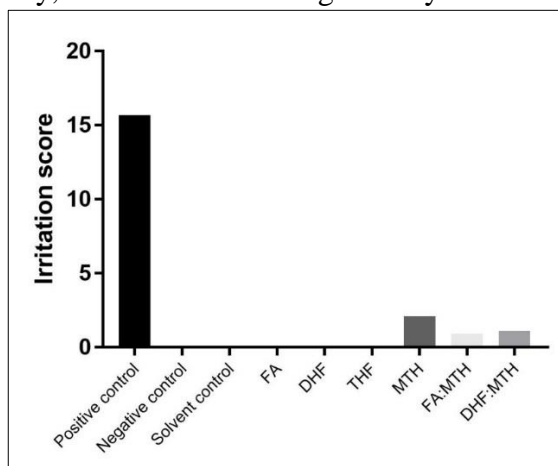
**Figure 6.** Cell viability of human fibroblasts after stimulation with the combinations between folates and antifolate methotrexat at 24h post-stimulation

The heart contains two major types of cells: cardiomyocytes - more than two-thirds of all cells and non-myocytes (fibroblasts, endothelial and peri-vascular cells) that are essential for normal cardiac homeostasis (ensures extracellular matrix, intercellular connection and specific vascularization) [9]. In other words, the cells from the organ respond to physiological and pathological stress requiring detailed evaluation in the context of particular cases.

The methotrexate tested on fibroblasts did not show toxicity according to the study conducted by Piegols *et al.* that obtained IC<sub>50</sub> values relevant to certain tumor cells (less than the maximum concentration 42 hours after the start of a high-dose methotrexate infusion required to avoid human toxicity - 1  $\mu$ M) and viability percentage greater than 80% at all concentrations tested on fibroblasts [29]. Fibroblast-like cells from inflammatory diseases in the presence of metrotrexate at concentrations between 0.01  $\mu$ M and 10  $\mu$ M (at 48 hours) had viability values of over 70%, the inhibition of cell growth being dose-dependent [30]. Methotrexate is an FDA approved drug applied as treatment in multiple cancers, which acts by inhibition of the folate cycle, effect also observed in different tumoral cells. Ross *et al.* demonstrated that methotrexate in combination with dabrafenib sensitizes the metastatic melanoma cells resistant to BRAF inhibitors, whereas used as single agent has no activity against melanoma cells [31].

### 3.3. *In ovo* activities of folates and antifolates

In order to elucidate the affective mechanism exerted by the compounds and the combination of these, the angiogenic effect was analyzed. The four compounds, along with carefully selected combinations, were tested *in vivo*, applying the CAM assay. Thus, the general tolerability, the effect on the chorioallantoic membrane in normal development (24 hours after the application of the compounds) was evaluated. The data obtained indicated that folates were best tolerated, not inducing changes on the normal angiogenic process, anti-folate methotrexate revealed a slightly higher vessel density, but without inducing toxicity on vascular architecture and functionality (Figure 7).



**Figure 7.** Irritation evaluation of folates and antifolates using HET-CAM assay, represented by Irritation scores (IS) of the tested compounds, alongside to positive control (PBS), negative control (SDS 1% in PBS) and solvent control (DMSO 1%)

The current context is affected by antibiotic resistance which has a decisive effect on health, and in the case of anti-folates this situation has been found in anticancer therapies but also in therapies applied in case of microbial infections. The present study involved the evaluation of cell viability in the presence of both folates, anti-folate but also some associations between them in order to be a starting point in elucidating the synergistic and / or antagonistic preliminary effects exerted. Anti-folates have been used for more than half a century in anticancer therapy due to the inhibition of dihydrofolate reductase and imply the formation of tetrahydrofolate (THF), a compound necessary for the synthesis of purine and pyrimidine. Tetrahydrofolate deficiency inhibits nucleic acids and protein synthesis, thus decisively influencing the growth and development of both normal and tumor cells [32]. During pregnancy there are various changes in the immune and glandular functions of the mother, which lead to various diseases and some of the most common are skin diseases. Taking into consideration the triggering factor, they can be inflammatory or glandular, more or less exacerbated depending on the reactions that take place, with the specification that the diseases involving the sebaceous and eccrine glands are often more pronounced compared to those with apocrine involvement [33]. The applied therapies are relatively safe but few in number and with certain limitations. The process of placental angiogenesis is crucial in maintaining adequate blood flow to the development of the fetus, altering the process leads to devastating effects for mother and fetus. Folates together with fatty acids modulate specific receptors during placental angiogenesis both directly (through angiogenic factors) and indirectly (through specific modulation) [34].

Folate deficiency and its cellular reconstitution remain a problem to be explained in terms of chemical reactions and mechanisms of action involved. At the same time, anti-folate drugs used especially in the treatment of cancer, but also in various autoimmune diseases can send false signals to the body, creating a basis for clonal selection [35]. Mandatory folate fortification has led to positive results in various parts of the world, but the cellular transformation in their presence / absence and the possible double effect exerted by both folate and anti-folate must be studied in detail, with more and more studies signaling the association of administration with possible malignant consequence [35].

In the case of the cell-methotrexate interaction, the folate receptor (FR) and the reduced folate carrier (RFC) are responsible for the absorption of the compound. The latter is ubiquitously expressed in healthy cells, is overexpressed in tumor cells, being a key mechanism of absorption of both folates and antifolates; Impairment of RFC synthesis or RFC protein mutations leads to therapeutic resistance due to possible cellular dysfunctions [5].

#### 4. Conclusions

Reactions involving folate are of major importance, especially if we consider that inhibition of transporters leads to devastating effects such as malformations in the fetus. Despite numerous studies in recent years involving the administration of folate to reduce defects in the fetus, and not only, there are more and more studies that associate folate with the progression of tumor processes. Thus, it is necessary to highlight the reactions involved and elucidate certain effects in the context of the association of several factors that are either beneficial for the correction of certain defects or devastating for cellular replication. The studies performed require a correlation with experiments involving oxidative stress for a better understanding of the importance of the administration of compounds for prevention but also of compounds for healing.

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