

## Review

# Cancer metabolism and oxidative stress: Insights into carcinogenesis and chemotherapy via the non-dihydrofolate reductase effects of methotrexate



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## ABSTRACT

Methotrexate has been in use as an anti-cancer agent for over 60 years. Though inhibition of dihydrofolate reductase is its best known mechanisms of action, its non-dihydrofolate reductase dependent mechanisms disrupt metabolic pathways resulting in a depletion of NAD(P)H and increasing oxidative stress. These mechanisms highlight a novel dependence of cancer cells on their metabolic abnormalities to buffer oxidative stress and chemotherapeutic agents interfere with these cellular abilities. Mitochondria appear to play a significant role in maintaining cancer cell viability and alterations in metabolism seen in cancer cells aid this mitochondrial ability. Further research is needed to understand the effects of other chemotherapeutic agents on these pathways.

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## 1. Introduction

The origins of modern day chemotherapy can be traced back to the early days of World War II. On December 2, 1943, the port of Bari, Italy was the target of a German air raid on Allied forces [1–3]. The casualties of this air raid far outnumbered those expected for the size of the attack. Though banned by the Geneva Protocol of 1925, Allied forces were concerned with the possibility that the German army would resort

to the use of chemical weapons. In August of 1943, President Roosevelt approved the shipment of a secret cargo of mustard gas via SS Harvey. The explosion of the ship during this raid released its contents onto the city inflicting an untold number of civilian casualties. With some variation in details depending on the source, investigations by Dr. Stewart Francis Alexander, a Lieutenant Colonel and expert in chemical warfare noted significant suppression of both lymphoid and myeloid tissues in those exposed to the chemical agent [1]. Later research with a related compound, mustine, led to successful reduction of tumor mass in a patient with non-Hodgkin's lymphoma and to a realization that pharmacotherapy of cancers was feasible.

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Following World War II, a second step forward in chemotherapy was made by a pathologist at Harvard Medical School. Sidney Farber had studied the stimulating effect of folic acid on the proliferation of acute lymphoblastic leukemia cells when given to children with this cancer [4]. At that time, knowledge of the role of folic acid was limited to its effect as a cofactor in the synthesis of purines. This observation led to one of the first attempts at rational drug design. In collaboration with the first chemists to successfully synthesize folic acid at Lederle Laboratories, Farber helped design the folate analog aminopterin [4,3]. Later, this collaboration led to the synthesis of the antibiotic trimethoprim and the chemotherapeutic agent amethopterin, more famously known as methotrexate [4]. Since that time, the intracellular biochemistries of both folic acid and methotrexate have been further explored and detailed.

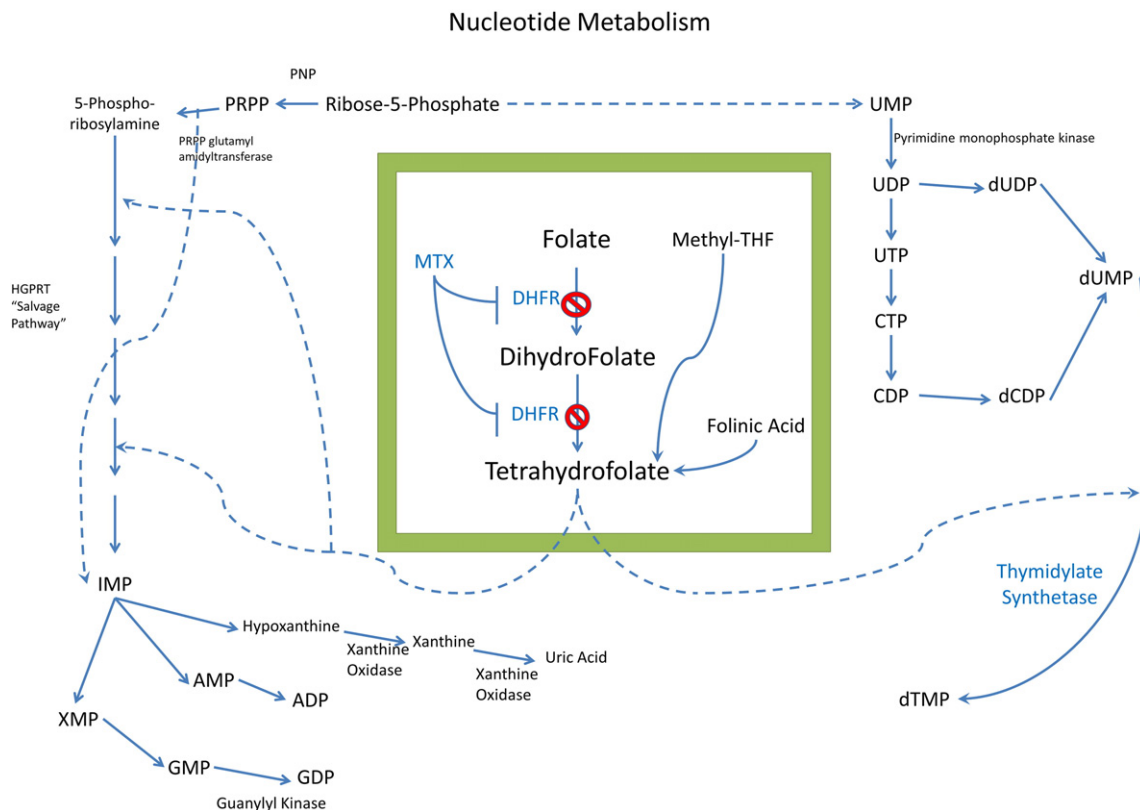
The classically taught mechanism of action for methotrexate is inhibition of dihydrofolate reductase (DHFR) to deplete cellular pools of tetrahydrofolate and stop the production of thymidylate. Cells lacking adequate thymidine are unable to synthesize DNA, which results in the arrest of cellular proliferation. The combined effect is thought to lead to the demise of rapidly dividing cell populations either through apoptosis or autophagy. In cancer therapy, sustained maximal DHFR inhibition is targeted and important for several reasons. First, it provides steady inhibition of DNA synthesis, and, second, it minimizes the risk of developing resistance to MTX [5]. With only 1% of the average cellular DHFR concentration required to maintain a sufficient reserve of reduced folate coenzymes, high doses of MTX are required to achieve this effect and are frequently limited by toxicities [6]. Major adverse effects of MTX therapy include dermatitis, elevated transaminases, mucositis, and myelosuppression. Leucovorin, a folate derivative that bypasses the

DHFR reaction, is used as a rescue agent to curb the incidence and severity of these effects and often found in high dose MTX protocols (see Fig. 1).

The in vitro mechanics of the anti-folate MTX on DHFR are well known. Newer studies reveal in vivo biochemical interactions of MTX with multiple enzymes. They describe interactions of MTX with multiple metabolic pathways ranging from cellular energy production, antioxidant regeneration, nucleotide synthesis and buffering of oxidative stress [7,8,6]. This review will describe the peculiarities of cancer metabolism and the non-DHFR mechanisms of MTX. These interactions support the hypothesis that mitochondria and this organelle's function in buffering of oxidative stress. In addition to dysfunctional glycolysis, this mitochondrial function appears to have a significant influence on the cancer cell's metabolism and the mechanisms of other chemotherapeutic agents.

## 2. Methods

Online literature searches of PubMed and Google Scholar were performed using the following terms and related biochemical pathways: autophagy, aerobic glycolysis, cancer metabolism, hydrogenosomes, glutamine metabolism, methotrexate, mitophagy, oxidative stress and cellular redox balance. No limitations were placed on the date of publication. Though the number of search results was not recorded, approximately 1800 publications were reviewed. The types of manuscripts included reviews, original research and letters to editor with both clinical and pre-clinical data. Historical texts and materials were reviewed in order to provide insight into the origins of chemotherapeutic agents.



**Fig. 1.** Effects of methotrexate on folate metabolism in relation to nucleotide metabolism. Via its inhibition of dihydrofolate reductase, methotrexate interferes with the generation of tetrahydrofolate and subsequently, DNA synthesis. Tetrahydrofolate is metabolized further for use as a cofactor in the synthesis of dTMP. Folinic acid, leucovorin, is often used to rescue cells from methotrexate and restore cellular stores of tetrahydrofolate. In addition to interference with DNA synthesis, this diagram superficially shows the involvement of tetrahydrofolate in the metabolism of purines. Legend: ADP, adenosine diphosphate; AMP, adenosine monophosphate; CDP, cytidine diphosphate; CTP, cytidine triphosphate; dCDP, deoxycytidine diphosphate; DHFR, dihydrofolate reductase; dTMP, deoxythymidine monophosphate; dUDP, deoxyuridine diphosphate; dUMP, deoxyuridine monophosphate; GDP, guanine diphosphate; GMP, guanine monophosphate; HGPRT, hypoxanthine-guanine phosphoribosyltransferase; IMP, inosine monophosphate; Methyl THF, methyltetrahydrofolate; MTX, methotrexate; PNP, purine nucleotide phosphorylase; PRPP, phosphoribosyl pyrophosphate; UDP, uracil diphosphate; UMP, uracil monophosphate; UTP, uracil triphosphate; XMP, xanthine monophosphate.

In cases in which animal studies were reported, attempts were made to search for human trials of similar therapeutic scenarios.

### 3. Metabolic dysfunction of the cancer cell and the Warburg effect

Otto Warburg was awarded the 1931 Nobel Prize for Physiology or Medicine as a result of his research on cellular respiration. He hypothesized that cancerous transformation of tissue was a result of an injured respiration, or metabolism. This hypothesis, referred to as “The Warburg Effect”, describes a dysfunctional metabolic state dominated by glycolysis ending in lactate production and present in a variety of cancers [9, 10]. Warburg postulated that this dysfunctional metabolism was the primary cause of cancer. Cancer cell research has since characterized a complex state of metabolic disarray involving glycolysis, the pentose phosphate pathway, the tricarboxylic acid (TCA) cycle, the electron transport chain and amino acid metabolism [11,6,12–14]. The derangements seen in cancer cells result from a combination of factors including hypoxia, poor total body and cellular antioxidant reserve, mitochondrial dysfunction and free radical production.

The elementary differences between normal and tumor cell metabolism are characterized by increased glycolytic flux even in the presence of adequate oxygen [14]. This change in cellular metabolism is oft considered a result, not an instigator, of cancerous transformation. However, the idea of metabolic disarray as an initiator of cancer has gained additional support in recent years [14]. The metabolic ratios of glycolysis and mitochondrial oxidative respiration in transformed cells show similarities to proliferating non-transformed cells but the ability of transformed cells to return to non-Warburg, or non-stressed, metabolic profile is hindered. Some may argue against this theory as oxygen consumption in these transformed cells continues despite the production of ATP via aerobic glycolysis [15]. The perturbed metabolism of transformed cells does not fully exclude oxidative phosphorylation as a source of ATP production but cellular oxygen tension, or lack thereof, does influence these cellular processes.

Under normal oxygen tension, hypoxia-inducible factor alpha (HIF-1 $\alpha$ ) is produced and degraded at an equal rate. The Von Hippel–Lindau tumor suppressor gene product, pVHL, has multiple functions, one of which is a recognition subunit of an E3 ubiquitin ligase complex. Prolyl hydroxylases (PHD) catalyze the hydroxylation of proline residues on the oxygen-dependent degradation site of HIF-1 $\alpha$  creating a docking site for pVHL directed degradation [16]. Degradation of HIF-1 $\alpha$  via pVHL is an oxygen-dependent process, converting oxygen and  $\alpha$ -ketoglutarate to carbon dioxide, and is a rate-limiting reaction dependent on the functional capacity of pVHL [17]. In the absence of sufficient reactants the HIF-1 $\alpha$  protein stabilizes, binds HIF-b and is transported to the nucleus where it stimulates the transcription of genes containing the hypoxia response element. Stabilization of HIF-1 $\alpha$  outside of a true hypoxic environment is referred to as pseudohypoxia. The pseudohypoxic phenotype has been seen in association with alterations in TCA associated enzymes and may lead to changes in epigenetic homeostasis with tumor promoting effects [14]. Both succinate dehydrogenase and fumarate dehydrogenase possess tumor suppressing functions and loss of these functions has been observed in many VHL-associated renal tumors [16]. The defects of these mitochondrial enzymes may be from inhibition exogenous to the enzyme, such as inactivation through reaction with free radicals, or a functional defect secondary to genetic mutation.

The HIF transcription cascade results in increased synthesis of glucose transporters GLUT1 and GLUT3, 6-phosphofructo-2-kinase, phosphoglycerate kinase 1, pyruvate kinase M2, monocarboxylate transporter MCT4 (a lactate efflux pump), and pyruvate dehydrogenase kinase (PDK1). When phosphorylated by PDK1, the pyruvate dehydrogenase enzyme is inactivated and this inhibits the conversion of pyruvate into acetyl-CoA for the TCA cycle [18]. Despite a lack of glycolysis derived acetyl-CoA, TCA cycle activity continues. In oxygen concentrations as low as 1%, NADH production maintains a normal rate with

mitochondrial ATP production significantly reduced [19]. As GLUT1 and GLUT3 maintain an insulin-independent influx of glucose, activation of insulin receptors would be associated with a much larger volume of raw materials for its metabolic processes. If the cell remains normoxic, aberrantly expressed PDK1 blocks delivery of glycolysis derived substrate to the TCA cycle. Mitochondria in the pseudohypoxic cell become starved for materials begin catabolizing amino acids, such as glutamine and asparagine, to supply the TCA cycle with intermediates,  $\alpha$ -ketoglutarate and oxaloacetate respectively [20,21,13].

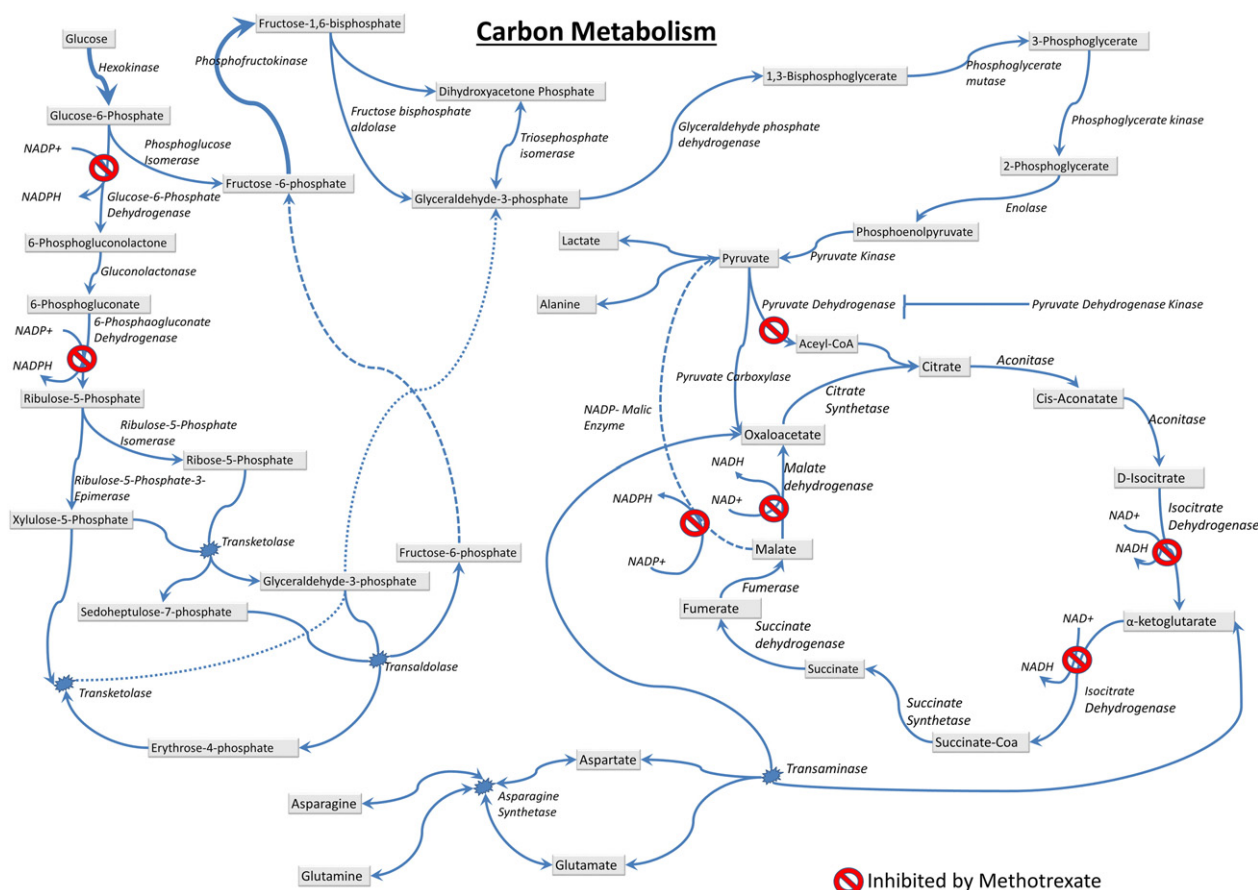
The change in fuel source and pathway may offer an explanation for the inverse relationship between the aggressiveness in tumor growth and proliferation and the drop in serum glutamine concentrations [22]. The conversion of amino acids to TCA intermediates has been seen as glutamine derived carbon exiting the TCA cycle as malic acid and being catalyzed by malic enzymes to produce NADPH; a reactant in the regeneration of free radical scavengers like glutathione [23]. The catabolism of glutamine and asparagine seen in highly metabolically active cancers is likely due to the ease with which they are transaminated to supply TCA intermediates. Additionally, high concentrations of pyruvate serve as a sink for nitrogen freed from amino acid transaminations [24].

### 4. Methotrexate

MTX is an inhibitor of dihydrofolate reductase and, more generally speaking, many NAD(P)H-dependent oxidoreductases. Unfortunately, its clinical use is associated with various toxicities. High doses of MTX are required to induce inhibition of the entire cellular pool of DHFR and are often associated with increased frequency and severity of its adverse effects [6]. Folinic acid, or leucovorin, has long been used to rescue cells from MTX toxicity and sidesteps the DHFR blockade in the cell. Beyond very high dose leucovorin rescues, few reports can be found of markedly reduced MTX efficacy with low dose leucovorin or concurrent folate supplementation. This suggests methotrexate toxicity and pharmacokinetics in chemotherapy may be secondary to its effects on cellular abilities to buffer oxidative flux via non-DHFR mechanisms. Multiple studies report various “antioxidant” therapies using quercetin, L-carnitine, lipoic acid, melatonin, and curcumin are effective in reducing MTX toxicity [25,11,26–28].

Dihydrofolate reductase is an NADPH-dependent oxidoreductase that oxidizes NADPH to NADP + while reducing dihydrofolic acid to tetrahydrofolic acid. Reversing the reaction, this same enzyme can be referred to as tetrahydrofolate dehydrogenase, an NADP +--dependent enzyme. In addition to DHFR, MTX inhibits many NAD(P)-dehydrogenase enzymes; glucose-6-phosphate dehydrogenase (G6PD), 6-phosphogluconate dehydrogenase (6PGD), pyruvate dehydrogenase, isocitrate dehydrogenase,  $\alpha$ -ketoglutarate dehydrogenase, and malate dehydrogenase [11,29,7,8,6,28] (see Fig. 2). There is a synergistic inhibitory effect on several enzymes more directly involved with maintenance of the cellular redox state via the regeneration of glutathione. Glutathione reductase, gamma-glutamylcysteine synthetase, glutathione peroxidase and lactate dehydrogenase are also inhibited on a concentration dependent manner by MTX with no effect from co-administered leucovorin [6] (see Table 1).

These NAD-dependent dehydrogenase enzymes have conserved super-secondary structures with varying subunits [30]. While all are related in regard to this structure, differences lie in individual enzyme subunits. These subunits may be responsible for the variable responses to methotrexate and its polyglutamates. The function of the enzymes in this study was measured by the rate of NAD(P)H oxidation at increasing MTX concentrations. Direct comparison of MTX's inhibitory effects on the activity of DHFR is difficult as studies using the same result measure could not be found. The serum concentrations of methotrexate seen in the table, however, are frequently reached in high dose methotrexate administrations.



**Fig. 2.** An overview of carbon metabolism with enzymes affected by methotrexate. MTX can be seen inhibiting many enzymes involved in glucose metabolism. The enzymes inhibited in this figure are all dehydrogenase enzymes and involved in reduction of NAD<sup>+</sup>/NADP<sup>+</sup> to NADH/NADPH. These reduced compounds are involved in multiple cellular reactions, the regeneration of reduced glutathione being one of them. Also seen in this figure is the incorporation of asparagine and glutamine as carbon sources for the production of TCA cycle intermediates. Legend: NAD<sup>+</sup>, nicotinamide adenine dinucleotide; NADP<sup>+</sup>, nicotinamide adenine dinucleotide phosphate; NADH, reduced nicotinamide adenine dinucleotide; NADPH, reduced nicotinamide adenine dinucleotide phosphate; star-shape indicates reaction of multiple reactants and products with various intermediates. Dashed lines indicate a metabolite is present in two locations along the metabolic pathway. Bold lines indicate an irreversible reaction.

**Table 1**  
Inhibitory effect of methotrexate on NADPH-dependent oxidoreductases [6].

Enzyme	[MTX] μM	Activity
2-Oxaloglutarate dehydrogenase	15	50%
	100	0%
Isocitrate dehydrogenase	100	50%
	500	0%
Malate dehydrogenase	150	50%
	400	0%
Succinate dehydrogenase	500	80%
Glutathione reductase		
CFE-HeLa cell enzyme	80	50%
	250	10%
Purified enzyme	110	50%
	200	20%
6-Phosphogluconate dehydrogenase		
CFE-Hela cell enzyme	50	50%
	200	10%
Purified enzyme	100	50%
Glucose-6-phosphate dehydrogenase		
CFE-Hela cell enzyme	100	50%
	250	10%
Purified enzyme	100	50%
	250	10%
Gamma-glutamylcysteine synthetase	100	46%
Glutathione peroxidase	100	50%

Values in this table were obtained directly from the text or via interpretation of figures in the reference.

Both G6PD and 6PGD enzymes catalyze the reduction of NAD(P)<sup>+</sup> to NAD(P)H in the pentose phosphate pathway for eventual use in either the reduction of oxidized glutathione, the production of a free radicals via NADPH oxidase, cholesterol production, steroidogenesis, etc. The remaining enzymes are largely involved in TCA cycle reactions resulting in the reduction of NAD<sup>+</sup> to NADH within the mitochondria. In proliferating cells, the pentose phosphate pathway and actions of malic enzyme are currently considered the most significant source of cellular NAD(P)<sup>+</sup> regeneration [6]. With the blockage of these enzymes, the cellular ability to regenerate glutathione, and deal with oxidative stress, is inhibited. Highlighting the dependence of cells on glutamine in dealing with oxidative stress, the loss of glutaminase-2 production leads to decreased NAD(P)H production, decreased glutathione production and sensitizes cancer cells to oxidative stresses like those induced by ionizing radiation [31]. As MTX doses increase, they impose a greater oxidant buffering handicap, proving fatal for both normal and abnormal cells. Cellular depletion of NAD(P)H leading to cell death is also seen in the treatment of infectious diseases. Antimicrobial therapy of both Gram negative and Gram positive organisms with bactericidal, but not bacteriostatic, antibiotics leads to a transient depletion of NADH, increased oxidative stress and ultimately cell death [32].

In vivo MTX metabolism follows a similar pathway to natural folates with polyglutamation via the enzyme folylpolyglutamyl synthetase [33, 34]. MTX polyglutamates, especially with three or more glutamate residues, are retained in cells longer, and with higher enzyme affinity,



than unmodified MTX [35]. Cellular concentrations of polyglutamated methotrexate rise even in the absence of circulating drug, until cellular efflux begins [33]. Animals fed a diet rich in supplemented glutamine showed not only a decreased incidence of MTX adverse effects, but also an enhanced anti-tumor effect in cancer cells [6]. This effect was also observed in the treatment of human tumors, and is likely secondary to MTX polyglutamylation [36].

Intracellular polyglutamated MTX concentrations are an important factor in achieving maximum pharmacologic effects in the treatment of malignancy [35]. Rodent studies have shown that administration of oral glutamine in conjunction with high dose MTX therapy was not only associated with decreases in enteric mucositis, but also a 300% increase in polyglutamated MTX concentrations within malignant cells when compared to normal cells [36]. In a phase I trial based on these rodent studies, nine patients with inflammatory breast cancer were placed on a similar regimen. In this human study, one case of grade I mucositis was reported and eight of the nine patients responded to the chemotherapy regimen with a median survival of 35 months [36].

Asparaginases are frequently used in combination with MTX. The process of MTX polyglutamylation influences the schedule dependent response to combination therapy with L-asparaginase. Pre-treatment of cancer cells with L-asparaginase leads to significant interference with MTX polyglutamylation [37]. While its primary enzymatic activity is focused on asparagine, L-asparaginases have varying levels of glutaminase activity that depends on the source of the enzyme. Post-MTX treatment with L-asparaginase, the deamination of glutamine has a synergistic effect on the activity of L-asparaginase [38,37]. Pretreatment with asparaginase decreases tumor cell retention, and lowers the tumoricidal effects, of MTX. The current literature suggests that this effect is largely due to a massive deamination of glutamine being required for optimal asparagine deamination [38].

Interference with cellular abilities to maintain the supply of glutamine, glutamate and glutathione is detrimental, and often fatal, to the cell. Glutaminase 2, a phosphate-activated mitochondrial enzyme, catalyzes the hydrolysis of glutamine to glutamate [39]. Loss of this enzyme results in an effect similar to methotrexate therapy: decreased NADH and glutathione with increased sensitivity to oxidant stress [39]. Combination chemotherapy with metabolically active agents, i.e., asparaginase, effectively starves the cancer cell of two sources of fuel used to maintain functional output of the TCA cycle. In settings of glutamine depletion, increased expression of asparagine synthetase has been observed in hamster cell lines [40]. Pretreatment of cancer cells with methotrexate significantly handicaps the regeneration of NAD(P)H. Glutamine and glutamate shift from supplying intermediates of the TCA cycle into a significant resource sink, de novo synthesis of glutathione. As intracellular levels of glutamine decrease, expression of asparagine synthetase increases with subsequent glutamine depletion and increased ammonia production. With an evolving depletion of glutamine and cellular shift toward increased asparagine synthesis, the addition of L-asparaginase induces a starvation of asparagine and glutamine with increased concentrations of aspartate, glutamate, and ammonia. The restriction of both asparagine and glutamine have been observed to induce expression of asparagine synthetase, glutamine synthetase, IGF-I and -II, IGFBP-1, CHOP, ornithine decarboxylase, oncogenes c-JUN and c-FOS and ribosomal proteins L17 and L25 [41]. In asparaginase-resistant cells, inhibition of glutamine synthetase has been shown to block proliferation and decrease cellular viability [42]. Additionally, as expression of asparagine and glutamine synthetase increases there is concomitant stabilization and up-regulation of the Myc and BCL-2 oncoproteins [41].

## 5. Mitochondrial roles in oxidative stress and carcinogenesis

Endosymbiotic theory is currently the most accepted process of mitochondrial origin. Two general assumptions accompany this theory. The first is that mitochondrial acquisition accompanied an

accumulation of atmospheric oxygen. The second marks a separation of aerobic and anaerobic (assumed lack of mitochondria) eukaryotes. The recent inclusion of various anaerobes to eukaryotic lineages is secondary to a much better understanding of variant “mitochondria-like” organelles – i.e., hydrogenosomes, mitosomes. Recent biological and geochemical theories propose an atmospheric oxygen increased long before a noticeable increase in oxygen tension in oceanic waters. This atmospheric oxygenation led to oxidation and solubilization of land bound sulfide deposits making ocean waters increasingly anoxic and sulfidic. In the light of this Proterozoic ocean chemistry theory for mitochondrial endosymbiosis, one can rationalize that the benefit of mitochondrial acquisition was not solely an ability to provide ATP via its utilization of oxygen but its ability to use electrons, as reduced molecules, to buffer oxidant stresses in the environment. As a more electronegative atom than sulfur, increasing concentrations of dissolved oxygen would make it a much more efficient recipient of those free electrons [43].

Contemporary mitochondria are very dynamic organelles. They fuse, divide, power metabolic detoxification, generate stored energy and serve many important regulatory and signaling functions. Recent literature describes a localizing mechanism of mitochondria with definitive influence by the oxygen tension of the cell [44,45]. In hypoxic states, mitochondria in pulmonary artery endothelial cells undergo inward migration, via microtubules, and localize around the nucleus [44]. Mitochondrial respiration is a significant producer of free radicals with the electron transport chain being the primary source. This movement is associated with a local increase in production of free radicals and reactive oxygen species in the vicinity of the nucleus [44].

Mechanisms to mitigate damage from oxidative stresses produced within the cell have evolved alongside the mitochondria. Superoxide dismutase (SOD), catalase, glutathione (GSH) and thioredoxin are well known scavengers of free radicals and their reactive metabolites [46]. Intermediates and products of glycolysis may also serve either antioxidant roles, such as pyruvate, or serve as a substrate for an antioxidant regenerating pathway as glucose-6-phosphate, glyceraldehyde-3-phosphate via NADH production [47,46]. The absence of adequate oxygen, presence of a dysfunctional electron transport chain, or a deficiency of cofactors places the mitochondria at risk of increased free radical production. A state of normal oxygen tension encourages mitochondrial localization in the cell periphery and a subsequent local increase in free radical concentrations. For a metabolically normal cell, injury induces the production of reactive oxygen species produced via NAD(P)H oxidase and then secretion into the extracellular matrix to initiate the process of healing. This peripheral increase in free radicals places cell membrane components at risk. The hydroxyl radical, regardless of its source, is one that may freely diffuse through cell membranes. One membrane component particularly vulnerable to hydroxyl radicals is arachidonic acid often esterified to membrane phospholipids. A class of eicosanoids called isoprostanes are formed upon reaction of arachidonic acid with free radicals; i.e., hydroxyl radicals [48]. Isoprostanes are isomers of the cyclo-oxygenase enzyme product prostaglandin F2 and will subsequently induce the same inflammatory cascade [49]. There is also some evidence to suggest the formation of thromboxane A2 via isoprostane endoperoxide rearrangement [50]. In a cell with poor antioxidant reserve, similar hypoxic events increase the likelihood of subsequent DNA damage from those free radicals.

Mitochondria are arguably the most significant physiologic players in chemical metabolism. Beyond the ETC., reactions catalyzed via the cytochrome P450 family of enzymes are a significant source of free radical production. Cytochrome P450 enzymes make up a superfamily of heme-thiolate proteins involved in steroid synthesis and are responsible for 75% of phase I drug metabolism [51–53]. The majority of these enzymes are associated with NAD(P)H-reductases and found in either the endoplasmic reticulum or the mitochondria [53]. An electron is obtained from NAD(P)H via the associated reductase enzyme and transferred to the substrate at the enzyme's binding site via its heme group

[51]. The overall efficiency of electron transfer from NAD(P)H to the cytochrome P450 enzyme and then to its substrate can vary. Although this reaction typically detoxifies a chemical, it may also transform a procarcinogen to its carcinogenic metabolite; i.e., 5-methylchrysene from tobacco smoke [54]. While some studies report an electron leakage flow of only 0.15%, in steroidogenic tissues the cytochrome P450 components outnumber electron transport chain components ten to one [53]. The “leaked” electrons can react with a variety of molecules to form free radicals, just as those from the electron transport chain.

In vitro studies show that MTX therapy decreases intracellular concentrations of reduced glutathione [55]. While NAD(P)H is required for regeneration of reduced glutathione, it is not required for de novo synthesis. In de novo synthesis, glutamate, cysteine, glycine and ATP are used in an enzyme catalyzed reaction. Depletion of glutathione in serine starved cells (a precursor to cysteine) has been associated with impaired glycolysis and an elevation in cellular ROS [56]. Tumor suppressor p53, in conjunction with the cyclin-dependent kinase inhibitor, p21, arrests cellular development and promoted cellular survival by rapid shunting of exogenous serine into the glutathione synthesis pathway [56]. In cells without p53, this shunt did not occur. The resulting serine depletion and oxidative stress lead to not only impaired proliferation, but also a reduction in cellular viability [56].

These rescue pathways must be supplied components in order to preserve the cell and maintain homeostasis. Catabolism of cellular components is a growing area of investigation into cancer metabolism and centers on autophagy. Autophagy is the process in which cells degrade and recycle macromolecules and organelles via two distinct pathways; the ubiquitin-proteasome system (UP system) and the lysosome [57]. Smaller, short-lived proteins are the typical degradation targets of the UP system. However, the size and stability large protein aggregates and organelles leave the lysosomal pathway as the most effective means of autophagy. In mammalian cells, lysosomal autophagy is directed by a chaperone system marking macromolecules and organelles for digestion [58]. The most apparent benefits of autophagy are resultant decreases in damaged, unneeded or toxic cellular components while producing raw materials for cellular metabolism [59].

While its pathways are not fully understood, the regulation of these processes is intimately linked with their function; i.e., recycling cellular components and providing fuel for cellular processes [60]. Cellular starvation is a powerful inducer of autophagy. A fall in intracellular amino acid concentrations is another stimulant of autophagy. Constructing a spectrum of starvation signals, nitrogen is an excellent marker of cellular nutrition. To clarify, in a very well supplied cell, amino acids would activate cellular anabolic pathways via conjugated tRNA activation of the Gcn protein kinases [61]. As amino acid concentrations drop, the number of transamination and deamination reactions in the cell increases to attenuate this lack of key amino acids. Cellular concentrations of ammonia, especially in concert with glutaminolysis, serve as a powerful regulator in the basal cellular autophagy rates [62]. Further along the spectrum, however, total nitrogen starvation is a more potent activator than either of the previous nitrogen-related stimulants [61].

Reactive oxygen species are also potent activators of autophagy. Superoxide, in particular, has been found to be a major player in the activation of reactive oxygen species induced autophagy. In properly functioning cells, it is reduced to hydrogen peroxide and then to water via SOD1, SOD2 and catalase or scavenged by one of many different antioxidants present in the cell or in circulation. It has been observed that not only do polymorphisms in the genes coding these proteins affect their efficacy, but chronic therapy with MTX also influences expression profiles with these genes [63]. When antioxidant systems are overwhelmed, mitochondrial components are at a very distinct geographical disadvantage to the effects of free radicals and reactive oxygen species. Although the GSH regenerating abilities of mitochondria are present, mitochondrial autophagy, or mitophagy, may ensue if sufficiently injured or damaged. This has been seen in association with significant decreases

in the ratio of GSH/GSSG inducing mitophagy, while ample concentrations of GSH are inhibitory to the process [64].

2-Deoxyglucose (2DG), and glucose starvation, have been observed to induce apoptosis in some cancer cells. Recent research has revealed a dichotomy of effect depending on cellular conditions. In hypoxic environments, 2DG and glucose starvation inhibit autophagic processes, possibly through significant decreases in ATP through PI3K III-Beclin1 complex blocking the effects of autophagy activating genes [65]. These same conditions will activate autophagy in a normoxic environment through separate pathways initiated by ROS formation [66,65]. This dual effect could be explained by the utilization of glycolysis intermediates and end-products in mitochondrial redox buffering roles.

## 6. Mitochondrial metabolism and the cell cycle

Senescent thymocytes obtain 88% of ATP production occurs from oxidative glucose metabolism. However, when stimulated to proliferate, 86% of ATP production is obtained without oxidative phosphorylation, by the conversion of glucose to lactate [10]. This shift to non-oxidative ATP production pathways results in significant increases, as much as 20-fold, in glucose utilization in proliferating cells [10,67]. This metabolic redirection to aerobic glycolysis and lactate production offers several benefits for the proliferating cell [10]. These include the production of numerous metabolic intermediates to serve as antioxidants, creation of an acidic environment to improve oxygenation and stimulation of angiogenesis via enzyme activation and the production of various inflammatory mediators – all of additional benefit to cancer cells in a hypoxic environment [10]. Outside of the process of cell division, this same metabolic redirection occurs during episodes of hypoxic stress. Prior to cell division, mitochondrial mass expands largely for two reasons. The first is in preparation for increased metabolic demands of replication. The second is the preparation for splitting of the parent cell's mitochondrial population between two daughter cells. As with mitochondrial localization induced by HIF, microtubules are involved in mitochondrial changes seen in preparation for cell division [68].

Cellular progression of G1- to S-phase is dependent on a favorable redox status in the cell and is a significant signaling pathway [69]. If we assume that the TCA cycle progresses at a constant rate and increased inner membrane mitochondrial volume correlates with total TCA cycle enzyme then the G1/S phase would have a high [NADH]/[NAD<sup>+</sup>] redox status [70]. In addition to mitochondrial morphologic changes, there is an associated decrease in ATP production due to decreased NADH flow through the electron transport change. High concentrations of NAD(P)H have been shown to have multiple effects on regulatory proteins, gene expression, directly or epigenetically, and cell cycle progression.

Mitochondrial inner membrane production in HeLa cells, with functional enzymatic capabilities, is completed by mid-S-Phase [70]. At this point in the cell cycle, there is no increase in mitochondrial membrane potential. Prior to entering M-Phase, there is a significant increase in mitochondrial mass and membrane potential, as much as 168% and 228% respectively, with less than a 20% increase in ATP [70]. There is a noted discrepancy, and delay, between the cessation of inner mitochondrial membrane accumulation, in mid-S phase, and the increase of mitochondrial membrane potential prior to M-phase. Metabolism during these transitional stages is highly dependent on glutamine for cellular metabolic demands. When starved for glutamine, synchronized HeLa cells do not progress from G1- to S-phase or S- to G2/M-phase [71]. When those same cells are placed in media with glutamine without glucose, the cell cycle does progress at these points, albeit at a slower rate [71].

The effects of folate depletion and methotrexate therapy on the cell cycle have been studied. Folic acid depleted HepG2 cells have been observed and they tend to arrest in an S-phase with notable inhibition of G2 phase progression [72]. Cell cycle arrest at these stages was observed to precede apoptosis in this study with those in S-phase arrest

particularly [72]. The literature also supports a methotrexate induced arrest the cell cycle S-Phase, i.e., an induced folate deficiency. The literature supports the observation of S-phase arrests in both low and high dose methotrexate therapies [73–75]. Interestingly, a thorough evaluation of these studies suggests a temporary blockade of G1 to S phase that occurs in the first few hours after administration of methotrexate and it appears to be dose related [74,75].

The historical cytotoxic mechanism of MTX is felt to be a disruption of DNA synthesis via its inhibition of DHFR. However, the antagonistic effect of MTX on other NADPH-dependent oxidoreductases resulting in decreasing NADH production and, subsequently, GSH depletion offers a slightly more accurate model of toxicity [11,29,7,8,6,28]. Depletion of reduced glutathione, GSH, has been observed to affect the cell cycle similarly to both folate depletion and methotrexate therapy. GSH depletion has not only been observed to restrict cell cycle progression from G1 to S-phase, but also restrict DNA synthesis arresting cells in S-Phase [76]. The authors of this study explain that the slightly less pronounced inhibition of G1 to S-phase may be due to pathways dependent on, in part, a separate pool of thiol proteins [76]. However, the mechanism by which methotrexate interferes with cell cycle progressions is not fully understood and studies are generally limited.

## 7. DNA damage and malignant transformation

Cellular hypoxia induces a peri-nuclear mitochondrial migration. Applying the same cellular state of fluctuating oxidative stress and free radical production in the vicinity of the nucleus would place the nuclear DNA at increased risk of damage. Beyond the risk for genetic damage and mutation during chromosomal replication, the current genome profile being expressed in a senescent cell would expose many regulatory and functional sites to an even higher risk of damage. Many commonly mutated tumor suppressor genes and oncogenes, P53, MYC and BCL as examples, induce metabolic changes in non-transformed cells in periods of high cellular stress [77–83]. Affecting amino acid metabolism, mitochondrial function, glycolysis and autophagy, the end function of these genes is to temporarily halt cellular self-destruction while processes attempt to overcome that stress. A Pyrrhic victory of sorts, these genes may prolong the life of the cell but at the cost of potential mutation if recovery fails, leading to cancerous transformation.

The mitochondrial chromosome is at a much higher risk for damage via this mechanism. Two important differences are present between nuclear and mitochondrial DNA, the first being that mitochondrial chromosomes are not protected via histones [84]. In nuclear DNA, genetic material is wrapped around histones and physically protected, to some degree, from the damaging effects of free radicals. The second factor is that the status of cellular oxygen tension would likely have no significant effect on the proximity of free radicals produced by mitochondria. Low level reactive oxygen species could be severe enough to induce mtDNA damage if not adequately buffered.

Combining the two factors, i.e., a state of cellular hypoxia and increased free radical production, would put the mitochondrial DNA at a much higher risk for damage than nuclear DNA. Periods of chronic reactive oxygen species exposure have been shown to damage mtDNA and other macromolecules more extensively and with longer lasting effect, than damage to nuclear DNA [85]. Whether its resultant acute damage or epigenetic changes through chronic FR exposure of nuclear or mitochondrial DNA, this process could be responsible for “locking” cancer cells into this abnormal metabolism [86–90].

## 8. Mitochondria, evolution and cancer development

Genetic predispositions aside, the literature supports the postulate that genetic mutations in most cancers are secondary to severe metabolic and mitochondrial dysfunction leading to inappropriate and inadequate buffering of oxidant stresses. A better understanding of the complex interactions of cellular metabolism, mitochondrial function,

and oxidative stress appears to have very widespread implications in the pathophysiology and treatment of disease [91–97]. Immediate application of this review falls on the subject of chemotherapeutics but applications may exist for the treatment or prevention of many pathologies.

While exact environmental parameters of Proterozoic oceans are under debate, an increased level of electrochemical redox stress from dissolving land bound sulfide deposits prior to oceanic oxygenation is certainly plausible [43]. Acquisition of an organelle with the sole purpose of buffering changes in redox stress would undoubtedly increase cellular fitness, expand the number of tolerable environments and allow for the evolution of more complex cellular processes [86–90]. Coincidentally or not, the acquisition of these organelles, and subsequent stabilization of environmental redox status, is followed by a massive increase in biodiversity known as the Cambrian Explosion that marks the end of the Proterozoic Era.

Current understandings describe the primary mitochondrial role as an oxygen dependent power plant with the primary role of ATP production via oxidative phosphorylation. Research into mitochondrial origins and the environmental redox status of early eukaryotic cells may further expose early roles of the ancestral organelle. Investigations of mitosomes and hydrogenosomes reveal organelles very similar to mitochondria, with reduced or non-existent ATP-producing functions [98]. Interestingly, all three organelles have in common the ability to create and store reduced molecules for later use by the cell. Some research suggests that molecular hydrogen, one product of hydrogenosomes, may serve as an effective antioxidant and has been shown to decrease area of infarction in rat brains affected by ischemia–reperfusion injuries [99]. The mitochondrial currency of stored reduced molecules, NAD(P)H, and its ratio to NAD<sup>+</sup> has been shown to have intimate interactions in the growth and metastasis of tumors. Excessive concentrations of NAD(P)H in relation to NAD<sup>+</sup> have been shown to have a positive effect on the growth and metastasis of breast cancers [100].

Many cancers originate in hypoxic microenvironments. Detailed studies have documented how characteristics of the tumor microenvironment can lead to significant changes in tumor phenotypes. Survival of these cells appears to be dependent, in part, upon mitochondrial abilities to regenerate free radical scavengers to buffer oxidant stress. Experimental studies have shown a dependence on mitochondrial and cytosolic properties to perpetuate tumors. By placing normal and tumor nuclei in tumor and normal cells respectively, normal daughter cells were produced upon division of the tumor nuclei and normal cell cytoplasm [101]. In the second population of cells, with normal nuclei and tumor cytoplasmic contents, division resulted in the production of more tumor cells or cell death [101].

The resultant metabolism of these cells becomes highly dependent on the catabolism of amino acids, particularly glutamine, to preserve the cell via regeneration of free radical scavengers. With this realization, the Warburg effect seemingly covers only part of the metabolic derangements seen in cancer. The increase in glycolysis and lactate production may serve as a depository for nitrogen freed from transamination reactions needed to fuel enzyme catalyzed reduction of NAD(P)<sup>+</sup>. Methotrexate interferes with many enzymes involved in reduction of NAD(P)<sup>+</sup> to NAD(P)H and, more significantly, cellular abilities to buffer oxidative stress.

## 9. Discussion

Acquisition of the early mitochondrial organelle led to increased cellular fitness, in part, due to its ability to buffer redox stresses secondary to changes in the cells' immediate environment. This buffering function increased cellular hardiness allowing survival during environmental changes. Although part of a whole, individual cells throughout the body are subject to changes and stresses in their own microenvironments. Cellular metabolites serve as intermediate signals of the extracellular environment to effect genetic expression. New understandings



of the pharmacologic mechanisms of methotrexate may suggest that cellular redox buffering, metabolism and mitochondrial function are more susceptible targets in cancer therapy than genomic attack. A better understanding of metabolic pathways and the effects of other anti-cancer agents on them may offer significant insight into paradoxical effects seen with many chemotherapeutic drugs. In consideration of the known and unknown mechanism of many current pharmaceutical agents in use today, it may be possible to fashion synergistic regimens of medications for a variety of cancers once the characteristic metabolic alterations of specific neoplasms are better understood.

The importance and function of the mitochondria and their changes during the cell cycle also offer insight into peculiarities of other chemotherapeutic agents. In regard to the antimetabolic chemotherapeutics, such as paclitaxel, a proliferation rate paradox is described by these agents selectively targeting of cells with high rates of division yet these same agents will induce regression in tumors with low rates of proliferation [102]. Beyond cell division, microtubules are highly utilized in the intracellular localization of mitochondria. Agents interfering with the proper function of microtubules likely interfere with the purposeful migration and localization of mitochondria throughout the cell cycle.

Recent targeting of cisplatin to increase mitochondrial uptake increases its efficacy in cancer cell lines that had previously grown resistant. The classically described mechanism of the highly reactive molecule, cisplatin, is based around the formation of adducts with proteins and DNA. Though the nuclear DNA adducts formed during cellular division are thought to be key markers of the anti-cancer mechanism of cisplatin, there is a paradoxical effect in regard to cytotoxicity of cisplatin in cells that are not actively dividing. Research has shown that an accumulation of cisplatin in mitochondria may be the responsible mechanism for its toxicity in non-mitotic cells. Adducts formed with mitochondrial DNA and proteins appear to increase rates of ROS production and have also been shown to activate caspases in enucleated cancer cells [103]. This mitochondrial pathway of toxicity is reinforced by the observation of increased cisplatin resistance in cells with dysfunctional mitochondria. As seen with methotrexate, there is an observed reduction in cisplatin toxicity in cells supplied with mitochondria-targeted antioxidants, particularly in regard to nephrotoxicity [103].

Despite the cell's extensive ability to neutralize free radicals via mitochondrially derived NAD(P)H, in a scenario of overwhelming oxidant stress the reserve capabilities of endogenous and exogenous antioxidants may be unable to defend the cell from the damaging effects of free radicals. These stresses may be environmental or nutrition related or due to the genetic predispositions of the cell. Iron, copper, various forms of radiation, inflammation, smoke, vehicle exhaust, anti-neoplastic drugs and ischemia/reperfusion injuries are all known to increase cellular oxidants [104]. Down syndrome is associated with increased oxidant stress secondary to over-expression of superoxide dismutase relative to catalase and glutathione peroxidase [105–107]. The SOD1 gene coding for superoxide dismutase is found on chromosome 21, 21q22.1 [107]. Individuals with Down syndrome have lower plasma total antioxidant status measured as reduced glutathione [107]. This inborn dysfunctional ratio leads to an increased susceptibility to cellular oxidant stress. It is well known that patients with Down syndrome are at an increased risk of not only malignancy, but also Alzheimer's disease and adverse drug reactions to many chemotherapeutic agents, including MTX. In addition to Down syndrome, similar mitochondrial and metabolic dysfunctions are accompanied by sensitivity to oxidant stresses in ataxia-telangiectasia, Fanconi anemia, and Werner syndrome [106].

By better understanding the biochemical pathways and metabolism of methotrexate and other chemotherapeutic agents, it may be possible to improve tumoricidal responses from anticancer radiotherapy. The mechanism of cellular toxicity from irradiation is the result of direct DNA damage secondary to free radical formation and its damage to

molecules in close proximity. It seems plausible that tumor cells could be "loaded" with methotrexate with co-administered glutamine supplementation prior to irradiation. By coordinating radiotherapy with peaking intracellular methotrexate polyglutamates with a serum methotrexate nadir it may be possible to increase the cytotoxic effect while decreasing radiation and collateral damage to healthy cells.

For millennia, the living cell has evolved mechanisms to preserve itself and protect its genome at all costs. The mitochondrial organelle appears to play a significant role not only in the evolution of complex life, but also in the development and maintenance of cancer cells. Despite overwhelming stresses ranging from extreme starvation to massive free radical attack, the cell persists. At the cost of growth and detriment to its host, the cancer cell persists. When its metabolism has "damaged" its cellular blueprints and reprogrammed cellular physiology enough to thrive in its new environment, the cancer cell proliferates, escapes its site of origin, settles elsewhere in its new world and, yet again, persists.

### Transparency documents

Transparency documents associated with this article can be found, in the online version.

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