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Review

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Abstract

Many chronic diseases are associated with mitochondrial dysfunction, and many of these same diseases are also associated with gut dysbiosis. In this paper, we link these two factors together by arguing that gut dysbiosis leads to mitochondrial dysfunction through an excess supply of deuterium to the mitochondrial ATPase pumps. We further argue that impaired one-carbon metabolism plays a central role in the disease process. One-carbon metabolism (also known as the methylation pathway) is a fundamental biochemical process involving the transfer of methyl groups from one molecule to another, to carry out many cellular functions, including DNA, RNA, and protein methylation and phosphatidylcholine synthesis. The pathway centers on S-adenosylmethionine (SAMe), the universal methyl donor. The methyl group is sourced from methyl-tetrahydrofolate, while methionine, glycine, serine, and formaldehyde are carriers of one-carbon units. An overlooked aspect is the crucial role that the gut microbiome plays in assuring that the one-carbon units are virtually free of deuterium. Deuterium is a natural heavy isotope of hydrogen, and it damages the ATPase pumps in the mitochondria. Microbes produce extremely deuterium depleted (deupleted) hydrogen gas which they use as a reducing agent to convert carbon dioxide into organic molecules, such as acetate, butyrate and formate. We show here through an in-depth review of methylation and demethylation processes how they are used to deliver deupleted protons to the mitochondria, to protect them from damage. Toxic exposures to organophosphate insecticides and deficiencies in nutrients such as methionine, choline, betaine, and serine can impair methylation pathways, causing disease through co-causality of mitochondrial dysfunction. We further suggest that synthetic versions of critical nutrients such as choline and methionine may be problematic because they supply methyl groups that are not deuterium depleted.

Keywords: gut dysbiosis; methanogens; acetogens; archaea; butyrate; methionine; choline; hydrogen gas; deuterium; mitochondria

1. Introduction

Myalgic encephalomyelitis/chronic fatigue syndrome (ME/CFS) is a debilitating chronic disease characterized by extreme fatigue, post-exertional malaise, impaired memory, pain, and gastrointestinal and sleep disturbances [1]. Its incidence may be increasing since the SARS-CoV-2 pandemic, because the condition known as long COVID has significant overlapping symptoms [2]. Mitochondrial dysfunction associated with oxidative stress is a major factor in both conditions [3,4].

A comprehensive multi-omics analysis involving 106 cases of ME/CFS and 91 healthy controls revealed major disturbances in the gut microbiome of patients compared to controls. Most notable was a reduction in the prevalence of the common butyrate-producing microbes *Faecalibacterium prausnitzii* and *Eubacterium rectale*, along with a deficiency in butyrate. The abundance of *F. prausnitzii* was inversely associated with fatigue severity. The acetate-producing bacterium *Coprobacter secundus* was also reduced in prevalence, along with reduced acetate production [5].

Ruminococcus gnavus, also known as *Mediterraneibacter gnavus*, a mucin-degrading bacterium [5], was elevated in the ME/CFS patients [6]. *R. gnavus* is strongly associated with Crohn's disease, and it produces an inflammatory polysaccharide called glucorhamnan, formed from the sugars rhamnose and glucose [7]. Another species that was elevated in association with ME/CFS was *Clostridium bolteae*, also known as *Enterocloster bolteae*, whose overgrowth has also been linked to autism [8], as well as IgG-4 related disease and systemic sclerosis [9], and fatty liver disease [10,11]. Like *R. gnavus*, it also produces an inflammatory immunogenic polysaccharide [8]. Interestingly, both *E. bolteae* and *R. gnavus* are alcohol producers, and increased levels of ethanol in the feces along with elevated levels of these species are associated with nonalcoholic steatohepatitis [11].

All the microbially produced nutrients mentioned above - lactate, ethanol, acetate, and butyrate - are low deuterium nutrients, and, for this reason, all are likely vital for the health of the mitochondria in the host [12]. Deuterium is a heavy isotope of protium, and it is a natural element found in seawater at 155 parts per million (ppm). Metabolic processes inherently favor a significantly deuterium depleted (deupleted) environment within the mitochondria, because deuterium damages the ATPase pumps, causing inefficiencies in ATP production and increasing the release of reactive oxygen species (ROS) [13]. The microbes play an essential role in the supply of deupleted nutrients to the host, and they accomplish this feat primarily through the synthesis of hydrogen gas that has lost 80% of the deuterium normally present in water [14]. This hydrogen gas is then used as a reducing agent to produce deupleted nutrients, especially acetate and butyrate, but also, as we shall see, formaldehyde, formate, glycine, serine, methionine, choline, and methyl-tetrahydrofolate.

While not derived directly from hydrogen gas, lactate does possess a deupleted proton. Lactate carries a "reducing equivalent" via the reversible reaction catalyzed by lactate dehydrogenase, which converts nicotinamide adenine dinucleotide (NADH) to NAD^+ to produce lactate, and reconverts NAD^+ back to NADH in the mitochondria when lactate is taken up from the medium [15]. Lactate dehydrogenase is a member of a large class of proteins called flavoproteins, which work with a cofactor flavin adenine dinucleotide (FAD) to facilitate the transfer of hydride ions, and these enzymes typically have a high deuterium kinetic isotope effect (KIE) due to their use of proton tunneling [16]. Deuterons are not nearly as efficient in this physical phenomenon as are protons due to their larger size. Sutcliffe et al. wrote in 2006: "It is now widely accepted that enzyme-catalyzed CH bond breakage occurs by quantum mechanical tunnelling" [16]. In the mitochondrial matrix, deupleted protons carried by NADH are released during the enzymatic action of NADH dehydrogenase (Complex II), another flavoprotein, which converts NADH back to NAD^+ [17]. This enzyme is possibly the most important direct supplier of deupleted protons to the mitochondrial ATPase pumps.

Ethanol plays a similar role in providing NADH to the mitochondria, through the activity of the mitochondrial enzyme acetaldehyde dehydrogenase. However, acetaldehyde, an intermediary in ethanol metabolism, is highly reactive, so ethanol is not an ideal nutrient for this reason [18,19].

F. prausnitzii is widely recognized as an important health-promoting butyrate-producing bacterium in the human colon, although efforts to produce *F. prausnitzii* probiotics are challenging due to its sensitivity to oxygen and dependence on other microbes for critical micronutrients [20,21]. Inflammatory conditions cause luminal oxygen levels to rise, and this is problematic for *F. prausnitzii*, a strict anaerobe [21,22]. Furthermore, *F. prausnitzii* are auxotrophic for folate, meaning that they depend upon either the diet or other microbes for its supply, and folate is essential for them to thrive [23]. Folate plays a central role in methylation pathways, the main topic of this paper, because, as methyl-tetrahydrofolate (CH_3-THF), it supplies methyl groups to S-adenosyl methionine (SAMe), the

universal methyl donor. The methyl groups will be highly unlikely to carry deuterium if they are derived from microbial metabolism.

In a study involving 60 patients with chronic fatigue, half of the patients were found to have serum levels of folate that were deficient (below 3.0 micrograms/liter) [24]. As early as 1968 it was already recognized that folate deficiency is a common feature in Crohn's disease [25], and this deficiency was later confirmed in a paper published in 2010 [26]. The human gut microbiome has a very efficient capacity to convert dietary folates into CH_3-THF, but the microbes are unable to reduce supplemental folic acid to folate and subsequently to CH_3-THF. Folic acid is delivered unmodified to the liver, which then reduces it to folate and converts it to CH_3-THF. Excessive doses of folic acid stress the liver, causing pseudo-MTHFR (CH_2-THF reductase) deficiency and liver injury in mice [27]. High doses of folic acid appear unmodified in the circulation and are likely not useful for supporting methylation pathways [28]. Gut dysbiosis is now recognized to be linked to a long list of debilitating chronic diseases, including type 2 diabetes, obesity, non-alcoholic fatty liver disease, hypertension, arthritis, autism, depression, anxiety, and sleep disorder [29]. A common thread may be decreased acetate and butyrate production, concurrent with insufficient methylation capacity and increased production of lactate, ethanol, and inflammatory polysaccharides.

2. Hydrogen Gas Recycling by Gut Microbes: A Critical Role for Formaldehyde

The production of hydrogen and methane gasses by gut microbes is an essential step in the recycling of organic matter from carbon dioxide (CO_2), through multiple organic intermediates, and back to CO_2. Gut microbes can use hydrogen gas to reduce CO_2 to both methane and acetate, and they can then oxidize methane in a series of steps yielding methanol, formaldehyde, and formate, and finally returning to CO_2 in a complete cycle. In the process, the hydrogen atoms originally in the hydrogen gas are retained in the biosynthesized small organic molecules, and they become valuable to the host because they are extremely deupleted. This is because of the under-appreciated fact that the hydrogen gas has much less deuterium than is typical in the natural world, probably in part because deuterons preferentially stay in the liquid phase. The microbes that produce abundant H_2 become enriched in deuterium internally as a consequence, but microbes are much more resilient to high deuterium load than mammals are [30]. In fact, it is possible to cultivate deuterium-tolerant strains of *Escherichia coli* via an adaptation protocol, and these microbes have proven to be useful for synthesizing highly deuterated biological molecules for experimental use. Via such methods, O Paliy et al. succeeded in creating three highly deuterium-tolerant strains of *E. coli* [31]. *E. coli* possesses four membrane-associated [Ni-Fe]-hydrogenases that can produce H_2 gas from glucose, formate, glycerol, and other small organic molecules, via anaerobic fermentation [32].

Strict anaerobes likely play an essential role in providing deupleted nutrients to the host. Interestingly, research comparing the deuterium content of lipids in sediments sourced from deep marine water vs shallow water revealed that certain lipids produced at depth by strict anaerobes had significantly less deuterium content, with δD values up to -348‰ (parts per thousand), which is about 100 ppm as compared to 155 ppm. This same study found significantly enriched deuterium in hopanoids and branched chain fatty acids, components of bacterial membranes produced by bacteria at the surface [33].

2.1. Production of Methane Gas by Archaea and the Recycling Between Methane and CO_2

Evolution has created the three domains of life, which are the Archaea, the Bacteria, and the Eukarya. Archaea are a remarkable group of microorganisms with unique capabilities that play an essential role in supporting the other domains. Methanogens are anaerobic archaea that can synthesize methane from carbon dioxide and hydrogen gas using a unique set of enzymes not present in the other two domains. Methane production (methanogenesis) is the only biochemical pathway that methanogens use to generate ATP [34]. The unique enzyme complex, methyl-coenzyme M reductase, catalyzes the final step that reduces methyl-coenzyme M to methane [35]. These

remarkable microbes can produce methane not only from CO_2 but also from formate, methanol, methylamines, and acetate by a pathway involving a unique set of coenzymes [36].

Table 1 shows the reaction sequence that begins with four molecules of deupleted hydrogen gas and, through a series of steps, converts CO_2 to a sequence of small organic molecules, finally returning to CO_2 production in a complete cycle. Formaldehyde dehydrogenase depends on the cofactor NAD^+, which it converts to NADH as another product of the reaction [37]. Methanol dehydrogenases also use NAD^+ as a cofactor in some cases, but other cofactors are also found, such as pyrroloquinoline quinone (PQQ) [38]. Formate dehydrogenase can also convert NAD^+ to NADH, but there is an alternative pathway that uses a hydrogenase to produce hydrogen gas. The formate hydrogenylase complex (FHL), expressed by *E. coli*, catalyzes the decomposition of formate into CO_2 and H_2 gas, restoring one of the four hydrogen molecules that were consumed in this cycle. This is the primary path by which hydrogen gas is originally produced by enteric bacterial fermentation processes that often begin with glucose [39].

The overall result of the cycle, as shown in Table 1, is the production of 2 molecules of deupleted NADH from NAD^+, two molecules of deupleted water, and 2 deupleted protons. All these products, accumulating in the gut lumen, become valuable in helping to maintain low deuterium in the host mitochondria.

Not all of the formaldehyde is converted to CO_2 -- some of it is siphoned off to convert tetrahydrofolate (THF) to methylene-tetrahdrofolate (CH_2-THF). The two protons in the methylene group will be extremely deupleted due to the fact that they are sourced from formaldehyde, which traces back to H_2 gas. Both methylotrophs and methanotrophs possess the necessary enzymes to conjugate formaldehyde with THF to produce CH_2-THF. This is an important point that we will return to later in this paper, because it implies that the methyl groups carried by methionine will be extremely deupleted. Formaldehyde can also react spontaneously with THF, although the enzymatic pathway is favored in the microbes [40].

Table 1. The reaction sequence that is common among gut microbes, which starts by converting carbon dioxide and extremely deupleted hydrogen gas into methane gas and water. A series of dehydrogenases extract protons from methane, ultimately converting it to formate, while reducing NAD^+ to NADH, or converting oxygen to water while oxidizing NADH to NAD+. The last step in the cycle restores carbon dioxide, to come full circle. In the end, two molecules of NAD^+ are reduced to NADH, two molecules of deupleted water are produced, and two deupleted protons are released into the medium. Some of the formaldehyde that is produced gets siphoned off to convert tetrahydrofolate to methylene-tetrahydrofolate, thus seeding methylation pathways with deupleted protons originally sourced from hydrogen gas.

Reaction	Enzymes involved
$4H_2 + CO_2 \rightarrow CH_4 + 2H_2O$	Methanogenesis by archaea
$CH_4 + O_2 + NADH + H^+ \rightarrow CH_3OH + NAD^+ + H_2O$	Methane monooxygenase
$CH_3OH + NAD+ \rightarrow HCHO + NADH + H^+$	Methanol dehydrogenase
$HCHO + NAD^+ + H_2O \rightarrow HCOOH + NADH + H^+$	Formaldehyde dehydrogenase
$HCOOH + NAD^+ \rightarrow CO_2 + NADH + H^+$	Formate dehydrogenase
$4H_2 + 2NAD^+ + O_2 \rightarrow 2NADH + 2H^+ + 2H_2O$	**Overall Reaction**

2.2. Synthesis of Deupleted Hydrogen Gas Through Fermentation

The hydrogen gas that archaea use to synthesize methane from CO_2 is produced by many species of anaerobic bacteria in the colon through fermentation of dietary fiber and sugars. The primary hydrogen-producing bacteria are members of the Bacteroides and Firmicutes phyla [41,42]. A highly significant finding is that hydrogen gas produced by microbes from small organic molecules is likely severely depleted in deuterium, compared to the typical ratio of deuterium to hydrogen in nature [12]. It has been found experimentally that microbially produced hydrogen gas had a deuterium fraction that was 80% lower than the reference amount normally found in seawater (155 parts per million) [14]. While this fractionation is partially due to the fact that deuterium tends to stay in the

liquid phase, hydrogenases, the class of enzymes that perform this feat, are members of the flavoprotein class of enzymes that exploit proton tunneling to favor hydrogen over deuterium in their reaction product [43]. Hydrogenases can have a very high deuterium kinetic isotope effect (KIE), as high as 43 in an acidic environment [44].

2.3. Other Pathways Besides Methane Production for H_2 Consumption

Besides methane production, H_2 can also be used to reduce CO_2 to acetate and to reduce sulfate to hydrogen sulfide gas, H_2S. Both the acetate and the H_2S are therefore likely to be sources of deupleted protons. Acetogenic bacteria are a specialized group of strictly anaerobic species that can convert two molecules of CO_2 to acetate using H_2 as a reducing agent in the Wood-Ljungdahl pathway. They are primarily members of the Firmicutes phylum [45].

While excessive H_2S is toxic to human cells, H_2S is also a signaling gas, and it can be utilized by mitochondria to facilitate ATP production. In fact, human cells endogenously synthesize H_2S from cysteine under stress conditions, and it is released into the mitochondrial intermembrane space, where it is metabolized to sulfate and thiosulfate [46]. H_2S oxidation in the mitochondria could be a mechanism to decrease the deuterium burden in the mitochondria through the supply of two deupleted protons sourced from H_2S.

Figure 1 illustrates the three primary pathways where bacteria consume H_2 to produce useful organic products. Sulfate-reducing bacteria convert sulfate to hydrogen sulfide gas, methanogens convert carbon dioxide to methane, and acetogens convert carbon dioxide to acetate. We argue here that all three of these products are useful sources of deupleted protons. Acetate can be converted to acetyl-Coenzyme A (acetyl-CoA) and metabolized in the citric acid cycle in the mitochondria to yield CO_2 and water. Acetate is also the precursor to butyrate, a short-chain fatty acid that is the primary energy source of the colonocytes [47]. Methane, through the intermediary formaldehyde, becomes a source of deupleted methyl groups that are ultimately transferred from CH_3-THF to homocysteine to form methionine, the universal methyl donor, via the cobalamin-dependent enzyme, methionine synthase [48]. The step that adds the third proton to convert CH_2-THF to CH_3-THF is catalyzed by the enzyme MTHFR, a flavoprotein that can select protons over deuterons through proton tunneling. Thus, all the protons in the methyl group attached to methionine's sulfur atom can be expected to be deupleted. H_2S, the product of the third pathway, can provide two deupleted protons to the intermembrane space of the mitochondria when it is oxidized to sulfate and thiosulfate.
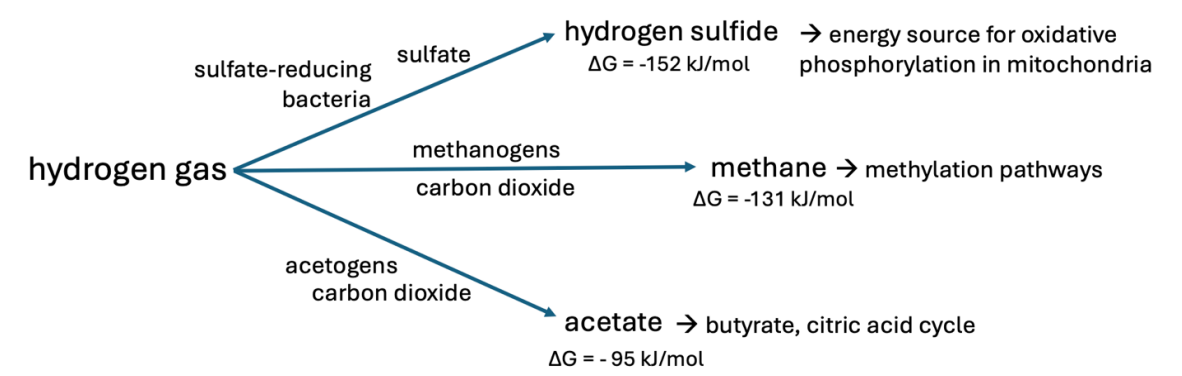
Figure 1. Schematic of three primary pathways where bacteria utilize hydrogen gas as a reducing agent. All three pathways yield valuable metabolites that can help the host mitochondria maintain low levels of deuterium. The Gibbs free energy (ΔG) values show that hydrogen sulfide production is the most thermodynamically favorable among the three pathways.
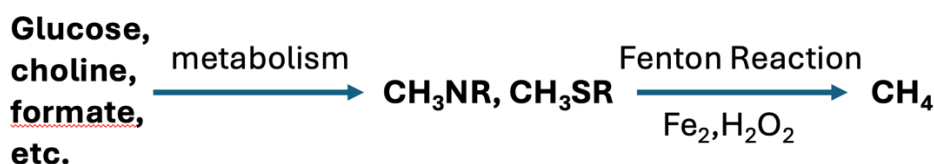
2.4. An Important Role for Methylotrophs

The human archaeome (archaeal microbes that colonize the human gut) is an underestimated contributor to the gut microbiome [49]. Thus far, we have shown that archaea can use hydrogen gas as a reducing agent to produce severely deupleted methane gas, which is then utilized by gut bacteria to produce formate, NADH, and deupleted water, and to supply methylene units to the methylation pathways. The methanogenic order of archaea called Methanomassiliicoccales are exclusively obligate anaerobes. These unique organisms produce methane by hydrogen-dependent reduction of methanol or methylamines [50]. Members of this clade inhabit the human gut, where they can metabolize tri-, di-, and monomethylamine to produce methane gas. The enzyme trimethylamine (TMA) methyltransferase (MttB) transfers a methyl group from TMA to a corrinoid protein, from which it is then transferred by coenzyme M methyltransferase to coenzyme M. Methyl Coenzyme M reductase then produces methane, the final product [51–53].

While *Methanobrevibacter smithii* is the dominant archaeal methanogen in the human gut, Methanomassiliicoccales are also found at significant levels, and it is intriguing that their levels rise with age [54]. It has been suggested that they would likely be beneficial as a probiotic because of their ability to clear TMA from the gut [34]. This would lead to a reduction in the production of trimethylamine oxide (TMAO) by the liver. Elevated serum levels of TMAO are linked to cardiovascular disease [34] and to fatty liver disease [55]. The pathway from choline to trimethylamine to methane gas may ultimately be a significant contributor to the supply of deupleted NADH and deupleted methyl groups in the methylation pathways.

While it is traditionally said that methane is produced mainly by enzymatic decomposition of organic matter by anaerobic archaea, it has been shown recently that many organisms, including bacteria, cyanobacteria, plants, fungi, algae, and even human cells, can produce methane *nonenzymatically* in the presence of reactive oxygen. Methane production by *Bacillus subtilis* and *E. coli* is triggered by free iron and reactive oxygen species (ROS) under conditions of oxidative stress. A seminal paper by Ernst et al. demonstrated that methyl groups bound to nitrogen and sulfur are substrates for this spontaneous reaction, where they are converted to reactive methyl radicals as an intermediary [56]. This process is shown schematically in Figure 2. An editorial on the Ernst et al. paper made bold predictions as follows: "An in-depth understanding of the various factors that control cellular methane formation and consumption and a thorough understanding of its dual function and bioactivity may bring significant benefits to the domain of human health and disease." [57].

It is tempting to speculate that oxidative stress induced by deuterium overload in the mitochondria leads to the productive release of methane gas derived from methyl groups that can then be recycled into useful sources of deupleted protons through the action of resident gut methanotrophs. Oxidative stress in the gut linked to inflammatory bowel disease would likely impair the activity of strictly anaerobic species [21,22], and it is therefore fortuitous that methane can be sourced from methyl groups under such conditions to compensate for the reduction in anaerobic methane production.

Glucose, choline, formate, etc. $\xrightarrow{\text{metabolism}}$ **CH$_3$NR, CH$_3$SR** $\xrightarrow[\text{Fe}_2,\text{H}_2\text{O}_2]{\text{Fenton Reaction}}$ **CH$_4$**

Examples: trimethylamine (CH$_3$)$_3$N, dimethylsulfoxide (DMSO)

Figure 2. Especially under conditions of oxidative stress, several gut microbial strains, and even human cells, can convert methyl groups attached to nitrogen or sulfur to methane gas. Possible substrates include methionine, trimethylamine (TMA) and dimethylsulfoxide (DMSO).

3. Choline Plays a Central Role in Methylation Pathways

In this section, we trace the metabolic pathways associated with choline, a nutrient that has been shown to have many benefits to human health.   Choline carries three methyl groups attached to its nitrogen atom, which are sourced from SAMe.   As phosphatidylcholine, it populates plasma membranes. As acetylcholine, it acts as an excitatory neurotransmitter in neurons. Gut microbes can break it down into interesting metabolites that likely serve to further deplete deuterium in derived nutrients. But the liver produces a metabolite called trimethylamine oxide (TMAO) whose elevation in the serum has been linked to many chronic diseases.
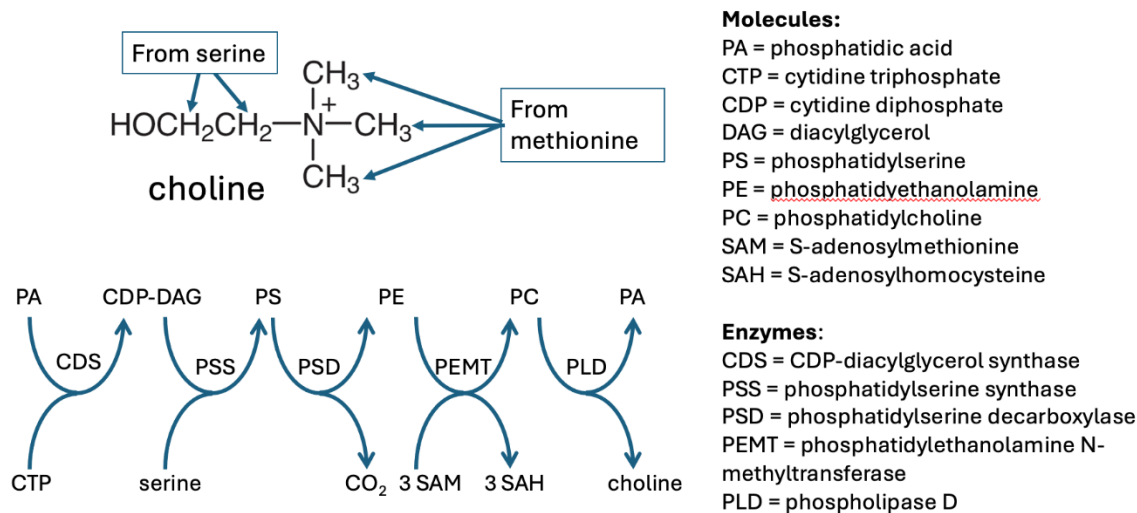
3.1. Choline Synthesis

Choline, also known as trimethylethanolamine, is a remarkable nutrient known to be essential for brain health. Phosphatidylcholine (PC) is the most abundant phospholipid in the outer leaflet of mammalian plasma membranes, and it serves as a storage form of choline. PC localizes to the outer layer of the lipid bilayer. PC hydrolysis by phospholipase D (PLD) releases free choline, which can then be taken up by neurons and converted to acetylcholine, a powerful neurotransmitter.

De novo choline synthesis as PC takes place primarily in the liver, in a series of steps that involve three phospholipids -- phosphatidyl serine (PS), phosphatidylethanolamine (PE) and phosphatidylcholine (PC), via the Kennedy pathway [58]. Phosphatidylserine decarboxylase (PSD) converts PS to PE, and then PE becomes PC via the transfer of three methyl groups from SAMe to PE, catalyzed by phosphatidylethanolamine N-methyl transferase (PEMT).   Since the methyl groups in SAMe are derived from CH_3-THF, they too will be deupleted. These pathways are illustrated in Figure 3.

PC can also be synthesized directly from dietary choline, which normally accounts for about 70% of the PC molecules in the body. Choline is an essential nutrient because the liver does not normally synthesize enough to serve the body's needs. Humans with insufficient dietary choline develop fatty liver disease [59]. However, PEMT plays an essential supportive role as well, since Pemt-/- mice develop non-alcoholic fatty liver disease and cholestasis when fed a high fat diet [60].

Plants synthesize choline via a somewhat different pathway, which begins with phosphoethanolamine instead of PE. But the enzyme that catalyzes the reaction, phosphoethanolamine N-methyltransferase (PEAMT) also transfers methyl groups from SAMe, just as in animal-based synthesis [61].

Molecules:
PA = phosphatidic acid
CTP = cytidine triphosphate
CDP = cytidine diphosphate
DAG = diacylglycerol
PS = phosphatidylserine
PE = phosphatidyethanolamine
PC = phosphatidylcholine
SAM = S-adenosylmethionine
SAH = S-adenosylhomocysteine

Enzymes:
CDS = CDP-diacylglycerol synthase
PSS = phosphatidylserine synthase
PSD = phosphatidylserine decarboxylase
PEMT = phosphatidylethanolamine N-methyltransferase
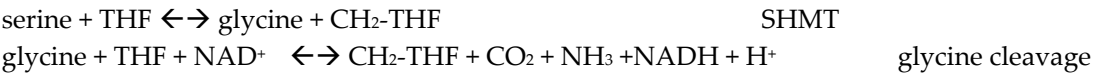PLD = phospholipase D

Figure 3. The series of reactions that lead to choline synthesis, beginning with PA. Three different membrane phospholipids (PE, PS, and PC) act as intermediaries. PE and PS localize mainly to the inner membrane of the lipid bilayer in the plasma membrane. Once three methyl groups are attached to ethanolamine via enzymatic

action of PEMT, the product PC translocates to the outer membrane. PLD is the enzyme that detaches choline from PA in the outer membrane to release it into the circulation.

All the protons bound to the carbon atoms in choline can be predicted to be deupleted, given their origins. This may be the primary reason why choline is so important in metabolism. Both the α carbon and the β carbon in serine can be traced back to formaldehyde because they are sourced from CH_2-THF via the glycine cleavage system and serine hydroxymethyltransferase (SHMT) (see Figure 4). Both reactions take place primarily in the mitochondrial inner membrane.

serine + THF $\longleftrightarrow$ glycine + CH_2-THF   SHMT

glycine + THF + NAD^+ $\longleftrightarrow$ CH_2-THF + CO_2 + NH_3 +NADH + H^+ glycine cleavage

The glycine cleavage system is widely expressed in animals, plants, and bacteria. The reaction is reversible, so it can be used both to synthesize and to degrade glycine [62]. Whether glycine is obtained from the diet or synthesized by gut microbes or human cells, the protons bound to its α carbon atom will be deupleted, because of their origin in formaldehyde. Glycine sourced from a synthetic supplement is an exception. The glycine cleavage system is the most prominent pathway in serine and glycine catabolism in humans and other vertebrates [62].
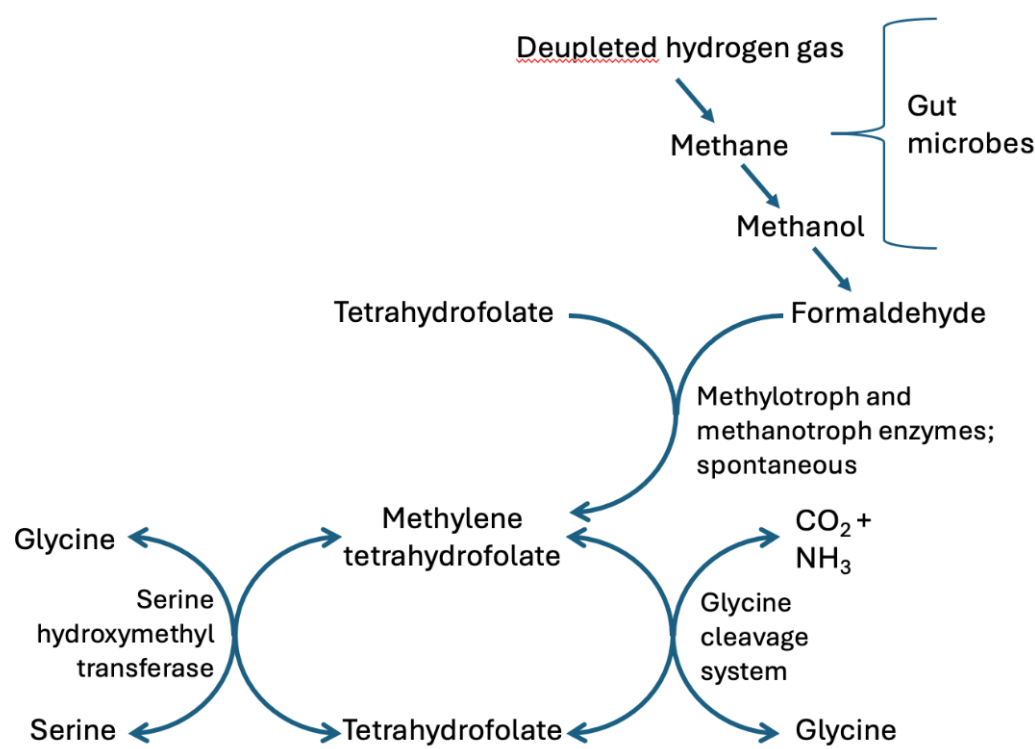
Figure 4. Glycine and serine are both mobile carriers of deupleted methylene groups transferred from methylene tetrahydrofolate. Both the α carbon and the β carbon of serine can be expected to be deupleted because they trace back to formaldehyde produced by gut microbes from methane gas, where the protons were sourced from extremely deupleted hydrogen gas. Formaldehyde feeds into the system either through a spontaneous reaction or through enzymatic catalysis by enzymes expressed by methylotrophs and methanotrophs.

3.2. Choline Metabolism

Dietary choline can be metabolized via several different pathways, many of which involve gut microbial enzymes. Up to 60% of the methyl groups in a typical human diet are derived from choline [63]. Choline is first converted to betaine (trimethylglycine) via the intermediary glycine betaine aldehyde (GlyBA). Choline oxidase, a microbial enzyme, converts choline to GlyBA, which is then converted to betaine by betaine-aldehyde dehydrogenase (BADH), primarily expressed in the liver.

Betaine then donates its three methyl groups to three molecules of homocysteine, regenerating three methionine molecules.

Another pathway for choline metabolism begins with the microbial enzyme choline trimethylamine-lyase (CutC), which anaerobic bacteria use to convert choline into trimethylamine (TMA) and acetaldehyde [64]. TMA can be further metabolized by gut microbes to produce either three molecules of methane (via the enzyme methyl-coenzyme M reductase (MCR) expressed in archaea), or three molecules of formaldehyde, via the microbial enzyme trimethylamine dehydrogenase (TMADH), found in methylotrophs. Both the methane and the formaldehyde potentially become excellent sources of deupleted protons to fuel the mitochondria in the colonocytes. Any TMA that does not get further metabolized by gut microbes makes its way to the liver, where it is oxidized to trimethylamine oxide (TMAO). Elevated levels of TMAO in the blood are associated with both fatty liver disease and increased risk to cardiovascular disease [4]. Archaea protect from the synthesis of TMAO in the liver by metabolizing TMA to produce methane gas [65].

Most intriguing is the fact that TMA dehydrogenase is a flavoprotein with a high deuterium KIE due to vibrationally assisted hydrogen tunneling [66]. A study on its activity on deuterated dimethylamine showed that the KIE for Kcat/Km had a strong dependency on pH and rose to as high as 8 at pH 9 or above [67]. What this could mean is that it selectively removes molecules of TMA that are free of deuterium, leaving behind a pool that is enriched in deuterium, which can then get oxidized in the liver to produce *deuterium-enriched* TMAO.

As shown in Figure 5, the metabolism of choline ultimately to glycine, via the gut microbes and the liver, results in the production of one molecule of NADH, one molecule of H_2O_2, and three molecules of methionine. All of these are useful sources of deupleted protons, assuming that the protons in choline are deupleted. Laboratory-synthesized choline is problematic because it is not expected to have deupleted protons.

Enzymes:
ChOx: Choline oxidase (microbial enzyme)
BADH betaine-aldehyde dehydrogenase
BHMT: betaine-homocysteine S-methyltransferase
CutC: choline trimethylamine-lyase (anaerobic microbial)
MCR: methyl-coenzyme M reductase (in archaea)
TMADH trimethylamine dehydrogenase (microbial enzyme)
FMO3: flavin-containing monooxygenase 3 (liver enzyme)
ChAT: choline acetyltransferase (in neurons)

Metabolites:
GlyBA: glycine betaine aldehyde
TMA: trimethylamine
TMAO: trimethylamine oxide
HCHO: formaldehyde
TMG: trimethylglycine (betaine)
DMG: dimethylglycine
MMG: monomethylglycine (sarcosine)
HCY: homocysteine
MET: methionine

Figure 5. Metabolic pathways in the gut, liver and brain that are involved in choline metabolism.   Anaerobic microbes convert choline to TMA.   When the archaeal methylogens that express MCR are deficient, the liver converts TMA to TMAO, a metabolite whose elevation in the circulation is linked to fatty liver disease and increased cardiovascular risk. Gut microbes convert choline to TMG, while releasing hydrogen peroxide and converting NAD^+ to NADH. The three methyl groups in TMG are metabolized sequentially in the liver and kidneys, leaving glycine as the final reaction product. This pathway regenerates three methionine molecules

from homocysteine. Choline is reversibly converted to acetylcholine in the brain, and acetylcholine is a powerful neurotransmitter that may also be significant for supplying acetate as a fuel source for the astrocytes. .

3.3. Dietary Versus Synthetic Supplemental Choline

The study of dietary and supplemental choline and its potential therapeutic impact came to prominence in the 1970s. A 1977 study was the first to show that choline supplements given to patients with tardive dyskinesia brought about a significant reduction in choreic movements in 9 of 20 patients over the course of 2 weeks of supplementation. The benefit was presumed to be due to an increase in brain acetylcholine in response to the supplemental choline [68].   Since that time, hundreds of studies have sought to clarify a wide range of potential health benefits of dietary or supplemental choline. The most consistently identified benefit has been with respect to improved memory and/or attention in patients with Alzheimer's or other forms of cognitive decline, which is again attributed to its ability to increase brain acetylcholine production [69].

The growing recognition of choline's role in maintaining health led to the National Institute of Health including it among the nutrients for which it established a Dietary Reference Intake (DRI) in 1998 [70].   Sales data for choline over time are not available, but sales of cognition-enhancing nutrients called "nootropics," of which choline is one, are projected to have a compounded annual growth rate globally of nearly 15% between 2023 and 2030 [71].

It is within this context that we feel it is important to bring to wider awareness the processes utilized for the manufacture of choline sold as a dietary supplement, and the potential that deuterium introduced incidentally through these purely synthetic manufacturing processes could impact the efficacy of these products, for the reasons explained in detail in this paper.

One method of producing choline at scale involves the application of solvents such as ethanol, hexane, or acetone, to natural choline sources such as arctic krill, soybeans, sunflower seeds, or egg yolks [72]. We would expect the choline derived from these natural sources to be relatively deupleted due to the KIEs of the enzymes involved in its biosynthesis as we have described throughout this paper. Consequently, TMA, a microbial metabolite of choline described previously, would also be deupleted in its three methyl groups. As mentioned, if the anaerobic methylotrophs are thriving in the gut, those methyl groups go on to contribute to methane gas or formaldehyde production, both of which act as substrates for the methylation cycle and SAMe production.

A purely synthetic method for choline production was patented as long ago as the 1950s, wherein trimethylamine was reacted with ethylene oxide to produce choline that was considered pure enough for human consumption [73]. Since that time, several other techniques have been developed for the production of various forms of choline salts sold as dietary supplements, such as choline chloride, choline bitartrate, and choline citrate. These salts are formed predominantly by reacting trimethylamine (which is itself synthetically produced) with the relevant acid to generate the salt, leaving behind trimethylamine oxide (TMAO) as a contaminant that must be removed in order to purify the choline salt before consumption.

It is interesting to note that supplementation with the synthetically produced choline bitartrate, but not dietary choline or naturally derived phosphatidylcholine, resulted in a significant increase in serum TMAO levels [74]. These authors wrote in the Conclusion of their abstract: "Despite high choline content in egg yolks, healthy participants consuming four eggs daily showed no significant increase in TMAO or platelet reactivity. However, choline bitartrate supplements providing comparable total choline raised both TMAO and platelet reactivity, demonstrating that the form and source of dietary choline differentially contributes to systemic TMAO levels and platelet responsiveness." The authors of that study hypothesized that phosphatidylcholine might be less available to the gut microbes for metabolism into TMA, compared to a choline salt. In a study aptly titled, "Gut microbe-generated trimethylamine N-oxide from dietary choline is prothrombotic in subjects", both vegetarians and omnivores were given a supplement containing 500 mg choline bitartrate twice a day over a period of two months.   Both groups showed a ten-fold increase in

TMAO levels already after one month of supplementation, and this correlated with increased platelet aggregation associated with thrombotic risk [75].

We suggest that the cause of these differences in choline's differential relationship with TMAO production may have to do with the relative excess of deuterium that would be introduced into the synthetically produced choline compared to the deuterium depletion inherent within a naturally derived choline source. The methyl groups associated with the TMA microbially generated from a high deuterium supplement would be less favored enzymatically for donation as methyl groups. Consequently, more would be transported from the gut to the liver to be oxidized into TMAO, resulting in the risks associated with that metabolite.   Furthermore, the TMA that does not get metabolized by the archaea (and is therefore converted to TMAO by the liver) becomes relatively enriched in deuterium, because the high KIE of the enzymes involved results in the selective removal of the deupleted molecules of TMA. It may be that TMAO is exploited by the body as a signal of deuterium overload in the methylation pathways, and metabolic policies are altered to reflect this issue.

3.4. The Pathogenic Mechanisms of TMAO that Induce Tissue Damage and Disease

Choline is considered an essential nutrient for human health. However, the metabolites of choline produced by the gut microbes and the liver enzymes, TMA and subsequently TMAO in the liver, respectively, are responsible for the development of human diseases such as those involving liver, kidney, and cardiovascular damage [76]. Elevated levels of TMAO in the blood stream result in systemic inflammation, endoplasmic reticulum (ER) stress, mitochondrial stress, and disruption of normal physiological functions [77]. TMAO causes its harmful effects on cells by modulating the autophagy mechanisms. Autophagy for normal cells is a housekeeping mechanism to degrade an excess of damaged organelles and through recycling to provide new cellular constituents for regeneration and self-sustainability. However, when dysregulated, autophagy can lead to cell death, tissue damage, and disease [78]. Nevertheless, autophagy is also a life-rescuing mechanism for cancer cells [79]. Therefore, a fine balance of autophagic mechanisms is required to maintain homeostasis.

TMAO's harmful effects caused by modulation of autophagy are bidirectional, even for the same organ. TMAO's inhibition of autophagy produces damage to the kidneys through the activation of NLRP3 inflammasome and mitochondrial dysfunction [80]. Overall, these two pathogenic processes are interrelated [81]. On the other hand, again regarding kidney disease, when it promotes autophagy, TMAO also promotes cell death and thus kidney tissue damage through the aggravation of overwhelming deposition of calcium oxalate crystals that induce the production of ROS and inflammation [82].

Regarding cardiometabolic disease, TMAO, by inhibiting autophagy, activates the p13K/AKT/mTOR pathway and interferes with the activity of p62 protein, provoking the expression of other interrelated genes that promote atherosclerosis [83]. Indeed, other studies on human aortic cells have shown that TMAO, apart from its action as an inflammatory pathway mediator (by phosphorylating numerous kinases in the cytosol) is a mitochondrial gene modulator, and a promoter of intracellular adhesion molecule (ICAM) expression that is tightly linked to atherosclerosis [84]. TMAO induces alterations in healthy metabolic processes and inflammation through the generation of ROS [85]. These mechanisms involve the ER and mitochondrial stress, and the disturbance of innate immunity.

On the other hand, as in the kidney tissue cases with TMAO pathogenic activity, TMAO, by increasing the autophagic degradation of an essential calcium transporter, the sarcoplasmic reticulum $Ca2+$-ATPase (SERCA2a), affects calcium homeostasis. TMAO induced cardiac hypertrophy in the animals tested in the B Lei et al. study by increasing the intracellular calcium concentrations [86]. Moreover, the reversal of autophagic (lysosomal) induction by TMAO restored the SERCA2a-mediated calcium influx and helped to alleviate cardiac hypertrophy. However, the pathogenic outcome was not only attributed to TMAO's promotion of autophagic degradative capacity, but also to the alteration of essential cellular regulators that protect from cardiac myopathy on a genetic level.

The response to TMAO in this study was found not only to downregulate the expression of the *SERCA-2a* gene but also to upregulate the expression of the *ANP* and *MYH7* genes. The myosin heavy chain 7 (*MYH7)* gene expression has been found to be responsible for severe cardiac and other skeletal myopathy complications [87], whereas the atrial natriuretic peptide (*ANP*) gene encodes for a hormone-like peptide, ANP, which is often released in conditions of heart failure in humans [88]. Moreover, the downregulation of p62 expression by TMAO in the same study indicates the loss of p62 cardioprotection. p62 has been found to protect from hypoxia and ischemic disease in other animal studies [89].

In summary, TMAO as a toxic metabolite is pathogenic for cardiac and kidney tissues. The pathology induced by TMAO in these organs is interrelated [90]. TMAO functions to induce pathology not only by influencing the fine balance between autophagy and cell death but also as a gene expression modulator that promotes heart myopathy that leads to heart failure. Finally, TMAO's role to promote atherosclerosis is a major contributor to vascular damage and heart disease. Recent studies attribute to TMAO an important pathogenic role in promoting cancer and metastasis [77]. The autophagic and gene expression modulations by TMAO that have been identified to produce the already mentioned diseases can prove to be important factors that also promote and lead to cancer. The mitochondrial dysfunction and accelerated aging induced by TMAO [91], like that found in cardiac mitochondria [92] can be the common link between the autophagy interference and the pathology promotion of the diseases caused by metabolic reprogramming [93].

4. Metabolism of Methyl Groups Supports Mitochondrial Health

We have seen that SAMe donates methyl groups to several different molecules, e.g., to produce phosphatidylcholine, and also to methylate proteins, DNA and RNA. We have also seen that phosphatidylcholine ultimately delivers its methyl groups back to the gut microbes, which can then recycle the methyl groups to restore CH_3THF, while further scrubbing deuterium from the molecules. The process of *demethylation* is not simply the removal of a methyl group to be attached to some other molecule. Instead, it results in the full metabolism of the methyl group, which directly or indirectly provides deupleted protons to the mitochondria.

In this section, we will critically examine the various ways in which methyl groups are removed from organic molecules. We will consider the removal of methyl groups from histones and from DNA, and the metabolism of the methyl group in methionine that results in its conversion back to formaldehyde. Finally, we will consider whether the studies in rats that found that dietary methionine restriction supports longevity were flawed in their design.

4.1. Histone Methylation and Demethylation

Histone methylation is an epigenetic modification that can regulate gene expression. Histones can be methylated through a transfer of a methyl group from SAMe to either their arginine or their lysine residues. Lysine can pick up as many as three methyl groups, and arginine can hold up to two. All these methyl groups are bound to nitrogen atoms in the residues [94].

Histones are demethylated via the enzymatic action of histone demethylases. But, in this reverse step, the methyl group is not simply attached to another carrier molecule but rather is released as formaldehyde. While formaldehyde is known to be a cancer risk due to its high reactivity, it is usually very quickly cleared through metabolism [95]. As has been described, in the gut, formaldehyde can donate its methylene unit to tetrahydrofolate (THF) to produce CH_2-THF, or it can be processed by anerobic bacteria to produce further deupleted hydrogen gas, which can then be used to synthesize SCFAs.

Besides the microbial pathways, human cells can exploit formaldehyde to reduce NAD+ to NADH. Alcohol dehydrogenase 5 metabolizes formaldehyde to formate, while converting NAD^+ to NADH. Formate is a precursor for *de novo* synthesis of purine nucleotides, in a folate-independent pathway [96]. Formate can also be further metabolized to produce CO_2 and H_2O, through the enzyme formate dehydrogenase, producing yet another molecule of NADH. Assuming that SAMe carried a

deupleted methyl group, both of these molecules of NADH can provide deupleted protons to the mitochondria.

All these reactions are beneficial as they support lower deuterium exposure in the mitochondria.

4.2. DNA Methylation and Demethylation

SAMe is also responsible for methylating the cytosine base in DNA, forming 5-methylcytosine (5mC), in both eukaryotes and bacteria, and this is a key epigenetic modification that alters gene expression, occurring mainly at CpG dinucleotides [97,98].

Just as for histones, the process of removing methyl groups from DNA does not involve a simple methyl transfer reaction. The process begins with a family of ten-eleven translocation (TET) enzymes, which remove the protons from the methyl groups, one by one, finally yielding 5-carboxylcytosine (5caC). Thymine DNA glycosylase then swaps out 5caC for a fresh intact cytosine base, initiating the process of base excision repair [99]. TET enzymes are key players in the control of cellular differentiation and transformation in mammalian cells [100]. It has only recently been demonstrated that 5caC can also be directly decarboxylated in place, without requiring the machinery that has to rely on DNA breaks and repairs. Direct decarboxylation is a rapid process that occurs in diverse mammalian cells [101].

An interesting aspect of the reaction catalyzed by TET enzymes is that other reaction products are succinate and CO_2, which are derived from α-ketoglutarate, a substrate [102]. The deupleted proton that is extracted is presumably released into the medium, helping to lower the deuterium content there. Each methyl group that is ultimately removed yields three succinate molecules and three free protons.

Mitochondrial DNA can also be methylated, and TET2 is expressed in mitochondria. A study demonstrated that TET2 levels were upregulated in mitochondria following acute brain ischemia, and the levels of hydroxymethylcytosine increased in parallel, peaking two days after the injury. There was a corresponding increase in the levels of ATP in the mitochondria, suggesting that TET activity facilitated ATP production [103]. The synthesis of succinate to support Complex II, as well as the release of deupleted protons into the medium, should both be beneficial to mitochondrial health. Defects in TET2 are linked to cancer. Mutations in TET2 are present in 7% to 28% of adult acute myeloid leukemia (AML) patients [104].

TET proteins are members of a class of proteins called dioxygenases that are known to have a high deuterium KIE for the removal of a proton from a CH bond. In a study by Jonasson and Daumann, an iron(IV)-oxo complex used as a model for the active site in TET enzymes (lacking the surrounding second coordination sphere), was shown experimentally to have a deuterium KIE of 9 for 5-methyl-cytosine, and 29 for 5-hydroxymethyl-cytosine [102]. Furthermore, a paper investigating the KIE for another dioxygenase that also produces succinate from α-ketoglutarate (taurine-α-ketoglutarate dioxygenase) found a deuterium KIE value close to 60 for abstraction of a proton from taurine, which the authors claimed was due to efficient quantum tunneling of the hydrogen atom [105].

Myelodysplastic syndrome is a hematopoietic disorder that frequently progresses to AML. Patients with TET2 mutations exhibit lower survival rates and a greater potential to progress to AML. TET2 is one of the most frequently mutated genes in myeloid malignancies. It has been proposed that pharmacological enhancement of TET2 activity might be a good strategy for blocking malignant transformation of leukemia cancer cells [106].

4.3. Methionine Metabolism to Methanethiol and Beyond

Several commensal bacteria in the human gut are capable of producing methanethiol as a breakdown product of methionine 107,108]. Methanethiol, also known as methylmercaptan, is a one-carbon organic sulfur compound with the formula CH_3SH. It has a distinctive unpleasant odor resembling rotten cabbage, and it is a major contributor to the smell of feces. Human cells express an enzyme called selenium-binding protein 1 (SELENBP1), which metabolizes methanethiol into H_2O_2,

H_2S, and formaldehyde [109]. This collaborative activity between the gut microbes and the human host is thus another way to produce formaldehyde, which can then be further metabolized, as we've discussed.　The reaction product H_2O_2 may also be significant. We have argued in previous research that H_2O_2 may be an important supplier of deupleted protons, as it is a gas that freely diffuses past the mitochondrial outer and inner membranes, and it is converted into two molecules of deupleted water by glutathione peroxidase, highly expressed in the mitochondria [110]. Being a gas, it is likely further stripped of any deuterium during its synthesis.

SELENBP1, a selenoprotein that uses copper as a cofactor, is highly expressed in the colon, liver, and lungs. SELENBP1 is a tumor suppressor involved in the regulation of cell proliferation, senescence, migration and apoptosis [111]. Many tumor cells express low levels of SELENBP1, and this reduced expression is often associated with tumor progression, poor prognosis, and resistance to anti-cancer therapy in many types of cancer [111,112]. It is conceivable that SELENBP1 protects from cancer through its ability to release small molecules that support the delivery of low-deuterium protons to the mitochondria.

Germ-free mice and germ-free pigs were observed to have elevated levels of methionine in their colon compared to controls [113]. This may reflect the fact that methionine is no longer being converted to methanethiol, due to the absence of gut microbes.

4.4. Dietary Methionine Restriction and Longevity

Senescence and aging are complex processes directly related to homeostasis during the human lifespan [114]. It has been experimentally proven for nematodes that increased growth rate through metabolic reprogramming comes at the cost of reduced life expectancy (longevity) [115]. Therefore, it would not be surprising for molecules involved in metabolic (aging) stress responses and that affect growth rate to also affect life expectancy [116].

In a paper published in 1993, Orentreich et al. reported on an experiment where rats fed a low-methionine diet experienced a 30% increase in life span [117]. This is surprising, since methionine is an essential amino acid. Variations on this experiment have been repeated many times over the years, and the results consistently show that methionine deficiency, while it slows growth rate, supports longevity in rats [117–120]. It appears that the suppression of growth hormone production by dietary methionine restriction is the primary mechanism by which it extends lifespan, since mice that overexpress growth hormone experience a 50% reduction in lifespan [121].

Eighty percent methionine restriction in rats and mice increases longevity and strongly improves mitochondrial function, but it also slows growth rate. Caro et al., in a paper published in 2009, were interested in assessing whether a 40% reduction in dietary methionine would be sufficient to improve the lifespan without causing slow growth. Indeed, their experiment demonstrated that this was possible. They quantified results specifically showing that the restricted rats had decreased ROS production and leak at complex I in the mitochondria and reduced oxidative stress in the kidneys and brain. In the abstract, these authors boldly concluded that "methionine is the only dietary factor responsible for the decrease in mitochondrial ROS production and oxidative stress, and likely for part of the longevity extension effect, occurring in DR [dietary restriction]" [118].

What is especially significant to us is that the authors specified in detail in the Methods section that both the methionine-restricted and the control rats were supplied with precise amounts of *synthetic* amino acids in lieu of natural proteins. They wrote: "The diets contained a mixture of amino acids instead of protein, and their precise composition is shown in Table 1" [118]. The restricted group received only 60% of the synthetic methionine compared to the control group.

While human cells cannot synthesize methionine from cysteine, gut microbes can, and they source the methyl group from CH_3-THF [122]. Nearly all bacterial species possess biosynthetic pathways for methionine synthesis [123]. The dietary deficiency in methionine would be expected to induce methionine synthesis from cysteine by the gut microbes to compensate for the deficiency. Assuming that the methyl group in CH_3-THF is derived from formaldehyde, then the microbial biosynthesis of methionine will yield a methionine molecule carrying deupleted protons in its methyl

group. This could explain why the rats with reduced dietary methionine had improved function of mitochondrial oxidative phosphorylation, due to the reduced deuterium burden in the mitochondria.

In a mouse study published in 2023, it was found that restricted dietary methionine could reduce the levels of TMAO resulting from a high-choline diet. A choline-rich diet combined with methionine restriction led to an alteration in the gut microbes resulting in increased production of butyrate and reduced TMA production by the bacteria [124]. This can be explained by the hypothesis that choline provided the precursors for methionine synthesis from homocysteine via the intermediary glycine beta aldehyde, to correct for methionine deficiency. This left behind less choline to get converted to TMA, while also allowing more hydrogen gas to be used as a reducing agent by acetogenic bacteria, to boost butyrate production.

5. Is Acetylcholine an Important Source of Acetate to Fuel Astrocytes?

Acetylcholine is a neurotransmitter that plays an important role in the synapse by signaling to acetylcholine receptors to effect various responses in the receptor neuron or muscle cell. Nicotinic acetylcholine receptors (nAChRs) are ligand-gated ion channels that transmit the signal from nerve to muscle that elicits muscle contraction. Acetylcholine is released from the axon of a motor neuron into the synaptic cleft, and acetylcholine then diffuses across the synaptic cleft and binds to the nAChRs on the muscle cell membrane. nAChRs are also expressed throughout the peripheral and central nervous system, where they are involved in fast synaptic transmission [125]. Muscarinic acetylcholine receptors are G-protein-coupled receptors with complex physiological responses, present in synapses in both central and peripheral nerves [126,127].

The enzyme acetylcholinesterase very quickly breaks down acetylcholine into acetate and choline, terminating the signaling response. Choline is then reabsorbed by the presynaptic neuron through a high-affinity choline transporter and reused for further acetylcholine synthesis. However, acetate is released into the medium, and it is plausible that it may be a significant source of fuel for the astrocytes. Astrocytes are highly efficient at taking up acetate from the extracellular space via the transport protein monocarboxylate transporter 1 (MCT1). They use it both as an energy source for ATP synthesis in the mitochondria and as a precursor for *de novo* glutamine synthesis [128]. Acetate serves as an alternative nutrient source under nutrient-deprived conditions. In fact, mitochondria in tumor cells differentially express the enzyme that converts acetate to acetyl-Coenzyme A, which allows them to reduce their dependence on glucose and glutamine [129]. It is interesting that synaptosomes do not readily take up acetate, compared to astrocytes, and this may be a strategy to assure that acetate remains in the synapse for its use by the astrocytes [130].

Several organophosphate insecticides induce their toxic effects by suppressing acetyl-cholinesterase, causing hyperstimulation of both muscarinic and nicotinic receptors. This overstimulation can result in a cholinergic crisis, characterized by symptoms that include muscle weakness, seizures, respiratory failure, and even death [131]. A popular organophosphate insecticide, pirimiphos-methyl, has been shown to disrupt spermatogenesis and reduce fertility in male rats [132]. It is conceivable that an aspect of their toxicity is that they deprive the synaptic astrocytes of an important fuel supply.

6. Benefits of a High Fiber Diet

A high fiber diet leads to increased fermentation by gut microbes, which can then promote the synthesis of deupleted SCFAs by the gut microbes. Of these, butyrate has garnered the most attention for its beneficial role not only in gut health but also throughout the organism. It is well established that butyrate is a histone deacetylase inhibitor, but it is also a ligand for G-protein coupled receptors and, of course, a valuable energy source for the mitochondria [133]. It is conceivable that much of the benefit of butyrate stems from its value as a supplier of low-deuterium protons to the mitochondria. There are several distinct gut-brain communication channels by which SCFAs could modulate brain function, including immune, endocrine, vagal, and humoral pathways [134].

A promising prebiotic containing wheat bran extract that is enriched in arabinoxylan oligosaccharides (AXOS) has been shown to have striking positive effects on the gut microbiome in an obese population. Arabinoxylan is a plant hemicellulose found in the cell walls of cereal grains. These oligosaccharides are not digested by human enzymes, but they are easily fermented by beneficial gut bacteria, notably Bifidobacteria. A multiomics study showed that this prebiotic supplement led to an increase in not only Bifidobacteria but also the beneficial strain Prevotella in the gut of overweight subjects with previous signs of metabolic syndrome. The authors observed an increase in the production of butyrate associated with the supplement, along with a boost in "a wide diversity of butyrate producers." Notably, there was an increase in plasma levels of phosphatidyl-choline, which they hypothesized was due to a greater availability of dietary choline following a reduction in the availability of microbial enzymes that convert choline to TMA. This implies a reduced synthesis of TMAO in the liver, and therefore protection from cardiovascular disease [135].

7. Butyrate is a Universal Protective/Essential Nutrient for the Human Organism

The short chain fatty acids, predominantly acetate, propionate, and butyrate, are produced by gut microbiota of animals and humans mainly through fermentation of dietary fiber. Human and animal cells do not possess the necessary enzymatic machinery for butyrate biosynthesis. The overall health effects of butyrate and how these are exerted are described in a study by K Hodkinson et al. [136]. Butyrate is readily produced in the gut, in multiple ways, by the gut colonizers [137]. A question arising, however, is whether butyrate produced by the gut microbiome remains localized to the GI tract, or if it is distributed throughout the organism. Undeniably, butyrate produced in mammals (including humans) is mainly the result of microbial fermentation in the large intestine [137]. Recent studies have shown that butyrate, apart from its anticancer effects, by controlling cellular proliferation of colonocytes, is readily transported by the blood stream to reach not only the hepatic and kidney tissues but far more distantly, reaching the human brain tissues and the skin [138].

In the human brain, butyrate can potentially reach adequate amounts from the gut to explain its beneficial health effects [139,140]. Adults that show low colonisation of butyrate microbial fermenters, and therefore low production of butyrate in the gut, have increased risk to develop inflammatory bowel disease (IBD) [141]. Moreover, the loss of butyrate production by gut fermenters has been linked to the development of various systemic autoimmune disorders, amongst them are rheumatoid arthritis, lupus erythematosus, and allergic asthma [142,143].

It is probably clinical cases involving infants that best indicate how important butyrate synthesis by the intestinal colonizers is, not only for GI tract health, but also for more distant organs. Butyrate produced in the large intestine is mainly metabolized by colonocytes, while the remainder is transported via the liver portal system through monocarboxylate transporters and metabolized by hepatocytes. Butyrate may extend its regulatory functions well beyond the GI tract in distant organs like the liver, the pancreas, the brain, the adipose tissue, and the immune system [140]. Moreover, there is evidence to suggest that butyrate is metabolized in organs distant from the GI tract; however more studies are needed to clarify this [144]. From infancy until the age of 2, butyrate production in the gut is a significant factor in conferring a reduced risk of developing atopic diseases in the respiratory tract and the skin [145.146]. Butyrate has found therapeutic value in treating depression, as well as neurodegenerative diseases and cognitive behavior [147]. Particularly for skin diseases, including psoriasis, the presence of gut-produced butyrate is essential for their prevention [148]. Moreover, the potential health effects of butyrate on human skin healing and re-epithelization are remarkable. A study of E Bachar-Wikstrom et al. indicates that butyrate exerts its healing activity on the skin by lowering the endoplasmic stress markers and thus producing re-epithelization of skin tissue [149].

Furthermore, the health-inducing activity of butyrate against brain tissue disorders that involve neural inflammation (e.g., cognitive impairment) and evolve from mitochondrial respiratory dysfunction, may be due to butyrate's direct deacetylation activity that affects the normalisation of gene transcription [133].

Summing up, all the above studies indicate that butyrate's production by the gut colonizers has a highly beneficial effect on human health. Some of these benefits come indirectly through sensitization and transportation of nerve signals from the GI tract. Other benefits stem from its direct transport to and action on distant tissues, far away from the GI tract. This establishes the production of butyrate in the gut as a far more essential factor for human health than was once thought. One of its basic properties seems to be the maintenance of mitochondrial respiratory chain health, as demonstrated in a study on cell lines taken from autistic boys, in order to combat neurodegeneration [150].

Sarcopenia is the loss of skeletal muscle mass during aging, which can lead to injury due to falls. A study on aged female mice demonstrated remarkable abilities of butyrate to protect against muscle atrophy in hindlimb muscles. It also increased markers of mitochondrial biogenesis in skeletal muscle, and it reduced markers of oxidative stress and apoptosis [151]. Butyric acid is severely reduced in the serum of patients with acetylcholine signaling deficiency due to myasthenia gravis. Butyrate's presence induces autophagy of the T regulatory cells in these patients by inhibiting the activity of mammalian target of rapamycin (mTOR) [152]. In turn, the autophagy induction activates their differentiation, and this enables the T cells to combat the myasthenia disease.

These studies indicate that butyrate's direct activity must be exerted in far distant organs from the gut. Butyrate's localized effect such as increased histone acetylation must involve many human tissues, although its transport from the intestinal lumen is minimal. Butyrate activity in alleviating neurological disorders (Alzheimer's disease (AD), Parkinson's disease (PD), autism spectrum disorder (ASD), and Huntington's disease (HD)), however, is a far more distant phenomenon [153]. Furthermore, as far as neural tissue is concerned, butyrate has proved to influence the production of acetylcholine by enhancing the activity of choline acetyltransferase, its synthesizing enzyme [154].

8. Creatinine Is a Waste Product, but Is It Really?

Creatine is a nutrient found only in animal-based foods. It has become quite popular as a supplement, especially for people seeking athletic fitness. Phosphocreatine acts as an energy buffer to support rapid synthesis of ATP during extreme physical activity. Creatine spontaneously converts to its metabolite creatinine, which forms a ring structure that may offer a unique opportunity to trap and secure deuterium. It is interesting that creatinine is not further metabolized by human cells but rather is simply excreted through the urine.

8.1. Creatine Synthesis is a Major Pathway by Which One-Carbon Units Are Lost

Creatine is an endogenously produced molecule that is also available from the diet which plays an important role in powering muscle fibers. It can only be sourced from animal- based foods, which can be an issue for vegetarians. Many athletes and fitness enthusiasts take high doses of creatine supplements to increase muscle strength and endurance [155]. Creatine is synthesized from glycine, methionine, and arginine, in a two-step process. An amidino group is first transferred from arginine to glycine to produce guanidinoacetate, catalyzed by L-arginine:glycine amidinotransferase (AGAT). Guanidinoacetate then picks up a methyl group from methionine catalyzed by the enzyme guanidinoacetate N-methyltransferase (GAMT). Creatine is released into the circulation by the liver and is readily taken up by muscle cells, the main consumers of creatine [156]. Both the methylene unit derived from glycine and the methyl group supplied by methionine will be deupleted, as we have previously argued in this paper. Creatine synthesis accounts for the loss of about 40% of all methyl groups derived from SAMe, and it also consumes 20-30% of arginine's amidino groups. Vegetarians obtain no creatine from the diet, putting greater strain on methylation pathways [157].

Creatine phosphate is the main high-energy phosphate-storage molecule in mammals, and it has recently been found that muscle cells synthesize a substantial amount of it themselves [158]. Creatine is converted to creatine phosphate in the mitochondria of muscle cells, via the activity of creatine kinase, while also converting ATP to ADP. When ATP levels drop, a high energy phosphate is transferred from creatine phosphate to ADP to reform ATP in a reverse reaction that replenishes ATP,

producing a waste product called creatinine. Creatinine can also form spontaneously from creatine, and it involves the loss of a water molecule and the formation of a ring structure called an imidazolidinone ring. Creatinine then gets excreted into the circulation and eventually is passed out through the kidneys. It is not further metabolized, so the one-carbon units originally coming from both glycine and methionine are lost. In the next section, we will hypothetically describe how, via its imidazolidinone ring, creatinine may play a useful role in trapping deuterium for excretion through the kidneys.

8.2. Can the Imidazolidinone Ring in Creatinine Sequester Deuterium?

Normally, nitrogen and oxygen atoms in organic molecules can freely exchange their bound protons with deuterons from the medium, but they are unable to secure a deuteron for very long because it quickly exchanges back. Carbon, on the other hand, rarely gives up its protons in exchange for deuterons. However, there are exceptions, and these may be significant for a potential to trap and sequester deuterium [159].

In previous published work, we have pointed out the unusual fact that certain carbon- and nitrogen-containing ring structures have a unique property that a carbon atom in the ring can exchange its bound protons with deuterons from the water-based medium. Rings with this property include the imidazole ring in histidine [5] and the pyrrolidine ring in proline [159,160]. The breakdown product of creatine, namely creatinine, is formed nonenzymatically along with the release of a water molecule. It reconfigures into a carbon- and nitrogen-containing ring called an imidazolidinone ring, which bears a strong family resemblance to an imidazole ring, since both are five-membered rings containing two nitrogen atoms. Interestingly, imidazolidinone derivatives have shown promise as anti-cancer agents [161].

The five-membered ring structure in imidazolidinone contains two nitrogen atoms and a carbon atom derived from glycine in a one-carbon unit bound to two protons. A study published in 2020 showed that the protons bound to this α carbon atom, under basic conditions, could freely exchange with deuterons when immersed in heavy water [162]. These authors wrote: "We presented a new method of synthesis of deuterated creatinine analogues, using organic base and D_2O. The study allowed us to obtain the Cre [creatinine] analogue, containing two deuterons, which were introduced after 60 min incubation in a 1% solution of TEA [trimethylamine] in D_2O at room temperature. The introduced deuterons did not undergo back-exchange under acidic and neutral conditions" [162]. If creatinine is then able to trap deuterium, this would justify it being a waste product, since it would no longer be suitable as a deupleted nutrient in the mitochondria.

A central aspect of the beneficial effects of creatine supplementation relates to its ability to improve mitochondrial health.   Creatine supplements have been shown to be beneficial in treating a long list of diseases that are associated with mitochondrial dysfunction, including both acute traumatic mitochondrial dysfunction and chronic atraumatic mitochondrial dysfunction.   These conditions include traumatic brain injury, fatty liver disease, diabetes, obesity, cardiovascular disease, neurodegenerative disorders, chronic fatigue syndrome, and long COVID [163].   It could be that the sequestering of deuterium in creatinine in the mitochondria plays a role in improving mitochondrial health, systemically.

Curiously, brown adipocytes in mice express a "futile creatine cycle" in their mitochondria that involves robust phosphocreatine phosphatase activity by tissue nonspecific alkaline phosphatase (TNAP). This thermogenic reaction wastes the energy in the phosphate anion by converting it to heat. Creatine kinase then restores creatine phosphate in the futile cycle. Inhibition of futile creatine recycling leads to obesity in mice [164]. We can therefore postulate that this recycling process may facilitate the acquisition of deuterium by creatinine inside the mitochondria, as creatine kinase has been shown to accelerate the phosphocreatine proton-deuterium exchange rate [165].

9. Conclusions

Gut dysbiosis is widely recognized as a contributing factor to a long list of chronic diseases and conditions. Mitochondrial dysfunction is also a common thread among many diseases. In this paper, we present evidence that these two pathologies are intimately intertwined, primarily because the gut microbiome is responsible for fueling the host with a multitude of deupleted nutrients. Furthermore, microbial ability to carry out this service is severely compromised when the microbes are disturbed by nutritional deficiencies and toxic exposures. The mitochondrial ATPase pumps are very sensitive to deuterium, which interferes with their production of ATP and induces the release of reactive oxygen species.

We have shown that the metabolite butyrate, derived from dietary fiber and produced by obligate anaerobes, is an essential nutrient for the colonocytes, and that butyrate deficiency has been found in association with many diseases. The microbes produce butyrate and other short chain fatty acids (SCFAs) through a process that involves recycling severely deupleted hydrogen gas: producing it from simple organic molecules such as glucose and formate and using it to reduce carbon dioxide to acetate and butyrate and to generate the one-carbon units that feed the methylation pathways. Inflammation in the gut interferes with the production of SCFAs.

Methylation pathways play many roles both in metabolism and in altering gene expression. We have described the methylation pathways in great detail, but through a lens that reflects on the way in which they can work to minimize the exposure of mitochondria to deuterium. Choline, methionine, and formaldehyde are central players in methylation pathways, and an intricate collaboration between the microbes and the host assures a healthy outcome, as long as nutritional support is sufficient and toxic exposures are minimized.

A novelty of our hypothesis is that it may be able to explain why dietary choline and dietary methionine are much more beneficial to health than synthetic versions of these molecules, simply because the biosynthesized dietary forms are deupleted and the synthetic forms are not. A diet that is rich in prebiotics (e.g., fiber), probiotics (e.g., fermented foods), and adequate natural sources of methionine and choline is likely to maintain healthy mitochondria and to promote longevity.

Data Availability: N/A (no new data).

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