Title: Causes and Clinical Sequelae of Riboflavin Deficiency

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Abstract

Riboflavin, in its cofactor forms flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN), plays fundamental roles in energy metabolism, cellular antioxidant potential and metabolic interactions with other micronutrients, including iron, vitamin B6 and folate. Severe riboflavin deficiency, largely confined to low-income countries, clinically manifests as cheilosis, angular stomatitis, glossitis, seborrheic dermatitis and severe anemia with erythroid hypoplasia. Sub-clinical deficiency may be much more widespread, including in high-income countries, but typically goes undetected because riboflavin biomarkers are rarely measured in human studies. There are adverse health consequences of low and deficient riboflavin status throughout the lifecycle, including anemia and hypertension, that could contribute substantially to the global burden of disease. This review will consider the available evidence on causes, detection and consequences of riboflavin deficiency, ranging from clinical deficiency signs to manifestations associated with less severe deficiency, and the related research, public health and policy priorities.

INTRODUCTION

Riboflavin is a water-soluble B vitamin, also known as vitamin B2. It is primarily found as an integral component of the coenzymes flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN) which serve as electron carriers in oxidation-reduction (redox) reactions for energy production, cellular antioxidant function and numerous metabolic pathways. Notably, riboflavin has important metabolic interactions with other nutrients where it is required in the synthesis of niacin from tryptophan and in the metabolism of iron, vitamin B6, vitamin B12 and folate. Thus, some of the reported deficiency signs may reflect perturbations in the metabolism of other nutrients that are dependent upon FAD or FMN. Apart from the clinical deficiency signs, low riboflavin status may be associated with a range of adverse health outcomes across the lifecycle, but in general these effects are not well recognized. Riboflavin biomarkers are rarely assessed in human studies, and thus neither deficiency nor sub-clinical deficiency (low riboflavin status without clinical signs) is well documented.

This review considers the causes and clinical sequelae of low and deficient riboflavin status across the lifecycle. The available evidence on causes, detection and consequences of riboflavin deficiency will be reviewed, ranging from clinical deficiency signs to manifestations associated with less severe deficiency, and the related research and public health priorities will be identified. To appreciate human riboflavin deficiency, it is first necessary to consider the functional and metabolic roles and interaction with other nutrients.

METABOLIC ROLES OF RIBOFLAVIN

Structure

Riboflavin (IUPAC name: 7,8-Dimethyl-10-[(2S,3S,4R)-2,3,4,5 tetrahydroxypentyl]benzo [g]pteridine-2,4-dione) consists of a tricyclic heteroaromatic isoalloxazine ring which is attached to a ribityl side chain (**Figure 1**). Riboflavin serves as a precursor of the important coenzymes FMN and FAD, which function in reactions involving a wide range of flavoenzymes, estimated to be around 400 in total in all organisms in nature (64) of which 80 have been identified in humans (66). The process of conversion of riboflavin to its coenzyme forms occurs predominantly in the cytoplasm (47, 74) but some reports show that conversion

to FAD may also take place in the mitochondria (9) and even in the nucleus (38). Initially riboflavin is subjected to ATP-dependent phosphorylation facilitated by Zn^{2+} and catalyzed by the enzyme flavokinase with FMN being the product of this reaction. A small fraction of FMN is directly utilized as a coenzyme but most is modified by addition of pyrophosphate-bridged adenyl moiety in a reaction using Mg^{2+} and the enzyme FAD synthetase which results in the generation of FAD. The process is controlled by the FAD content of tissues and an excess of FAD inhibits this conversion as demonstrated in rats (122). Some of the generated FAD is further altered to forms that are bound covalently to specific enzymes which have important roles in metabolism (112). Most flavoproteins contain a tightly but noncovalently bound flavin, though an estimated 10% of all flavoproteins contain a covalently bound flavin, including sarcosine dehydrogenase, succinic dehydrogenase and monoamine oxidase A and B. The steps of the synthesis of flavin coenzymes and formation of covalently bound flavins are under the control of thyroid hormones (27, 61, 83, 94). Most human flavoproteins are FAD-dependent (around 84%) whereas only 16% use FMN as a cofactor (62). **Figure 1** to go here. Structures of riboflavin FMN and FAD. Figure adapted, with permission from Editor, from Pentieva (80).

Functional roles

The tricyclic heteroaromatic isoalloxazine ring of FMN and FAD can reversibly accept and donate one or two electrons in a wide variety of oxidation and reduction reactions. Complex I (NADH dehydrogenase) and Complex II (succinate dehydrogenase) requiring FMN and FAD, respectively, have a central role in energy production within the mitochondrial respiratory chain. Complex I and II translocate electrons across the inner mitochondrial membrane that creates the electrochemical potential difference necessary for ATP production. In addition, FAD is a coenzyme for various dehydrogenases, hydrolases, oxidases and transferases that are involved in other redox reactions of intermediary metabolism such as the first step of fatty acid β-oxidation, oxidative decarboxylation of pyruvate and α-ketoglutarate, choline catabolism, purine catabolism, sphingosine synthesis, synthesis of cholesterol and steroid hormones. As a coenzyme for several cytochrome P-450 components, FAD is also important for the metabolism of drugs and various xenobiotics (96).

Riboflavin is involved in the antioxidant defense system through the FAD-dependent enzyme glutathione reductase, which regenerates reduced glutathione from its oxidized form. Glutathione reductase is an essential part of the glutathione redox cycle and is responsible for

the maintenance of an optimal concentration of reduced glutathione that protects living cells from the damaging effect of reactive oxygen species (ROS). Riboflavin administration in animal models of chronic degenerative diseases associated with ROS such as diabetes (1), renal toxicity (3) and hepatotoxicity (2) showed increased activities of antioxidant enzymes including glutathione reductase or reduced glutathione, indicating that riboflavin influences antioxidant status. In vitro mechanistic studies showed that riboflavin may exert these effects by altering both gene expression and signaling pathways associated with antioxidant enzymes (53). In addition to the key role of riboflavin in the glutathione redox cycle, FAD is also involved indirectly in protection against ROS by acting as a coenzyme for xanthine oxidase, which catalyzes the oxidation of hypoxanthine and xanthine to uric acid, that in turn is one of the most effective water-soluble antioxidants. Thus, decreased activity of xanthine oxidase and reduced blood uric acid concentrations have been reported in riboflavin deficiency (18). Despite the well-recognized antioxidant properties of riboflavin, it can also act as prooxidant under exposure to ultraviolet (UVA) light. The isoalloxazine ring of riboflavin absorbs energy from UVA light through a photosensitization reaction which involves the production of singlet oxygen and subsequently other ROS that could damage the adjacent molecules (46, 78). Thus, riboflavin could be damaging to tissues exposed to high intensity light such as skin and eyes. However, there are reports that other antioxidants such as ascorbate, carotenoids, tocopherols and polyphenols can counteract any adverse effect of riboflavin exposed to light (25, 49). Nonetheless, the photosensitizing properties of riboflavin may have implications for its use as an antimicrobial agent (58) and in treating conditions such as keratoconus (99) and cancer (59).

Riboflavin is also considered to function as a regulator of cryptochromes which are photosensitive flavoproteins located in the ganglion cells in the retina of the eye and responsible for setting circadian rhythm in response to daylight (45, 119). One study however provided a molecular basis for the distinct circadian roles of different animal cryptochromes and found that the type of cryptochromes in vertebrates lack the structural features to securely bind the photoactive flavin, indicating that riboflavin might have lost its functional importance for circadian photoreception in vertebrate animals in the course of the evolution (60). Further studies are required to clarify the role of riboflavin in circadian biology.

Interactions of riboflavin with other nutrients

Riboflavin coenzymes are involved in the metabolism of other B-vitamins (folate, vitamin B12, vitamin B6 and niacin) and iron.

In relation to other B vitamins, FAD is required for methylenetetrahydrofolate reductase (MTHFR), a key folate metabolizing enzyme responsible for the conversion of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate. FMN and FAD act together as coenzymes for methionine synthase reductase (MTRR) which is necessary for the regeneration of methylcobalamin, the biologically active form of vitamin B12 that is required by methionine synthase (MS; (120)). Both MTHFR and MS are important players in one-carbon metabolism and are involved in the remethylation of homocysteine to methionine, which is subsequently activated by ATP to form S-adenosylmethionine (SAM), the universal methyl donor for a wide range of substrates such as DNA, proteins, phospholipids, neurotransmitters and hormones, all of which are regulators of essential biological processes (7). In addition, FMN acts as a cofactor for pyridoxine (pyridoxamine) phosphate oxidase (PPO), a key enzyme in vitamin B6 metabolism that converts the 5-phosphates of both pyridoxine and pyridoxamine to pyridoxal-5′-phosphate (PLP), the biologically active B6 derivative required for numerous reactions of amino acid metabolism, one-carbon metabolism, glycogenolysis and gluconeogenesis. Although PLP can also be generated by phosphorylation of pyridoxal via pyridoxal kinase, the rate of production of PLP through PPO is higher and this pathway is considered more efficient (73), making riboflavin an essential nutrient for the maintenance of adequate vitamin B6 status. Finally, the conversion of tryptophan to niacin involves FAD-dependent kynurenine hydroxylase. As a result of its role in these pathways involving folate, vitamin B12, vitamin B6 and niacin, deficient or low riboflavin status may cause metabolic disturbances leading to functional deficiencies of these vitamins.

Riboflavin also interacts with iron. Free non-protein bound reduced riboflavin is involved in the mobilization of iron from its storage protein ferritin in the cytoplasm. Both FMNH2 and FADH2 participate in electron transfer pathways accompanied by specific protein structural arrangements of ferritin that reduce Fe^{3+} to Fe^{2+} and facilitate the physiologically important process for iron mobilization from ferritin (20). Other agents such as ascorbate and glutathione are also capable of mobilizing Fe^{2+} cations from ferritin but the reduced riboflavin forms have been shown to be more effective in this process (105). The mobilization of iron from ferritin is relevant for all the tissues but is considered especially important for the gastrointestinal mucosa as it may have implications for iron absorption (84). Animal and *in-vitro* mechanistic studies provide evidence that riboflavin status can influence both gastrointestinal iron absorption (22, 89, 90) and iron loss (89). Although two human studies using iron stable isotope (^{58}Fe) showed no measurable changes in iron absorption after riboflavin supplementation, in both

investigations the participants had improvements in different hematological parameters, indicating that the effect is more likely to be driven by the mobilization of endogenous iron from ferritin (34, 88).

RIBOFLAVIN DEFICIENCY IN HUMANS

Deficiency signs

Riboflavin deficiency, known as ariboflavinosis, typically presents with cheilosis, angular stomatitis, glossitis, , redness and swelling of the mouth and throat and severe anemia with erythroid hypoplasia (**Figure 2**). Other symptoms, such as corneal vascularization, seborrheic dermatitis, and neurological alterations may also occur, but these are not considered specific for ariboflavinosis (77) and may be the result of other vitamin deficiencies due to the metabolic interaction of riboflavin with these nutrients (96). Clinical signs of riboflavin deficiency in humans appear after several months of dietary intakes of less than 0.5–0.6 mg/d (50). Of concern, one case report just published describes a life-threatening condition of hypoglycemia and lactic acidosis due to severe riboflavin deficiency in a neonate caused by riboflavin deficiency resulting from a maternal vegan diet (51).

Figure 2 to go here. Riboflavin deficiency in humans

Riboflavin deficiency signs have been documented predominantly in populations from low-income countries where the consumption of foods rich in riboflavin such as milk and dairy products is limited, notably among women and children in The Gambia (11, 15), elderly persons in Guatemala (19), children in Côte d'Ivoire (97) and in adolescent refugees from Bhutan living in southeastern Nepal (17). Infants of mothers with a low riboflavin status during pregnancy are also likely to be born riboflavin deficient (14). As will be discussed below, an accumulating body of evidence from high-income countries shows that deficient and low riboflavin status may be more common than previously recognized, particularly affecting adolescent girls and young women (4, 10, 52, 54, 114).

With the onset of riboflavin deficiency, several metabolic adaptations occur in order to preserve critical functions of the vitamin. One of these adaptations is a considerable drop in hepatic free riboflavin to almost unmeasurable levels, with no detectable changes in FAD and FMN concentrations required for the functioning of flavoenzymes (35). In the early stages of

riboflavin deficiency, there is also prioritization in relation to the various redox reactions in energy production and intermediary metabolism, with a well-maintained electronic transfer for the synthesis of ATP and a substantial reduction in β-oxidation of fatty acids (79, 98). Adaptation to riboflavin deficiency is also reported to occur in the maintenance of the glutathione redox cycle, with an increase in de novo synthesis of reduced glutathione from its amino acid precursors in response to the diminished conversion of oxidized glutathione back to its reduced form by the FAD-dependent glutathione reductase (95).

Functional deficiencies of other micronutrients secondary to riboflavin deficiency

The low hemoglobin concentrations and hypochromic anemia observed in riboflavin deficiency are mainly a result of the impaired mobilization of iron from intracellular stores (i.e. hepatic ferritin) that requires reduced riboflavin forms (4, 86). Studies conducted in population groups with a deficient or sub-optimal riboflavin status inlcuding school children (23, 26), men (34), pregnant (30, 63, 110), lactating (87) and women of reproductive age (88), have shown that riboflavin supplementation leads to improved hematological status.

Consistent with riboflavin's role in vitamin B6 metabolism, a compromised B6 status is a common consequence of riboflavin deficiency. Correspondingly, our small intervention trial in older adults with insufficiency of either vitamin at baseline showed that riboflavin supplementation resulted in, not only improved riboflavin status, but also increased plasma PLP, the biologically active B6 derivative (65). In addition, observational studies have found that biomarkers of both B vitamins are strongly correlated and indicate that riboflavin may be the limiting nutrient for the maintenance of adequate vitamin B6 status (52, 54). Such evidence is entirely consistent with much earlier human studies showing that riboflavin supplementation led to an increase in the activity of FMN-dependent PPO in erythrocytes, the enzyme required for the generation of PLP (12).

Riboflavin deficiency interferes with the metabolism of folate, most notably in individuals homozygous for the common C677T polymorphism in *MTHFR*, with important functional and health consequences. In individuals with the variant *MTHFR* 677TT genotype, there is an increased risk of hypertension, but supplementation with riboflavin, the MTHFR cofactor, can effectively lower blood pressure independent of use or type of antihypertensive medications (71). Molecular studies show that in affected individuals, there is an increased propensity for

the riboflavin (FAD) cofactor to dissociate from the enzyme's active site, rendering it inactive, and in turn leading to impaired folate metabolism (121). Also, low riboflavin status is associated with elevated homocysteine, the well-recognized phenotype for this polymorphism, whilst riboflavin supplementation of individuals with the variant TT genotype results in marked lowering of homocysteine concentrations (69), perhaps suggesting that optimal riboflavin can stabilize the variant enzyme and thus restore MTHFR activity *in vivo*. Also, whilst most studies linking this folate polymorphism with cardiovascular disease focus on homocysteine, the more recently recognized blood pressure phenotype may be the more relevant to cardiovascular risk. The totality of evidence from genome-wide association studies (33) and clinical studies (93, 123) shows that the *MTHFR* C677T polymorphism is associated with an increased risk of hypertension and hypertension in pregnancy by up to 87%. Importantly, randomized trials from our center demonstrated lowering of systolic blood pressure by 6 to 13 mmHg in response to riboflavin when targeted at hypertensive patients with the variant *MTHFR* 677TT genotype (44, 118). This novel role of riboflavin in modulating blood pressure in a genotype-specific manner may have important public health implications. The potential to prevent or treat hypertension in sub-populations worldwide could be considerable, considering that this genotype affects 10% of people globally, ranging 4-26% in Europeans (increasing north to south), 20% in Northern China, to as high as 32% in Mexico (117).

Other health effects that are possibly associated with riboflavin deficiency have been described. Riboflavin deficiency has been implicated as a risk factor for cancer in animal studies (86), but there is inconsistency in the epidemiological evidence, including from meta-analyses, in relation to human colorectal cancer (16, 124) and breast cancer (125, 127). Phototherapy with blue light used to treat neonatal hyperbilirubinemia has been shown to cause various adverse effects, including degradation of riboflavin and deficiency of the vitamin (113). However, riboflavin supplementation in this case is contraindicated since the products of riboflavin photolysis can lead to DNA damage (106). Studies from malaria endemic regions showed that individuals with riboflavin deficiency are relatively resistant to malaria and have lower rates of parasitemia, but the course of the disease may be more severe than in people with adequate riboflavin status (29).

Causes of riboflavin deficiency

Insufficient dietary intake of riboflavin is considered to be the primary cause of deficiency. Nutritional considerations for healthy adults, including food sources and typical dietary intakes

in populations globally, will be considered below. In patients, however, several conditions, including alcoholism, diabetes mellitus, liver disease, thyroid and adrenal insufficiency, and gastrointestinal and biliary obstruction, may precipitate or exacerbate riboflavin deficiency (96). Alcohol appears to cause deficiency by interfering both with the digestion and intestinal absorption of riboflavin (82, 109). Other patient groups at greater risk of riboflavin deficiency include those with anorexia nervosa due to extremely low dietary intakes and those with lactose intolerance owing to treatment involving the exclusion of milk and dairy products, the major dietary sources of riboflavin. Antiepileptic and psychotropic drugs such as chlorpromazine, imipramine, and amitriptyline (6, 81), as well as some antimalarial drugs such as quinacrine (31), can inhibit the conversion of riboflavin to its active coenzyme derivatives. Drugs, such as tetracycline, theophylline, and caffeine, as well as metals, such as zinc, copper, and iron, may chelate or form complexes with riboflavin and thus affect its bioavailability (102).

ASSESSMENT OF RIBOFLAVIN STATUS

Riboflavin status can be assessed in erythrocytes, urine and plasma using various methodological approaches (**Table 1**).

Erythrocyte glutathione reductase activation coefficient (EGRac)

The measurement of the activity of the erythrocyte enzyme glutathione reductase (EGR) (EC 1.6.4.1), an enzyme that is dependent on the riboflavin cofactor FAD, is considered the gold-standard method for assessment of long-term status (43). Glutathione reductase, a nicotinamide adenine dinucleotide phosphate (NADPH)-dependent flavoprotein, and the major flavoprotein in erythrocytes, catalyzes the oxidative cleavage of the disulfide bond of oxidized glutathione (GSSG) to form reduced glutathione (GSH): $GSSG + NADPH + H^+ \rightarrow 2GSH + NADP^+$.

The activity of EGR is calculated by measuring the oxidation of NADPH to NADP spectrophotometrically, with and without the presence of added FAD coenzyme to generate a ratio of FAD stimulated to unstimulated enzyme activity, with higher values reflective of lower riboflavin status (80). The degree of *in vitro* stimulation of EGR activity depends on the FAD saturation of the apoenzyme, which, in turn, depends on the availability of riboflavin. An EGR activity-coefficient (AC) ratio of 1.0 indicates no stimulation by FAD and suggests more than adequate concentrations of FAD (and riboflavin) in erythrocytes. In those with riboflavin

deficiency, the basal EGRac falls and the *in vitro* stimulation by FAD rises; thus, higher values of EGRac are indicative of more deficient riboflavin status.

In a systematic review of randomized controlled trials, Hoey et al. in 2009 (43), found EGRac to be an effective biomarker of severe deficient-to-normal riboflavin status, and sensitive to riboflavin supplementation at doses ranging from 1.0 to 5.0 mg/d, for periods of at least 4 weeks. It should be noted however that EGRac is not considered to be a sensitive marker of riboflavin status in replete adults (85). Although there are no universally accepted EGRac cut-off values to define an optimal or low status, a coefficient of ≥ 1.40 is widely accepted as the cut-off value indicative of riboflavin deficiency (32, 50, 68). Sufficient riboflavin status is generally recognized as an EGRac of <1.2, while insufficiency is very broadly recognized as an EGRac value of between 1.2 and 1.4 (100). The 2017 European Food Safety Authority (EFSA) report on Dietary Reference Values for riboflavin concluded that an EGRac value <1.3 was appropriate to define adequate riboflavin status based on data from Boisvert et al. in 1993 indicating that riboflavin tissue saturation occurred in this range, as measured from urinary excretion values (19). More recent analysis of data generated at this center supports a slightly lower coefficient of ≤ 1.26 as the cut-off for adequate riboflavin status, with a value of between 1.27 and 1.39 indicative of suboptimal status (54). These cut-offs were generated based on the distribution of EGRac values, measured in response to a 16-week riboflavin intervention at a level of 1.6mg/d, within the range of typical dietary intakes of riboflavin from food sources. Further evidence to support the use of EGRac as a reliable biomarker of status comes from analysis of data from the National Adult Nutrition Survey (NANS), showing significant correlations of dietary riboflavin intake with EGRac in this representative sample of Irish adults (r =–0.275, P < 0.001), an association which was strengthened by the inclusion of riboflavin supplement users (52). A number of factors (**Table 2**) are known to affect the performance of the EGRac and should be considered when interpreting data from different populations (80).

Urinary riboflavin excretion

Urinary excretion of riboflavin is recognized as a reliable measure of short-term status, that reflects dietary intake when tissues are saturated, indicated by the inflection in the urinary excretion curve in relation to riboflavin intake. As recently reviewed, experimental balance studies indicate that urinary riboflavin excretion rates increase slowly with increasing intakes until the point of tissue saturation, after which any further increase of riboflavin intake leads to a corresponding steep elevation of the excretion rate (80). Riboflavin concentrations in urine

can be determined in fasted, random, or 24-hour urine samples, with or without adjustment for creatinine concentration (101). Assessment methods include fluorometric high performance liquid chromatography (HPLC), microbiological assays, or competitive-binding protein assays which offer the advantage of being sensitive, quick and requiring no pre-treatment steps (80). Cut-off values for deficiency and adequacy/sufficiency have been proposed by Sauberlich et al. in 1974 (101) based on controlled depletion/repletion studies, with deficiency indicated as total 24-h urinary excretion of riboflavin below 40mg/day (or 27mg/g creatinine), while values between 40 and 120 mg/day (or 80mg/g creatinine) indicated insufficiency, and values exceeding 120mg/day represented sufficiency in adults (**Table 1**). A number of factors are known to interfere with riboflavin excretion, including oral contraceptive agents and pregnancy as well as certain antibiotics and psychotropic drugs (**Table 2**).

Direct riboflavin biomarkers

A limited number of studies have measured riboflavin status directly using plasma (considered a short-term measure of status) and erythrocytes. Of the plasma biomarkers, riboflavin and FMN have yielded more promising results and been shown to respond to low dose riboflavin supplementation, compared with FAD concentrations where only modest responses to intervention were observed (48). These direct measures, analyzed using liquid-chromatography-tandem-mass-spectrometry (LC-MS/MS), offer some advantages over EGRac, primarily related to the convenience of using a plasma sample in contrast with the rather tedious preparation of washed red blood cells, making them much more accessible for population surveys.

Erythrocyte riboflavin is considered a longer-term marker of riboflavin intake and has been reported to be significantly lower in individuals with clinical signs of riboflavin deficiency compared with apparently healthy adults (8). Supplementation with low-dose riboflavin for 12wks was shown to increase erythrocyte FMN and FAD concentrations, with the greatest response (87%) observed in erythrocyte FMN compared to a modest, albeit, significant response in FAD (48). Also, a strong association between erythrocyte riboflavin markers and EGRac (as the reference method) was reported in older British adults (13). Other studies, comparing riboflavin assessment as measured using erythrocyte riboflavin (or FMN) concentrations and EGRac in pregnant women (40), and responses in older adults pre-and post-intervention with low-dose riboflavin (48), also show good agreement between these methods

and generate comparable estimates of riboflavin deficiency. Further studies are however needed to more fully validate these direct biomarkers.

Thus, a number of reliable markers of riboflavin status are available, with EGRac offering the advantage of providing a highly sensitive, specific and long-term biomarker of status. EGRac does however require very specific pre-analysis processing and therefore there is a need to further investigate the utility of more accessible biomarkers in plasma and erythrocytes that respond sensitively, specifically and predictably to changes in the supply of dietary riboflavin or riboflavin stores, and more importantly, can be directly related to health outcomes. The lack of accessible biomarkers has resulted in very limited available data on riboflavin status in populations globally. Because of the reliance on dietary data only without corresponding biomarker evidence in population-based cohorts, low riboflavin status may go undetected and there are concerns that it may be a much more widespread problem than is generally recognized. The transnational DERiVE project (2017-2021), funded by the JPI-HDHL scheme and coordinated by Ulster University, aimed to address this research gap and focussed on developing accessible riboflavin biomarkers and demonstrating functional, gene-nutrient and health effects of riboflavin status in Canadian, Irish and UK human cohorts.

DIETARY INTAKES AND STATUS OF RIBOFLAVIN

Dietary recommendations

Worldwide dietary recommendations for riboflavin range from 1.1 to 1.6 mg/d for adults. An increment of 0.3 mg/d is recommended during pregnancy to cover increased tissue synthesis for fetal and maternal demands, and an additional 0.4–0.5 mg/d during lactation (**Table 4**). The most recent 2017 guidelines issued by the EFSA Panel on Dietetic Products, Nutrition and Allergies (NDA), recommended 1.3mg/d as the average requirement (AR) and 1.6mg/d as the population reference intake (PRI) for European adults aged >18yrs. These recommendations were formulated on the basis that the inflection point in the urinary riboflavin excretion curve in relation to dietary riboflavin intake reflects body saturation and can be used as a biomarker of adequate riboflavin status (32).

Dietary sources

The primary dietary sources of riboflavin include milk, milk products, eggs and offal according to the European Nutrient Composition Database of EFSA, which includes food composition

data from 13 national dietary surveys including Ireland (National Adult Nutrition survey; NANS) and the UK (National Diet and Nutrition Survey; NDNS). Similarly, in the US, the largest contributors to total riboflavin intake are milk and milk drinks, bread and bread products, mixed foods whose main ingredient is meat, ready-to-eat cereals, and mixed foods whose main ingredient is grain (36, 50). Vegetables and plant foods contribute much smaller amounts of riboflavin in populations following western dietary patterns (50, 75, 92), although have been reported to be significant contributors of intake in a convenience sample of Canadian adults of mixed ethnicity (4). The main dietary sources of riboflavin in the Irish diet (based on analysis of NANS data), are shown in Table 3; dairy products are estimated to contribute 22% of total riboflavin intake, while ready-to-eat breakfast cereals contribute between 5.9 in and 13.8% across different age / sex categories (56). Similar patterns are reported in the most recent UK NDNS data, here milk and milk products contributed 27% and breakfast cereals contributed 9% to total riboflavin intakes in 19- to 64-year-old adults (92).

In the US and Canada, mandatory riboflavin enrichment policies are in place to replace the riboflavin lost from starch during milling, at levels of 0.40 mg/100g for wheat starch and 4 mg/kg for starch, as regulated by the US Food and Drug Administration (111) and the Canadian Food Inspection Agency (24), respectively. Riboflavin in food occurs mainly as FAD, although in eggs and milk it occurs primarily as free riboflavin (86). The bioavailability of all forms of riboflavin from food is estimated to be as high as 95%, up to a maximum intake of approximately 27 mg per meal, with little or no absorption at higher intakes (126). Food sources (i.e. animal v plant) do not appear to influence bioavailability, based on available, albeit limited, evidence. The riboflavin content of cooked foods is however influenced by cooking method, with a doubling in riboflavin losses reported when foods are subjected to boiling compared to alternative methods such as steaming or microwaving (28). At high doses, riboflavin supplementation results in bright yellow urine (‘flavinuria’), a harmless side effect. No adverse or toxic effects of high riboflavin intake in humans have been reported (67) and a tolerable upper intake level for riboflavin was not considered necessary by the Institute of Medicine in the USA when the RDA was revised in 1998.

Riboflavin intakes and status in populations globally

Few population-based studies have access to riboflavin biomarker data and instead rely on estimates of dietary intake to report status. The UK and Ireland are among the very few countries worldwide to include riboflavin biomarkers in their national nutrition surveys.

Results from the UK NDNS rolling program (2014/15-2015/16) reported mean riboflavin intakes of 1.8 mg/d for men and 1.4 mg/d for women aged 19-64yrs, with corresponding mean EGRac values of 1.33 and 1.38, while 47% of men and 61% of women had an EGRac value >1.3 (32). Recent analysis of population data from Ireland (NANS) observed significantly better riboflavin status (i.e. lower EGRac values) in older (≥65yrs) compared with younger (<65yrs) males and females. In NANS, mean intake was 2.2 and 1.6mg/d in males and females aged 18-64yrs, with lower intakes of 1.8 and 1.6 mg/d reported in males and females aged ≥ 65yrs (52). The higher intakes observed in males compared to females aged 18-64yrs were reflected in the corresponding EGRac values, which were 1.37 in males and 1.40 in females respectively, while values of 1.34 were reported for all adults aged ≥ 65yrs (52). Further analysis identified B-vitamin supplement use, dietary riboflavin intake, fortified food, milk consumption, age, smoking status and hemoglobin, as most strongly associated with riboflavin status in the Irish adults (52).

No population-based riboflavin biomarker data are available for the US or Canada where riboflavin enrichment of all foods made from white flour is mandated. In the US National Health and Nutrition Examination Survey (NHANES) dietary intakes of 2.5 mg/day for men and 1.8 mg/day for women were found and less than 6% of the US population had an intake of riboflavin from foods and supplements below the EAR (36, 50). In Canada, dietary intakes of 2.2mg/d in males and 1.7mg/d in females aged 19-50yrs were reported (41). Although no population-based riboflavin biomarker data are available for Canada, EGRac values >1.40 were reported in 40% of a convenience sample of women of child-bearing age who were participating in a study aimed at investigating the association of riboflavin biomarkers with hemoglobin concentration and anemia (4, 5). Dairy products contributed the most to riboflavin intake and energy-adjusted dietary riboflavin intake was found to be inversely associated with EGRac (B = −0.04, 95% CI: −0.07, −0.01).

Much lower dietary riboflavin intakes are found in populations with typically low intakes of milk and dairy products, the richest dietary sources of the vitamin. In a population-based sample of 1,253 in China who participated in the Jiangsu Nutrition Study, among adults aged 18–45 years, a mean riboflavin intake of 0.80mg/d was reported and 97% of participants had an intake below the Chinese EAR values of 1.2 mg/d for women and 1.4 mg/d for men (104). It should be noted however that riboflavin excretion rates appear to be much lower in Chinese adults, which may have implications for dietary requirements in this population (21), although

further confirmation of this finding is required. High rates of biochemical deficiency have also been observed in populations from low- and middle-income countries with poor dietary riboflavin intakes, including women from urban and rural Cambodia (55, 116), and elderly free-living adults in Guatemala where the prevalence of riboflavin deficiency (using an EGRac cut-off value of >1.30) was reported to range from 50 to 75% and dietary riboflavin intake was strongly correlated with milk consumption (19).

HEALTH EFFECTS OF RIBOFLAVIN INADEQUACY ACROSS THE LIFECYCLE

Pregnancy

As described earlier, riboflavin deficiency can alter iron metabolism though various mechanisms, including impairing iron absorption, increasing intestinal loss of iron, and/or reducing the utilization of iron for the synthesis of hemoglobin. Riboflavin deficiency can thus contribute to iron deficiency anemia in pregnancy, while riboflavin supplementation of women of reproductive age was shown to enhance circulating hemoglobin concentrations and improve the response of iron deficiency anemia to iron therapy (88). Notably, a randomized trial conducted in pregnant women with anemia in China showed that the inclusion of riboflavin (along with retinol) decreased the prevalence of anemia compared to supplementation with iron and folic acid only (63).

There is also some, albeit limited, evidence linking riboflavin deficiency with an increased risk of hypertension in pregnancy (70). Hypertensive disorders during pregnancy carry significant risks for both the mother and child and are implicated in the development of one-third of severe maternal morbidity (115) and one-tenth of preterm births (107) which are the leading cause of neonatal morbidity and mortality (39). Pre-eclampsia, a condition unique to human pregnancy, is characterized by the presence of hypertension and proteinuria or hypertension and end-organ dysfunction with or without proteinuria (76). Of concern, the prevalence of hypertension and pre-eclampsia has increased in the recent years (103) and the costs associated with managing a pregnancy complicated by pre-eclampsia are estimated to be more than double compared to those associated with an uncomplicated pregnancy (37). The total cost burden of pre-eclampsia during the first year after delivery was an estimated $2.18 billion in the United States alone in 2012 (108). Thus, hypertension in pregnancy is of major concern (91) and the potential role played by riboflavin in its prevention warrants further investigation.

Childhood and adolescence

As in adults, there are very limited published data on riboflavin status in children and adolescents. One notable exception are data from the population-based NDNS survey conducted in the UK which showed a decline with age in riboflavin status, along with folate and B12 biomarkers, and a corresponding increase in plasma homocysteine, from childhood to adolescence (57). Of note, in this and other (though not population-based) studies in Dutch and Greek children (7), dietary intakes as measured by using validated methodologies in general were found to compare favorably with dietary reference values across all age groups and were not lower in the older children. The mechanism for the decline in riboflavin, folate and B12 status with age through adolescence, despite no corresponding decline in dietary intakes, is not clear but may be an indication that riboflavin and other B vitamin requirements of older children are increased due to higher metabolic demands for growth from childhood to adolescence (7).

Young, middle age and older adulthood

The importance of riboflavin inadequacy in adulthood likely relates in part to its interactive effects with folate and vitamin B6. Riboflavin plays a key role in one-carbon metabolism where, as FAD, it acts as a cofactor for the folate metabolizing enzyme MTHFR. The importance of riboflavin in folate metabolism is perhaps most evident in individuals homozygous for the C677T polymorphism in *MTHFR*, resulting in a thermolabile enzyme with reduced activity, thus impairing folate recycling. The homozygous variant *MTHFR* 677TT genotype affects about 10% of people globally, but this figure is much higher in some countries, including Mexico where a reported 32% of the population have the TT genotype (70).

Although the health concerns in relation to this polymorphism have predominantly focused on homocysteine as the well described phenotype, arguably of greater relevance to public health is the more recent emergence of a blood pressure phenotype, and a modulating role of riboflavin (as the MTHFR co-factor), in determining the risk of hypertension in affected individuals (70). Notably, three randomized trials to date in hypertensive patients showed that intervention with low-dose riboflavin (1.6 mg/day) results in lowering systolic blood pressure (by 6 to 14 mmHg), specifically in those with the variant TT genotype in *MTHFR* (70). Moreover, recent evidence from a large cohort of over 6000 Irish adults, showed that the variant TT genotype in *MTHFR* was associated with higher blood pressure and an increased risk of hypertension from 18 years, whilst better biomarker status of riboflavin reduced this genetic risk (114). Thus,

intervention with riboflavin may have a role in preventing the development of hypertension in adulthood, before and in pregnancy, and potentially hypertensive disorders including preeclampsia linked with this common folate polymorphism (91), but this remains to be demonstrated.

Also relevant to riboflavin's roles across the lifecycle is its critical metabolic interaction with vitamin B6. Specifically, the conversion of vitamin B6 to the metabolically active form in tissues, PLP, is dependent upon riboflavin in the cofactor form FMN. Riboflavin is thus an important determinant of PLP, with recent evidence indicating that it is the limiting nutrient for maintaining vitamin B6 status in adulthood, and particularly so in individuals with the *MTHFR* 677TT genotype (52). PLP, in turn, functions as a coenzyme of 5-aminolevulinic acid synthase, which is involved in the synthesis of heme, the iron-containing component of hemoglobin. Low vitamin B6 secondary to riboflavin deficiency may thus impair hemoglobin synthesis and lead to microcytic anemia.

Public health considerations

Riboflavin deficiency is a significant problem in low-middle income countries. Across the developed world also, deficient riboflavin status may be widespread, but this is largely undocumented as biomarker status is rarely measured in population-based studies (72). The UK and Ireland are the only countries worldwide to routinely include a riboflavin biomarker as part of their national nutrition surveys; other countries rely on dietary riboflavin intakes only for the assessment of riboflavin status. Thus, low and deficient riboflavin status may go undetected in countries worldwide. Riboflavin status should be more widely assessed in nutrition surveys so that deficiency can be identified, and the appropriate public health measures implemented to prevent deficiency.

Riboflavin deficiency in women of reproductive age and in pregnancy may be a particular concern across countries globally. Whilst riboflavin is provided in rich supply in milk and dairy foods, other foods sources do not appear to provide adequate amounts to meet typical dietary needs, particularly in pregnancy where requirements are highest. Riboflavin-fortified foods such as breakfast cereals can make an important contribution to riboflavin biomarker status (42), albeit if fortification is undertaken on a voluntary basis (i.e. at the discretion of the food manufacturer), the benefit will be confined only to those women choosing to consume fortified foods. Given emerging evidence that riboflavin deficiency contributes to a higher risk both of

anemia and hypertension in pregnancy, appropriate riboflavin interventions, may need to be considered such as supplementation (for individual women before and during pregnancy) or food fortification (at a population-wide level).

SUMMARY AND RESEARCH PRIORITIES

Riboflavin plays a fundamental role in energy metabolism and supporting cellular antioxidant potential and has important metabolic interactions with other nutrients, including iron, vitamin B6 and folate. Given its numerous functional roles, riboflavin is essential in maintaining health and preventing disease throughout the lifecycle. Correspondingly, riboflavin deficiency can contribute to a number of adverse health outcomes, including anemia.

The emergence of a novel interaction of riboflavin with the *MTHFR* C677T polymorphism with impacts for blood pressure control is potentially important in preventing hypertension and its clinical sequalae in sub-populations globally affected by this genetic risk factor. Notably, in adults genetically at risk of developing hypertension owing to the variant *MTHFR* 677TT genotype, supplemental riboflavin (the co-factor for MTHFR) was shown in randomized trials to lower systolic blood pressure by up to 13 mmHg. A blood pressure response to intervention of this magnitude could have important clinical impacts, given that a reduction in systolic blood pressure of 10 mmHg is estimated to decrease stroke risk by 40%. However, neither the link of this common folate polymorphism (affecting 10% of populations worldwide) with hypertension, nor the important role of riboflavin in modulating the blood pressure phenotype, are well recognized and warrant further investigation. Mechanistic studies to better understand how this polymorphism is linked with hypertension, and how riboflavin lowers blood pressure in affected individuals, are also needed.

Riboflavin deficiency in pregnancy may be a particular concern across countries globally. Given the importance of riboflavin in both iron and folate metabolism, further research is needed investigating riboflavin-related anemia in pregnancy and the potential role of riboflavin deficiency in hypertensive disorders of pregnancy.

Riboflavin deficiency may be much more widespread than is generally perceived but typically goes undetected in almost all countries because biomarkers are rarely measured. Riboflavin status should be assessed in human studies, so that deficiency can be identified, and the

appropriate public health measures implemented to prevent it. The availability of accessible and validated riboflavin biomarkers, particularly for use in population-based nutrition surveys, would facilitate riboflavin status being more widely assessed and this should be prioritized.

Whilst riboflavin is provided in rich supply in milk and dairy foods, other foods sources do not appear to provide adequate amounts to meet typical dietary needs and thus appropriate interventions, such as food fortification with riboflavin, may need to be considered. Finally, dietary riboflavin recommendations may need to be reconsidered in light of emerging scientific evidence of previously unrecognized functional and health effects of riboflavin within the dietary intake range.

DISCLOSURE STATEMENT

H McNulty and M Ward hold an international patent on the use of riboflavin in the treatment of blood pressure. K Pentieva has no conflict of interest to declare.

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Table 1. Biomarkers of riboflavin status and cut points indicative of deficiency

Biomarker	**Strengths**	**Limitations**
EGRac Enzyme erythrocyte glutathione reductase coefficient Activation Coefficient (ac) is expressed as the ratio of enzyme activity *with added coenzyme* to basal enzyme activity *without added coenzyme* Cut-off values: • ≤ 1.26 Adequate • 1.27-1.39 Sub-optimal • ≥1.4 Deficient	• Considered gold-standard • Functional assay • Stable for several years • Sensitive to small changes	• Specific treatment required • Lack of standardisation • Less reliable in deficiency of glucose 6-phosphate dehydrogenase or ß-thalassemia • High sensitivity to small degrees of cofactor (FAD) desaturation
24h Urinary Riboflavin Cut-off values: • <40mg/day (<27mg/g creatinine): indicating deficiency • 40-120mg/day (<80mg/g creatinine) indicating insufficiency • ≥ 120mg/day (≥ 80mg/g creatinine) Indicating sufficiency	• Direct test • High variability within and between subjects • Reflects recent dietary intake	• Reflects recent dietary intake only • 24hr samples not convenient
Serum/Plasma/Erythrocyte Riboflavin FAD FMN No official cut-off values	• Direct test • Accessible • Vitamers (riboflavin, FAD, FMN) are stable	• Affected by many factors • High variability in plasma/erythrocyte riboflavin within and between-subjects

Table 2. Factors affecting biomarkers of riboflavin assessment

EGRac: Enzyme erythrocyte glutathione reductase coefficient	**Urinary Riboflavin**
• **The length of the pre-incubation of reagents with EGR enzyme:** Too short a pre-incubation period may underestimate EGRac values. • **Age of erythrocytes** may influence EGR activity as concentrations decline with aging of cells. • **Age of the subject** may affect EGR activity. • **Genetic conditions,** including glucose-6-phosphate dehydrogenase deficiency and heterozygous β-thalassemia, are associated with disorders in erythrocyte flavin metabolism which may result in misleading EGRac test results. • **Pyridoxine deficiency** interferes with the EGRac test, resulting in a decrease in erythrocyte glutathione reductase activity • **Disease states,** including iron-deficiency anemia, severe uremia, cirrhosis of the liver, and hypothyroidism, lead to increased erythrocyte glutathione reductase activity. • **Conditions of negative nitrogen balance** lead to a fall in EGRac values.	• **Physical activity and sleep** can decrease riboflavin excretion. • **Negative nitrogen balance and infection** induce a breakdown of tissue protein and, hence, increase urinary excretion of riboflavin. • **Drugs,** including some antibiotics and psychotropic drugs such as phenothiazines, can increase the excretion of riboflavin. • **Oral contraceptive agents and pregnancy:** riboflavin excretion can decrease when oral contraceptive agents are used and during the third trimester of pregnancy, while during the second trimester, excretion increases. • **Within-subject variability** can be high for 24h urinary riboflavin excretion.

Adapted from Pentieva et al 2021 (80)

Table 3. Food sources of riboflavin in Irish adults

Food	Riboflavin (mg/100g)	Riboflavin (mg/serving)
Cow's milk	0.23	0.46/200ml glass
Fortified breakfast cereal (average)	1.30	0.39/30g bowl
Fortified cereal bars	0.78	0.35/1 bar
Yoghurt	0.27	0.34/125g pot
Eggs	0.47	0.24/1 egg
Chicken (breast, grilled)	0.19	0.23/120g
Cheese	0.43	0.13/30g

Source: The National Adult Nutrition Survey (NANS); 2008-2010 (75)

Table 4. Dietary reference values for Riboflavin (mg/d) for adults (>19yrs)

RDA (mg/day)	**UK, DOH (1991) RNI**	**US, IOM (1998) RDA**	**EFSA, NDA panel (2017) PRI**	**WHO / FAO (2004) RNI**
Male	1.3	1.3	1.6	1.3
Female	1.1	1.1	1.6	1.1
Pregnancy	1.4	1.4	1.9	1.4
Lactation	1.6	1.6	2.0	1.6

UK Department of Health (Reference Nutrient Intake, RNI); US Institute of Medicine (Recommended Dietary Allowance, RDA); European Food Safety Authority (Dietary Reference Value, DRV); World Health Organization/Food and Agriculture Organization of the United Nations (RNI).