Nelson Alexandre Rodrigues Tavares

Riboflavin status and effects of supplementation on biomarkers of cardiovascular disease in the elderly

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Supervised by:

Professor Pedro Alexandre Afonso de Sousa Moreira

Faculty of Nutrition and Food Sciences,

University of Porto

Portugal

Professor Teresa Maria de Serpa Pinto Freitas do Amaral

Faculty of Nutrition and Food Sciences,

University of Porto

Portugal

Aos meus pais

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Contents

1. Introduction

In this introduction the state of the art knowledge concerning the impact of age-related nutrition issues promoting health and reducing the risk of the most common elderly diseases, particularly cardiovascular diseases, will be reviewed.

In the developed world, cardiovascular diseases are the predominant cause of disability and death in elderly people[1]. Some nutritional causes are implied in the cardiovascular diseases, one of which is a low B vitamin intakes[2]. Evidence exists that low B vitamin intakes and high homocysteine concentrations are common problems among older adults in the United States[3].

The importance of riboflavin is less clear; however, act as cofactor in the enzymatic breakdown of homocysteine and supplementation might play a role in ensuring maximal reductions in homocysteine levels[4].

Portuguese people have one of the lowest levels of life expectancy in the Eur-A, the reference group as designated by World Health Organization, which includes 27 european countries, despite a gain of 4.5 years over the past 20 years. In Portugal, girls born in 2002 can expect to live almost 81 years and boys slightly less than 74 years, which is 1.5 and 2.0 years less than their Eur-A counterparts, respectively [5]. Although we currently are seeing the results of the baby boom, the Portuguese population aged 65 years and older is expected to grow from about 17% of the population in 2003 to projected 23% in 2030 [5]. In addition, World Health Organization estimates that, on average, Portuguese people can expect to be healthy for about 90% of their lives. They lose on

average 7.8 years to illness – the difference between life expectancy and healthy life expectancy [6].

Due to medical treatment advances, it is reasonable to expect the average life span to reach or exceed 100 years in the 21st century [7]. However, because the average age at chronic disease onset has not risen to the same extent as life expectancy, an elderly currently aged 75 years can look forward to only 4 more years of active health followed by more than 7 years of disability. If the age of chronic disease onset does not increase commensurate to the added years of life still to come, a growing number of centenarians will spend the last 2 decades of their lives living with the serious and debilitating consequences of chronic disease. This is not a future that anyone is happy to contemplate [8].

Over the recent decades the health status of the Portuguese population has improved [5]. This can be explained both to more significant progress in 30% of the municipalities and to the diminishing disparities among regions between 1991 and 2001. Both effects are related to social and health factors and behaviours. However, there is still some concern over regional disparities, particularly between urban-coastal and rural-interior regions. The latter had, and still have, the worst health condition. Rural regions are also the poorest in the country. Health inequalities are associated with economic and social factors, such as income, living conditions, unemployment, health care and illiteracy [9]. The exact relation between literacy and health is still unclear, being recognized that people with low literacy are more likely to report having poor health, and are more likely to have diabetes and heart failure, than those with adequate literacy[10].

Similar to the other Eur-A countries, most Portuguese die from noncommunicable diseases. Mortality from cardiovascular diseases is higher in Portugal than in the Eur-A, and they are the main killer causing 36% of all deaths [(5)]. For Portuguese females, cardiovascular diseases deaths are the most consistently in excess mortality by 10-80%. Portuguese people die 12% less often from cancer than in the Eur-A, but mortality is not declining as rapidly as in the Eur-A. Portugal has the highest mortality rate for diabetes in the Eur-A, with a sharp increase since the late 1980s [(5)].

Mortality rates have declined in virtually all countries due to progress in preventing infectious diseases and improving hygiene, sanitation and overall social development and living standards. This decline in mortality was accompanied more recently by an equally sharp fall in birth rates, the only exception being most of sub-Saharan Africa. Ultimately, the demographic transition leading to population ageing can be summarized as a shift from high mortality/high fertility to low mortality/low fertility. As fertility declines and more people live longer, the relative weight of society's main dependent groups - children and older persons – is shifting towards older persons [(11)].

With the increasing emphasis on health and the progressive lengthening of the average life span among adult men and women, both the scientific community and the general public have been examining ways to improve well-being and to prevent disease at every stage of life. It is fair to say that the earlier in life one starts healthy nutritional and lifestyle measures, the more likely these measures are to be effective in the long run. The evidence is that even when measures

are begun in one's 60s and 70s, definite benefits occur in many categories of chronic disease. There are many ways in which nutrition can prevent major categories of chronic disease and thereby promote health and vigor when initiated at a later age [12].

Preventive care, delivered through a country's primary care system, can improve all-cause mortality and premature mortality, particularly from cardiovascular diseases [5].

The medical treatment for the management of such conditions as obesity, hyperlipidemia, diabetes mellitus, osteoporosis, hypertension and other cardiovascular diseases requires pharmacopterapy to achieve optimal disease control for some of this long-term conditions [13]. Elderly people consume a large proportion of health care, including drugs, and evidence shows that prescribing to this group is often inappropriate [14], and they can affect the absorption and metabolism of certain nutrients.

One of this examples is of antacids containing aluminum ion which presents an effect on gastro intestinal motility and influence gastric emptying on the absorption of riboflavin from the gastro intestinal tract [15]. Anticholinergic drugs may affect riboflavin absorption, this is attributed to delayed gastric emptying and slower transit of riboflavin through the small intestinal lumen [16].

Enhanced urinary excretion of vitamins induced by drugs is a major factor in development of vitamin deficiencies. In addition to increasing urinary excretion, drugs can induce vitamin deficiencies by altering their intestinal absorption,

transport, storage, and/or metabolic conversions. Alterations in various aspects of flavin metabolism have been observed following administration of certain drugs, namely antimalarial, antimicrobial, anticancer, and some tricyclic antidepressant and antipsychotic agents [17]. Phenytoin, phenothiazine and tetracycline as well as thiazide and tricyclic are antidepressants that could contribute to riboflavin depletion, because inhibits the incorporation of riboflavin into flavin adenine dinucleotide and flavin adenine mononucleotide.

There are many ways in which nutrition can prevent major categories of chronic disease and thereby promote health and vigor when initiated at a later age [12]. Nutrition also has a major role in protecting health and slowing disease progression. Paradigms that promote the nutritional components of healthy aging are needed to increase the age of chronic degenerative disease onset and to maintain healthy, functional lives for as long as possible [8].

Good nutrition may contribute significantly to the health and well being of older individuals, and their ability to recover from illness. However there is no gold standard for diagnosing undernutrition, and most clinically available nutrition screening instruments lack sensitivity and specificity. In addition, abnormal nutritional indicators may simply reflect effects of age, functional disability or severe underlying disease [18, 19]. Furthermore, the difficulties in detecting early signs of undernutrition are similar to those encountered in the early recognition of many age related diseases [18]. However, in the case of nutritional deficiency there are two further difficulties: for almost every nutrient there is a long latency period a low intake leads to overt clinical manifestations, and early diagnosis depends upon the findings of abnormalities of special tests, including

biochemical and hematological investigations. Second, the true significance of abnormal results of these tests are not fully understood in the elderly [20].

Undernutrition affects about 4% of community-dwelling elderly subjects [21]. This prevalence is due to age-related diseases together with aging processes. Developing countries have a substantial higher prevalence of undernutrition [21], and it is well known that chronic energy deficiency is a risk factor for adult low productivity, morbidity, and mortality [22-27]. Moreover, elderly undernutrition increases morbidity and mortality. Therefore needs to be diagnosed and treated quickly to avoid these negative consequences [28]. The key predictors of undernutrion are loss of appetite and anorexia, and older adults exhibit less hunger and earlier satiety. Impared appetite contributes to the undernutrition seen in elderly in both community and institutionalized settings[13]. A decreased food intake could be attributed to changes in taste and flavor sensations, such as the decline in odor perception with age [29].

A prevention-oriented, life cycle approach reduces the population's cardiovascular disease risk status with primary prevention strategies, such as proper nutrition. To prevent cardiovascular disease a decrease in risk factors related with diet such as obesity, lipemic disorders and diabetes mellitus are important. Coffee consumption was associated with reduced circulating B-vitamin concentrations[30]. Other dietary habits that may lower cardiovascular risk include cereals, small fish, hardtack and olive oil intake[31]. Various international and professional organizations have made recommendations for fish or docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) consumption, n-3-fatty acid supplements and n-3 enriched foods are therefore

becoming increasingly important dietary options[32]. A lower ratio of n-6/n-3 fatty acids is more desirable in reducing the risk of many of the chronic diseases of high prevalence in Western societies, as well as in the developing countries[33]. It can result in delayed cardiovascular disease progression and ultimately reduced disease incidence. Secondary and tertiary prevention involving a combined high-risk and population approach identifies those at high risk and protects or treats them, keeping as many as possible at low risk, and continues to shift the entire population to lower risk [8].

Measures of nutritional status are usually valuable as may be predictive of health outcomes. The complex nature of diet has posed a difficult challenge to its measurements in nutritional epidemiological studies. Food frequency questionnaires can be used for categorizing persons accurately according to intake and for identifying subjects at the extremes of intake. They are easy to administer and measure long-term intake[34].

Fruits and vegetables are linked to reduction in cardiovascular disease in a variety of ways. Firstly, they provide nutrients, such as fiber, folate, potassium, and carothenoids and other phytochemicals that may reduce cardiovascular risk. Secondly, certain nutrients may directly improve conditions wich are established, diet-related cardiovascular disease risk factors, such as blood pressure, hyperlipidemia, and diabetes. Thirdly, the consumption of fruits and vegetables may be associated with a reduced intake of saturated fat and cholesterol rich foods[35].

Several laboratory studies have shown that compounds in fruit and vegetables, which have an antioxidant effect, provide beneficial effect on atherosclerosis and cardiovascular disease [36]. On the other hand, a diet high in fruit and vegetables has another advantage, namely, that of lowering the dietary glycemic potency and energy density [37].

In recent years there has been much interest in the importance of plasma homocysteine as a graded risk factor for cardiovascular disease. Riboflavin intake also emerged as a factor influencing plasma total homocysteine in men and women from the Framingham Offspring Cohort. Riboflavin is a cofactor for methylenetetrahydrofolate reductase, an enzyme involved in the remethylation of homocysteine to methionine may contribute to higher fasting tHcy concentrations. As part of the Fifth Offspring Cohort examination, fasting blood samples were not obtained for determination of riboflavin and a modest association between dietary riboflavin and tHcy was observed[38].

Recently it was confirmed a folate-riboflavin interaction in determining plasma homocysteine [39]

Serum total homocysteine concentration is a strong predictor for incident ischemic stroke among patients at increased risk because of chronic coronary heart diseases. The graded association observed is independent of traditional risk factors or inflammatory markers and indicates the importance of serum homocysteine levels in patients with preexisting vascular disease [40].

To become old is not programmed. Life span is the result of the interactions between genes and the environment in which we live. The environment also

determines how a gene influencing life span is expressed over a lifetime [41]. Biological ageing is the progressive decline in physiological ability to meet demands that occurs over time. It is due to the accumulation of damage at the cellular level and his rate is determined by both environmental and genetic factors. There is increasing evidence that many known factors influence the rate of cellular damage accumulation and hence biological ageing and that the pathogenesis of some important diseases is related to biological ageing [42]. It is fair to say that the earlier in life one starts healthy nutritional and lifestyle measures, the more likely these measures are to be effective in the long run.

The periconceptional use of folic acid to prevent neural tube defects is a good example of the importance of early nutrition, even before birth, playing a role as a factor governing future health. Randomized controlled trials[43-47] studies of women with or without a prior pregnancy affected by neural tube defects have shown that folic acid supplements during the periconceptual period significantly reduce the risk of further neural tube defects-affected pregnancies [8]. The importance of nutrition during pregnancy has been well documented, with evidence suggesting a protective effect against various adverse birth outcomes for pregnant women following professional recommendations [48].

Studies in both humans and animals have shown that the events during gestation and early post-natal stages may have long term consequences for health[49]. Fetal undernutrition is linked to an increase risk of cardiovascular disease, obesity, type II diabetes and hypertension, amongst other diseases. Human epidemiologic and experimental animal studies suggest that early nutrition affects susceptibility to chronic diseases in adulthood. These studies

provide evidence that biological mechanisms may exist to ''memorize'' the metabolic effects of early nutritional environments [49]. As an adaptation to undernutrition in foetal life, permanent metabolic and endocrine changes occur; this would be beneficial if nutrition remained scarce after birth. If nutrition becomes plentiful, however, these changes predispose to obesity and impaired glucose tolerance [50]. The evidence for the association of adverse adult outcomes with lower birth weight is strong for blood pressure and impaired glucose tolerance[50].

Routine physical activity and good nutrition help reduce the risk of chronic diseases related to aging and facilitate the ability to remain independent at home [13]. Food insecurity occurs whenever the availability of nutritionally adequate and safe food, or the ability to acquire foods in socially accepted way is limited or uncertain and rates were higher in households in which the elderly adults lived alone [13]. Cultural background, behaviour and values should be taken into account to reinforce traditional family support for elderly. Olders with chronic diseases benefit more from family involvement [51]. Many people, as they age, remain fully independent and actively engaged in their communities, however, others fare less well and need more support. A broad array of appropriate, culturally sensitive food and nutrition services, physical activities and health and supportive care customized to the population of older adults are necessary.

Maintenance of oral health is an important yet often neglected component to successful aging. When elder's oral health is compromised, they tend to avoid difficult-to-chew foods such as fruits, vegetables and whole grains – all nutrient-dense foods recommended in the prevention of chronic diseases[36].

It is, therefore, imperative to prevent tooth loss through preventive dentistry and nutrition education and to integrate dental health within nutrition assessment for older adults [36]. Edentulism is prevalent among older people all over the world and is highly associated with socio-economic status [52], could cause reduced nutritional intake and has been implicated as a factor causing malnutrition in elderly patients [29, 53]. Tooth loss is independently associated with onset of disability and mortality in old age and may be an early indicator of accelerated aging [54].

Maintenance of a normal plasma glucose concentration requires precise matching of glucose utilization and endogenous glucose production or dietary glucose delivery [55]. Hypoglycemia could result from unbalanced glucose level[56] and may also go unrecognized in the elderly population because of restrict communication, cognitive impairment, and possibly fewer adrenergic symptoms [57]. The choice of carbohydrate-rich foods in the habitual diet should take into account not only their chemical composition but also their ability to influence postprandial glycemia. Fiber-rich foods generally have a low glycemic index, although not all foods with a low glycemic index necessarily have high fiber content [22]. Several beneficial effects of low-glycemic index, high-fiber diets have been shown, including lower postprandial glucose and insulin responses, an improved lipid profile, and, possibly, reduced insulin resistance. In nondiabetic persons, suggestive evidence is available from epidemiologic studies that a diet based on carbohydrate-rich foods with a low-glycemic index, high-fiber content may protect against diabetes or cardiovascular disease [58].

World Health Organization recommends limiting non-milk extrinsic sugars consumption to less than or 10% energy to reduce the risk of unhealthy weight gain and dental caries, and to restrict frequency of intake to less than or 4 times per day to reduce risk of dental caries [23]. Older adults especially those from low-income backgrounds, are at increased risk of dental caries [59].

More recently, in controlled feeding studies, fructose has been used to elevate daylong serum triacylglycerol concentrations in healthy and diabetic subjects, an event that could lead to an accumulation of lipoprotein remnants, which could be atherogenic[60] and considered as a cardiovascular disease factor.

Bioavailability or digestibility of a protein or the capacity to provide metabolically available nitrogen and amino acid to tissues and organs is important. The food matrix in which a protein is consumed can have significant impact on the bioavailability of amino acid for metabolic needs.

It is clear that protein plays a role in promoting optimal health. Many avenues are emerging for exploring protein's potential and elucidating the mechanisms at play in lean body mass retention, weight control, reduced inflammation, insulin sensitivity, and bone and cardiovascular health. The evidence available to date suggests that quality is important not only at the Dietary Reference Intakes but also at higher intakes. It is also evident that quality at higher compared with lower intakes is important for different reasons. Examination of the increasingly complex roles emerging for protein reveals these differences [61].

A high ileal digestibility of proteins is also relevant for reducing the amount of dietary nitrogen entering the colon. Protein fermentation by the intestinal flora may result in the formation of toxic compounds, including ammonia, dihydrogen

sulfide, indoles, and phenols that could irritate the colonic epithelial cells and increase the risk of colon cancer [61].

A decline in protein synthesis, particularly in the synthesis of myosin heavy chains is strongly connected with a parallel increase in fat mass and this mechanism may lead to the concomitant presence of sarcopenia[62]. The adoption of a more sedentary lifestyle and a less than optimal diet could contribute to sarcopenia, a complex multifactorial process facilitated by a combination of factors in olders[63].

Saturated fat intake is positively associated with cardiovascular disease, results in plasma elevated ratio of LDL to HDL cholesterol and LDL and total cholesterol concentrations. It is associated with an increased risk of coronary heart disease [64]. Higher habitual intakes of saturated and *trans* fats are independently associated with increased subclinical atherosclerosis [64]. The limit intake of saturated fat should be <7% of the total daily energy [65].

Dietary *trans* fatty acids come mostly from the industrial hydrogenation of unsaturated vegetable oils; they are found in manufacturing products such as cookies, pastries, and salad dressings. These deleterious effects of industrial trans fatty acids on cardiovascular health are well established [66] and have been associated with a greater risk, on decreasing plasma HDL cholesterol and increasing LDL cholesterol [67]. The consumption of trans fat should be <1% of the total daily energy [65].

Dietary patterns and lifestyle pratices are associated with chronic diseases in elderly people [13] and their analysis provides a characterization of recurrent dietary behaviour in olders, and can be used to provide tangible dietary advice[68].

Nutritionists can work with community aging services programs in alerting them to the importance of nutrition for elderly, making targeted nutrition information messages available and providing nutrition counseling [13]. For elders, who were at heightened vulnerability of nutritional inadequacy meals should meeting at least 33% of daily nutrient requirements[69] moderate in energy, adequate nutrient intake according Modified Food Guide Pyramid for people over seventy years of age[70] and also according to the New Food Guide for the Portuguese Population where a range of recommended food portions was established for the groups of fats and oils; milk and dairy products; meat, fish, seafood, and eggs; pulses; potatoes, cereals and cereal products; vegetables; fruits; and water for hydration balance [71].

Chemosensory deficits experienced by the elderly generally cannot be reversed. However, sensory interventions including intensification of the taste of food can compensate for age-related perceptual losses in ageusia (absence of taste) or hypogeusia (diminished sensitivity of taste) [72]. Sodium chloride became increasingly utilized as taste enhancer. Elders also suffer chronically from the absence of a chronic low-grade, diet-induced, non-chloride-depleted, potassium-replete, metabolic alkalosis, an acid-base, potassium, and chloride state for which natural selection appears to have optimized human physiology. Elders also suffer chronically from the adverse cardiovascular effects of an

inverted dietary ratio of potassium to sodium, inverted in respect to the ratio in the diets consumed by *Homo sapiens* until the agricultural and industrial revolutions began, with the consumption of superphysiologic amounts of sodium chloride. Both decreasing sodium chloride intake and increasing potassium - and bicarbonate - rich precursors may likely not just help the aging skeleton but provide other potential health benefits as well [(73)]. Portugal in general has a high salt intake diet, and four different Portuguese samples showed similarly high mean daily salt intake levels, almost the double of the recommended by the World Health Organization [(74)].

Fluid intake is critical for maintaining vascular volume, regulating body temperature, removing waste from the body, supporting cellular homeostasis, and is more important to emphasize in older than in younger adults, because compromised homeostatic mechanisms such as loss of the thirst sensation can result in dehydration. Fluid needs in healthy older people are variable and greatly influenced by level of physical activity, ambient temperature, and medication use [(75)].

Moderate alcohol consumption is associated with a reduced risk of coronary hearth disease. This is believed to occur through alcohol's antithrombotic properties and its ability to increase high-density lipoprotein levels. It remains unclear whether polyphenol compounds in red wine make it an especially cardioprotective alcoholic beverage[(76)]. These compounds are proposed to act by inhibiting low-density lipoprotein oxidation and thrombosis independently of alcohol. Moderate alcohol consumption is not associated with any significant

morbidity; however, three or more drinks per day are associated with hypertriglyceridemia, cardiomyopathy, hypertension and stroke[76]. Alcohol impairs intestinal absorption of riboflavin [77].

Older, obese, and sedentary individuals are at high risk of developing diabetes and cardiovascular disease. Exercise training improves metabolic anomalies associated with such diseases, resting substrate oxidation and creates a metabolic milieu that appears to promote lipid utilization in sketetal muscle, thus facilitating a reversal of insulin resistence [78]. Walking and a combination of strength-building and flexibility exercise can improve physical performance and positive changes in quality of life in olders [79]. Exercise regulates the brain function. Regular exercise can enhance cognitive and functional activity scores in dementia patients and may improve it by participating in a regular exercise program [80].

Elderly persons are a nutritionally at-risk group for poorer vitamin and mineral status based on studies of plasma biomarkers of dietary exposure[37]. Micronutrient status deteriorates with increasing age. There is evidence to consider the supply of some important dietary antioxidants as critical in some elderly. In light of age-related decreases in the activities of antioxidant enzymes, a sufficient supply of dietary antioxidants is important, especially as oxidative damage is thought to contribute to the deteriorating process associated with aging and promote cardiovascular disease [81]. Calcium and vitamin D are two other examples of nutrients whose absorption and status is influenced by age.

For both, an age related decrease of receptor expression in the duodenum was shown in women [60].

Two large surveys have added to our knowledge riboflavin status in portuguese people: the ''Inquérito Alimentar Nacional''[82] and the Survey in Europe on Nutrition and the Elderly, a Concerted Action (SENECA) [83]. Results from the ''Inquérito Alimentar Nacional'', showed a high prevalence of low riboflavin intake (1.216 mg/day) and in Portuguese arm of the SENECA study (ages 70-75 years), high prevalences of riboflavin intakes below the lowest European recommended dietary intakes[84], (1.0 mg/day) were found. More recently ''Consumo Alimentar do Porto'' showed that the prevalence of low riboflavin intakes were higher in older people [85]. A poor riboflavin status with elevated homocysteine may be more common in population with poor nutrition or whose food supply is not fortified with folate [86].

There is little understanding of the matter that up to what extent the riboflavin has a protective role in disease prevention, and the data on riboflavin status and serum folate in elderly population in Portugal is nonexistent. This dissertation is aimed at filling the gap that is found in the researches conducted so far around the above mentioned topics, and to assess circulating riboflavin and folate with independent biomarkers. The dissertation is aimed at examining the impact of improved riboflavin status on biomarkers of cardiovascular disease in the elderly.

The dissertation is developed on the rationale of the gap point out in literature that there are few researches that discover the issues related with the riboflavin and folate status and its effects on elderly.

The main objectives of the study are: firstly to review the current knowledge of riboflavin as protective against diseases, secondly to assess in elders riboflavin and folate concentrations and the validity of its estimates from a semiquantitative food-frequency questionnaire currently used in Portugal, in comparision with independent biomarkers and thirdly to find the evidence about the benefit of riboflavin supplementation investigating the effects of oral riboflavin supplementation on plasma total homocysteine, ferritin, uric acid and C-reactive protein concentration in elderly people with a low riboflavin status. A four-week randomized, placebo-controlled, double-blind trial of riboflavin supplementation in seven Portuguese day social centers was performed.

Hence the dissertation is based on the aim of unfolding the impact of riboflavin status with the help of biomarkers, SFFQ, supplementation as well as review of concerned literature.

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2. Putative role of riboflavin in disease prevention

Nelson R Tavares

The Center for Human Nutrition, The University of Sheffield, United Kingdon
The Faculty of Nutrition and Food Sciences of the University of Porto, Portugal

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In the early part of the twentieth century, pioneering studies on the deficiency state of pellagra in experimental animals showed that water-soluble tissue extracts could be effective in treating diseases. Further studies showed that one part of the heat-stable fraction from the mentioned extract, called yellow growth factor, had fluorescent properties. This was later purified and named riboflavin. Until 1932, when the landmark discovery of the ‘’yellow enzyme’’ containing an isoalloxazine ring and a phosphate group was made, the physiological role of the yellow growth factor remained obscure. The synthesis of riboflavin, accomplished in 1935, was followed by the identification of the two active coenzyme forms, flavin mononucleotide (FMN) in 1937 and the clarification of the structure of flavin adenine dinucleotide in 1938, this formed from FMN.

As a water-soluble vitamin, riboflavin plays a part in a variety of oxidation-reduction reactions. Flavin mononucleotide and flavin dinucleotide act as active coenzyme forms of riboflavin that participate in a variety of reactions in the human body. Riboflavin has an important role in the fat metabolism disturbances. Through deficiency and supplementation studies and effects on the structure and function of the small intestine, riboflavin has a role in iron handling. Riboflavin is associated with compromised oxidant defense. Flavin

adenine dinucleotide acts as the co-factor for 5,10 Methylenetetrahydrofolate reductase, an important enzyme, which participates in the remethylation pathway for homocysteine metabolism. Homocysteine is located at a critical metabolic crossroad and therefore both pathways, remethylation and transsulfuration; and directly and indirectly impacts all methyl and sulphur group metabolism occurring in the body. Poor vitamin status could promote higher homocysteine levels. In addition, high levels of homocysteine could be considered conditional risk factors for cardiovascular diseases. Riboflavin has also been ascribed a role in the protection against certain cancers and cataracts.

Key-words: Riboflavin, homocysteine, iron handling

INTRODUCTION

Through its cofactor role in numerous reactions in the human body, riboflavin has been implicated as protective against diseases or conditions as diverse as cataract, oesophageal cancer, and cardiovascular disease.

Evidence comes from animal studies, epidemiological studies in humans, and a few experimental intervention studies in humans. Mechanisms for an apparent role for riboflavin as protection against certain diseases are not always understood.

Riboflavin, structure and active forms

Riboflavin, vitamin B2, is a water-soluble vitamin, a yellow, fluorescent compound, defined chemically as 7,8-dimethyl-10-(1'-D-ribityl) isoalloxazine. The planar isoalloxazine ring provides the basic structure not only for vitamin B2 but also for the naturally occurring phosphorylated coenzymes that are derived from riboflavin. The primary form of the vitamin is as an integral component of the coenzymes flavin mononucleotide (FMN) and flavin-adenine dinucleotide (FAD) [1-4]. Flavin mononucleotide and flavin-adenine dinucleotide linked covalently to specific tissue proteins, generally at the 8-αmethyl position of the isoalloxazine ring [5].

Dietary sources

Abundant in omnivorous diets, the Recommended Nutrient Intake (RNI) for riboflavin per day are:

Dietary reference Values for Riboflavin (mg/day) (6)

0-6 months	7-10 months	1-3 years	4-8 years	Males 9-13 years	Females 9-13 years	Males 14+ years	Females 14-18 years	Females 19 + years	Pregnancy	Lactation
0,3	0,4	0,5	0,6	0,9	0,9	1,3	1,0	1,1	1,4	1,6

The richest sources are yeast extract and offal products especially those based on liver [7]. Beer is a good source as are milk, cheese and eggs. Nuts, avocados, green and yellow vegetables contain reasonable amounts; important for vegetarians who need a varied diet, in order to avoid deficiency [8]. Diets based on rice are normally riboflavin deficient. As a heat-stable vitamin,

riboflavin losses in cooking are small, but it is easily destroyed by sunlight when in liquid form. Since dietary sources of riboflavin are largely in the form of coenzyme derivatives, these must be hydrolysed prior to absorption. Most of the vitamin B2 in foods occurs as riboflavin, flavin mononucleotide or flavin-adenine dinucleotide, all protein bound. In the Western diet dairy products, meats, poultry, fish are good sources. Grain products, contain relatively low levels of riboflavin; however, when enriched or fortified grains, cereals, and bakery products supply larger amounts. Broccoli, turnip greens, asparagus, and spinach are also good sources.

Bioavailability of riboflavin

Dietary sources of riboflavin are largely in the form of coenzyme derivatives, mainly flavin-adenine dinucleotide and, to a lesser degree, flavin mononucleotide all protein bound. These must be hydrolysed prior to absorption, in the acidic conditions of the stomach, from pyrophosphatase to phosphatase, and then absorbed as riboflavin in the small intestine by a sodium-dependent and saturable process, within the enterocyte, and phosphorylated to flavin mononucleotide. A reasonable estimation of bioavailability is approximately 95 percent of food flavin, up to a maximum of about 27 mg absorbed per single dose [(9)].

The composition of the diet appears to influence the riboflavin requirements. A lower ratio of fat to carbohydrate decreased the requirement [(10)]. This relationship was examined in elderly people.

Perhaps no more than 7 percent of food flavin is found as covalently attached 8α-flavin-adenine dinucleotide, bound to heterocyclic atoms of proteins that

function catalytically. Although some portion of the 8α-(aminoacid) riboflavins are released by proteolysis they do not have vitaminic activity [11].

Riboflavin is found inside cells, in the matrix, inner and outer mitochondrial membrane, liver and kidney microsomes, predominantly as flavin-adenine dinucleotide, enzyme covalently bound [12].

Conserved through an enterohepatic cycle, riboflavin is excreted in the urine by active tubular secretion.

Deficiency symptoms

Riboflavin deficiency has been documented in developed and developing countries and across various demographic groups [13,14]. The signs of ariboflavinosis are sore throat, hyperemia and edema of the pharyngeal and oral mucous membranes; cheiliosis, angular stomatitis and in a later stage glossitis, seborrheic dermatitis, photophobia and corneal vascularization, anaemia and brain dysfunction. Deficiency is most often accompanied by other nutrient deficiencies and may impair the metabolism of vitamin B6 by limitating the amount of FMN required by pyridoxine (pyridoxamine) 5-phosphate oxidase and the conversion of tryptophan to functional forms of niacin [15]. Diabetes mellitus [16,17], cancer [18] and cardiac diseases [19] are known to exacerbate riboflavin deficiency.

Assessment of riboflavin status

For assessment of riboflavin and its derivatives, a variety of available methods exist. These include Fluorometric procedures based on inherent fluorescent

properties of flavins [20], competitive protein binding [21] and binding to specific apoenzymes such as D-amino acid oxidase.

Currently, high-pressure liquid chromatography (HPLC) is the method most widely employed for determination of flavins in biological fluids and tissues [22]. After mild hydrolysis to convert flavin-adenine dinucleotide to the more stable flavin mononucleotide, high-pressure liquid chromatography leads to a more exact determination of flavin mononucleotide plus traces of riboflavin; and could be considered a useful indicator that reflects the functional cellularly trapped forms of riboflavin.

Riboflavin nutrition status could be generally evaluated, in both individuals and specific groups, by determining its urinary excretion and the erythrocyte gluthatione reductase activity coefficient (EGRAC) [23]. Urinary riboflavin can be measured by fluorometric high-pressure liquid chromatography methods [24,25] as well as by microbiological procedures.

High-pressure liquid chromatography techniques permit easier separation of other fluorescent flavin catabolites such as 7- and 8- hydroxymethylriboflavins [26] from riboflavin and were found useful in relating the recent dietary intake to urinary output. Urinary riboflavin determinations are directly affected by several factors such as renal excretion [27], physical activity, elevated body temperature, treatment with certain drugs, and other stress conditions associated with negative nitrogen balance [28]. Urinary riboflavin has been shown to increase with the administration of certain psychotropic drugs such as phenothiazine [29] antibiotics and under conditions causing negative nitrogen balance. A decrease in urinary riboflavin excretion with an increase in physical activity [30,31] was demonstrated in some studies.

Some dietary catabolites, like 10-formylmethyl and 2'-hydroxyethyl-flavins can interfere with the response of bacteria used for assay of urinary flavin.

For adults, a low urinary concentration of riboflavin is considered to be 19 to 27 µg/g creatinine and a deficient concentration to be below 19 µg creatinine.

Erythrocyte gluthatione reductase activity coefficient is based on the degree of saturation of the apoenzyme with its coenzyme flavin-adenine dinucleotide that reflects the body stores of flavin-adenine dinucleotide.

Results are expressed as an activity coefficient which is the ratio of activities in the presence of added flavinadenine dinucleotide and without its addition, and suggested guidelines to interpreting those are as follows: 1.2 or less indicates adequate riboflavin status, 1.2-1.4 borderline- to-show status, and greater than 1.4 a clear riboflavin deficiency [32]. However an upper limit of normality has been establish at 1.34 based on the mean plus 2 standard deviations of the Erythrocyte gluthatione reductase activity coefficient value of healthy elderly individuals aged 60 years and older [33]. A number of physiological variables could influence the results of this determination. Erythrocyte gluthatione reductase activity coefficient is not a valid test for individuals with glucose 6-phosphate dehydrogenase deficiency, because glutathione reductase in the erythrocytes has increase avidity for flavin-adenine dinucleotide [34]. In both hypothyroidism and hyperthyroidism glutathione reductase activity is affected promoting coefficient variations [35].

Functions of riboflavin

The major function of riboflavin is to be useful as the precursor of flavin-adenine dinucleotide and flavin mononucleotide, and of covalently bound flavins.

Riboflavin is primarily involved in energy-yielding metabolism. Its function is that of an electron carrier in the oxidation and reduction reactions of the flavin coenzymes. The redox functions of flavin coenzymes include both one-electron transfers and two-electron transfers from substrate to the flavin coenzyme [(36)]. These enzymes play a fundamental role in the mitochondrial electron transport chain.

Some reactions involve transfer of a single hydrogen to a flavin, forming flavin-H˚; which is then recycled in a separate reaction. Sometimes two molecules of flavin each accept one hydrogen atom from the substrate to be oxidized. Other reactions involve the sequential transfer of two hydrogens onto the flavin, forming first the flavin-H˚ radical, then fully reduced flavin-H2. The reoxidation of reduced flavins in enzymes that react with oxygen is a major source of potentially damaging oxygen radicals [(37)].

Cofactor in Macronutrient Metabolism

Flavin adenine dinucleotide (FAD) is one of the molecules considered ubiquitous throughout metabolism as a carrier of hydrogen atoms.

Riboflavin as flavin-adenine dinucleotide, is required in an important central metabolic pathway, the citric acid cycle, which provides the link between amino acid, carbohydrate and fat metabolism.

For each mole of acetyl CoA oxidized in this pathway, there is a yield of one FADH (flavoprotein reduced), equivalent to two ADP.

Riboflavin and disturbances to fat metabolism

Four studies point to the positive effect of riboflavin on disturbances in fat metabolism. In the first, the effect of riboflavin deficiency was tested on cerebrum and cerebellum of developing rat brain. The myelin lipids, cerebrosides, and sphingomyelin, as well as phosphatidylethanolamine, were considerably reduced in proportion. It is considered that riboflavin plays some role in the metabolism of essential fatty acids in brain lipids and the pathological effect of its deficiency is similar to that of fatty acid deficiency [(38)]. The second study showed that treatment with riboflavin abolished headaches and abnormal behaviour and normalised the plasma free carnitine, when measurement of [9,10(n)-3H] palmitate oxidation by cultured fibroblasts suggested a multiple acyl-CoA dehydrogenation disorder, in a 29 years old woman with severe hyperemesis gravidarum and atypical migraine [(39)]. A further study, after supplementation of riboflavin, clinical improvement occurred in acyl-CoA dehydrogenase deficiency; in which infants present recurrent hypoglycemia and lipid storage myopathy and increased urinary excretion of organic acids. The final study in this group, two patients with multiple acyl coenzyme A dehydrogenase deficiency, after riboflavin supplementation, flavin adenine dinucleotide and flavin mononucleotide concentrations in muscle and isolated mitochondria, and the activity of mitochondrial flavin adenine dinucleotide pyrophosphatase were total or partly corrected to normal levels and activity [(40)]. This highlights the importance of riboflavin in fat metabolism [(41)]. These four papers clearly illustrate the importance of riboflavin in fat metabolism disturbances.

ROLE IN IRON HANDLING

Riboflavin deficiency studies

It has been shown that riboflavin affects the integrity of red blood cells, haemoglobin and reduced glutathione [42]. Because of its oxidation-reduction potential, mainly due to nucleotides flavin-adenine dinucleotide and flavin mononucleotide, riboflavin plays an important role in the metabolism of iron, riboflavin-responsive anaemia in man having been shown, in early studies to have such characteristics features as an erythroid hypoplasia and reticulocytopenia [43]. Studies on animals with severe riboflavin deficiency suggest that body handling of iron appears altered during deficiencies states [44]. Thus, it seemed highly probable that the mobilization of the second major body source for iron reduction, ferritin, would be affected. In fact observation of differences in ferritin iron accumulation between control and riboflavin-deficient rats, with controlled inanition, overcamed some potencial objections to the earlier investigations on reduced absorption or transport of iron [45-47].

Supplementation studies

The inclusion of riboflavin in a supplementation study undertaken in rural Gambia of men and children, with microcytic anaemia, improved recovery, particularly in those individuals with unusually low levels of haemoglobin at the beginning [48]. Following study on rats showed that depleted ferritin stores and increased demand for iron turnover in rapid growth and pregnancy may impair iron mobilisation [49]. In a subsequent study, lactating women receiving iron and riboflavin supplementation a significant increase, relative to placebo, was shown in circulating plasma iron and in iron stores [50]. A further study on children with

subclinicae vitamin deficiencies supplemented with multivitamin and iron, also showed marked improvements in riboflavin status, and prevented deterioration on running performance [51].

Riboflavin and iron absorption and excretion

Beyond these activities and based on the marked effects on the normal structural development of gastrointestinal tract it was suggested that riboflavin plays a part in the absorption of iron and in excretion [52]. In the growing rat, liver ferritin-Fe concentrations were significantly lower in riboflavin-deficient rats after three weeks on the respective diets and remained lower until the conclusion of the experiment [53].

Riboflavin deficiency is associated with reduced in vitro iron-mobilization activity at the gastro-intestinal mucosa. Impact on iron mobilization from body stores and iron absorption may depend upon a number of factors, such as existing hepatic iron stores and the demand for rapid iron turnover [54]. Early studies in the rural community in the Gambia reported anaemia in certain sections of the population, and riboflavin deficiency appears to impose some limitations on the absorption and utilization of iron [55]. In a further study iron Fe absorption measured in the rat by monitoring whole-body retention of a dose of 59Fe using a small-animal gamma-counter, showed that riboflavin deficiency could be associated with a reduction in the percentage of the dose absorbed and an increase in the rate of loss of iron post absorption [56]. In a study of riboflavin deficiency and iron absorption in adult Gambian men the results indicate that the efficacy of iron utilization is impaired but that iron absorption is unaffected [57].

THE EFFECT OF RIBOFLAVIN ON THE STRUCTURE AND FUNCTION OF THE SMALL INTESTINE

Riboflavin deficiency is thought to be one of the factors, which elicit iron loss from the body because in gastrointestinal mucosa, one of the major absorption sites, the flavin-dependent iron mobilization from ferritin, an increased rate of turnover of epithelial cells will increase this loss. Another effect of riboflavin deficiency on gastrointestinal Fe distribution and loss was studied in weaning rats; the reported enhanced iron loss was due, predominantly, to an accelerated rate of small-intestinal epithelial turnover [(58)]. Depletion of riboflavin was associated with increased villus length and a proportional increase in the number of cell positions along villi in rats, and did not influence the number of mucus-producing goblet cells or the amount of mucosal glycoprotein in the small intestine [(59)].

The effects on the development of the gastrointestinal tract

The cytokinetics and structure of the small intestine may be altered in weaning rats in riboflavin deficiency induced at weaning and this impairs the normal increase in villus number. Prolonged deficiency leads to an adaptative increase in length of villi and depth of crypts [(60)]. Reversibility was studied, also in weaning rats, and the results show that the small intestine cannot readily recover from a period of riboflavin deficiency induced at weaning. These results highlight the critical nature of the weaning period for gastrointestinal development and the importance of adequate nutrition during infancy [(61)]. The earliest point at which riboflavin deficiency affects postweaning bowel development in rats, was studied and the results observed that developmental

changes to the duodenal crypt arise shortly after circulating riboflavin measurements show evidence of deficiency [62]. Participating in oxygen transport and oxidative phorylation, iron limits the synthesis of haem and controls the activity of the electron transport chain. Anaemia reduces work performance in humans and produces an effect on the efficiency of the electron transport chain in generating energy through oxidative phosphorylation.

Antioxidant activity

Often neglected as an important dietary antioxidant, riboflavin in its role as a precursor to flavin mononucleotide and flavin-adenine dinucleotide is extremely powerful. The Glutathione redox cycle provides a protective action against the scavenging of lipid peroxides [63]. Glutathione reductase requires flavin-adenine dinucleotide. A diminished conversion of oxidized glutathione occurs as a result of decreased activity of glutathione reductase [64], which leads to diminished concentrations of the substract for glutathione peroxidase and S-transferase, limiting the rate of degradation of lipid peroxides and xenobiotic materials [65]. NADPH provides reducing equivalents, another substract required by glutathione reductase, primarily generated by glucose-6-phosphate dehydrogenase, the activity of which is significantly diminished during riboflavin deficiency. Riboflavin deficiency as widely reported, is associated with compromised oxidant defense. The use of riboflavin, on the other end, as a reducing agent for 48 hours, decreased the parasite methemoglobin level, food vacuole size and inhibited asexual parasite growth in cultures of Plasmodium falciparum [66]. Combination of riboflavin with mefloquine, pyrimethamine, and quinine showed a marked potentiation of the actives of these drugs against

asexualstage parasites in vitro [67]. Reduced glutathione (GSH) is one of the most important endogenous antioxidants in the cell. Its synthesis is dependent on the activity of γ-glutamylcysteine synthase, and the availability of the substract, cysteine, which is either derived from the diet or protein catabolism, or synthesized from methionine in the liver by the transsulfuration pathway. An increasing supply of cysteine for reduced glutathione synthesis is produced when homocysteine transsulfuration is favoured over remethylation.

The role of riboflavin in homocysteine metabolism

The possible role of homocysteine, a thiol-containing amino acid which is naturally found in the body as an intermediary product in methionine metabolism in the pathogenesis of vascular disease, is at present an important subject of study [68].

Transsulfuration pathway for homocysteine metabolism

In this pathway, methionine is sequentially converted into cysteine via several enzymatic steps, the first step it is catalysed by methionine adenosyltransferase, and ATP-dependent activation, to S-adenosylmethionine. Subsequent demethylation and removal of the adenosyl moiety yields homocysteine. To form cystathionine, homocysteine condenses with serine in a reaction catalysed by cystathionine synthase and dependent on pyridoxal 5-phosphate (the active form of vitamin B6). Free cysteine is released after cleavage of cystathionine, catalysed by cystathionine γ−lyase. Methionine and homocysteine are readily interconvertible, but the subsequent step, the formation of cystathionine, is irreversible. The regulating activity of

remethylation and the transsulfuration pathway appears to depend on the availability of methionine. It is on homocysteine levels that the main regulatory control appears to be exerted. Homocysteine is remethylated by methionine synthase or betaine-homocysteine methyltransferase to yield methionine, when needed. Its metabolism is accelerated via the cystathionine β-synthase reaction when methionine is in excess [(69)]. Serum homocysteine concentrations are sensitive to blood levels of folate. Flavin-adenine dinucleotide acts as the co-factor for 5,10 Methylenetetrahydrofolate reductase, an important enzyme, which participates in the remethylation pathway for homocysteine metabolism. Recognized for its importance as coenzyme of cystathionine β-synthase and cystathioninase, vitamin B6 participates in the inactivation of homocysteine.

Remethylation pathway of homocysteine metabolism

In a vitamin B12-dependent reaction, a methyl group is donated to homocysteine by 5-Methyltetrahydrofolate; or by betaine, to regenerate methionine. Homocysteine catalise the reaction, with methylcobalamin as coenzyme. Folate and vitamin B12, are both, independent in the methyl donation of betaine. Adequate riboflavin nutrition is required for efficient utilization of dietary folic acid. The intermediate metabolite homocysteine is located at a critical metabolic crossroad and therefore both pathways, directly and indirectly impacts all methyl and sulphur group metabolism occurring in the body. Riboflavin is considered, among others, as a determinant of plasma total homocysteine concentration in the Framingham Offspring cohort [(70)].

Evidence for a role for riboflavin as protective against diseases

Literature regarding evidence for a role for riboflavin, as protective against diseases is not so crowded, and landmark studies may be not difficult to recognize. Trials are generally concise and include important information that is provided in adequate detail for careful evaluation. But, some data are of interest to some parts of the scientific community and not to others. Recent analysis deserves public dissemination and it is believed that an enormous amount of useful knowledge can be derived from carefully conducted studies. Full dissemination of obtained results is likely to be helpful to the scientific community.

RIBOFLAVIN AND CATARACTS

Cataract pathophysiology

Detoxification of xenobiotics is one of the major functions of reduced glutathione. High concentrations of reduced glutathione protect against oxidation and crosslinking the protein-bound Sulfhydryl-groups of crystallins, the transparent tissues of the eye, which have a relatively xenobiotic metabolism. Ageing generally reduces the lens metabolic efficiency, thus increasing its susceptibility to noxious factors; and could also promote the interactions for cataract noxae to induce the formation of a variety of cataract, associated with high protein-related light scattering and discoloration. Vitamins with antioxidant properties have been implicated in the development and progression of cataract. In numerous studies riboflavin was found to have benefits arising from utilization.

Epidemiology of cataract

Riboflavin deficiency was first postulated to be involved in the formation of cataract by Day, Langston & O'Brien [71] in 1931. Since then, there have been several human studies into the effects of riboflavin deficiency, and it is now widely agreed that riboflavin deficiency appears to be a significant predisposing factor for cataract [72]. Studies in animals and also epidemiological and interventional studies in humans have revealed the important role of riboflavin [73]. In the Lens Opacities Case-Control Study, which assessed the risk factors for various types of cataract among participants aged 40 to 79 years, the risk factors for age-related cataracts was evaluated. The results support a role for nutrition in cataractogenesis [74]. Lens opacities were associated with lower levels of riboflavin, and are compatible with the dietary intake and medical history results of Lens Opacities Case-Control Study [75]. It was found an increased risk with low levels of several nutrients including riboflavin. This kind of study could be considered an efficient design for the evaluation of potential factors for cataract. But unlike the cohort study, with few exceptions a case-control study cannot demonstrate the risk of developing the disease in individuals with a suspected risk factor. The Blue Mountains Eye Study [76], a cross-sectional study, where 79% of the participants aged 49 to 97 years attending, reports on the relationship between the three principal types of cataract, nuclear, cortical and posterior subcapsular, and a wide range of dietary macronutrients and micronutrients, including riboflavin, a very important element supporting the above mentioned hypothesis. Results showed a statistically significant dose trend for riboflavin supplements on cortical cataract; riboflavin (odds ratio 0.8, confidence interval 0.6 to 1.0, P=.05) and niacin (odds

ratio 0.7, confidence interval 0.1 to 1.0, P=.04) both shown to exert a weaker protective influence. The cross-sectional nature of this study, the selection bias, confounding and multiple comparisons could be considered as limitations for out coming results. The low statistical power may also have contributed to random error, because many subgroups had relatively small number of supplement users. The full assessment of the temporal relationship between vitamin supplementation use and cataract was not permitted due to the nature of the study, which is important in such analysis. The dietary vitamin intake was not corrected, which could contribute also for confounding the findings.

Riboflavin appears to be protective in isolation or as constituent of multivitamins preparations. The Lixian Cataract Study [77], a large intervention trial, was probably the first randomised population trial in the world; suggesting a general benefit and support the general notion that riboflavin supplementation were beneficial. Examinations at the end of the intervention showed a reduction of 36% in the prevalence of nuclear cataract for individuals aged 65-74 years, who received riboflavin plus niacin compared to those who did not. Although exists some inconsistencies with the results from other clinical trials like The Physician's Health Study, The Lixian Study provide a hopeful sign that riboflavin supplementation may lower the risk of nuclear cataract, and suggest lines of further research to confirm the protective effect of riboflavin [78].

Riboflavin and cardiovascular disease

Cardiovascular diseases (CVD) remain a major cause of death and morbidity in developed countries. Since the aetiology of cardiovascular disease is influenced by environmental and genetic factors [79], studies at cellular, molecular and

genetic levels are important to understand cardiac dysfunction. Advances in understanding vascular smooth-muscle and endothelial cells are also important; endothelial cells are quite active metabolically and normally produce substances that affect the vascular lumen. Flavin-adenine dinucleotide could prevent the decrease in ventricular multiple response threshold and the disturbance of mitochondrial function[80]. Riboflavin supplementation has cardioprotective effects in cardiac reoxygenation damage and these effects are mediated by flavin reductase [81]. Vitamin B2 could be considered an active supplement to the antioxidant and vitamin status of patients with hypertension and ischemic heart disease [82]. Riboflavin is one of B-vitamins on which the remethylation pathway of homocysteine is dependent; as is the trans-sulfuration pathway. Vitamin B2 is required for intracellular homocysteine metabolism [83]. To date, even though riboflavin plays a crucial role in both the trans-sulfuration and remethylation pathways of homocystein metabolism, the relationship between riboflavin status and homocysteine levels has been investigated in few studies.

Mechanisms of damage

Some reactive oxygen species (ROS), such as hydrogen peroxide, H_2O_2, produced mainly in mitochondria, after oxygen reduction to water in the electron transport chain [84], react with divalent cations in the Fenton reaction, thereby forming the very reactive hydroxyl radical (OH˚). Reactive oxygen species produced through the metabolism of homocysteine, could promote endothelial damage [85], but the actual mode is still unclear. Homocysteine facilitates the generation of hydrogen peroxide [86]. By creating oxidative damage to LDL

cholesterol and endothelial cell membranes, hydrogen peroxide can catalyse injury to vascular endothelium [87]. The platelet aggregation induced by hydrogen peroxide could be inhibited by flavin adenine dinucleotide via the glutathione reductase and peroxidase system [88]. The isolated rat mesenteric arterial bed was used to examine the activity of flavin-adenine dinucleotide, in the study of pivotal role of phosphate chain length in vasoconstrictor actions of adenine dinucleotides in rat mesenteric arteries, and showed those three or less phosphates are vasodilators [89]. By reacting with homocysteine, nitric oxide released by endothelial cells protects them from damage, forming S-nitrohomocysteine, which inhibits hydrogen peroxide formation. This protective mechanism can became overloaded, as homocysteine levels increase, allowing damage to endothelial cell to occur [90]. Atherosclerotic plaque formation could result from the combination of oxidative damage and endothelial collagen promoted by high levels of homocysteine.

Hyperhomocysteinaemia

High levels of homocysteine could be considered conditional risk factors for cardiovascular diseases [91]. Homocysteine-related abnormalities are also thought to contribute to birth defects and dementia, and there are many common acquired diseases, drugs and genetic disorders which adversely affect the metabolism of homocysteine. The major circulating forms of homocysteine are protein bound, with twenty per cent unbound. Elevated levels on plasma of total homocysteine in concentrations greater than 16 (mol/l, result from certain drugs utilisation such as folate, B6 and B12 antagonists. Another factor to be considered could be age increase. A thermolabile polymorphism of MTHFR is a

more recent approach to high plasma homocysteine levels investigation, with a range in homozygous state varying between 5 to 15 per cent, depending on populations, and where heterozygotes are meaningful [92]. Studies among coronary artery disease and premature vascular disease suggest that this genetic defect could contribute to development of cardiovascular diseases. Maintained by enzymes, the methionine metabolism could promote high levels of Hcy if folate, vitamine B6, vitamine B12 are not deficient. Adequate riboflavin nutrition is required for efficient utilization of dietary folate [93]. While the role of reactive oxygen species in the vasculature is far from clear, evidence suggests that homocysteine is a contributing source of reactive oxygen species in endothelial cells, vascular smooth muscle cells and intact aortas [94]. The importance of riboflavin in homocysteine metabolism is recognized. The first study, in humans, showing the above mentioned relationship was Determinants of Plasma Concentration in The Framingham Offspring cohort. In this cohort was observed an association between plasma tHcy and nutritional factors where riboflavin was included. The geometric mean tHcy was 23% higher in persons aged ≥65 years and 11% higher in men. Also the use of vitamin B supplements was associated with significantly lower tHcy concentrations [95]. In order to examine the association of a single tHcy measurement on subsequent hospitalisations due to cardiovascular diseases, a population-based prospective cohort study was conducted in Norway; also known as The Hordaland Homocysteine Study. Risk of cardiovascular diseases hospitalisations increased significantly with increasing baseline tHcy only in the oldest aged group, 65 to 67 years at baseline. Plasma tHcy levels is a strong predictor of cardiovascular diseases hospitalisations only in elderly individuals, and

especially among those pre-existing cardiovascular diseases [96]. Association between B vitamin and plasma homocysteine concentration in the general Dutch population aged 20-65 years [97] was the first study in which the association between dietary intake of riboflavin was investigated for a large population-based sample. The population of this study consisted of a random sample of subjects aged 20- 65 years from 3 cities in the Netherlands. Riboflavin intakes were calculated by using an extended version of the 1996 computerized Dutch food-composition table. Folate data were derived from a validated high-pressure liquid chromatography method with which Dutch foods were analysed. Nonfasting venous blood samples were used.

Folate was the most important dietary determinant of the plasma tHcy, and high dietary folate can make a substantial contribution to a reduction in plasma tHcy in the general population, which is important because each 1µmol/L decrease in tHcy may be associated with a 10% reduction risk of cardiovascular disease. No association between plasma tHcy and riboflavin was observed. As a cross-sectional study was limited the identification of causal relations. In previous studies, as above mentioned, subjects were older. More recently, the effect of riboflavin supplementation on plasma homocysteine in elderly people with low riboflavin status was studied, in a double blind, randomised, placebo-controlled riboflavin supplementation trial.

McKinley et al [98] found that despite the metabolic dependency of tHcy on riboflavin, it did not prove to be an effective homocysteine-lowering agent, even in the face of sub-optimal riboflavin status. A supplementation level of 1.6 mg/day riboflavin for 12 weeks was chosen based on a previous riboflavin intervention [99]. Even when the response to intervention was examined

separately in those subjects deemed to have inadequate biochemical status of either nutrient at baseline, supplementation with riboflavin resulted in a significant response in the status of whichever nutrient was low, riboflavin or vitamin B6. But the small number of subjects classified as having low status for both vitamins (n=7), riboflavin supplementation appeared to improve the status of each vitamin, but no firm conclusions can be drawn from such a small sample.

Low dose of riboflavin was also used in order to make the results more relevant for prevention of hyperhomocysteinemia through modification of dietary intakes, via fortification of foods. Fortification of foods can be a useful tool in combating micronutrient deficiencies, and successful application of food fortification technology is based largely on compatibility of vehicle, fortification and process. Riboflavin is unstable in alkaline medium; it is very sensitive to light, particularly in presence of ascorbic acid. The requirement regarding riboflavin should be considered looking for a bigger amount used for fortification and probably a homocysteine lowering effect will be effective. Evidence that homocysteine is an independent risk factor for vascular disease is growing, but is important to understand the determinants of plasma homocysteine. Low folate status could be considered the most important determinant of mild-to-moderate hyperhomocysteinemia.

It is well known that the folic acid fortification of enriched grain products in the United States dramatically altered the prevalence of elevated plasma homocysteine concentrations associated with low folate. Other determinants, like riboflavin, could assume greater importance and efforts to identify additional risk factors for mild-to moderate hyperhomocysteinemia needs to continue.

Riboflavin and esophageal cancer

Recent studies confirm that nitrosamines, well known carcinogens, may increase carcinogenesis due to dietary riboflavin deficiency [(100)]. Damage of DNA from its coenzyme action with enzymes of cytochrome P450 could be avoided by riboflavin protection [(101)]. In the aetiology of oesophageal cancer, a nutritional imbalance could be considered an important factor. To test this hypothesis, two large intervention trials using vitamins and minerals supplementation, were conducted in a population with the highest rate of esophageal cancer in the world, in Linxian, China (1985-1991). From the General Population Trial, results showed that riboflavin plus niacin decreased the incidence of oesophageal cancer by 14 per cent approaching significance. In the Dysplasia Trial results showed that in the supplementation group compared to the placebo group the risk of oesophageal cancer mortality is 16 per cent lower and oesophageal/gastric cardia cancer mortality 8 per cent lower. The suggestion of benefit from vitamin and mineral use should be observed in a longer follow up; although the results were not statistically significant. The measurement of cell proliferation with the utilization of tritiated thymidine labelling evaluated in 512 endoscoped patients showed that the participants who were supplemented were significantly more likely to revert to non-dysplastic cytology at both 30 and 72 months. The results can be considered very important because this was the first randomised trial in the world where a positive correlation between nutritional supplementation and reduction of incidence from human cancer was shown.

CONCLUSIONS

Riboflavin has an important role as cofactor in metabolism, participates in the antioxidant activity and in iron metabolism. Has a recognized importance as a determinant of plasma total homocysteine, and evidences for a role for riboflavin as protective against diseases appear from studies in humans. A hopefull sign that riboflavin supplementation may lower the risk of nuclear cataract comes form the Lixian study. Through a randomised trial, a positive correlation between riboflavin and oesophageal cancer was suggested. Few studies estimating the riboflavin requirements on the elderly, have been conducted to date.

Not only do they form an increasingly large proportion of the population, but also constitute the population group that consumes the largest number of prescribed and over-the-counter medications. This could interfere with riboflavin utilization by impairment of the conversion from vitamin through its coenzymes. Malabsorption and poor diet, which the elderly are more prone to, may interfere also, and may be intensified if alcohol abuse exists.

Dietary habits need to be altered in order to improve vitamin supply, particularly in elderly. A strong case can be made for preventing the marginal or manifest riboflavin deficiency states that may contribute substantially to this potentially important risk factor for cardiovascular disease, the largest cause of mortality among elderly. Persons undergoing haemodialysis or peritoneal dialysis are likely to require extra riboflavin along with other B vitamins. The efficacy of riboflavin supplementation for the prevention and treatment of anaemia must be further evaluated since there is strong evidence that riboflavin deficiencies affect iron utilization from supplements and are important on a large scale. No

cases of toxicity from ingestion of riboflavin have been reported, since the capacity of the normal human gastrointestinal tract to absorb this soluble vitamin is rather limited. Priority should be given to studies for setting Estimated Average Requirements for riboflavin for the elderly, defining values for clinical adequacy and inadequacy.

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3. Riboflavin, serum folate and its dietary correlates estimated from a semiquantitative food-frequency questionnaire in elderly

Nelson R Tavares[1*], Pedro S Moreira[1,2], Teresa F Amaral[1,3]

[1] Faculdade de Ciências da Nutrição e Alimentação da Universidade do Porto, Portugal

[2] Centro de Investigação em Actividade Física, Saúde e Lazer da Universidade do Porto, Portugal

[3] UISPA Unidade de Integração de Sistemas e Processos Automatizados do Instituto de Engenharia Mecânica da Universidade do Porto, Portugal

Abstract

Background: Since data regarding riboflavin in red blood cells and serum folate status in the elderly are limited, a food frequency questionnaire is often used to estimate riboflavin and folate status. However, its performance amongst this age group has not been evaluated. Objective: To assess in elders riboflavin and folate concentrations and the validity of its estimates from a semiquantitative food-frequency questionnaire (SFFQ). Design: A cross-sectional study to investigate riboflavin in red blood cells and serum folate and its dietary correlation. Subjects and Methods: Eighty-eight elders (66.7% female), ages between 60 and 94 years were recruited from seven day social care centers in Porto, Portugal, and volunteered after an interview. All subjects completed a SFFQ. EGRAC (erythrocyte glutathione reductase activation coefficient) in red blood cells and folate concentration in serum were estimated from one fasting blood sample.

Results: Were found forty-six subjects with an EGRAC <1.2. Mean EGRAC concentration was 1.174, median 1.10 (range 1.00-2.10) and mean daily riboflavin dietary intake from SFFQ was 3.344 mg, median 3.373 mg and range 0.662-4.806 mg. Spearman correlation coefficient between these two measures was r=0.073, P=0.497 and Pearson correlation, after adjustment for energy, was r=0.263, P=0.013. All participants where above of 7nmol/L serum folate cut-off. Mean serum folate concentration was 28.3±17.4 nmol/L, median 24.9nmol/L (range 9.7-126.9 nmol/L) and mean daily folate dietary intake from SFFQ was 477.7±122.9 µg, median 483.3 µg and range 143.3-782.1 µg. Spearman correlation coefficient between these two measures was r = -0.10, P=0.359, and Pearson correlation, after adjustment for energy, following $\log_e$

transformation was r = –0.58, *P*=0.593. Riboflavin and folate intakes estimated by SFFQ correlated poorly with EGRAC and folate serum values. Conclusions: In this low-literacy group of elderly men and women, in a day social care centers living situation, the SFFQ provided poor estimates of riboflavin and folate intake. However, further studies are needed to confirm or refute our findings.

Key Words: Folate intake; folic acid; biomarkers; SFFQ; validity; riboflavin.

Introduction

Nutritional problems are the root of many major chronic diseases in both developed and developing countries[1]. In epidemiological studies riboflavin is recognized as a determinant of plasma homocysteine concentrations[2-6]. Subjects who frequently consumed typical sources of riboflavin like dairy products have low plasma homocysteine concentrations[7]. Folate deficiency has been identified as a risk factor for cardiovascular disease[2], certain cancers[3] and neuropsychiatric conditions[4]. Folate plays a key role in single carbon transfer reactions, leading to the synthesis of DNA, neurotransmitters, phospholipids, hormones and it is also involved in homocysteine metabolism[5].

Riboflavin plays a prominent role in folate metabolism. Flavin adenine dinucleotide, a metabolite of riboflavin, serves as a cofactor for methylenetetrahydrofolate reductase (MTHFR)[8-10]. MTHFR is an important enzyme in folate metabolism; it catalyzes the conversion of 5,10-

methylenetetrahydrofolate to 5-methyltetrahydrofolate[11]. Flavin adenine dinucleotide was found to modify MTHFR activity in healthy subjects[12].

The reliability of habitual riboflavin and folate intake estimates remains a critical issue. The problems related with the assessment of habitual food intake are well documented[6].

Due to their ease of administration and low burden on the subject, the dietary intake in epidemiological studies is often assessed by food frequency questionnaires. Because there are errors associated with this type of dietary assessment, such methods have been validated by and complemented with measurement of biomarkers[13-17].

National surveys of the dietary intake and nutritional status of various groups in the United Kingdom have reported some discrepancy between the estimated intake of riboflavin in the diet and measures of riboflavin status[18].

Folate is particularly prone to such error, because it is derived from a variety of foods of both animal and plant origin, not all of which can be included in a SFFQ. Age-related factors such as the impaired memory and the cognitive capacity to correctly categorize the frequency of food items could limit the ability of a SFFQ to categorize the folate intake. Although several studies compared folate intake measured by SFFQ and biochemical measures of nutrient status to assess its validity most of them were performed in adults (Green *et al.,*[14]; Pufulete *et al*.,[15]; Yen *et al*.[16]) and none has been conducted specifically in the elderly. The studies of Jacques *et al*.,[13] in the United States of America and van de Rest *et al*.,[17] in the Netherlands, were conducted in mixed samples of adults and elders, showing a good agreement between serum folate and FFQ estimates of folate intake (respectively, r = 0.63, and r = 0.14).

These may not hold in other cultures and deserves further investigation, particularly in aged populations with different socio-economic and educational background.

Estimates of dietary intake of riboflavin and folate are currently a topic of considerable interest with several published studies addressing this topic[19-21]. Information on the relative contribution of those foods that are the main sources of riboflavin and folate intake and supplements consumption among Portuguese elderly is scarce. Data on the red blood cells riboflavin and serum folate in free--living elderly population in Portugal is also nonexistent.

From both epidemiologic and public health perspective, the assessment of the riboflavin and folate status and the study of a SFFQ validity in the elderly are of great importance. The purpose of our study was to assess in elders the circulating riboflavin and folate and to evaluate the validity of a SFFQ currently used in Portugal to estimate riboflavin and folate intake in comparison with independent biomarkers.

Material and Methods

Study population

Elderly people from seven day social care centers in Porto, aged ≥ 60 years, were recruited in order to participate in this cross-sectional study.

Exclusion criteria were not providing a blood sample, the use of riboflavin and folic acid or multivitamin supplements, using drugs known to interfere with folate metabolism such as nonsteroidal anti-inflammatory drugs, anticonvulsants, methotrexate, or other drugs with antifolate activity; or drugs that affect

riboflavin metabolism (antacids containing magnesium or ioniazid). Further exclusion criteria were the presence of hematological disorders or gastrointestinal or cardiovascular, hepatic or renal disease or impaired cognitive function with a Mini-Mental State Examination (MMSE)[22] score <18.

All participants were interviewed by nutritionists and a specially trained interviewer to provide information about demographics (including years of education), general health conditions and dietary intake information from SFFQ.

Dietary intake

The SFFQ was used to measure individual energy and nutrient intake, including riboflavin and folate, during the past year. The choice of relevant food items to be included in the SFFQ was based on their contribution to the between-person variance of intake of total energy, fat, carbohydrates, cholesterol, dietary fiber, vitamin A, carotenoids, vitamins C and E, calcium, alcohol, and caffeine, for people aged 40 years or older[23].

It was developed and validated by Lopes *et al.*[24, 25] from the Epidemiology Department, Faculty of Medicine, University of Porto, Portugal, and it is based on the Willett[26] model, and allowing the subjects to indicate their daily, weekly or monthly intake of 89 foods. The food list was adapted to include foods commonly consumed in Portugal. For each food item, participants were asked to indicate their usual consumption from 9 frequency categories, ranging from never or <1 time per month to 6 per day. The SFFQ did not include questions on portion size but rather specified medium servings, defined by natural (eg. orange, slice of bread) or household units (eg. cup, spoon table). For seasonal

foods, participants were asked to estimate their average intake (frequency) when the food was in season.

Blank spaces were available for recording any foods that were consumed more than once a week but were not listed as a food item on the questionnaire. The gram weights of medium servings were obtained from other published values[24]. The estimates of foods consumed per day were computed by multiplying the frequency of consumption of food items by standard portion weights. The food items (g/d) were subsequently converted into nutrients by using Food Processor Plus program 5.0 (ESHA Research, USA), that estimates total riboflavin and folate intake based on values from the US Department of Agriculture. Folate values in foods have been analysed using microbiological assay after trienzyme extraction[27]. As the Portuguese Food Composition Table did not provide estimates for riboflavin and folate composition of foods[28], the US Department of Agriculture folate values were used. Nutritional data about Portuguese foods composition[29] were added to the program if they were available.

The data entry was performed by optical reading and checked by a trained supervisor. All of study's participants had filled more than 70 items on the SFFQ, and reported a total daily energy intake inside the range of 947-5442 kcal on the SFFQ.

The SFFQ was previously validated with four 7-day Food Records (FR), in 75 female and 71 male community participants with a mean age of 60.3 years (Standard Deviation (SD)=11.2)[23]. In another study, the validity of this SFFQ to estimate the intake of several nutrients, including riboflavin and folate, was also assessed in university students aged 18-29 years old by comparing SFFQ with

4-day FR. Mean folate intake from SFFQ were significantly higher than those from the FR. Pearson correlation coefficients between folate intake from SFFQ and FR ranged in males and females, respectively, from 0.50 to 0.57 (P<0.001), and 0.38 to 0.53 (P<0.001); Pearson correlation coefficients between riboflavin intake from SFFQ and FR ranged in males and females, respectively, from 0.66 to 0.60 (P<0.001), and 0.59 to 0.71 (P<0.001) after total energy intake adjustment[30].

Eighty-eight elders were involved in the study. In accordance with the current revision of the Helsinki Declaration of 2008[31], the participants were informed of the purpose and risks of the experimental protocol and informed consent was obtained from the individuals before the start of the study.

Blood sampling and analysis

After subjects in the seven social day care centers had fasted overnight, 4 mL samples of blood were collected by venipuncture. A research nurse visited the participants at the day social care centers in the week after the interview for SFFQ. Blood was collected in previously frozen containers with no anticoagulant for serum folate measurement, and containing EDTA for the measurement of EGRAC (red blood cells). Samples were kept on ice at all times. Were processed and analysed the same day for serum folate and processed and stored at –20 ºC for batch analysis for EGRAC.

EDTA-treated hemolysates were prepared from blood samples and the FAD--dependent enzyme, EGRAC was measured using *RX DAYTONA* (Randox, UK)[32] to determine riboflavin status. EGRAC was calculated as the ratio of FAD-stimulated to unstimulated enzyme activity[33]. Concentrations of serum

folate were measured by a chemiluminescence immunoassay on ADVIA Centaur (Bayer Corporation). Intraassay coefficient of variation observed was lower than 6%, and laboratory follow Randox International Quality Assessment for quality assurance.

Data analysis

Data analysis was performed using Statistics Package for the Social Sciences 13.0, Chicago, USA. Estimated mean EGRAC and serum folate concentrations by quartile of dietary riboflavin and folate intakes and an investigation of whether mean riboflavin and folate concentrations across quartiles show a linear trend were calculated. The ability of the FFQ to correctly classify participants into quartiles of EGRAC and serum folate concentrations was evaluated. ANOVA was performed to determine whether gender was determinant of riboflavin and folate intake. Because there was no gender difference, data for men and women were combined for all data analysis. Group median and interquartile range were calculated. Spearman's correlation was calculated from SFFQ riboflavin folate and serum folate biomarkers concentration.

Energy-adjust folate and riboflavin intake was calculated by residuals method[34], where residuals are computed from regression of energy intake as the independent variable and the riboflavin and folate intakes as the dependent variable.

Riboflavin showed normal distribution values and the association between EGRAC and reported SFFQ riboflavin was expressed as Pearson's correlation coefficient.

Folate distribution was skewed toward higher mean values and were $\log_e$ (natural) transformed to improve their distribution toward normality. After this transformation, the association between serum folate biomarker and reported SFFQ folate was expressed as Pearson's correlation coefficients.

Agreement between methods was expressed as proportion agreement according to quartiles of red blood cells EGRAC, as Weighted kappa according to quartiles of serum folate, and intakes distributions in the total sample.

Results

Volunteers were 88 elderly (66.7% women) with mean age 77.2 years (SD 7.5). All were Caucasian, retired, 26.1% with zero education years (Table 1) and 89.5% with less than 5 years education. Mean BMI was 27.2 kg/m^2 (SD 5.1) in men and 29.8 kg/m^2 (SD 4.5) in women, with 35 women (60% total participants) between 25-34.9 kg/m^2 .

Median (interquartile range) dietary riboflavin intake from SFFQ were 3.373 mg (0.947). and median (interquartile range) energy were 3653 kcal (926.5). Biomarker median concentration was 1.1 (0.3) and forty-two elderly subjects with an EGRAC ≥1.2.

The mean EGRAC concentrations by quartile of dietary riboflavin intakes were calculated: first quartile, 1.168 (SD 0.064); second quartile, 1.109 (SD 0.177); third quartile, 1.123 (SD 0.218); and fourth quartile, 1.295 (SD 0.321).

No linear trend has been found for EGRAC values across quartiles of dietary riboflavin, showing that SFFQ was not able to correctly classify participants into quartiles of EGRAC concentrations. The ability of the SFFQ to correctly classify participants into quartiles riboflavin intake was further evaluated by agreement

proportion and a 21.59% (95% CI 13.81%-31.9%) was observed. Because observed concordance is smaller than mean-chance concordance, Weighted Kappa was not calculated.

Spearman's correlation coefficient between EGRAC concentration and estimated intake of riboflavin was r = 0.073, p = 0.497 and after adjustment for energy were r = 0.263, p = 0.013.

Median (interquartile range) dietary folate intake from SFFQ were 483.3 µg (151.4). Biomarker median concentration was 24.95 nmol/L (21.0).

As <7nmol/L indicates a negative folate balance[23-25], all participants were above this cut-off.

The mean folate concentrations by quartile of dietary folate intakes were calculated: first quartile, 25.5 nmol/L (SD 16.4); second quartile, 22.8 nmol/L (SD 4.7); third quartile, 22.5 nmol (SD 6.8); and fourth quartile, 11.2 nmol/L (SD 6.6).

No linear trend has been found for serum values across quartiles of dietary folate, showing that SFFQ was not able to correctly classify participants into quartiles of serum folate concentrations.

The ability of the SFFQ to correctly classify participants into quartiles folate intake was further evaluated by agreement proportion, a 25% (95% CI, 13.81%-31.9%) value was observed and Weighted Kappa = -0.05 (0, 0.09).

Spearman's correlation coefficient between serum concentration of folic acid and estimated intake of folate was r = -0.10, p = 0.359. After adjustment for energy and $\log_e$ (natural) folate intake and serum values from Pearson correlation were r = –0.58, p = 0.593. The SFFQ provided poor estimates of riboflavin and folate intake.

Table 1. Individual characteristics of study participants

Variable	Men (n=30) n (%)	Women (n=58) n (%)
Age (years)		
60-70	4 (13.3)	14 (24.1)
≥70	26 (86.7)	44 (75.9)
Body Mass Index (kg/m ^{2})		
≤24.9	14 (46.7)	6 (10.3)
25-29.9	11 (36.7)	28 (48.3)
30-34.9	3 (10.0)	17 (29.3)
35-39.9	2 (6.6)	6 (10.3)
≥40	0 (0.0)	1 (1.8)
Education (number of completed school years)		
0	3 (10.0)	20 (34.5)
1-4	21 (70.0)	35 (60.3)
5-9	6 (20.0)	3 (5.2)
Living arrangements		
Alone	8 (26.7)	21 (36.2)
With others	22 (73.3)	37 (63.8)
Mini-Mental State Exam (scores)		
18-23	9 (30.0)	20 (34.5)
24-30	21 (70.0)	38 (65.5)

Discussion

The validity of SFFQ estimates of riboflavin and folate intake versus EGRAC and serum folate biomarkers among a low-literacy elderly population showed no agreement between EGRAC and serum folate and its dietary intakes obtained from SFFQ.

According to a previous nutritional study of riboflavin status[35] 47.8% of elders in our sample showed an EGRAC ≥ 1.2. As found in another study[36], all the elders in our sample reached the cut-off for serum folate concentration.

As there are no Portuguese Dietary Reference Intakes, riboflavin and folate intakes were compared to Dietary Reference Intakes (DRI) = Estimated Average Requirements (EAR) recommendations[29]. Only 1 man (1.1%) was below the EAR (DRI) cut-offs for riboflavin (1.3g/d). Only six women (20.7%) and 3 men (5.1%) were below the EAR (DRI) cut-offs for folates (320 µg/d).

The correlation between EGRAC biomarker and reported SFFQ riboflavin (r = 0.26) although statistically significant, may be generally described as weak. Relationships between vitamin B_2 concentrations in erythrocytes and other indices have been investigated in cross-sectional studies[37-41], and in general, associations between vitamin B_2 and riboflavin intake[39-41] and between vitamin B_2 and EGRAC[37, 38] have been weak or absent.

The correlation between serum folate biomarker and reported SFFQ folate (r = -0.10) is non statistically significant and beyond the range reported in other studies[18, 19] which used more expensive and different dietary assessment methods. Garry *et al.,* [19], using weighed FR in elders found a correlation of *r* = 0.50; Hickling *et al.,*[18], in mixed sample of adults and elders using the folate intake tool, had a correlation coefficient of *r* = 0.54. Van de Rest *et al.*,[16]

compared dietary folate intakes with blood folate concentrations to assess the relative validity of a FFQ specifically designed to measure folate intake over the previous 3 months in elderly people (mean age 60 years Standard Deviation (SD) 6). The correlation between folate intake and serum folate concentration in this Dutch study was very low but reached statistical significance (Spearman correlation coefficient of 0.14, (P<0.01). Although statistically significant, a correlation coefficient of r = 0.14 may be generally described as weak.

The mean intake of riboflavin and folate was surprisingly high, namely after considering previously reported intakes in Portuguese elderly. Using the same SFFQ it was previously referred in a probabilistic sample of male adults, aged >70 years, at the same geographical region were this study was carried out (Porto, Portugal), a mean folate intake of 319.4 µg/d (SD 166.3).

However, we should consider the possibility that self-reported dietary intake have been biased toward higher values by several factors including demographic characteristics (older adults, social desirability)[42] which we did not measure. High social desirability has been previously associated with higher intake of some fruits and vegetables in Portuguese subjects from both genders (mean age of 20.7 years (SD 1.8) in females, and 21.2 years (SD 3.9) in males), and higher estimates of folate intake in male subjects[34].

The lack of validity to estimate folate intake using the SFFQ administered in the present study contrasts with previous attempts in a mixed sample of adults and elders[8], using FR, and is also different from other validation studies with similar methodology conducted in adults, being the correlations for estimated folate intake r = 0.33 for Longnecker (n=138)[27] and r = 0.58 for Bonifacj (n=150)[28] study.

The finding in this study that the SFFQ provided poor estimates of folate intake in this elderly group, could be considered not entirely unexpected. Serum folate is indicative of the folate status at the time the blood sample is collected, being influenced by recent changes in folate intake. Although we used serum folate concentrations to validate the dietary estimates of folate intake, as done previously[13, 14]. The methods need to be sensitive enough to detect early and correctable warnings of health risk. The first stage of early negative folate balance is assessed by measuring serum levels, which drop prior to tissue depletion, and which are paralleled by a reduction in red blood folate. The margin of safety between folate intake and requirement is small, so the serum folate concentration becomes subnormal after only 3 weeks of negative balance[26]. In a previous study performed in adults using serum folate concentrations as the sole biochemical criterion, the correlation coefficient between estimated folate intakes using the SFFQ and serum folate was r = 0.48 [13], indicating good agreement between the SFFQ and biomarker.

An advantage of using biomarkers is that they provide an objective measure of nutrient intake whose measurement errors are essentially independent of the errors associated with dietary intake measures based on self-report[43] and errors related to food tables biochemical measurements[44]. However, biomarkers may also be influenced by a number of physiologic, environmental, genetic and lifestyle factors[44].

A peculiar limitation for application of dietary methods in elderly, is literacy[30]. Participants in the present study presented a low-literacy level, with 89.8% in the range 0 to 5 years of completed school years. As the SFFQ requests a generic representation of intake in order to provide an accurate picture of the

recent usual intake, recalling foods frequently consumed may pose a challenge to elderly.

The validity of a SFFQ is also highly dependent on the correct selection of foods on the questionnaire[38, 39], in addition to the correct estimation of portion sizes and food consumption frequency. These limitations are well recognized and several attempts have been made previously to overcome these potential inaccuracies including the use of colour photographs of food portion size[39] and in the inclusion of summary questions (such as ''fruits'' or ''vegetables'' intake) to reduce chances of over-reporting in relation to specific questions[40].

The present study has several strengths. The cognitive status was evaluated by MMSE and was considered an exclusion criteria when MMSE<18, avoiding participants with impaired cognitive function, which could a priori limited the application for this SFFQ. We included only subjects who had fasted at least 10 hours because riboflavin and folate concentration measured in a fasted state is a better indicator of riboflavin and folate status.

According to our knowledge, this is the first study evaluating the performance of the riboflavin and folate intake from a SFFQ with biomarkers in a sample restricted to elders (60 years or older, which is the United Nations agreed cut-off to refer to older population). However, it remains to be known if the low validity that we have found is linked to the low level of literacy in the elderly or if this method is inappropriate for estimating riboflavin and folate intake in this age group.

In summary, results from this study showed 47% participants with an EGRAC ≥ 1.2 and were above the threshold for serum folate levels and suggest that intake estimates from this SFFQ for riboflavin and folate may be less reliable

and should be interpreted with particular caution, when assessing the riboflavin and folate intakes of elderly with a low education level. However, further studies are needed to confirm or refute our findings.

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4. Riboflavin supplementation and biomarkers of cardiovascular disease in the elderly

Nelson R Tavares[1], Pedro A Moreira[1,2], Teresa F Amaral[1,3]

[1] Faculdade de Ciências da Nutrição e Alimentação da Universidade do Porto, Portugal

[2] Centro de Investigação em Actividade Física, Saúde e Lazer da Universidade do Porto, Portugal

[3] UISPA, Unidade de Integração de Sistemas e Processos Automatizados do Instituto de Engenharia Mecânica da Universidade do Porto, Portugal

Abstract

Background: High levels of total plasma homocysteine are potentially harmful in the elderly. Objective: To investigated the effects of oral riboflavin supplementation on plasma total homocysteine, ferritin, uric acid and C-reactive protein concentration in elderly people with a low riboflavin status. We performed a four-week randomized, placebo-controlled, double-blind trial of riboflavin supplementation in seven Portuguese day social centers. Design: Eighty-eight individuals (66.7% female), aged between 60 and 94 years, volunteered to participate in the study following interview. Forty-two subjects, with an erythrocyte glutathione reductase activation coefficient (EGRAC) ≥1.2, were included in the intervention trial. All subjects gave informed consent. Study subjects were administered 10 mg riboflavin (n=21) or placebo (n=21) each day for 28 days. Results: Riboflavin supplementation significantly decreased plasma tHcy (*P*=0.005) and EGRAC (*P*=0.014), but not plasma ferritin, uric acid or C-reactive protein. Conclusions: In this elderly group, we found that 10 mg/day oral riboflavin supplementation lowered plasma homocysteine concentrations in subjects with low riboflavin status.

Keywords: riboflavin; homocysteine; uric acid; C-reactive protein; ferritin; elderly.

Introduction

Cardiovascular diseases are the leading cause of morbidity and mortality in Europe[1] and in the United States [2]. Biomarkers can be used to both identify patients at risk and stratify them according to prognosis[2]. Major biomarkers related to cardiovascular disease include homocysteine levels[3], uric acid[4], ferritin,[5] and C-reactive protein[6] which, in turn, could be related to riboflavin metabolism.

Riboflavin is a water-soluble vitamin present in a wide variety of foods, and its most important biologically active forms, flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN), participate as electron carriers in a range of redox reactions, some of which are absolutely key to the function of aerobic cells. Inadequate intake of riboflavin would therefore be expected to lead to disturbances in steps in intermediary metabolism, with functional implications. Riboflavin deficiency is also appointed as a risk factor for certain types of cancer [7, 8] and may modulate other conditions such as dark adaptation through cryptochromes, riboflavin-dependent photoreceptors, identified in the retina[9], and iron handling.

In recent years there has been much interest in the importance of plasma homocysteine as a graded risk factor for cardiovascular disease and riboflavin intake also emerged as a factor influencing plasma total homocysteine in men and women from the Framingham Offspring Cohort [10].

It has been suggested that riboflavin might act by increasing 5,10 methylenetetrahydrofolate reductase (MTHFR) activity and therefore lower plasma homocysteine concentrations [11-13].

Uric acid is one of the most effective water-soluble antioxidants in the blood. Xantine oxidase, a FAD-dependent enzyme, catalyzes the oxidation of hypoxanthine and xantine to uric acid. Riboflavin deficiency can result in decreased xantine oxidase activity, reducing blood uric acid levels[14].

Ferritin is a protein found inside cells that stores iron. Ferritin iron could be reduced and mobilized by reduced flavins in a variety of tissues[15, 16]. Riboflavin supplementation could improve riboflavin status and had a positive effect on iron absorption or iron mobilization from existing stores[9].

C-reactive protein (CRP), one of the major acute-phase reactants, as well as cytokines regulating its plasma level, have been used to predict the risk of cardiovascular events. High C-reactive protein plasma levels have been implicated in the development of ischemic heart disease in seemingly healthy subjects[6]. Riboflavin might have protective effects against the tissue damage associated with ischemia-reperfusion, probably mediated by flavin reductase and the reduction by dihydroriboflavin (produced from riboflavin by NADPH-dependent flavin reductase), of oxidized heme proteins[9]. The effect of riboflavin supplementation on C-reactive protein levels remain to be investigated.

In epidemiological studies riboflavin is recognized as a determinant of plasma homocysteine concentrations[10, 17-20]. Subjects who frequently consumed typical sources of riboflavin like dairy products have low plasma homocysteine concentrations[21].

Two interventions studies have shown no significant plasma homocysteine concentration change after supplementation with riboflavin. Lakshmi & Ramalakshmi, 1998[22] found in a not blinded and no placebo group study, a

significant improvement in riboflavin status but no significant change in plasma homocysteine concentration after riboflavin supplementation. McKinley et al., 2002[11] found in elderly subjects with an erythrocyte glutathione reductase activation coefficient (EGRAC, an indicator of riboflavin status) value less than 1.4, that riboflavin did not prove to be an effective homocysteine-lowering agent. Low riboflavin status is frequent among older populations, previously detected in 49 to 78% of elderly subjects from different populations[23, 24] and could show EGRAC values greater than 1.4[25].

Therefore, we hypothesized that homocysteine, uric acid, ferritin and C-reactive protein levels will be responsive to improved riboflavin status in elderly people. Serum folic acid levels were also investigated.

Methods

Setting and participants

Subjects aged 60 years or over were recruited from seven day social care centers in Porto, Portugal. All potential volunteers were interviewed by questionnaire about general health conditions, supplementation and drug use. Cognitive status was evaluated by the mini-mental state examination (MMSE)[26]. Subjects taking B-vitamin supplements or drugs that affect riboflavin metabolism (antacids containing magnesium or ioniazid), or who have hematological disorders or gastrointestinal, or cardiovascular, hepatic or renal disease or impaired cognitive function (MMSE <18), were excluded from the study. Eighty-eight elders volunteered after the interview, and were recruited in accordance with the current revision of the Helsinki Declaration of 2004[27]. Participants were informed of the purpose and risks of the experimental

protocol. Informed consent was obtained from the individuals before the start of the trial.

All volunteers were screened for suboptimal riboflavin status. According to a previous nutritional study of riboflavin deficiency[28] low riboflavin status is frequent among older subjects, with at least 49% of elderly subjects having an EGRAC value ≥ 1.2. We used this threshold value to define low riboflavin status for inclusion of subjects in our study, and 47% (n=42) had EGRAC≥1.2 .

Study design

We performed a 4-week, randomized, double-blind, placebo-controlled intervention trial. Subjects were randomly assigned to each of two groups. Subjects in the supplementation group received a daily capsule containing 10 mg riboflavin (manufactured by Lecifarma, Loures, Portugal), and all subjects in the placebo group received an identical capsule (similar shape and colour, with riboflavin replaced by microcrystalline celulose) for 28 days. We chose the dose of 10 mg/day of riboflavin because this dose led to significant improvement of riboflavin status in a previous study[22]. A supervisor in the day social care centers checked the number of remaining capsules daily to monitor compliance. We aimed to reduce plasma Hcy levels by 2 µmol/L, based on a previously reported association between mild-to-moderate hyperhomocysteinemia and ischemic stroke[29]. This target reduction corresponds to ⅔ of one standard deviation in the normal range[30] and gives at least 80% power (α=0.05, β=0.20, SD=3 µmol/L, one tailed) for detecting statistically significant changes. Plasma Hcy level determined the end point of the trial and the adequacy of sample size

was established from power calculations using typical variances from similar previous investigations[11, 23, 24, 31].

Blood collection procedures

Subjects in the day social care centers fasted overnight. Nurses then collected 10 ml blood samples by venepuncture. Samples were obtained one week before the trial was started (pre-supplementation) and after four weeks of supplementation (post-supplementation). Blood was collected in previously frozen containers containing EDTA for the measurement of EGRAC (red blood cells), plasma Hcy, plasma uric acid and high-sensitivity plasma C-reactive protein. Collection tubes for blood samples assayed for serum folic acid had no anticoagulant. Collection tubes for samples assayed for plasma ferritin contained heparin. Samples were kept on ice at all times, and were processed and analyzed on the same day. Those used for the measurement of EGRAC were processed and stored at –20 ºC for batch analysis.

Biochemical measurements

Biochemical analyses of blood samples were performed before and after supplementation. EDTA-treated hemolysates were prepared from blood samples and the FAD-dependent enzyme, EGRAC was measured using *RX DAYTONA* (Randox, UK)[32] to determine riboflavin status. EGRAC was calculated as the ratio of FAD-stimulated to unstimulated enzyme activity[33]. Serum folic acid, plasma ferritin and plasma Hcy concentrations were measured by a chemiluminescence immunoassay with an *ADVIA Centaur* (Bayer Corporation). Plasma uric acid was measured by colorimetric enzyme assay

with a *Hitachi* system (Roche). Plasma high-sensitivity C-reactive protein levels were measured by immunonephelometry on a *BN II Nephelometer* (Dade Behring). EGRAC assays were performed within one month of obtaining the sample.

Intra-assay variation coefficients were lower than 6% for all measurements. Both laboratories used Randox International Quality Assessment for external quality assurance.

Concentrations and activity coefficients of the variables (blood measurements), and baseline data for placebo and supplement groups are shown in Table 1. Defined cutoffs were as follow: serum folate concentrations < 7 nmol/L indicated low folate status[34]; normal homocysteine concentration was defined as ≤12 μmol/L, considering the increased concentrations with age[35]; depleted iron stores were defined by plasma ferritin <12 μg/L[36]; plasma uric acid values <7 mg/dL were considered normal[37]; subjects were classified as being high or low risk, based on plasma C-reactive protein levels, considering plasma C-reactive protein <1 mg/L as low[38].

Statistical analysis

Statistical analyses were performed using the Statistics Package for the Social Sciences 13.0, Chicago, USA. Data for men and women were combined for all analyses. The distributions of many of the blood variables were non-normal and could not be normalized by simple transformations; thus, data are presented as medians, minimum, maximum and percentiles. Placebo and supplementation groups were compared at baseline and post intervention using the Mann-Witney U test. The differences between the two groups were investigated and

differences in the levels of each variable were calculated. The Wilcoxon signed ranks test was used to assess the differences between treatment groups. *P* values <0.05 were considered significant.

Results

Volunteers (n = 88; 66.7% women) had a median age of 78.5 years (10^{th} to 90^{th} percentiles, 67.0 to 88.0). They were all Caucasian, retired and had MMSE > 18.

There was no significant correlation between plasma Hcy and EGRAC, serum folic acid, plasma ferritin, plasma uric acid or plasma high-sensitivity C-reactive protein at baseline between deficient and non-deficient groups (data not shown).

Forty-two volunteers (66.7% female) had low riboflavin status, defined by an EGRAC value ≥ 1.2, and were invited to participate in the intervention. **Table 1** shows the results of blood sample analyses for EGRAC, plasma homocysteine, serum folic acid, plasma ferritin, plasma uric acid, and plasma C-reactive protein. Baseline median plasma Hcy concentrations were 12.60 µmol/L (10^{th} to 90^{th} percentiles, 9.82 to 22.74) in the placebo arm of the study, and 11.40 µmol/L (10^{th} to 90^{th} percentiles 8.34 to 26.30) in the active arm of the study (*P*=0.320). Mean for Hcy concentration was reduced by 1.31 µmol/L (15.01 µmol/L (SD 9.38) before and 13.70 µmol/L (SD 9.36) 4 weeks in the active arm of the study (*P*=0.037).

Three subjects dropped out of the study due to unexpected illness by the second week. Thirty-nine subjects completed the study. Throughout the study, all the remaining subjects demonstrated 100% compliance, assessed by direct observation of capsule intake.

We analyzed plasma Hcy, high-sensitivity C-reactive protein and plasma uric acid in elderly men and women with low riboflavin status (EGRAC ≥ 1.2) following four weeks of 10 mg/day riboflavin supplementation, and there were significant differences in EGRAC, Hcy, ferritin and uric acid in the active arm of the study (**Table 2**)**.** Riboflavin supplementation significantly decreased plasma tHcy (p=0.005) and EGRAC (p=0.014), but not plasma ferritin, uric acid and C-reactive protein (**Table 3**).

Table 1. Baseline blood measurements[a]

Blood variable	Cut-off values	Placebo group	Riboflavin group
		n=21	n=21
EGRAC (µmol/L)	≥1.2	1.30 (1.25-1.50)	1.20 (1.20-1.35)
Plasma Hcy (µmol/L)	>12	12.60 (10.55-16.00)	11.40 (9.55-16.85)
Serum folic acid (nmol/L)	<7	25.50 (14.15-37.50)	21.40 (14.40-32.10)
Plasma ferritin (µg/L)	<12	124.00 (71.50–240.00)	110.00 (63.00-165.00)
Plasma uric acid (mg/dL)	≤7	5.60 (4.30–7.15)	5.10 (4.45–6.50)
Plasma high-sensitivity C-reactive protein (mg/L)	<1	0.37 (0.23–0.49)	0.18 (0.11–0.49)

[a] Values are median and percentiles (10^{th} - 90^{th})

[b] EGRAC, erythrocyte glutathione reductase activation coefficient

[c] Homocysteine

Table 2. Plasma homocysteine, plasma uric acid and C-reactive protein levels before and after four weeks of supplementation with 10 mg riboflavin per day in healthy elderly people with low riboflavin status (EGRAC ≥ 1.2)

	Placebo group			Riboflavin group		
	Before (week 0)	After (week 4)		Before (week 0)	After (week 4)	
	(n=21)	(n=18)	*p* value[a]	(n=21)	(n=20)	*p* value[a]
EGRAC(µmol/L)[b]	1.3 (1.2-1.7)[c]	1.4 (1.1-2.9)	0.753	1.2 (1.2-1.5)	1.1 (1.0-1.3)	<0.001
Hcy (µmol/L)[d]	12.6 (9.8-22.7)	13.1(8.9-32.2)	0.122	11.4 (8.3-26.3)	11.3(6.4-26.3)	0.012
Folic Acid (nmol/L)	25.5 (11.9-43.5)	23.4(14.2-45.3)	0.632	21.4(10.6-44.4)	28.6(14.3-45.4)	0.079
Ferritin (µg/L)	124.0(33.4-489.2)	84.0(26.3-368.2)	0.035	110.0(20.8-301.4)	89.5(17.6-261.5)	0.007
Uric Acid (mg/dL)	5.6 (3.4-8.8)	5.7(4.2-10.7)	0.815	5.1 (3.9-7.7)	5.9 (4.4-9.7)	0.033
C-reactive protein(mg/dL)[e]	0.4(0.1-1.5)	0.3 (0.1-2.7)	0.372	0.2 (0.1-1.1)	0.2(0.1-0.8)	0.876

[a] Response to intervention determined with the Wilcoxon Signed Ranks Test (*P*<0.05 considered statistically significant)

[b] EGRAC, erythrocyte glutathione reductase activation coefficient

[c] Values are median and percentiles (10^{th}-90^{th})

[d] Homocysteine

[e] Plasma high-sensitivity C-reactive protein

Table3. Comparison of variable values in placebo and riboflavin groups before and after supplementation.

	p^a
EGRAC (µmol/L)	0.014
Plasma Hcy (µmol/L)	0.005
Serum folic acid (nmol/L)	0.090
Plasma ferritin (µg/L	0.924
Plasma uric acid (mg/dL)	0.099
Plasma high-sensitivity C-reactive protein (mg/dL)	0.680

[a] Significance of the difference between the placebo and riboflavin groups with the Mann-Whitney U Test; $P<0.05$ considered statistically significant.

Discussion

We found that supplementation with 10 mg/day riboflavin lowered fasting Hcy concentrations in elderly subjects. We observed this effect in a randomized double-blind and placebo-controlled trial, with patient compliance closely monitored over the four week intervention period.

Riboflavin status and homocysteine

Furthermore, the reduction in EGRAC, that is sensitive in the short term to dietary intake of riboflavin[39], confirmed a significant improvement in riboflavin status, reflecting compliance with the supplementation regimen.

Lowering homocysteine by 1.31 μmol/L was a small decrease and did not bring Hcy concentration into the normal range, defined by <12 μmol/L. However, it can be considered as having biological significance. Meta-analysis of prospective and retrospective case-control studies predict that lowering homocysteine by 1.31 μmol/L (or ≈11% for an average of 12 μmol/L) would reduce the risk of coronary heart disease by 5% to 7% and stroke by 8% to 11%[40, 41].

C-reactive protein

Although plasma high-sensitivity C-reactive protein median concentration values were slightly lower after riboflavin supplementation, the difference did not reach statistical significance. Lack of response could be related with the C-reactive protein low levels at baseline. A previous study have demonstrated that this reduction is most pronounced in patients with elevated C-reactive protein levels at baseline (> or =1.0 mg/L)[42]. This can be explained also, by the fact that we established adequacy of sample size for riboflavin,

providing less power for detecting statistically significant changes for C-reactive protein levels. A number of studies[43-46] have demonstrated that C-reactive protein is a highly sensitive marker for cardiovascular disease in both men and women being possible that small differences in study parameters may contribute to disparity between findings. These may result from the inherent variability of biological measurements, differences in the rate of absorption between subjects, or other unidentified factors.

Plasma ferritin

Plasma ferritin concentrations were lower after intervention than those before intervention in both active and placebo arm of the study, although differences between groups did not reach statistical significance. Although the values are within the normal range, the median value is high for this age group. In developing countries where prevalence of inflammation is high, ferritin concentrations increase in the presence of an acute phase response and in parallel with C-reactive protein[47]. A CRP concentrations decrease is not paralleled by a decrease in plasma ferritin, possibly because the half-life-plasma CRP is 5-7 hours, whereas ferritin is closer to 30 hours[47]. Low serum ferritin results could have been influenced by CRP levels or by inadequacy of sample size for detecting changes that are statistically significant.

Uric acid

Although plasma uric acid levels median concentration values were slightly higher after riboflavin supplementation, the difference did not reach statistical significance. Plasma uric acid levels may affect vascular function[48, 49]. The health effects of the moderate increase

in uric acid observed in some studies but not in others are unknown[50]. Slightly higher plasma uric acid levels could have been influenced by increased xantine oxidase activity.

Serum folate

All the participants in our study were above the threshold for normal serum folate concentration at baseline. After riboflavin supplementation the difference did not reach statistical significance in both active and placebo arm of the study.

In populations with a high folate intake, the effect of the TT (homozygous) genotype on homocysteine levels is overcome, with a less pronounced increase in the risk of ischemic heart disease than in populations with lower folate intake[51].

Homozygosity for the C677T polymorpism

The protective effect of riboflavin is not dependent on the effect of MTHR genotype on homocysteine concentration. Indeed, the homocysteine response to riboflavin in our study was unlikely to be specific to individuals homozygous for the MTHFR 677T allele.

Homozygosity for the C677T polymorphism in the MTHFR-encoding gene is associated with an increased risk for cardiovascular diseases[52].

At the end of the study the active arm showed lower homocysteine and higher EGRAC values. Serum folate increases but the differences were not statistically significantly. One could argue if finding of lower homocysteine in the active arm of the study is only due to riboflavin supplementation. In two Portuguese studies, the minor allelic frequency for this polymorphism was 33%, indicating that this polymorphism is probably common in our population[53, 54]. At this frequency, we would expect to find five to ten subjects with this polymorphism in our study population. People who are homozygous for this polymorphism

have reduced MTHFR activity, resulting in elevated homocysteine levels; this effect is particularly marked among those with a lower folate status.

1C metabolism

Riboflavin and folate among other B-vitamins are coenzymes necessary for several reactions in one-carbon metabolism, a network of interrelated biochemical reactions that involve the transfer of one-carbon groups from one site to another. There has been renewed interest in this metabolism recently because modest dietary inadequacies of above mentioned vitamins can leads to a rise in blood levels of homocysteine[55]. At least one study in humans suggests that one-carbon metabolism can be interrupted by diminished riboflavin status[56]. Riboflavin is a co-factor for the critical folate-dependent enzyme, MTHFR, can impair activity of this enzyme and therefore interconversion of the different forms of folate.

In a previous study[11], 1.6 mg/day riboflavin failed to lower fasting Hcy concentrations in elderly subjects. This may be because riboflavin was not a limiting factor for one-carbon metabolism in these patients, and thus supplementation would have no effect on homocysteine. Hcy may therefore not be an appropriate biomarker of aberrant one-carbon metabolism in these patients.

Riboflavin intake

The prevalence of inadequate intake of riboflavin is high among elderly people, and they have a greater than average probability of being riboflavin deficient[9]. The Survey in Europe on Nutrition and the Elderly, a Concerted Action (SENECA) study reported that the

proportion of men with inadequate riboflavin intake was 55% for men with energy intake of 1300 kcal or less, and 30% for those with an energy intake of 1900 kcal or less; for women, the proportions were 68% and 43%, respectively[57], and high prevalence of riboflavin intakes below the lowest European dietary intakes were found in Portugal. Results from the National Dietary Survey[58] showed a high prevalence of riboflavin deficient intake. More recently Dietary Assessment in Porto, Portugal[59] showed that the prevalence of riboflavin deficient intakes were higher in older people.

In countries with folic acid supplementation in food, the results of clinical trials designed to examine the lowering Hcy effect of folic acid and vitamins B6 and B12 on mortality, have yielded somewhat neutral results[60, 61]. Riboflavin supplementation is currently not included in fortification programs in European countries, but could be considered in the future due to its lowering effects on homocysteine levels.

In conclusion, we found that 10 mg/day oral riboflavin supplementation lowered plasma homocysteine concentrations in elderly subjects. Identifying factors that can mediate Hcy levels, a possible risk factor for vascular disease, is particularly important in the elderly. Future studies should explore the mechanisms underlying the effects of riboflavin in flavin-mediated folate metabolism and the possible synergistic protective effect of these two vitamins. The combination of B vitamins that most effectively lowers Hcy concentrations has yet to be identified.

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5. Conclusions and future perspectives

The study was conducted with the aim to finding the answers to the main objectives decided and identified in introduction part.

After review the literature, the data gathered from Part 2 clearly indicated that riboflavin has an important role as cofactor in macronutrient metabolism, in iron absorption and excretion, has a possible role in homocysteine metabolism, and a relationship with cataracts, cancer and cardiovascular diseases.

The first main objective was to examine the validity of the SFFQ currently used in Portugal in comparision with independent biomarkers, for riboflavin and folate. In order to find the results regarding this matter, it is revealed from Part 3 that all participants, elders from seven day social care centers, were amongst the normal range for serum folate concentrations. Were found 47% elderly men and women with inadequate intake of riboflavin (EGRAC≥1.2), in line with previously found in elderly population.

The validity of SFFQ estimates of folate intake versus serum folate biomarker was assessed and the results showed a poor agreement between serum folate and dietary intakes obtained from the SFFQ. Similar conclusion was observed for SFFQ estimates riboflavin intake versus EGRAC biomarker. However, it remains to be known if the low validity that was found is linked to the low level of literacy in the elderly or if this method is inappropriate for estimating riboflavin and folate intake in this age group. Results obtained using this SFFQ for riboflavin and folate intakes should be interpreted with particular caution. Reasons for the poor correlation between folate and riboflavin intakes and serum folate and EGRAC should be explored and could lead to improved estimation of intakes.

For future validation other dietary methods such as the 24-hour recall should be investigated in order to reduced low literacy related problems for intake data quality.

The second main objective was to find the evidence about the benefit of riboflavin supplementation in elders. To get the result to this objective in Part 4 it was found that supplementation with 10 mg/day riboflavin, during four weeks, lowered fasting Hcy concentrations in riboflavin deficient elderly subjects. A significant improvement in riboflavin status reflecting compliance with the supplementation regimen was confirmed.

We did not observe an effect of riboflavin supplementation on uric acid serum, plasma ferritin and C-reactive protein concentration in our subjects. However, further studies are needed to confirm or refute our findings.

This dissertation is based on a methodological approach and completed by utilizing quantitative research methods. In this regards the statistical data analysis is conducted and finnaly the dissertation come up with the results respecting the main objectives.

It is revelead that riboflavin supplementation presents benefits in elderly people with a low riboflavin status.

Evidence came from effects of oral riboflavin supplementation on plasma total homocysteine, in a four-week randomized, placebo-controlled, double-blind trial of riboflavin supplementation in seven Portuguese day social centers performed. Riboflavin status appears to be a modulator in risk factors of CVD in elderly.

The SFFQ used could not categorized elderly people accordingly to intake level of folate and riboflavin.

Future research should explore the mechanisms underlying the effects of riboflavin in flavin-mediated folate metabolism and the possible synergistic protective effect of these two vitamins. These studies may permit in a near future developing functional foods to prevent and manage disease risk in elders, in the context of opportunities that exist at the nutrigenetic and pharmacogenetic interphase leading to personalized nutrition.

6.Abstract

As a water-soluble vitamin, riboflavin plays a part in a variety of oxidation-reduction reactions. Through its cofactor role in numerous reactions in the human body, riboflavin has been implicated as protective against diseases or conditions as diverse as cataract and cardiovascular disease (the leading cause of mortality in Europe and in the United States). Mechanisms for an apparent role for riboflavin as protection against certain diseases are not always understood.

Inadequate intake of riboflavin in older populations, could be expected to lead to disturbances in steps in intermediary metabolism, with functional implications.

Biomarkers can be used to both identify patients at risk and stratify them according to prognosis and major biomarkers related to cardiovascular disease include homocysteine levels, uric acid, ferritin and C-reactive protein, which, in turn, could be related to riboflavin metabolism. Folate deficiency has been identified as a risk factor for cardiovascular disease, and folate plays a key role in single carbon transfer reactions and it is involved in homocysteine metabolism. From both epidemiological and public health perspective, the assessment of the folate and riboflavin status as well as the study of a semi quantitative food-frequency questionnaire validity in the elderly are of great importance.

After describing the putative role of riboflavin in disease prevention the aims of this thesis were: firstly to investigate riboflavin in red blood cells and serum folate and the validity of a semi quantitative food-frequency questionnaire, in elderly people. Secondly to examinate

the effects of oral riboflavin supplementation on plasma total homocysteine, ferritin, uric acid and C-reactive protein concentration in elderly people with a low riboflavin status.

This thesis is based on the papers, which are referred in the text by their Roman numerals. Paper I, Putative Role of Riboflavin in Disease Prevention, a review. Paper II, EGRAC, serum folate and its dietary correlates estimated from a semi quantitative food-frequency questionnaire in elderly, a cross-sectional study to investigate riboflavin and serum folate and its dietary correlation. Paper III, Riboflavin supplementation and biomarkers of cardiovascular disease in the elderly, a four-week randomized, placebo-controlled, double-blind trial of riboflavin supplementation in seven Portuguese day social centers.

Riboflavin has an important role as cofactor in metabolism, and has a recognized importance as a determinant of plasma total homocysteine. Eighty-eight elders, were assessed, from those 66.7% women, with a median age of 78.5 years, Caucasian and retired. Forty-two had a low riboflavin status. In a four-week randomized, placebo-controlled, double-blind trial of riboflavin supplementation significantly decreased plasma homocysteine and EGRAC, but not plasma ferritin, uric acid or C-reactive protein. In this elderly group, we found that 10 mg/day oral riboflavin supplementation increased riboflavin status and lowered plasma homocysteine concentrations.

In the same sample, with low literacy (89.8% with less than 5 years education), serum folate concentrations were amongst the normal range. The semi quantitative food-frequency questionnaire has no validity to estimate folate and riboflavin intakes in this elderly group.

Taken together, the review and the present studies provide further support to the role of riboflavin supplementation in elders with low riboflavin status.

It was found some evidence suggestive for an effect of riboflavin on homocysteine levels and this may indicate that the beneficial effects of riboflavin supplementation should be considered for elderly people.

As the semi quantitative food-frequency questionnaire requests a generic representation of intake in order to provide an accurate picture of recent usual intake, recalling foods frequently consumed may pose more of a challenge to elderly. The differences observed between riboflavin and folate intake and riboflavin in erythrocytes and serum folate highlights the importance of biomarkers to investigate the role of diet in the development of chronic diseases, in elderly peolpe.

7. Resumo

Como uma vitamina hidrosolúvel, a riboflavina está presente numa variedade de reacções de oxidação-redução. Através dos seus co-factores participa em numerosas reacções no corpo humano, sendo considerada como um factor protector para certas doenças como as cataratas e as doenças cardiovasculares, que são a principal causa de morte na Europa e nos E.U.A. Contudo, não estão completamente esclarecidos os mecanismos de acção da riboflavina no seu aparente papel protector para certas doenças.

Pode esperar-se que a ingestão inadequada de riboflavina, em populações idosas, possa conduzir a distúrbios em fases do metabolismo intermédio, com implicações funcionais.

Os biomarcadores são utilizados para identificar pacientes em risco e estratificá-los de acordo com os prognósticos. Os principais biomarcadores relacionados com a doença cardiovascular incluem os níveis de homocisteina, de ácido úrico, de ferritina e de proteína C-reactiva, os quais, por sua vez, podem estar relacionadas com o metabolismo da riboflavina. A deficiência de folato tem sido identificada como um factor de risco para a doença cardiovascular, tendo os folatos um papel chave nas reacções de transferência dos átomos de carbono e estando também envolvidos no metabolismo da homocisteina. De ambas as perspectivas epidemiológica e de saúde pública, a avaliação dos níveis de folatos e da riboflavina assim como a validação de um questionário semi-quantitativo de frequência alimentar nos idosos é de grande importância.

O objectivo desta tese é, para além de descrever o papel putativo da riboflavina na prevenção das doenças, conhecer os níveis séricos de folatos e de riboflavina nos eritrócitos, em idosos. Também tem como objecto estudar a validade de um questionário semi-quantitativo de frequência alimentar através da avaliação dos valores obtidos resultantes da ingestão de folatos e riboflavina e da sua correlação com os níveis séricos de folatos e de riboflavina nos eritrócitos, em idosos. Também se investigou os efeitos da suplementação de riboflavina nos níveis séricos de homocisteína, ferritina, ácido úrico e da proteína C-reactiva, em idosos com baixos níveis de riboflavina.

Esta tese baseia-se nos estudos que estão referidos no texto com numeração romana. Artigo I, Papel putativo da riboflavina na prevenção da doença, uma revisão. Artigo II, EGRAC, folato sérico e a sua correlação com as estimativas obtidas a partir de um questionário semi-quantitativo de frequência alimentar, um estudo transversal em idosos. Artigo III, Suplementação com riboflavina e biomarcadores de doença cardiovascular, um estudo randomizado, com grupo placebo, duplamente cego, realizado durante quatro semanas em idosos de sete centros de dia Portugueses.

A riboflavina tem um papel importante como cofactor do metabolismo e a sua importância é reconhecida como determinante da homocisteina total plasmática. Foram avaliados oitenta e oito voluntários, dos quais 66.7% mulheres, com a mediana de idade de 78.5 anos, todos caucasianos e reformados. Quarenta e dois apresentavam baixos níveis de riboflavina. Num estudo experimental, aleatorizado, duplamente cego, com grupo placebo, a suplementação com 10 mg de riboflavina durante quatro semanas diminuiu

significativamente a homocisteina plasmática e o EGRAC, mas não a ferritina, o ácido úrico ou a proteína C-reactiva. Neste grupo de idosos, a suplementação oral de riboflavina de 10 mg/dia aumentou os níveis de riboflavina nos eritrócitos e baixou as concentrações de homocisteina plasmática.

No mesmo grupo de idosos com baixos níveis de literacia, em que 89.8% tinha menos que 5 anos de formação escolar, as concentrações de folato sérico estavam dentro dos níveis considerados normais. O questionário semi quantitativo de frequência alimentar não é válido para estimar a ingestão de folatos e riboflavina neste grupo de idosos.

A realização do artigo de revisão e os nossos estudos fornecem apoio adicional ao papel da suplementação da riboflavina em idosos com níveis baixos de riboflavina.

Foram encontradas algumas evidências que sugerem um efeito da riboflavina, nas concentrações plasmáticas de homocisteina indicando que pode ser benéfico para os idosos a suplementação com riboflavina.

Como um questionário semi-quantitativo de frequência alimentar fornece uma representação genérica da ingestão habitual recente de modo a fornecer uma imagem actual, a recolha de informação sobre a frequência da ingestão dos alimentos pode tornar-se um desafio para os idosos. As diferenças encontradas entre a ingestão de riboflavina e folatos e os níveis séricos de folatos e de riboflavina nos eritrócitos destacam a importância de utilizar biomarcadores nos estudos de validação e na identificação de padrões de ingestão alimentar associadas à deficiência de riboflavina e folatos que possam estar associados à redução dos riscos de doenças crónicas, em idosos.

8. Resumé

Comme une vitamine hydrosoluble, la riboflavine joue a part dans la variété des réactions oxydation-reduction. A travers de son rôle cofacteur dans des numérales réactions dans l'organisme humain, la riboflavine a été considérée comme une protection contre des maladies ou conditions tel que la cataracte et la maladie cardiovasculaire, la principale cause de mortalité en Europe et aux Etats-Unis. Des mécanismes pour un apparent rôle de la riboflavine comme protection contre certaines maladies ne sont pas toujours bien entendu.

Insuffisant prise de riboflavine peut conduire, dans les populations le plus âgées, à perturbations dans le métabolisme intermédiaire avec des implications funcionalles.

Les biomarqueres sont utilisés pour identifier des patients à risque et les stratifier avec leurs pronostiques et biomarqueres rapportés avec la maladie cardiovasculaire incluent les niveaux de homocysteine, acide urique, ferritin et protéine C-reactive qui à son tour peut être rapportés avec le métabolisme de la riboflavine. La carence en folate qui à été identifié comme un facteur de risque pour la maladie cardiovasculaire, joue un importante rôle dans les réactions de transference de le charbonne 1 et est impliqué dans le métabolisme de la homocysteine. Du point de vue de la perspective épidémiologique et de la santé publique, l'évaluation des niveaux de folate et de la riboflavine dans les plus âgées, est de grande importance.

L'objective de cette thèse était décrire le rôle putatif de la riboflavine dans la prévention de la maladie, aussi la validité d'un questionnaire semi quantitative de fréquence alimentaire

à travers l'évaluation des valeurs obtenues résultantes de l'ingestion de folates et riboflavine et les corréler avec les niveaux sériques de folates e de riboflavine dans les érythrocytes, dans les personnes âgées. Évaluer les effets de la supplémentation de la riboflavine dans les niveaux sériques de la homocysteine, la ferritine, acide urique et protéine C-reactive dans des personnes âgées avec bas niveaux de riboflavine.

Cette thèse se base sur les études qui sont rapportées dans le texte avec numération Romaine. Article I, "Rôle putatif de la riboflavine dans la prévention de la maladie, une révision. Article II, "EGRAC, folate sérique et sa corrélation avec les estimations obtenues à partir d'un questionnaire semi quantitatif de fréquence alimentaire, une étude transversale dans des personnes âgées. Article III, "Supplémentation avec riboflavine et biomarquers de la maladie cardiovasculaire, une étude doublement aveugle, contre placebo et randomisé réalisé pendent quatre semaines dans des personnes âgées de sept centres de jour portugais''.

La riboflavine joue un rôle important comme cofacteur dans le métabolisme et à une importance reconnue comme déterminant de la homocysteine. Quatre-vingt-huit volontaires on été évalué, desquels 66.7% femmes, avec le moyenne 78.5 années d'âge, tous caucasiens et retraités. Quarante-deux présentait des baisses niveaux de riboflavine. Dans une étude expérimentale, randomisé, en double aveugle, avec le group placebo, la supplémentation avec riboflavine a diminué significativement la homocysteine plasmatique et EGRAC, mais non ferritin, acide urique ou la protéine C-rective. Dans ce groupe de personnes âgées, nous avons trouvé que la supplémentation de riboflavine de 10 mg/jour a abaissé, après quatre semaines, les concentrations de la homocysteine plasmatique.

Dans le même groupe de personnes âgées avec de bas niveaux d'alphabétisme, où 89.8% avait moins que 5 ans de formation scolaire, les concentrations de folate sérique étaient dans des niveaux considérés normaux. Le questionnaire semi quantitatif de fréquence alimentaire n'est pas valide pour les estimations d'ingestion de folates que les niveaux de l'ingestion pour la riboflavine, dans ce groupe de personnes âgées.

La réalisation de l'article de révision et nos études fournissent aide supplémentaire au rôle de la supplémentation de la riboflavine dans des personnes âgées avec des niveaux bas de riboflavine.

Étions trouvé des preuves l'effet de riboflavine sur les concentrations plasmatiques de homocysteine indiquant que peut être bénéfique pour âgées la supplémentation avec riboflavine.

Comme le questionnaire semi quantitatif de fréquence alimentaire il fournit une représentation générique de l'ingestion habituelle récente afin de fournir une image actuelle, la collecte d'informations sur la fréquence de l'ingestion des aliments peut se devenir un défi pour les personnes âgées. Les différences constatées entre l'absorption de la riboflavine et folates et les taux sériques de folates et de la riboflavine dans les érythrocytes souligner l'importance de l'utilisation de biomarqueurs dans les études de validation et l'identification des modes de consommation alimentaire handicapées que la riboflavine et folate peut être associés à un risque réduit de maladies chroniques chez les personnes âgées.