Efficacy and safety of guanidinoacetic acid supplementation in healthy men and women

Chapter · January 2015

CITATIONS
0

READS
1,071

2 authors:

Sergej Ostojic

373 PUBLICATIONS **16,321** CITATIONS

SEE PROFILE

Marko D Stojanovic

University of Novi Sad

77 PUBLICATIONS **1,080** CITATIONS

SEE PROFILE

Sergej M. Ostojic, M.D., Ph.D.
Editor

Human Health and Nutrition

New Research

Nova Biomedical

HUMAN HEALTH AND NUTRITION

NEW RESEARCH

FOOD AND BEVERAGE CONSUMPTION AND HEALTH

Additional books in this series can be found on Nova's website under the Series tab.

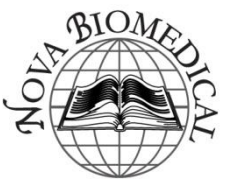
Additional e-books in this series can be found on Nova's website under the e-book tab.

FOOD AND BEVERAGE CONSUMPTION AND HEALTH

HUMAN HEALTH AND NUTRITION

NEW RESEARCH

SERGEJ M. OSTOJIC, MD, PHD
EDITOR



New York

Library of Congress Cataloging-in-Publication Data

ISBN: 978-1-63482-853-6 (e-Book)

Library of Congress Control Number: 2015939088

Published by Nova Science Publishers, Inc. † New York

Contents

Preface

In the past decade or so, nutrition research has grown into one of the most challenging and innovative health-related scientific disciplines in the world. New advances in basic and applied nutritional research help emphasize several *health challenges of the population such as obesity, chronic diseases (e.g., diabetes, cardiovascular disease, cancer) and aging. On the other hand, research focused on the healthy population with specific nutritional needs (e.g., athletes, elderly, children and adolescents) highlights new questions and dilemmas. The volume of information grows exponentially with new studies and challenging old paradigms. In this book,* authors explore selected topics that have implications for human health and nutrition, from epidemiological approaches to basic and applied trials.

In *Chapter 1*, authors discuss the importance of obesity and iron deficiency, as two most common nutritional disorders in the world. Authors challenge a new hypothesis: whether being overweight or obese is associated with an increased risk of iron deficiency anemia. It is already known that obesity is associated with an increased risk of low serum iron levels (the risk increases even more in the morbidly obese population). On the other hand, obese people tend to have higher hemoglobin and serum ferritin concentrations compared to normal weight people. Due to the fact that adipose tissue may act as an endocrine organ and produce several types of cytokines, authors report that greater adiposity could regulate iron metabolism through hepcidin-related effect of cytokines on iron storage and erythropoiesis.

Chapter 2 reviews selected conditions and diseases that might influence the transport of zinc in human nutrition. This essential mineral is involved in numerous structural, catalytic and regulatory functions of the body, with its role highlighted recently in diabetes mellitus Type 2 and cardiovascular disease. Specifically, low-grade inflammation that is presented in diabetes and cardiovascular disease can negatively affect zinc metabolism in the body. Furthermore, exercise might enhance the physiological demand for zinc, which is particularly relevant in chronic diseases when physical activity is prescribed as an element of primary or secondary prevention programs. Authors describe interconnected roles of exercise, inflammation and chronic disease on cellular zinc transporters in multiple cell types and cellular zinc homeostasis.

Sport nutrition is well-grounded scientific discipline, yet several questions are still debatable or challenging, such as protein requirements among athletes and/or performance-enhancing effects of protein supplements. In *Chapter 3*, authors discuss fundamental issues regarding protein supplementation and athletic performance through extensive overview of scientific literature. They provide comprehensive guidelines on daily protein requirements in

athletes and non-athletes, and describe benefits and drawbacks of protein consumption from different sources (animal proteins vs. vegetable proteins). In addition, updated information has been provided regarding the optimal timing of protein intake and the importance of leucine for muscle protein synthesis, as well as safety issues concerning high-protein intake in athletic environment.

In *Chapter 4*, authors investigate the efficacy and safety of novel nutritional supplement guanidinoacetic acid. Guanidinoacetic acid is a natural precursor of creatine, the latter playing an important role as an energy carrier in the cell. Authors suggest that dietary guanidinoacetic acid induces a significant increase in lean body mass, grip strength and upper body strength in 52 young men and women when administered for 6 weeks. On the other hand, treatment with three different oral doses of guanidinoacetic acid has no major effect on aerobic and anaerobic endurance, neither cardiovascular nor lactate responses after maximal exercise. It seems that 1.2 g/day of guanidinoacetic acid can be considered as a minimal dose with performance-enhancing properties. The effects are most consistently seen in participants receiving 2.4 grams of guanidinoacetic acid per day. Except for the dose of 4.8 grams of guanidinoacetic acid, reported side effects of guanidinoacetic acid administration are rather mild (e.g., weight gain, mild gastrointestinal distress). Furthermore, during guanidinoacetic acid loading liver and muscle enzymes remain within the normal clinical ranges, as well as serum antioxidant capacity and hematological indices.

Increased methylation after guanidinoacetic acid ingestion could affect metabolism of homocysteine and induce hyperhomocysteinemia. Due to the fact that elevated plasma homocysteine is discussed as a risk factor for a number of important diseases, it seems reasonable to employ different nutritional agents (e.g., methyl group donors) as additives during guanidinoacetic acid loading to suppress or counterbalance hyperhomocysteinemia. In *Chapter 5*, authors analyze whether dietary intake of methyl donors (e.g., betaine, choline and B vitamins) during guanidinoacetic acid loading affected metabolic and clinical markers, and increased the incidence of side effects after 8 weeks of administration. It seems that methyl group donors co-administered during guanidinoacetic acid supplementation largely prevented an increase in plasma homocysteine induced by pure guanidinoacetic acid. A powerful *homocysteine-balancing* effect has been noted in subjects co-supplemented with choline and betaine. All nutritional interventions seemed to be comparably effective for improving skeletal muscle mass as well as decreasing body fat, with no major changes in weight throughout the study. Considering the negligible disturbances in clinical markers of health status, the intake of guanidinoacetic acid together with methyl group donors and B vitamins during eight weeks is relatively safe if the substance is taken in the recommended amount.

Creatine is among the most popular dietary supplements in the world in terms of health and disease. In *Chapter 6*, authors evaluate the use of creatine in human nutrition from its metabolism and utilization to supplemental creatine effects in sports and medicine. Authors extensively review the physicochemical properties (e.g., solubility, stability in solid forms and solutions) of creatine monohydrate and several advanced creatine formulas (e.g., creatine ethyl ester, creatine chelate, creatine salts, buffered creatine) that are currently available in the market. In addition, creatine monohydrate and new forms of creatine are compared concerning ergogenic effects and bioavailability issues. With the final goal of increasing solubility of creatine, authors highlight future challenges of synthesizing creatine in the form of hydrophilic room temperature ionic liquids.

Chronic inflammation and oxidative stress accompanies many diseases and conditions (e.g., obesity, metabolic syndrome, diabetes mellitus, cancer, cardiovascular disease). Therefore, the use of nutritional agents with anti-inflammatory and antioxidant properties might be beneficial. In *Chapter 7*, authors present the results from three pilot intervention studies that evaluated multi-target biological activities of two medicinal plants in humans. In particular, the effects of extracts from *Sambucus ebilus* and *Agrimonia eupatoria* are tested on healthy volunteers. Results obtained indicate changes in selected markers for anti-oxidative and anti-inflammatory activity of two medicinal plants. Due to its favorable characteristics, authors consider supplementation with agrimony and elderberry teas as an option to combat oxidative stress related conditions, including low-grade inflammation and metabolic disturbances.

In *Chapter 8*, authors describe the role of different edible wild plants in nutrition indigenous to the Umbria region (Italy). It seems that gathering and consuming edible wild plants is still very much alive in Umbria as one aspect of an age-old ethno-botanical folk tradition although in most cases their nutritional value is unknown. Local names, parts used, folk medicinal properties and variations in culinary use linked to local traditions are still very important while conventions, exhibitions and themed courses provide information and promote these species as an environmental resource. It was found that the quality and quantity of the various components of the four species under examination (*Bellis perennis*, *Bunias erucago*, *Chondrilla juncea*, *Sanguisorba minor*) could make an excellent contribution to balancing and rationalizing diet as well as preventing metabolic pathologies. In particular, high content of micronutrients, polyphenols and antioxidants might offer advanced protection against degenerative processes and advances health of the consumer.

Chapter 9 discusses the significance of fruit and vegetable intake for health prevention in adolescent population. It seems that many dietary habits, such as fruit and vegetable intake, established during this sensible period of life continues into adulthood. World Health Organization reported that the daily fruit and vegetable intake in adolescents was below the recommended, despite the health benefits associated with optimal intake of fruits and vegetables. Several factors affect intake of fruits and vegetables during adolescence. Authors identify three key factors that might influence intake of fruits and vegetables in adolescents. These factors include individual factors (e.g., gender, age, knowledge, self-efficacy, taste preference), social factors (e.g., parents intake and modeling, parents and family support), and environmental factors (e.g., income, school availability, neighborhood, television). Authors also discuss several development strategies and effective intervention programs aimed to increase fruit and vegetable intake to promote adolescents' healthy dietary behaviors.

The number of people in Europe over the age of 65 years is expected to double over the next 50 years. It seems that sustained periods of physical inactivity and concomitant loss of muscle mass (sarcopenia) are more common in older people. The consequences of physical inactivity together with an unhealthy diet present an aggravating factor for chronic diseases and sarcopenia. In *Chapter 10*, authors discuss the importance of developing tools and interventions that reverse the negative consequences associated with physical inactivity and/or aging. Four studies evaluated adaptive responses to physical inactivity of 37 younger males and 16 older adults who received several interventions (nutritional, physical exercise or cognitive) during bed rest or rehabilitation that followed.

In particular, use of tensiomyography might be applicable novel approach to evaluate and manage sarcopenic muscle; the procedure is reliable, inexpensive and simple to use, and sensitive enough to detect early atrophic changes.

In: Human Health and Nutrition
Editor: Sergej M. Ostojic
ISBN: 978-1-63482-823-9

Chapter I

Obesity and Iron Deficiency Anemia: A Review of Population Based Studies

Jung-Su Chang[1,*], Eddy Owaga[2], Joseph Jordan Keller[3] and Chyi-Huey Bai[4]
[1]School of Nutrition and Health Sciences,
College of Public Health and Nutrition, Taipei Medical University, Taiwan
[2]Institute of Food and Bioresources Technology,
Dedan Kmiathi University of Technology, Nyeri, Kenia
[3]School of Public Health and Nutrition, Taipei Medical University, Taiwan
[4]Department of Public Health, College of Medicine,
Taipei Medical University, Taiwan

Abstract

Obesity and iron deficiency are amongst the most common nutritional disorders in the world. However, whether being overweight or obese is associated with increased risk of iron deficiency anemia (IDA) remains undefined. Obesity is associated with increased risk of hypoferremia (low serum iron levels) and the risk may increase further in morbid obesity (BMI > 40 kg/m2). On the other hand, obese people tend to have higher hemoglobin and serum ferritin concentrations compared to normal weight people. Since the sites for iron storage and erythropoiesis are remote from the intestine, the circulating peptide hepcidin is thought to act as the communicating signal between these organs. Adipose tissue is an active endocrine organ which can produce numerous cytokines, adipokines and hormones leading to hepcidin perturbation. Hence, greater adiposity may, directly or indirectly, regulate iron metabolism. The recent advances in the molecular mechanism of iron regulation have also led to the development of alternative iron therapeutic methods for both iron restrictive anemia and/or iron overload. This chapter discusses recent advances of obesity and iron metabolism with special focus on population based studies.

* E-mail: susanchang@tmu.edu.tw.

Keywords: Obesity; Iron deficiency; Hepcidin; Diet; Sex hormone; Adipocytes; Treatment

Introduction

Both iron deficiency (ID) and obesity are global epidemics affecting billions with regional disparities [1]. Increasing evidence suggests ID and obesity are causally associated at the molecular level [2]. The association was first observed in the 1960-s among obese children with decreased serum iron being as frequent as 36% and 18% in obese males and females, respectively [3]. This observation was later confirmed in obese adolescents [4, 5] and adults [6, 7]. Currently, it is hypothesized that the low-grade inflammatory phenotype may be contributory to ID. While the possible relationship between obesity and hypoferremia has been described in children [4, 5, 8] and women [9-15], it is still debatable whether being overweight or obese is associated with an increased risk of anemia or iron deficiency anemia (IDA) [12-19]. One systematic review of 25 studies investigating iron status in obese populations reported that obese people tend to have higher hemoglobin (Hb) and ferritin concentrations, but lower transferrin saturation compared to non-obese controls [20]. This conclusion adds credence to the view that hypoferremia is associated with obesity, but that the higher Hb levels observed among the overweight and obese indicates that sufficient iron is available for erythropoiesis [20]. Iron metabolism is highly regulated and involves cross-talk signals between multi-organs, hormones and cytokines. This chapter discusses recent advances of obesity and iron metabolism with special focus on factors that influence iron metabolism such as diet, adipokines, inflammation and sex hormone.

Physiology of Iron Metabolism

Iron is an essential trace element for all microorganisms. Iron is also a potent pro-oxidant which can donate electrons leading to reactive oxygen species (ROS). However, there is no known mechanism for iron excretion. Thus, as a primitive defense mechanism, iron metabolism is tightly regulated in humans.

The average adult human contains 2-4 g of iron in the body (approximately 0.06 g iron/kg body weight) [21]. About two-thirds of this iron (60-70%) is stored in the hemoglobin; the other third is contained in the iron-binding proteins such as ferritin and transferrin [22]. Ferritin uses as much as 20% of the iron. Myoglobin and iron containing enzymes use about 10%. Only about 1-2% is in transit in plasma. The major site of iron utilization is the bone marrow (~20 -25 mg iron/day) where it is used in heme synthesis for the red blood cells (RBCs) [23]. Recycling of heme iron from senescent RBC is the primary source of erythropoiesis [22]. This process is mediated by macrophage and accounts for 90% of the daily iron requirement in adults. In healthy adults, only 10% (1-2 mg) of iron is absorbed from diet to replace the iron losses (e.g., menstrual or dead cell loss). The absorbed iron is transported across the basolateral membrane of intestinal enterocytes into the circulation via ferroportin (FPN). Transferrin carries two ferric iron (Fe^{3+}) and transport iron throughout the body. All iron-requiring cells express transferrin receptor on the cell surface including RBCs. Increased iron requirement triggers transcriptional upregulation of transferrin receptor as well

as shedding of transferrin receptor. Hence, soluble transferrin receptor (sTfR) reflects the body's iron status. The excess iron is stored in the liver in the form of ferritin (<20%). Thus, the amount of ferritin in circulation normally reflects the amount of iron stored in the body in healthy subjects. When iron stores diminishes, little ferritin is made, which allows greater amounts of iron to enter the mucosal iron pool for transport into the bloodstream.

Hepcidin is the principle regulator of systemic iron homeostasis [24]. Hepcidin mRNA is primarily synthesized in the liver. But it is also expressed at low steady-state levels in other organs including the heart [25], brain [25], lung [25], macrophage rich tissues [25] and adipocytes [26]. The amount of circulating hepcidin contributed by the non-liver organs remains unknown. A recent study showed hepcidin mRNA expression was increased in adipose tissue of obese patients [26]. This finding suggests that adipose tissue may be directly involved in iron metabolism via regulating hepcidin secretion. As proposed by Nemeth and Ganz [21], hepcidin controls plasma iron concentration by physically binding to FPN leading to FPN internalization, degradation, and thus to blockage of cellular iron export from enterocytes to body cells. FPN is the sole known cellular iron exporter in mammals. Taken together, hepcidin-FPN axis maintain iron homeostasis via: 1) decreasing dietary iron absorption in duodenum, 2) inhibiting the release of recycled iron by macrophages to peripheral, 3) and inhibiting iron mobilization from hepatic or splenic stores [21, 27].

Pathophysiology of Iron Metabolism Related to Obesity

Several mechanisms concerning obesity and dysregulated iron metabolism have been identified in humans. As described above, the iron homeostasis is maintained by the iron absorption in the gut (1-2 mg absorbed iron/day) and iron sequestration (20 -25 mg recycled iron/day) [23]. The bioavailability of iron in the diet is influenced by (i) iron status, (ii) form of iron in the diet (heme and non-heme iron), (iii) diet composition (inhibitor and enhancer), and (iv) iron exporter hepcidin-FPN axis. However, most of the dietary iron bioavailability data have been derived from malnutrition subjects. Therefore, whether obese people exhibit the same iron absorption rate as malnutrition people remains unclear. The recent discovery of hepcidin-FPN axis has provided new aspects on how our body responds to iron status, particularly, in the inflammatory state. Adipose tissue is a very active endocrine organ that produces numerous cytokines, adipokines and hormones [28]. Hence, greater adiposity may, directly or indirectly, regulate hepcidin secretion resulting in altered iron homeostasis. As discussed below, several factors are known to influence iron metabolism in relation to obesity, namely diet, adipocytes, leptin, inflammation, macrophage, erythropoiesis and sex hormone.

Diet

The iron requirements of an individual are proportional to growth (~0.2 -0.6 mg iron/day), basal (~0.6 -1 mg iron/day) and menstrual (~0.5 mg iron/day) losses [29]. For adults, approximately 1-2 mg of iron (male and postmenopausal female: 1 mg/day and

reproductive aged female: 1.5-2 mg/day) must be absorbed daily to replace the basal and menstrual losses. Typical Western diets contain 6 mg/1000 kcal of iron while Chinese diet contains 7 mg/1000 kcal of iron [30]. Iron absorption rate is regulated by the iron status of an individual: 5-10% in individuals with normal iron status and 20-30% in individuals with Iron Deficiency Anemia (IDA). In patients with IDA, iron bioavailability has been estimated to be 14–18% for mixed diets and 5–12% for vegetarian diets [31]. These data have been used to generate dietary reference values for all population groups [31]. However, whether these values can apply to overweight and obese subjects remain unclear.

Literature shows a poor correlation between dietary iron intake and obesity-related hypoferremia [32]. Aeberli *et al.*investigated dietary iron intake in 121 children aged 6-14 years (yrs) and reported no differences in dietary iron intakes nor iron bioavailability between overweight and normal weight children [33]. The average iron intake in Taiwanese adult women is 15.2 mg/day, reaching 100% of the dietary recommended iron intake for reproductive-aged women [34]. Dietary iron intake (animal or plant iron) did not differ between anemic status nor did BMI status in Taiwanese women [34]. Obese people tend to replace carbohydrate (CHO) with fat and protein as sources of energy compared with normal weight [33, 34]. Animal protein such as red meat, poultry and fish are good sources of heme iron. In addition, animal iron accounts for 10-30% of total iron intake (approximately 15% is absorbed). CHO foods such as red bean and grains are also good sources of non-heme iron (approximately 3-8% is absorbed). The absorption rate of plant-based iron depends largely on the concomitant presence of iron absorption-regulating factors (e.g., vitamin C and vitamin A) and iron-absorption inhibitors (e.g., calcium, tea, coffee, and polyphenol) in the meal.

Menzie *et al.*compared dietary factors that affect iron absorption in obese and non-obese adults and found no association between obesity-related hypoferremia and dietary factors [35]. Dietary composition may also affect iron bioavailability. Using radioactively labeled ^{59}ferric citrate, animal study showed mice fed on high-fat diet had lower iron absorption rate in the duodenum and the high-fat diet induced hypoferremia was independent of hepcidin level [36]. Another study showed that mice fed with high-fat and high-fructose diet developed hepatic iron overload and this was, in part, due to decreased hepatic hepcidin levels [37]. Upon administration of 4 mg of isotopically labeled fortification iron to healthy premenopausal Thai women, Zimmermann *et al.*observed an inverse relationship between Body Mass Index (BMI) and fractional iron absorption [38]. However, these authors did not measure hepcidin levels in Thai women. Taken together, more studies are needed to clarify the role of dietary factors in the development of obesity-related IDA, particularly the effects of high fat/low CHO diet on hepcidin and FPN expression.

Adipocytes

In healthy normal weight subjects, iron homeostasis is primarily controlled by hepcidin produced in the liver. There are conflicting evidences concerning the role of adipose-derived hepcidin in obesity-related hypoferremia or IDA. Bekri *et al.*first reported the possible role of adipocytes in hepcidin secretion [26]. Hepcidin mRNA expression was increased in subcutaneous adipose tissue (SCAT) in severe obese patients and its expression was positively correlated with inflammatory signals, IL-6 and C-reactive protein (CRP) [26]. By examining adipose tissue derived from patients who underwent cardiac surgery, Vokurka *et*

*al.*observed the basal expression of hepcidin mRNA was higher in epicardial visceral adipose tissue (VAT) than in the SCAT. Hepcidin expression in SCAT increased significantly at the end of surgery but not in the VAT. However, Tussing-Humphreys *et al.*demonstrated that SCAT derived from obese or lean adults does not release hepcidin *in vivo* [39]. The notion is that hepcidin expression is much higher in the liver than in the adipose tissue (approximately 700 times greater than in VAT and SCAT) [26, 40]. Hence, it is difficult to conclude to what extent adipocytes-secreted hepcidin contributes to the obesity-related hypoferremia.

Leptin

Leptin is produced primarily by the white adipose tissue to signal fat storage in the body. It controls food intake via regulating neuropeptides in hypothalamus [41]. Plasma leptin levels are positively correlated with BMI. A positive association between serum hepcidin and leptin was found in obese children [42]. Later, the same researchers conducted a 6-months weight reduction program in obese children and found an association between decreased BMI and decreased leptin and hepcidin levels in children [43]. *In vitro* study in HuH7 human hepatoma cell line showed leptin upregulates hepatic hepcidin expression through the JAK2/STAT3 signaling pathway [44]. These data suggest that leptin may regulate iron homeostasis via controlling hepatic hepcidin expression. However, most obese people develop leptin resistance and have high leptin levels in the body [45].

Inflammation

Obesity is characterized by a low grade systemic inflammation. Adipose tissue secretes many pro-inflammatory cytokines such as interleukin 6 (IL-6), tumor necrosis factor (TNF-α), interleukin 1β (IL-1β) and monocyte chemoattractant protein-1 (MCP-1). The effects of pro-inflammatory cytokines on iron status have been extensively reviewed [21, 23, 28, 46-51]. For example, it is well reported that hepcidin is transcriptionally upregulated by pro-inflammatory cytokines such as IL-6 [52]. This condition is commonly referred to as anemia of inflammation (AI) or anemia of chronic disease (ACD) [52, 53].

Macrophage

The pathological hallmark of obesity is characterized by the infiltration of macrophage into the adipose tissue, leading to increased release of proinflammatory cytokines [54]. P-selectin glycoprotein ligand-1 (PSGL1) is a critical adhesion molecule for the recruitment of leukocytes into adipose tissues [55, 56]. Macrophage play a key role in both host defense and iron metabolism [23].

By recycling heme iron from senescent RBCs, splenic and hepatic macrophages provide sufficient iron to bone marrow for erythropoiesis. Deletion of iron exporter gene ferroportin-1 ($Fpn1^{-/-}$) in macrophage resulted in increased TNF-α and IL-6 secretions in iron-loaded macrophage [57]. FPN mutations have also been identified in different ethnic groups.

Furthermore, disruption in FPN may lead to hyperferritinemia, iron overload in macrophage and/or hepatocellular iron deposition [58].

Erythropoiesis: Role of Hypoxia

Hypoxia profoundly affects erythropoiesis via activation of the hypoxia inducible factor (HIF)/hypoxia response element (HRE) [59]. The HIF system senses oxygen levels and plays a key role in anemia induced hypoxia [60]. HIF-1α is thought to be a negative regulator of hepcidin by binding to the HRE in the hepcidin [59]. The HIF system can also induce erythropoietin (EPO) production in the kidney thereby promoting the proliferation and differentiation of erythroid progenitor cells [61]. The HIF/EPO system is also the negative regulator of hepcidin [49]. By suppressing hepcidin level, EPO can enhance blood iron levels and supply iron to the bone marrow for erythropoiesis.

Pathological adipose tissue expansion may cause adipocyte hypertrophy, which creates areas of local adipose tissue micro-hypoxia [54]. It may also upregulate adipokines and pro-inflammatory cytokines that are associated with transcriptional upregulation of hepcidin such as leptin, IL-6, interferon-alpha (IFN) and TNF-α. As hypothesized by Means [50], the presence of inflammatory cytokines may interfere with erythropoiesis through the following mechanisms: (1) inhibited erythroid progenitors response to EPO (e.g., IL-1, IFN and TNF-α); (2) impaired iron mobilization or utilization through upregulation of serum ferritin and downregulation of TfR on progenitor cells; and (3) decreased RBC survival (e.g., IL-1 and TNF-α).

Sex Hormone

Association between obesity and the reproductive hormones have been noted in humans, however this relationship may differ by menopausal status [62].

A positive association between blood estrogen (estradiol and estrone) and BMI existed in postmenopausal women [63] but a reverse relationship was found in premenopausal obese women [62]. The mechanisms underlying this association are complex and undefined. However, the source of estrogen may play a key role in this relationship.

There are three types of estrogen in the human body: estrone (E1), estradiol (E2) and estriol (E3). In premenopausal women, estradiol is the major estrogen that is predominantly secreted by the ovary. During menopause, estrone replaces estradiol and becomes the predominant estrogen. Estrone is secreted by both ovary and peripheral tissue such as adipose tissue and muscle. Ovarian estrogen production decreases after menopause and adipocytes-mediated aromatase conversion of androstenedione to estrone is dominant in obese postmenopausal women [64]. Estrone can be further converted to estradiol by 17β hydroxysteroid dehydrogenase (17β-HSD) [65]. In 1987, Siiteri [64] proposed obese people maybe associated with: (i) increased androgen precursors available for conversion to estrogen; (ii) increased conversion efficiency of androstenedione to estrone ; and (iii) decreased plasma sex hormone-binding protein (SHBG) levels, which binds estradiol (E2), leading to increased levels of free serum estradiol.

It has long been speculated that there are cross-talk signals between sex hormone and iron profile at the systemic level [66]. In female, body iron status is closely associated with menopausal status. Ovariectomized mice, an animal model of postmenopausal osteoporosis, showed elevated body iron stores [67-69] and this was, in part, due to increased FPN expression in the duodenum and decreased hepcidin and bone morphologic protein 6 (BMP-6) expression in the liver [69].

Using *in vitro* and *in vivo* models, several studies demonstrate estrogen directly regulates hepcidin expression [66, 68, 69]. Though, these findings are inconclusive.

Yang *et al.*first reported 10 and 100 nM 17β-estradiol (E2) inhibited hepcidin expression in HepG2 and HuH7 cells and this inhibition was mediated through estrogen response element (ERE) that is located between -2474 and -2462 upstream from the start of hepcidin gene [66]. This effect was also observed in wild-type mice that received 100 nM E2/kg body weight for 24 hours [66]. Hou *et al.*identified another ERE, located on -1244 to -1232 upstream from the start of hepcidin transcription site, that plays a key role in E2-mediated (10^{-7} M) downregulation of hepcidin expression [68]. However, Ikeda and colleagues reported E2 treatment (10^{-8}-10^{-6} M) upregulated hepatic hepcidin expression in HepG2 cells, which was mediated through a distinct GRP30-BMP6-dependent mechanism [69]. Administration of testosterone to wild-type mice showed increased iron incorporation into RBCs and this effect was through BMP/Smad- mediated downregulating of hepcidin transcription [70].

A recent population based study of Chinese men observed a negative association between testosterone and serum ferritin levels [71]. Taken together, these data provide first evidence that sex hormone may directly be involved in iron homeostasis.

Treatment of Obesity-Related Iron Deficiency

Treatment of obesity-related iron deficiency can be achieved through two main approaches: (i) energy-restricted diet; and (ii) hepcidin antagonists. Furthermore, incidences of iron overload can also be managed by use of hepcidin agonists.

Energy-Restricted Diet

Information on the role of life-style interventions on iron status are scarce. Traditionally, iron supplementation is recommended for individuals who have iron deficiency or IDA. However, recent publications show the treatment efficacy of iron supplementation may vary significantly between normal weight and overweight/obese individuals [38, 72, 73]. The notion is that elevated serum hepcidin levels may reduce iron bioavailability in obese children and adults. Weight reduction, on the other hand, may improve obesity-related hypoferremia [43, 72, 73] even in the absence of elevated serum hepcidin [74]. Weight reduction, particularly loss of adipose tissue, is associated with change of adipokines, pro-inflammatory cytokines and sex hormone. It has been suggested that decreasing adipose tissue-derived signals may lead to decreased hepcidin expression, hence, a better iron status in obese individuals. Although serum hepcidin is positively correlated with the degree of BMI, a few

studies found normal or lower serum hepcidin levels in mild overweight/obese children and adults [74, 75]. Chang and colleagues investigated 648 children aged 7-13 yrs living in Taipei and New Taipei city and reported obese and overweight children had lower serum hepcidin concentrations compared with their normal weight counterparts [75]. Cheng *et al.*conducted a 6 to 12-months weight reduction program (high protein and low protein diet: 5600 kJ/day) on young Australian women (18-25 yrs; BMI $\geq$ 27.5 kg/m^2) and observed a normal serum hepcidin and CRP levels at baseline among participants [74]. These authors found that loss of > 10% initial body weight was associated with a better iron status regardless of the type of diet and hepcidin levels [74].

Taken together, these data suggest that weight loss induced by energy-restricted diet may be used as a means to correct obesity-related hypoferremia in spite of restricted heme and non-heme iron intake.

Hepcidin As a Potential Iron Therapy Target

Recent advances in the molecular mechanism of iron regulation suggest that hepcidin deficiency or excess play a pathogenic role in iron disorder. Thus, hepcidin agonists and antagonists have been developed as a means of iron therapy. The hepcidin-based therapy is an area of intensive research and in-depth reviews have been published elsewhere [76-82]. However, of note, the available data are primarily based on animal models. Although several hepcidin-targeted therapies have already been administered in human clinical trials, no clinical data has been reported yet [83].

Hepcidin Antagonists for Treatment of Iron Restrictive Anemia

Iron restrictive anemia is associated with elevated serum hepcidin due to the presence of chronic inflammation. If the cause of anemia in obese individuals is due to inflammation-driven hepcidin synthesis, hepcidin antagonists can be employed as a means of iron therapy. However, as noticed by Chang [75] and Cheng [74], normal hepcidin levels may occur in healthy young overweight/obese individuals.

In this circumstance, hepcidin antagonists would only be suitable for patients with elevated serum hepcidin such as severe obese [43, 84] or those burdened with comorbid conditions [84, 85].

Two small-molecule antagonists of hepcidin have been developed including fursultiamine [86] and NOX-H94 [87], which target hepcidin-FPN axis. Fung *et al.*reported fursultiamine, a Food and Drug Administration (FDA)-approved thiamine derivative, directly interfered with hepcidin binding to FPN, by inhibiting FPN C326 thiol residue which is essential for hepcidin binding [86]. Schwoebel *et al.*identified a novel anti-hepcidin compound NOX-H94, a structured L-oligoribonucleotide that binds human hepcidin with high affinity and reported that NOX-H94 protects FPN from hepcidin-induced degradation in an acute cynomolgus monkey model of IL-6-induced hypoferremia [87]. Cooke and colleagues developed human anti-hepcidin antibodies (Abs) to neutralize hepcidin expression and reported that 12B9m Abs increase the serum iron availability leading to enhanced red cell hemoglobinization in a mouse and cynomolgus monkeys model of AI [77]. Anti-cytokine therapeutic approaches have also been developed to down-regulate inflammation-associated hepcidin synthesis. Briefly, these include anti-IL-6 antibodies [88], AG490 which is a STAT3

inhibitor [89], and carbon monoxide-releasing molecules [90]. The bone morphogenetic protein (BMP) pathway plays a key role in stimulating hepcidin transcription. Thus, iron availability can be restored by BMP therapeutic agents such as microRNA miR-130a [91] and heparin [82].

Hepcidin Agonists for Treatment of Iron Overload

Iron overload disorders may also occur in obese individuals with hereditary iron disorders. This includes hereditary hemochromatosis (HH) and iron loading anemia associated with ineffective erythropoiesis (e.g., β-thalassemia and sickle cell disease). Hepcidin insufficiency is the common clinical feature. However, iron overload of HH is results from the combination of genetic disorders in the genes encoding hepcidin or its regulator, leading to excessive dietary iron absorption and iron deposition in the body [78]. By contrast, hepcidin production is suppressed due to ineffective erythroid regulators (e.g., GDF15, TWSG1) in the β-thalassemia major. In practice, phlebotomy is an effective treatment for patients with hereditary hemochromatosis. In β-thalassemia major, iron chelation therapy is used to prevent iron overload, however, the treatment is is less well tolerated due to intolerance of parenteral chelating agents [79]. Tissue iron overload is a primary focus of β-thalasemia management and it can be fatal in both transfused and non-transfused patients. Gardenghi et al.tested the hypothesis that β-thalasemic mice absorb more iron than is needed for erythropoiesis. By placing β-thalasemic mice on a normal (35 ppm iron) and low iron diet (2.5 ppm iron) for 5 months, the authors found that the β-thalasemic mice that received low iron diet did not exhibit a worsening anemia despite relative systemic iron deficiency [92]. By contrast, β-thalasemic mice on a normal iron diet (35 ppm iron) showed decreased hemoglobin, RBCs and reticulocyte count but increased iron storage in the liver and spleen [92]. It has been speculated that the reversal of ineffective erythropoiesis in β-thalasemic mice is, in part, due to decreased formation of toxic unpaired α-globin chains and heme iron-reactive oxygen species mediated membrane damage in RBCs [76].

Schmidt *et al.*delivered TMPRSS6 siRNA formulated in lipid nanoparticles (LNP) to Hfe (-/-) and thalassemia intermedia (Hbb (th3/+)) mice and reported LNP-TMPRSS6 siRNA induces hepcidin expression and diminishes tissue and serum iron levels in transgenic mice [93]. Furthermore, LNP-TMPRSS6 siRNA treatment of Hbb (th3/+) mice substantially improved the anemia by altering RBCs survival and alleviating ineffective erythropoiesis. TMPRSS6 is the negative modulator of hepcidin and by silencing TMPRSS6 expression thereby leading to increased hepcidin synthesis. Overall, as suggested by Bartnikas and Fleming [76], there is very narrow therapeutic window of hepcidin modulation as expression of very high levels of hepcidin in β-thalasemic mice may lead to more severe anemia [92].

Conclusion

Mechanisms underlying obesity-related hypoferremia are different from those of conventional iron deficiency associated with under weight or normal weight individuals. Given the fast rising number of overweight and obesity persons, an insight on the interaction between obesity-related inflammation and iron metabolism is of greater importance towards developing public health strategies for preventing dysmetabolic iron overload syndrome. The

recent advances in the molecular mechanism of iron regulation have also led to the development of alternative iron therapeutic methods for both iron restrictive anemia and/or iron overload. Although hepcidin-based therapeutic methods remain to be tested in clinical trials, it is probably more relevant for obese patients with comorbidities. By contrast, life-style modification (e.g., weight reduction and exercise) may help to restore normal body iron circulation in healthy overweight and obese people with normal hepcidin levels.

Acknowledgments

No conflicts of interest apply in this work. Dr. Jung-Su Chang was supported by the Ministry of science and technology, Taiwan (103-2320-B-038-015) and Taipei Medical University (103TMU-TMUH-11).

References

[1] Low S., Chin M. C., Deurenberg-Yap M. Review on epidemic of obesity. *Ann. Acad. Med. Singapore,* 2009; 38:57.

[2] Datz C., Felder T. K., Niederseer D., Aigner E. Iron homeostasis in the metabolic syndrome. *Eur. J. Clin. Invest.,* 2013; 43:215-224.

[3] Wenzel B., Stults H., Mayer J. Hypoferraemia in obese adolescents. *Lancet,* 1962; 280:327-328.

[4] Pinhas-Hamiel O., Newfield R., Koren I., Agmon A., Lilos P., Phillip M. Greater prevalence of iron deficiency in overweight and obese children and adolescents. *Int. J. Obes.,* 2003; 27:416-418.

[5] Nead K. G., Halterman J. S., Kaczorowski J. M., Auinger P., Weitzman M. Overweight children and adolescents: a risk group for iron deficiency. *Pediatrics,* 2004; 114:104-108.

[6] Micozzi M. S., Albanes D., Stevens R. G. Relation of body size and composition to clinical biochemical and hematologic indices in US men and women. *Am. J. Clin. Nutr.,* 1989; 50:1276-1281.

[7] Lecube A., Carrera A., Losada E., Hernández C., Simó R., Mesa J. Iron deficiency in obese postmenopausal women. *Obesity,* 2006; 14:1724-1730.

[8] del Giudice E. M., Santoro N., Amato A., Brienza C., Calabro P., Wiegerinck E. T., Cirillo G., Tartaglione N., Grandone A., Swinkels D. W. Hepcidin in obese children as a potential mediator of the association between obesity and iron deficiency. *J. Clin. Endocrinol. Metab.,* 2009; 94:5102-5107.

[9] Cepeda-Lopez A. C., Osendarp S. J., Melse-Boonstra A., Aeberli I., Gonzalez-Salazar F., Feskens E., Villalpando S., Zimmermann M. B. Sharply higher rates of iron deficiency in obese Mexican women and children are predicted by obesity-related inflammation rather than by differences in dietary iron intake. *Am. J. Clin. Nutr.,* 2011; 93:975-983.

[10] Yanoff L., Menzie C., Denkinger B., Sebring N., McHugh T., Remaley A., Yanovski J. Inflammation and iron deficiency in the hypoferremia of obesity. *Int. J. Obes.*, 2007; 31:1412-1419.

[11] Menzie C. M., Yanoff L. B., Denkinger B. I., McHugh T., Sebring N. G., Calis K. A., Yanovski J. A. Obesity-related hypoferremia is not explained by differences in reported intake of heme and nonheme iron or intake of dietary factors that can affect iron absorption. *J. Am. Diet Assoc.*, 2008; 108:145-148.

[12] Gartner A., El Ati J., Traissac P., Bour A., Berger J., Landais E., El Hsaïni H., Rayana C. B., Delpeuch F. A double burden of overall or central adiposity and anemia or iron deficiency is prevalent but with little socioeconomic patterning among Moroccan and Tunisian urban women. *J. Nutr.*, 2013;144:87-97.

[13] Ausk K. J., Ioannou G. N. Is obesity associated with anemia of chronic disease? A population-based study. *Obesity*, 2008; 16:2356-2361.

[14] Neymotin F., Sen U. Iron and obesity in females in the United States. *Obesity*, 2011; 19:191-199.

[15] Kozai D., Kabasawa Y., Ebert M., Kiyonaka S., Otani Y., Numata T., Takahashi N., Mori Y., Ohwada T. Transnitrosylation directs TRPA1 Selectivity in N-nitrosamine activators. *Mol. Pharmacol.*, 2014; 85:175-185.

[16] Karl J. P., Lieberman H. R., Cable S. J., Williams K. W., Glickman E. L., Young A. J., McClung J. P. Poor iron status is not associated with overweight or overfat in non-obese pre-menopausal women. *J. Am. Coll. Nutr.*, 2009; 28:37-42.

[17] Eckhardt C., Torheim L., Monterrubio E., Barquera S., Ruel M. The overlap of overweight and anaemia among women in three countries undergoing the nutrition transition. *Eur. J. Clin. Nutr.*, 2007; 62:238-246.

[18] Fanou-Fogny N., Saronga N., Koreissi Y., Dossa R., Melse-Boonstra A., Brouwer I. Weight status and iron deficiency among urban Malian women of reproductive age. *Br. J. Nutr.*, 2011; 105:574-579.

[19] Kordas K., Centeno Z. Y. F., Pachón H., Soto A. Z. J. Being overweight or obese is associated with lower prevalence of anemia among Colombian women of reproductive age. *J. Nutr.*, 2013; 143:175-181.

[20] Cheng H., Bryant C., Cook R., O'Connor H., Rooney K., Steinbeck K. The relationship between obesity and hypoferraemia in adults: a systematic review. *Obes. Rev.*, 2012; 13:150-161.

[21] Nemeth E., Ganz T. Regulation of iron metabolism by hepcidin. *Annu. Rev. Nutr.*, 2006; 26:323-342.

[22] Pietrangelo A. Hereditary hemochromatosis-a new look at an old disease. *N. Engl. J. Med.*, 2004; 350:2383-2397.

[23] Ganz T. Macrophages and systemic iron homeostasis. *J. Innate Immun.*, 2012; 4:446-453.

[24] Nicolas G., Bennoun M., Devaux I., Beaumont C., Grandchamp B., Kahn A., Vaulont S. Lack of hepcidin gene expression and severe tissue iron overload in upstream stimulatory factor 2 (USF2) knockout mice. *Proc. Natl. Acad. Sci. U S A*, 2001; 98:8780-8785.

[25] Krause A., Neitz S., Magert H. J., Schulz A., Forssmann W. G., Schulz-Knappe P., Adermann K. LEAP-1, a novel highly disulfide-bonded human peptide, exhibits antimicrobial activity. *FEBS Lett.*, 2000; 480:147-150.

[26] Bekri S., Gual P., Anty R., Luciani N., Dahman M., Ramesh B., Iannelli A., Staccini-Myx A., Casanova D., Ben Amor I., et al. Increased adipose tissue expression of hepcidin in severe obesity is independent from diabetes and NASH. *Gastroenterology*, 2006; 131:788-796.

[27] Chaston T., Chung B., Mascarenhas M., Marks J., Patel B., Srai S. K., Sharp P. Evidence for differential effects of hepcidin in macrophages and intestinal epithelial cells. *Gut*, 2008; 57:374-382.

[28] Coimbra S., Catarino C., Santos-Silva A. The role of adipocytes in the modulation of iron metabolism in obesity. *Obes. Rev.*, 2013; 14:771-779.

[29] UNICEF, World Health Organization. Iron Deficiency Anaemia: assessment, prevention, and control - A guide for programme manager. WHO, 2001.

[30] Chang J. S., Lin S. M., Huang T. C., Chao J. C., Chen Y. C., Pan W. H., Bai C. H. Serum ferritin and risk of the metabolic syndrome: a population-based study. *Asia Pac. J. Clin. Nutr.*, 2013; 22:400-407.

[31] Hurrell R., Egli I. Iron bioavailability and dietary reference values. *Am. J. Clin. Nutr.*, 2010; 91:1461S-1467S.

[32] Cheng H. L., Bryant C., Cook R., O'Connor H., Rooney K., Steinbeck K. The relationship between obesity and hypoferraemia in adults: a systematic review. *Obes. Rev.*, 2012; 13:150-161.

[33] Aeberli I., Hurrell R. F., Zimmermann M. B. Overweight children have higher circulating hepcidin concentrations and lower iron status but have dietary iron intakes and bioavailability comparable with normal weight children. *Int. J. Obes. (Lond).*, 2009; 33:1111-1117.

[34] Chang J. S., Chen Y. C., Owaga E., Palupi K. C., Pan W. H., Bai C. H. Interactive effects of dietary fat/carbohydrate ratio and Body Mass Index on Iron Deficiency Anemia among Taiwanese women. *Nutrients*, 2014; 6:3929-3941.

[35] Menzie C. M., Yanoff L. B., Denkinger B. I., McHugh T., Sebring N. G., Calis K. A., Yanovski J. A. Obesity-related hypoferremia is not explained by differences in reported intake of heme and nonheme iron or intake of dietary factors that can affect iron absorption. *J. Am. Diet Assoc.*, 2008; 108:145-148.

[36] Sonnweber T., Ress C., Nairz M., Theurl I., Schroll A., Murphy A. T., Wroblewski V., Witcher D. R., Moser P., Ebenbichler C. F., et al. High-fat diet causes iron deficiency via hepcidin-independent reduction of duodenal iron absorption. *J. Nutr. Biochem.*, 2012; 23:1600-1608.

[37] Tsuchiya H., Ebata Y., Sakabe T., Hama S., Kogure K., Shiota G. High-fat, high-fructose diet induces hepatic iron overload via a hepcidin-independent mechanism prior to the onset of liver steatosis and insulin resistance in mice. *Metabolism*, 2013; 62: 62-69.

[38] Zimmermann M. B., Zeder C., Muthayya S., Winichagoon P., Chaouki N., Aeberli I., Hurrell R. F. Adiposity in women and children from transition countries predicts decreased iron absorption, iron deficiency and a reduced response to iron fortification. *Int. J. Obes. (Lond).*, 2008; 32:1098-1104.

[39] Tussing-Humphreys L., Frayn K. N., Smith S. R., Westerman M., Dennis A. L., Nemeth E., Thomson J., Pusatcioglu C. Subcutaneous adipose tissue from obese and lean adults does not release hepcidin in vivo. *Sci. World J.*, 2011; 11:2197-2206.

[40] Tussing-Humphreys L. M., Nemeth E., Fantuzzi G., Freels S., Guzman G., Holterman A. X., Braunschweig C. Elevated systemic hepcidin and iron depletion in obese premenopausal females. *Obesity (Silver Spring),* 2010; 18:1449-1456.

[41] Zhang Y., Proenca R., Maffei M., Barone M., Leopold L., Friedman J. M. Positional cloning of the mouse obese gene and its human homologue. *Nature,* 1994; 372:425-432.

[42] del Giudice E. M., Santoro N., Amato A., Brienza C., Calabro P., Wiegerinck E. T., Cirillo G., Tartaglione N., Grandone A., Swinkels D. W., Perrone L. Hepcidin in obese children as a potential mediator of the association between obesity and iron deficiency. *J. Clin. Endocrinol. Metab.,* 2009; 94:5102-5107.

[43] Amato A., Santoro N., Calabro P., Grandone A., Swinkels D. W., Perrone L., del Giudice E. M. Effect of body mass index reduction on serum hepcidin levels and iron status in obese children. *Int. J. Obes. (Lond.),* 2010; 34:1772-1774.

[44] Chung B., Matak P., McKie A. T., Sharp P. Leptin increases the expression of the iron regulatory hormone hepcidin in HuH7 human hepatoma cells. *J. Nutr.,* 2007; 137:2366-2370.

[45] Hamann A., Matthaei S. Regulation of energy balance by leptin. *Exp. Clin. Endocrinol. Diabetes,* 1996; 104:293-300.

[46] Dongiovanni P., Fracanzani A. L., Fargion S., Valenti L. Iron in fatty liver and in the metabolic syndrome: a promising therapeutic target. *J. Hepatol.,* 2011; 55:920-932.

[47] Ganz T. Hepcidin and iron regulation, 10 years later. *Blood,* 2011; 117:4425-4433.

[48] Kemna E. H., Kartikasari A. E., van Tits L. J., Pickkers P., Tjalsma H., Swinkels D. W. Regulation of hepcidin: insights from biochemical analyses on human serum samples. *Blood Cells Mol. Dis.,* 2008; 40:339-346.

[49] Kemna E. H., Tjalsma H., Willems H. L., Swinkels D. W. Hepcidin: from discovery to differential diagnosis. *Haematologica,* 2008; 93:90-97.

[50] Means R. T., Jr. Hepcidin and anaemia. *Blood Rev.,* 2004; 18:219-225.

[51] Pinto J. P., Ribeiro S., Pontes H., Thowfeequ S., Tosh D., Carvalho F., Porto G. Erythropoietin mediates hepcidin expression in hepatocytes through EPOR signaling and regulation of C/EBPalpha. *Blood,* 2008; 111:5727-5733.

[52] Nemeth E., Rivera S., Gabayan V., Keller C., Taudorf S., Pedersen B. K., Ganz T. IL-6 mediates hypoferremia of inflammation by inducing the synthesis of the iron regulatory hormone hepcidin. *J. Clin. Invest.,* 2004; 113:1271-1276.

[53] Nemeth E., Valore E. V., Territo M., Schiller G., Lichtenstein A., Ganz T. Hepcidin, a putative mediator of anemia of inflammation, is a type II acute-phase protein. *Blood,* 2003; 101:2461-2463.

[54] Sun K., Kusminski C. M., Scherer P. E. Adipose tissue remodeling and obesity. *J. Clin. Invest.,* 2011; 121:2094-2101.

[55] Russo H. M., Wickenheiser K. J., Luo W., Ohman M. K., Franchi L., Wright A. P., Bodary P. F., Nunez G., Eitzman D. T. P-selectin glycoprotein ligand-1 regulates adhesive properties of the endothelium and leukocyte trafficking into adipose tissue. *Circ. Res.,* 2010; 107:388-397.

[56] Sato C., Shikata K., Hirota D., Sasaki M., Nishishita S., Miyamoto S., Kodera R., Ogawa D., Tone A., Kataoka H. U., et al. P-selectin glycoprotein ligand-1 deficiency is protective against obesity-related insulin resistance. *Diabetes,* 2011; 60:189-199.

[57] Zhang Z., Zhang F., An P., Guo X., Shen Y., Tao Y., Wu Q., Zhang Y., Yu Y., Ning B., et al. Ferroportin1 deficiency in mouse macrophages impairs iron homeostasis and inflammatory responses. *Blood*, 2011; 118:1912-1922.

[58] Kasvosve I. Effect of ferroportin polymorphism on iron homeostasis and infection. *Clin. Chim. Acta*, 2013; 416:20-25.

[59] Hintze K. J., McClung J. P. Hepcidin: A critical regulator of iron metabolism during hypoxia. *Adv. Hematol.*, 2011; 2011:510304.

[60] Nicolas G., Chauvet C., Viatte L., Danan J. L., Bigard X., Devaux I., Beaumont C., Kahn A., Vaulont S. The gene encoding the iron regulatory peptide hepcidin is regulated by anemia, hypoxia, and inflammation. *J. Clin. Invest.*, 2002; 110:1037-1044.

[61] Hodges V. M., Rainey S., Lappin T. R., Maxwell A. P. Pathophysiology of anemia and erythrocytosis. *Crit. Rev. Oncol. Hematol.*, 2007; 64:139-158.

[62] Freeman E. W., Sammel M. D., Lin H., Gracia C. R. Obesity and reproductive hormone levels in the transition to menopause. *Menopause*, 2010; 17:718-726.

[63] Olson M. B., Shaw L. J., Kaizar E. E., Kelsey S. F., Bittner V., Reis S. E., Smith K., Braunstein G. D., Berga S. L., Johnson B. D., et al. Obesity distribution and reproductive hormone levels in women: a report from the NHLBI-sponsored WISE study. *J. Womens Health (Larchmt)*, 2006; 15:836-842.

[64] Siiteri P. K. Adipose tissue as a source of hormones. *Am. J. Clin. Nutr.*, 1987; 45:277-282.

[65] Purohit A., Newman S. P., Reed M. J. The role of cytokines in regulating estrogen synthesis: implications for the etiology of breast cancer. *Breast Cancer Res.*, 2002; 4:65-69.

[66] Yang Q., Jian J., Katz S., Abramson S. B., Huang X. 17beta-Estradiol inhibits iron hormone hepcidin through an estrogen responsive element half-site. *Endocrinology*, 2012; 153:3170-3178.

[67] Liu G., Men P., Kenner G. H., Miller S. C. Age-associated iron accumulation in bone: implications for postmenopausal osteoporosis and a new target for prevention and treatment by chelation. *Biometals*, 2006; 19:245-251.

[68] Hou Y., Zhang S., Wang L., Li J., Qu G., He J., Rong H., Ji H., Liu S. Estrogen regulates iron homeostasis through governing hepatic hepcidin expression via an estrogen response element. *Gene*, 2012; 511:398-403.

[69] Ikeda Y., Tajima S., Izawa-Ishizawa Y., Kihira Y., Ishizawa K., Tomita S., Tsuchiya K., Tamaki T. Estrogen regulates hepcidin expression via GPR30-BMP6-dependent signaling in hepatocytes. *PLoS One*, 2012; 7:e40465.

[70] Guo W., Bachman E., Li M., Roy C. N., Blusztajn J., Wong S., Chan S. Y., Serra C., Jasuja R., Travison T. G., et al. Testosterone administration inhibits hepcidin transcription and is associated with increased iron incorporation into red blood cells. *Aging Cell*, 2013; 12:280-291.

[71] Liu Z., Ye F., Zhang H., Gao Y., Tan A., Zhang S., Xiao Q., Zhang B., Huang L., Ye B., et al. The association between the levels of serum ferritin and sex hormones in a large scale of Chinese male population. *PLoS One*, 2013; 8:e75908.

[72] Baumgartner J., Smuts C. M., Aeberli I., Malan L., Tjalsma H., Zimmermann M. B. Overweight impairs efficacy of iron supplementation in iron-deficient South African children: a randomized controlled intervention. *Int. J. Obes. (Lond.)*, 2013; 37:24-30.

[73] Sanad M., Osman M., Gharib A. Obesity modulate serum hepcidin and treatment outcome of iron deficiency anemia in children: a case control study. *Ital. J. Pediatr.,* 2011; 37:34.

[74] Cheng H. L., Griffin H. J., Bryant C. E., Rooney K. B., Steinbeck K. S., O'Connor H. T. Impact of diet and weight loss on iron and zinc status in overweight and obese young women. *Asia Pac. J. Clin. Nutr.,* 2013; 22: 574-582.

[75] Chang J. S., Li Y. L., Lu C. H., Owaga E., Chen W. Y., Chiou H. Y.: Interleukin-10 as a potential regulator of hepcidin homeostasis in overweight and obese children: a cross-sectional study in Taiwan. *Nutrition,* 2014; 30:1165-1170.

[76] Bartnikas T. B., Fleming M. D. A tincture of hepcidin cures all: the potential for hepcidin therapeutics. *J. Clin. Invest.,* 2010; 120:4187-4190.

[77] Cooke K. S., Hinkle B., Salimi-Moosavi H., Foltz I., King C., Rathanaswami P., Winters A., Steavenson S., Begley C. G., Molineux G., Sasu B. J. A fully human anti-hepcidin antibody modulates iron metabolism in both mice and nonhuman primates. *Blood,* 2013; 122:3054-3061.

[78] Fung E., Nemeth E. Manipulation of the hepcidin pathway for therapeutic purposes. *Haematologica,* 2013; 98:1667-1676.

[79] Ganz T., Nemeth E. The hepcidin-ferroportin system as a therapeutic target in anemias and iron overload disorders. *Hematology Am. Soc. Hematol. Educ. Program.,* 2011; 2011:538-542.

[80] Kong W. N., Gao G., Chang Y. Z.: Hepcidin and sports anemia. *Cell Biosci.,* 2014; 4:19.

[81] Silvestri L. Inhibiting the hepcidin inhibitor for treatment of iron overload. *Blood,* 2013; 121:1068-1069.

[82] Wozney J. M., Rosen V., Celeste A. J., Mitsock L. M., Whitters M. J., Kriz R. W., Hewick R. M., Wang E. A. Novel regulators of bone formation: molecular clones and activities. *Science,* 1988; 242:1528-1534.

[83] Nemeth E. Anti-hepcidin therapy for iron-restricted anemias. *Blood,* 2013; 122:2929-2931.

[84] Tussing-Humphreys L. M., Nemeth E., Fantuzzi G., Freels S., Holterman A. X., Galvani C., Ayloo S., Vitello J., Braunschweig C. Decreased serum hepcidin and improved functional iron status 6 months after restrictive bariatric surgery. *Obesity (Silver Spring),* 2010; 18:2010-2016.

[85] Martinelli N., Traglia M., Campostrini N., Biino G., Corbella M., Sala C., Busti F., Masciullo C., Manna D., Previtali S., et al. Increased serum hepcidin levels in subjects with the metabolic syndrome: a population study. *PLoS One,* 2012; 7:e48250.

[86] Fung E., Sugianto P., Hsu J., Damoiseaux R., Ganz T., Nemeth E. High-throughput screening of small molecules identifies hepcidin antagonists. *Mol. Pharmacol.,* 2013; 83:681-690.

[87] Schwoebel F., van Eijk L. T., Zboralski D., Sell S., Buchner K., Maasch C., Purschke W. G., Humphrey M., Zollner S., Eulberg D., et al. The effects of the anti-hepcidin Spiegelmer NOX-H94 on inflammation-induced anemia in cynomolgus monkeys. *Blood,* 2013; 121:2311-2315.

[88] Song S. N., Tomosugi N., Kawabata H., Ishikawa T., Nishikawa T., Yoshizaki K. Down-regulation of hepcidin resulting from long-term treatment with an anti-IL-6

receptor antibody (tocilizumab) improves anemia of inflammation in multicentric Castleman disease. *Blood,* 2010; 116:3627-3634.

[89] Zhang S. P., Wang Z., Wang L. X., Liu S. J. AG490: an inhibitor of hepcidin expression in vivo. *World J. Gastroenterol.,* 2011; 17:5032-5034.

[90] Shin D. Y., Chung J., Joe Y., Pae H. O., Chang K. C., Cho G. J., Ryter S. W., Chung H. T. Pretreatment with CO-releasing molecules suppresses hepcidin expression during inflammation and endoplasmic reticulum stress through inhibition of the STAT3 and CREBH pathways. *Blood,* 2012; 119:2523-2532.

[91] Zumbrennen-Bullough K. B., Wu Q., Core A. B., Canali S., Chen W., Theurl I., Meynard D., Babitt J. L. MicroRNA-130a is up-regulated in mouse liver by iron deficiency and targets the bone morphogenetic protein (BMP) receptor ALK2 to attenuate BMP signaling and hepcidin transcription. *J. Biol. Chem.,* 2014; 289:23796-23808.

[92] Gardenghi S., Ramos P., Marongiu M. F., Melchiori L., Breda L., Guy E., Muirhead K., Rao N., Roy C. N., Andrews N. C., et al. Hepcidin as a therapeutic tool to limit iron overload and improve anemia in beta-thalassemic mice. *J. Clin. Invest.,* 2010; 120:4466-4477.

[93] Schmidt P. J., Toudjarska I., Sendamarai A. K., Racie T., Milstein S., Bettencourt B. R., Hettinger J., Bumcrot D., Fleming M. D. An RNAi therapeutic targeting Tmprss6 decreases iron overload in Hfe(-/-) mice and ameliorates anemia and iron overload in murine beta-thalassemia intermedia. *Blood,* 2013; 121:1200-1208.

In: Human Health and Nutrition
Editor: Sergej M. Ostojic
ISBN: 978-1-63482-823-9

Chapter II

Determinants of Zinc Transport in Humans: Zinc Status, Exercise, Inflammation and Chronic Diseases

***Anna Chu*[1], *Meika Foster*[1] *and Samir Samman*[1,2,*]**
[1]Department of Human Nutrition, University of Otago, New Zealand
[2]Discipline of Nutrition and Metabolism, School of Molecular Bioscience, University of Sydney, Australia

Abstract

Zinc is involved in numerous structural, catalytic and regulatory functions of the body. The role of zinc in chronic diseases, such as Type 2 diabetes mellitus (DM) and cardiovascular disease (CVD) and associated aspects such as inflammation, has attracted much research interest. Recent findings also suggest that the prescription of exercise in the prevention and management of chronic diseases enhances the physiological demand for zinc. The present review aims to explore the determinants of whole body and cellular zinc transport in humans, as related to chronic diseases, inflammation and exercise. Whole body zinc homeostasis is maintained primarily at the level of the gastrointestinal tract and liver. The bioavailability and amount of dietary zinc consumed directly influence zinc uptake and endogenous zinc secretion in the gut. With no defined zinc store within the body, the assessment of zinc status in humans is complicated. The search for a sensitive zinc biomarker may benefit from the study of cellular zinc homeostasis. Zinc transporters and metallothionein act in a coordinated fashion to mediate zinc transport within the cell. Perturbations in cellular zinc homeostasis have been linked to CVD and Type 2 DM, as part of the disease pathologies. In a similar vein, low-grade inflammation that co-exists with chronic diseases has been shown to mediate changes in zinc homeostasis. While exercise has shown beneficial effects on inflammation, it can also induce suboptimal zinc status, which exacerbates the deleterious effects of deranged zinc homeostasis in chronic diseases. Limited studies have explored the cellular zinc

* E-mail: samir.samman@otago.ac.nz.

transport under exercise stress in humans, hence further research is required to ascertain the connection between whole body zinc homeostasis and cellular zinc transport network under conditions of health and chronic diseases.

Keywords: Zinc; Inflammation; Exercise: Diabetes mellitus; Cardiovascular disease

Introduction

The interest in metabolism and function of zinc in humans was amplified by the recognition of zinc deficiency in the 1960s [1]. Since then, zinc has been implicated in numerous metabolic functions, including energy metabolism, immunity and antioxidant activity. Almost 10% of the proteins found in the human body have been predicted to bind zinc *in vivo* [2]. The extensive involvement of zinc in biology can be categorized into structural, catalytic and regulatory roles. The importance of zinc ions in gene transcription and protein structure was clarified further through the discovery of 'zinc fingers'. 'Zinc fingers' are protein motifs characterized by a zinc ion coordinated with a combination of cysteine and histidine residues which maintain structural integrity of the protein [3]. In addition, 'zinc fingers' also have the ability to serve as sensors of cellular zinc ions. An example of an intracellular zinc sensor in humans is metal-responsive transcription factor-1 (MTF-1). This transcription factor represents the primary regulatory control of cellular zinc homeostasis at the transcription level [4]. Although proteins containing 'zinc fingers' were initially recognized for their interaction with nucleic acids, recent evidence suggests that proteins with 'zinc finger' motifs also play a crucial role in other biological functions, for instance, in mediating protein-protein interactions [5].

In humans, zinc serves a catalytic role in over 50 metalloenzymes, spanning across all six classes [6]. Carbonic anhydrase (CA) was the first discovered zinc metalloenzyme; other enzymes include carboxypeptidase, alkaline phosphatases (ALP), transferases, ligases, lyases, isomerases, DNA/RNA polymerase, reverse transcriptase and superoxide dismutase (SOD) [7]. Recent findings also suggest that zinc can modulate the activities of proteins that were not identified as zinc proteins. For example, transient fluctuations in cytoplasmic zinc concentration can activate or inhibit protein tyrosine phosphatases [8]. Many proteins that can be modulated by zinc are mediators of cellular signaling pathways, supporting the role of zinc ions as second messengers within the cell, akin to calcium. Examples of the major signaling pathways in which zinc participates include the redox signaling pathways [9] where zinc, despite being a redox inert metal, provides an overall antioxidant effect.

Overt zinc deficiency often manifests with clinical signs, such as impaired immunity and growth retardation. In low- and middle-income countries (LMIC), zinc deficiency in young children contributes to the rates of morbidity and mortality arising from persistent diarrhea and pneumonia [10]. Other manifestations of zinc deficiency include diverse forms of skin lesions, impaired wound healing, hypogeusia, behavioral disturbances and night blindness. In contrast, marginal zinc deficiency presents with a spectrum of ambiguous signs and symptoms. The consequences of suboptimal zinc status are suggested to impact many chronic diseases, including Type 2 diabetes mellitus (DM) [11] and cardiovascular diseases (CVD) [12]. The insidious effects of marginal zinc deficiency are reflective of the numerous physiological roles in which zinc is involved.

Whole Body Zinc Homeostasis

Absorption and Excretion

Of the dietary zinc that is absorbed, the majority is delivered through the small intestine, primarily at the distal duodenum or proximal jejunum. The mode of zinc absorption involves both saturable and passive mechanisms. Current evidence suggests that the saturable pathway is responsible for zinc absorption of up to 7-9 mg of daily ingested zinc [13] and requires the expression of specific zinc transporters and divalent metal transporters. At dietary zinc levels beyond 9 mg elemental zinc/d, passive transport involving paracellular zinc diffusion becomes the prominent mean of zinc absorption [14]. The fractional absorption of zinc appears to be dependent on total dietary zinc intake, rather than the host's intrinsic zinc status [15].

In addition to dietary zinc intake, a major factor in the determination of zinc absorption within the gut is the bioavailability of zinc amongst the chyme formed from digestion. Factors that influence zinc bioavailability and their effects on fractional zinc absorption were reviewed recently [17]. The absorption of zinc can be hindered through direct competition with other nutrients or by the presence of anti-nutrients, such as phytate [18], which form insoluble zinc complexes within the lumen. *In vitro* studies suggest that calcium and magnesium can form precipitates of phytate, enabling high affinity binding of zinc and thereby decreasing the availability of zinc for absorption.

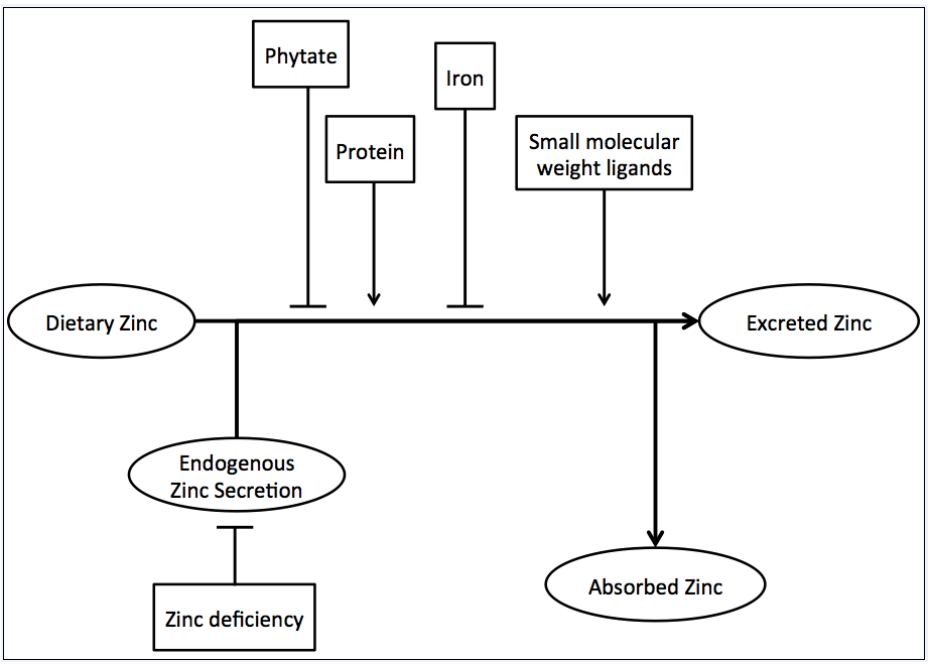


Figure 1. Factors affecting bioavailability and absorption of zinc within the gastrointestinal tract [16]. Zinc absorption in the lumen is determined by exogenous factors such as phytate, protein, iron and small molecular weight ligands, such as citrate and histidine. Endogenous zinc secretion also contributes to the luminal zinc concentration. Compensatory mechanisms in zinc deficiency modulate the amount of zinc secreted in mucosal cells, bile and pancreatic secretions.

Moreover, high iron to zinc ratio in the diet, typically achieved by iron supplementation, has been shown to decrease zinc bioavailability significantly and ultimately impact on zinc status [19]. In contrast, zinc absorption may be enhanced by the formation of coordination complexes between zinc and small molecular weight ligands. Specifically, the amino acids histidine and cysteine can form stable complexes with zinc in the lumen, which improves the efficiency of zinc absorption [20]. The introduction of animal protein into plant-based meals can significantly improve the bioavailability and absorption of zinc [21]. Figure 1 summarizes zinc homeostasis in the gastrointestinal tract with emphasis on the factors influencing the bioavailability of zinc.

In order to estimate zinc bioavailability in the diet, the World Health Organization (WHO) identified 3 categories of zinc bioavailability according to the food source of zinc, phytic acid:zinc (PA:Zn) molar ratio and total calcium intake [23]. Figure 2 shows zinc content and PA:Zn molar ratios in common foods. A high fractional zinc absorption of 50% dietary zinc is estimated for a highly refined diet with PA:Zn molar ratio of less than 5. In contrast, an unrefined diet, which corresponds with a PA:Zn molar ratio of greater than 15, reduces estimated zinc absorption by 70%. However, most of the studies identified from the WHO report derived fractional zinc absorption from single meals, which may not be representative of the total diet.

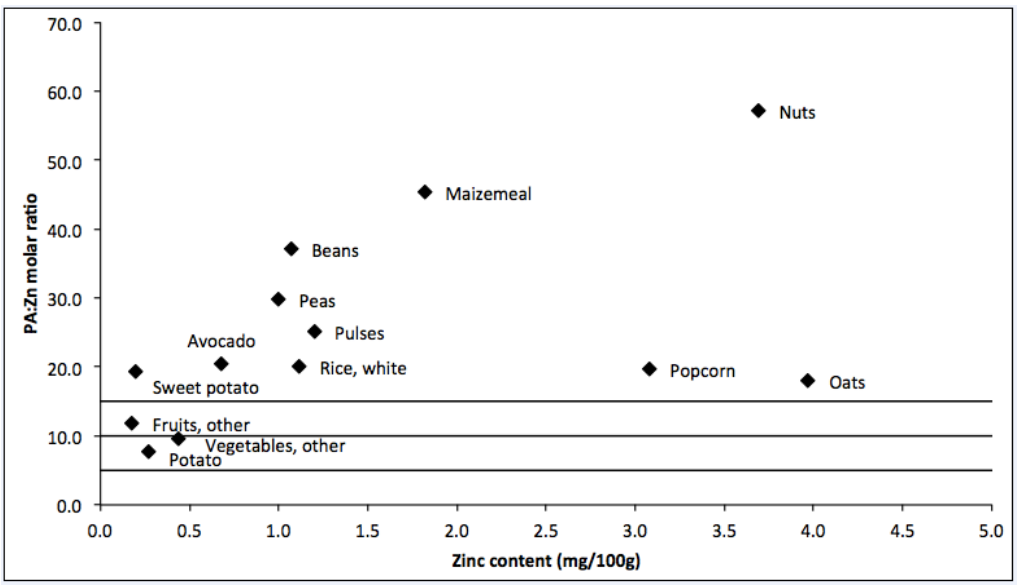


Figure 2. Zinc content (mg/100 g edible portion) and phytic acid:zinc (PA:Zn) molar ratios of selected foods. PA:Zn molar ratios of 5, 10 and 15 are shown to represent high, moderate and low zinc bioavailability foods, respectively. While foods with higher PA:Zn molar ratio are predicted to provide a lower percentage of bioavailable zinc in the gut, higher total zinc content in these foods may compensate for the reduced efficiency of zinc absorption, resulting in a greater amount of total zinc absorbed. Data adapted from [22].

In 2004, the International Zinc Nutrition Consultative Group developed two categories for estimating dietary zinc absorption in the context of the total diet [24]. Mixed diets and refined vegetarian diets, with PA:Zn molar ratio of 4-18, were grouped into a single category, with fractional zinc absorption predicted to be 26% in men and 34% in women. The second category, of lower zinc bioavailability, consists of unrefined, cereal-based diets with PA:Zn

molar ratio greater than 18. However, the limited number of studies identified in the latter category limits the interpretation of the cut-offs.

The excretion of zinc in humans is primarily through fecal zinc originating from dietary sources, as well as endogenous zinc released in bile, pancreatic secretions and the turnover of mucosal cells. Regulation of fecal excretion is crucial in the maintenance of whole body zinc homeostasis [25]. In zinc depletion and supplementation studies, the amount of fecal zinc excreted is inversely correlated to dietary zinc intake, through changes in fractional zinc absorption and endogenous zinc secretion. Under marginal habitual dietary zinc intakes (< 8 mg/d Zn), a reduction in endogenous zinc excretion appears to be the initial point of homeostatic adjustment [26]. It is unclear whether the mechanism of action is through increased efficiency in the reabsorption of zinc or a decrease in endogenous zinc secretion. In comparison to fecal zinc losses, urinary zinc represents a negligible route of excretion, which remains largely unchanged with small fluctuations in dietary zinc intakes, but responds to increased zinc exposure [27]. However, urinary zinc losses become significant in conditions that increase urine production, such as uncontrolled DM [11], or induce catabolism, for example severe burns, trauma/surgery.

Tissue Distribution

Zinc is found in all body organs and tissues, approximating to 1.2-2.3 grams of zinc in a human adult [7, 28]. Zinc primarily exists intracellularly, with close to 95% of total body zinc found within different body tissues [29]. The remainder of zinc resides in extracellular fluid, such as plasma or serum zinc, which represents 0.1% of total zinc within the body. While the highest concentrations of zinc are observed in the choroid of the eye and the prostate gland, the majority of zinc within the body is found in the musculoskeletal system [7]. Zinc concentration within erythrocytes is a number of magnitudes higher than the concentration of blood plasma. The majority of erythrocyte zinc is associated with CA, with some found incorporated in the cell membrane [14].

After absorption in the intestinal tract, zinc is bound to albumin and delivered to the systemic circulation via the hepatic portal system [30]. Approximately 30-40% of absorbed zinc in the portal blood exchanges with the liver, where zinc can be sequestered by hepatic metallothionein (MT). The liver represents a pool of readily exchangeable zinc in the body. The presence of hormones and inflammation can initiate the redistribution of zinc, leading to changes in hepatic zinc store [14].

The systemic zinc concentration is maintained within 10-18 μmol/L with diurnal variations across the day [24]. Albumin binds to ~75% of zinc found in plasma due to its abundance in blood and high affinity to zinc. Recent observations suggest that the binding and release of zinc from albumin may be modulated by fatty acids in the circulation [31]. The remaining zinc found in plasma is bound to α-2 macroglobulin (~25%) or with low molecular weight complexes (~1%), such as cysteine and histidine. The uptake of zinc into peripheral tissue appears to be dependent on the properties of zinc carriers. From the systemic circulation, zinc is transported into interstitial fluid through the endothelium via one of two transport systems [32]. Zinc-albumin complex is transported mainly via a saturable pathway by receptor-mediated transcytotic vesicles. The alternative endothelial transport system is a non-saturable pathway where zinc complexed with albumin or histidine is transported through

the intercellular junctions or by nonselective, bulk-fluid transcytosis. Under sufficient zinc status, plasma zinc is predicted to turn over ~150 times, which increases by one third when challenged with very low dietary zinc intake (< 5 μmol/d) [33]. Zinc isotopic studies have identified a rapidly exchangeable zinc pool which represents the entire pool of zinc in the plasma in addition to a portion located within the liver [34]. This kinetically defined metabolic zinc pool reflects habitual dietary zinc intake and may be useful in predicting total absorbed zinc in the gastrointestinal tract [25].

The musculoskeletal system contains 90% of total zinc in the body, of which two-thirds reside in skeletal muscles [29]. The majority of zinc in muscles is found within protein complexes, where zinc provides structural stability and enzymatic activities of metalloenzymes such as lactate dehydrogenase, SOD and CA. At rest, the turnover of zinc within muscles and bone has been suggested to be slower than the liver and other metabolically active tissues [34]. During exercise, muscular contractions can disrupt cellular structures [35] which leads to the release of proteins and ions, such as zinc, from myocytes. In recovery from exercise, the initial stages of muscle repair occur with monocytes and leukocytes infiltrating muscle cells, initiating cytokine production and stimulating the subsequent inflammatory response [36]. Inflammatory cytokines have been shown to regulate the expression of cellular zinc transporters in a number of tissues and thereby alter whole body zinc homeostasis [37].

Biomarkers of Zinc Status

Systemic zinc concentration, analyzed in serum or plasma, is currently the recommended biomarker when assessing zinc status within a population [38, 39]. In a recent meta-analysis which explored the relationship between dietary and/or supplemental zinc and plasma zinc concentration in healthy populations, a doubling of zinc intake corresponded with a 6% increase in plasma or serum zinc concentration [40]. However, it is well-known that the systemic zinc concentration can be confounded by factors other than zinc status, which limits its usefulness as a diagnostic biomarker in individuals. Diurnal variation and interaction between zinc and other nutrients or drugs, including oral contraceptive agents, are examples of factors known to impact serum zinc concentration in healthy populations [41]. Fluctuations in hormones, such as insulin and leptin as a result of fasting or the post-prandial state, can also influence zinc concentration in the circulation. Inflammation and metabolic stress due to exercise, infection and chronic diseases are conditions which may cause redistribution of zinc in the body, typically causing a decrease in plasma zinc concentration [42].

Zinc concentrations in various blood components, such as erythrocytes, mononuclear cells, polymorphonuclear cells and platelets were determined to be poor indicators of zinc status in meta-analyses of zinc supplementation and depletion studies [38]. Moreover, there is limited evidence to support the use of other biological tissues, such as saliva and nail, in the assessment of zinc status in humans. In contrast, data from zinc supplementation studies suggests that urinary zinc excretion may be an effective biomarker of extreme dietary zinc intakes [38], with a dose-response effect observed for zinc supplementation between 15 and 50 mg/d. Urinary zinc excretion responds well during severe zinc depletion (< 2 mg/d) [43, 44] or higher intakes [27], however lacks the sensitivity to differentiate dietary zinc intakes around the recommended dietary zinc intakes (8-14 mg/d) [45]. Similar to urinary zinc

excretion, hair zinc appears to respond to zinc supplementation with limited data supporting its validity in zinc depletion.

Functional biochemical tests, specifically enzymatic activities of zinc-dependent metalloenzymes, also have been explored in the estimation of zinc status. Commonly analyzed enzymes include plasma ALP and erythrocyte Cu-Zn SOD. Meta-analysis of zinc supplementation and depletion trials revealed plasma ALP activity to be a poor zinc biomarker [38]. Zinc supplementation generally decreases erythrocyte Cu-Zn SOD activity, primarily due to an antagonistic relationship between dietary zinc intake and copper absorption [46]. Similar to the systemic zinc concentration, a number of factors influences enzymatic activities, which complicates the delineation of relationship between zinc status and activities of enzymes.

Given the limitations of the current zinc biomarkers, recent attention in this field has focused on the assessment of novel biomarkers, in both global genomic [47] and proteomic [48] studies. Recent investigations into the responses of serum microRNAs in zinc depletion and subsequent repletion have revealed possible novel biomarkers of zinc status [49]. The expression of zinc transporter and MT genes have been used in zinc supplementation and depletion studies to determine the relationships between mediators of cellular zinc homeostasis and whole body zinc status. *In vitro* studies have shown changes in cellular zinc transporter and MT expressions in response to different concentrations of zinc incubated in the media [50, 51]. However, *in vitro* regulatory response in zinc transporter and MT expressions does not appear to correspond with the effects observed in humans after zinc supplementation [51]. Hence, further studies are required to determine the usefulness of zinc transporter and MT measurements as zinc biomarkers in humans.

The use of the peripheral blood mononuclear cells (PBMC) transcriptome as a marker of interest in nutrigenomic studies has gained momentum recently, due to the ease of sampling and analyses in humans. To date, two distinct approaches have been used to explore the changes in PBMC gene expression in nutritional intervention studies. In the first approach, the analysis of PBMC provides an insight into the role of inflammation that forms part of the pathology of many chronic diseases. Examples of studies that are pertinent include supplementation trials of immunomodulating nutrients, such as zinc and polyphenols, and studies that explore acute immune responses to nutritional challenges [52]. The alternative approach uses PBMC as a surrogate model for other biological tissues that are difficult to obtain in humans, such as those of the liver and pancreas. The underlying assumption of the latter approach is that the transcriptomic responses of PBMC and the target tissue are similar. While this relationship is reasonably established in genes regulating lipid metabolism [53, 54], it remains largely unexplored in the mediators of cellular zinc homeostasis. Given that zinc mostly resides intracellularly within the liver and musculoskeletal system, the search for a novel zinc biomarker will benefit from future studies that aim to identify similarities in cellular zinc metabolism between PBMC and peripheral tissues.

Whole body zinc homeostasis is influenced by dietary factors, such as zinc bioavailability in the gut lumen, and intrinsic determinants, such as inflammation and zinc status. As alluded to above, the assessment of zinc status in humans is complicated by the tight regulatory control of zinc homeostasis. The study of the mediators of cellular zinc homeostasis may shed light on the regulatory mechanism of zinc transport, in addition to advancing the discovery of novel zinc biomarkers in humans.

Cellular Zinc Homeostasis

The regulation of cellular zinc is under tight homeostatic control. While the circulating zinc concentration in zinc-sufficient conditions is maintained within the micromolar range (around 10-18 µmol/L) [24], intracellular free zinc concentrations have been suggested to be multiple of magnitudes lower, i.e. in picomolar concentrations. Fluorescence spectroscopy of free zinc ions within the cell has identified compartmentalisation of zinc in subcellular locations, such as the Golgi apparatus and mitochondria. The accumulation of zinc within different subcellular organelles is important in eliciting rapid changes in cytoplasmic zinc concentration. Given the extensive role of zinc in modulating the activities of proteins within many cellular signaling pathways, the maintenance of cytoplasmic zinc concentration is important in the propagation of cellular signals, among other functions of zinc. Cellular zinc homeostasis is regulated primarily by two families of zinc transporters and MT. In addition, recent evidence suggests that a minor route of zinc transport can be mediated through calcium-conducting channels.

Zinc Transporters

To date, a total of 24 zinc transporters have been identified, classified into two families of solute carriers (SLC) according to their proposed function and structure. The mechanism of zinc transport through plasma and subcellular membranes is via secondary active transport, whereby the co-transport of solutes occurs without direct coupling of adenosine triphosphate, suggesting that the zinc transport process is energy independent. Both hydrogen (H^+) and bicarbonate (HCO_3^-) ions are proposed to counter-transport with zinc via the action of zinc transporters; hence acid-base balance is possibly intrinsically linked to cellular zinc homeostasis.

Members from the Zinc Transporter (ZnT; SLC30) family are responsible largely for decreasing cytoplasmic zinc concentration by zinc efflux into extracellular space or subcellular compartments, such as the Golgi apparatus and endoplasmic reticulum. Thus far, most of the 10 identified ZnTs reside primarily on the subcellular membranes (Table 1). Members from the ZnT family initially were predicted to form homodimers, with the exception of ZnT5 and ZnT6, which form heterodimers. However, a recent *in situ* study using bimolecular fluorescence complementation and visualization of intracellular free zinc demonstrated the ability of various ZnTs, specifically ZnT1-4, to form heterodimers [55]. Although the crystal structure is yet to be resolved, the structure for ZnT proteins can be estimated from YiiP, a cation-diffusion facilitator of zinc arising from *Escherichia coli* [56]. ZnT proteins are depicted typically with six transmembrane domains, with both the N- and C-termini located intracellularly. Multiple sites of zinc binding have been suggested, one of which is located on the cytosolic domain and can trigger conformational change, facilitating zinc transport through the protein [57]. To date, only ZnT10 out of the ZnT family appears to have the ability to transport divalent metals other than zinc, specifically manganese.

While dietary zinc intake appears to regulate the expression and activity of some ZnTs, other factors also have been shown to modulate ZnT proteins, in a tissue specific manner.

Table 1. Localization, transcriptional regulation and association with pathology of Zinc Transporters (ZnT)[1]

Zinc transporter (ZnT; SLC30)	Tissue distribution	Subcellular localization	Transcriptional regulation (response)	Single nucleotide polymorphism (SNP) or mutations of genes and their association with pathology in humans
ZnT1	Ubiquitous	Plasma membrane, vesicles	All: zinc depletion (↓) Blood cells: zinc excess (↑)	
ZnT2	Mammary gland, prostate, retina, pancreas, small intestine, kidney	Endosomal / lysosomal / secretory vesicles, plasma membrane	Mammary cells: prolactin (↑), glucorticoid hormone (↑), zinc excess (↑) Others: zinc depletion (↓)	Mammary epithelial cell zinc secretion (H54R mutation) [75]
ZnT3	Brain, testes, pancreas	Synaptic vesicles, intracellular vesicles	Pancreatic cells: glucose (↑), zinc depletion (↑), IL-1β (↓), IFN-γ (↓), IL-1β + TNF-α + IFN-γ (↓) [76]	Schizophrenia in females (rs11126936, rs6547521, rs2083363, and rs11126931) [77] Verbal learning memory (rs11126936) [78]
ZnT4	Ubiquitous	Secretory vesicle, plasma membrane	Small intestine: zinc depletion (↓), cell differentiation (↑) T-cells: PHA (↑) Pancreatic cells: IFN-γ (↓) [76]	
ZnT5	Ubiquitous	Golgi apparatus, secretory vesicles, plasma membrane	Small intestine: zinc excess (↓)	
ZnT6	Ubiquitous	Golgi apparatus, vesicles	Small intestine: zinc depletion (↓), zinc excess (↑) Pancreatic cells: IL-1β (↓), IFN-γ (↓) [76]	
ZnT7	Ubiquitous	Golgi apparatus, vesicles	Peritoneal mesothelial cells: glucose (↑) [79]	
ZnT8	Pancreas, thyroid, adrenal gland, testes	Secretory granules	Pancreatic cells: IL-1β (↓), IFN-γ (↓), IL-1β + TNF-α + IFN-γ (↓) [76]	Type 2 diabetes mellitus (rs13266634, rs11558471, rs3802177) [80–82] Intraocular pressure (rs7815720) [83]
ZnT9	Ubiquitous	Cytoplasm, nucleus	Blood cells: ultraviolet-B radiation (↓) [84]	
ZnT10	Brain, retina, liver	Golgi apparatus, endosomes, plasma membrane [85]	Neurons: IL-6 (↓) [86]	Manganese toxicity and associated symptoms i.e. hepatic cirrhosis, polycythemia, dystonia, and Parkinsonism [87]

[1]Adapted from [37, 61, 88] and updated [75–87].

Abbreviations: IL, interleukin; IFN, interferon; TNF, tumor necrosis factor; PHA, phytohaemagglutinin.

The function of secretory pathways in specialized cells, such as prostate, mammary and pancreatic cells, are reliant on the expression and activity of ZnT members [58]. Hormones have been shown to influence cellular zinc transport, for instance the regulation of ZnT2 expression by prolactin in mammary cells during lactation [59]. Furthermore, zinc secretion into breast milk is further enhanced by the subcellular relocalization of ZnT2 as a result of post-translational regulation [60].

In contrast to the role of ZnT proteins, the 14 members of the ZIP (Zrt-, Irt-like Protein; SLC39) family of transporters function to increase cytoplasmic zinc concentration by transporting zinc from subcellular organelles or extracellular space (Table 2). The three dimensional structure of ZIP proteins has not been resolved. Most ZIP transporters are predicted to have eight transmembrane domains, with the N- and C- termini located away from the cytoplasm. The exact process of zinc transport through ZIPs remains unclear. Current evidence suggests that the transport of zinc by ZIP1 and ZIP2 is independent of energy [61]. With the exception of ZIP7, ZIP11 and ZIP13, homodimers formed by ZIPs appear to function at the plasma membrane, either primarily or inducible by other biological factors [57].

External HCO_3^- can induce zinc transport through ZIP2, ZIP8 and ZIP14 [62, 63]. Other divalent metals, such as iron, cadmium and manganese, can also influence the activity of ZIPs by direct competition for binding sites. In particular, ZIP8 and ZIP14 have been shown to transport iron, cadmium and manganese under physiological conditions, in addition to zinc [64]. While tissue zinc concentration and zinc status play a role in determining the activities of ZIPs, other factors, such as inflammation and hormonal changes, may have a greater influence on specific ZIP transporters [61]. Furthermore, current evidence suggests that zinc transporters localise in different subcellular components, subject to tissue specificity [65].

Tables 1 and 2 summarize the localization, transcriptional regulation, and association with pathology of zinc transporters in humans. The regulatory mechanism of zinc transporters can be categorized into those that occur at the transcriptional or post-translational level. The classic zinc-inducible transcription factor, MTF-1, is confirmed to regulate the transcription of ZnT1, ZnT2, Zip10 and Zip11 in murine models and MT in humans [66]. 'Zinc fingers' and the acidic activation domains within MTF-1 are proposed to mediate zinc sensing in the cytoplasm, which promotes the localization of the activated transcription factor to the nucleus. Once within the nucleus, MTF-1 can bind to the metal-responsive element of regulated genes, thereby modulating their expression. Moreover, the activation of a novel zinc finger protein, ZNF658, is suggested to repress the transcription of ZnT5 and ZnT10 when challenged with excess zinc [67]. Mutations and single nucleotide polymorphisms (SNP) in some zinc transporters have been associated with different pathology and diseases in humans. The interaction among zinc transporters, nutrient status and disease remains to be determined.

The multiple levels of regulation on zinc transporter activities reflect differences in the requirements of intracellular zinc signaling compared to other functions of zinc. While transcriptional regulation of zinc transporters represents a viable system to modulate the cytoplasmic zinc concentration in time frames of hours or days, post-translational modifications that modulate the activity of zinc transporters can initiate rapid zinc flux within minutes. [68]. Hence, most post-translational regulation of zinc transporters observed to date has been related to the propagation of cellular signals. External stimuli, such as extracellular zinc, can result in ZIP7 phosphorylation on the endoplasmic reticulum, initiating a rapid increase in cytoplasmic zinc [69].

Table 2. Localization, transcriptional regulation and association with pathology of Zrt-, Irt-like Proteins (ZIP)[1]

Zrt, Irt-like Protein (ZIP; SLC39)	Tissue distribution	Subcellular localization	Transcriptional regulation (response)	Single nucleotide polymorphism (SNP) or mutations of genes and their association with pathology in humans
ZIP1	Ubiquitous	Plasma membrane, intracellular vesicles, endoplasmic reticulum	Osteoblasts: cell differentiation (↑) Prostate: prolactin (↑), testosterone (↑) Liver: IL-6 (↑)	
ZIP2	Ubiquitous	Plasma membrane	Leukocytes: zinc chelator TPEN (↑)	
ZIP3	Ubiquitous	Plasma membrane, lysosomes	Blood cells: zinc excess (↓) Mammary cells: prolactin (↑)	Bipolar disorder (rs4806874) [89]
ZIP4	Gastrointestinal tract, kidney, hippocampal neurons	Plasma membrane, apical surface of enterocytes, lysosomes	Small intestine/colon: zinc depletion (↑)	Acrodermatitis enteropathica (reviewed in [90])
ZIP5	Pancreas, kidney, liver, stomach, intestine	Plasma membrane, basolateral surface of enterocytes	Liver: IL-6 (↑) Pancreatic: IL-1β (↓)[76]	High myopia (rs199624584) [91]
ZIP6	Ubiquitous	Plasma membrane	Liver: IL-6/IL-1 (↑) Dendritic cells: LPS (↑) Pancreatic cells: IL-1β (↓); IL-1β + TNF-α + IFN-γ (↓) [76]	Length of survival in esophageal squamous-cell carcinoma (rs7242481) [92]
ZIP7	Ubiquitous	Endoplasmic reticulum, Golgi apparatus, intracellular vesicles	Skeletal muscles: cell differentiation (↓) [93]	
ZIP8	Ubiquitous	Plasma membrane, lysosomes, mitochondria	Leukocytes: LPS (↑), immune activation (↑) Lung: TNF-α (↑)	HDL-cholesterol (rs13107325) [94] Blood pressure (rs13107325) [95] Schizophrenia (rs13107325) [96]
ZIP9	Ubiquitous	*trans*-Golgi apparatus, plasma membrane[97]	Prostate and breast: androgen (↑) [98]	

Table 2. (Continued)

Zrt, Irt-like Protein (ZIP; SLC39)	Tissue distribution	Subcellular localization	Transcriptional regulation (response)	Single nucleotide polymorphism (SNP) or mutations of genes and their association with pathology in humans
ZIP10	Brain, liver, erythroid, testes	Plasma membrane	Brain, liver, erythroid progenitor cells: zinc depletion (↑) Intestine: thyroid hormone (↑)	
ZIP11	Stomach, testes, intestine [99]	Golgi apparatus [100]		Serum selenium concentration (rs891684) [101]
ZIP12	Brain, lung, testes, retina			
ZIP13	Ubiquitous	Intracellular vesicles, Golgi apparatus		Spondylocheiro dysplastic form of Ehlers–Danlos syndrome (rs121434363) [102]
ZIP14	Ubiquitous	Plasma membrane	Liver: IL-6/IL-1 (↑), nitric oxide (↑) Neurons: IL-6 (↑) [86]	

[1]Adapted from [37, 61, 88] and updated [76, 86, 89–102].

Abbreviations: IL, interleukin; TPEN, N,N,N',N'-tetrakis(2-pyridylmethyl)ethane-1,2-diamine; LPS, lipopolysaccharide; TNF, tumor necrosis factor; IFN, interferon; HDL, high-density lipoprotein.

The transient increase in free zinc concentration phosphorylates Akt and extracellular regulating kinase (ERK)-1/2, thereby activating downstream cellular signaling pathways. Multiple potential sites for glycosylation have been identified in ZIP8 and ZIP14, however they do not appear to affect the movement of metals through these transporters [70]. It is unclear whether other post-translational modifications are able to modulate the activity of zinc transporters.

Zinc Transport by Calcium-Conducting Channels

Thus far, four families of calcium-conducting channels have been shown to transport zinc into the cell, specifically voltage-gated calcium channels (VGCC), glutamate receptors, acetylcholine receptors and transient receptor potential (TRP) channels. The uptake of zinc by VGCC was identified in murine muscle cells initially and confirmed subsequently in *in vitro* studies of the heart, brain and pancreatic β-cells [71]. The transport of zinc in the brain appears to be reliant on both VGCC and glutamate receptors under depolarized conditions [72]. To date, the largest family of calcium-conducting channels capable of zinc transport is the TRP channels, of which seven members have been reported to transport zinc under

physiological conditions [73]. The activation and depolarization of TRPM3 channels to transport zinc into pancreatic β-cells can activate other VGCC, which potentiate the influx of zinc [74]. The biological significance and relative contribution of zinc transport through calcium-conducting channels in humans is not known.

Extracellular Zinc Sensor

Fluctuations in extracellular zinc also have been shown to modulate cellular function and processes. The mechanism of action is proposed to be mediated through an extracellular zinc receptor, G protein-coupled receptor 39 (GPR39). GPR39 is a novel receptor that can trigger changes in intracellular calcium in response to extracellular zinc via the inositol triphosphate pathway [103]. Increases in intracellular calcium have a flow-on effect in mediating cellular signals, such as those involved in secretion, cell proliferation and growth [104], through the modulation of ERK-1/2 and Akt pathways. The significance of GPR39 in cellular function is established in the pancreas, intestinal tract and the brain. GPR39 knockout (KO) mice display reduced ability to secrete insulin and deregulation of normal gastrointestinal function [105]. A lack of human studies limits the translation of GPR39 functions to whole body zinc homeostasis.

Metallothionein

MT represents a large group of metal-binding proteins which have been shown to buffer cytoplasmic zinc concentration. In humans, multiple isoforms of MT have been found, of which only those in the MT-1 and MT-2 subtypes are central to mediating cellular zinc homeostasis [106]. Structurally, MT is defined by the unique amino acid sequence of cysteine residues that provide binding sites for metals, such as zinc. The seven metal binding sites of MT have discretely different affinities for zinc, with dissociation constants (K_d) ranging over four orders of magnitude [107]. This chemical property of MT complements its function as a chaperone and cellular storage protein for zinc, mediating the movement and availability of zinc to other proteins [108]. In addition to the maintenance of cellular zinc homeostasis and related activities, the release of zinc from thiol groups in MT confers the indirect antioxidant effects of zinc [109].

While MT is found ubiquitously at basal levels in all tissues, the expression of MT genes can be induced by metal ions, oxidative stress, hormones (e.g., glucocorticoids) and cytokines (e.g., interleukin (IL)-6). The multiple promoter regions upstream of the MT structural gene are reflective of the various stimuli that can regulate MT expression. Zinc excess and depletion in humans and *in vitro* studies have shown MT expression to be highly inducible and reflective of the availability of zinc [49, 110]. In a recent study that determined the effects of zinc supplementation on the expression of zinc transporter and MT genes, *MT-2A* gene expression was found to increase substantially within 2 days of zinc supplementation (20 mg elemental Zn/d), without significant concomitant change in plasma zinc concentration [111]. Over the course of the trial, the change in *MT-2A* gene expression was determined by the expression of zinc transporter genes. Specifically, the change in *ZIP1* gene expression was a significant predictor of *MT-2A* gene expression.

Upregulation of MT gene expression has been proposed to be mediated through the co-activation of multiple transcription factors by zinc transported into the cytoplasm via ZIP1. A novel transcription factor, CCAAT/enhancer-binding protein α (C/EBPα), can induce MT expression, both independently and synergistically with MTF-1 [112]. Activation of C/EBPα by phosphorylation can be mediated through the phosphoinositide 3-kinase/Akt pathway, one of the many signaling pathways in which zinc plays a regulatory role as a second messenger [9]. The activation of MTF-1 and C/EBPα is consistent with the role of MT in counteracting increases in intracellular zinc concentrations.

Numerous routes and proteins play a role in maintaining cellular zinc homeostasis, as summarized in Figure 3. Coordination among various zinc transporters and MT has been documented in humans previously [113]. However, the mechanisms and organization by which the novel mediators of cellular zinc homeostasis and zinc-binding proteins coordinate cellular responses to biological challenges, such as exercise, inflammation and chronic diseases, remain to be elucidated.

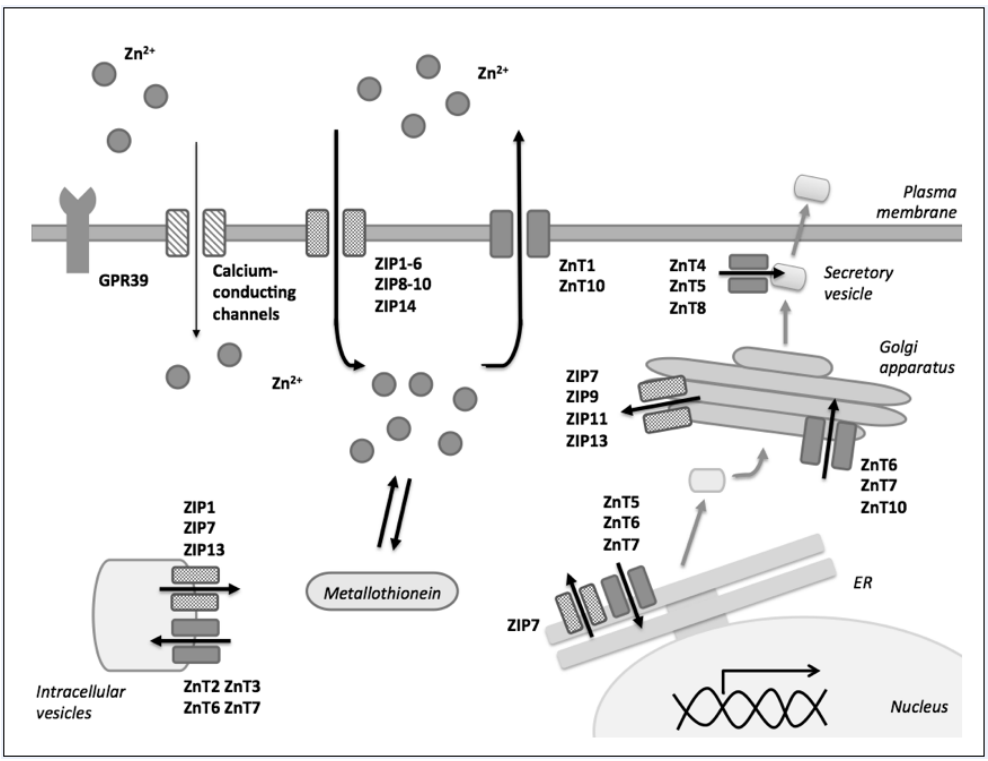


Figure 3. Schematic showing the mediators of cellular zinc homeostasis, their proposed sites of action and their influence on cellular function. The primary mediators of cellular zinc homeostasis include the two families of zinc transporters (ZnT and ZIP) and metallothionein (MT). Recent evidence suggests calcium-conducting channels provide a minor route of cellular zinc import. Changes in zinc content in subcellular locations have been linked to cellular processes, such as the production of secretory proteins. In a similar vein, the action of extracellular zinc sensor (GPR39) can mediate cellular signaling pathways through fluctuations in extracellular zinc. GPR39, G protein-coupled receptor 39; ZIP, Zrt-, Irt-like Protein; ZnT, Zinc Transporter; ER, endoplasmic reticulum.

Zinc and Chronic Diseases

There is increasing evidence that zinc is implicated in the pathogenesis and management of chronic diseases, such as Type 2 DM and CVD. Patients with Type 2 DM and CVD often present with perturbed zinc status as a result of disease state and/or suboptimal dietary zinc intake. Recent studies investigating the interrelationship of zinc and chronic diseases have identified novel interactions between mediators of cellular zinc homeostasis and disease pathology.

Type 2 Diabetes Mellitus

The discovery of zinc's role in the biosynthesis, storage and secretion of insulin initiated interest in zinc metabolism in the context of Type 2 DM. Individuals with Type 2 DM are of marginal zinc status due to higher urinary zinc excretion and lower serum zinc concentration [11]. Suboptimal zinc status affects glycemic control by compromising the production and secretion of insulin in the pancreas [114] and impacting insulin sensitivity in peripheral tissues [11]. Increasing dietary zinc intake through diet or supplementation has been proposed as an adjunct therapy in Type 2 DM [115], which is supported by recent meta-analyses that reveal improved measures of glycemic control [116] and lipidemia [117] following zinc supplementation in Type 2 DM.

The beneficial effects of improved zinc status in the management of Type 2 DM may be related to modulated expression of zinc transporter genes. Genome-wide association studies have identified SNP variants in the *ZnT8* gene to be associated with risk of developing Type 2 DM in numerous ethnic groups [118]. ZnT8 protein is highly expressed in pancreatic β-cells and functions to provide zinc to the secretory vesicles, contributing to the storage and secretion of insulin. A recent study in ZnT8 KO mice revealed that increased insulin secretion from the pancreas corresponded with lower systemic insulin concentration. This paradoxical relationship between insulin secretion and systemic concentration was explained by the role of ZnT8 in regulating hepatic insulin clearance [119]. A recent trial found that, while individuals with the risk allele of *ZnT8* gene had lower hepatic insulin clearance relative to those in the non-risk allele group, there was a substantial improvement in their insulin processing after zinc supplementation [120]. Larger studies have shown that the adverse effects of *ZnT8* SNP on glycemic control can be moderated by improvement in zinc status through increases in dietary zinc intake [81] and plasma zinc concentration [121].

Current evidence suggests that attenuation of the insulin signaling pathway, at least in part, contributes to the pathology of Type 2 DM. *In vitro* studies have shown that zinc ions can inhibit protein tyrosine phosphatase activity, thereby promoting the propagation of the insulin signal [122]. Increased glucose uptake was observed in 3T3-L1 adipocytes upon incubation with zinc, in both the presence and absence of insulin. The insulinomimetic effect of zinc appears to be dose responsive at concentrations of 0, 20, 50, 100 and 200 μmol/L of zinc chloride ($ZnCl_2$) in the media [123]. Taken together, the evidence suggests that zinc is capable of increasing glucose uptake in the absence of insulin and synergistically with insulin. Molecular analysis from *in vitro* studies revealed that improvements in cellular glucose uptake were mediated through improved tyrosine phosphorylation of the insulin receptor and

insulin receptor substrate (IRS)-1 [124]. Table 3 shows selected studies that report on the effects of zinc supplementation on fasting insulin concentration and/or Homeostatic Model Assessment-Insulin Resistance.

ZnT7 is responsible for the translocation of cytoplasmic zinc into the Golgi apparatus and intracellular vesicles; the expression of ZnT7 has been associated with the regulation of glycemic control. ZnT7 KO mice were found to be more susceptible than their wild-type counterparts to high fat diet-induced postprandial hyperglycemia and insulin resistance [131]. This may be due to the crucial roles that ZnT7 plays in insulin secretion and the activation of the insulin-signaling pathway. ZnT7 KO mice display down-regulation of *Irs2* gene expression, reduced Akt activation and subsequent lowered efficiency of the insulin-signaling pathway. The interaction between zinc transporters and Type 2 DM pathology is further demonstrated in the relationships between expression of zinc transporter genes and measures of glycaemic control. In a 12-week zinc supplementation trial [132], changes in glucose and insulin concentrations were related to fold changes of *Zip10* and *ZnT6* genes, respectively. Further studies in humans are required to ascertain the intricacies and altered function of the cellular zinc transport system in Type 2 DM.

Table 3. Effects of zinc supplementation on insulin sensitivity in humans

Study	Study population	Supplementation (dose, duration)	Fasting insulin concentration	HOMA-IR
[125]	Obese women (Zinc, *n*=28; Placebo, *n*=28)	30 mg elemental zinc 30 days	Decreased in zinc group	Decreased in zinc group
[126]	Obese (Zinc, n=30; Placebo, *n*=30)	30 mg elemental zinc 4 weeks	Decreased in zinc group	Decreased in zinc group
[127]	Pre-pubertal obese children (Zinc, *n*=30; Placebo, *n*=30)	20 mg elemental zinc 8 weeks	Decreased in zinc group	Decreased in zinc group
[128]	Obese women (Zinc, *n*=20; Placebo, *n*=20)	30 mg elemental zinc 8 weeks	NS	NS
[129]	Type 2 DM (Zinc, *n*=20; Placebo, *n*=20)	240 mg elemental zinc 12 weeks	NS	Not reported
[130]	Type 2 DM (Zinc; *n*=44, Control, *n*=32) Healthy (Zinc, *n*=32; Control, *n*=40)	50 mg elemental zinc 4 weeks	NS	Not reported

Abbreviations: HOMA-IR, Homeostatic Model Assessment-Insulin Resistance; NS, not significant; DM, diabetes mellitus.

Cardiovascular Diseases

One of the principal underlying causes of CVD is atherosclerosis, which co-exists with oxidative stress. Zinc plays a role in attenuating the atherosclerotic process and optimizing redox balance through moderation of apoptotic, nitric oxide and nuclear factor-kappa B (NF-κB)-related signaling mechanisms and the oxidative modification of low-density lipoprotein-cholesterol [9].Conversely, the pathology of atherosclerosis contributes to perturbation in zinc metabolism [133]. Alteration in the distribution of zinc among its plasma carriers has been observed in patients with atherosclerosis [134], possibly as a result of the interaction between circulating fatty acids and binding affinity of zinc and albumin [31]. In addition, oxidative stress induced by hydrogen peroxide (H_2O_2) can significantly increase intracellular zinc in endothelial cells, which promotes apoptotic signals; when endothelial cells were over-expressed with MT-1 protein or treated with a zinc chelator, a reduction in oxidative stress-induced apoptosis was observed [135]. While favorable effect of zinc supplementation on high density lipoprotein (HDL)-cholesterol has been observed for patients with Type 2 DM [117], the overall effect of zinc on CVD risk, as related to lipid profile and atherosclerosis, is currently unclear.

Hypertension is another key contributor to the risk of CVD. The pathogenesis of arterial hypertension has been associated with changes in the distribution of zinc between extracellular and intracellular spaces [136]. In addition, a lower level of plasma zinc concentration combined with higher erythrocyte zinc content in hypertensive patients suggests perturbed whole body zinc homeostasis. Untreated arterial hypertension appears to increase gastrointestinal uptake of zinc, which is attenuated upon anti-hypertensive treatment [137]. It is unclear which mechanisms of cellular transport mediate the changes in zinc homeostasis as a result of arterial hypertension. In genome-wide association studies, a SNP variant in the *ZIP8* gene has been associated with blood pressure [95], HDL-cholesterol level and coronary heart disease risk [94], suggesting that zinc transporters may play a role in perturbed zinc homeostasis that co-exists with the pathogenesis of CVD.

Determinants of Zinc Transport

Established first-line strategies for the prevention and treatment of chronic diseases, such as Type 2 DM and CVD, include dietary manipulation and exercise prescription. Exercise training has been used to induce beneficial effects on the low-grade inflammatory state [138] that coexists with many chronic diseases. While exercise has proved to be an effective mean in modifying chronic disease risk and management, it also poses alteration in zinc homeostasis by modulating inflammation, promoting zinc loss and redistributing zinc in the body.

Inflammation

Inflammation is the programmed immune response to stimuli that are potentially harmful. As part of the innate immune system, acute inflammatory response is characterized by the

transmittance of cellular signals within the immune cell network which, in part, requires changes in cellular zinc concentration. The intricate management of zinc concentration in the cytoplasm of immune cells serves functional purposes, i.e. cellular signal propagation and activation of zinc-dependent proteins [139], while at the same time minimizing the supply of zinc to intracellular microbes [140]. At the systemic level, the acute phase response of inflammation includes a transiently rapid decline in plasma zinc concentration as a result of the redistribution of zinc to other tissues [141]. This is facilitated primarily by altered zinc transporter and MT expressions that are instigated by systemic pro-inflammatory molecules, such as C-reactive protein and cytokines [76, 142]. For example, the redistribution of zinc from plasma to the liver occurs as a result of the upregulation of ZIP14 and MT induced by IL-6 [143]. Known transcriptional regulatory control of zinc transporters by specific inflammatory signals are summarized in Tables 1 and 2.

Extracellular stimuli, such as lipopolysaccharide, activate toll-like receptors that initiate acute immune responses through the activation of cellular signaling pathways. The classic transcription factor, NF-κB, represents the master regulatory protein that facilitates immune function in cells. In addition to modulating the expression of cytokines, such as tumor necrosis factor (TNF)-α and IL-1β, *in vitro* studies have confirmed that NF-κB has direct transcriptional regulation of ZIP8 [144]. NF-κB activation induces ZIP8 expression which contributes to cellular zinc uptake in immune cells. In turn, the increase in cytoplasmic zinc inhibits the activation of NF-κB, thereby providing a potent negative feedback loop of NF-κB signaling pathways in acute immune response. The role of zinc in immune function is further supported by the recent observation of higher expression and secretion of IL-1β in zinc-depleted macrophages through the activation of NACHT, LRR and PYD domains-containing protein 3 [145].

Despite acute localized inflammation being a crucial part of the immune defense system, the chronic activation of the inflammatory processes forms an underlying feature of many chronic diseases, for example Type 2 DM. Impaired immune function, as part of the Type 2 DM pathology, can ultimately contribute to the deterioration of pancreatic β-cells. Zinc supplementation in Type 2 DM can alter the gene expression of cytokines, such as TNF-α [146]. In addition, relationships among systemic inflammatory markers and gene expression of zinc transporters and cytokines [146, 147] suggest an improvement of immune response in Type 2 DM after zinc supplementation through increased efficiency of signaling pathways mediated by zinc. For instance, *IL-1β* gene expression in PBMC was predicted by the expression of zinc transporter and MT genes in both the presence and absence of zinc supplementation in Type 2 DM.

Exercise

Exercise has been prescribed as a treatment strategy for chronic diseases, due in part to its ability to modulate low-grade inflammation. However, exercise can also alter zinc homeostasis, which may negate the favorable effects of exercise [148]. Zinc loss during exercise, in particular through sweat, is well documented [149, 150]. The magnitude of zinc loss in sweat is dependent on training status, duration of exercise and ambient temperature. In prolonged exercise, conservation of sweat zinc is evident after an hour of aerobic activity and this adaptation is enhanced further in heat-acclimatized individuals [151]. In contrast, urinary

zinc loss as a result of exercise has been difficult to quantify due to differences in exercise test conditions [152, 153].

Reports of the change in plasma zinc concentration immediately after an aerobic exercise bout are mixed [152, 154, 155]. Table 4 summaries selected studies reporting on changes in plasma zinc concentrations immediately after exercise and during exercise recovery. In studies that report increases in plasma zinc immediately after exercise, a decrease in plasma zinc concentration is observed during exercise recovery [152, 156]. Furthermore, lower levels of zinc and CA-I in erythrocytes were found immediately after high intensity cycling, which returned to baseline levels after 30 minutes of rest [157]. Taken together, the results suggest a shift of zinc from plasma to erythrocytes; the redistribution of zinc between different body compartments highlights the rapid flux of zinc when challenged by exercise.

Localized exercise-induced muscle inflammation and its sequel have been proposed to explain the flux of zinc observed during exercise recovery. In a study where ^{70}Zn was infused into subjects after a maximal aerobic exercise bout, zinc shifted from plasma into the interstitial fluid and the liver, presumably due to the acute phase response and/or changes in oncotic pressure associated with exercise [161]. The acute stress of exercise induces the production of inflammatory cytokines, such as IL-6, which can sequester zinc in the liver via increases in hepatic MT and differential regulation of zinc transporters [143].

Table 4. Effect of an acute exercise bout on plasma zinc concentration immediately after exercise and during recovery of exercise

Study	Study population	Intervention	Plasma zinc concentration	
			Immediately after exercise	**During exercise recovery**
[152]	Moderately trained male runners (*n*=9)	Running, maximal field test	NS	Decreased at 120 min
[154]	Untrained healthy males (*n*=5)	Cycling, maximal $\dot{V}O_2$max test	Increased	Not reported
[155]	Untrained healthy males (*n*=20)	Running, progressive exercise test till fatigue	Decreased	Not reported
[156]	Untrained males (*n*=9)	Running at 80-90% $\dot{V}O_2$max for 30 min	Increased	Decreased at 30 min
[158]	Untrained males and females (*n*=8)	Step test, 40 min	Decreased	Not reported
[159]	Untrained males (*n*=10)	Swimming, 400 m	Increased	Decreased at 60 min
[160]	Untrained males (*n*=32)	Boxing training, 60 min	Decreased	Not reported

Abbreviations: NS, not significant.

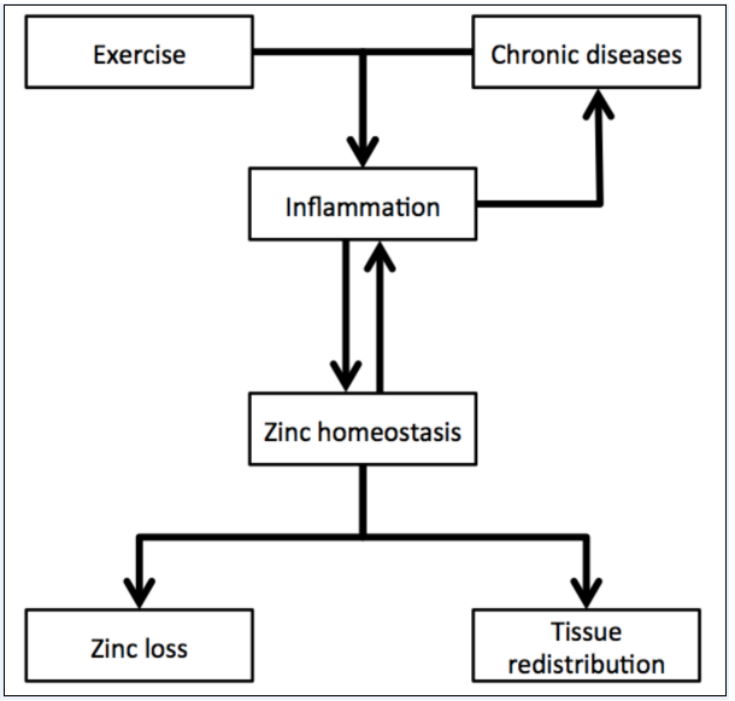


Figure 4. Determinants of zinc transport. Exercise and chronic diseases can influence the inflammatory state of the body, which in turn initiate changes in zinc homeostasis through redistribution or loss of zinc. Altered zinc status, as a result of zinc depletion or supplementation, can affect the immune system and its inflammatory processes, thereby impacting the pathogenesis of chronic diseases, such as Type 2 DM and CVD.

For inactive individuals who undergo repeated bouts of exercise, additional zinc losses and transfer between body compartments are hypothesized to compromise zinc status. In previously inactive individuals, a decline in serum zinc concentration was observed after several weeks of aerobic training [162]. In addition, Ohno *et al.* reported a reduction in albumin-bound plasma zinc in men after a 10-week running program [163]. Collectively, these observations suggest an increased requirement for zinc when previously inactive individuals are under chronic exercise stress.

Training status of the individual is implicated in the regulation of zinc homeostasis during exercise, however the details of this process remain uncertain. Smaller fluctuations in serum zinc concentration during exercise was found in endurance-trained individuals with higher aerobic thresholds, when compared to inactive individuals [153]. Longitudinal studies that followed athletes over a competitive season report contradictory changes to blood zinc concentration [164, 165], however the failure of some studies to assess dietary zinc intake during the study period limits the interpretation of the results. In cross-sectional studies, there appear to be no significant differences in plasma zinc level between athletes and the general population [166]. In contrast, athletes in aerobic disciplines, such as triathletes or long distance runners, are more likely to display signs of zinc redistribution from plasma to erythrocytes when compared to their anaerobically-trained counterparts [167]. Moreover, erythrocyte-SOD appears to be upregulated as a result of adaptation to aerobic exercise. It is plausible that, over time, adaptations associated with exercise can modulate the redistribution

of zinc within the body, however the details of this process are unknown. Correlations between erythrocyte zinc concentration and activity of erythrocyte-MT and -SOD in elite athletes further emphasize the requirement for zinc in the development of antioxidative adaptation as a result of exercise [167].

To date, no studies have investigated the cellular network of zinc transport under exercise stress in humans. However, *in vitro* studies suggest that the loss of ZIP7 expression in muscle cells contributes to a reduction in the expression of principal mediators of glucose metabolism, specifically glucose transporter 4, the insulin receptor and downstream signaling molecules [93]. Thus, ZIP7 is implicated in glycolysis and glycogen synthesis within the muscle cell, suggesting a link between zinc homeostasis, cellular metabolism in muscle and exercise performance. Similarly, variants in *ZnT8* SNP have been associated with undesirable exercise-related functional outcomes, such as lower muscle size and strength [168]. How exercise influences cellular zinc transporters and their effects on exercise performance in humans require further investigation, possibly by measuring multiple aspects of zinc transport network to capture cellular zinc flux in tissues, such as skeletal muscles.

The interrelationship between inflammation and zinc homeostasis as related to exercise and chronic diseases is summarized in Figure 4.

Conclusion

The maintenance of zinc homeostasis at the whole body and cellular levels is critical in optimizing the biological functions of zinc. In comparison to the investigation of cellular zinc transport, the mechanisms which maintain whole body zinc homeostasis are well established. Factors, such as exercise, inflammation and chronic diseases, are significant determinants of zinc homeostasis in humans. The management of chronic diseases, such as Type 2 DM and CVD, often involves the provision of exercise training regimes, which impose additional requirements for dietary zinc to maintain optimal zinc status. In the dietary management of chronic diseases, clinicians should consider the provision of bioavailable dietary zinc to maximize zinc uptake from the gut.

While earlier studies with zinc isotopes have confirmed the major sites of zinc homeostatic regulatory control in the gastrointestinal tract and liver, the exact cellular zinc transport mechanisms remain to be determined. The current knowledge of cellular zinc homeostasis is based on fluorescence techniques and the expression of mediators of cellular zinc homeostasis in cell culture studies, thereby limiting the translation of results to humans. A sensitive zinc biomarker to detect marginal zinc deficiency in humans remains to be discovered. Future research that ascertains the connection between whole body zinc homeostasis and the cellular zinc transport network under conditions of health and diseases will extend the current knowledge and provide a more comprehensive evidence base for clinical practice.

References

[1] Prasad A. S., Miale A., Farid Z., Sandstead H. H., Schulert A. R. Zinc metabolism in patients with the syndrome of iron deficiency anemia, hepatosplenomegaly, dwarfism, and hypognadism. *J. Lab. Clin. Med.*, 1963; 61: 537–49.

[2] Andreini C., Banci L., Bertini I., Rosato A. Counting the zinc-proteins encoded in the human genome. *J. Proteome Res*., 2006; 5: 196–201.

[3] Klug A. The discovery of zinc fingers and their applications in gene regulation and genome manipulation. *Annu. Rev. Biochem.*, 2010; 79: 213–31.

[4] Choi S., Bird A. J. Zinc'ing sensibly: controlling zinc homeostasis at the transcriptional level. *Metallomics*, 2014; 6: 1198-215.

[5] Laity J. H., Lee B. M., Wright P. E. Zinc finger proteins: new insights into structural and functional diversity. *Curr. Opin. Struct. Biol.*, 2001; 11: 39–46.

[6] Vallee B. L., Falchuk K. H. The biochemical basis of zinc physiology. *Physiol. Rev.*, 1993; 73: 79–118.

[7] Samman S. Zinc. In: Mann J., Truswell A. S., editors. Essentials of Human Nutrition. 4th ed. Oxford: Oxford University Press; 2012. p. 171–5.

[8] Wilson M., Hogstrand C., Maret W. Picomolar concentrations of free zinc(II) ions regulate receptor protein-tyrosine phosphatase β activity. *J. Biol. Chem.*, 2012; 287: 9322–6.

[9] Foster M., Samman S. Zinc and redox signaling: perturbations associated with cardiovascular disease and diabetes mellitus. *Antioxid. Redox. Signal*, 2010; 13: 1549–73.

[10] Fischer Walker C. L., Ezzati M., Black R. E. Global and regional child mortality and burden of disease attributable to zinc deficiency. *Eur. J. Clin. Nutr.*, 2009; 63: 591–7.

[11] Jansen J., Karges W., Rink L. Zinc and diabetes - clinical links and molecular mechanisms. *J. Nutr. Biochem*., 2009; 20: 399–417.

[12] Little P. J., Bhattacharya R., Moreyra A. E., Korichneva I. L. Zinc and cardiovascular disease. *Nutrition*, 2010; 26: 1050–7.

[13] King J. C. Does zinc absorption reflect zinc status? *Int. J. Vitam. Nutr. Res.*, 2010; 80: 300–6.

[14] Holt R. R., Uriu-adams J. Y., Keen C. L. Zinc. In: Erdman Jr. J. W., MacDonald I. A., Zeisel S. H., editors. Present Knowledge in Nutrition. 10th ed. London: Wiley-Blackwell; 2012. p. 521–39.

[15] Chung C., Stookey J., Dare D. Current dietary zinc intake has a greater effect on fractional zinc absorption than does longer term zinc consumption in healthy adult men. *Am. J. Clin. Nutr*., 2008; 87: 1224–9.

[16] Sandström B. Bioavailability of zinc. *Eur. J. Clin. Nutr.*, 1997; 51: S17–9.

[17] Bel-Serrat S., Stammers A.-L., Warthon-Medina M., Moran V. H., Iglesia-Altaba I., Hermoso M., Moreno L. A., Lowe N. M. Factors that affect zinc bioavailability and losses in adult and elderly populations. *Nutr. Rev.*, 2014; 44: 1–19.

[18] Foster M., Karra M., Picone T., Chu A., Hancock D. P., Petocz P., Samman S. Dietary fiber intake increases the risk of zinc deficiency in healthy and diabetic women. *Biol. Trace Elem. Res*., 2012; 149: 135–42.

[19] Zaman K., McArthur J. O., Abboud M. N., Ahmad Z. I., Garg M. L., Petocz P., Samman S. Iron supplementation decreases plasma zinc but has no effect on plasma fatty acids in non-anemic women. *Nutr. Res.*, 2013; 33: 272–8.
[20] Schölmerich J., Freudemann A. Bioavailability of zinc from zinc-histidine complexes. I. Comparison with zinc sulfate in healthy men. *Am. J. Clin. Nutr*., 1987; 45:1480–6.
[21] Samman S. *Zinc. Nutr. Diet*, 2007; 64: S131–4.
[22] Wessells K. R., Singh G. M., Brown K. H. Estimating the Global Prevalence of Inadequate Zinc Intake from National Food Balance Sheets: Effects of Methodological Assumptions. *PLoS One*, 2012; 7: e50565.
[23] World Health Organization. Trace elements in human nutrition and health. 1996.
[24] Brown K. H., Rivera J. A., Bhutta Z., Gibson R. S., King J. C., Lönnerdal B., Ruel M. T., Sandtröm B., Wasantwisut E., et al. International Zinc Nutrition Consultative Group (IZiNCG) technical document #1. Assessment of the risk of zinc deficiency in populations and options for its control. *Food Nutr. Bull*., 2004; 25: S94–203.
[25] Krebs N. E., Hambidge K. M. Zinc metabolism and homeostasis: the application of tracer techniques to human zinc physiology. *Biometals*, 2001; 14: 397–412.
[26] Sian L., Mingyan X., Miller L. V., Tong L., Krebs N. F., Hambidge K. M. Zinc absorption and intestinal losses of endogenous zinc in young Chinese women with marginal zinc intakes. *Am. J. Clin. Nutr.*, 1996; 63: 348–53.
[27] Verus A. P., Samman S. Urinary zinc as a marker of zinc intake: results of a supplementation trial in free-living men. *Eur. J. Clin. Nutr.*, 1994; 48: 219–21.
[28] Thompson R. Assessment of zinc status. *Proc. Nutr. Soc.*, 1991; 50: 19–28.
[29] Jackson M. Physiology of zinc: general aspects. In: Mills C., editor. Zinc in Human Biology. New York, NY: Springer-Verlag; 1989. p. 1–14.
[30] Smith K. T., Failla M. L., Cousins R. J. Identification of albumin as the plasma carrier for zinc absorption by perfused rat intestine. *Biochem. J.*, 1979; 184: 627–33.
[31] Lu J., Stewart A. J., Sadler P. J., Pinheiro T. J. T., Blindauer C. A. Albumin as a zinc carrier: properties of its high-affinity zinc-binding site. *Biochem. Soc. Trans.*, 2008; 36: 1317–21.
[32] Tibaduiza E. C., Bobilya D. J. Zinc transport across an endothelium includes vesicular cotransport with albumin. *J. Cell Physiol.*, 1996; 167: 539–47.
[33] King J. C., Shames D. M., Woodhouse L. R. Zinc homeostasis in humans. *J. Nutr.*, 2000; 130: 1360S–6S.
[34] Miller L. V., Hambidge K. M., Naake V. L., Hong Z., Westcott J. L., Fennessey P. V. Size of the zinc pools that exchange rapidly with plasma zinc in humans: alternative techniques for measuring and relation to dietary zinc intake. *J. Nutr*., 1994; 124: 268–76.
[35] Overgaard K., Lindstrøm T., Ingemann-Hansen T., Clausen T. Membrane leakage and increased content of Na+ -K+ pumps and Ca2+ in human muscle after a 100-km run. *J. Appl. Physiol.*, 2002; 92: 1891–8.
[36] Clarkson P. M., Hubal M. J. Exercise-induced muscle damage in humans. *Am. J. Phys. Med. Rehabil.*, 2002; 81: S52–69.
[37] Lichten L. A., Cousins R. J. Mammalian zinc transporters: nutritional and physiologic regulation. *Annu. Rev. Nutr*., 2009; 29: 153–76.
[38] Lowe N., Fekete K., Decsi T. Methods of assessment of zinc status in humans: a systematic review. *Am. J. Clin. Nutr.*, 2009; 89: 2040–51.

[39] International Zinc Nutrition Consultative Group. IZiNCG Technical Brief 2: Assessing population zinc status with serum zinc concentration. 2007.

[40] Lowe N. M., Medina M. W., Stammers A.-L., Patel S., Souverein O. W., Dullemeijer C., Serra-Majem L., Nissensohn M., Moran V. H. The relationship between zinc intake and serum/plasma zinc concentration in adults: a systematic review and dose-response meta-analysis by the EURRECA network. *Br. J. Nutr.*, 2012; 108: 1962–71.

[41] Samman S. Challenges and opportunities in the assessment of zinc status. *Nutr. Diet.*, 2011; 68: 95–6.

[42] Foster M., Samman S. Zinc and regulation of inflammatory cytokines: implications for cardiometabolic disease. *Nutrients*, 2012; 4: 676–94.

[43] Lowe N. M., Woodhouse L. R., Sutherland B., Shames D. M., Burri B. J., Abrams S. A., Turnlund J. R., Jackson M. J., King J. C. Kinetic parameters and plasma zinc concentration correlate well with net loss and gain of zinc from men. *J. Nutr.*, 2004; 134: 2178–81.

[44] Johnson P. E., Hunt C. D., Milne D. B., Mullen L. K. Homeostatic control of zinc metabolism in men: Zinc excretion and balance in men fed diets low in zinc. *Am. J. Clin. Nutr*., 1993; 57: 557–65.

[45] National Health and Medical Research Council. Nutrient reference values for Australia and New Zealand: including recommended dietary intakes. Canberra, Australia: Commonwealth of Australia; 2006.

[46] Hughes S., Samman S. The effect of zinc supplementation in humans on plasma lipids, antioxidant status and thrombogenesis. *J. Am. Coll. Nutr*., 2006; 25: 285–91.

[47] Moore J. B., Blanchard R. K., Cousins R. J. Dietary zinc modulates gene expression in murine thymus: results from a comprehensive differential display screening. *Proc. Natl. Acad. Sci. U S A*, 2003; 100: 3883–8.

[48] Beattie J. H., Gordon M.-J., Rucklidge G. J., Reid M. D., Duncan G. J., Horgan G. W., Cho Y.-E., Kwun I.-S. Aorta protein networks in marginal and acute zinc deficiency. *Proteomics*, 2008; 8: 2126–35.

[49] Ryu M., Langkamp-Henken B., Chang S.-M., Shankar M. N., Cousins R. J. Genomic analysis, cytokine expression, and microRNA profiling reveal biomarkers of human dietary zinc depletion and homeostasis. *Proc. Natl. Acad. Sci. U S A*, 2011; 108: 20970–5.

[50] Overbeck S., Uciechowski P., Ackland M. L., Ford D., Rink L. Intracellular zinc homeostasis in leukocyte subsets is regulated by different expression of zinc exporters ZnT-1 to ZnT-9. *J. Leukoc. Biol.*, 2008; 83: 368–80.

[51] Andree K., Kim J., Kirschke C. Investigation of lymphocyte gene expression for use as biomarkers for zinc status in humans. *J. Nutr.*, 2004; 134: 1716–23.

[52] Afman L., Milenkovic D., Roche H. M. Nutritional aspects of metabolic inflammation in relation to health-insights from transcriptomic biomarkers in PBMC of fatty acids and polyphenols. *Mol. Nutr. Food Res.*, 2014; 58: 1708–20.

[53] Powell E. E., Kroon P. A. Low density lipoprotein receptor and 3-hydroxy-3-methylglutaryl coenzyme A reductase gene expression in human mononuclear leukocytes is regulated coordinately and parallels gene expression in human liver. *J. Clin. Invest*., 1994; 93: 2168–74.

[54] Rudkowska I., Raymond C., Ponton A., Jacques H., Lavigne C., Holub B. J., Marette A., Vohl M.-C. Validation of the use of peripheral blood mononuclear cells as surrogate model for skeletal muscle tissue in nutrigenomic studies. *OMICS*, 2011; 15: 1–7.

[55] Golan Y., Berman B., Assaraf Y. G. Heterodimerization, Altered Subcellular Localization and Function of Multiple Zinc Transporters in Viable Cells Using Bimolecular Fluorescence Complementation. *J. Biol. Chem.*, 2015; 290: 9050-63.

[56] Lu M., Fu D. Structure of the zinc transporter YiiP. *Science*, 2007; 317: 1746–8.

[57] Hogstrand C., Fu D. Zinc. In: Maret W., Wedd A., editors. Binding, Transport and Storage of Metal Ions in Biological Cells. Cambridge: Royal Society of Chemistry; 2014. p. 666–94.

[58] Kelleher S. L., McCormick N. H., Velasquez V., Lopez V. Zinc in specialized secretory tissues: roles in the pancreas, prostate, and mammary gland. *Adv. Nutr*., 2011; 2: 101–11.

[59] Qian L., Lopez V., Seo Y. A., Kelleher S. L. Prolactin regulates ZNT2 expression through the JAK2/STAT5 signaling pathway in mammary cells. *Am. J. Physiol. Cell Physiol*., 2009; 297: C369–77.

[60] Lopez V., Kelleher S. L. Zinc transporter-2 (ZnT2) variants are localized to distinct subcellular compartments and functionally transport zinc. *Biochem. J.*, 2009; 422: 43–52.

[61] Jeong J., Eide D. J. The SLC39 family of zinc transporters. *Mol. Aspects Med.*, 2013; 34: 612–9.

[62] Gaither L. A., Eide D. J. Functional expression of the human hZIP2 zinc transporter. *J. Biol. Chem*., 2000; 275: 5560–4.

[63] Girijashanker K., He L., Soleimani M., Reed J. M., Li H., Liu Z., Wang B., Dalton T. P., Nebert D. W. Slc39a14 gene encodes ZIP14, a metal/bicarbonate symporter: similarities to the ZIP8 transporter. *Mol. Pharmacol.*, 2008; 73: 1413–23.

[64] Jenkitkasemwong S., Wang C.-Y., Mackenzie B., Knutson M. D. Physiologic implications of metal-ion transport by ZIP14 and ZIP8. *Biometals*, 2012; 25: 643–55.

[65] Hennigar S. R., Kelleher S. L. Zinc networks: the cell-specific compartmentalization of zinc for specialized functions. *Biol. Chem.*, 2012; 393: 565–78.

[66] Günther V., Lindert U., Schaffner W. The taste of heavy metals: gene regulation by MTF-1. *Biochim. Biophys. Acta*, 2012; 1823: 1416–25.

[67] Ogo O. A., Tyson J., Cockell S. J., Howard A., Valentine R. A., Ford D. The zinc finger protein ZNF658 regulates the transcription of genes involved in zinc homeostasis and affects ribosome biogenesis through the zinc transcriptional regulatory element (ZTRE). *Mol. Cell Biol.*, 2015; 35: 977–87.

[68] Kambe T. Introduction: "Zinc Signaling"–The Blossoming Field of Zinc Biology. In: Fukada T., Kambe T., editors. Zinc Signals in Cellular Functions and Disorders. Toyko: Springer; 2014. p. 1–6.

[69] Taylor K. M., Hiscox S., Nicholson R. I., Hogstrand C., Kille P. Protein kinase CK2 triggers cytosolic zinc signaling pathways by phosphorylation of zinc channel ZIP7. *Sci. Signal*, 2012; 5: ra11.

[70] Wang C.-Y., Jenkitkasemwong S., Duarte S., Sparkman B. K., Shawki A., Mackenzie B., Knutson M. D. ZIP8 is an iron and zinc transporter whose cell-surface expression is up-regulated by cellular iron loading. *J. Biol. Chem.*, 2012; 287: 34032–43.

[71] Bouron A., Oberwinkler J. Contribution of calcium-conducting channels to the transport of zinc ions. *Pflugers Arch.*, 2014; 466: 381–7.

[72] Colvin R. A., Davis N., Nipper R. W., Carter P. A. Zinc transport in the brain: routes of zinc influx and efflux in neurons. *J. Nutr.,* 2000; 130: 1484S–7S.

[73] Bouron A., Kiselyov K., Oberwinkler J. Permeation, regulation and control of expression of TRP channels by trace metal ions. *Pflugers Arch. Eur. J. Physiol.*, 2014 (in press).

[74] Wagner T. F. J., Drews A., Loch S., Mohr F., Philipp S. E., Lambert S., Oberwinkler J. TRPM3 channels provide a regulated influx pathway for zinc in pancreatic beta cells. *Pflugers Arch. Eur. J. Physiol.*, 2010; 460: 755–65.

[75] Chowanadisai W., Lönnerdal B., Kelleher S. L. Identification of a mutation in SLC30A2 (ZnT-2) in women with low milk zinc concentration that results in transient neonatal zinc deficiency. *J. Biol. Chem.*, 2006; 281: 39699–707.

[76] Egefjord L., Jensen J. L., Bang-Berthelsen C. H., Petersen A. B., Smidt K., Schmitz O., Karlsen A. E., Pociot F., Chimienti F., et al. Zinc transporter gene expression is regulated by pro-inflammatory cytokines: a potential role for zinc transporters in beta-cell apoptosis? *BMC Endocr. Disord.*, 2009; 9: 7.

[77] Perez-Becerril C., Morris A., Mortimer A., McKenna P. J., de Belleroche J. Allelic variants in the zinc transporter-3 gene, SLC30A3, a candidate gene identified from gene expression studies, show gender-specific association with schizophrenia. *Eur. Psychiatry*, 2014; 29: 172–8.

[78] Da Rocha T. J., Blehm C. J., Bamberg D. P., Fonseca T. L. R., Tisser L. A., De Oliveira Junior A. A., De Andrade F. M., Fiegenbaum M. The effects of interactions between selenium and zinc serum concentration and SEP15 and SLC30A3 gene polymorphisms on memory scores in a population of mature and elderly adults. *Genes Nutr.*, 2014; 9: 377.

[79] Zhang X., Liang D., Guo B., Sun L., Chi Z.-H., Cai Y., Wang L., Ma J. Zinc transporter 7 induced by high glucose attenuates epithelial-to-mesenchymal transition of peritoneal mesothelial cells. *Biol. Trace Elem. Res.*, 2013; 151: 138–47.

[80] Cauchi S., Del Guerra S., Choquet H., D'Aleo V., Groves C. J., Lupi R., McCarthy M. I., Froguel P., Marchetti P. Meta-analysis and functional effects of the SLC30A8 rs13266634 polymorphism on isolated human pancreatic islets. *Mol. Genet. Metab.*, 2010; 100: 77–82.

[81] Kanoni S., Nettleton J. A., Hivert M.-F., Ye Z., van Rooij F. J. A., Shungin D., Sonestedt E., Ngwa J. S., Wojczynski M. K., et al. Total zinc intake may modify the glucose-raising effect of a zinc transporter (SLC30A8) variant: a 14-cohort meta-analysis. *Diabetes*, 2011; 60: 2407–16.

[82] Horikawa Y., Miyake K., Yasuda K., Enya M., Hirota Y., Yamagata K., Hinokio Y., Oka Y., Iwasaki N., et al. Replication of genome-wide association studies of Type 2 diabetes susceptibility in Japan. *J. Clin. Endocrinol. Metab.*, 2008; 93: 3136–41.

[83] Ozel A. B., Moroi S. E., Reed D. M., Nika M., Schmidt C. M., Akbari S., Scott K., Rozsa F., Pawar H., et al. Genome-wide association study and meta-analysis of intraocular pressure. *Hum. Genet.*, 2014; 133: 41–57.

[84] Jung E. J., Kawai T., Park H. K., Kubo Y., Rokutan K., Arase S. Identification of ultraviolet B-sensitive genes in human peripheral blood cells. *J. Med. Invest.*, 2008; 55: 204–10.

[85] Kambe T., Hashimoto A., Fujimoto S. Current understanding of ZIP and ZnT zinc transporters in human health and diseases. *Cell Mol. Life Sci.*, 2014; 71: 3281–95.

[86] Fujishiro H., Yoshida M., Nakano Y., Himeno S. Interleukin-6 enhances manganese accumulation in SH-SY5Y cells: implications of the up-regulation of ZIP14 and the down-regulation of ZnT10. *Metallomics*, 2014; 6: 944–9.

[87] Quadri M., Federico A., Zhao T., Breedveld G. J., Battisti C., Delnooz C., Severijnen L. A., Di Toro Mammarella L., Mignarri A., et al. Mutations in SLC30A10 cause parkinsonism and dystonia with hypermanganesemia, polycythemia, and chronic liver disease. *Am. J. Hum. Genet.*, 2012; 90: 467–77.

[88] Huang L., Tepaamorndech S. The SLC30 family of zinc transporters - A review of current understanding of their biological and pathophysiological roles. *Mol. Aspects Med*., 2013; 34: 548–60.

[89] Baum A. E., Hamshere M., Green E., Cichon S., Rietschel M., Noethen M. M., Craddock N., McMahon F. J. Meta-analysis of two genome-wide association studies of bipolar disorder reveals important points of agreement. *Mol. Psychiatry*, 2008; 13: 466–7.

[90] Schmitt S., Küry S., Giraud M., Dréno B., Kharfi M., Bézieau S. An update on mutations of the SLC39A4 gene in acrodermatitis enteropathica. *Hum. Mutat*., 2009; 30: 926–33.

[91] Guo H., Jin X., Zhu T., Wang T., Tong P., Tian L., Peng Y., Sun L., Wan A., et al. SLC39A5 mutations interfering with the BMP/TGF-β pathway in non-syndromic high myopia. *J. Med. Genet*., 2014; 51: 518–25.

[92] Wu C., Li D., Jia W., Hu Z., Zhou Y., Yu D., Tong T., Wang M., Lin D., et al. Genome-wide association study identifies common variants in SLC39A6 associated with length of survival in esophageal squamous-cell carcinoma. *Nat. Genet.*, 2013; 45: 632–8.

[93] Myers S. A., Nield A., Chew G.-S., Myers M. A. The zinc transporter, slc39a7 (Zip7) is implicated in glycaemic control in skeletal muscle cells. *PLoS One*, 2013; 8: e79316.

[94] Waterworth D. M., Ricketts S. L., Song K., Chen L., Zhao J. H., Ripatti S., Aulchenko Y. S., Zhang W., Yuan X., et al. Genetic variants influencing circulating lipid levels and risk of coronary artery disease. *Arterioscler. Thromb. Vasc. Biol*., 2010; 30: 2264–76.

[95] Ehret G. B., Munroe P. B., Rice K. M., Bochud M., Johnson A. D., Chasman D. I., Smith A. V., Tobin M. D., Verwoert G. C., et al. Genetic variants in novel pathways influence blood pressure and cardiovascular disease risk. *Nature*, 2011; 478: 103–9.

[96] Carrera N., Arrojo M., Sanjuán J., Ramos-Ríos R., Paz E., Suárez-Rama J. J., Páramo M., Agra S., Brenlla J., et al. Association study of nonsynonymous single nucleotide polymorphisms in schizophrenia. *Biol. Psychiatry*, 2012; 71: 169–77.

[97] Pascal L. E., Wang Z. Unzipping Androgen Action Through ZIP9: A Novel Membrane Androgen Receptor. *Endocrinology*, 2015; 155 : 4120–3.

[98] Thomas P., Dong J., Berg A. H., Pang Y. Identification and characterization of membrane androgen receptors in the ZIP9 zinc transporter subfamily: II. Role of human ZIP9 in testosterone-induced prostate and breast cancer cell apoptosis. *Endocrinology*, 2014; 155: 4250–65.

[99] Yu Y., Wu A., Zhang Z., Yan G., Zhang F., Zhang L., Shen X., Hu R., Zhang Y., et al. Characterization of the GufA subfamily member SLC39A11/Zip11 as a zinc transporter. *J. Nutr. Biochem.*, 2013; 24: 1697–708.

[100] Kelleher S. L., Velasquez V., Croxford T. P., McCormick N. H., Lopez V., MacDavid J. Mapping the zinc-transporting system in mammary cells: molecular analysis reveals a phenotype-dependent zinc-transporting network during lactation. *J. Cell Physiol.*, 2012; 227: 1761–70.

[101] Gong J., Hsu L., Harrison T., King I. B., Stürup S., Song X., Duggan D., Liu Y., Hutter C., et al. Genome-wide association study of serum selenium concentrations. *Nutrients*, 2013; 5: 1706–18.

[102] Giunta C., Elçioglu N. H., Albrecht B., Eich G., Chambaz C., Janecke A. R., Yeowell H., Weis M., Eyre D. R., et al. Spondylocheiro Dysplastic Form of the Ehlers-Danlos Syndrome-An Autosomal-Recessive Entity Caused by Mutations in the Zinc Transporter Gene SLC39A13. *Am. J. Hum. Genet.*, 2008; 82: 1290–305.

[103] Hershfinkel M. The Zinc-Sensing Receptor, ZnR/GPR39: Signaling and Significance. In: Fukada T., Kambe T., editors. Zinc Signals in Cellular Functions and Disorders. Toyko: Springer; 2014. p. 111–33.

[104] Cohen L., Sekler I., Hershfinkel M. The zinc sensing receptor, ZnR/GPR39, controls proliferation and differentiation of colonocytes and thereby tight junction formation in the colon. *Cell Death Dis.*, 2014; 5: e1307.

[105] Popovics P., Stewart A. J. GPR39: a Zn(2+)-activated G protein-coupled receptor that regulates pancreatic, gastrointestinal and neuronal functions. *Cell Mol. Life Sci.*, 2011; 68: 85–95.

[106] Blindauer C. A. Metallothioneins. In: Maret W., Wedd A., editors. Binding, Transport and Storage of Metal Ions in Biological Cells. Cambridge: Royal Society of Chemistry; 2014. p. 606–65.

[107] Colvin R. A., Holmes W. R., Fontaine C. P., Maret W. Cytosolic zinc buffering and muffling: their role in intracellular zinc homeostasis. *Metallomics*, 2010; 2: 306–17.

[108] Maret W. Metals on the move: zinc ions in cellular regulation and in the coordination dynamics of zinc proteins. *Biometals*, 2011; 24: 411–8.

[109] Oteiza P, I. Zinc and the modulation of redox homeostasis. *Free Radic. Biol. Med.*, 2012; 53: 1748–59.

[110] Aydemir T. B., Blanchard R. K., Cousins R. J. Zinc supplementation of young men alters metallothionein, zinc transporter, and cytokine gene expression in leukocyte populations. *Proc. Natl. Acad. Sci. U S A*, 2006; 103: 1699–704.

[111] Chu A., Ward S., Zaman K., Foster M., Petocz P., Samman S. Metallothionein (MT-2A) gene expression is upregulated after zinc supplementation in healthy individuals. *Proceedings of the Nutrition Society of Australia*, 2014; 38: 69.

[112] Datta J., Majumder S., Kutay H., Motiwala T., Frankel W., Costa R., Cha H. C., MacDougald O. A., Jacob S. T., Ghoshal K. Metallothionein expression is suppressed in primary human hepatocellular carcinomas and is mediated through inactivation of CCAAT/enhancer binding protein alpha by phosphatidylinositol 3-kinase signaling cascade. *Cancer Res.*, 2007; 67: 2736–46.

[113] Foster M., Hancock D., Petocz P., Samman S. Zinc transporter genes are coordinately expressed in men and women independently of dietary or plasma zinc. *J. Nutr.*, 2011; 141: 1195–201.

[114] Huber A. M., Gershoff S. N. Effect of zinc deficiency in rats on insulin release from the pancreas. *J. Nutr.*, 1973; 103: 1739–44.

[115] Ruz M., Carrasco F., Rojas P., Codoceo J., Inostroza J., Basfi-fer K., Valencia A., Vásquez K., Galgani J., et al. Zinc as a potential coadjuvant in therapy for Type 2 diabetes. *Food Nutr. Bull.*, 2013; 34: 215–21.

[116] Capdor J., Foster M., Petocz P., Samman S. Zinc and glycemic control: a meta-analysis of randomised placebo controlled supplementation trials in humans. *J. Trace Elem. Med. Biol.*, 2013; 27: 137–42.

[117] Foster M., Petocz P., Samman S. Effects of zinc on plasma lipoprotein cholesterol concentrations in humans: a meta-analysis of randomised controlled trials. *Atherosclerosis*, 2010; 210: 344–52.

[118] McCarthy M. I. Genomics, Type 2 diabetes, and obesity. *N. Engl. J. Med.*, 2010; 363: 2339–50.

[119] Tamaki M., Fujitani Y., Hara A., Uchida T., Tamura Y., Takeno K., Kawaguchi M., Watanabe T., Ogihara T., et al. The diabetes-susceptible gene SLC30A8/ZnT8 regulates hepatic insulin clearance. *J. Clin. Invest.*, 2013; 123: 4513–24.

[120] Maruthur N. M., Clark J. M., Fu M., Linda Kao W. H., Shuldiner A. R. Effect of zinc supplementation on insulin secretion: interaction between zinc and SLC30A8 genotype in Old Order Amish. *Diabetologia*, 2014; 58: 295–303.

[121] Shan Z., Bao W., Zhang Y., Rong Y., Wang X. Interactions between zinc transporter-8 gene (SLC30A8) and plasma zinc concentrations for impaired glucose regulation and Type 2 diabetes. *Diabetes*, 2014; 63: 1796–803.

[122] Haase H., Maret W. Protein tyrosine phosphatases as targets of the combined insulinomimetic effects of zinc and oxidants. *Biometals*, 2005; 18: 333–8.

[123] Tang X., Shay N. Zinc has an insulin-like effect on glucose transport mediated by phosphoinositol-3-kinase and Akt in 3T3-L1 fibroblasts and adipocytes. *J. Nutr.*, 2001; 131: 1414–20.

[124] Miranda E. R., Dey C. S. Effect of chromium and zinc on insulin signaling in skeletal muscle cells. *Biol. Trace Elem. Res.*, 2004; 101: 19–36.

[125] Marreiro D. D. N., Geloneze B., Tambascia M. A., Lerário A. C., Halpern A., Cozzolino S. M. F. Effect of zinc supplementation on serum leptin levels and insulin resistance of obese women. *Biol. Trace Elem. Res.*, 2006; 112: 109–18.

[126] Payahoo L., Ostadrahimi A., Mobasseri M., Bishak Y. K., Jafarabadi M. A. Effects of zinc supplementation on serum leptin level and insulin sensitivity in obese people. *Trace Elem. Electrolytes*, 2014; 31: 27–33.

[127] Hashemipour M., Kelishadi R., Shapouri J., Sarrafzadegan N., Amini M., Tavakoli N., Movahedian-Attar A., Mirmoghtadaee P., Poursafa P. Effect of zinc supplementation on insulin resistance and components of the metabolic syndrome in prepubertal obese children. *Hormones (Athens)*, 2009; 8: 279–85.

[128] Kim J., Lee S. Effect of zinc supplementation on insulin resistance and metabolic risk factors in obese Korean women. *Nutr. Res. Pract.*, 2012; 6: 221–5.

[129] Seet R. C. S., Lee C.-Y. J., Lim E. C. H., Quek A. M. L., Huang H., Huang S. H., Looi W. F., Long L. H., Halliwell B. Oral zinc supplementation does not improve oxidative stress or vascular function in patients with Type 2 diabetes with normal zinc levels. *Atherosclerosis*, 2011; 219: 231–9.

[130] Oh H.-M., Yoon J.-S. Glycemic control of Type 2 diabetic patients after short-term zinc supplementation. *Nutr. Res. Pract*., 2008; 2: 283–8.

[131] Huang L., Kirschke C. P., Lay Y.-A. E., Levy L. B., Lamirande D. E., Zhang P. H. Znt7-null mice are more susceptible to diet-induced glucose intolerance and insulin resistance. *J. Biol. Chem*., 2012; 287: 33883–96.

[132] Foster M., Chu A., Petocz P., Samman S. Zinc transporter gene expression and glycemic control in post-menopausal women with Type 2 diabetes mellitus. *J. Trace Elem. Med. Biol*., 2014; 28: 448–52.

[133] Foster M., Samman S. Zinc and atherosclerosis: clinical observations and poten- tial mechanisms. In: Rink L, editor. Zinc in Human Health. Amsterdam: IOS Press; 2011. p. 347–72.

[134] Malavolta M., Piacenza F., Costarelli L., Giacconi R., Muti E., Cipriano C., Tesei S., Spezia S., Mocchegiani E. Combining UHR-SEC-HPLC-ICP-MS with flow cytometry to quantify metallothioneins and to study zinc homeostasis in human PBMC. *J. Anal. At Spectrom.*, 2007; 22: 1193–8.

[135] Wiseman D. A., Wells S. M., Hubbard M., Welker J. E., Black S. M. Alterations in zinc homeostasis underlie endothelial cell death induced by oxidative stress from acute exposure to hydrogen peroxide. *Am. J. Physiol. Lung Cell Mol. Physiol*., 2007; 292: L165–77.

[136] Tubek S. Role of zinc in regulation of arterial blood pressure and in the etiopathogenesis of arterial hypertension. *Biol. Trace Elem. Res.*, 2007; 117: 39–51.

[137] Tubek S. Increased absorption of zinc from alimentary tract in primary arterial hypertension. *Biol. Trace Elem. Res*., 2001; 83: 31–8.

[138] Nimmo M., Leggate M., Viana J., King J. The effect of physical activity on mediators of inflammation. *Diabetes Obes. Metab.*, 2013; 15: 51–60.

[139] Haase H., Rink L. Functional significance of zinc-related signaling pathways in immune cells. *Annu. Rev. Nutr.*, 2009; 29: 133–52.

[140] Stafford S. L., Bokil N. J., Achard M. E. S., Kapetanovic R., Schembri M. A., McEwan A. G., Sweet M. J. Metal ions in macrophage antimicrobial pathways: emerging roles for zinc and copper. *Biosci. Rep.*, 2013; 33: 541–54.

[141] Gaetke L. M., McClain C. J., Talwalkar R. T., Shedlofsky S. I. Effects of endotoxin on zinc metabolism in human volunteers. *Am. J. Physiol.*, 1997; 272: E952–6.

[142] Besecker B. Y., Exline M. C., Hollyfield J., Phillips G., Disilvestro R. A., Wewers M. D., Knoell D. L. A comparison of zinc metabolism , inflammation , and disease severity in critically ill infected and noninfected adults early after intensive care. *Am. J. Clin. Nutr.*, 2011; 93: 1356–64.

[143] Liuzzi J. P., Lichten L. A., Rivera S., Blanchard R. K., Aydemir T. B., Knutson M. D., Ganz T., Cousins R. J. Interleukin-6 regulates the zinc transporter Zip14 in liver and contributes to the hypozincemia of the acute-phase response. *Proc. Natl. Acad. Sci. U S A*, 2005; 102: 6843–8.

[144] Liu M.-J., Bao S., Gálvez-Peralta M., Pyle C. J., Rudawsky A. C., Pavlovicz R. E., Killilea D. W., Li C., Nebert D. W., et al. ZIP8 regulates host defense through zinc-mediated inhibition of NF-κB. *Cell Rep.*, 2013; 3: 386–400.

[145] Summersgill H., England H., Lopez-Castejon G., Lawrence C. B., Luheshi N. M., Pahle J., Mendes P., Brough D. Zinc depletion regulates the processing and secretion of IL-1β. *Cell Death Dis.*, 2014; 5: e1040.

[146] Chu A., Foster M., Hancock D., Bell-Anderson K., Petocz P., Samman S. TNF-α gene expression is increased following zinc supplementation in Type 2 diabetes mellitus. *Genes Nutr*., 2015; 10: 440.

[147] Foster M., Petocz P., Samman S. Inflammation markers predict zinc transporter gene expression in women with Type 2 diabetes mellitus. *J. Nutr. Biochem.*, 2013; 24: 1655–61.

[148] Chu A., Samman S. Zinc Homeostasis in Exercise: Implications for Physical Performance. *Vitam. Miner*., 2014; 3: 40–2.

[149] DeRuisseau K. C., Cheuvront S. N., Hyames E. M., Sharp R. G. Sweat iron and zinc losses during prolonged exercise. *Int. J. Sport Nutr.*, 2002; 12: 428–37.

[150] Hoshi A., Watanabe H., Chiba M., Inaba Y., Kobayashi M., Kimura N., Ito T. Seasonal variation of trace element loss to sweat during exercise in males. *Environ. Health Prev. Med*., 2002; 7: 60–3.

[151] Montain S. J., Cheuvront S. N., Lukaski H. C. Sweat mineral-element responses during 7 h of exercise-heat stress. *Int. J. Sport Nutr. Exerc. Metab.*, 2007; 17: 574–82.

[152] Anderson R. A., Polansky M. M., Bryden N. A. Strenuous running - Acute effects of chromium, copper, zinc and selected clinical variables in urine and serum of male runners. *Biol. Trace Elem. Res*., 1984; 6: 327–36.

[153] Anderson R. A., Bryden N. A., Polansky M. M., Deuster P. A. Acute exercise effects on urinary losses and serum concentrations of copper and zinc of moderately trained and untrained men consuming a controlled diet. *Analyst*, 1995; 120: 867–70.

[154] Lukaski H. C., Bolonchuk W. W., Klevay L. M., Milne D. B., Sandstead H. H. Changes in plasma zinc content after exercise in men fed a low-zinc diet. *Am. J. Physiol. – Endocrinol. Metab.*, 1984; 247: E88–93.

[155] Kaczmarski M., Wójcicki J., Samochowiec L., Dutkiewicz T., Sych Z. The influence of exogenous antioxidants and physical exercise on some parameters associated with production and removal of free radicals. *Pharmazie*, 1999; 54: 303–6.

[156] Bordin D., Sartorelli L., Bonanni G., Mastrogiacomo I., Scalco E. High intensity physical exercise induced effects on plasma levels of copper and zinc. *Biol. Trace Elem. Res.*, 1993; 36: 129–34.

[157] Ohno H., Hirata F., Terayama K., Kawarabayashi T., Doi R., Kondo T., Taniguchi N. Effect of short physical exercise on the levels of zinc and carbonic anhydrase isoenzyme activities in human erythrocytes. *Eur. J. Appl. Physiol.*, 1983; 51: 257–68.

[158] Gleeson M., Almey J., Brooks S., Cave R., Lewis A., Griffiths H. Haematological and acute-phase responses associated with delayed-onset muscle soreness in humans. Eur. J. Appl. Physiol. Occup. Physiol., 1995; 71: 137–42.

[159] Döker S., Hazar M., Uslu M., Okan İ., Kafkas E., Boşgelmez İ. İ. Influence of training frequency on serum concentrations of some essential trace elements and electrolytes in male swimmers. *Biol. Trace Elem. Res.*, 2014; 158: 15–21.

[160] Karakukcu C., Polat Y., Torun Y. A., Pac A. K. The effects of acute and regular exercise on calcium, phosphorus and trace elements in young amateur boxers. *Clin. Lab.*, 2013; 59: 557-562.

[161] Volpe S. L., Lowe N. M., Woodhouse L. R., King J. C. Effect of maximal exercise on the short-term kinetics of zinc metabolism in sedentary men. *Br. J. Sports Med.*, 2007; 41: 156–61.

[162] Kara E., Akil M., Yalçinkaya Ö. The effect of aerobic exercise programme on trace element levels of young men. *African J. Microbiol. Res.*, 2012; 6: 165–8.

[163] Ohno H., Sato Y., Ishikawa M., Yahata T., Gasa S., Doi R., Yamamura K., Taniguchi N. Training effects on blood zinc levels in humans. *J. Sports Med. Phys. Fitness*, 1990; 30: 247–53.

[164] Peake J. M., Gerrard D. F., Griffin J. F. T. Plasma zinc and immune markers in runners in response to a moderate increase in training volume. *Int. J. Sports Med*., 2003; 24: 212–6.

[165] Couzy F., Lafargue P., Guezennec C. Y. Zinc metabolism in the athlete: influence of training, nutrition and other factors. *Int. J. Sports Med.*, 1990; 11: 263–6.

[166] Córdova A., Navas F. J. Effect of training on zinc metabolism: changes in serum and sweat zinc concentrations in sportsmen. *Ann. Nutr. Metab.*, 1998; 42: 274–82.

[167] Koury J., de Oliveira A., Portella E. S., de Oliveira C. F., Lopes G. C., Donangelo C. M. Zinc and copper biochemical indices of antioxidant status in elite athletes of different modalities. *Int. J. Sport Nutr. Exerc. Metab.*, 2004; 14: 358–72.

[168] Sprouse C., Gordish-Dressman H., Orkunoglu-Suer E. F., Lipof J. S., Moeckel-Cole S., Patel R. R., Adham K., Larkin J. S., Hubal M. J., et al. SLC30A8 nonsynonymous variant is associated with recovery following exercise and skeletal muscle size and strength. *Diabetes*, 2014; 63: 363–8.

In: Human Health and Nutrition
Editor: Sergej M. Ostojic
ISBN: 978-1-63482-823-9

Chapter III

Protein Supplementation and Athlete Performance

Jay R. Hoffman[1,*], Jeffrey R. Stout[1] and Daniel S. Moran[2,3]
[1]Institute of Exercise Physiology and Wellness,
University of Central Florida, Orlando, FL 3, US
[2]School of Health Science, Ariel University, Ariel, Israel
[3]Givat Washington College of Education,
Hevel Yavne Regional Council, Israel

Abstract

To optimize athletic performance and enhance recovery from high intensity training, the competitive athlete needs to ensure adequate energy and protein consumption. Differences in protein requirements for athletes, non-athletes and different types of athletes (i.e., endurance vs. strength/power) are well acknowledged. This has resulted in many athletes using protein supplements as a means of achieving their recommended protein intake and often to exceed the recommended amounts. In the past few years, a number of studies have focused on whether the timing of protein ingestion as it relates to a workout enhances protein synthesis and muscle recovery. This chapter focuses on protein requirements for different types of athletes and whether protein timing provides any advantage on affecting performance gains and recovery. Considering the high protein intake for many athletes, a discussion will also be directed to the safety aspects of high protein consumption.

Keywords: Supplementation; Protein timing; Nutrition; Athletes

[*] Corresponding author: Email: jay.hoffman@ucf.edu.

Introduction

Proteins are nitrogen-containing substances that are comprised of amino acids. They form the major structural component of muscle and other tissues in the body. Proteins are also used to produce hormones, enzymes, and hemoglobin. Although proteins are not the primary or desired source of energy, during periods of nutrient deprivation they can also be used to produce energy. For proteins to be used by the body, they are first degraded to their simplest form known as amino acids. There are twenty amino acids identified that are needed for growth and metabolism, and they are categorized as either being essential or nonessential. Nonessential amino acids can be synthesized by the body and do not need to be consumed in the diet, whereas essential amino acids cannot be synthesized endogenously and must be consumed in the diet. However, there are non-essential amino acids that are considered to be 'conditionally essential'; this means that there may be times when obtaining some of these nonessential amino acids becomes vital for maintaining health. For example, during periods of extreme physiological stress the endogenous production of the nonessential amino acid glutamine may not be sufficient for maintaining proper immune function [1]. Absence of any of the essential amino acids from the diet can limit the ability for muscle to grow or be repaired.

Daily Protein Requirements

Maintaining high levels of protein and preserving lean body mass in the body is important during periods of intense physical training and training overload. In order to sustain muscle protein balance during periods of stress and/or high intensity activity, diet alone may not be sufficient. The use of protein supplements can or perhaps should be used to attenuate proteolysis and promote muscle protein accretion [2-5]. Daily protein needs are determined by the ability to maintain a positive balance between protein degradation and protein synthesis. If protein degradation exceeds protein synthesis the body is considered to be in a catabolic state. In contrast, if protein synthesis exceeds protein degradation the body is considered to be in an anabolic state. Thus, protein balance is the outcome between an increase in skeletal muscle protein synthesis (anabolism) and a reduction in muscle protein breakdown (catabolism). A positive balance results in an increase in skeletal muscle mass as opposed to a negative balance, which results in a loss of skeletal muscle. In general, the protein requirement for an adult to maintain a positive protein balance is 0.8 $g \cdot kg \cdot day^{-1}$ body mass. However, for individuals who are active their protein needs are likely greater and dependent on the type of physical activity being performed. Individuals performing strength and power sports have greater daily protein need than individuals involved in sub-maximal but sustained activities (e.g., endurance activities). Both groups of active individuals though require a greater daily protein intake than their sedentary colleagues.

Resistance exercise has been shown to stimulate both protein synthesis and protein degradation, but in a fasted state protein degradation will still exceed protein synthesis [6,7]. When protein is ingested following exercise, the increase in muscle protein synthesis is between 50% and 100% greater than that seen from resistance exercise only [8]. Other investigators have reported that the combination of oral ingestion of amino acids and

resistance exercise may produce an even greater increase (3.5-fold) in muscle protein synthesis [9]. Although resistance exercise and protein intake can each increase muscle protein synthesis, the combination of the two is clearly superior in eliciting significant gains. Evidence is quite compelling that demonstrates that strength/power athletes have a greater daily protein requirement than other segments of the population. In studies examining high versus low daily protein intakes, higher protein consumption was associated with greater gains in protein synthesis, muscle size, and body mass. Fern and colleagues [10] compared two daily doses of protein ingestion (3.3 vs. 1.3 $g \cdot kg^{-1} \cdot day^{-1}$) in subjects performing a 4-week resistance training program. Significantly greater gains in protein synthesis and body mass were observed in the subjects consuming the higher daily protein intake. Similar results were noted by others that reported greater rates of protein synthesis in novice resistance-trained individuals consuming 2.62 $g \cdot kg^{-1} \cdot day^{-1}$ versus 0.99 $g \cdot kg^{-1} \cdot day^{-1}$ [11]. However, in the latter study no differences in muscle hypertrophy or strength were observed between the two groups. Considering that subjects were previously untrained, the significant gains in strength were likely related to neural adaptations and not to any muscle structural changes. Other investigators examining competitive strength/power athletes (collegiate football players), reported that trained athletes consuming 2.0 $g \cdot kg^{-1} \cdot day^{-1}$ for 12 weeks showed greater gains in strength than a similar group of strength/power athletes consuming 1.2 $g \cdot kg^{-1} \cdot day^{-1}$ [12].

Protein intakes between 1.2 and 1.7 $g \cdot kg^{-1} \cdot day^{-1}$ are recommended for strength/power athletes to maintain a positive nitrogen balance [13]. However, there have been only limited studies conducted on competitive strength/power athletes. One study examining college football players compared three different daily protein intakes (1.2 $g \cdot kg^{-1} \cdot day^{-1}$, 1.7 $g \cdot kg^{-1} \cdot day^{-1}$, and 2.4 $g \cdot kg^{-1} \cdot day^{-1}$) and found no significant differences in strength or lean body mass between the groups, but the greatest gains in strength (1-RM squat and bench press) were seen in the group consuming the highest daily protein intake [14]. Additional research on daily protein intakes in competitive strength-trained athletes still appears to be warranted.

To maintain a positive protein balance endurance athletes are required to consume 1.2 to 1.4 $g \cdot kg^{-1} \cdot day^{-1}$ [13]. Although maximizing muscle size and strength is not the primary goal for these athletes, loss of lean tissue from high energy expenditure and inadequate energy intake can have a significant detrimental effect on endurance performance. Therefore, these athletes also have a greater daily protein requirement than the sedentary individual.

Protein Supplementation

In regards to protein and amino acid supplementation, the leading dietetic and sports medicine organizations generally take a conservative approach to supplementation [13]. Consensus among these organizations is that protein needs can generally be met through food intake. However, these organizations also acknowledge the role that protein and amino acids have in optimizing the training response and enhancing recovery, and how the timing of ingestion may provide significant benefits. Further, the most convenient and efficient method for providing immediate protein needs to enhance recovery may be through supplementation.

As discussed earlier, protein synthesis is elevated by about 100% from resting concentrations following a resistance training session [6]. Early investigations examining the benefits of protein supplements infused amino acids directly into the body, a method of ingestion that is not comparable to how most athletes (competitive, recreational or tactical) consume protein. This may result in unrealistic expectations or incorrect extrapolations considering that evidence exists suggesting that between 20% and 90% of the amino acids ingested orally are removed from the circulation during the initial pass through the liver [15-17], and further exacerbated by exercise [18, 19]. However, others have demonstrated comparable changes in net muscle protein balance (synthesis − degradation) in comparisons between oral ingestion and infused essential amino acids following resistance exercise [20], indicating that oral consumption of protein, typical for individuals consuming protein supplements, is effective in maintaining a positive protein balance following exercise.

A recent meta-analysis was performed examining the ability of protein supplementation to enhance the adaptive responses of skeletal muscle to resistance exercise [21]. Data from 22 randomized controlled studies that included 680 participants were included in the analysis. The inclusion criteria for consideration was that each study have a supplementation group that consumed a minimum of 1.2 $g \cdot kg \cdot day^{-1}$ of protein taken in combination with a prolonged resistance training program of at least 6-weeks or longer. Results revealed that protein supplementation in combination with resistance training can significantly augment the gains in lean body mass, cross-sectional area of both type I and type II muscle fibers and strength. These results appeared to be consistent for both younger (23 ± 3 y) and older (62 ± 6 y) adults.

One of the benefits associated with protein consumption following an intense workout is in its ability to enhance the recovery and remodeling processes within skeletal tissue [22]. Several studies have reported a decrease in the extent of muscle damage, attenuation in force decrements, and an enhanced recovery from protein ingestion following resistance exercise [2, 23-26]. Hulmi and colleagues [2] have shown that when protein is consumed prior to, and immediately following a bout of resistance exercise an increase in messenger RNA (mRNA) expression is observed, preventing a post-exercise decrease in myogenin mRNA expression. This is thought to accelerate muscle adaptation and enhance muscle recovery from the training session. Thus, the timing of the protein supplement may take on greater importance in stimulating muscle adaptations that occur during prolonged training. This will be discussed in further detail later.

Protein Sources

Protein consumption from normal dietary intake can be consumed from a variety of dietary sources that can be from animal and/or plant origin. Protein can also be ingested as a supplement, which can also provide protein from the same variety of sources. Which protein is most effective in maximizing performance gains is of interest to all athletes. Protein effectiveness is accomplished by determining its quality and digestibility. Quality refers to the availability of amino acids that it supplies, and digestibility considers how the protein is best utilized [27]. A protein that contains all of the essential amino acids is considered to be a complete protein. Proteins from animal sources are considered to be complete proteins. In

contrast, proteins from vegetable sources are incomplete in that they are generally lacking one or two essential amino acids. Thus, someone who desires to get their protein from vegetable sources (i.e. vegetarians) will need to consume a variety of vegetables, fruits, grains, and legumes to ensure consumption of all essential amino acids. As such, individuals are able to achieve necessary protein requirements without consuming beef, poultry, or dairy. Protein digestibility refers to how the body can efficiently utilize dietary sources of protein. Proteins from vegetable sources are generally less efficient than animal proteins [27].

Animal Proteins

The common animal proteins typically found in protein supplements include whey, casein and bovine colostrum. Whey is a general term that typically denotes the translucent liquid part of milk that remains following the process (coagulation and curd removal) of cheese manufacturing. Whey is one of the two major protein groups of bovine milk, accounting for 20% of the milk. Whey contains high levels of the essential and branched chain amino acids. There are several varieties of whey protein that result from various processing techniques used to separate whey protein. These include whey powder, whey concentrate, whey isolate, and whey hydrolysate. Whey protein powder is more commonly used in the food industry as an additive in food products. It has several different varieties including sweet whey, acid whey (seen in salad dressings), demineralized (seen primarily as a food additive including infant formulas), and reduced forms. The demineralized and reduced forms of whey are not seen in sports supplements. In contrast, whey concentrate, whey isolate and whey hydrolysate contains more biologically active components and proteins, which make them a very attractive supplement for the athlete [27, 28]. Whey concentrate contains a protein concentration of 70% – 80% protein and is the most common form of whey protein found in sport supplements [28]. Whey isolates though contains protein concentrations of 90% with minimal amounts of lactose or lipids making it ideal for individuals who are lactose intolerant. Whey hydrolysate is thought to provide an accelerated rate of absorption as the manufacturing process of creating a hydrolysate (i.e., partially digested) is thought to enhance absorption and utilization of amino acids [28]. There is limited to no evidence demonstrating enhanced gastric emptying or plasma appearance of amino acids following whey hydrolysate ingestion. However, whey hydrolysates have been shown to enhance the insulin response compared to whey concentrate or isolate [28], and may improve recovery compared to whey isolate [29].

Casein is the major component of protein found in bovine milk accounting for nearly 70-80% of the total protein, and is responsible for the white color of milk. Similar to whey, casein is a complete protein. It exists in milk in the form of a micelle, which is a large colloidal particle. Once ingested it forms a gel or clot in the stomach. The ability to form this clot makes it very efficient in nutrient supply. The clot is able to provide a sustained slow release of amino acids into the blood stream, sometimes lasting for several hours [30]. This provides better nitrogen retention and utilization by the body.

Bovine colostrum is the “pre” milk liquid secreted by mothers in the first few days following birth. This nutrient-dense fluid is important for the newborn for its ability to provide immunities and assist in the growth of developing tissues in the initial stages of life. Evidence exists that bovine colostrum contains growth factors that stimulate cellular growth

and DNA synthesis [31]. Although it is not as common as whey and casein as a supplement, it does present interesting potential. Colostrum ingestion has been demonstrated to significantly elevate insulin-like-growth factor 1 (IGF-1) [32] and enhance lean tissue accruement [33, 34]. However, athletic performance improvements have been less conclusive. No changes in vertical jump or strength performance have been reported following 2- [32] and 8-weeks [34] of supplementation, respectively.

Vegetable Proteins

To provide for all of the essential amino acids various types of vegetable proteins need to be combined. Popular sources include legumes, nuts and soy. One advantage of vegetable protein is a likely reduction in the intake of saturated fat and cholesterol. Soy, from the legume family, is the most widely used vegetable protein source. Soy is a complete protein with a high concentration of branched chained amino acids. There have been many reported benefits related to soy proteins associated with health and performance (including reducing plasma lipid profiles, increasing LDL-cholesterol oxidation and reducing blood pressure) [27]. The soybean can be separated into three distinct categories; flour, concentrates, and isolates. Soy flour is the least refined form, and is commonly found in baked goods. Soy concentrate is made from defatted soybeans, is more palatable and has a high degree of digestibility. It is often found in nutrition bars, cereals, and yogurts. Isolates are the most refined soy protein product containing the greatest concentration of protein, but unlike flour and concentrates, contain no dietary fiber. Soy isolates are very digestible and easily introduced into foods such as sports drinks and health beverages.

Amino Acids versus Whole Protein Which Has Greater Benefit?

The use of either essential amino acids or whole protein ingestion appears to be beneficial in stimulating muscle protein synthesis. However, the question of whether one type of protein is more advantageous than the other is interesting. A study by Kersick et al., [35] suggested that the combination of whey and casein may be more beneficial in eliciting lean tissue gains when compared to a whey and essential amino acids combination. Unfortunately, there is not sufficient data to provide any conclusion. Interestingly, one study examined pre- and post-exercise whey protein ingestion and reported no benefits in regards to stimulating muscle protein synthesis from a pre-exercise ingestion of whey protein compared to a 1 h post-exercise ingestion [36]. This contrasts with the studies examining pre-exercise amino acid ingestion, which indicate that pre-exercise intake has greater benefit than post-exercise consumption. These differences are likely related to the difference in absorption rates and subsequent delivery of amino acids to exercising muscle. Tipton et al., [36] showed that arterial amino acid concentrations are approximately 100% higher than resting levels following ingestion of essential amino acids but only 30% following whey protein ingestion, indicating a greater amino acid availability to active muscle. In addition, the effect of the added carbohydrate to the amino acid blend (no carbohydrate was included with the whey

protein) likely enhanced the uptake of amino acids into the muscle by stimulating a greater insulin response.

Soy Protein vs. Animal Protein Consumption

The use of soy protein to fulfill daily protein requirements is popular among vegetarians and athletes who at times may prefer an alternative to animal-based proteins. The primary question regarding the consumption of soy is whether it provides the similar effect as an animal-based protein in stimulating protein synthesis and muscle protein accretion. Tang and colleagues [37] compared equivalent content of essential amino acids (10 g) as either whey hydrolysate, micellar casein or soy protein isolate consumed following 4-sets per exercise of a 10 – 12 repetition maximum (RM) on both unilateral leg press and knee extension exercises. Whey protein ingestion resulted in a significantly greater increase in plasma leucine concentrations during the 3-h recover period than both casein and soy proteins. In addition, whey protein ingestion stimulated muscle protein synthesis to a greater extent than both casein and soy. These differences were attributed to the faster absorption of whey protein and the faster increase of leucine in the circulation acts as a leucine 'trigger' to stimulate muscle protein synthesis [37]. Anthony et al. [38] fed carbohydrate + soy or carbohydrate + whey in treadmill exercised rats and reported that both soy and whey proteins were able to significantly enhance muscle protein synthesis to a greater extent than rats fed carbohydrate only. However, whey feedings appeared to enhance the phosphorylation of S6K1 and mTOR to a greater degree than soy, suggesting a potential differential response in mTOR signaling between the two types of proteins. Subsequent research in older men (60 – 75 y) comparing soy and whey protein after resistance exercise reported that both proteins increased p70S6K phosphorylation at 2 hours post exercise [39]. However, participants ingesting whey protein were able to maintain phosphorylation of p70S6K up to 4 hours post-exercise, whereas participants ingesting soy did not.

Volek and colleagues [40] compared the effects of either a daily whey or soy protein ingestion in untrained men and women participating in a 9-month periodized resistance training program. No significant differences were noted between the groups in the change in 1-RM bench press or squat strength. However, the change in lean body mass was significantly higher in whey than soy following 3-, 6- and 9-months of training. In addition, fasting leucine concentrations were significantly elevated (20%) and post-exercise plasma leucine increased more than 2-fold in the whey group. An additional study compared soy and whey protein supplementation in resistance trained men [41]. Participants consumed 20 g of either soy or whey on a daily basis for two weeks. Following the supplementation period, participants performed 6 sets of 10 repetitions of the squat exercise at 80% of the participant's 1-RM. Participants consuming the soy protein were shown to have an attenuated testosterone response to an acute training program, while whey may blunt the cortisol response to exercise [41]. Although others have reported no differences in the resting testosterone response following 12-weeks of soy and whey protein supplementation with resistance exercise [42], the response to exercise was not assessed.

Present understanding appears to support the use of milk, or animal-based proteins to maximize muscle protein synthesis and changes in lean body mass. This is likely related to

differences in protein quality as milk proteins contain a greater concentration of leucine. Further research appears warranted whether soy ingestion attenuates the anabolic response to exercise.

Importance of Leucine for Muscle Protein Synthesis

Leucine is one of the branched chain amino acids that appear to be a potent stimulator of muscle protein synthesis. Increases in muscle protein synthesis occur quite rapidly in response to an oral dose of leucine, with synthesis peaking between 30 – 60 min following ingestion in some reports [43], or 45 – 90 minutes in others [44]. This appears to be facilitated by a transient increase in insulin. Although leucine appears to independently stimulate an increase rate of muscle protein synthesis, the duration of stimulation does appear to be prolonged when accompanied by elevations in insulin [43]. A leucine and carbohydrate combination may appear to provide a sustained elevation in muscle protein signaling, though the precise role that carbohydrate plays is still not well understood. Others have demonstrated that essential amino acids (including leucine) can stimulate muscle protein synthesis independent of insulin [45]. Drummond and colleagues [46] indicated that the essential amino acids stimulate muscle protein stimulus, but the hyperinsulinemia resulting from carbohydrate ingestion may be more involved with attenuating protein breakdown. Thus, carbohydrates may have a greater role in muscle protein synthesis indirectly by reducing the AMP activated protein kinase (AMPK) inhibition of the mammalian target of rapamycin (mTOR) protein signaling pathway [47].

When leucine is provided in combination with resistance exercise the stimulus for muscle protein synthesis appears to be enhanced. Dreyer et al. [48] infused leucine enriched essential amino acids with carbohydrates in subjects performing 10 sets of 10 repetitions of their 1-RM in the leg extension exercise. They reported a 145% increase in muscle protein synthesis in the supplement + resistance exercise group compared to only a 41% increase in muscle protein synthesis in the resistance exercise group only. Elevations in protein synthesis appear to be partially explained by increases in the phosphorylation of the mTOR-signaling pathway. Specifically, leucine enriched essential amino acids provide following exercise were demonstrated to enhance phosphorylation of mTOR, S6K1 and 4E-BP1 signaling molecules.

Increases in muscle protein synthesis appear to occur in a dose-dependent manner [44, 45, 49]. Norton and colleagues [44] compared different dosing's of leucine in an animal study. Rats were fed a meal that contained 10%, 20% or 30% whey or wheat protein. The whey protein meals contained 47 mg, 94 mg and 142 mg of leucine, respectively, while the wheat protein (e.g., soy) meals contained 29 mg, 60 mg and 89 mg of leucine, respectively. Feedings of 10% wheat protein, containing 29 mg of leucine did not stimulate muscle protein synthesis, but feedings of 10% whey protein containing 47 mg of leucine did initiate protein synthesis. As leucine content increased from whey or wheat ingestion, an increase in muscle protein synthesis was noted. An interesting aspect of this study is that it was the first report indicating that a specific threshold may be needed to initiate mRNA translation resulting in muscle protein synthesis, and that if protein content is not sufficient (i.e., not reaching this threshold) then the anabolic processes may be blunted.

Protein Timing – Acute Effects

When protein is consumed following a workout the anabolic response is greater the closer the protein was consumed to the workout. Rasmussen and colleagues [50] provided untrained subjects 6 g of essential amino acids with 35 g sucrose following a resistance training workout. No differences in net muscle protein synthesis were seen in comparisons of protein ingestion periods of 1 or 3 h post-workout. However, when this same combination of essential amino acids and carbohydrate was infused immediately before exercise, the increase in muscle protein synthesis was significantly greater compared to infusion occurring immediately post-exercise [51]. Amino acid infusion immediately prior to the training session resulted in a 46% increase in amino acid concentration within skeletal muscle immediately post-exercise and an 86% elevation 1 h following exercise, which was significantly greater than those values seen from the same amino acid and carbohydrate infusion occurring immediately following the training session [51]. The primary benefit from pre-exercise ingestion of amino acids is likely related to an increased rate of delivery and subsequent uptake by skeletal muscle during exercise. A 2.6-fold greater increase in the rate of amino acid delivery to skeletal muscle is reported when the protein was consumed before exercise compared to post-exercise [51].

The composition of the amino acids provided in many of these studies was predicated on their proportional importance to stimulate protein synthesis. Evidence from several investigations have demonstrated that only the essential amino acids are necessary for stimulating protein synthesis, and that increases in muscle protein synthesis were relative to the essential amino acid composition of the supplement [20, 52, 53]. There is little to no evidence to support the use of nonessential amino acids as part of any nutritional supplement. Borsheim and colleagues [52] have also noted that differences in the clearance rate of amino acids following ingestion appears to alter the composition of the essential amino acids absorbed by the muscle. Leucine and isoleucine appear to have a more potent effect than the other essential amino acids on muscle protein synthesis [48, 52].

Effect of Timing – Whole Proteins

The two most common whole proteins used in dietary supplements are casein and whey. The differences in these proteins stem to a large extent to differences in digestive properties and the amino acid composition. As discussed earlier, when casein is ingested it forms a gel or clot in the stomach which slows down absorption. As a result, casein provides a sustained but slow release of amino acids into the bloodstream, sometimes lasting for several hours [30]. Whey protein is the translucent liquid part of milk, and contains higher amounts of the essential and branched chain amino acids [27,28]. In addition, whey protein has been shown to have a faster absorption capability than casein, which may have important implications for increasing the rate of protein synthesis following a training session [30].

One of the first comparisons between casein and whey protein supplementation examined protein synthesis rates following a 30 g feeding [30]. The investigators reported that ingestion of whey protein resulted in a rapid appearance of amino acids in the plasma, while ingestion of casein resulted in a slower rate of absorption, but provided for a more sustained elevation

in plasma amino acid concentrations. As a result of the fast absorption rate of whey, a more rapid increase in protein synthesis (68%) was observed within approximately two hours following ingestion. Casein ingestion though stimulated a more sustained elevation in protein synthesis, with a peak synthesis rate of approximately 31% above baseline. However, the sustained effect of casein resulted in a significantly higher leucine balance 7 hours following ingestion with no change from baseline seen at that time point following whey consumption. The fast and slow increase in muscle protein synthesis occurring from whey and casein ingestion, respectively, was supported by a subsequent investigation by Tipton et al. [22]. They suggested that although whey protein consumption may result in a more rapid increase in protein synthesis, a large part of this protein is oxidized (used as fuel), while casein consumption, due to its slower absorption rate, may result in a greater protein accretion over a longer duration. Interestingly, Dangin and colleagues [54] compared multiple ingestions of whey protein (over 4 h) to a single serving of whey or casein (total protein consumed was equivalent). The multiple ingestion periods resulted in a greater net leucine oxidation than a single feeding of either casein or whey. Whey protein's fast rate of absorption and high concentrations of leucine may make it the appropriate protein to consume immediately following a workout. Recent work by Burd and colleagues [55] compared whey and casein protein at rest and after exercise and found that whey protein significantly increased muscle protein synthesis significantly greater than casein in both conditions. Considering that there may be a heightened sensitivity in skeletal tissue following a workout, [24, 56, 57] ingestion of whey protein immediately following the training session may enhance muscle remodeling and recovery.

Studies on performance effects of whey versus casein ingestion in athletic or trained populations are limited. Kerksick and colleagues [35] examined resistance trained men for 10-weeks. Subjects were provided either a carbohydrate placebo, 40 g of whey protein and 8 g of casein, or 40 g of whey protein and 8 g of amino acids (5 g of branch chain amino acids and 3 g of glutamine) per day. The group ingesting the whey and casein combination experienced the greatest increase in lean body mass, but no differences were noted between the groups in strength gains. Wilborn and colleagues [58] compared 24 g of daily whey ingestion to 24 g of daily casein supplementation in collegiate female basketball players for 8-weeks. Significant improvements were noted in both groups in lean tissue accruement, and strength and power improvements. However, no differences between the groups were observed in any of the body compositional or performance measures suggesting that both proteins are beneficial.

Protein Timing: Training Response

The initial training study indicating the importance of protein timing examined older adults (74.1 ± 1 years) ingesting a liquid protein supplement (10 g protein, 7 g carbohydrate, and 3 g fat) immediately after or 2 h following each resistance training session (three times per week) for 12 weeks [57]. The investigators reported that muscle cross-sectional area and individual muscle fiber area were significantly increased in the participants who consumed the supplement immediately following each workout, but were not altered in the participants who ingested it 2 h following the workout. Willoughby and colleagues [59] examined the effects of pre and post protein feedings (20 g blend of whey and casein 1 h before and the

same 20 g supplement immediately post-workout) in a group of young (19 ± 2 y), untrained men. The placebo group consumed a similar amount of carbohydrate at the same time points. Following 10-weeks of training the protein supplement group realized significantly greater gains in strength, lean body mass than the placebo group. In addition, although both groups experienced significant gains in myofibrillar protein content, the gains observed in the protein group were significantly greater than that seen in the placebo group. Cribb and Hayes [56] examined the effects of protein timing in young (21-24 years) recreational male bodybuilders, 40 g of whey isolate and 43 g of carbohydrate (glucose) were provided either immediately before and after each resistance training session or in the morning and evening. Significantly greater gains in lean body mass, cross-sectional area of type II fibers, contractile protein content, and strength were seen in the pre- and post-workout feeding group compared to the morning and evening feeding group.

Research examining the effects of protein timing in a group of experienced, resistance trained athletes is quite limited. One study investigated the effects of protein timing in experienced, competitive college football players [60]. Participants were randomized into three groups. The first group consumed a 42 g protein supplement pre- and post-workout; the second group consumed the same supplement, albeit in the morning and evening; and the third group were not provided the supplement and served as the control group (e.g., they performed the same workout, but were not provided any protein supplement). Significant strength and power improvements were reported in all three groups, with no between-group differences observed. The average daily protein intake for all three groups ranged from 1.6 – 2.3 $g \cdot kg^{-1}$ body mass. The results of this study suggested that if dietary protein intake is at, or exceeds recommended levels for a strength–power athlete (1.6 $g \cdot kg \cdot day^{-1}$), additional protein from a supplement, regardless of when it is ingested, may not bring about further performance gains. In addition, all three study groups were in a positive nitrogen balance, suggesting that protein intakes were sufficient in meeting the athlete's protein needs. Considering that this study was only 10 weeks in duration, it may not have been long enough to see differences in performance gains from nutrient timing in experienced, competitive strength/power athletes. Another potential factor influencing the results of that study is that the supplement contained only collagen protein and no carbohydrate. This may have delayed nutrient absorption, and subjects may have missed the window of adaptation. Additional research still appears warranted in examining the potential benefits associated with protein ingestions surround a workout in experienced, resistance trained athletes, who consume a relatively high daily intake of protein.

How Much Protein Should Be Consumed per Ingestion?

Research on protein supplementation has used various quantities of protein per ingestion. These studies range from 6 g of amino acids to more than 40 g of whole protein, amino acids or proprietary blends in various combinations. However, little research has been conducted on whether there is a ceiling on the effectiveness of the quantity of protein that can be effectively used per ingestion. One study examined post-exercise protein drinks containing 0, 5, 10, 20, or 40 g protein [61]. Protein was ingested following an acute bout of leg extension exercise,

while whole-body leucine oxidation was measured over 4 h. The results indicated that muscle protein synthesis increased with each increase of protein quantity up to 20 g. No difference in protein synthesis was seen between the 20 g and 40 g dose. Whether a multi-joint structural exercise such as the squat, or a normal training routine (6 – 7 exercises using 3 – 4 sets per exercise), would stimulate further increases in protein synthesis at higher doses is not known. A recent study characterized the dose-response relationship between various amounts of whey protein ingestion on myofibrillar protein synthesis in experienced, resistance trained men who were not competitive athletes [62]. Doses of 0, 10, 20 and 40 g of whey protein isolate were ingested 10-min following resistance exercise. Results indicated that ingestion of moderate (20 g) and high (40 g) doses of whey protein stimulated a greater response of myofibrillar muscle protein synthesis than the low (10 g) dose. However, no additional benefit was noted in myofibrillar muscle protein synthesis when comparing 20 g to 40 g feedings, confirming previous research suggesting that an upper limit of muscle protein synthesis with 20 g feedings. Important to note though is that the body mass of the participants of this study was approximately 80 kg. Whether larger individuals can utilize greater amounts of protein remains largely unknown. Still, how much protein is consumed per ingestion may be less important that the pattern of protein ingestion.

Recent studies have examined the pattern of daily protein intake [63, 64]. Moore and colleagues [64] provided 80 g of whey protein per day to young, resistance trained men. Participants were randomized into three different dosing pattern groups. One group consumed the protein in a pulse fashion (8 x 10 g of whey protein every 1.5 h); another group used an intermediate ingestion fashion (4 x 20 g every 3 h); and the final group consumed the protein in a bolus fashion (2 x 40 g every 6 h). Ingestion occurred following an acute bout of knee extension exercise (4 set of 10 repetitions using 80% 1RM). Whole-body protein turnover was significantly greater (~ 19%) during the pulse ingestion format than the bolus ingestion format, and trended towards being greater than the intermediate format (~ 9%). Rates of protein synthesis were significantly greater for the pulse ingestion format compared to the intermediate and bolus formats (32% and 19%, respectively). Further inferential analysis showed likely small and moderate increases in whole-body protein turnover for pulse and intermediate ingestion formats compared to the bolus ingestion format. Thus, the pattern of protein ingested appears to impact whole-body protein metabolism. Areta and colleagues [63] examining myofibrillar protein synthesis, cell signaling and mRNA abundance using the same research methodology as the previous study reported that all three ingestion protocols increased myofibrillar protein synthesis, throughout the 12 h recovery period (ranging from 88% - 148%). However, the intermediate ingestion pattern elicited the greatest levels of myofibrillar protein synthesis than the other two ingestion patterns. Thus, it does appear that protein ingestion every three hours has the potential to maximize muscle mass development. Figure 1a-c provides theoretical feeding patterns of protein. Figure 1a depicts a typical feeding pattern, while Figures 1b and 1c provide optimal protein feedings to ensure reaching the leucine threshold and maximizing muscle protein synthesis.

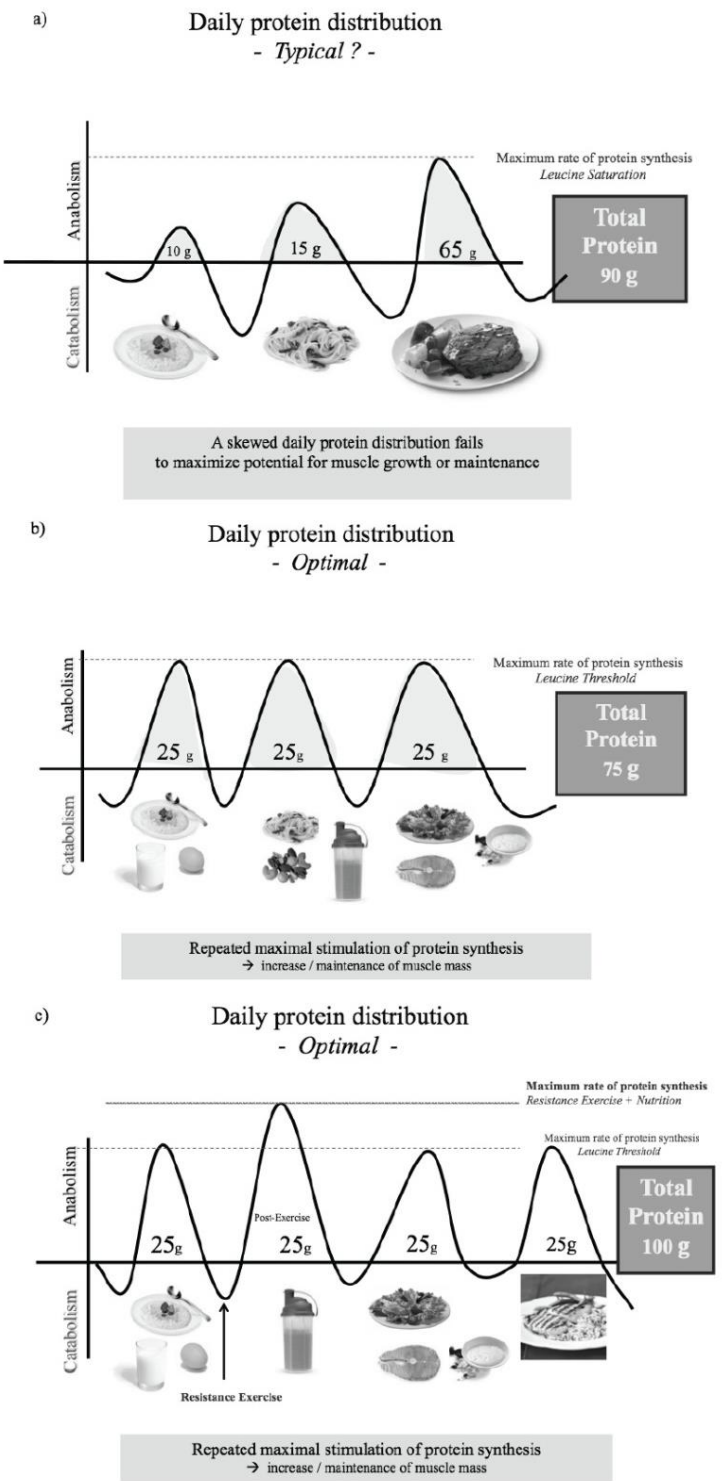


Figure 1. Daily protein distribution.

Safety

The use of high protein diets and protein supplements has been suggested to have potential health risks related to cardiovascular, renal, hepatic and bone function. Most of these concerns have proven to be without any scientific merit. Regarding cardiovascular disease risk, if protein intake comes primarily from meats, dairy products and eggs, without regard to fat intake, an increase in the consumption of saturated fat and cholesterol would likely occur. However, with making certain dietary modifications (removing skin from chicken, substituting fish for meat), the risk for cardiovascular disease will be reduced. Interestingly, Jenkins and colleagues [65] reported a decrease in lipid profiles in individuals consuming a high protein diet, while others have reported on an inverse relationship with protein intake and blood pressure [66].

The major concern associated with renal function is the role that the kidneys have in nitrogen excretion and the potential of a high protein diet to over-stress the kidneys. In healthy individuals there does not appear to be any adverse effects associated with a high protein diet. Poortsman and Dellalieux [67] examining bodybuilders consuming 2.8 $g \cdot kg^{-1}$ of protein per day reported no changes in any kidney function tests. However, for individuals with existing kidney disease it may be prudent to maintain a lower protein intake to reduce the progression of renal disease. High protein diets are also associated with an increase in calcium excretion. This is likely related to the ingestion of high amounts of animal protein, which is higher in sulfur-based amino acids than vegetable proteins [68,69]. Sulfur-based amino acids are thought to be the primary cause of calciuria (calcium loss). This can potentially result in kidney stones, but may also increase in acid secretion due to the elevated protein consumption. If the kidneys are unable to buffer the high endogenous acid levels, other physiological systems will need to compensate, such as bone. Calcium released from bone can be used to buffer high acidic levels and restore acid-base balance. However, Creedon and Cashman [70] reported no change in calcium concentrations or bone resorption in rats consuming 500 mg of casein per day. Others have demonstrated that animal protein intake in an older population, several times greater than the RDA requirement, results in a bone density accruement and significant decrease in fracture risk [71]. Hanley and Whiting [72] recently concluded that insufficient protein intake is a much greater problem for bone health than protein excess.

High protein diets have also been suggested to increase the risk for hepatic disease primarily due to the liver being stressed from metabolizing a greater protein intake [13]. Although one study by Jorda and colleagues [73] did indicate morphological changes in liver mitochondria consequent to high protein diets in rats, the investigator indicated that these changes were not pathological, but represented a positive hepatocyte adaptation to a metabolic stress. There is little to no scientific evidence regarding protein intake and hepatic disease [27]. More relevant is that protein is believed to be important for liver function by providing lipotropic agents such as methionine and choline for the conversion of fats to lipoprotein for removal from the liver [74]. In addition, high protein diets has been suggested to offset the elevated protein catabolism seen with liver disease [75], while a high protein diet has also been shown to improve hepatic function in individuals suffering from alcoholic liver disease [76]. In summary, there is little to no scientific evidence indicating any increased health risk from protein supplementation.

Summary

The benefits of elevating protein intake in trained athletes are well accepted. Most dieticians would emphasize the use of dietary protein to achieve these goals; however, the ability to provide protein at specific time points surrounding the workout may be best achieved from a supplement. Recent emphasis on the importance of protein timing is interesting with some evidence to support its efficacy. Data though is not conclusive in regards to the experienced athlete. Recent information strongly indicates the importance of multiple protein feedings per day to maintain a sustained elevation of muscle protein synthesis.

References

[1] Walsh, NP; Blannin, AK; Robson, PJ; Gleeson, M. Glutamine, exercise and immune function. *Sports Med*., 1998, 26, 177-191.

[2] Hulmi, JJ; Kovanen, V; Selanne, H; Kraemer, WJ; Häkkinen, K; Mero, AA. Acute and long-term effects of resistance exercise with or without protein ingestion on muscle hypertrophy and gene expression. *Amino Acids*, 2009, 37, 297-308.

[3] Koopman, R; Wagenmakers, AJ; Manders, RJ; Zorenc, AH; Senden, JM; Gorselink, M; Keizer, HA; van Loon, LJ. Combined ingestion of protein and free leucine with carbohydrate increases postexercise muscle protein synthesis in vivo in male subjects. *Am J Physiol Endocrinol Metab*., 2005, 288, E645-E653; 2005.

[4] Pitkänen, HT; Nykanen, T; Knuutinen, J; Lahti, K; Keinanen, O; Alen, M; Komi, PV; Mero, AA. Free amino acid pool and muscle protein balance after resistance exercise. *Med Sci Sports Exerc*., 2003, 35, 784-792.

[5] Tang, JE; Manolakos, JJ; Kujbida, GW; Lysecki, PJ; Moore, DR; Phillips, SM. Minimal whey protein with carbohydrate stimulates muscle protein synthesis following resistance exercise in trained young men. *Appl Physiol Nutr Metab*., 2007, 32, 1132-1138.

[6] Biolo, G; Maggi, SP; Williams, BD; Tipton, KD; Wolfe, RR. Increased rates of muscle protein turnover and amino acid transport after resistance exercise in humans. *Am J Physiol*., 1995, 268, E514-520.

[7] Phillips, SM; Tipton, KD; Aarsland, A; Wolf, SE; Wolfe, RR. Mixed muscle protein synthesis and breakdown after resistance exercise in humans. *Am J Physiol Endocrinol Metab*., 1997, 273, E99-E107.

[8] Biolo, G; Tipton, KD; Klein, S; Wolfe, RR. An abundant supply of amino acids enhances the metabolic effect of exercise on muscle protein. *Am J Physiol Endocrinol Metab*., 1997, 273, E122-E129.

[9] Miller, SL; Tipton, KD; Chinkes, DL; Wolf, SE; Wolfe, RR. Independent and combined effects of amino acids and glucose after resistance exercise. *Med Sci Sports Exerc*., 2003, 35, 449-455.

[10] Fern, EB; Bielinski, RN; Schutz, Y. Effects of exaggerated amino acid and protein supply in man. *Experientia*, 1991, 47, 168-172.

[11] Lemon, PWR; Tarnopolsky, MA; MacdougaL, JD; Atkinson, SA. Protein requirements and muscle mass/strength changes during intensive training in novice bodybuilders. *J Appl Physiol*., 1992, 73, 767-775.

[12] Hoffman, JR; Ratamess, NA; Kang, J; Falvo, MJ; Faigenbaum, AD. Effects of protein supplementation on muscular performance and resting hormonal changes in college football players. *J Sport Sci Med.*, 2007, 6, 85-92.

[13] Rodriguez, NR; DiMarco, NM; Langley, S. Nutrition and athletic performance. Position stand of the American College of Sports Medicine: American Dietetics Association and Dieticians of Canada. *Med Sci Sports Exerc.*, 2009, 41, 709-731.

[14] Hoffman, JR; Ratamess, NA; Kang, J; Falvo, MJ; Faigenbaum, AD. Effect of protein intake on strength: body composition and endocrine changes in strength/power athletes. *J Int Soc Sports Nutr*., 2006, 3, 12-18.

[15] Cortiella, J; Mathews, DE; Hoerr, RA; Bier, DM; Vernon, VR. Leucine kinetics at graded intakes in young men: quantitative fate of dietary leucine. *Am J Clin Nutr*., 1988, 48, 998-1009.

[16] Matthews, DE; Marano, MA; Campbell, RG. Splanchnic bed utilization of leucine and phenylalanine in humans. *Am J Physiol Endocrinol Metab*., 1993, 264, E109-E118.

[17] Matthews, DE; Marano, MA; Campbell, RG. Splanchnic bed utilization of glutamine and glutamic acid in humans. *Am J Physiol Endocrinol Metab*., 1993, 264, E848-E854.

[18] Halseth, A; Flakoll, PJ; Reed, EK; Messina, AB; Krishna, MG; Lacy, DB; Williams, PE; Wasserman, DH. Effect of physical activity and fasting on gut and liver proteolysis in the dog. *Am J Physiol Endocrinol Metab*., 1997, 273, E1073 – E1082.

[19] Williams, BD; Wolfe, RR; Bracy, D Wasserman, DH. Gut proteolysis provides essential amino acids during exercise. *Am J Physiol Endocrinol Metab*., 1996, 270, E85-E90.

[20] Tipton, KD; Ferrando, AA; Phillips, SM; Doyle D; Jr. Wolfe, RR. Postexercise net protein synthesis in human muscle from orally administered amino acids. *Am J Physiol.*, 1999, 276, E628-E634.

[21] Cermak, NM; Res, PT; de Groot, L; Saris, WHM; van Loon, LJC. Protein supplementation augments the adaptive response of skeletal muscle to resistance-type exercise training: a meta-analysis. *Am J Clin Nutr*., 2012, 96, 1454-1464.

[22] Tipton, KD; Elliot, TA; Cree, MG; Wolf, SE; Sanford, AP; Wolf, RR. Ingestion of casein and whey proteins result in muscle anabolism after resistance exercise. *Med Sci Sports Exerc*., 2004, 36**,** 2073-2081.

[23] Cooke, MB; Rybalka, E; Stathis, CG; Cribb, PJ; Hayes, A. Whey protein isolate attenuates strength decline after eccentrically-induced muscle damage in healthy individuals. *J Int Soc Sports Nutr*., 2010, 7, 30.

[24] Hoffman, JR; Ratamess, NA; Tranchina, CP; Rashti, SL; Kang, J; Faigenbaum, AD. Effect of protein ingestion on recovery indices following a resistance training protocol in strength/power athletes. *Amino Acids*, 2010, 38, 771-778.

[25] Kraemer, WJ; Ratamess, NA; Volek, JS; Hakkinen, K; Rubin, MR; French, DN; Gomez, AI; McGuigan, MR; Scheet, TP; Newton, RU; Spiering, BA; Izquierdo, M; Dioguardi, FS. The effects of amino acid supplementation on hormonal responses to overreaching. *Metabolism*, 2006, 55, 282-291.

[26] Ratamess, NA; Kraemer, WJ; Volek, JS; Rubin, MR; Gomez, AL; French, DN; Sharman, MJ; McGuigan, MR; Scheet, TP; Hakkinen, K; Newton, RU; Dioguardi, FS.

The effects of amino acid supplementation on muscular performance during resistance training overreaching. *J Strength Cond Res.*, 2003, 17, 250-258.

[27] Hoffman, JR; Falvo, MJ. Protein – Which is best? *J Sports Sci Med*., 2004, 3, 118-130.

[28] Hulmi, JJ; Lockwood, CM; Stout, JR. Effect of protein/essential amino acids and resistance training on skeletal muscle hypertrophy: A case of whey protein. *Nutr Metab*., 2010, 7, 51.

[29] Buckley, JD; Thomson, RL; Coates, AM; Howe, PRC; DeNichilo, MO; Rowney, MK. Supplementation with a whey protein hydrolysate enhances recovery of muscle force-generating capacity following eccentric exercise. *J Sci Med Sport.*, 2010, 13, 178-181.

[30] Boirie, Y; Dangin, M; Gachon, P; Vasson, MP; Maubois, JL; Beaufrere, B. Slow and fast dietary proteins differently modulate postprandial protein accretion. *Proc Natl Acad Sci USA*, 1997, 94, 14930-14935.

[31] Kishikawa, Y; Wantanabe, DS; Watanabe, T; Kubo, S. Purification and characterization of cell growth factor in bovine colostrum. *J Vet Med Sci*., 1996, 58, 47-53.

[32] Mero, A; Miikkulainen, H; Riski, J; Pakkanen, R; Aalto, J; Takala, T. Effects of bovine colostrum supplementation on serum IGF-I, IgG, hormone, and saliva IgA during training. *J Appl Physiol.*, 1997, 83, 1144-1151.

[33] Antonio, J; Sanders, M; Van Gammeren, D. The effects of bovine colostrum supplementation on body composition and exercise performance in active men and women. *Nutrition*, 2001, 17, 243-247.

[34] Brinkworth, GD; Buckley, JD. Bovine colostrum supplementation does not affect plasma buffer capacity or haemoglobin content in elite female rowers. *Eur J Appl Physiol*., 2004, 91, 353-356.

[35] Kerksick, CM; Rasmussen, CJ; Lancaster, SL; Magu, B; Smith, P; Melton, C; Greenwood, M; Almada, AL; Earnest, CP; Kreider, RB. The effects of protein and amino acid supplementation on performance and training adaptations during ten weeks of resistance training. *J Strength Cond Res*., 2006, 20, 643-653.

[36] Tipton, KD; Elliot, TA; Cree, MG; Aarsland, AA; Sanford, AP; Wolfe, RR. Stimulation of net muscle protein synthesis by whey protein ingestion before and after exercise. *Am J Physiol Endocrinol Metab*., 2007, 292, E71-E76.

[37] Tang, JE; Moore, DR; Kujbida, GW; Tarnopolsky, MA; Phillips, SM. Ingestion of whey hydrolysate, casein, or soy protein isolate: effects on mixed muscle protein synthesis at rest and following resistance exercise in young men. *J Appl Physiol*., 2009, 107, 987-992.

[38] Anthony, TG; McDaniel, BJ; Knoll, P; Bunpo, P; Paul, GL; McNurlan, MA. Feeding meals containing soy or whey protein after exercise stimulates protein synthesis and translation initiation in the skeletal muscle of male rats. *J Nutr*., 2007, 137, 357-362.

[39] Mitchell, CJ; Della Gatta, PA; Petersen, AC; Cameron-Smith, D; Markworth, JF. Soy protein ingestion results in less prolonged p70S6 kinase phosphorylation compared to whey protein after resistance exercise in older men. *J Int Soc Sports Nutr*., 2015, 12, 6.

[40] Volek, JS; Volk, BM; Gomez, AL; Kunces, LJ; Kupchak, BR; Freidenreich, DJ; Aristizabal, JC; Saenz, C; Dunn-Lewis, C; Ballard, KD; Quann, EE; Kawiecki, DL; Flanagan, SD; Comstock, BA; Fragala, MS; Earp, JE; Fernandez, ML; Bruno, RS; Ptolemy, AS; Kellogg, MD; Maresh, CM; Kraemer, WJ. Whey protein supplementation during resistance training augments lean body mass. *J Am Coll Nutr*., 2013, 32, 122-135.

[41] Kraemer, WJ; Solomon-Hill, G; Volk, BM; Kupchak, BR; Looney, DP; Dunn-Lewis, C; Comstock, BA; Szivak, TK; Hooper, DR; Flanagan, SD; Maresh, CM; Volek, JS. The effects of soy and whey protein supplementation on acute hormonal responses to resistance exercise in men. *J Am Coll Nutr*., 2013, 32, 77-74.

[42] Kalman, D; Feldman, S; Martinez, M; Krieger, DR; Tallon, MJ. Effect of protein source and resistance training on body composition and sex hormones. *J Int Soc Sports Nutr*., 2007, 4, 4.

[43] Anthony, JC; Lang, CH; Crozier, SJ; Anthony, TG; MacLean, DA; Kimball, SR; Jefferson, LS. Contribution of insulin to the translational control of protein synthesis in skeletal muscle by leucine. *Am J Physiol Endocrinol Metab*., 2002, 282, E1092-E1101.

[44] Norton, LE; Layman, DK; Bunpo, P; Anthony, TG; Brana, DV; Garlick, PJ. The leucine content of a complete meal directs peak activation but not duration of skeletal muscle protein synthesis and mammalian target of rapamycin signaling in rats. *J Nutr*., 2009, 139, 1103-1109.

[45] Cutherbertson, D; Smith, K; Babraj, J; Leese, G; Waddell, T; Atherton, P; Wackerhage, H; Taylor, PM; Rennie, MJ. Anabolic signaling deficits underlie amino acid resistance of wasting: aging muscle. *FASEB J*., 2005, 19, 422-424.

[46] Drummond, MJ; Dreyer, HC; Fry, CS; Glynn, EL; Rasmussen, BB. Nutritional and contractile regulation of human skeletal muscle protein synthesis and mTORC1 signaling. *J Appl Physiol.*, *2009*, 106, 1374-1384.

[47] Hardie, DG; Sakamoto, K. AMPK: a key sensor of fuel and energy status in skeletal muscle. *Physiology*, 2006, 21, 48-60.

[48] Dreyer, HC; Drummond, MJ; Pennings, B; Fujita, S; Glynn, EL; Chinkes, DL; Dhanani, S; Volpi, E; Rasmussen, BB. Leucine-enriched essential amino acid and carbohydrate ingestion following resistance exercise enhances mTOR signaling and protein synthesis in human muscle. *Am J Physiol Endocrinol Metab.*, 2008, 294, E392-E400.

[49] Bohe, J; Low, A; Wolfe, RR; Rennie, MJ. Human muscle protein synthesis is modulated by extracellular, not intramuscular amino acid availability: a dose-response study. *J Physiol*., 2003, 552, 315-324.

[50] Rasmussen, BB; Tipton, KD; Miller, SL; Wolf, SE; Wolfe, RE. An oral essential amino acid-carbohydrate supplement enhances muscle protein anabolism after resistance exercise. *J Appl Physiol*., 2000, 88, 386-392.

[51] Tipton, KD; Rasmussen, BB; Miller, SLl; Wolf, SE; Owens-Stovall, KK; Petrini, BE; Wolfe, RR. Timing of amino acid-carbohydrate ingestion alters anabolic response of muscle to resistance exercise. *Am J Physiol Endocrinol Metab*., 2001, 281, E197-E206.

[52] Borsheim, E; Tipton, KD; Wolf, SE; Wolfe, RR. Essential amino acids and muscle protein recovery from resistance exercise. *Am J Physiol Endocrinol Metab*., 2002, 283, E648-E657.

[53] Tipton, KD; Gurkin, BE; Matin, S; Wolfe, RR. Nonessential amino acids are not necessary to stimulate net muscle protein synthesis in health volunteers. *J Nutr Biochem*., 1999, 10, 89-95.

[54] Dangin, M; Boirie, Y; Guillet, C; Beaufrere, B. Influence of the protein digestion rate on protein turnover in young and elderly subjects. *J Nutr*., 2002, 132**,** 3228S-3233S.

[55] Burd, NA; Yang, Y; Moore, DR; Tang, JE; Tarnopolsky, MA; Phillips, SM. Greater stimulation of myofibrillar protein synthesis with ingestion of whey protein isolate v.

micellar casein at rest and after resistance exercise in elderly men. *Br J Nutr*., 2012, 108, 958-962.

[56] Cribb, PJ; Hayes, A. Effects of supplement timing and resistance exercise on skeletal muscle hypertrophy. *Med Sci Sports Exerc.*, 2006, 38, 1918-1925.

[57] Esmarck, B; Andersen, JL; Olsen, S; Richter, EA; Mizuno, M; Kjaer, M. Timing of postexercise protein intake is important for muscle hypertrophy with resistance training in elderly humans. *J Physiol.*, 2001, 535, 301-311.

[58] Wilborn, CD; Taylor, LW; Outlaw, J; Williams, L; Campbell, B; Foster, CA; Smith-Ryan, A; Urbine, S; Hayward, S. The effects of pre- and post-exercise whey vs. casein protein consumption on body composition and performance measures in collegiate female athletes. *J Sports Sci Med*., 2013, 12, 74-79.

[59] Willoughby, DS; Stout, JR; Wilborn, CD. Effects of resistance training and protein plus amino acid supplementation on muscle anabolism, mass and strength. *Amino Acids*, 2007, 32, 467-477.

[60] Hoffman, JR; Ratamess, NA; Tranchina, CP; Rashti, SL; Kang, J; Faigenbaum, AD. Effect of protein supplement timing on strength; power and body compositional changes in resistance-trained men. *Int J Sport Nutr Exerc Metab.*, 2009, 19, 172-185.

[61] Moore, DR; Robinson, MJ; Fry, JL; Tang, JE; Glover, EI; Wilkinson, SB; Prior, T; Tarnopolsky, MA; Phillips, SM. Ingested protein dose response of muscle and albumin protein synthesis after resistance exercise in young men. *Am J Clin Nutr*., 2009, 89, 161-168.

[62] Witard, OC; Jackman, SR; Breen, L; Smith, K; Selby, A; Tipton, KD. Myofibrillar muscle protein synthesis rates subsequent to a meal in response to increasing doses of whey protein at rest and after resistance exercise. *Am J Clin Nutr*., 2014, 99, 86-95.

[63] Areta, JL; Burke, LM; Ross, ML; Camera, DM; West, DW; Broad, EM; Jeacocke, NA; Moore, DR; Stellingwerff, T; Phillips, SM; Hawley, JA; Coffey, VG. Timing and distribution of protein ingestion during prolonged recovery from resistance exercise alters myofibrillar protein synthesis. *J Physiol*. 2013, 591, 2319-2331.

[64] Moore DR; Areta J; Coffey VG; Stellingwerff T; Phillips SM; Burke LM; Cléroux M; Godin JP; Hawley JA. Daytime pattern of post-exercise protein intake affects whole-body protein turnover in resistance-trained males. *Nutr Metab* (Lond)., 2012, 16, 91.

[65] Jenkins, DJA; Kendall, CWC; Vidgen, E; Augustin, LSA; van Erk, M; Geelen, A; Parker, T; Faulkner, D; Vuksan, V; Josse, RG; Leiter, LA; Connelly, PW. High protein diets in hyperlipidemia: effect of wheat gluten on serum lipids; uric acid and renal function. *Am J Clin Nutr*., 2001, 74, 57-63.

[66] Obarzanek, E; Velletri, PA; Cutler, JA. Dietary protein and blood pressure. *JAMA*. 1996, 275, 1598-1603.

[67] Poortmans, JR; Dellalieux, O. Do regular high protein diets have potential health risks on kidney function in athletes? *Int J SportNutr Exerc Metab.*, 2000, 10, 28-38.

[68] Barzel, US; Massey, LK. Excess dietary protein can adversely affect bone. *J Nutr*. 1998, 128, 1051-1053.

[69] Remer, T; Manz, F. Estimation of the renal net acid excretion by adults consuming diets containing variable amounts of protein. *Am J Clin Nutr*. 1994, 59, 1356-1361.

[70] Creedon, A; Cashman, KD. The effect of high salt and high protein intake on calcium metabolism, bone composition and bone resorption in the rat. *Br J Nutr*. 2000, 84, 49-56.

[71] Hannan, MT; Tucker, KL; Dawson-Hughes, B; Cupples, LA; Felson, DT; Kiel, DP. Effect of dietary protein on bone loss in elderly men and women: The Framingham Osteoporosis Study. *J Bone Mineral Res*., 2000, 15, 2504-2512.

[72] Hanley, DA; Whiting, SJ. Does a high dietary acid content cause bone loss, and can bone loss be prevented with an alkaline diet? *J Clin Densitom*., 2013, 16, 420-425.

[73] Jorda, A; Zaragosa, R; Portoles, M; Baguena-Cervellera, R; Renau-Piqueras, J. Long-term highprotein diet induces biochemical and ultrastructural changes in rat liver mitochondria. *Arch Biochem Biophy*., 1998, 265, 241-248.

[74] Navder, K; Lieber, CS. Nutritional support in chronic disease of the gastrointestinal tract and the liver. In: *Nutritional Aspects and Clinical Management of Chronic Disorders and Diseases*. Ed:Bronner, F. Boca Raton, FL: CRC Press. 2003, 45-68.

[75] Navder, K; Lieber, CS. Nutrition and alcoholism. In: *Nutritional Aspects and Clinical Management of Chronic Disorders and Diseases*. Ed: Bronner, F. Boca Raton, FL: CRC Press. 2003, 307-320.

[76] Mendenhall, CL; Moritz, TE; Roselle, GA; Morgan, TR; Nemchausky, BA; Tamburro, CH; Schiff, ER; McClain, CJ; Marsano, LS; Allen, JI. A study of oral nutrition support with oxadrolone in malnourished patients with alcoholic hepatitis: results of a Department of Veterans Affairs Cooperative Study. *Hepatology.*, 1993, 17, 564-575.

In: Human Health and Nutrition
Editor: Sergej M. Ostojic
ISBN: 978-1-63482-823-9

Chapter IV

Efficacy and Safety of Guanidinoacetic Acid Supplementation in Healthy Men and Women

Sergej M. Ostojic[1,2,*] and Marko Stojanovic[1,2]
[1]Center for Health, Exercise and Sport Sciences, Belgrade, Serbia
[2]Faculty of Sport and Physical Education, University of Novi Sad, Serbia

Abstract

Guanidinoacetic acid (GAA) is a natural precursor of creatine and under investigation as a novel dietary supplement. However, its use in human nutrition is hampered by limited knowledge on its physiological effectiveness and safety after supplementation. The main aims of the present study were: (a) to identify if oral GAA affects human performance and body composition; (b) to determine the most effective dose regimen of GAA; and (c) to analyze the incidence and severity of adverse effects of GAA supplementation. Fifty two (n = 52) male and female college athletes who were experienced in exercise training (> 2 years), and who were between 20 and 25 years of age were included in the study. Participants were randomized in a double-blind design to receive three different dosages of GAA (1.2 g/day, 2.4 g/day, and 4.8 g/day) or placebo (inulin) by oral administration for 6 weeks. Two-way mixed model ANOVA revealed significant increase in lean body mass (P = 0.006), handgrip strength (P = 0.03), and bench press performance (P = 0.014) in participants supplemented with GAA. Supplementation with GAA for 6 weeks had no major effect on indices of anaerobic power and capacity. Low-dose GAA (1.2 g/day) can be considered as the minimum effective dose for improving performance characteristics, while the effects are most consistently seen in participants receiving 2.4 g/day of GAA. Except for the dose of 4.8 g/day of GAA, reported side effects of GAA administration are rather mild (e.g., weight gain, gastrointestinal distress). The findings of this study demonstrate that oral GAA is an effective performance-enhancing agent with dose-dependent effects and mild side effects experienced when ingested over 6 weeks.

* Corresponding author: Email: sergej.ostojic@chess.edu.rs.

Keywords: Creatine; Ergogenic; Side effects; Hyperhomocysteinemia; Supplement

Introduction

Guanidinoacetic acid (GAA; also known as glycocyamine or guanidinoacetate) is the biochemical precursor of creatine, the latter being phosphorylated and playing an important role as an energy carrier in the cell. Creatine irreversibly degrades to creatinine, which is excreted via the kidneys with approximately 1.6% of the creatine pool being daily degraded to creatinine and excreted [1]. The daily creatine loss must be replaced either through the body's own creatine biosynthesis or via exogenous supply. Creatine and GAA deficiency can occur due to impairment in endogenous synthesis [2] and/or increased requirements during energy-demanding circumstances [3]. This deficit may impede cellular bioenergetics, which suggests a need for creatine or GAA replenishment from exogenous sources. Since the uptake of GAA through the diet is negligible [4], provision of concentrated GAA in oral form as a precursor of creatine is rather unexplored in human nutrition. Preliminary studies that evaluated the digestibility of GAA suggested that the bioavailability after oral administration of GAA was nearly 100% [5], making GAA eligible as a potent dietary additive. According to the research in animals [6-8] and preliminary reports in humans [9-11] short-term oral administration of GAA increases serum level of creatine. This 'creatine-delivering' effect of supplemental GAA has been advocated in both athletic environment and patients [9, 12-15], although the clear physiological and clinical benefits of GAA administration are yet to be determined. So far, neither human study evaluated the physiological effectiveness of oral GAA, nor has the dose-response effect of supplemental GAA been determined. Currently, there is only little information available about the adverse effects of GAA supplementation in humans [9]. Therefore, the main aims of the present study were: (a) to identify if the GAA improves human performance and body composition, (b) to determine the most effective dose regimen of GAA, and, (c) to analyze the incidence and severity of adverse effects of GAA supplementation.

Methods

Study Design

The original randomized, double-blind, placebo-controlled study was initiated in 2009 to examine the safety and physiological outcomes after medium-term supplementation of oral GAA in healthy men and women [16]. During the study, additional analyses of the original data set were introduced to explore specific aspects of GAA administration [9-11]. Herein, we report results on the physiological effectiveness and additional information on the safety profile of GAA observed throughout the 6-week intervention period.

Participants

Male and female college athletes who were experienced in exercise training (> 2 years) and who were between 20 and 25 years of age, were candidates for inclusion in the study. They were not admitted to the study if any of the following criteria were present: (1) a history of metabolic disease; (2) musculoskeletal dysfunction; (3) known heart disease; (4) smoking; (5) use of any performance-enhancing drugs or dietary supplements within the past 60 days; (6) an impaired response to exercise test; (7) residence outside the city of Novi Sad, or unwillingness to return for follow-up; and, (8) pregnancy in case of women. Both men and women were participants in the study. All participants were fully informed verbally and in writing about the nature and demands of the study as well as the known health risks. They gave their informed consent regarding their voluntary participation in the study. Approval of the University's ethical advisory commission was obtained, with all procedures performed in accordance with the Declaration of Helsinki. All participants completed a health history questionnaire, prescreening blood and urine profiling, and general pre-participation examination at the initial recruitment. If any of specific markers were above the reference values, subjects were excluded from the study. Subjects were obliged to maintain their normal physical activity patterns and dietary regimen throughout the duration of the study. Upon initial recruitment, fifty two (n = 52) participants met the criteria to take part in the study with number of participants was in accordance with optimal sample size [17]. The mean physical characteristics of participants were: age 22 ± 2 years, weight 71 ± 14 kg, height 176 ± 10 cm.

Intervention

Participants were randomized according to a computer generated randomization list in a double-blind design to receive three different dosages of GAA (1.2 g, 2.4 g, 4.8 g) or placebo (inulin) at a dose of 8 capsules per day by oral administration for 6 weeks. Groups were matched for subjects' age, weight, and exercise performance, with women had an equal probability of assignment to the groups. Study personnel were blinded to the treatment assignment for the duration of the study. GAA and placebo were provided in non-transparent soft capsules Interventions were supplied by AlzChem AG (Trostberg, Germany). Interventions were delivered in numbered (coded) plastic bags and the code was revealed once recruitment, data collection and laboratory analyses were completed. All subjects received supplementation package for in-between-tests period at every visit to the lab. Subjects collected coded treatment directly from the research staff at the lab and self-administered capsules during the study. Supplementation period lasted for 6 weeks. Participants were instructed to take daily dose of eight capsules in the morning upon waking before the breakfast with plenty of water. The primary endpoint with respect to the efficacy in exercise performance was the increase of muscle power on leg press test achieving a significant (5%) change in number of repetitions. Additional analyses were done on the isometric and isotonic muscle strength, anaerobic and aerobic performance indicators, body composition variables, selected biochemical profiles in blood and urine, and incidence of side effects. All testing procedures were conducted at baseline and at the end of the first, second, fourth, and sixth week. In the 24 hours before the performance tests, the subjects did not participate in any prolonged exercise or drinking alcoholic and/or caffeine beverages. In

general, except for the pre-testing day, habitual caffeine intake was not restricted during the study. All participants performed regular exercise for the whole duration of the study (four sessions per week for 60 minutes/session at 70-85% of maximal heart rate). Subjects were strongly instructed to limit exercise to the prescribed training regimen. Most of the training sessions were performed at the faculty's athletic training facility.

Experimental Protocol

Participants visited the laboratory on 5 occasions: before starting receiving the intervention and after one, two, four and six weeks during the supplementation period. All measurements were taken between 9 and 12 a.m. after an overnight fast of between 10 and 12 h. For this report, fasting blood was analyzed for complete blood count (Coulter blood counter, Model S-plus II, Coulter Electronics Inc., Hialeah, Florida, USA), and yielded values for red blood cell (RBC) count, white blood cell (WBC) count, platelets, hemoglobin (Hb), hematocrit (Hct), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC). The serum total antioxidant capacity (TAC) was analyzed by the procedure of chemiluminescence (Boehringer Mannheim GmbH, Germany). Glucose, urea, total cholesterol, triglycerides, and lipoprotein levels were analyzed by standard enzymatic methods and an automated analyzer (Hitachi 704, Tokyo, Japan). Serum activities of aspartate aminotransferase (AST)**,** alanine aminotransferase (ALT)**,** *alkaline phosphatase (ALP)***,** gamma-glutamyl transaminase (γ-GT), and creatine kinase (CK) along with calcium level *were analyzed* by an automated analyzer (RX *Daytona*, *Randox* Laboratories Ltd., Crumlin, UK). Blood and urine *sodium and potassium levels were analyzed* by ISE direct with ILyte analyzer (Kaunas, Lithuania). Routine urinalysis was completed by standard screening test (Machery-Nagel GmbH & Co. KG, Duren, Germany), with urine pH assessed by standard pH meter (Radiometer PHM82, Copenhagen, Denmark). The urinary *protein* was measured by a Randox commercial kit with a *Bayer opeRA* autoanalyser (Bayer Diagnostics, Leverkusen, Germany). All samples for each subject were assayed in the same run. For all values, the first reading was discarded and the mean of the next three consecutive readings with a coefficient of variation below 15% was used in the study.

Height was measured using a stadiometer (Seca 202, Hanover, MD, USA) while body mass was obtained using a calibrated balance beam scale (W & T Avery Ltd, Model 3306 ABV, Smethwick, West Midlands, UK). The subjects were measured nude, in the same state of hydration and nourishment after voiding. Total body resistance was measured with a foot-pad bioelectrical impedance analyzer (BF-662W, Tanita Corporation, Tokyo, Japan) at a fixed signal frequency of 50 kHz and 500μA. When these preliminary measurements were done subjects completed a general warm up (15-min of stretching and individual exercise). After a general warm up, each subject underwent a series of strength tests. Isometric strength of forearm muscles was assessed by hand grip test [18] with hydraulic hand dynamometer (Jamar J00105, Lafayette Instrument Company, Lafayette, IN, USA). Afterwards, subjects were instructed to perform the maximal number of full repetitions for the supine free weight bench press (75% of body mass) and leg press (45°, 150% of body mass) exercises (Pro-Fitness Exercise Systems, Phoenix, AZ, USA), respectively. Single and repetitive maximal vertical jump performance was assessed using a contact mat (Just Jump System, Probotics,

Huntsville, AL, USA) [19]. Finally, subjects were instrumented for submaximal-to-maximal interval endurance running test and blood lactate sampling. Gas-exchange data were collected throughout the test using a breath-by-breath metabolic system (Vacu-Med CPX, Ventura, CA, USA), while lactate concentration was determined using reflectance photometer (Accutrend, Roche Deutschland Holding GmbH, Mannheim, Germany). Exercise test was performed according to incremental protocol using a treadmill system (Trackmaster TMX425C, Newton, KS, USA). During and after the test heart rate (HR) was also recorded using a HR monitor at beat-to-beat interval (Polar S810, Kempele, Finland). All subjects were assessed on the same day with the tests performed in the same order. The participants were familiar with testing procedures as part of their regular training process. A week before the testing, the subjects performed a 10 min familiarization trial on the treadmill along with vertical jump assessment and isodynamic strength tests. To ensure that the testing environment was appropriately controlled, the laboratory was kept as quiet as possible during all measurements. The testing room was maintained at 22.0 ± 2.0°C and 25 ± 5% relative humidity.

In order to assess potential side effects to the supplementation regimen, all subjects were instructed to report any adverse effects of supplementation (e.g., diarrhea, nausea, weight gain, muscle cramps) at every visit to the lab. An open-ended questionnaire for self-assessment of well-being and side-effects was administered during the study. During the first week of intervention, all participants were interviewed by phone to closely monitor acute adverse effects of supplementation. In case of severe effects reported (e.g., vomiting, headache, vision impairment, numbness), subjects were closely monitored by phone and in person at a daily basis, and if another episode occurred which impaired the study participation or subject's health, participants were excluded from the study.

Statistical Analyses

The data are expressed as means ± SD. Two-way mixed model analysis of variance (ANOVA) with repeated measures was used to establish if any significant difference existed between participants' responses over time. Where significant differences were found, the Tukey test was employed to identify the differences. The rates of side-effects occurrence between the groups were compared using the Fisher exact probability test. *P* values of less than 0.05 were considered to be statistically significant. The data were analyzed using the statistical package SPSS, PC program, version 14.0 (SPSS Inc., Chicago, Illinois, USA).

Results

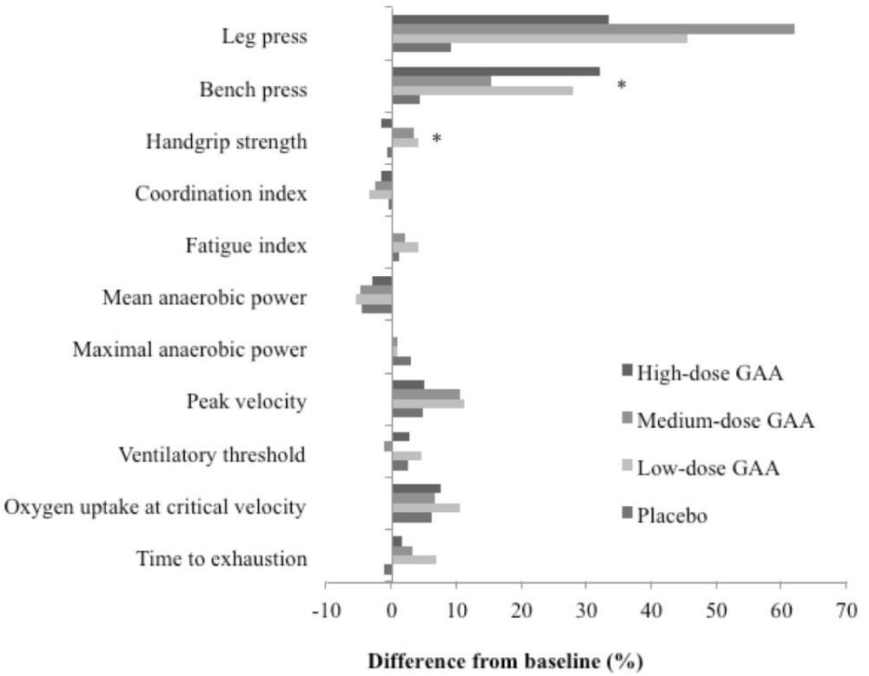
Fifty-two participants (n = 52) underwent randomization and received at least one dose of a study supplement. Twelve participants per group were randomly assigned to placebo, low-dose and medium-dose GAA group, and 16 to high-dose GAA group. Seven volunteers withdrew before the end of the study; one female from placebo group, and one female from low-dose GAA group were lost due to follow-up. Five females from high-dose GAA group were excluded from the study due to side effects experienced during the intervention. Most participants received all interventions regularly but a few omitted some quantity of capsules.

The total compliance with the regimen was 86 ± 5% for the all GAA groups, and 95 ± 2% for the placebo group.

Table 1. Incidence of side effects reported after placebo and GAA administration

Symptoms	Placebo (*n* = 12)	1.2 g GAA (*n* = 12)	2.4 g GAA (*n* = 12)	4.8 g GAA (*n* = 16)	*P*
Swallowing problems	5 (41.7)	4 (33.3)	4 (33.3)	6 (37.5)	0.99
Heartburn	4 (33.3)	3 (25.0)	4 (33.3)	4 (25.0)	0.93
Intestinal cramping	2 (16.7)	2 (16.7)	3 (25.0)	7 (43.7)	0.41
Diarrhea	2 (16.7)	1 (8.3)	0 (0)	4 (25.0)	0.25
Muscle cramping	0 (0)	1 (8.3)	2 (16.7)	3 (18.8)	0.47
Appetite increase	1 (8.3)	0 (0)	1 (8.3)	3 (18.8)	0.53
Weight gain	1 (8.3)	1 (8.3)	3 (25.0)	4 (25.0)	0.57
Bloadtdness	0 (0)	0 (0)	3 (25.0)	3 (18.8)	0.11
Nausea	0 (0)	0 (0)	2 (16.7)	10 (62.5)	0.0001
Abdominal pain	0 (0)	0 (0)	1 (8.3)	6 (37.5)	0.008
Vomiting	0 (0)	0 (0)	0 (0)	6 (37.5)	0.001
Numbness	0 (0)	0 (0)	0 (0)	1 (6.3)	0.99
Tingling	0 (0)	0 (0)	0 (0)	1 (6.3)	0.99
Headache	0 (0)	0 (0)	0 (0)	3 (18.8)	0.06
Vision impairment	0 (0)	0 (0)	0 (0)	1 (6.3)	0.99

Values are presented as actual numbers with percentage in parentheses



Asterisk (*) indicates significant interaction effect (trial vs. group) at $P < 0.05$.

Figure 1. Percentage change in exercise performance end points 0 vs. 6 week.

Twenty nine participants reported different side-effects of supplementation (Table 1). Side effects were experienced in 3 females and 2 males in placebo group, 2 females and 2 males in low-dose GAA group, 3 males and 4 females in medium-dose GAA group, and 3 males and 10 females in high-dose GAA group, respectively. The rates of most subjectively reported adverse effects were not different between the groups, except for nausea, abdominal pain and vomiting that were more frequent in participants supplemented with high-dose GAA ($P < 0.05$).

Changes in weight and body composition outcomes from the baseline to the end of the study are presented in Table 2. No significant between-group changes were observed at the end of the intervention (week 6) in body weight, nor for fat percentage or total body water. In addition, intervention affected lean body mass ($P = 0.006$), with participants receiving 2.4 g of GAA per day, and 4.8 g of GAA per day significantly elevated their lean body mass compared to placebo ($P < 0.05$).

Changes in exercise performance end points from baseline to week 6 are presented in Figure 1. Significant differences in handgrip strength were seen between the groups ($P = 0.03$).

Following 6-weeks of GAA ingestion, participants receiving low-dose GAA and medium-dose GAA significantly improved their handgrip strength compared to placebo ($P < 0.05$). Furthermore, muscle endurance expressed as the change from baseline in repetitions performed in the bench press exercise was significantly greater in the 1.2 g/day dose of GAA ($P = 0.01$), and the 4.8 g/day dose ($P = 0.01$) compared to placebo. No significant between-group changes were observed at week 6 in lower body muscle endurance, nor anaerobic or aerobic performance. In addition, no changes in heart rate measures neither exercise-related lactate responses have been found (not presented).

Blood glucose and lipid profiles (triglycerides, total cholesterol, LDL cholesterol, and HDL cholesterol) were not affected by the intervention. In addition, no interaction effect of intervention has been found for AST, ALT, CK, ALP and γ-GT. On average, liver and muscle function enzymes were within the normal clinical ranges from baseline throughout the study termination. GAA intervention affected RBC ($P = 0.03$), with low-dose GAA induced a decrease in RBC at post-administration as compared to the baseline ($5.4 \pm 0.4 \times 10^{12}$/L *vs.* $5.2 \pm 0.5 \times 10^{12}$/L; $P = 0.02$). On the other hand, GAA loading for 6 weeks had no effect on serum Hgb, Hct, erythrocyte indices, platelets and WBC count (not presented). None of the GAA-supplemented participants with GAA experienced anemia or macrocytic erythrocytes (e.g., MCV > 90 fL); all hematological indices were well within their respective normal range at all times. Serum electrolytes, albumin and total antioxidant capacity were not affected by GAA; no effect of intervention (except for urinary sodium) has been reported for urine electrolytes, urea nitrogen, proteins and urine pH.

Discussion

This study has evaluated the effectiveness and safety of six-week GAA supplementation in young physically active men and women. Dietary GAA induced significant increase in lean body mass, grip strength and upper body strength. Treatment with three different oral doses of GAA for 6 weeks had no major effect on aerobic and anaerobic endurance, neither

cardiovascular and lactate responses after maximal exercise. It seems that 1.2 g/day of GAA can be considered as a minimal dose with ergogenic properties, while the effects are most consistently seen in participants receiving 2.4 grams of GAA per day. Except for the dose of 4.8 grams of GAA, reported side effects of GAA administration are rather mild (e.g., weight gain, mild gastrointestinal distress). Furthermore, during GAA loading liver and muscle enzymes remained within the normal clinical ranges, as well as serum antioxidant capacity and hematological indices.

Body Composition and Exercise Performance

According to authors' knowledge, no mammalian or human studies investigated the short- or medium-term effects of exogenous GAA on weight and body composition. Although its relevance is questionable with regards to humans, the European Food Safety Authority [4] authorized GAA as a feed additive for chickens for fattening, with GAA significantly improved weight gain at 600 and 800 mg per kg feed for an experimental period of 42 days. Although no body composition was assessed, GAA at doses of 800 mg/kg and above increased breast weight and reduced the amount of abdominal fat, particularly when chicken were supplemented with doses of 1000 mg GAA per kg feed and above. The results of the present study indicated that supplemental GAA increased lean body mass in healthy men and women, while no effect of intervention has been found for weight, total body water and fatness. However, the exact mechanism of action is not revealed so far. Weather an increase in lean body mass after GAA administration is due to the influx of water into the muscle cell and/or enhanced protein synthesis should be further analyzed employing onward body composition assessment techniques (e.g., DEXA, magnetic resonance imaging).

No human studies known to authors examined the effects of GAA on exercise performance, although few studies from the 1950-s indicated a beneficial effect of oral GAA (co-administered with betaine) on wellness and general muscular strength in clinical patients [12]. In the present study we found beneficial effects of supplemental GAA on maximal voluntary strength during handgrip performance, and muscular endurance as assessed during isodynamic bench press exercise. Although we did not measure muscular concentration of phosphocreatine after GAA intervention, enhanced synthesis of creatine and improved energy balance might explain purported ergogenic effect of GAA loading. Since the upper body strength is less developed in general population as compared to the lower body strength [20], it seems that GAA supplementation is particularly effective for enhancing muscular performance for muscle groups with lower initial level of strength. Therefore, GAA supplementation (low-dose and high-dose of GAA in particular) may have a greater effect on the relative strength gains for the upper body muscle groups (e.g., chest, shoulders, arms) in novice athletes or subjects with lower level of fitness. In addition, aerobic and anaerobic performance indicators were not affected by GAA administration. This is probably due to the fact that performance tasks of longer duration primarily rely on anaerobic glycolysis and/or oxidative phosphorylation instead of ATP-PCr energy system [21].

Table 2. Body composition changes during the study. Values are presented as mean ± SD

	Baseline	1st week	2nd week	4th week	6th week	*P*
Weight (kg)						
Placebo	67.8 ± 12.8	67.9 ± 12.8	68.5 ± 13.5	68.4 ± 13.3	68.7 ± 13.5	0.32
1.2 g GAA	73.4 ± 19.3	73.9 ± 19.6	74.9 ± 19.4	75.1 ± 19.4	75.2 ± 19.3	
2.4 g GAA	70.3 ± 12.4	70.6 ± 12.5	71.1 ± 12.0	71.3 ± 12.0	71.6 ± 12.2	
4.8 g GAA	72.1 ± 10.8	73.9 ± 10.8	74.1 ± 11.2	74.5 ± 11.3	74.9 ± 11.3	
Body fat (%)						
Placebo	19.8 ± 6.4	19.6 ± 6.0	18.6 ± 5.5	18.3 ± 5.5	18.0 ± 5.5	0.63
1.2 g GAA	20.6 ± 4.9	19.9 ± 4.9	19.3 ± 4.6	18.9 ± 4.7	18.4 ± 4.7	
2.4 g GAA	19.1 ± 6.6	18.0 ± 6.0	17.1 ± 5.4	17.2 ± 5.6	17.1 ± 5.4	
4.8 g GAA	20.1 ± 7.7	18.5 ± 7.8	18.1 ± 7.2	18.2 ± 7.1	17.5 ± 7.1	
Total body water (%)						
Placebo	53.7 ± 5.2	53.8 ± 4.5	54.2 ± 4.5	54.1 ± 3.8	53.8 ± 2.3	0.19
1.2 g GAA	53.2 ± 3.4	54.4 ± 3.6	54.7 ± 3.5	55.6 ± 3.6	55.8 ± 3.2	
2.4 g GAA	54.4 ± 4.6	55.1 ± 4.3	55.9 ± 4.2	55.8 ± 4.0	56.8 ± 3.8	
4.8 g GAA	53.8 ± 4.6	56.1 ± 4.7	55.9 ± 4.4	55.9 ± 4.1	56.7 ± 4.2	
Lean body mass (kg)						
Placebo	51.0 ± 11.5	51.1 ± 11.5	51.8 ± 11.8	51.7 ± 11.7	51.9 ± 11.6	0.006
1.2 g GAA	55.6 ± 17.4	56.5 ± 18.1	57.9 ± 18.0	58.3 ± 17.6	58.5 ± 17.7	
2.4 g GAA	54.1 ± 12.7	54.8 ± 12.7	55.6 ± 12.7	55.6 ± 13.2	55.8 ± 12.8 *	
4.8 g GAA	54.4 ± 11.2	56.6 ± 11.3	56.8 ± 11.0	56.9 ± 11.0	57.9 ± 11.5 *	

Note. * - Indicates significant change 0 vs. 6 week as compared to the placebo at $P < 0.05$

Clinical Markers

The effects of GAA supplementation on muscle and liver enzyme efflux, hematological indices, serum electrolytes and lipid profiles in humans were not profoundly investigated in the past. Although poorly designed, previous human studies from the 1950-s [12, 14, 15, 22-24] indicated no major changes in markers of clinical status after extensive blood and urine analysis in patients supplemented with GAA and betaine. However, concerns have been raised that GAA supplementation may induce muscle and/or liver damage [6]. Recent report from our lab found that 2.4 g/day of GAA had an acceptable side-effects profile, with a low incidence of biochemical abnormalities [9]. The present study reported that GAA supplementation has no clinically significant effect on clinical enzymes (AST, ALT, ALP, CK, γ-GT). All reported values throughout the study were well within the normal range. Although no other human studies on GAA were reported, according to the results of previous studies we could conclude that GAA does not significantly affect liver and muscle enzymes in healthy humans.

Since muscle creatine uptake is sodium dependent [25], and since there have been some anecdotal reports that creatine may promote dehydration, fluid retention and alter serum and/or urine electrolyte status (through dilution), there has been interest in determining whether administration of GAA (as an agent that stimulates creatine synthesis) affects blood volume and/or electrolytes in body fluids. The European Food Safety Authority [4] reported

elevated potassium level in male rats fed with high-dose GAA (5%) for 90 days, and the difference disappeared in the animals kept for four weeks after the end of the treatment. According to the results of the present study, it seems that GAA supplementation for 6 weeks does not significantly affect Hct, Hgb or serum and urine potassium and calcium levels. We noticed significantly lower urine sodium in participants supplemented with medium-dose GAA after intervention, which could indicate increased sodium uptake by the cell. However, the effect was rather transient and clinically insignificant. Findings from the present investigation support results of previous studies that GAA supplementation does not considerably alter electrolyte status.

Clinical chemistry analyses in rats fed GAA for 90 days showed slightly reduced serum triglycerides and cholesterol levels for the three highest-dose groups, while no significant differences for total cholesterol were found in chickens fed with GAA for 35 days [4]. It has been reported [26] that creatine supplementation positively affects lipid profiles in hypertriglyceridemic patients but the exact mechanism of action is not known. One theory of this phenomenon is that creatine may enhance the quality of exercise thereby accentuating the positive effects of exercise on blood lipid profiles. The present study demonstrates that despite possible lipid-lowering effect of creatine, GAA supplementation did not change blood lipid profiles in healthy young men and women. These findings suggest that the possible influence of GAA on lipid profiles in physically active men and women with normal blood lipids in either transient or non-existent.

In vitro studies have shown that guanidino compounds might be responsible for the increased hemolysis in uremic patients [27, 28]. Moreover, several studies reported that guanidino compounds could also affect platelet count and aggregation in chronic renal failure [29,30]. It seems that the accumulation of several experimentally proven toxic guanidino compounds (e.g., guanidinosuccinic acid, guanidinobutyric acid, guanidine, methylguanidine) could contribute to the hematological complications seen in renal insufficiency [31]. Yet, plasma GAA concentrations were significantly decreased in this particular subjects and its role in hemolysis is questionable [28]. Besides toxic hemolysis and possible anemia induced by guanidino compounds, another possible mechanism affecting hematological status of subjects receiving GAA could be related with methylation reactions during creatine synthesis. The European Food Safety Authority [4] indicated that MCV was significantly increased in chickens by 1500 mg GAA kg^{-1} feed, which could be a sign of deficiency of vitamin B_{12} and folic acid. However, we reported that GAA ingestion did not deplete B vitamins pool available for remethylation in healthy men and women, as assessed by serum folates, vitamin B_6 and vitamin B_{12} [11]. During the present study we found that GAA intervention affected RBC, while GAA loading had no effect on serum Hgb, RBC indices, Hct, platelet count and WBC count. None of the GAA-supplemented participants with GAA experienced anemia or macrocytic erythrocytes (e.g., MCV > 90 fL); all hematological indices were well within their respective normal range at all times. Although the effects of GAA on hematological status were not deeply examined in the past in neither healthy humans nor animals, results of the present study indicate that medium-term GAA supplementation does not appear to adversely affect clinical markers of hematological status in physically active men and women.

Side Effects of GAA

According to the historical seminal studies from the 1950-s, no significant adverse effects were noted after GAA loading in clinical environment [12-15, 23]. Authors mainly combined GAA with betaine, with oral GAA administered in dosages from 0.2 to 5 grams per day for up to 6 months. Recent reports from our laboratory described mild to moderate hyperhomocysteinemia in healthy men and women after GAA supplementation, while only minimal subjective side effects were reported (e.g., gastrointestinal distress, weight gain, muscle cramping) [9-11]. Herein, we reported cumulative results on the safety profile of GAA, suggesting similar incidence of most side effects reported after GAA intervention. No participants from low-dose and medium-dose have being excluded from the study due to vexatious side effects. However, it seems that the risk of different detrimental adverse effects is increased following intake of 4.8 grams of GAA per single serving, with nausea marked as a most frequent side-effect (> 60%) in both men and women. Moreover, five female volunteers who received 4.8 g/day of GAA were permanently excluded from the study during the first week of administration due to repeated episodes of vomiting. Although orally ingested GAA is probably absorbed completely [4, 5], there may be an upper limit to intestinal absorption of GAA in humans, and the excess GAA ingested may serve to cause moderate to severe GI distress (e.g., abdominal pain, nausea, vomiting) we found in participants receiving 4.8 grams of GAA per day. Another possible explanation is an inhibitory effect of excess GAA acting as $GABA_A$ agonist on duodeno-jejunal motility [34], which requires further investigation. In addition, participants from all treatment groups (including placebo) reported swallowing problems, heartburn and intestinal cramping as most frequent GI complaints. These effects are probably not related to GAA intervention, and happened due to large quantity of capsules (size 00) taken at once. We could presume that such effects will probably disappear or be reduced if the formulation is liquid or the daily dose is divided into two or three units.

It is puzzling why only females experienced intrusive gastrointestinal side effects (e.g., severe nausea, vomiting) during the supplementation with 4.8 g/day of GAA. It could be due to one of the following factors: 1) more sensitive gastrointestinal system in females, particularly during the menarche [35]; 2) females have higher incidence of cyclic vomiting syndrome [36]; 3) higher dose of GAA per kg bodyweight in females; and, 4) the unknown influence of GAA on gut in women, which requires more investigation. At long last, severe continuous signs of intolerance were not observed in young healthy men and women supplemented with GAA for 6 weeks. Although long-term studies are not available at the moment, GAA could be considered as relatively safe nutritional supplement for physically active population.

Limitations of the Study

Despite the evidence that GAA enhanced exercise performance in healthy male and female young recreational athletes, the present study has several limitations. Firstly, we did not consider other possible factors that could be responsible for the observed differences in exercise performance between the groups, such as individual daily variation responses to exercise (particularly for maximal tests). Second, although we selected subjects with similar

age and training status, training routine seems to be quite heterogeneous, with gender differences observed. The size of the experimental samples along with concomitant high coefficient of variability (CV) for certain parameters could be considered partly limited. Consequently, although of moderate magnitude, the observed differences between the groups in several exercise performance indicators (e.g., anaerobic performance indicators) could not reach the statistically significant level. Moreover, we partially controlled extraneous factors such as nutrition, yet strict prescription of dietary regimen and advanced monitoring of food consumed were not conducted for the present study.

Conclusion

The administration of GAA for six weeks led to the progress in lean body mass and muscular strength performance in healthy men and women. Low-dose GAA (1.2 g/day) seems to be minimal dosage for improving exercise performance. Supplemental GAA had no effect on clinical markers of health (e.g., liver and muscle enzymes, lipid status, hematological indices), with values were within reference ranges during the intervention. There is no reason to believe that medium-term GAA supplementation has any severe prolonged detrimental subjective side-effect if taken in a recommended amount, particularly for 1.2 g/day and 2.4 g/day of GAA. The risk of gastrointestinal distress may be increased, however, following intake of 4.8 grams of GAA per single serving during supplementation in female participants. In addition, the risk of hyperhomocysteinemia after GAA ingestion may require long-term monitoring and countermeasures (e.g., addition of methyl donors or serine) before its regular supplementation on a daily basis.

Acknowledgments

No conflicts of interest apply in this work. This study was supported by the Serbian Ministry of Science (Grant No. 175037), and by AlzChem AG, Trostberg, Germany (Grant No. AN_85E_S09). Trial identification: www.clinicaltrials.gov number NCT01133899. The authors thank the research participants for their dedication, and Dr. Barbara Niess for the technical and scientific advice.

References

[1] Williams, MH; Branch, JD. Creatine supplementation and exercise performance: an update. *J Am Coll Nutr*., 1998, 17(3), 216-34.

[2] Tsubakihara, Y; Hayashi, T; Shoji, T. Guanidinoacetic acid (GAA) in patients with chronic kidney disease (CKD) and diabetes mellitus (DM). *Kid Res Clin Pract*., 2012, 31(2), A81.

[3] Sotgia, S; Carru, C; Caria, MA; Tadolini, B; Deiana, L; Zinellu, A. Acute variations in homocysteine leves are related to creatine changes induced by physical activity. *Clin Nutr*., 2007, 26(4), 444-9.

[4] European Food Safety Authority. Safety and efficacy of guanidinoacetic acid as feed additive for chickens for fattening. *EFSA J*., 2009, 988, 1-30.

[5] Lemme, A; Tossenberg, J; Ringel, J. Digesibility and availability of the creatine source guanidinoacetic acid in broilers. *J Anim Sci.*, 2007, 85(Suppl.), 153.

[6] Stead, LM; Au, KP; Jacobs, RL; Brosnan, ME; Brosnan, JT. Methylation demand and homocysteine metabolism: effects of dietary provision of creatine and guanidinoacetate. *Am J Physiol Endocrinol Metab*., 2001, 281(5), E1095-100.

[7] Edison, EE; Brosnan, ME; Meyer, C; Brosnan, JT. Creatine synthesis: production of guanidinoacetate by the rat and human kidney in vivo. *Am J Physiol Renal Physiol*., 2007, 293(6), F1799-804.

[8] da Silva, RP; Nissim, I; Brosnan, ME; Brosnan, JT. Creatine synthesis: hepatic metabolism of guanidinoacetate and creatine in the rat in vitro and in vivo. *Am J Physiol Endocrinol Metab*., 2009, 296(2), E256-61.

[9] Ostojic, SM; Niess, B; Stojanovic, M; Obrenovic, M. Creatine metabolism and safety profiles after six-week oral guanidinoacetic acid administration in healthy humans. *Int J Med Sci*., 2013, 10(2), 141-7.

[10] Ostojic, SM; Niess, B; Stojanovic, M; Idrizovic, K.. Serum creatine, creatinine and total homocysteine concentration-time profiles after a single oral dose of guanidinoacetic acid in humans. *J Funct Foods*., 2014, 6(1), 598-605.

[11] Ostojic, SM; Stojanovic, MD; Drid, P; Hoffman, J. Dose-response effects of oral guanidinoacetic acid on serum creatine; homocysteine and B vitamins levels. *Eur J Nutr*., 2014 (in press). doi 10.1007/s00394-014-0669-0.

[12] Borsook, ME; Borsook, H. Treatment of cardiac decompensation with betaine and glycocyamine. *Ann West Med Surg*., 1951, 5(10), 830-55.

[13] Graybiel, A; Patterson, CA. Use of betaine and glycocyamine in the treatment of patients with heart disease: preliminary report. *Ann West Med Surg*., 1951, 5(10), 863-75.

[14] Higgins, AR; Harper, HA; Kline, EF; Merrill, RS; Jones, RE; Smith, TW; Kimmel, JR. Effects of creatine precursors in arthritis; clinical and metabolic study of glycocyamine and betaine. *Calif Med*., 1952, 77(1), 14-8.

[15] Dixon, HH; Dickel, HA; Shanklin, JG; Peterson, RD; West, ES. Therapy in anxiety states and anxiety complicated by depression. *West J Surg Obstet Gynecol*., 1954, 62(6), 338-41.

[16] Ostojic, SM. Guanidinoacetic acid administration in physically active men and women. 2010. (Available at, http, //www.clinicaltrials.gov/ct2/ show/NCT01133899).

[17] Julious, SA. Sample sizes for clinical trials with normal data. *Stat Med*., 2004, 23(12), 1921-86.

[18] Hoffman, JR. *Norms for Fitness; Performance and Health*. HK: Champaign: IL, 2006.

[19] Bosco, C; Luhtanen, P; Komi, PV. A simple method for measurement of mechanical power in jumping. *Eur J Appl Physiol*., 1983, 50(2), 273–82.

[20] Jones, EJ; Bishop, PA; Woods, AK; Green, JM. Cross-sectional area and muscular strength, a brief review. *Sports Med*., 2008, 38(12), 987-94.

[21] Herda, TJ; Beck, TW; Ryan, ED; Smith, AE; Walter, AA; Hartman, MJ; Stout, JR; Cramer, JT. Effects of creatine monohydrate and polyethylene glycosylated creatine supplementation on muscular strength, endurance, and power output. *J Strength Cond Res.*, 2009, 23(3), 818-26.

[22] Van Zandt, V; Borsook, H. New biological approach to the treatment of congestive heart failure. *Ann West Med Surg*., 1951, 5(10), 856-62.

[23] Borsook, ME; Billing, HK; Goklseth, JG. Betaine and glycocyamine in the treatment of disability resulting from acute anterior poliomyelitis. *Ann West Med Surg.*, 1952, 6(7), 423-27.

[24] Baron, H. Some effects of DL-methionine and glycocyamine on growth and nitrogen retention in rats. *J Nutr*., 1958, 64(2), 229-39.

[25] Brault, JJ; Abraham, KA; Terjung, RL. Muscle creatine uptake and creatine transporter expression in response to creatine supplementation and depletion. *J Appl Physiol*., 2003, 94(6), 2173-80.

[26] Earnest, CP; Almada, A; Mitchell, TL. High-performance capillary electrophoresis-pure creatine monohydrate reduced blood lipids in men and women. *Clin Sci.*, 1996, 91(1), 113-8.

[27] Giovanneti, S; Cioni, L; Balestri, PL; Biagini, M. Evidence that guanidines and some related compounds cause hemolysis in chronic uremia. *Clin Sci.*, 1968, 34(1), 141-8.

[28] Tanaka, A; Takaihashi, Y; Mizokuci, M; Shimada, N; Koide, H. Plasma, urinary, and erythrocyte concentrations of guanidino compounds in patients with chronic renal failure. *Ren Fail.*, 1999, 21(5), 499-514.

[29] Davis, JW; McField, JR; Phillips, PE; Graham, BA. Guanidinosuccinic acid on human platelet. Effects of exogenous urea, creatinine, and aggregation in vitro. *Blood.*, 1972, 39(3), 388-97.

[30] Maejima, M; Takahashi, S; Hatano, M. Platelet aggregation in chronic renal failure - whole blood aggregation and effect of guanidino compounds. *Nippon Jinzo Gakkai Shi*., 1991, 33(2), 201-12.

[31] De Deyn, PP; Marescau, B; Cuykens, JJ; Van Gorp, L; Lowenthal, A; de Potter, WP. Guanidino compounds in serum and cerebrospinal fluid of non-dialyzed patients with renal insufficiency. *Clin Chim Acta*., 1987, 167(1), 81-8.

[32] Robinson, TM; Sewell, DA; Casey, A; Steenge, G; Greenhaff, PL. Dietary creatine supplementation does not affect some hematological indices, or indices of muscle damage and hepatic and renal function. *Br J Sports Med*., 2000, 34(4), 284-8.

[33] Kreider, RB; Melton, C; Rasmussen, CJ; Greenwood, M; Lancaster, S; Cantler, EC; Milnor, P; Almada, AL. Long-term creatine supplementation does not significantly affect clinical markers of health in athletes. *Mol Cell Biochem.*, 2003, 244(1-2), 95-104.

[34] Fargeas, MJ; Fioramonti, J; Bueno, L. Central and peripheral action of GABAA and GABAB agonists on small intestine motility in rats. *Eur J Pharmacol.*, 1988, 150(1-2), 163-9.

[35] Beattie, WS; Lindblad, T; Buckley, DN; Forrest, JB. The incidence of postoperative nausea and vomiting in women undergoing laparoscopy is influenced by the day of menstrual cycle. *Can J Anaesth.*, 1991, 38(3), 298-302.

[36] Abell, TL; Adams, KA; Boles, RG; Bousvaros, A; Chong, SK; Fleisher, DR; Hasler, WL; Hyman, PE; Issenman, RM; Li, BU; Linder, SL; Mayer, EA; McCallum, RW;

Olden, K; Parkman, HP; Rudolph, CD; Taché, Y; Tarbell, S; Vakil, N. Cyclic vomiting syndrome in adults. *Neurogastroenterol Motil.*, 2008, 20(4), 269-84.

[37] Groeneveld, GJ; Beijer, C; Veldink, JH; Kalmijn, S; Wokke, JH; van den Berg, LH. Few adverse effects of long-term creatine supplementation in a placebo-controlled trial. *Int J Sports Med*., 2005, 26(4), 307-13.

[38] Ostojic, SM; Ahmetovic, Z. Gastrointestinal distress after creatine supplementation in athletes:are side effects dose-dependent? *Res Sports Med*., 2008, 16(1), 15-22.

In: Human Health and Nutrition
Editor: Sergej M. Ostojic
ISBN: 978-1-63482-823-9

Chapter V

Supplementation with Methyl Donors during Guanidinoacetic Acid Loading in Humans

***Sergej M. Ostojic*[1,2,*], *Marko Stojanovic*[1,2] *and Patrik Drid*[1,2]**

[1]Center for Health, Exercise and Sport Sciences, Belgrade, Serbia
[2]Faculty of Sport and Physical Education, University of Novi Sad, Serbia

Abstract

The main aim of this study was to investigate whether dietary intake of methyl donors (e.g., betaine, choline and/or B vitamins) during guanidinoacetic acid (GAA) loading affected metabolic and clinical markers, and increased the incidence of side-effects after 8 weeks of administration. Forty (n = 40) healthy men and women aged 18 to 30 years were recruited for a GAA-controlled, double-blind and parallel-group intervention study. Subjects were allocated to four randomly assigned trials: group A was supplemented with pure GAA, group B with a formulation containing GAA, choline, and B vitamins, group C with GAA, betaine, and B vitamins, and group D with GAA and B vitamins. Addition of methyl donors to GAA largely precluded the elevation of plasma total homocysteine caused by the GAA administration alone ($P < 0.05$). The intake of B vitamins in groups B, C and D significantly increased levels of plasma folates, vitamin B_{12} and holo-transcobalamine ($P < 0.05$). Reported side-effects during the intervention were transient and clinically irrelevant, and no major disturbances of clinical enzymes and other biochemical indicators of health status have been observed. It seems that addition of methyl donors in combination with dietary GAA supplementation effectively compensated for methylation requirements, such that the administration can be considered as a relatively safe nutritional intervention.

Keywords: Methyl donors, Homocysteine, Side-effects, Vitamin B_{12}, Vitamin B_6

[*] Corresponding author: Email: sergej.ostojic@chess.edu.rs.

Introduction

Guanidinoacetic acid (GAA; also known as glycocyamin or guanidinoacetate, chemical formula $NH_2C(=NH)NHCH_2COOH$) is an amino acid like compound that is naturally present in the human body in low concentration. GAA is considered as a possible dietary supplement due to its role in the synthesis of creatine, a fundamental compound in cellular bioenergetics. Beneficial effects of dietary GAA have been found in both patients and a healthy population, yet its use in human nutrition is still hampered by limited data on GAA clinical features when administered separately or co-administered with other nutritional agents. In particular, since creatine synthesis is considered to be the major user of methyl groups [1], increased methylation after GAA intake could affect metabolism of homocysteine and induce hyperhomocysteinemia [2-4]. Several mechanisms for the hyperhomocysteinemic effect of GAA have been proposed: (a) accelerated conversion of *S*-adenosylmethionine to *S*-adenosylhomocysteine through the reaction catalyzed by GAA *N*-methyltransferase (GAMT) [5]; (b) decreased activity of cystathionine β-synthetase partly due to a decrease in hepatic *S*-adenosylmethionine [6]; and, (c) decreased homocysteine remethylation due to a decrease in hepatic betaine concentration [7]. Due to the fact that elevated plasma homocysteine is discussed as a risk factor for a number of important diseases [8], it seems reasonable to employ different nutritional agents (e.g., methyl group donors) as additives during GAA loading to suppress or counterbalance hyperhomocysteinemia. Supplementation with betaine and B vitamins seemed to be an effective counter strategy to cope with an increased methylation demand and hyperhomocysteinemia induced by GAA administration in humans [9]. However, no studies contrasted the metabolic effects of betaine, choline (betaine precursor) and B vitamins during GAA loading. Comparing possible metabolic effects of folate supplementation with betaine-dependent remethylation might help to determine the most effective additive for GAA to prevent hyperhomocysteinemia. Moreover, the complex effects of GAA and methyl donors on other metabolic markers, and body composition indicators are largely unknown. Finally, the side effects of such an intervention on clinical markers of health status, and subjective adverse events are yet to be determined. Therefore, the main goals of the present study were to evaluate biochemical and physiological behavior of different dietary methyl donors dispensed during GAA loading, and to analyze the incidence and severity of adverse events throughout the intervention.

Methods

Participants

Male and non-pregnant female moderately physically active subjects, aged 18 to 30 years, were recruited from February to April 2011 through digital laboratory database. The study protocol was approved by the local IRB according to the Declaration of Helsinki. The subjects were not admitted to the study if any of the following criteria were present: (1) elevated total serum homocysteine above 15 μmol/L; (2) a history of metabolic disease; (3) known heart disease; (4) use of any dietary supplement (e.g., creatine, B complex vitamins, choline/lecithin, proteins, amino acids) within the past 60 days; and, (5) residence outside the

city of Belgrade, or unwillingness to return for follow-up. Females were strongly suggested not to get pregnant during the study, with no women got pregnant throughout the course of the study. During the study usage of hormonal contraceptives was strongly discouraged. All subjects were volunteers and gave their informed written consent to take part in the study. They were in good health, as assessed by health questionnaire and general pre-participation examination including blood and urine screening profile (including blood glucose, lipid profile, aspartate aminotransferase (AST), alanine aminotransferase (ALT), urea, creatinine, total homocysteine (T-Hcy), hemoglobin, hematocit, red and white blood cell count, routine urinalysis, human chorionic gonadotropin (hCG) [women only], urine creatinine, proteins, urea nitrogen, sodium, calcium and potassium). Subjects were moderately physically active (average of 4 hours of exercise per week). They were informed that they could withdraw from the study at any time, even after giving their written consent. The stopping rules for participants included: (a) refuse to participate in this research at any time, (b) inability to follow prescription procedure, (c) pregnancy or significant change of health status, (d) serious side-effects (clinical or subjective). Withdrawal of participation did not include withdrawal of data compiled up to that point. Subjects were obliged to maintain their normal physical activity patterns and dietary regimen (including no use of any other supplements or medicaments) throughout the duration of the study. Compliance was monitored at each visit to the lab by analyzing 5-day food records with average daily energy intake, macronutrient content, and B complex vitamins intake calculated (Nutribaze, Phoenix, AR, USA) by certified dietitian. Two days prior to the baseline testing subjects met a nutritionist who instructed them how to maintain a normal dietary pattern through self-selected diets throughout the study. In the 48-h before baseline and subsequent assessment points, all subjects were advised by a nutritionist to restrict from caffeine and/or alcoholic beverages due to the possible relation to serum T-HCy. Subjects were asked not to change their physical activity patterns during the study yet no habitual or programmed physical activity was monitored throughout the study.

Supplementation Protocol

Subjects were allocated in a GAA-controlled, double-blind and parallel-group design to four randomly assigned trials, with treatment lasting for 8 weeks. The four intervention trials included group A (supplemented with 2.4 g/day of GAA), group B (2.4 g/day of GAA + 3.0 g/day of choline dihydrogencitrate + 5 µg/day of B_{12} + 10 mg/day of B_6 + 600 µg/day of folic acid), group C (2.4 g/day of GAA + 1.6 g/day of betaine HCl + 5 µg/day of B_{12} + 10 mg/day of B_6 + 600 µg/day of folic acid) and group D (2.4 g/day of GAA + 5 µg/day of B_{12} + 10 mg/day of B_6 + 600 µg/day of folic acid). Groups were matched for weight, and women had an equal probability of assignment to the groups. The unique treatment formulation was provided by AlzChem AG (Trostberg, Germany) in powder form in coded sachets to be stirred into a liquid (0.5 L of lukewarm water) by the participant right before consumption, with all drinks similar in appearance, texture and taste. A person not further involved in the study assigned codes to the study treatments and randomly allocated the selected participants according to a computer-generated list and kept the key in a sealed envelope. The code was revealed to the researchers once recruitment, data collection and laboratory analyses were completed. All subjects received the supplementation packages for in-between-tests period at

every visit to the lab directly from the research staff. Subjects were instructed to take one portion of drink in the morning upon waking before breakfast and another portion in the evening before the last meal, with sachet counts used to determine subject compliance. Subjects were required to return unused supplements at the end of the study (if any). The primary endpoint with respect to the efficacy in affecting blood metabolites was the change of serum T-HCy level achieving a significant change in concentration from baseline to 2 weeks (first assessment point after intervention) in order to detect a possible clinically relevant alteration of serum T-HCy as early as possible and stop the intervention if needed. Additional analyses were done on blood and urinary biochemical profiles, body composition variables, and prevalence of side effects.

Experimental Design

All testing were conducted at pre-supplementation (baseline), after 2, 4, 6, and 8 weeks of supplementation. All subjects were assessed on two consecutive days with the tests performed in the same order. Blood samples were obtained between 9 and 10 a.m. after an overnight fast (10 to 12 h). Both 24-h and morning urine samples were provided in plastic containers at baseline and at every lab visit throughout the end of the study. All biochemical urinalysis were done on whole urine samples (24 h volume with morning samples included). Morning samples were provided in separate plastic container for HCG analyses (females only), and added to the rest of the urine collected for complete biochemical analyses. From 10 p.m. until after the fasting measurements the next morning, participants were not allowed to smoke, eat, or drink anything except for water. In the 24 hours before each testing session, the subjects did not participate in any prolonged exercise due to a possible relationship between physical activity and plasma T-HCy levels [10]. At each of the visits to the lab, volunteers provided a fasting blood sample from a radial vein into different evacuated vacutainer test tubes (Greiner Bio-One, Basel, Switzerland) while seated. Blood was collected in following tubes: a) two gel vacutainers for biochemical variables; b) 7.5% K3-EDTA vacutainers for homocysteine; and, c) Na-citrate vacutainers for coagulation and ESR. The K3-EDTA tubes were centrifuged within the next 10 minutes at 3000 × g for 10 min. Plasma was separated, frozen at -20°C, stored and analyzed for homocysteine and other metabolites after 4 weeks of intervention and after the completion of the study. Gel vacutainers were centrifuged within the next 30 minutes at 3000 × g for 15 min, with serum stored at -20°C and the analysis completed at the same day, after 4 weeks of intervention and after the completion of the study for selected outcomes individually. T-Hcy was measured with chemiluminescent immunoassay method using chemistry analyzer (DPC Immulite 2000, Siemens, Germany), while folate and vitamin B_{12} were measured by electrochemiluminescence using the automatic analyzer (Cobas E411, *Roche* Diagnostics, Indianapolis, IN, USA). Serum vitamin B_6 concentrations were analyzed by HPLC with fluorimetric detection (Hewlett-Packard, Palo Alto, CA, USA). Plasma holo-transcobalamin was measured by microparticular enyzmatic imunoanalysis (Axsyme, *Abbott* Laboratories, Abbott Park, IL, USA), with the serum proteins and *albumin measured* by Aeroset BCG (*Abbott* Laboratories, Abbott Park, IL, USA). Creatine and GAA in both plasma and urine samples were assessed by HPLC with fluorimetric detection (Hewlett-Packard, Palo Alto, CA, USA), with creatinine in serum and urine determined by kinetic Jaffa method (RX *Daytona*, *Randox* Laboratories Ltd., Crumlin, UK). Level of choline was

assayed by ELISA and quantitative real time RT-PCR (Microplate Reader Rayto RT 2100C, Rayto Ltd, Shenzhen, China). Methionine and arginine were assessed by AccQ-TagUltra pre-column derivization UPLC (Waters Copr., Milford, MA, USA). The complete blood count was performed using a Coulter blood counter (Model S-plus II, Coulter Electronics Inc., Hialeah, Florida, USA) and yielded values for red blood cell count (RBC), white blood cell count (WBC), platelets, hemoglobin (Hb), hematocrit (Hct), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC). Glucose, total cholesterol, triglycerides, and lipoprotein levels were analyzed by standard enzymatic methods and an automated analyzer (Hitachi 704, Tokyo, Japan). Serum activities of AST**,** ALT**,** *alkaline phosphatase (ALP)***,** gamma-glutamyl transaminase (γ-GT), and creatine kinase (CK) *were analyzed* by an automated analyzer (RX *Daytona, Randox* Laboratories Ltd., Crumlin, UK*).* Urine *sodium, calcium and potassium levels were analyzed* by ISE direct with ILyte analyzer (Kaunas, Lithuania). Volume of urine was monitored for 24-urine diuresis, with routine urinalysis was completed by standard screening test (Machery-Nagel GmbH & Co. KG, Duren, Germany), and urine pH assessed by standard pH meter (Radiometer PHM82, Copenhagen, Denmark). Urine hCG was qualitatively analyzed by standard method of immunochromatography (BTNX Inc., Coral Springs, FL, USA). The urinary *protein* was measured by a Randox commercial kit with a *Bayer opeRA* autoanalyser (Bayer Diagnostics, Leverkusen, Germany). All samples for each subject were assayed in the same run. For all values, the first reading was discarded and the mean of the next three consecutive readings with a coefficient of variation below 15% was used in the study. All measurements were controlled by certified senior biochemists at referenced biochemistry laboratories. After the biochemical sampling was finished, anthropometrical variables were obtained at each visit to the lab, with height measured using a stadiometer (Seca 217, Hamburg, Germany) only at baseline testing point. Advanced body composition assessment including body weight was conducted by direct segmental multi-frequency bioimpedance spectroscopy analyzer (Inbody 720S, Biospace, Tokyo, Japan). Recorded outputs included intracellular and extracellular water, total body protein, total mineral and osseous content, body fat percentage, weight, and skeletal muscle mass. Subjects were measured in underwear in the same state of hydration and nourishment after voiding. The same trained technician did the anthropometric assessment in aim to minimize the testing error. To ensure that the testing environment was appropriately controlled, the testing room was maintained at 23.0 ± 2.0°C and 25 ± 10% relative humidity during all measurements.

In order to assess potential adverse events of the intervention, all subjects were instructed to report any side effects throughout the study. An open-ended questionnaire for self-assessment of well-being and deleterious side effects was administered and discussed with each participant during the study at every visit to the lab. During the first week of intervention, all subjects were interviewed by phone to closely monitor acute adverse events of supplementation. Side effects severity and frequency of occurrence, and relation to intervention were noted and analyzed in detail. Each adverse event reported was closely evaluated, with affected subject additionally interviewed by another investigator in aim to prevent inter-examiner bias. The laboratory end point committee evaluated adverse event reports in a blinded fashion, and end decisions for inclusion into the final analysis were determined unanimously.

Statistical Analyses

From the pool of 216 subjects initially selected for the present study, and 87 checked for eligibility, we finally recruited 40 subjects (twenty males and twenty females), according to power analysis (power = 0.90, $\alpha = 0.05$) for the primary outcome measure, and allocated them to four different treatment groups. The required sample of 10 subjects in each of the four groups was estimated with the goal of rejecting the null hypothesis if the means of the serum T-Hcy, with equal standard deviations of 2.0 μmol/L, differed by at least 1.0 μmol/L according to Mann-Whitney U test, with a type I error of 0.05 (two-sided) and 90% percent power. The data were reported as means ± SD. Differences within and between groups were tested by one-way analysis of variance (ANOVA). Two-way ANOVA with repeated measures was used to establish if any significant differences existed between subjects' responses over time of intervention (0 *vs.* 8 weeks). Where significant differences were found, the Tukey test was employed to identify the differences. Effects-sizes in two-way ANOVA with replication after 8 weeks of administration were assessed by advanced Cohen statistics. The rates of side-effects occurrence between the groups were compared using the Fisher exact probability test. *P* values of less than 0.05 were considered to be statistically significant, with $\eta^2 > 0.22$ indicated a large effect of drink, time or mixed factors. The data were analyzed using the statistical package SPSS 21.0, PC program (IBM SPSS Data Collection, New York, NY, USA).

Results

Subject baseline characteristics are presented in Table 1. No differences were found for baseline characteristics between the groups. One female participant (from group A) left the study before baseline testing due to a significant change of health status not related to the current study. Three participants (one male and two females) left the study due to follow up. Results are presented for intention-to-treat population ($n = 39$).

Most participants consumed the entire intervention, but few omitted some quantity of drinks. The total compliance with the supplementation regimen was 99.5%. No participant left the study due to adverse events of intervention. Several adverse events were recorded during the study (Table 2), but no differences were found between groups with regards to the frequency of subjectively reported side effects. Side effects were reported as mild, single-episodic, and clinically non-significant. They were experienced in two males and one female from group A, two females from group B, and one male from group C. No participant from group D reported any side-effect of the intervention throughout the study.

Changes in plasma B-vitamins and guanidino-related compounds during the study are presented in Figure 1. The results of two-way mixed model ANOVA found significant interactions (trial *vs.* group) for plasma T-Hcy, folates, vitamin B_{12}, holo-transcobalamine, GAA and creatinine ($P < 0.05$). Moderate to large size-effects for mixed factors (time *vs.* drink interaction) were found for plasma T-Hcy ($\eta^2 = 0.43$), folates ($\eta^2 = 0.36$), vitamin B_6 ($\eta^2 = 0.26$), and GAA ($\eta^2 = 0.56$). No relevant size-effects of intervention were found for changes in plasma amino acids (e.g., methionine, arginine, choline), plasma lipids, clinical enzymes, and urinary outcomes (not presented), except for protein excretion ($\eta^2 = 0.25$).

Table 1. Baseline characteristics of study participants
Values are mean ± SD

	Group A (*n* = 9)	Group B (*n* = 10)	Group C (*n* = 10)	Group D (*n* = 10)	*P*
Age (years)	23.7 ± 3.0	23.9 ± 3.5	21.6 ± 2.8	23.2 ± 3.8	0.41
Weight (kg)	72.6 ± 14.3	69.7 ± 9.4	68.9 ± 10.6	68.5 ± 8.8	0.85
Height (cm)	175.0 ± 9.1	174.4 ± 6.9	173.4 ± 6.7	175.4 ± 7.3	0.94

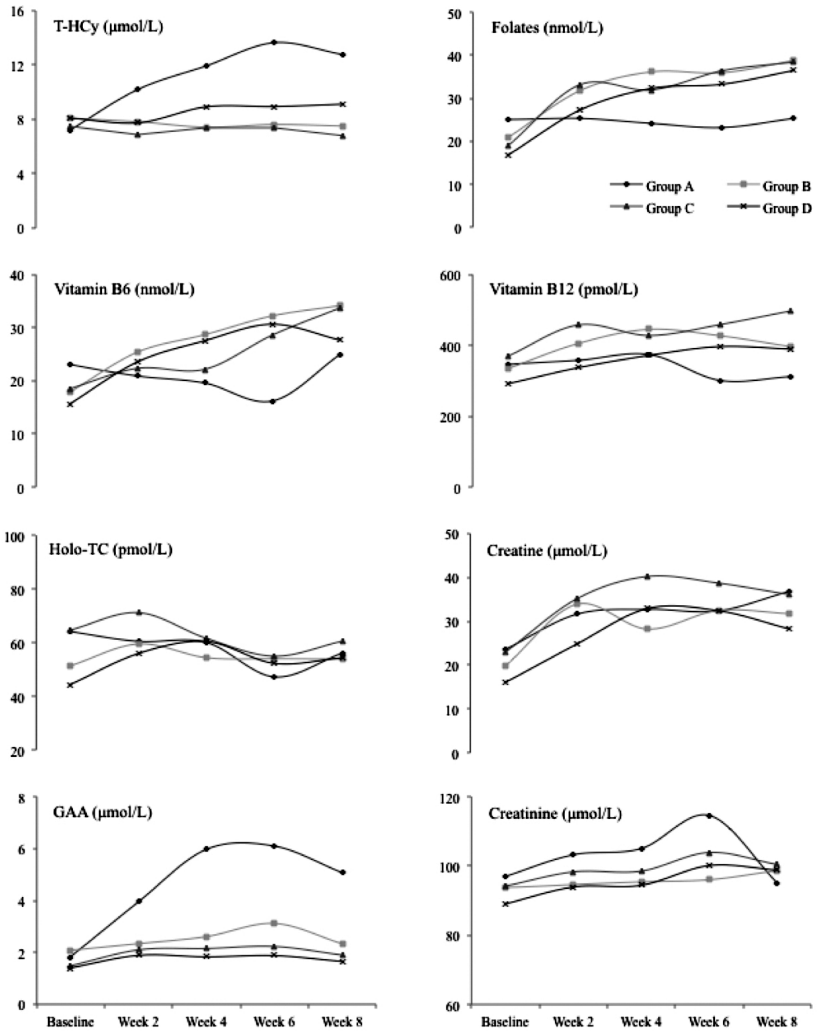


Figure 1. Changes in plasma B-vitamins and guanidino-related compounds. Error bars are removed for more clarity. *Abbreviations:* T-Hcy – total homocysteine; Holo-TC – holo-transcobalamine; GAA – guanidinoacetic acid.

Table 2. Incidence of subjectively reported side effects
Values are presented as actual numbers with percentage in parentheses

	Group A (*n* = 9)	**Group B (*n* = 10)**	**Group C (*n* = 10)**	**Group D (*n* = 10)**	***P***
Mild nausea	2 (22.2)	0	0	0	0.05
Change in saliva taste	1 (11.1)	0	0	0	0.23
Change in body odor	1 (11.1)	0	0	0	0.23
Stomach burning	1 (11.1)	0	0	0	0.23
Frequent urination	0	1 (10.0)	1 (10.0)	0	0.99
Appetite decrease	0	1 (10.0)	0	0	0.99

Hemathological indices during the study are presented in Figure 2. Intervention affected blood hematocrit and RBC count ($P < 0.05$), with notable fall-off in participants from group B and C. No differences were found for changes in other hemathological indices between the groups throughout the study. In addition, no relevant size-effects for mixed factors (time *vs.* drink interaction) were found for hematological markers after 8 weeks of administration.

Changes in body composition outcomes are presented in Figure 3. Two-way mixed model ANOVA revealed similar responses for all regimens concerning changes in weight, body fat, intracellular and extracellular water, total body protein, skeletal muscle, and osseous and total mineral content during 8-week intervention. Furthermore, no relevant size-effects of the intervention were found for changes in above outcomes after 8 weeks of administration. In addition, no differences between groups have been reported in nutritional outcomes throughout the study (not presented).

Discussion

For the first time the biochemical and physiological effects of methyl group donors supplementation during 8-week GAA administration have been evaluated in humans, along with side-effects incidence and severity of such a nutritional intervention. The results of the present study indicate that the addition of methyl donors to dietary GAA largely precluded the elevation of plasma T-HCy caused by GAA administration alone. Plasma T-HCy remained essentially unchanged during the study in participants supplemented with GAA along with methyl donors, while the group supplemented with sole GAA experienced ~ 80% elevation in T-HCy from baseline level to post-administration. A particularly powerful size-effect for this 'homocysteine-balancing' action has been noted in subjects receiving supplemental choline and betaine, and both formulations could be considered as equally effective in preventing GAA-provoked hyperhomocysteinemia. All interventions homogenously produced an increase in skeletal muscle mass, accompanied by an elevation in total protein synthesis and intracellular hydration (average gain in skeletal muscle mass was about 2 kg), at the same time as reducing body fatness (2% on average) in healthy young subjects after 8 weeks of supplementation. It seemed that subjectively reported side-effects during 8 weeks of administration of different GAA formulations were rather transient, minor and clinically

irrelevant, with no major disturbances of clinical enzymes and other biochemical indicators of health status.

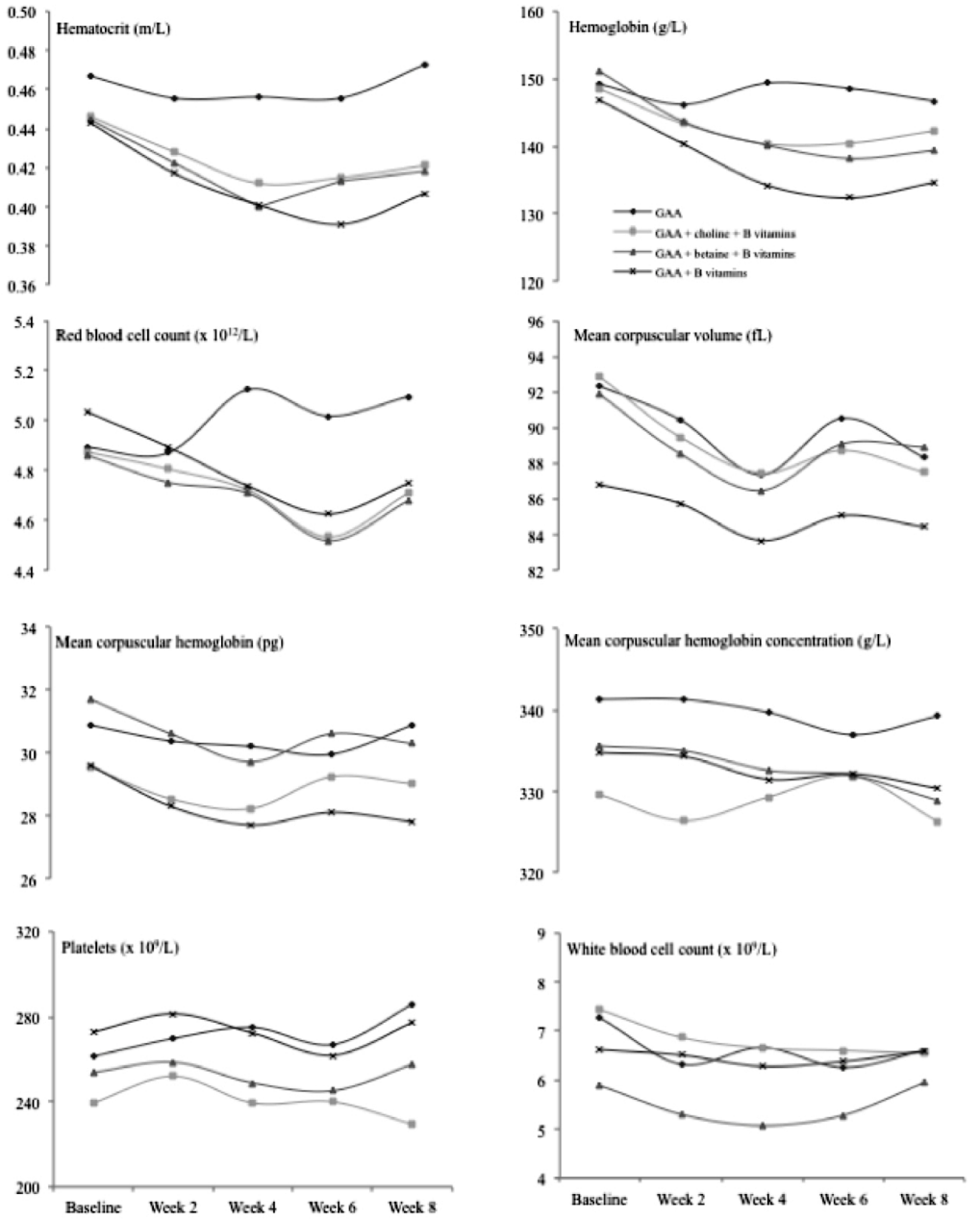


Figure 2. Changes in hematological indices during the study. Error bars are removed for more clarity.

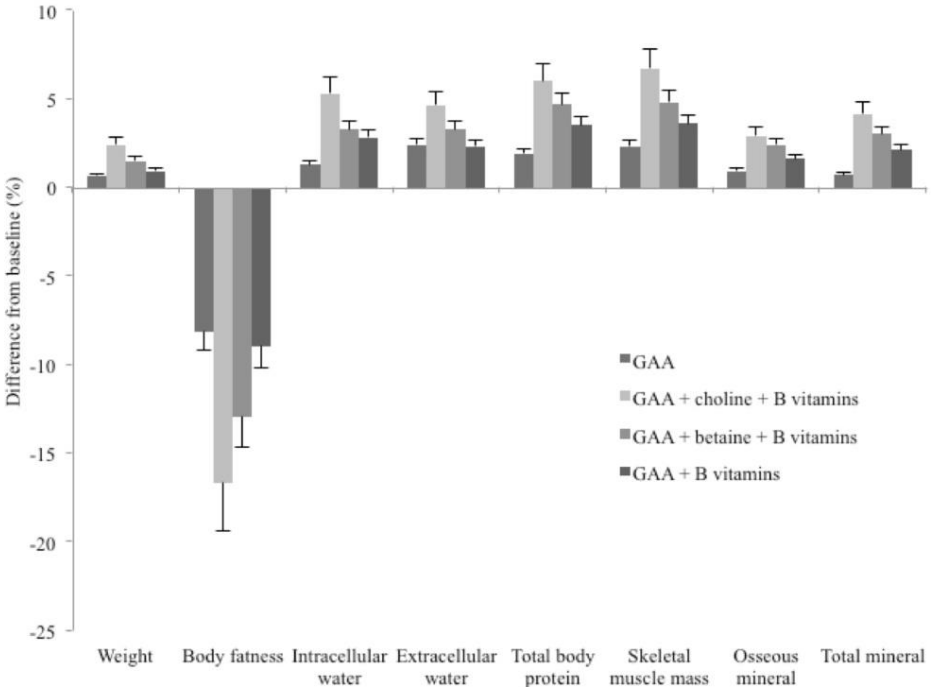


Figure 3. Percentage change in body composition outcomes 0 *vs.* 8 week.

Homocysteine Dynamics

It is apparent that biological methylations and homocysteine metabolism are intimately linked and that alterations in one my affect the other [5, 6]. GAA as a methyl group acceptor has a substantial hyperhomocysteinemic effect probably by accelerating the conversion of SAM to SAH and further to homocysteine [7]. For the present study, an increase in T-HCy after GAA administration has been noted from week 2 onwards, with the peak level of T-HCy found after 6 weeks of supplementation (approximately 82% above the baseline level). In addition, splitting the dosage into two portions seemed to amplify the rise in T-Hcy induced by GAA as compared to a previous study (an increase of ~ 28% at post-administration) [3]; this may indicate enhanced conversion of GAA into creatine. Yet, superior rise in T-Hcy is maybe overrated due to the small sample size. On the other hand, co-administration of GAA and methyl group donors effectively prevented or attenuated upturn of serum T-HCy. Choline and betaine groups influenced T-HCy in a similar fashion, keeping plasma T-HCy low at all sampling points during the intervention as compared to the baseline. These results seem to be in accordance with a previous GAA-loading study in animals, confirming a homocysteine-lowering effect of choline (and betaine) [7], although in that study the effect of choline was most pronounced when applied in an excess amount in relation to the amount of GAA. The comparable magnitude of the homocysteine-balancing effect between choline and betaine probably indicates that production of betaine from supplemental choline by choline oxidase (mainly in kidney and liver) was substantial and quick. Compared to the administration of sole GAA, the formulation with GAA and B vitamins effectively attenuated the rise in T-HCy, yet the power of this effect was smaller when compared to the effect of choline and

betaine protocols. Furthermore, besides an initial drop in T-HCy level after two weeks as compared to the pre-administration level (for about 4%) in group D, we found a steady increase in T-HCy, although minor and clinically insignificant, throughout the rest of the intervention period. One might speculate that this increase would have continued if the intervention would have been further extended, possibly due to the insufficient capacity of such formulation (folic acid, vitamin B_6, vitamin B_{12}) to provide abundant methyl groups for a prolonged methylation demand [12].

Several randomized placebo-controlled trials have evaluated the effects of multivitamin and/or betaine/choline supplementation on fasting T-HCy or hyperhomocysteinemia in humans. In overview of several clinical trials [13-15], folic acid lowered fasting plasma T-HCy levels by 26% and addition of vitamin B_{12} lowered T-HCy by another 7%, but addition of vitamin B_6 was not associated with further reductions in T-HCy concentrations. Reductions in T-HCy were larger at higher pretreatment T-HCy levels and at lower pretreatment folates level [13]. Furthermore, when elevation in T-HCy was induced in animals by nutritional manipulation (such as methionine or GAA loading) the effectiveness of B-complex vitamins was less pronounced [7]. On the other hand, betaine or phosphatidylcholine seemed to effectively lower both, fasting or post-methionine T-HCy from 20 to 50% [15]. For the present study, the notable differences in effectiveness for balancing T-HCy levels after loading with GAA were probably due to the application of different formulations of methyl group donors. Since the additive formulations were matched for folic acid, vitamin B_{12} and vitamin B_6 content, the additional effect on T-HCy balance was mostly due to the betaine-dependent remethylation of homocysteine to methionine. Furthermore, transsulfuration of HCy through activation of β-cystathionine synthase induced by an increased hepatic concentration of SAM should be considered as well [16]. Yet, an alternative mechanism for the T-HCy lowering effect of choline (although probably limited) could involve a reduction in the endogenous production of phopshatidylcholine via the phopshatidylethanolamine N-methyltransferase pathway [17, 18], but the relevance of this effect would require more investigation. In short, supplementation with a multi-formula containing methyl group donors (e.g., betaine/choline with B vitamins) together with GAA effectively reduced the increase in T-HCy during medium-term supplementation whereas GAA plus B vitamins had only a secondary effect in this setting.

Metabolic Markers of the Intervention

Folates, vitamin B_6 and vitamin B_{12} are all involved in the metabolism of homocysteine [19], and GAA through its hyperhomocysteinemia-inducing effect could alter B vitamins status in blood plasma [7], and even induce folate and cobalamine deficiencies [20]. On the other hand, supplementation with B vitamins in subjects receiving GAA could provide additional methyl groups and positively affect B vitamin status in plasma and liver, although this pathway might not be as functional as the betaine pathway for donating methyl-groups to homocysteine [Setoue et al. unpublished data]. In the present study, we found that GAA co-administered with betaine and choline along with B complex vitamins (over RDA, yet lower than UL), strongly increased blood B vitamins concentration from week 2 of the intervention throughout the rest of the intervention, as compared to sole GAA. No major differences in the effectiveness of the different methyl donors used has been noted. Yet, it seems that the

power-size of the formulations to affect particular serum B vitamins was largest for folates ($\eta^2 = 0.36$) and vitamin B_6 ($\eta^2 = 0.26$), which could be related to the balance between nutrient use through metabolic processes and supply through oral intake. The ability to affect the status of a particular vitamin could imply that either more vitamin is supplemented than utilized, or more vitamin is utilized then provided. Both ways would require more investigation. Although a trend of mild decline for serum B vitamins could be noted in subjects supplemented with pure GAA (with lowest points attained after six weeks of supplementation), we did not find signs of a clinically significant decline for serum B-vitamins after supplementation. Finally, since folic acid-dependent remethylation and betaine-dependent remethylation are interrelated (e.g., betaine/choline intake affects folate metabolism and *vice versa*) [21, 22], it is hard to isolate the effect of each additive on plasma B vitamins profiles in the present study.

A number of amino acids and amino acid derivates play key roles in creatine and/or methionine metabolism, with arginine, glycine and serine are among the most important. For the present study, plasma levels of arginine and methionine were particularly monitored as individual indicators of amino acids metabolism during loading with GAA alone and GAA plus methyl group donors. Since GAA, a precursor of creatine, is formed from arginine and glycine by *L*-Arginine:glycine amidinotransferase (AGAT) mainly in the kidney, addition of exogenous GAA (and concomitant increase in creatine synthesis) could lead to down-regulation of AGAT and an increase in arginine concentration, both in the kidney and in plasma [23, 24], and concomitant enhanced utilization of arginine as a substrate for secondary pathways [25] such as formation of urea and ornithine, guanidinosuccinate and argininic acid [26]. Furthermore, since insulin plays a role in cellular transport of arginine [27], and GAA could alter insulin secretion *in vivo* [28], it could be hypothesized that exogenous GAA might influence arginine cellular uptake and plasma levels, although this mechanism may have peripheral importance for the present study. On the other hand, exogenous GAA could affect fasting serum methionine through consuming more SAM than under normal physiological conditions and cause plasma methionine and/or protein shortfall [5]. For the methyl group donors, additional resynthesis of methionine through remethylation of SAH should be counted for total plasma methionine yield as well [29]. Therefore, the physiological role and relationship between the pathways of arginine and methionine synthesis and catabolism after GAA loading could be complex and difficult to analyze, owing to diet, hormones and cytokines [26], and the effects of methyl donors or accompanied co-factors (e.g., betaine, choline, folic acid, vitamin B_{12}) on the metabolism of these two amino acids are largely unknown. Although one would expect a decrease of endogenous GAA synthesis and thus an arginine-sparing effect as a consequence of GAA-loading, in the present study we noticed a homogeneous drop in serum arginine after 2 weeks of intervention for all groups, yet the values observed for other sampling points are close to baseline values. Furthermore, no relevant size-effects of the different formulations were found for plasma arginine during intervention. Consequently, no clear conclusion can be drawn from these results, and measurement of other indicators of arginine metabolism (e.g., plasma citrulline clearance, arginine excretion) could possibly help to elucidate the effects of different GAA formulations on arginine metabolism. In accordance with previous animal studies [7], we did notice a mild reduction in serum methionine during intervention as compared to the baseline, yet the disturbances are rather small, with no relevant size-effects throughout the intervention period. It seems that all formulations equally provoked a vague drop in blood methionine (as well as

total serum proteins), starting from week 2 of administration. We could hypothesize that GAA enhanced the rate of methionine utilization, either for the methylation pathway or for protein anabolism. Yet, due to the fact that several aspects of methionine metabolism were not monitored during the study (e.g., methionine levels in diet, sarcosine excretion, SAM levels in serum and/or liver) full understanding of the methionine metabolism during GAA plus additives application is not clear at the moment. Finally, due to the fact that choline, as an important dietary nutrient and source of labile methyl groups, plays an important role in homocysteine metabolism [15], serum choline was used as an indicator of choline provision/consumption for vitamin B_{12} independent remethylation during the GAA-loading intervention. Although we did notice slightly depressed serum choline in the group A and D (which probably indicates enhanced consumption of betaine by loading of a methyl-group acceptor such as GAA, although no signs of choline deficiency were found such as serum level below 5 µmol/L in hemorrhagic kidney necrosis), and increased serum choline level in group B (likely due to additional choline oral application and/or lesser remethylation through betaine-related pathway), we did not find a relevant size-effect throughout the intervention period ($\eta^2 < 0.09$). It seems that GAA with or without methyl group donors did not adversely affect choline metabolism during supplementation.

GAA, Creatine and Creatinine Profiles

GAA can be eliminated from the blood via two parallel pathways. The first pathway is the saturable uptake into the liver and potentially other organs (kidney, pancreas, spleen, heart, skeletal muscle) where GAA turns into creatine by GAMT. The second pathway is renal elimination. The clearance of GAA is closely interrelated to creatine due to the premise that both metabolites are competing for the identical specialized receptor (CrT1) for the transport across the cell membrane, and that the GAA uptake could be inhibited by creatine if the concentration of creatine was high enough to completely saturate CrT [31] (or maybe *vice versa* for critically high GAA). It has been reported that several agents (e.g., insulin, catecholamines, exercise, IGF-1, taurine) can affect the creatine and/or GAA uptake and turnover [30, 31], but the effects of betaine/choline and/or B complex vitamins on the guanidino compounds cycle are largely unknown. For the present study we found that GAA, creatine and creatinine measured in both urine and serum at baseline largely corresponded to the reference values found in the literature [32]. As expected, all formulations homogenously induced an increase in plasma creatine level from week 2 throughout the rest of the intervention period, with a similar trend noticed in creatine excretion by the kidney. Yet, no differences were found between groups for changes in serum and urine creatine during the intervention; obviously, creatine uptake and excretion acted in a similar fashion for all groups. On the other hand, relevant size-effects were found for both serum and urine GAA, showing a large main effect of time vs. intervention ($\eta^2 = 0.56$). So far, we could hypothesize that the degree of creatine cycling by various tissues (including liver and kidney) and excretion are similar for all groups, and none of the methyl group donors affected creatine uptake or clearance dynamics. In contrast, the application of GAA and methyl group donors somehow affected the conversion of GAA by the liver (or potentially other tissues) and/or absorption in the gut or kidney, with an attenuated rise in serum GAA during administration which is maybe due to more efficient transformation to creatine, and a lesser rate of GAA

urine excretion, as compared to pure GAA. Additionally, very low GAA excretion through the kidney for all groups during the present study, may indicate either high utilization and/or excretion through other organs (e.g., bile, feces, second-pass liver kinetics). To fully understand the distribution, metabolism and excretion of GAA additional studies with labeled GAA would be required. Finally, no relevant effect-sizes were found for serum and urine creatinine (along with other urine outcomes) during the intervention, and the observed values were well within the reference limits, indicating no clinically significant renal damage. The possibility that GAA alone or in combination with methyl group donors may increase renal stress during medium-term supplementation is unlikely.

Clinical Markers

The effects of GAA and homocysteine-lowering nutrients on serum lipid profiles seem to be complex and controversial, with data in healthy individuals are lacking. Although animal studies [33] showed a mild effect of GAA on reducing serum triglycerides and cholesterol levels, the previous study [3] suggested that a possible influence of GAA on lipid profiles in physically active subjects with normal blood lipids is either transient or non-existent. On the other hand, it has been reported that betaine (trimethylglycine) and phosphatidylcholine could raise blood cholesterol and triacylglycerol, while folic acid had no effect on lipid concentrations. This cholesterol-elevating effect of betaine/choline is probably due to an increased export of lipids from the liver into the circulation [34]. No significant disturbances in lipid profiles from the normal ranges has been noted throughout the present study. In addition, no participants experienced clinically significant changes in AST, ALT, ALP, CK and γ-GT as compared to the baseline during the present study, suggesting no muscular and liver damage during the intervention.

Previous studies have shown that different guanidino compounds might affect hematopoiesis [35, 36]. However, the effect of GAA on hematological indices seemed to be rather transient or clinically insignificant [3]. On the other hand, it was reported that loading with betaine, choline and/or B complex vitamins can have complex and sometimes opposed effects on different hematological indices. In short, studies have shown that betaine loading may decrease measures of the red blood cell volume and hemoglobin concentration (e.g., MCV, MCH, MCHC) [37], while vitamin B_{12} and folates supplementation could enhance hematopoieses and treat anemia [38]. However, data examining combined effects of GAA and methyl group donors are lacking for healthy humans. In the present study, we did find mild fall-off in hemathological indices (e.g., Hct, RBC) during the intervention, yet no relevant size-effects for mixed factors of administration were noted, suggesting possible clinical irrelevance of above findings. Drop in RBC and Hct was particularly noticeable in groups B and C, stressing a possible cumulative effect of GAA and betaine on hemolysis. In addition, MCV below normal reference range (80 -100 fL) was found in less than 5% of samples, and none of the subjects experienced MCV above 100 fL throughout the intervention. Although scientifically interesting and controversial, the effect of GAA and methyl group donors on hematological indices for the present studies remains clinically irrelevant.

Body Composition Outcomes

So far, combined effects of GAA and methyl donors on body composition outcomes were not examined in humans. Studies from the 1950-s reported an increase in body weight in subjects with chronic illnesses treated with combination of betaine and GAA [39-41]. It seems that medium-term intake of pure GAA could affect body composition indicators, such as lean body mass, in physically active individuals but the clear mechanism of action is yet to be determined (SM Ostojic, et al., unpublished results). On the other hand, the influence of B complex vitamins (e.g., folates, vitamin B_6, vitamin B_{12}) to affect body composition in humans is less evident, if present at all [42], while betaine supplementation could have an effect on cellular hydration [43]. Here we found that GAA intervention did affect body composition in healthy humans. It seems that the intervention induced an elevation in extracellular and intracellular water, with the magnitude of effects is similar among groups. Thus, no additional effect of betaine/choline and B vitamins was noted for hydration status as compared to pure GAA. The similar trend was noted for total body protein as well; all formulations induced gains in total body protein from week 2 onwards, but the effects of the different formulations were similar with no additional effect of supplemental methyl group donors. Despite the relatively short duration of the intervention, we found a mild elevation of total mineral and osseous mineral content during the study, which is in accordance with previous creatine studies [44]. Yet, no relevant differences in magnitude of changes were found between formulations, implying equivalent power of dietary GAA and added methyl donors to affect mineral accumulation. Interestingly, no significant changes were found for body weight within or between the groups during the supplementation regimen, while body fat was reduced in equal manner in all groups, and the ability of different treatment formulations to reduce body fatness was basically the same ($\eta^2 < 0.09$). As a consequence of both enhanced intracellular hydration and protein uptake, skeletal muscle mass upraised consistently in all groups from week 2 onwards. The possible mechanisms of GAA action to increase skeletal muscle mass could include: 1) an increase in intramuscular water as a consequence of osmotic activity of newly-synthesized creatine [45]; 2) anabolic proliferation due to intracellular hyperhydration [46]; 3) an improved nitrogen balance [47], 4) or an increased satellite cell activity after elevated creatine uptake [48]. Furthermore, it could be postulated that an increase in skeletal muscle mass would provide an increase in person's metabolic rate and, in turn, more body fat would be burned (muscle tissue is burning more calories than fat tissue); in the state of caloric balance, body weight will be largely unaffected which was actually obtained in the present study. Another mechanism for the indirect fat-reducing effect of GAA could be the higher training load enabled by the increase in muscular creatine [49]. Although subjects were strongly advised not to change their regular physical activity patterns during the present study, habitual physical activity was not monitored so it could be possible that elevated creatine (after GAA loading) affected training intensity and accelerate favorable changes in body composition.

Subjective Adverse Effects

For the present study, subjectively reported side-effects during 8 weeks of GAA loading and methyl donors supplementation were rather transient, minor and clinically irrelevant, with

no major disturbances of indicators of health status. Furthermore, no single subject was excluded from the study due to side-effects. When compared to the previous study [3], it seems that splitting the dosage of GAA in two portions, using a lower dose along with a different dosage form (e.g., capsules *vs.* drink), significantly reduced the prevalence of gastrointestinal side-effects after GAA administration. In addition, the incidence of side-effects was similar among groups. Gastrointestinal effects (e.g., mild nausea, change in saliva taste, stomach burning) were reported occasionally (especially during the first week of administration), right after the drink consumption, but the effects disappeared throughout the rest of the monitoring period. These effects are probably due to the individual gastrointestinal sensitivity to the formulation. Change of body odor was reported in a single male participant supplemented with pure GAA. At the moment, there is no clear physiological mechanism known to the authors that relates GAA administration or metabolism with sweat production and changes in body odor. Frequent urination and mild polyuria were experienced during the first week of administration in two subjects (male and female) from group B and group C, but the effect fully disappeared during the rest of the monitoring period. This transient and individual effect found in hypersensitive subjects could be related to simple overhydration not related to the intervention, ingestion of a hyperosmolar substance or cellular hydration changes induced by intervention containing choline/betaine or a creatine precursor such as GAA [50, 51]. Furthermore, one female from group B reported appetite reduction following the intervention. Although this effect is single-case and insignificant, it could be related to either a GAA effect on gastrointestinal motility [52], or a choline and/or B vitamin effect on satiety [53]. Although long-term studies are not available at the moment, nutritional intervention containing GAA and methyl donors could be considered as a relatively safe procedure for physically active men and women during medium-term supplementation.

Limitations of the Study

Although we used a longitudinal design and controlled interventional strategies to gain optimal insight into the association between intake of GAA and methyl donors, and various metabolic and physiological outcomes, several study limitations should be noted. Firstly, the results (particularly for body composition outcomes) could be influenced by differences in habitual physical activity or training routine among participants, which was not monitored throughout the present study. Although we have the baseline values as reference nutritional values and instructed the participants to maintain their dietary habits, we did not monitor other relevant dietary factors (e.g., nutrient bioavailability, choline nutritional intake, composition of dietary proteins, coffee and/or tea consumption) that could have additionally affected metabolites profiles (particularly for plasma level of methionine, arginine, albumin, choline), and the effects of ingested amino acids (e.g., serine, cysteine) and proteins on the post-prandial T-HCy metabolism were not considered as well. It should be noted that responses after GAA loading with methyl group donors supplementation in the present study were noted in young and healthy subjects, while other population groups could have responded to the treatment in different pattern.

Conclusion

Methyl group donors (e.g., choline, betaine, B vitamins) co-administered during GAA supplementation largely prevented an increase in plasma T-HCy induced by pure GAA, and a powerful 'homocysteine-balancing' effect has been noted in subjects co-supplemented with choline and betaine. All nutritional interventions seemed to be comparably effective for improving skeletal muscle mass as well as decreasing body fatness, with no major changes in weight throughout the study. Considering the negligible disturbances in clinical markers of health status, the intake of GAA together with methyl group donors and B vitamins during 8 weeks is relatively safe if the substance is taken in the recommended amount. However, long-term studies in lager groups are necessary to corroborate the results obtained in this pilot trial.

Acknowledgments

No conflicts of interest apply in this work. This study was supported by the Serbian Ministry of Science (Grant No. 175037), and by AlzChem AG, Trostberg, Germany (Grant No. BN_214E_S10). Trial identification: www.clinicaltrials.gov number NCT01371357. The authors thank Dr. Barbara Niess for the technical and scientific advice.

References

[1] Edison, EE; Brosnan, ME; Meyer, C; Brosnan, JT. Creatine synthesis: production of guanidinoacetate by the rat and human kidney in vivo. *Am J Physiol Renal Physiol*., 2007, 293(6), F1799-804.

[2] Stead, LM; Brosnan, JT; Brosnan, ME; Vance, DE; Jacobs, RL. Is it time to reevaluate methyl balance in humans? *Am J Clin Nutr*., 2006, 83(1), 5-10.

[3] Ostojic, SM; Niess, B; Stojanovic, M; Obrenovic, M. Creatine metabolism and safety profiles after six-week oral guanidinoacetic acid administration in healthy humans. *Int J Med Sci*., 2013, 10(2), 141-7.

[4] Ostojic, SM; Niess, B; Stojanovic, M; Idrizovic, K. Serum creatine, creatinine and total homocysteine concentration-time profiles after a single oral dose of guanidinoacetic acid in humans. *J Funct Foods*., 2014, 6(1), 598-605.

[5] Stead, LM; Au, KP; Jacobs, RL; Brosnan, ME; Brosnan, JT. Methylation demand and homocysteine metabolism: effects of dietary provision of creatine and guanidinoacetate. *Am J Physiol Endocrinol Metab*., 2001, 281(5), E1095-100.

[6] Fukada, S; Shimada, Y; Morita, T; Sugiyama, K. Suppression of methionine-induced hyperhomocysteinemia by glycine and serine in rats. *Biosci Biotechnol Biochem*., 2006, 70(10), 2403-9.

[7] Setoue, M; Ohuchi, S; Morita, T; Sugiyama, K. Hyperhomocysteinemia induced by guanidinoacetic acid is effectively suppressed by choline and betaine in rats. *Biosci Biotechnol Biochem*., 2008, 72(7), 1696-703.

[8] Lewington, S; Bragg, F; Clarke, R. A review on metaanalysis of biomarkers: promises and pitfalls. *Clin Chem*., 2012, 58(8), 1192-204.

[9] Ostojic, SM; Niess, B; Stojanovic, M; Obrenovic, M. Co-administration of methyl donors along with guanidinoacetic acid reduces the incidence of hyperhomocysteinemia compared to guanidinoacetic acid administration alone. *Br J Nutr*., 2013, 110(5), 865-70.

[10] Sotgia, S; Carru, C; Caria, MA; Tadolini, B; Deiana, L; Zinellu, A. Acute variations in homocysteine leves are related to creatine changes induced by physical activity. *Clin Nutr*., 2007, 26(4), 444-9.

[11] Fowler, B. Homocysteine: overview of biochemistry, molecular biology, and role in disease processes. *Sem Vasc Med*., 2005, 5(2), 77-86.

[12] Verhoef, P; Katan, MB. A healthy lifestyle lowers homocysteine, but should we care? *Am J Clin Nutr*., 2004, 79(5), 713-4.

[13] Verhoef, P; de Groot, LC. Dietary determinants of plasma homocysteine concentrations. *Sem Vasc Med*., 2005, 5(2), 110-23.

[14] Ciaccio, M; Bellia, C. Hyperhomocysteinemia and cardiovascular risk: effect of vitamin supplementation in risk reduction. *Curr Clin Pharmacol*., 2010, 5(1), 30-6.

[15] Ueland, PM. Choline and betaine in health and disease. *J Inherit Metab Dis*., 2011, 34(1), 3-15.

[16] Purohit, V; Abdelmalek, MF; Barve, S; Benevenga, NJ; Halsted, CH; Kaplowitz, N; Kharbanda, KK; Liu, QY; Lu, SC; McClain, CJ; Swanson, C; Zakhari; S. Role of S-adenosylmethionine, folate, and betaine in the treatment of alcoholic liver disease: summary of a symposium. *Am J Clin Nutr*., 2007, 86(1), 14-24.

[17] Reo, NV; Adinehzadeh, M; Foy, BD. Kinetic analyses of liver phosphatidylcholine and phosphatidylethanolamine biosynthesis using (13)C NMR spectroscopy. *Biochim Biophys Acta*., 2002, 1580(2-3), 171-88.

[18] Noga, AA; Stead, LM; Zhao, Y; Brosnan, ME; Brosnan, JT; Vance, DE. Plasma homocysteine is regulated by phospholipid methylation. *J Biol Chem*., 2003, 278(8), 5952-5.

[19] Blom, HJ; Smulders, Y. Overview of homocysteine and folate metabolism. With special references to cardiovascular disease and neural tube defects. *J Inherit Metab Dis*., 2011, 34(1), 75-81.

[20] Horne, DW; Cook, RJ; Wagner, C. Effect of dietary methyl group deficiency on folate metabolism in rats. *J Nutr*., 1989, 119(4), 618–621.

[21] Niculescu, MD; Zeisel, SH. Diet, methyl donors and DNA methylation: interactions between dietary folate, methionine and choline. *J Nutr*., 2002, 132(8), 2333S-5S.

[22] Olthof, MR; Brink, EJ; Katan, MB; Verhoef, P. Choline supplemented as phosphatidylcholine decreases fasting and postmethionine-loading plasma homocysteine concentrations in healthy men. *Am J Clin Nutr*., 2005, 82(1), 111-7.

[23] Wyss, M; Kaddurah-Daouk, R. Creatine and creatinine metabolism. *Physiol Rew*., 2000, 80(3), 1107-213.

[24] Jahangir, E; Vita, JA; Handy, D; Holbrook, M; Palmisano, J; Beal, R; Loscalzo, J; Eberhardt, RT. The effect of L-arginine and creatine on vascular function and homocysteine metabolism. *Vasc Med*., 2009, 14(3), 239-48.

[25] Derave, W; Marescau, B; Vanden, Eede, E; Eijnde, BO; de Deyn, PP; Hespel, P. Plasma guanidino compounds are altered by oral creatine supplementation in healthy humans. *J Appl Physiol.*, 2004, 97(3), 852-7.

[26] Wu, G; Morris, SM; Jr. Arginine metabolism: nitric oxide and beyond. *Biochem J.*, 1998, 336(Pt I), 1-17.

[27] Sobrevia, L; Gonzalez, M. A role for insulin on L-arginine transport in fetal endothelial dysfunction in hyperglycaemia. *Curr Vasc Pharmacol.*, 2009, 7(4), 467-74.

[28] Alsever, RN; Georg, RH; Sussman, KE. Stimulation of insulin secretion by guanidinoacetic acid and other guanidine derivatives. Endocrinology., 1970, 86(2), 332-6.

[29] Dudman, NP; Wilcken, DE; Wang, J; Lynch, JF; Macey, D; Lundberg, P. Disordered methionine/homocysteine metabolism in premature vascular disease. Its occurrence, cofactor therapy, and enzymology. *Arterioscler Thromb Vasc Biol.*, 1993, 13(9), 1253-60.

[30] Wyss, M; Schulze, A. Health implications of creatine: can oral creatine supplementation protect against neurological and atherosclerotic disease? *Neuroscience.*, 2002, 112(2), 243-60.

[31] Tachikawa, M; Kasai, Y; Yokoyama, R; Fujinawa, J; Ganapathy, V; Terasaki, T; Hosoya, K. The blood-brain barrier transport and cerebral distribution of guanidinoacetate in rats: involvement of creatine and taurine transporters. *J Neurochem.*, 2009, 111(2), 499-509.

[32] Carducci, C; Birarelli, M; Leuzzi, V; Carducci, C; Battini, R; Cioni, G; Antonozzi, I. Guanidinoacetate and creatine plus creatinine assessment in physiologic fluids: an effective diagnostic tool for the biochemical diagnosis of arginine:glycine amidinotransferase and guanidinoacetate methyltransferase deficiencies. *Clin Chem.*, 2002, 48(10), 1772-8.

[33] European Food Safety Authority. Safety and efficacy of guanidinoacetic acid as feed additive for chickens for fattening. *EFSA J.*, 2009, 988, 1-30.

[34] Olthof, MR; van, Vliet, T; Verhoef, P; Zock, PL; Katan, MB. Effect of homocysteine-lowering nutrients on blood lipids: results from four randomised, placebo-controlled studies in healthy humans. *PloS Med.*, 2005, 2(5), e135.

[35] Maejima, M; Takahashi, S; Hatano, M. Platelet aggregation in chronic renal failure - whole blood aggregation and effect of guanidino compounds. *Nippon Jinzo Gakkai Shi.*, 1991, 33(2), 201-12.

[36] Tanaka, A; Takaihashi, Y; Mizokuci, M; Shimada, N; Koide, H. Plasma, urinary, and erythrocyte concentrations of guanidino compounds in patients with chronic renal failure. *Ren Fail.*, 1999, 21(5), 499-514.

[37] Hayes, KC; Pronczuk, A; Cook, MW; Robbins, MC. Betaine in sub-acute and sub-chronic rat studies. *Food Chem Toxicol.*, 2003, 41(12), 1685-700.

[38] Morris, MS; Jacques, PF; Rosenberg, IH; Selhub, J. Folate and vitamin B-12 status in relation to anemia, macrocytosis, and cognitive impairment in older Americans in the age of folic acid fortification. *Am J Clin Nutr.*, 2007, 85(1), 193-200.

[39] Borsook, ME; Borsook, H. Treatment of cardiac decompensation with betaine and glycocyamine. *Ann West Med Surg.*, 1951, 5(10), 830-55.

[40] Borsook, ME; Billing, HK; Goklseth, JG. Betaine and glycocyamine in the treatment of disability resulting from acute anterior poliomyelitis. *Ann West Med Surg*., 1952, 6(7), 423-27.

[41] Dixon, HH; Dickel, HA; Shanklin, JG; Peterson, RD; West, ES. Therapy in anxiety states and anxiety complicated by depression. *West J Surg Obstet Gynecol*., 1954, 62(6), 338-41.

[42] Lukaski, HC. Vitamin and mineral status: effects on physical performance. *Nutrition*., 2004, 20(7-8), 632-44.

[43] Armstrong, LE; Casa, DJ; Roti, MW; Lee, EC; Craig, SA; Sutherland, JW; Fiala, KA; Maresh, CM. Influence of betaine consumption on strenuous running and sprinting in a hot environment. J Strength Cond Res., 2008, 22(3), 851-60.

[44] Antolic, AM; Roy, BD; Tarnopolsky, MA; Zernicke, RF; Wohl, GR; Shaughnessy, SG; Bourgeois, JM. Creatine monohydrate increases bone mineral density in young Sprague-Dawley rats. *Med Sci Sports Exerc*., 2007, 39(5), 816-20.

[45] Brosnan, JT; Brosnan, ME. Creatine: endogenous metabolite, dietary, and therapeutic supplement. *Annu Rev Nutr*., 2007, 27, 241-61

[46] Deldicque, L; Theisen, D; Bertrand, L; Hespel, P; Hue, L; Francaux, M. Creatine enhances differentiation of myogenic C2C12 cells by activating both p38 and Akt/PKB pathways. *Am J Physiol Cell Physiol*., 2007, 293(4), C1263-71.

[47] Craig, SAS. Betaine in human nutrition. *Am J Clin Nutr*., 2004, 80(3), 539-49.

[48] Dangott, B; Schultz, E; Mozdziak, PE. Dietary creatine monohydrate supplementation increases satellite cell mitotic activity during compensatory hypertrophy. *Int J Sports Med*., 2000, 21(1), 13-6.

[49] Volek, JS; Duncan, ND; Mazzetti, SA; Staron, RS; Putukian, M; Gómez, AL; Pearson, DR; Fink, WJ; Kraemer, WJ. Performance and muscle fiber adaptations to creatine supplementation and heavy resistance training. *Med Sci Sports Exerc*., 1999, 31(8), 1147-1156.

[50] Schwab, U; Törrönen, A; Meririnne, E; Saarinen, M; Alfthan, G; Aro, A; Uusitupa, M. Orally administered betaine has an acute and dose-dependent effect on serum betaine and plasma homocysteine concentrations in healthy humans. *J Nutr*., 2006, 136(1), 34-8.

[51] Atkinson, W; Elmslie, J; Lever, M; Chambers, ST; George, PM. Dietary and supplementary betaine: acute effects on plasma betaine and homocysteine concentrations under standard and postmethionine load conditions in healthy male subjects. *Am J Clin Nutr*., 2008, 87(3), 577-85.

[52] Fargeas, MJ; Fioramonti, J; Bueno, L. Central and peripheral action of GABAA and GABAB agonists on small intestine motility in rats. *Eur J Pharmacol*., 1988, 150(1-2), 163-169.

[53] Melanson, KJ; Angelopoulos, TJ; Nguyen, VT; Martini, M; Zukley, L; Lowndes, J; Dube, TJ; Fiutem, JJ; Yount, BW; Rippe, JM. Consumption of whole-grain cereals during weight loss: effects on dietary quality, dietary fiber, magnesium, vitamin B-6, and obesity. *J Am Diet Assoc*., 2006, 106(9), 1389-90.

In: Human Health and Nutrition
Editor: Sergej M. Ostojic
ISBN: 978-1-63482-823-9

Chapter VI

New Forms of Creatine in Human Nutrition

Milan B. Vranes [*] ***and Snezana Papovic***
Faculty of Sciences, University of Novi Sad, Novi Sad, Serbia

Abstract

Creatine is one of the best-known and most studied ergogenic supplements among athletes. Besides its performance-enhancing power, creatine has significant clinical potential in patients with neurological and neuromuscular diseases. The most frequently used form of creatine is creatine monohydrate. The utilization of creatine monohydrate seems to be somewhat limited due to its physico-chemical characteristics such as poor water solubility, instability in aqueous solutions (because of its tendency to cyclize into biologically inactive creatinine), and finite capacity of creatine transporters. Therefore, the pharmaceutical industry strives to develop novel forms of creatine that will diminish or overcome aforementioned limitations. New formulations of creatine seem to appear in the market on a daily basis while no sufficient research is conducted regarding their physico-chemical characteristics and safety in humans. In this chapter, authors reviewed recent literature on advanced creatine formulations (e.g., creatine salts, chelates, esters and alkaline buffered forms). The purpose and goal for the use of new creatine formulations have been discussed as well as their advantages and disadvantages compared to creatine monohydrate.

Keywords: Creatine monohydrate, Sport supplement, Ethyl ester Salts, Buffered creatine

[*] Corresponding author: Email: milan.vranes@dh.uns.ac.rs.

Introduction

The human body is a myriad of independent systems working symbiotically to sustain life. Throughout these systems, many mechanisms exist to provide and regenerate energy that is required for the body to function properly. The energy that is generated in the process of food digestion is used partially for maintaining body temperature while the larger part of that energy is stored as adenosine triphosphate (ATP). This is a high-energy molecule that is transported to all parts of the cell where energy is needed. It represents the basic transport form of chemical energy that is being released during transport of the phosphate group (PO_4^{3-}) to specific receptors.

A man of average body weight (70 kg) has 50 g of ATP. The energy that is being released from all the molecules of ATP during hydrolysis of his one PO_4^{3-} group into energetically inferior molecule such as adenosine diphosphate (ADP) is not sufficient for performing a heavy workout lasting 0–1.5 seconds. However, there are other compounds stored in the muscles such as energetically rich N-phosphato derivatives of guanidine that serve as a energy source when needed. The phosphorylated form of creatine (Cr) is phoshpocreatine (PCr); it is used for replenishing ATP molecules used immediately during high intensity exercise. The ability of PCr to quickly regenerate ATP under anaerobic conditions in the muscle cell have made creatine the most researched molecule in the area of sport nutrition.

The future of creatine research is quite promising. Considering the fact that Cr supplementation (CrS) is beneficial for improving physical performance, particularly for tasks requiring muscular strength and power, scientists are determined to find ways to increase Cr buildup in the muscle after oral administration by discovering new forms of Cr. The safety of long term CrS in athletic and clinical environment, especially in patients who lack Cr due to disruptions in its biosynthesis due to neurological and neuromuscular diseases, has been extenivelly studied in the past decade. All this lead to development of new forms of creatine, while their effect on athletic performance and health has not been researched thoroughly. In this chapter, the authors will overview scientific studies on Cr in order to answer the question whether the new forms of Cr are more efficient in comparison to its most used form so far – creatine monohydrate (CrMH).

History of Creatine

Creatine (*κρέας*, Greek for meat) was isolated from meat for the first time in 1835 [1], while almost a century later (1927) the phosphorylate form, phosphocreatine, a high-energy phosphate compound [2, 3] was discovered (Figure 1).

Lundsgaard [4] proved that PCr plays the central role in energy production for muscular contraction. In 1934 Lohmann [5] reported that in the case of high availability of ATP, the phosphate group (PO43–) is transferred to Cr and then stored in the form of PCr. Once the level of ATP drops, the phosphate group is transferred from PCr to ADP through the reaction catalyzed by the enzyme creatine kinase (CK) [5]. Lohnmann had unequivocally determined the importance of PCr in maintaining high levels of ATP during physically demanding anaerobic activities. In scientific literature, this was described as PCr/Cr shuttle system [6, 7].

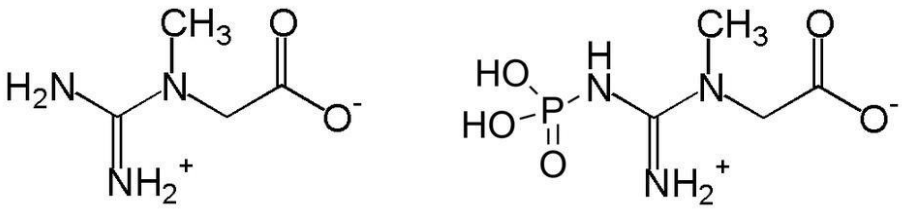


Figure 1. Chemical structure of creatine, Cr (left) and phosphocreatine, PCr (right).

Metabolism of Creatine

Biosynthesis of Creatine

Creatine (Cr; (*α*-methylguanido)acetic acid; *N*-(aminoiminomethyl)-*N*-methyl-glycine), is synthesized in two steps in the kidney and the liver, from where it gets transported through blood by the means of active transport system to its final destination: tissues with high energy consumption such as skeletal muscle [8] (Figure 2).

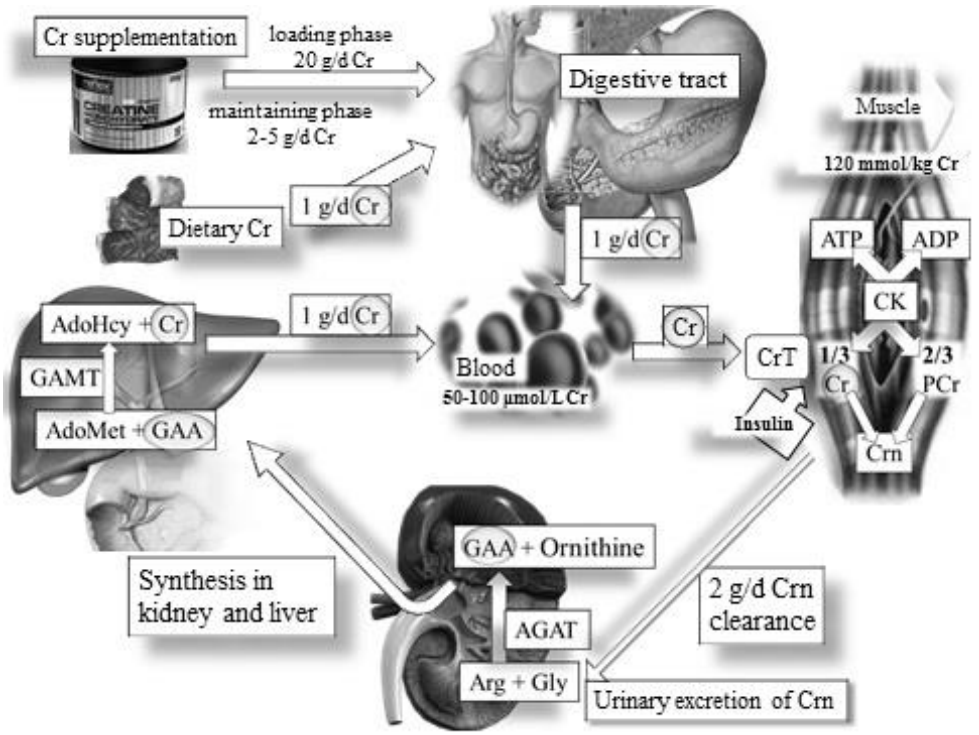


Figure 2. Schematic representation of Cr biosynthesis and metabolism.

The body produces Cr from L-arginine, glycine and L-methionine that are supposed to be plentiful in human diet [9]. The synthesis begins in the kidney [10] by the reaction in which the amino group of L-arginine [11] gets transferred onto glycine, thus forming ornithine and

guanidino-acetic acid (GAA). This reaction is catalyzed by glycine-amidinotransferase (AGAT) [12,13]. The kidney-formed GAA gets transported through blood to the liver where the enzyme guanidinoacetate-methyltransferase (GAMT) catalyzes methylation of amidino group of GAA to Cr with S-adenosyl-L-methionine (SAM) as the methyl donor. During this reaction, S-adenosylhomocisteine is being released [14].

Newly formed Cr is actively transported from the liver through the cell membrane into the circulation. Absorption of Cr from blood into the muscles occurs in the opposite direction of the concentration gradient of intracellular concentration of Cr (in plasma c(Cr) = 50-100 μmol/l while intracellular c(Cr +PCr) = 40 mmol/l) [15]. The process is carried out by the NaCl dependent Cr transporter. The mechanism of Cr uptake seems to be stimulated by insulin [15-21]. Muscle cells store 95% of Cr [22], while the remaining 5% is present in the heart, brain and testes [23].

PCr As a Regulator of Energy Processes

Creatine phosphate (PCr) as an energy regulator of muscle contraction plays fundamental multifaceted role in relation to exercise metabolism. Storage capacity of PCr is relatively small. Through reverse activity of the enzyme CK, during muscular contraction [6] the PO_4^{3-} group is quickly transferred to ADP in aim to maintain high levels of ATP [5, 24] (Figure 3). In this process, one proton is being absorbed which means that Cr plays a role in increasing pH in the muscles [25, 26] and therefore neutralizes the effects of exercise-induced metabolic acidosis [27]. Transfer of PO_4^{3-} group from PCr onto ADP and formation of ATP is thermodynamically spontaneous process (ΔG^0= -8,6 kJ/mol) [25].

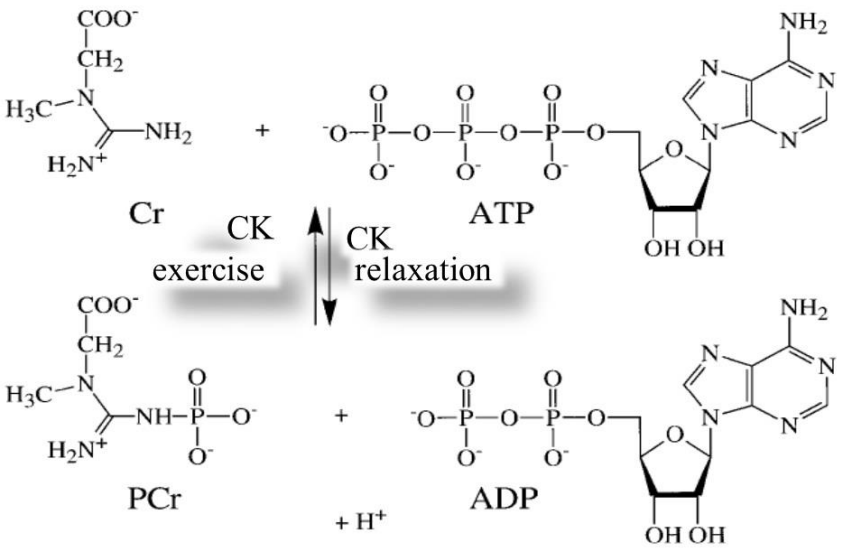


Figure 3. Conversion of PCr into Cr with a release of one molecule of ATP.

During periods of rest, when ATP levels are restored by aerobically generated oxidative phosphorylation, CK acts in reverse direction, restoring 95% of PCr during 3-4 minutes [28].

An increase in the level of PCr (during Cr supplementation for example) reduces intramuscular lactate accumulation and delays the onset of fatigue [27, 29].

Creatine Kinase

There are several isoforms of CK that are named after the tissue in which they exert their biocatalytic effect. Cytosolic CK is composed of subunits MM-CK that is expressed in sarcomeric (skeletal and cardiac) muscle [30], BB-CK is found in smooth muscle and non-muscle tissues [31-33], while MB-CK isoform expressed in cardiac muscle. Isoenzyme CK known as mitochondrial CK (Mi-CK) is found in the intermembrane space of mitochondria [34, 35]. ATP that is formerly generated by oxidative phosphorylation in the mitochondrial matrix and then transported in the intermembrane mitochondrial space through adenine nucleotide translocator (ATN), plays an important role in the phosphorylation of Cr (imported from the cytosol through protein pores) in the presence of the enzyme Mi-CK [24, 36, 37].

Newly formed PCr diffuses into cytosol through protein pores. It then becomes available for ATP formation in the reaction with ADP and PCr catalyzed by CK. Dephosphorylated Cr is being transported from the cytosol back to mitochondria [38]. Mitochondrial Mi-CK and cytosolic CK (CKc) are linked in a so-called PCr/Cr-shuttle [6]. Inside the cell (were PCr/Cr and ATP/ADP are located), the cytosolic form of CK (CKc) is activated in order to maintain the equilibrium between Cr and PCr. During the periods of rest, the PCr stores are being replenished by the ATP formed by glycolysis [38]. In this reaction the glycolytic form of CK (CKg) phosphorylates Cr into PCr and increases its availability for periods of physical activity [28].

Absorption of Cr in the Gastrointestinal System

Creatine can be obtained through an omnivorous diet. Because Cr is found in food, it is not surprising that Cr is absorbed from the gastrointestinal system (GI) via a process similar to other nutrients (amino acids, glucose, vitamins), so it can be absorbed through amino acid or peptide transporters in the small intestine. The mechanism of absorption of Cr in GI was elucidated after identifying mRNA of Cr transporter (CrT) [39]. Transporters mediating Cr flux through the intestinal wall have been identified in rodents mostly in the ileum [40], jejunum [41], on the apical [41, 42] and basolateral membranes of enterocytes [43]. Studies have shown that CrT has an important role in the export of Cr from the enterocyte across the basolateral membrane [43].

Transfer of Cr and Cr Transporter (CrT)

Creatine that was provided through biosynthesis or oral intake is transported into the skeletal muscles against a concentration gradient via a NaCl dependent transporter [15]. The structure of this transporter is similar to dopamine or GABA transporters [15, 39]. It requires at least 2 Na^+ and one Cl^- to transport single molecule of Cr into the cell. The function of CrT

is determined by insulin, exercise and the content of Cr in the muscles [44]. The CrT is highly specific for Cr, neither creatinine (Crn) nor PCr are substrates. Branched chain amino acids (BCAA) that are frequently used in supplementation do not affect Cr transport, although they used to be considered competitive substrates for CrT [18, 45]. However, there is one competitive substrate, beta-guanidinopropionic acid [39, 46]. The rate-limiting step in muscle Cr uptake is intracellular unphosphorylated Cr content, which makes about 1/3 of the muscle Cr pool [47, 48], rather than extracellular Cr concentration. The increase of extracellular concentration is known to down-regulate the Cr in striated muscle cells [18]. It means that after achieving maximum concentrations of Cr in the muscle cells by consuming large amounts of Cr in shorter period of time (e.g., loading phase) this concentration in the cells can be maintained by further taking much smaller concentration of Cr (e.g., maintenance phase). This will be discussed in further details later. CrT expression and its regulation may differ in disease with insufficient activity of CrT, as it is described later.

Bioavailability and Clearance

When Cr is administered non-intravenously through oral, sublingual and transdermal route, it is transported to the GI tract and then further through the bloodstream to the target cells in its unchanged form. Uptake of Cr into the bloodstream and target tissue depends on its bioavailability. Based on the research so far, bioavailability is diminished due to (1) insufficient solubility when administered by oral route; (2) degradation of Cr to Crn in the stomach and gastrointestinal tract (GI); (3) increased fecal excretion of Cr after oral intake; and (4) degradation of Cr by the gut flora.

It is primarily necessary to have Cr supplement (e.g., powder, tablet, capsule) completely soluble before use. Limited bioavailability of Cr is evident in lozenges, as they require disintegration and dissolution, while suspension requires dissolution of the suspended particles [49]. Unlike these forms, bioavailability from solutions and meat is practically maximal (99%) [50]. Although absorption of Cr after meat consumption is slower than from a solution, its bioavailability is not diminished. Therefore the limiting factor for CrS is solubility. Cr supplement that has a good solubility provides optimal bioavailability. In order to achieve maximum solubility, new forms of Cr are being synthesized and tested. This will be discussed in further details later.

Spontaneous non-enzymatic cyclization of Cr into Crn depends on pH value of the solution, with reaction fastest at pH = 3.4 [51-54]. When Cr is ingested in oral forms, conversion of Cr into Crn is negligible in the pH environment of the stomach (pH = 2) [52, 55], while the level of Cr in the plasma increases considerably [49].

In other segments of gut, Cr spends more time than in the stomach, but even higher pH in jejunum and ileum (pH = 6-7), no significant conversion of Cr into Crn has been reported. This can be inferred by a non-measurable amount of Crn in feces. As absorption of Cr in small intestine is mediated by CrT, with the process could be saturated by continuous CrS. Fecal excretion of Cr increases with higher Cr administered [56]. It used to be thought that the gut flora has the ability to metabolize Cr into Crn [57], but the latest studies shown that is not the case [42, 43, 54, 58, 59]. In order to determine bioavailability of Cr it is necessary to determine the levels of Cr in the muscle cells before and after CrS by using muscle biopsy

and/or whole body Cr retention estimate by measuring the difference in Cr intake and urinary excretion.

Studies have shown that Cr monohydrate (CrMH) does not degrade during the digestive process, and nearly 99% of orally ingested Cr is being either stored in the muscles or excreted by urine [50, 55, 60, 61]. Cr uptake by the muscles as well as urinary clearance by the kidneys diminishes through supplementation. Creatine is irreversibly trapped in the muscle because its polar nature prevents its passive efflux back into the circulation. During the early phase of the supplementation (the initial 1-3 days) clearance is top-most. The levels of Cr in urine increases progressively as large doses of Cr are being continuously ingested [62, 63]. After initial supplementation (e.g., loading phase) saturated muscular pool of Cr [64], Cr being to eliminate from the body through kidneys. After the loading phase, CrS is reduced to 2-5 g/d as daily excretion of Cr in the form of Crn is about 2 grams on average [44].

Therefore, daily intake of large doses of Cr results in large quantities of Cr/Crn being excreted through urine, a situation that can potentially lead to kidney problems [50]. As Cr pharmacokinetics changes over time, so should the dosage [44]. In order to deliver the highest possible amount of Cr into the muscles, it has been shown that it is necessary to maintain 50-100 μmol/L of Cr in plasma [65]. These levels are easily achieved by consuming 2 grams per day of Cr. After 2 hours the plasma Cr levels will return to the baseline yet the degree of Cr uptake by the muscles is probably low. From this perspective, it is more efficient to take 20 g of Cr and the return to the baseline values happens after more than 10 hours. Plasma levels of Cr higher than 100 μmol/L will not further increase Cr uptake by the muscle cells so the saturation limit of CrT is close to 100 μmol. The effect of carbohydrates (CHO) administered during CrS is attributed to the more efficient removal of Cr from the blood caused by the CHO-medicated stimulation of CrT that ensures better absorption and accumulation of Cr [66]. In people who do not exercise, there was no effective increase of intramuscular PCr levels during the course of CrS [65], due to limited conversion of Cr into PCr in the cytosol and mitochondria [67]. Physical activity increases the content of Mi-CK i CKc [68], so in non-athletic population muscles cannot generate proper response to CrS due to diminished activity of CK. Also, as the consequence of exercise, insulin levels in the blood increase leading to the increased uptake of Cr by the muscles [69-72].

Creatine in Sport

Dietary Creatine

Daily utilization of Cr for an average human is ~ 2 g. This is because Cr cyclizes into its metabolite creatinine (Crn) that is being excreted daily in the amounts of 2 g [22]. Half of the daily dose of Cr is available through diet (exogenous source of Cr) [73], while the remaining amount of Cr is supplied by endogenous biosynthesis [74]. Various kinds of meat are particularly rich in Cr [23] (Table 1).

Table 1. Cr content in selected foods (data adapted from Balsom et al. [23])

Food	g / kg
Herring	6.5-10
Beef	4.5
Salmon	4.5
Pork	5
Cod	3
Tuna	4
Milk	0.1
Cranberries	0.02
Shrimp	Trace

A balanced diet and biosynthesis of Cr can maintain the constant amount of 120 g of Cr in a 70 kg human. The range of Cr in skeletal muscles is 110-160 mmol/kg of dry mass [75-77], of which 60% is found in the form of PCr, while the remaining 40% is available as Cr [78, 64]. The amount of Cr varies due to several factors, such as dietary habits, the type of muscle fibers used, and gender [49, 79]. Vegetarians represent a distinct group with low Cr availability since only the endogenously Cr is available [80]. Although the levels of Cr in vegetarians plasma is considerably lower in comparison to meat eaters, the concentration of Cr in muscles is mostly within the normal range [66, 78, 81, 82]. Large amounts of fish and meat are required to obtain gram quantities of Cr, but this diet will also include additional amounts of fat and protein. It is necessary to ensure a more efficient way of increasing dietary availability of Cr. Exogenous Cr can also be obtained through CrS.

Creatine Supplementation

The intracellular stores of PCr are small, meaning that PCr is quickly depleted during maximal exercise (10-20 seconds). Depletion of ATP and PCr in the muscle during exercise causes a drop in exercise intensity. Therefore, CrS (e.g., CrMH, novel forms) is a way to increase the amounts of PCr in the muscles, leading to muscle mass growth and increased perfoemance (ergogenic effect) [29, 54, 64, 69, 78, 83-86]. Following paragraphs focuses nutritive procedures and dosing strategies for CrS that are effective in promoting either an acute physiological response that may improve exercise performance, or influence chronic training adaptations. It is necessary to consider all the advantages of CrS, to minimize possible side effects, and count issues related to absorption, distribution and relevant pharmacokinetic parameters of CrS such as clearance, bioavailability, half life, and elimination from the body [44].

Supplementation Protocols and Retention of Cr in the Skeletal Muscles

During CrS, the typical dosage pattern was divided into two phases: a loading phase and a maintenance phase. The method of increasing Cr stores in the muscles is described in scientific literature as a loading phase. It consists of ingesting 20 g of CrMH (divided in 4 x 5

g of daily doses in the course of 4-6 days or 0.3 g/kg body weight) [69, 87, 88]. After 6 days of loading, Cr stores in the muscles seems to be filled up. Afterwards, it is necessary to take only 2-5 g of CrMH daily as a single dose (or 0.03 g/kg) in order to maintain higher Cr stores levels [87, 89] (Figure 4). This mode of supplementation may promote fast ergogenic effect. If CrS in terminated after the loading phase that lasted for 6 days, a decrease in muscle Cr levels has been found, and after 4 weeks muscle Cr will revert back to pre-supplementation levels. There is no clear consensus on how much Cr a person should ingest per day, because individuals have different weights and body muscle content. Cr is stored in the form of total Cr (TCr = Cr + PCr). Above protocol optimizes intramuscular Cr content and whole body Cr retention [82].

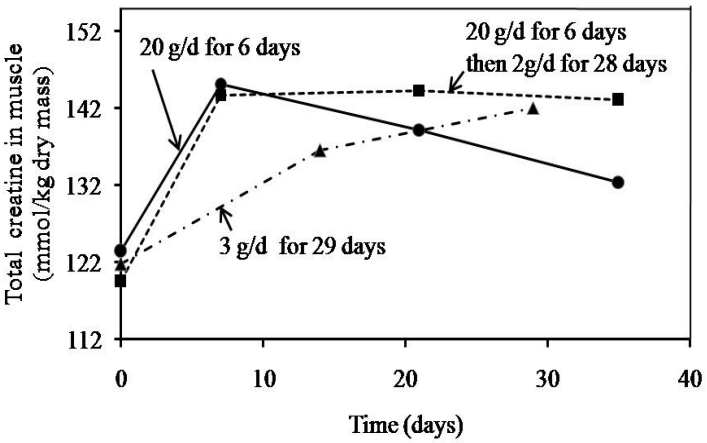


Figure 4. Content of total creatine (Cr + PCr) in subjects taking different forms of supplementation over the course of 4 weeks (data adapted from [78]).

Hultman [78] suggested a "cycling" strategy that does not require the loading phase. During constant supplementation with 3 g/d over 29 days there is no fast saturation of Cr stores in the muscles as with the loading phase. During the “cycling” protocol levels of Cr are being increased gradually, and after 4 weeks they are the same as during the loading and maintaining phase (Figure 4). In comparison to the loading phase, the increase of Cr muscle stores is more gradual and thus the ergogenic effect does not occur as quickly [62, 90].

In studies where the loading phase is neglected (6 g/d CrMH for 12 weeks), it has been reported that the increase of muscle size and strength is far slower than in the case of protocols with the loading phase [91, 92]. Exogenous intake of small amounts of Cr (2 g/d, a value equivalent to the concentration of Cr that degrades into Crn) [22] did not show any beneficial effect on aerobic or anaerobic metabolism during endurance exercise [93, 94]. Therefore, if the goal is to increase muscle Cr levels, the minimal daily dose of Cr is 3 g.

The amount of Cr that gets retained in the muscles after CrS depends on the initial muscle Cr content [95]. Individuals with lower Cr content in the muscles before CrS (e.g., vegetarians) can increase Cr muscle stores for 20-40%, while those individuals with relatively high levels of Cr before CrS can achieve only 10-20% increase [29, 84, 87]. During CrS a decrease in endogenously formed Cr has been demonstrated [22]. Upon termination of CrS, a concentration of Cr reverts back to the baseline levels [62]. Cr accumulation in the muscle

might be depressed by the presence of several drugs (e.g., ouabain, digoxin) [96] or vitamin E deficiency [97].

There are 20-30% of subjects that are unresponsive to CrS (e.g., non-responders), with muscle levels of Cr $\leq$ 10 mmol/kg dry muscle after 5 days of 20 g/day of CrS [98]. Differences in efficacy of CrS can occur due to insufficient amount of Cr that was ingested, formulation of the supplement, the type of training as well as the activity of CrT and its mode of transport to muscle cells. In aim to increase Cr uptake by the muscle, numerous commercial forms of Cr has been developed [15,19]. Research efforts are also directed toward finding the ways to transport Cr to muscle cells independently of Cr transporters, such as facilitating passive diffusion [99,100].

Effects of Insulin and Glucose on Cr Utilization

Upon intense physical activities, insulin is being secreted [101, 102] and this facilitates Cr uptake by the muscle [103]. This effect is attributed to insulin's ability to indirectly stimulate NaCl pump that enhances Cr transport. Therefore, Cr uptake into skeletal muscle can be stimulated by the compounds that induce insulin secretion [20], such as glucose, CHO [104], and short chain glucose polymers (e.g., maltodextrin). For that reason, above compounds are often co-administered with CrMH [105]. It is necessary to avoid high-fructose components (such as fruit juices) because fructose does not stimulate insulin secretion [65, 106, 107]. Studies on CrS with CHO were done in wide range of CHO amounts, in order to achieve optimal amounts of CHO that stimulates Cr uptake into the muscle. Based on the collective evidence presented, to each dose of CrS (20 g/d during loading and 5 g/d CrMH during maintaining phase) a 93 g of CHO should be added in aim to achieve optimal uptake [66]. This protocol of CHO-CrS enables higher concentration of Cr in the muscle to be achieved, when compared to CrS alone. Preen et al. [108] established that CrS combined with 1 g of glucose per kg body mass twice per day increased muscle total Cr by 9% more as compared to CrS alone. Another study investigated the effects of protein co-administered during CrS. A half of the amount of CHO (93 g) was replaced with protein (PRO, whey protein isolate), so the combination of CrMH, 47 g of CHO and 50 g of PRO has been evaulated [103]. The results showed similar increase in muscle Cr as in the case of CrS with CHO [103, 109, 110]. Concentration of Cr in plasma was the same like in the previous studies, which indicates that the added PRO does not affect absorption of Cr through GI tract. Another study suggests that β-glucan bars (polysaccharides rich in dietary fibers) facilitate Cr retention by decreasing the velocity of intestinal absorption rate [50]. Additional studies evaluated the combination of CrMH with CHO but with lower dose of CHO while its efficacy remained the same (e.g., CrMH followed by ingesting 18 g of glucose per dose) [105]. This supplementation protocol is particularly important in people who are not supposed to ingest large amounts of CHO (e.g., diabetes patients) or with limited energy consumption [66]. A combination of CrMH with CHO is administered four times per day during the loading phase. Study revealed that it is not recommended to mix CrMH and CHO in the same bowl, since CHO or PRO reduces solubility of CrMH in the solution, which will be discussed in further details later.

Regardless of which protocol is chosen, Cr should be administered close to exercise session (60 minutes prior to and/or immediately after) [111]. The reason for this is to provide a higher degree of Cr accumulation and therefore promote better gains in strength, body composition (increase lean mass with no increase in fat mass), training adaptations and muscle mass [70, 112, 113].

Influence of Caffeine on Ergogenic Properties of Cr

Caffeine is used in sport supplementation with the goal of keeping focus during workout [114], and thus improving athletic performance [115]. Researchers and athletes have long known that caffeine and Cr independently improve performance so a combination would be the next logical step. In light of this, there have been studies designed to determine if the two agents (caffeine and Cr) could work together to increase exercise performance. The findings from several studies suggest that caffeine impairs the advantages of Cr loading [116, 117], whereas other studies reported significant elevations in muscle Cr and improved athletic performance after Cr-caffeine co-administration [64, 84, 118]. Further studies are needed to clarify ergogenic potential of caffeine-Cr formulation.

Effect of Cr on Water Retention

Cr is osmotically active compound, so an increase in its concentration in muscle cells requires the formation of equilibrium inside and outside the cells. The equilibrium is achieved by water uptake into the cell. This results in water retention and overall increase in muscle mass [119-121]. This is usually expected during the initial 5-7 days of CrS (e.g., loading phase with 20 g of Cr per day) with an increase in intramuscular concentration of Cr and water. As an osmotically active compound, Cr increases cell volume that appears to be a proliferative, anabolic signal that may enhance protein synthesis [121-124] which suggests a method by which extended CrS may promote muscular hypertrophy [125].

Effect of Cr on Muscle Metabolism

Beneficial effects of Cr are achievable after an increase in muscular TCr to 20 mmol/kg dry muscle (dm), which requires CrS for 5 days with 20 g of Cr per day [64, 69, 98]. That amount is needed in order to significantly attenuate the drop in ATP levels during intense exercise, as well as to elicit a proliferative anabolic signal [126] and cell mitotic activity [127]. This is partly attributed to cell volumization via induced water retention [128] and muscle insulin-like growth factor-1 (IGF-1) signaling [86, 129]. CrS and resistance training has been shown to stimulate the rate of synthesis of two major contractile proteins, actin and myosin heavy chains [91, 130]. These studies utilized standard Cr supplementation protocol over the course of 6-8 weeks [131-133]. Increases in muscle cell diameter and increase in fat-free body mass by approximately 2.8-3.2 kg has been reported as well [134].

Effect of Cr on Athletic Performance and Health

Many activities have a high dependence on the PCr/Cr system [29]. Success in team sports [131, 135, 136], weight lifting [137], field events (e.g., shot put and discus throwing [138], knee extensions [131], jumping squats [139]), swimming [141] requires short-term singular or a limited number of repeated intense muscle contractions. Athletes who require sudden, high intensity bursts of power and strength are ideal candidates for Cr supplementation [132, 133].

The main energy source during short term, high intensity exercise is PCr. An increase in Cr and PCr availability achieved through CrS affects workout and training adaptations. Larger concentration of PCr in muscles contributes to fast ATP regeneration under anaerobic conditions. This is how high levels of ATP are maintained during the course of demanding anaerobic activities such as sprinting or weight lifting. Increasing the availability of PCr may help speed recovery between sprints or bouts of intense exercise. It enables athletes to do more work over a series of sprints or sets of exercise, which leads to improves in maximal strength (as measured by 1 repetition maximum), power, increase muscle mass or benefit in sport performance.

Although Cr supplements are typically marketed as bodybuilding and strength-boosting supplements, there are some assumptions that may prove beneficial for endurance athletes as well. For aerobic activities there is a less evidence that CrS might be helpful. Cr may improve endurance, but the magnitude of improvement seems to be dependent on two issues: the duration of the endurance event, which in most cases is dictated by the intensity of exercise, and the mode of exercise. Based on time to exhaustion measurements and average work achieved, CrS demonstrates good effects on short-duration, high-intensity endurance events that last up to approximately 3-4 min. Potential ergogenic effects are diminished as duration increases.

Short-term CrS (5-7 days) promotes an increase in total intramuscular Cr that might improve maximal power/strength (for 5–15%) [140], work performed during sets of maximal effort muscle contractions (for 5–15%) [139], single-effort sprint performance (for 1–5%) [142], work performed during high-intensity sprints or endurance training repetitive sprint performance (for 5–15%) [143, 144]. In addition, long term CrS (5 g/day during 21 months) does not negatively affect athletes' health nor caused any clinically significant change in serum metabolic markers, muscle and liver enzyme efflux, serum electrolytes, blood lipid profiles, red and white whole blood cell hematology, or quantitative and qualitative urinary markers [145]. In addition, this research supports previous reports from short-term studies (5 days-12 weeks) and long term retrospective studies of athletes (up to 5 years) that found no adversely effects in athletes, healthy individuals and patient populations [60, 136, 146-150].

Creatine As a Therapeutic Agent

Most Cr studies were done on primarily healthy volunteers and well-trained athletes [95,133]. There is a small number of studies relevant for specific patient populations such as elderly people [151, 152], sick children and adolescents, people with muscle dystrophy, hypercreatinemia and creatinuria with lower Cr and PCr levels [8]. There are also patients

with a deficit of endogenously produced Cr [153-155], who suffer from cerebral Cr deficiency syndromes (CCDS). Studies have shown that the inability of biosynthesis of Cr is due to congenital deficiency of the enzyme AGAT and/or GMAT [156] as well as deficiency in transport of Cr through cell membranes through CrT [157, 158]. These specific patient populations were subjected to CrS in combination with dietary restrictions and/or additional interventions. Benefits of CrS have been reported overall. CrS provides therapeutic benefit for patients with metabolic disorders (myophosphorylase deficiency) [159], GAMT deficency [160], neuromuscular diseases [161,162], during recovery following immobilization [163]. A confirmation that Cr and CK are of vital importance in normal brain development was obtained from Cr deficient patients [153, 164]. Those patients suffer from severe developmental disabilities with language delay, extrapyramidal syndrome, behavioral disorders and epileptic seizures [153, 154, 165]. If Cr treatment starts early, it could prevent the development of clinical symptoms [153].

Brain has become an increasingly popular tissue with respect to Cr disposition in the investigation of neurological disease. To date there is a little evidence available concerning brain Cr uptake and saturation. Several studies have found low brain Cr levels in patients with Parkinson's disease [166] and Huntington's disease [167]. These clinically relevant discoveries support the importance of the CK system and CrS for normal physiological function of the human body [168-170] and brain disorders [171], thus offering a new perspective on Cr/PCr system.

Physicochemical Properties of Creatine Monohydrate

Creatine crystallizes as monoclinic prisms [172, 173] with one molecule of water. By using X-ray crystallograhpy, Kato et al. [174] have determined that molecules of Cr exist in the zwitterionic form [175] (Figure 5). The carboxyl group is found in the deprotonated (anionic) form while the guanidine group is in the protonated (cationic) form [174].

These two groups interact electrostatically through strong *Coulomb* interactions, but formation of hydrogen bonds also takes place (Figure 5). Strong electrostatic interactions between the molecules of Cr are the reason for its low solubility in polar solvents such as water (17 g/l at 25^{o} C), while in non-polar solvents, CrMH is practically insoluble. When CrMH is heated at temperatures above 102^{o} C, water evaporates while Cr is converted in its anhydrous form [176]. Water solution of CrMH is practically pH-neutral (between 7.0 and 7.4 depending on concentration), while the acid dissociation constants are: $pK_1 = 2.79$ and $pK_2 = 12.1$ [177]. Isoelectric point (pI) is the arithmetic mean of acid dissociations constants, and its value is 7.4.

Depending on pH of the solution, Cr can take different forms. When pH is lower than 2.79 it is found primarily in the cationic form (Figure 6) while at pH above 12.1 it takes the anionic form. Zwitterionc form is present at all other pH values. Solvent pH and subsequently the form in which Cr is found in the solution are the main determinants of its physico-chemical characteristics (e.g., solubility, stability etc.) as well as its biological characteristics such as bioavailability, permeability etc.

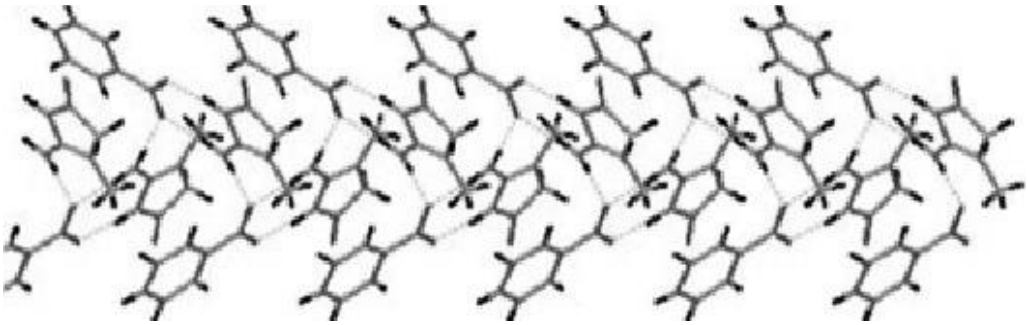


Figure 5. Hydrogen bonds between Cr molecules in crystal of CrMH (adapted from [175]).

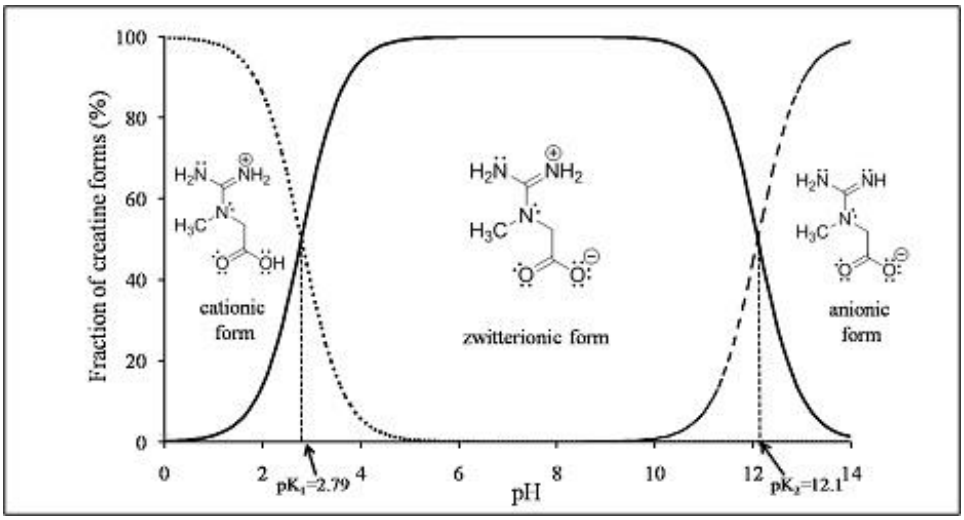


Figure 6. Fraction of Cr forms at various pH values.

Solubility of Cr Monohydrate

The biggest deficiency of CrMH is its low water solubility. It is also the reason for synthesis of various forms of Cr that are more soluble (e.g., salts, complexes etc.). Water solubility of Cr increase with temperature in a linear manner. One liter of water dissolves 6 g of Cr at 4^{o} C, 14 g at 20^{o} C, 17 g at 25^{o} C, 34 g at 50^{o} C, and 45 g at 60^{o} C. High water intake that is necessary for solubility can lead to water retention and gastrointestinal discomfort.

Low solubility of Cr is the consequence of its pH-dependent structure. In the pH range between 4.79 (pK_1+2) and 10.1 (pK_2-2) as much as 99% of the molecules are zwitterionic (Figure 6) where the nitrogen atom from the guanidine functional group is positively charged while the oxygen atom from the carboxyl group is negatively charged. This results in strong electrostatic attraction between these two oppositely charged groups casing aggregation of the molecules, as independent Cr molecules cannot be solvatized by water further causing low solubility. Saturated water solution of CrMH has pH = 7.4 because as its concentration increases, the pH of the solution is getting close to the pI of the zwitterion. However, at that

exact pH value the percentage of zwitterionic form is at maximum, hence its solubility is minimal.

Lowering the pH of Cr solution to values below 4.79 (by adding some acid or buffer) protonation of carboxyl group takes place, and the zwitterionic form turns into the cationic form (creatinium ion) (Figure 6). As these are positively charged ions, they repel each other leading to higher solubility. This is in line with previously reported experimental results [178], where solubility of Cr is being tested in the pH range from 1 to 9 at 25^{o} C.

Data shows that solubility of CrMH in the pH = 4 to pH = 9 range (dominant zwitterionic form) is low (around 16 g/L) while after lowering pH below 4 it starts to increase abruptly (at pH = 1, it is ~ 52 g/l). This practically means that if all CrMH is solubilized and ingested, there would be no concern as to whether the acidic environment of the stomach would lower its solubility and bioavailability. This is the exact reason for synthesizing numerous commercial forms of Cr containing inorganic and organic acids as solubilizing these forms would ensure much lower pH as opposed to CrMH alone thus increasing its solubility as it will be discussed later in further detail. Also, increasing pH of the solution to values above 10.1 leads to the formation of anionic structures of Cr (creatinate ion) and increase of solubility. Lastly, it is important to mention that in the presence of other molecules and ions certain percentage of molecules of water will be engaged in solvation of these molecules leaving less water molecules available for solvation of Cr causing decrease in solubility. This phenomenon is called "salting out" effect. For this reason preparing Cr solutions together with say amino acid supplements or carbohydrates should be avoided, as they will lead to decrease in solubility of CrMH.

Stability of Cr Monohydrate

Stability in Solid Forms

CrMH powder is very stable for long periods of time, even at higher temperatures [55]. Kept at 60^{o} C for 44 months leads to decomposition of only 0.0106% of molecules. At temperatures of 100^{o} C dehydration on one molecule of water takes place, while at temperatures above, 230° C one more water molecule is lost leading to formation of Crn [176]. This is an extremely important piece of information because after cooking meat at temperatures above 230^{o} C, the option of delivering Cr this way is completely lost.

Stability in Solutions

Unlike in solid state, Cr is not stable in aqueous solution due to non-enzymatic intra-molecular cyclization to Crn [179] (Figure 7). The velocity of Cr degradation is not dependent on its concentration, but it depends on pH and temperature.

In the pH range between 2.79 and 12.1 the largest percentage of Cr is found in it zwitterionic form that is still balanced with small concentration of neutral molecule (Figure 7, (a)). Although concentration of the neutral molecule in the solution in negligible in comparison to the concentration of zwitterion structure [180] it is thought that this form of Cr plays a crucial role in cyclization to Crn [179].

This is a two-step reaction: first, there is a nucleophilic attack of the free electron pair from the nitrogen atom in the guanidine functional group onto the electrophilic carbon from

carboxyl group (Figure 7, step 1) leading to the formation of cyclical transient state. This reaction phase does not take place in very acidic conditions (pH<2.5 [55]), because the protonated atom of nitrogen possesses no nucleophilic properties (Figure 7, (b)), therefore making Cr very stable in highly acidic conditions such as stomach environment [59]. On the other hand, this phase of the reaction is greatly reduced in highly alkaline environments (Figure 7, (c)) because the deprotonated carboxyl group has less pronounced electrophilic properties. The next phase of the reaction, starts with proton attachment to the OH-group from the transient cyclical intermediate leading to formation of one molecule of water and formation of Crn. Since this phase requires free H^+ ions, it practically does not happen in alkaline conditions. It means, that even if certain amount of cyclical intermediate is formed in the first phase of the reaction, its further conversion to Crn is blocked, thus making Cr more stable in alkaline conditions. For this very reason, many buffered Cr products have appeared on the market as it will be discussed in further details later.

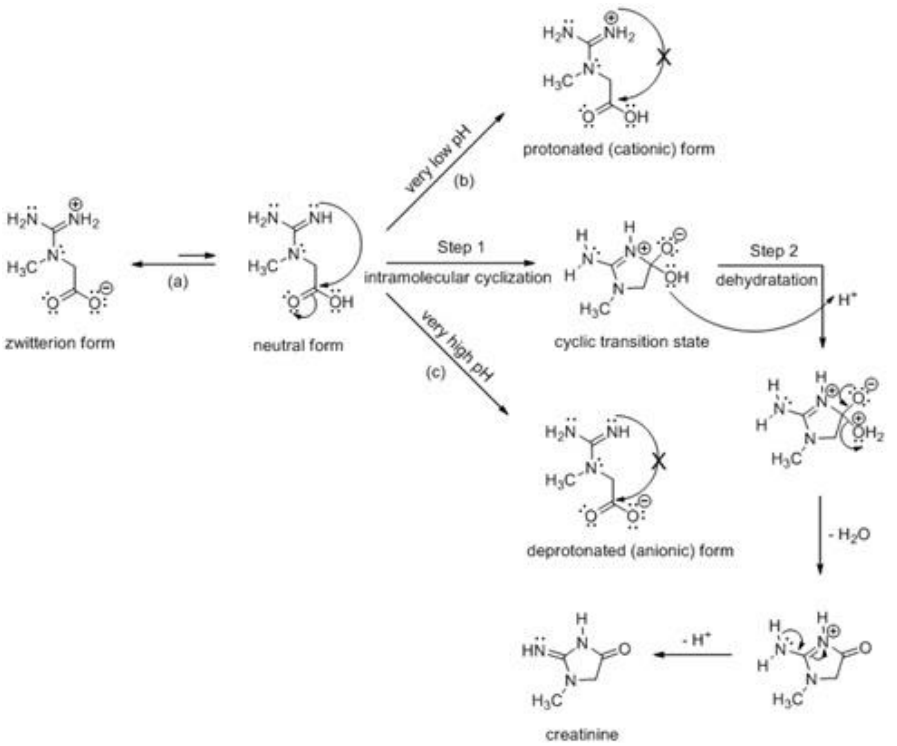


Figure 7. Mechanism of Cr cyclization to Crn (adapted from [179]).

Now, we could ask the question: "At what pH is the conversion of Cr into Crn the fastest?" The first papers that dealt with this problem had appeared at the beginning of the last century [52, 53] while more recent research is also available [179, 181, 182]. These results lead to the conclusion that the rate of transformation of Cr into Crn is the fastest at pH = 3.4 (Figure 8) while both the increase and the decrease in pH abruptly slow down the speed of cyclization.

However, one could ask the following question:" What percentage of Cr is lost due to the conversion in Crn at that pH in certain time frame under specific reaction conditions?"

Howard and Harris [181] have demonstrated that at pH = 3.5 and 3 days at 25° C, about 21% of Cr is being converted while in the first 8 hours of the reaction that percentage is negligible.

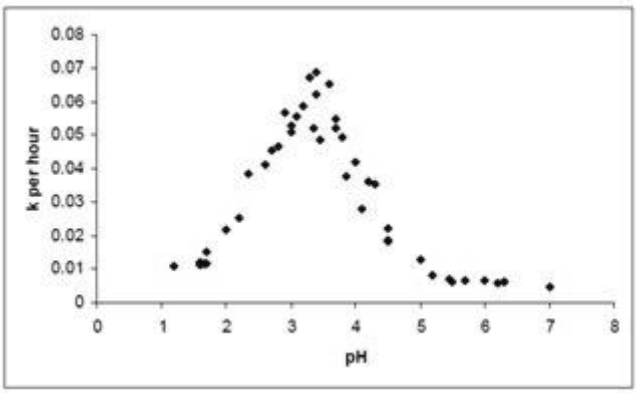


Figure 8. Cyclization rate of Cr into Crn versus pH (According to [182]).

In solutions with neutral pH (6.5-7.5) Cr decomposition is negligible even after three days. As water solution of CrMH has pH = 7.4, we can predict that the solution will certainly be stable for a longer period of time. Another factor that affects stability of water solution of Cr is temperature. In general, as temperature gets lower, stability of Cr increases, or in other words, its conversion rate to Crn is slower [61, 179, 181]. Figure 9 shows that the percentage of decomposed Cr at 4° C after one months is very small even at pH = 3.5. This is in line with the results reported by Ganguly et al. [61] who came to the conclusion that decomposition of Cr is much slower when kept in a refrigerator, therefore this mode of preservation is recommended when Cr solution is not consumed right after preparation.

Table 2. pH of the often used solutions

Product	pH value
Coca-Cola	2.52
Sprite	3.29
Orange juice	2.90
Red Bull	3.3
Milk	6.7
Yoghurt	3.9-4.5
Fruit yoghurt	4.5
Fruit cocktail	3.6-4.0
Ice tea	3.86
Grapefruit juice	3.0-3.7
Diet Cola	3.39
Carbonated water	3-4

Likewise, dissolving Cr in soft energy drinks, fruit juices, carbonated water etc., should be avoided because these products are usually acidic therefore leading to faster loss of Cr (Table 2). It is important to mention that besides the effects of pH and temperature, other

factors that affect Cr stability have been investigated. Uzzan et al. [183] tested water activity on stability of Cr at various temperatures, while adjusting water activity by mixing it in different proportions with glycerol. They came to the conclusion that in solutions with reduced water activity (solutions with lower percentage of water), stability of Cr is higher, especially at lower temperatures.

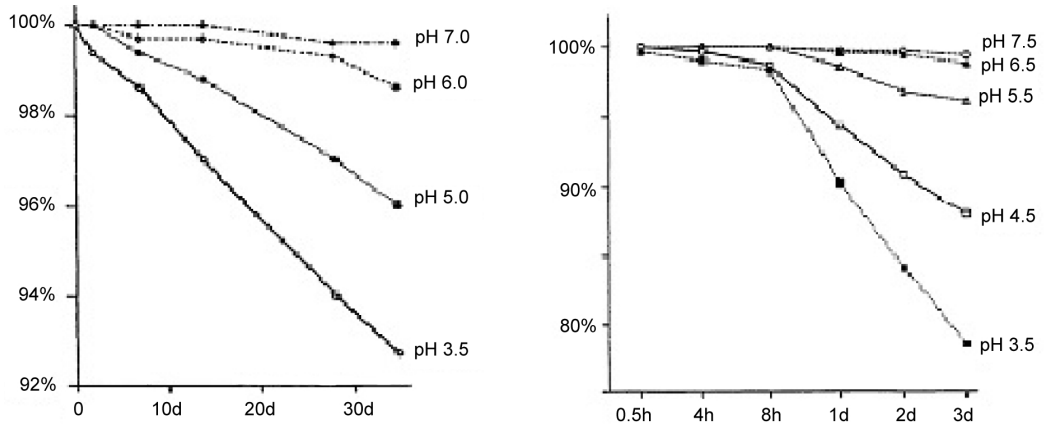


Figure 9. Effect of pH on Cr stability in solution at 25°C (left) and 4°C (right) (Adapted from [181]).

It means that by adding certain substances such as glucose or protein supplements it is possible to reduce water activity as one percentage of water molecules will be bound to these molecules in the process called solvation. Although this method might increase stability of water solution of Cr, it is not recommended because addition of other substances can decrease its solubility in water.

Novel Forms of Creatine

Cr Ethyl Ester

Esterification is a common procedure used for reducing polarity and increasing solubility and bioavailability of pharmaceutical products. Ethyl ester of Cr is formed through esterification reaction between carboxyl group from the Cr molecule and ethyl alcohol in the presence of hydrochloric acid. The product is a stable crystalline form called creatine ethyl ester hydrochloride (CEE·HCl) [184] (Figure 10).

Newly formed ethyl-ester Cr should have two huge advantages over Cr alone. First, the electrostatic attraction between molecules of CEE is far weaker because this molecule cannot exist in the form of a bipolar zwitterion that increases water solubility. Its solubility is further increased by synthesizing commercial forms such as hydrochloride salts (see chapter Creatine salts) making CEE·HCl about 13 times more soluble than CrMH [184].

Second, converting the carboxyl group to a more lipophilic ester reduces polarity in comparison to Cr which is something that should make transport by passive diffusion from GI into blood and blood into muscle cells possible, independently from the Cr transport

molecules. These two advantages over Cr should significantly increase bioavailability of Cr and therefore its ergogenic effects in comparison to CrMH.

Although results have shown that permeability of CEE molecules throughout lipophilic membranes is significantly higher in comparison to molecules of Cr [185], research results based on patients with CrT deficiency in brain cells have shown that one-year supplementation did not increase Cr levels in the brain [100] and that CEE is not lipophilic enough after all in order to be transported through cell membrane by passive diffusion [99].

However, a much bigger deficiency of CEE is its low stability under biological conditions. Stability of CEE is significantly reduced as pH of the environment gets higher [185,186], during which time the half-life at pH=1 is about 22 days, while at pH=7.4 (blood pH) half-life is only 1 minute. This means that CEE is stable in acidic environment such as stomach conditions, while in blood, 99% of CEE is decomposed within 7 minutes [186], making the amount of Cr that finally reaches the muscles very low. Giese and Lecher used 1H NMR to show that molecules of CEE in plasma do not hydrolyze into Cr and ethanol by the action of the enzyme esterase, as it was originally thought [187], but rather gets converted into inactive Crn [188]. This reaction is spontaneous and happens even without the enzyme esterase. The aforementioned results indicate that under physiological conditions almost all CEE is converted to Crn. This has been confirmed by the increase of Crn in plasma upon consumption of CEE–based supplements [189, 190]. The effect of CEE on body composition and sport performance has been reported by Spillane et al. [190]. The researchers randomly assigned in a double-blind manner 30 male resistance-trained athletes to ingest 0.30 g/kg per day fat-free mass (about 20 g/day) of either a placebo, CrMH, or CEE for 42 days. The authors reported that Cr ethyl-ester did not show any benefit in regards to muscle mass increase in comparison to CrMH or maltodextose placebo. Other parameters such as total body mass, fat mass, fat-free mass and thigh muscle mass did not increase in the group taking CEE when compared to the control groups. The CEE group showed a large increase in serum Crn levels while the levels of Cr in serum and muscles did not increase. These results can be explained by degradation of CEE in the GI tract.

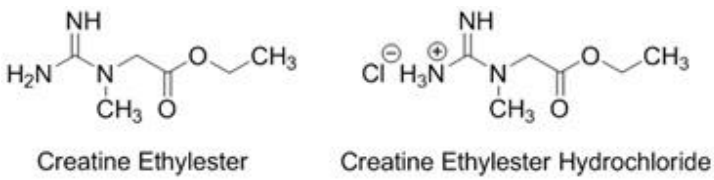


Figure 10. Chemical structure of Cr ethyl ester (CEE) and Cr ethyl ester hydrochloride (CEE·HCl).

Cr Chelate

A large number of organic and biologically active molecules have atoms with the free electron pair (nitrogen, sulfur, oxygen) that can be used to form bonds with other molecules. Those molecules with electron donor atoms are called ligands and they can form complexes with metals ions. When a ligand has multiple electron donor atoms or functional groups that can form multiple bonds with metal ions, they are called polydentate ligads. Complexes that

are formed this way are called chelates. Chelate complexes are much more stable of those formed though only one bond (monodentate ligands).

As it has already been mentioned, a molecule of Cr is found in zwitterionic form in a wide range of pH values and therefore, has multiple electron-donor groups such as negatively charged carboxyl group and the free electron pair on the nitrogen atoms. The negatively charged atom of oxygen from the carboxylic group forms ionic bond with the metal ion, while the nitrogen atom participates in formation of the weaker coordination-covalent bond (Figure 11). The formation of ionic bond between the metal ion and the oxygen from the carboxyl group results in charge neutralization. With the loss of negative charge in the molecule of Cr, its zwitterionic structure is disrupted therefore reducing the electrostatic attraction between the molecules. This results in increase of water solubility. On the other hand, the loss of charge on the Mg^{2+}-ion makes its absorption through the GI tract optimal. Also, high stability of chelates would *protect* molecules of Cr from its conversion into Crn in the acidic stomach environment [191]. Even though all this sounds quite promising, the track record of magnesium chelate with Cr was not very convincing. There are several problems that do not back up the claims of the manufacturers who claim that this formula is better than CrMH. First, the structure of chelate of Mg^{2+}-ion with Cr is not known. It is not even known with how many molecules of Cr does Mg form the complex therefore making the *recipe* for preparation of these forms of complexes questionable. However, a far greater problem is the stability of these chelates. As the stability constant for Mg-Cr has not been determined experimentally, we can only assume its value. It is known that Mg^{2+}-ion forms complexes of low stability even with much more efficient chelate ligands such as ethylen-ediaminetetraacetic acid (EDTA) (Figure 11). Molecules of EDTA have the same kind of electron donor groups as Cr, but unlike Cr that can form only two bonds with a metal ion, the molecules of EDTA can simultaneously form as many as six (hexadentate ligand). Based on this, we can conclude that a molecule of EDTA forms a much more stable complex with Mg^{2+}-ions when compared to Cr. As the stability constant for Mg-EDTA chelate is knows and at pH=1.5 it is $K_{st}=1.2\cdot10^{-7}$ it means that at the pH levels in the stomach this chelate would decompose quite fast. As Cr forms a far less stable chelate with Mg^{2+}-ion than it is the case with EDTA, its form in stomach environment is practically unsustainable, meaning that it decomposes instantly into Mg and Cr. The metabolic fate of the components is practically identical as in the case of ingestion of pure CrMH. The paper published by Hageböck and Bader [59] shows that the rate of conversion of Cr into Crn in GI track for both forms is very similar and negligible.

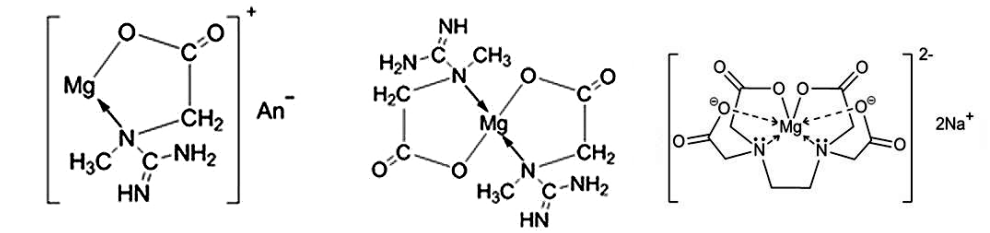


Figure 11. Structure of magnesium-Cr chelate forms (a,b) (adapted from [191]) and Mg-EDTA chelate (c).

In the end we can ask ourselves the following question: How many ions of Mg^{2+} do we ingest at the stage of Cr loading that is done with 20 g of Cr per day? One mole of Cr molecules has the mass of 131.1 grams, while one mole of Mg ions has the mass of 24.3 grams. Since in the suggested formula for the chelate the molar ratio 1:1, it would mean that 20 grams of creatine comes with 3.7 grams of Mg^{2+}- ions when ingested as chelates. This is 9 times higher than the daily-recommended dose for this element (The Recommended Daily Allowance (RDA) of magnesium for adults is 420 mg/day for men and 320 mg/day for women). When it comes to studying the effect of Cr-Mg chelate on physical performance, the number of publications is quite low.

Selsby et al. studied the effect of supplementation with Cr-Mg chelate and CrMH on the peak performance at a bench press test [192]. They have concluded that both forms have a positive effect, with no significant distinction between the two. Another study done by Brilla et al. [193] tested the effects of Cr magnesium chelate by comparing it with Cr mixed with magnesium oxide. The third group received placebo in the form of maltodextrine. The volunteers were healthy 19 to 24 year old subjects. Both groups experienced an increase in power and body water when compared to placebo, while the Cr magnesium chelate group also showed a greater increase in intracellular water [193]. The difference in p values for the peak torque was negligible as it was 0.06 in the Cr magnesium chelate group and 0.04 in the magnesium chelate group. This means that Mg chelate intake does not results in improvement of athletic performance when compared to taking the components separately. Unfortunately, the study does not include a group receiving pure CrMH.

Creatine Salts

One of the most important reasons for making salts of organic molecules is the increase in solubility [194]. It is mostly done by protonation with strong inorganic acids (such as HCl) that turn neutral organic molecules into their cationic forms and since the products are positively charged, the repulsive electrostatic interactions between them make them more soluble in water. The same principle can be used in the case of Cr. By protonation of carboxylate anion, molecules of Cr are converted from their zwitterionic form into the cationic form, a so-called creatinium ion (Figure 12). As the low water solubility of Cr is the main issue, this lead to creation of large number of its commercially available salt forms. The simplest way for synthesizing these salts with Cr it its cationic form is protonation of the carboxylate anion. However, since carboxyl group is quite acidic (pK = 2.79) it means that this group can be protonated only by stronger acids, such as those with pK < 2.79. Those are HCl (pK = -7), HNO_3 (pK = -1.4) and other strong inorganic acids. Some forms of commercial Cr such as Cr hycrochloride (Figure 12) [195], Cr nitrate [196] are made through the reaction of equimolar amounts of creation and strong acids. Water solutions of these salts have very low pH values that allow the molecule of Cr to be found in the cationic form and therefore the solubility is much higher when compared to CrMH. The best example is Cr-hydrochloride. Its saturated solution has pH = 0.3. At that pH value all the molecules of Cr are in the cationic form that is very soluble. At 25^{o} C, the solubility is a high as 709 mg/ml. This is 40 times more than the solubility of CrMH at the same temperature [178].

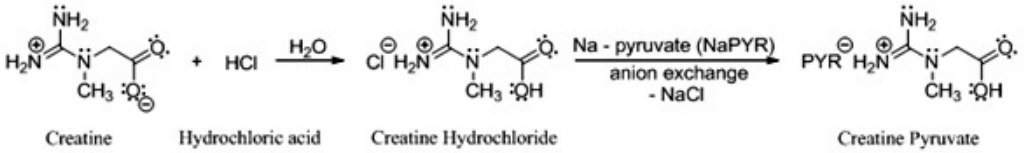


Figure 12. Scheme of Cr hydochloride and Cr pyruvate synthesis.

Besides the salts with strong inorganic acids, there are numerous commercially available forms of Cr salts with weak organic acids. Most frequently those are intermediates in the process of energy extraction from the molecules of glucose whether it is glycolysis (such as pyruvate) or in åå cycle (citrate, malate, fumarate, oxalate and α-ketoglutarate). The goal of synthesizing these compounds is creating forms that have synergistic effect of both cation and anion, and thus additionally increasing net energy in the body of an athlete as well as improving performance. However these acids are too weak to protonate the carboxyl group on Cr, so they are mostly synthesized by reactions between Cr-hydrochloride and sodium or calcium salts of the same acids (sodium citrate, calcium pyruvate). By replacing chloride ions with corresponding anions of those acids, many commercially available forms have been created like Cr pyruvate [197], Cr-malate [198], Cr-trihydrooxycitrate [199] and others. Although some authors assume that it is possible to create Cr salts with anions of weak organic acids such as citric (pK = 3.1, 4.8, 6.4) by simple mixing off their solutions [200], NMR studies have shown that the compound that is formed is not Cr tricitrate but rather physical mixture of Cr and citric acid [55, 178]. Solubility of Cr salts with weak acids is much lower in comparison to Cr salts with strong inorganic anions, but it is still much larger than solubility of CrMH. For example, Cr pyruvate and dicreatine citrate have about 5 times larger solubility than CrMH at 25^{o} C [61, 178]. As far as the stability of water solutions of Cr salts is concerned, they are mostly dependent on pH of the solution. When pH value of the solution prepared according to the manufacturer's direction is very low, such as the case of Cr hydrochloride (pH = 2.4) and Cr pyruvate (pH = 2.6) [59], molecules of Cr are found in their cationic form (see Figure 7, (b)) and that reduces its conversion to Crn.

On the other hand, water solution of Cr-citrate has the pH value of 3.3 that is *dangerously close* to the values at which cyclization into Crn takes place (pH=3.4). However, as it was previously mentioned, the amount of Cr that gets converted into Crn is small within 8 hours (even at pH=3.4) [181], so if Cr is consumed within reasonable period of time, there is no concern as whether it would be lost.

This is in line with studies that have shown that the amount of Crn appearing in the GI tract during the period of 8 hours whether it be from Cr or its salts practically negligible [59]. When it comes to permeability of Cr salts through cell membrane, it is relatively low, frequently even lower than permeability of Cr alone [178]. On the other hand, by measuring the concentration of Cr in plasma after intake in the form of CrMH, Cr pyruvate and tricreatine citrate (all doses contained 4.4 grams of Cr) it was shown that the maximum concentration is achieved after 1 hour, and that it diminishes in the following order pyruvate>citrate>monohydrate [201]. Higher concentrations of Cr salts in plasma when compared to CrMH can be the consequence of poor absorption of these forms in the muscle but also due to the change in pH of the plasma because of the presence of pyruvate and citrate as well as their effect on insulin. The only way to confirm the true bioavailability of

commercially available forms of Cr is to determine the levels in the muscle before and after Cr consumption. However this has not been investigated in the studies so far.

The effect of Cr salts on performance was studied with Cr-pyruvate and Cr-citrate. Some studies have shown that supplementation with pyruvate and citrate can lead to body weight reduction and fat mass reduction in overweight individuals [202], and also improves athletic performance [203, 204]. Therefore, the assumption is that these salts would have the most profound effect. However, the results showing the effect of these two forms of Cr on athletic performance are not always in accord. Two studies investigating the same outcome (endurance after short-term supplementation with Cr-Pyr) showed conflicting results. One study was investigating the effects of 7 g per day of Cr-Py for 7 days in the subjects who were all well-trained cyclists [205] and found that it did not beneficially affect the subjects. The other study performed for 5 days with 7.5 g of Cr-Py per day showed that canoeists had increased paddling speed and decreased concentrations of lactate. This suggested possible ergogenic effect of Cr-Py for aerobic performance [206]. Studies focused on Cr-citrate imply that high-doses of short-term supplementation can increase anaerobic performance in women [207] and delay neuromuscular fatigue during cycle ergometry [208]. Another double-blind study with placebo investigated the effects of Cr pyruvate, tricreatine citrate and placebo by measuring endurance capacity and handgrip strength. Daily doses of 5 g/day of Cr-Pyr or Cr-Cit (approximately 3 g of Cr) were designed to slowly load muscles with Cr. The results showed that the supplements increased mean power [209]. However it is important to mention that in none of the aforementioned studies there was a comparison with CrMH. One of the rare studies with such comparison concluded that the combination of CrMH and calcium pyruvate did not show improvement in athletic performance of college football players as compared to CrMH alone [210]. From all this we can conclude that the biggest advantage of Cr salts in comparison to CrMH is their superior solubility and therefore simpler preparation. However, after consumption, molecules of Cr regardless of whether they were ingested as salts or CrMH, are transformed into the cationic form in the stomach, so the only advantage of Cr salt over CrMH can be derived from the anions only, although this has not been confirmed in the studies so far. It is necessary to mention that the effect of Cr salts with inorganic anions (such as chloride or nitrate) on athletic performance has not been the subject of scientific studies.

Buffered Cr

Higher stability of Cr under alkaline conditions made the manufacturers of sport supplements to create a so called *buffered* or *pH-correct* form of Cr under the brand name of Kre-Alcalyn® [KA] [211]. Its production is based on the patent of Jeffrey M. Golini [191] and in essence it is the combination of alkaline powders (such as sodium carbonate) and CrMH. This combination increases the pH and therefore stability, so the latest formulations of KA, as the manufacturer claims, have pH that is around 12 [212].

However, data shown in the patent [191] that refer to stability of CrMH in acidic environment are not in line with the data obtained by other authors [59, 178, 179, 182]. The patent claims that the speed of conversion of Cr into Crn increases exponentially when pH is reaching zero, while numerous other results indicate high stability of Cr at under highly acidic conditions. Also, in the patent there is a clam that 1 gram of Cr dissolved in 1 liter of water is

being converted to Crn within 43 minutes that is also not in line with the results in previous publications that show high Cr stability at that pH value [59, 178]. Water solution of CrMH has pH of about 7.4 at which Cr is very stable (only 1% gets degraded in 30 days, Figure 9) so after increasing pH to over 12 there is probably no further increase in the stability. On the other hand, when it arrives in the stomach, small amount of the base that was added to KA in order to increase the pH cannot change the pH in the stomach, so the stability of the formulation is very similar as stability of CrMH [59]. According to the manufacturer, it is enough to take 2 capsules per day (1.5 g total) and there is no need for the loading phase. It means that this daily amount can replace 20 g of CrMH in the loading and 5 g in the maintenance phase, also meaning that according to the manufacturer, KA is about 10 times more efficient than CrMH. In order to confirm these claims, Jagim et al. [213] compared the results of muscle Cr content, body composition and training adaptations based on three groups of healthy resistance-trained males. The first group of participants received KA according to manufacturer guidelinesß (1.5 g/d for 28-days), while the second group took KA at Cr equivalent loading (4 x 5 g/d for 7-days) and maintenance doses (5 g/d for 21-days) as CrMH. Finally the third group was supplemented with normal loading (4 x 5 g/d for 7-days) and maintenance doses (5 g/d for 21-days) of CrMH. The conclusion of this study was that the KA brand Cr did not promote greater increase in muscle Cr content when compared to CrMH.

The Future of Creatine

There is no doubt that Cr will be the most important energy enhancing sport supplement for a long time. For that reason, the pharmaceutical industry will continue with production of novel forms of Cr with the final goal of making its use most comfortable through increasing its potency. With the final goal of increasing solubility of Cr, the future will certainly bring attempts of synthesizing Cr in the form of hydrophilic room temperature ionic liquids (RTIL) [214, 215]. Ionic liquids are ionic compounds that are mostly composed of a large asymmetrical organic cation and inorganic (or organic) anion that due to their dimensions are unable to form a perfect crystal lattice, therefore they stay liquids at room temperature [216-222]. As Cr can be both cation (creatinium ion) and anion (creatinate ion), there is a possibility to synthesize an ionic liquid. Of course, it is necessary to find the corresponding counter ion which is not easy as it needs to be non-toxic at large concentrations, have large biological availability, unobstructed GI resorption and in the best case demonstrate some form of synergistic effect when combined with Cr. The advantages of ionic liquid would be complete solubility and also the possibility of consumption without making a solution first [221]. This would mean that only a few drops of ionic liquid based on Cr could replace the lengthy procedure of dissolving CrMH before consumption. On the other hand, there are increasing number of studies showing that deficiency of Cr in the brain [171] and other organs can lead to numerous diseases such as CCDS [153], developmental disabilities with language delay [155], Huntington's [158] and Parkinson's disease [157]. Above diseases might be at least partly due to the inability of Cr transport through the usual pathways. For that reason further research will be directed toward finding lipophilic forms of Cr such as

dodecyl Cr ester [169, 170] that will allow transport of Cr through cells by the means of passive diffusion, a process where CrT is not needed.

Conclusion

Bioavailability of CrMH is almost 100% and at first sight it seems that there is not need for creation of new forms of Cr. However, the fact that the new formulas appear on the markat al.most daily, raise the following question: *Why those new formulas are better than CrMH*? One of the biggest problems of CrMH, low solubility in water, has been successfully solved by the synthesis of the novel products in the forms of chelates and ions with metals. The salts with inorganic acids such as Cr chloride and Cr nitrate have shown a particularly good solubility, but so far there has not been a single study showing their effect on athletic performance. On the other hand, research based on Cr salts like biologically active anions, such as citrate and pyruvate, have not shown that their synergistic effect with Cr is superior to CrMH. Creatine chelates with Mg^{2+}-ions have shown better solubility in water in comparison to CrMH but their stability under physiological conditions is under question, and requires further studies and better physico-chemical characterization. For now, there is no scientific proof that by using Cr in the form of Mg-chelate there is any improvement in performance when compared to CrMH.

The second big problem of CrMH is its instability in water solutions as it has the tendency for cyclization in its biologically inactive form Crn. Formulations of Cr with alkaline compounds, a so called *'buffered Cr* are designed to increase pH values of the solution and prevent the cyclization reaction. However, recent studies have shown that water solution of CrMH is particularly stable and that within 30 days there is less than 1% loss of Cr. Also, alkaline forms did not demonstrate higher stability under physiological conditions nor better results when it comes to body composition or training adaptations in comparison to CrMH.

The third and maybe the biggest problem that CrMH is facing (along with other forms of Cr) is its transport into the cells through the specific transporters that have limited capacity. Synthesis of more lipophilic forms of Cr such as esters could make passive transport possible and therefore form large deposits of Cr in the cells. So far there has been only one commercially available formulation of Cr with increased lipophilic properties. This form is Cr ethyl ester. However, this molecule turned out to be insufficiently lipophilic for passive transport through cell membrane, and also has a pronounced tendency for cyclization into Crn therefore exerting a much lower effect than CrMH.

From everything that has been said so far, studying the effects of the new forms of Cr such as salt, chelates, esters and alkaline-buffered forms have not shown better results in body composition, strength, endurance and training adaptations in comparison to CrMH.

Acknowledgments

No conflicts of interest apply in this work. This study was financially supported by the Ministry of Education, Science and Technological Development of Serbia (No. ON172012),

and the Provincial Secretariat for Science and Technological Development of APV. The authors would like to thank Dr. Sergej Ostojic on great collaboration and the chance to make contribution to this book.

References

[1] Chevreul, E. Sur la composition chimique du bouillon de viandes. *J Pharm Sci Access*., 1835, 21, 231-42.

[2] Fiske, CH; SubbaRow, Y. The nature of the 'inorganic phosphate' in voluntary muscle. *Science*, 1927, 65(1686), 401-3.

[3] Eggleton, P; Eggleton, GP. The inorganic phopshate and a labile form of organic phosphate in the gastrocnemius of the frog. *Biochem J*., 1927, 21(1), 190-5.

[4] Lundsgaard, E. Weitere Untersuchungen über Muskelkontraktionen ohne Milchsäurebildung. *Biochem Z*., 1930, 227, 51-83.

[5] Lohmann, K. Über die enzymatische Aufspaltung der Kreatinphosphorsäure: zugleich ein Beitrag zum Chemismus der Muskelkontraktion. *Biochem Z*., 1934, 271, 264-77.

[6] Bessman, SP; Geiger, PJ. Transport of energy in muscle: the phosphorylcreatine shuttle. *Science* 1981, 211(4481), 448-52.

[7] Tombes, RM; Shapiro, BM. Metabolite channeling: a phosphorylcreatine shuttle to mediate high energy phosphate transport between sperm mitochondrion and tail. *Cell* 1985, 41(1), 325-34.

[8] Fitch, CD; Lucy, DD; Bornhofen, JH; Dalrymple, GV. Creatine metabolism in skeletal muscle: II. creatine kinetics in man. *Neurology* 1968, 18(1 Pt 1), 32-42.

[9] Fitch, CD; Shields, RP; Payne, WF; Dacus, JM. Creatine metabolism in skeletal muscle. 3. Specificity of the creatine entry process. *J Biol Chem*., 1968, 243(8), 2024-7.

[10] Borsook, H; Dubnoff, JW. The formation of glycocyamine in animal tissues. *J Biol Chem*., 1941, 138, 389-403.

[11] Carlson, M; Van, Pilsum; JF. S-adenosylmethionine: guanidinoacetate N-methyltransferase activities in livers from rats with hormonal deficiencies or excesses. *Proc Soc Exp Biol Med*., 1973, 143(4), 1256-9.

[12] McGuire, DM; Gross, MD; Van, Pilsum, JF; Towle, HC. Repression of rat kidney L-arginine: glycine amidinotransferase synthesis by creatine at a pretranslational level. *J Biol Chem*., 1984, 259(19), 12034-8.

[13] McGuire, DM; Gross, MD; Elde, RP; van, Pilsum, JF. Localization of L-arginine-glycine amidinotransferase protein in rat tissues by immunofluorescence microscopy. *J Histochem Cytochem*., 1986, 34(4), 429-35.

[14] Wyss, M; Kaddurah-Daouk, R. Creatine and creatinine metabolism. *Physiol Rev*., 2000, 80(3), 1107-213.

[15] Snow, RJ; Murphy, RM. Creatine and the creatine transporter: a review. *Mol Cell Biochem*., 2001, 224(1-2), 169-81.

[16] Daly MM, Seifter S. Uptake of creatine by cultured cells. *Arch Biochem Biophys*., 1980, 203(1), 317-24.

[17] Dai, WX; Vinnakota, S; Qian, XJ; Kunze, DL; Sarkar, HK. Molecular characterization of the human CRT-1 creatine transporter expressed in Xenopus oocytes. *Arch Biochem Biophys*., 1999, 361(1), 75-84.

[18] Loike, JD; Somes, M; Silverstein, SC. Creatine uptake, metabolism, and efflux in human monocytes and macrophages. *Am J Physiol*., 1986, 251(1 Pt 1), C128-35.

[19] Loike, JD; Zalutsky, DL; Kaback, E; Miranda, AF; Silverstein, SC. Extracellular creatine regulates creatine transport in rat and human muscle cells. *Proc Natl Acad Sci USA*., 1988, 85(3), 807-11.

[20] Odoom, JE; Kemp, GJ; Radda, GK. The regulation of total creatine content in a myoblast cell line. *Mol Cell Biochem*., 1996, 158(2), 179-88.

[21] Guimbal, C; Kilimann, MW. A $Na^{(+)}$-dependent creatine transporter in rabbit brain, muscle, heart, and kidney. cDNA cloning and functional expression. *J Biol Chem*., 1993, 268(12), 8418-21.

[22] Walker, J. Creatine: biosynthesis, regulation, and function. *Adv Enzymol Relat Areas Mol Biol*., 1979, 50, 117-242.

[23] Balsom, PD; Soderlund, K; Ekblom, B. Creatine in humans with special reference to creatine supplementation. *Sports Med*., 1994, 18(4), 268-80.

[24] Clark, IF; Field, ML; Ventura-Clapier, R. An introduction to the cellular creatine kinase system in contractile tissue. In: Conway MA, Clark F, cds. Creatine and Creatine Phosphate: Scientific and Clinical Perspectives. San Diego, Academic Press, 1996, 51-64.

[25] Wallimann, T; Wyss, M; Brdiczka, D; Nicolay, K; Eppenberger, HM. Intracellular compartmentation, structure and function of creatine kinase isoenzymes in tissues with high and fluctuating energy demands: the 'phosphocreatine circuit' for cellular energy homeostasis. *Biochem J*., 1992, 281(Pt 1), 21-40.

[26] Wallimann, T; Tokarska-Schlattner, M; Neumann, D; Epand, RM; Epand, RF; Andres, RH; Widmer, HR; Hornemann, T; Saks, VA; Agarkova, I; Schlattner, U. The phospho-creatine circuit: molecular and cellular physiology of creatine kinases, sensitivity to free radicals and enhancement by creatine supplementation. In: Molecular Systems Bioenergetics: Energy for life, Basic Principles, Organization and Dynamics of Cellular Energetics, Saks, VA, ed, Wiley-VCH, Weinheim, Germany., 2007, 195-264.

[27] Quistorff, B; Johansen, L; Sahlin, K. Absence of phosphocreatine resynthesis in human calf muscle during ischaemic recovery. *Biochem J*., 1993, 291(Pt 3), 681-6.

[28] Rawson, ES; Stec, MJ; Frederickson, SJ; Miles, MP. Low-dose creatine supplementation enhances fatigue resistance in the absence of weight gain. *Nutrition*, 2011, 27(4), 451-5.

[29] Greenhaff, PL; Bodin, K; Soderlund, K; Hultman, E. Effect of oral creatine supplementation on skeletal muscle phosphocreatine resynthesis. *Am J Physiol*., 1994, 266(5 Pt 1), E725-30.

[30] Yagi, K; Mase, R. Coupled reaction of creatine kinase and myosin A-adenosine triphosphatase. *J Biol Chem*., 1962, 237, 397–403.

[31] Eppenberger, ME; Eppenberger, HM; Kaplan, NO. Evolution of creatine kinase. *Nature* 1967, 214(5085), 239-41.

[32] Dawson, DM; Eppenberger, HM; Kaplan, NO. The comparative enzymology of creatine kinases. II. Physical and chemical properties. *J Biol Chem.*, 1967, 242(2), 210-7.

[33] Eppenberger, HM; Dawson, DM; Kaplan, NO. The comparative enzymology of creatine kinases. I. Isolation and characterization from chicken and rabbit tissues. *J Biol Chem.*, 1967, 242(2), 204-9.

[34] Jacobs, H; Heldt, HW; Klingenberg, M. High activity of creatine kinase in mitochondria from muscle and brain and evidence for a separate mitochondrial isoenzyme of creatine kinase. *Biochem Biophys Res Commun*., 1964, 16(6), 516-521.

[35] Saks, VA; Rosenshtraukh, LV; Smirnov, VN; Chazov, EI. Role of creatine phosphokinase in cellular function and metabolism. *Can J Physiol Pharmacol*., 1978, 56(5), 691-706.

[36] Clark, IF. Creatine and phosphocreatine: a review of their usc in exercise and sport. *J Athl Train.*, 1997, 32(1), 45-50.

[37] Ma, TM; Friedman, DL; Roberts, R. Creatine phosphate shuttle pathway in tissues with dynamic energy demand. In: Conway MA, Clark IF, eds. Creatine and Creatine Phosphate: Scientific and Clinical Perspectives. San Diego: Academic Press, 1996, 17-32.

[38] van Deursen, J; Heerschap, A; Oerlemans, F; Ruitenbeek, W; Jap, P; Wieringa, B. Skeletal muscles of mice deficient in muscle creatine kinase lack burst activity. *Cell*, 1993, 74(4), 621-31.

[39] Nash, SR; Giros, B; Kingsmore, SF; Rochelle, JM; Suter, ST; Gregor, P; Seldin, MF; Caron, MG. Cloning, pharmacologi cal characterization, and genomic localization of the human creatine transporter. *Receptors Channels*, 1994, 2(2), 165-74.

[40] Peral, MJ; Gálvez, M; Soria, ML; Ilundáin, AA. Developmental decrease in rat small intestinal creatine uptake. *Mech Ageing Dev*., 2005, 126(4), 523-30.

[41] Tosco, M; Faelli, A; Sironi, C; Gastaldi, G; Orsenigo, MN. A creatine transporter is operative at the brush border level of the rat jejunal enterocyte. *J Membr Biol.*, 2004, 202(2), 85-95.

[42] Peral, M; Garcia-Delgado, M; Calonge, ML; Durán, JM; M., De, La, Horra, MC; Wallimann, T; Speer, O; Ilundáin, A. Human, rat and chicken small intestinal Na^+ - Cl^- -creatine transporter: functional, molecular characterization and localization. *J Physiol*., 2002, 545(1), 133-44.

[43] Orsenigo, MN; Faelli, A; De Biasi, S; Sironi, C; Laforenza, U; Paulmichl, M; Tosco, M. Jejunal creatine absorption, what is the role of the basolateral membrane? *J Membr Biol*., 2005, 207(3), 183-95.

[44] Persky, AM; Brazeau, GA; Hochhaus, G. Pharmacokinetics of the dietary Supplement Creatine, *Clin Pharmacokinet*., 2003, 42(6), 557-74.

[45] Möller, A; Hamprecht, B. Creatine transport in cultured cells of rat and mouse brain. *J Neurochem*., 1989, 52(2), 544-50.

[46] Gori, Z; De, Tata, V; Pollera, M; Bergamini, E. Mitochondrial myopathy in rats fed with a diet containing beta-guanidine propionic acid, an inhibitor of creatine entry in muscle cells, *Br J Exp Pathol*., 1988, 69(5), 639-50.

[47] Meyer, RA; Sweeney, HL; Kushmerick, MJ. A simple analysis of the "phosphocreatine shuttle". *Am J Physiol*., 1984, 246(5 Pt 1), C365-77.

[48] Meyer, RA; Brown, TR; Krilowicz, BL; Kushmerick, MJ. Phosphagen and intracellular pH changes during contraction of creatine-depleted rat muscle. *Am J Physiol*., 1986, 250(2 Pt 1), C264–74.

[49] Harris, RC; Nevill, M; Harris, DB; Fallowfield, JL; Bogdanis, GC; Wise, JA. Absorption of creatine supplied as a drink, in meat or in solid form. *J Sports Sci*., 2002, 20(2), 147-51.

[50] Deldicque, L; Décombaz, J; Zbinden, Foncea, H; Vuichoud, J; Poortmans, JR; Francaux, M. Kinetics of creatine ingested as a food ingredient. *Eur J Appl Physiol*., 2008, 102(2), 133-43.

[51] Borsook, H; Dubnoff, J. The hydrolysis of phosphocreatine and the origin of urinary creatinine. *J Biol Chem*., 1947, 168(2), 493-511.

[52] Cannan, R; Shore, A. The creatine-creatinine equilibrium: the apparent dissociation constants of creatine and creatinine. *Biochem J*., 1928, 22(4), 921-9.

[53] Edgar, G; Shiver, H. The equilibrium between creatine and creatinine, in aqueous solution: the effect of hydrogen ion. *J Am Chem Soc*., 1925, 47, 1170-88.

[54] Chanutin, A; Guy, LP. The fate of creatine when administered to man. *J Biol Chem*., 1926, 67, 29-41.

[55] Jäger, R; Purpura, M; Shao, A; Inoue, T; Kreider, RB. Analysis of the efficacy, safety, and regulatory status of novel forms of creatine. *Amino Acids*, 2011, 40(5), 1369-83.

[56] Wixom, RL; Davis, GE; Flynn, MA; Tsutakawa, RT; Hentges, DJ. Excretion of creatine and creatinine in feces of man. *Proc Soc Exp Biol Med*., 1979, 161(4), 452-7.

[57] Twort, F; Mellanby, E. On creatine-destroying Bacilli in the intestine and their isolation. *J Physiol*., 1912, 44(1-2), 43-9.

[58] Poortmans, J; Kumps, A; Duez, P; Fofonka, A,Carpentier, A; Francaux, M. Effect of oral creatine supplementation on urinary methylamine, formaldehyde, and formate. *Med Sci Sports Exerc*., 2005, 37(10), 1717-20.

[59] Hageböck, M; Stahl, U; Bader, J. Stability of creatine derivatives during simulated digestion in an in vitro model. *Food Funct*., 2014, 5(2), 359-63.

[60] Buford, TW; Kreider, RB; Stout, JR; Greenwood, M; Campbell, B; Spano, M; Ziegenfuss, T; Lopez, H; Landis, J; Antonio, J. International Society of Sports Nutrition position stand: creatine supplementation and exercise. *J Int Soc Sports Nutr*., 2007, 4, 6.

[61] Ganguly, S; Jayappa, S; Dash, AK. Evaluation of the stability of creatine in solution prepared from effervescent creatine formulations. *AAPS Pharm Sci Tech*., 2003, 4(2), E25.

[62] Vandenberghe, K; Goris, M; Van, Hecke, P; Van Leemputte, M; Vangerven, L; Hespel, P. Long-term creatine intake is beneficial to muscle performance during resistance training. *J Appl Physiol*., 1997, 83(6), 2055-63.

[63] Maganaris, CN; Maughan, RJ. Creatine supplementation enhances maximum voluntary isometric force and endurance capacity in resistance trained men. *Acta Physiol Scand*., 1998, 163(3), 279-87.

[64] Casey, A; Constantin-Teodosiu, D; Howell, S; Hultman, E; Greenhaff, PL. Creatine ingestion favorably affects performance and muscle metabolism during maximal exercise in humans. *Am J Physiol*., 1996, 271(1 Pt 1), E31-7.

[65] McCall, W; Persky, AM. Pharmacokinetics of creatine. *Subcell Biochem*., 2007, 46, 261-73.

[66] Green, AL; Hultman, E; Macdonald, IA., Sewell, DA; Greenhaff, PL. Carbohydrate ingestion augments skeletal muscle creatine accumulation during creatine supplementation in humans. *Am J Physiol*., 1996, 271(5 Pt 1), E821-6.

[67] Schedel, JM; Tanaka, H; Kiyonaga, A; Shindo, M; Schutz, Y. Acute creatine ingestion in human: Consequences on serum creatine and creatinine concentrations. *Life Sci*., 1999, 65(23), 2463-70.

[68] Menshikova, EV; Ritov, VB; Fairfull, L; Ferrell, RE; Kelley, DE; Goodpaster, BH. Effects of exercise on mitochondrial content and function in aging human skeletal muscle. *J Gerontol A Biol Sci Med Sci*., 2006, 61(6), 534-40.

[69] Harris, RC; Söderlund, K; Hultman, E. Elevation of creatine in resting and exercised muscle of normal subjects by creatine supplementation. *Clin Sci (Lond)*., 1992, 83(3), 367-74.

[70] Robinson, TM; Sewell, DA; Hultman, E; Greenhaff, PL. Role of submaximal exercise in promoting creatine and glycogen accumulation in human skeletal muscle. *J Appl Physiol*., 1999, 87(2), 598-604.

[71] Tarnopolsky, MA; MacLennan, DP. Creatine monohydrate supplementation enhances high-intensity exercise performance in males and females. *Int J Sport Nutr Exerc Metab*., 2000, 10(4), 452-63.

[72] Chilibeck, PD; Stride, D; Farthing, JP; Burke, DG. Effect of creatine ingestion after exercise on muscle thickness in males and females. *Med Sci Sports Exerc*., 2004, 36(10), 1781-8.

[73] Hoberman, HD; Sims, EA; Engstrom, WW. The effect of methyltestosterone on the rate of synthesis of creatine. *J Biol Chem*., 1948, 173(1), 111-6.

[74] Hoogwerf, BJ; Laine, DC; Greene, E. Urine C-peptide and creatinine (Jaffe method) excretion in healthy young adults on varied diets: sustained effects of varied carbohydrate, protein, and meat content. *Am J Clin Nutr*., 1986, 43(3), 350-60.

[75] Greenhaff, P. The nutritional biochemistry of creatine. *J Nutr Biochem*., 1997, 8(11), 610-8.

[76] Bemben, MG; Lamont, HS. Creatine supplementation and exercise performance: recent findings. *Sports Med*., 2005, 35(2), 107-25.

[77] Mesa, JL; Ruiz, JR; Gonzales-Gross, MM; Sainz, A; Garzon, MJ. Oral creatine supplementation and skeletal muscle metabolism in physical exercise. *Sports Med*., 2002, 32(14), 903-44.

[78] Hultman, E; Soderlund, K; Timmons, JA; Cederblad, G; Greenhaff, PL. Muscle creatine loading in men. *J Appl Physiol*., 1996, 81(1), 232-7.

[79] Snow, RJ; Murphy, RM. Factors influencing creatine loading into human skeletal muscle. *Exerc Sports Sci Rev*., 2003, 31(3), 154-8.

[80] Venderley, AM; Campbell, WW. Vegetarian diets: nutritional considerations for athletes. *Sports Med*., 2006, 36(4), 293-305.

[81] Maughan RJ. Creatine supplementation and exercise performance. *lnt J Sport Nutr*., 1995, 5(2), 94-101.

[82] Burke, DG; Chilibeck, PD; Parise, G; Candow, DG; Mahoney, D; Tarnopolsky, M. Effect of creatine and weight training on muscle creatine and performance in vegetarians. *Med Sci Sports Exerc*., 2003, 35(11), 1946-55.

[83] Bogdanis, GC; Nevill, ME; Boobis, LH; Lakomy, HK. Contribution of phosphocreatine and aerobic metabolism to energy supply during repeated sprint exercise. *J Appl Physiol*., 1996, 80(3), 876-84.

[84] Greenhaff, PL; Casey, A; Short, AH; Harris, R; Soderlund, K; Hultman, E. Influence of oral creatine supplementation of muscle torque during repeated bouts of maximal voluntary exercise in man. *Clin Sci (Lond).*, 1993, 84(5), 565-71.

[85] Balsom, PD; Söderlund, K; Sjödin, B; Ekblom, B. Skeletal muscle metabolism during short duration high-intensity exercise: influence of creatine supplementation. *Acta Physiol Scand*., 1995, 154(3), 303-10.

[86] Hespel, P; Derave, W. Ergogenic effects of creatine in sports and rehabilitation. *Subcell Biochem*., 2007, 46, 245-59.

[87] Kreider, RB; Leutholtz, BC; Greenwood, M. Creatine. In: Wolinsky I, Driskell 1, eds. Nutritional Ergogenic Aids. Boca Raton, FL: CRC Press LLC, 2004, 81-104.

[88] Williams, MH; Kreider, R; Branch, JD. Creatine: The power supplement. Champaign, IL: Human Kinetics Publishers, 1999, 252.

[89] Burke, DG; Smith-Palmer, T; Holt, LE; Head, B; Chilibeck, PD. The effect of 7 days of creatine supplementation on 24-hour urinary creatine excretion. *J Strength Cond Res*., 2001, 15(1), 59-62.

[90] Candow, DG; Chilibeck, PD; Chad, KE; Chrusch, MJ; Davison, KS; Burke, DG. Effect of ceasing creatine supplementation while maintaining resistance training in older men. *J Aging Phys Act*., 2004,12(3), 219-31.

[91] Willoughby, DS; Rosene, JM. Effects of oral creatine and resistance training on myosin heavy chain expression. *Med Sci Sports Exerc*., 2001, 33(10), 1674-81.

[92] Willoughby, DS; Rosene, JM. Effects of oral creatine and resistance training on myogenic regulatory factor expression. *Med Sci Sports Exerc*., 2003, 35(6), 923-9.

[93] Thompson, CH; Kemp, GJ; Sanderson, AL; Dixon, RM; Styles, P; Taylor, DJ; Radda, GK. Effect of creatine on aerobic and anaerobic metabolism in skeletal muscle in swimmers. *Br J Sports Med*., 1996, 30(3), 222-5.

[94] Wilder, N; Gilders, R; Hagerman, F; Deivert, RG. The Effects of a 10-week, Periodized, Off-Season Resistance-Training Program and Creatine Supplementation Among Collegiate Football Players. *J Strength Cond Res*., 2002, 16(3), 343-52.

[95] Volek, JS; Rawson, ES. Scientific basis and practical aspects of creatine supplementation for athletes. *Nutrition* 2004, 20(7-8), 609-14.

[96] Bennett, SE; Bevington, A; Walls, J. Regulation of intracellular creatine in erythrocytes and myoblasts: influence of uraemia and inhibition of Na, K-ATPase. *Cell Biochem Funct*., 1994, 12(2), 99-106.

[97] Gerber, GB; Gerber, G; Koszalaka, TR; Emmel, VM. Creatine metabolism in vitamin E deficiency in the rat. *Am J Physiol*., 1962, 202, 453-60.

[98] Greenhaff, PL. Creatine supplementation: recent developments. *Br J Sports Med*., 1996, 30(4), 276-7.

[99] Adriano, E; Garbati, P; Damonte, G; Salis, A; Armirotti, A; Balestrino, M. Searching for a therapy of creatine transporter deficiency: Some effects of creatine ethyl ester in brain slices in vitro. *Neuroscience* 2011, 199, 386-93.

[100] Fons, C; Arias, A; Sempere, A; Póo, P; Pineda, M; Mas, A; Lópes-Sala, A; Garcia-Villoria, J; Vilaseca, MA; Ozaez, L; LluchM; Artuch, R; Campistol, J; Ribes, A. Response to creatine analogs in fibroblasts and patients with creatine transporter deficiency. *Molec Genet Metabol*., 2010, 99(3), 296-9.

[101] Miller, WJ; Sherman, WM; Ivy, JL. Effect of strength training on glucose tolerance and post-glucose insulin response. *Med Sci Sports Exer*. 1984, 16(6), 539-43.

[102] Douen, AG; Ramlal, T; Rastogi, S; Bilan, PJ; Cartee, GD; Vranic, M; Holloszy, JO; Klip, A. Exercise Induces Recruitment of the "Insulin-responsive Glucose Transporter". Evidence for distinct intracellular insulin- and exercise-recruitable transporter pools in skeletal muscle. *J Biol Chem*., 1990, 265(2), 13427-30.

[103] Steenge, GR; Lambourne, J; Casey, A; Macdonald, IA; Greenhaff, PL. Stimulatory effect of insulin on creatine accumulation in human skeletal muscle. *Am J Physiol*., 1998, 275(6 Pt 1), E974-9.

[104] Brand-Miller, J. Glycemic index and body weight. *Am J Clin Nutr*., 2005, 81(3), 722-3.

[105] Greenwood, M; Kreider, R; Earnest, C; Rassmussen, C; Almada, A. Differences in creatine retention among three nutritional formulations of oral creatine supplements. *J Exerc Physiol Online* 2003, 6, 37-43.

[106] Tappy, L; Randin, JP; Felber, JP; Chiolero, R; Simonson, DC; Jequier, E; DeFronzo, RA. Comparison of thermogenic effect of fructose and glucose in normal humans. *Am J Physiol*., 1986, 250(6 Pt 1), E718-24.

[107] Truswell, AS. Glycaemic index of foods. *Eur J Clin Nutr*., 1992, 46 Suppl 2, S91-101.

[108] Preen, D; Dawson, B; Goodman, C; Beilby, J; Ching, S. Creatine supplementation: a comparison of loading and maintenance protocols on creatine uptake by human skeletal muscle. *Int J Sport Nutr Exerc Metab* 2003, 13(1), 97-111.

[109] Steenge, GR; Simpson, EJ; Greenhaff, PL. Protein- and carbohydrate-induced augmentation of whole body creatine retention in humans. *J Appl Physiol*., 2000, 89(3), 1165-71.

[110] Beck, TW; Housh, TJ; Johnson, GO; Coburn, DW; Malek, MH; Cramer, JT. Effects of a drink containing creatine, amino acids, an protein, combined with ten weeks of resistance training on body composition, strength, and anaerobic performance. *J Strength Cond Res*., 2007, 21(1), 100-4.

[111] Antonio, J; Ciccone, V. The effects of pre versus post workout supplementation of creatine monohydrate on body composition and strength. *J Int Soc Sports Nutr*., 2013, 10(1), 36.

[112] Cribb, PJ; Hayes, A. Effects of supplement timing and resistance exercise on skeletal muscle hypertrophy. *Med Sci Sports Exerc*., 2006, 38(11), 1918-25.

[113] Cribb, PJ; Williams, AD; Hayes, A. A creatine-protein-carbohydrate supplement enhances responses to resistance training. *Med Sci Sports Exerc*., 2007, 39(11), 1960-8.

[114] Burke, LM. Caffeine and sports performance. *Appl Physiol Nutr Metab*., 2008, 33(6), 1319-1334.

[115] Jones, G. Caffeine and other sympathomimetic stimulants: modes of action and effects on sports performance. *Essays Biochem*., 2008, 44, 109-123.

[116] Vandenberghe, K; Gillis, N; Van, Leemputte, M; Van, Hecke, P; Vanstapel, F; Hespel, P. Caffeine counteracts the ergogenic action of muscle creatine loading. *J Appl Physiol*., 1996, 80(2), 452-7.

[117] Hespel, P; Op't Eijnde, B; Van Leemputte, M. Opposite actions of caffeine and creatine on muscle relaxation time in humans. *J Appl Physiol*., 2002, 92(2), 513-8.

[118] Hultman, E; Greenhaff, PL. Skeletal muscle energy metabolism and fatigue during intense exercise in man. *Sci Prog*., 1991, 75(298 Pt 3-4), 361-70.

[119] Kramer, WJ; Volek, JS. Creatine supplementation: Its role in human performance. *Clin Sports Med*., 1999, 18(3), 651-66.

[120] Volek, J; Mazzetti, S; Farquhar, W; Barnes, B; Gomez, A; Kraemer, W. Physiological responses to short-term exercise in the heat after creatine loading. *Med Sci Sports Exerc*., 2001, 33(7), 1101-08.

[121] Powers, ME; Arnold, BL; Weltman, AL; Perrin, DH; Mistry, D; kahler, DM; Kraemer, W; Volek, J. Creatine Supplementation Increases Total Body Water Without Altering Fluid Distribution. *J Athl Train*, 2003, 38(1), 44-50.

[122] Haussinger, D. The role of cellular hydration in the regulation of cell function. *Biochem J*., 1996, 313(Pt 3), 697-710.

[123] Pasantes-Morales, H; Lezama, RA; Ramos-Mandujano, G; Tuz, KL. Mechanisms of cell volume regulation in hypo-osmolality. *Am J Med*., 2006, 119(7 Suppl 1), S4–11.

[124] Ritz, P; Salle, A; Simard, G; Dumas, JF; Foussard, F; Malthiery, Y. Effects of changes in water compartments on physiology and metabolism. *Eur J Clin Nutr*., 2003, 57 *Suppl* 2, S2-5.

[125] Kelly, VG; Jenkins, DG. Effect of oral creatine supplementation on near maximal strength and repeated sets of high-intensity bench press exercise. *J Strength Cond Res*., 1998, 12(2), 109-15.

[126] Safdar, A; Yardley, N; Snow, R; Melov, S; Tarnopolsky, M., Global and targeted gene expression and protein content in skeletal muscle of young men following short-term creatine monohydrate supplementation. *Physiol Genomics*., 2008, 32, 219-28.

[127] Saremi, A; Gharakhanloo, R; Sharghi, S; Gharaati, M; Larijani, B; Omidfar, K. Effects of oral creatine and resistance training on serum myostatin and GASP-1. *Mol Cell Endocrinol*., 2010, 317, 25-30.

[128] Hickner, R; Dyck, D; Sklar, J; Hatley, H; Byrd, P., Effect of 28 days of creatine ingestion on muscle metabolism and performance of a simulated cycling road race. *J Int Soc Sports Nutr*., 2010, 7, 26.

[129] Deldicque, L; Louis, M; Theisen, D; Nielens, H; Dehoux, M; Thissen, JP; Rennie, MJ; Francaux, M. Increased IGF mRNA in human skeletal muscle after creatine supplementation. *Med Sci Sports Exerc*., 2005, 37, 731-6.

[130] Ingwall, JS; Weiner, CD; Morales, MF; Davis, E; Stockdale, FE. Specificity of creatine in the control of muscle protein synthesis. *J Cell Biol*., 1974, 62(1), 145-51.

[131] Stout, J; Eckerson, J; Noonan, D. Effects of 8 weeks of creatine supplementation on exercise performance and fat-free weight in football players during training. *Nutr Res*., 1999, 19, 217–25.

[132] Earnest, CP; Snell, PG; Rodriguez, R; Almada, AL; Mitchell, TL. The effect of creatine monohydrate ingestion on anaerobic power indices, muscular strength and body composition. *Acta Physiol Scand*., 1995, 153, 207–9.

[133] Kreider, RB; Klesges, R; Harmon, K; Grindstaff, P; Ramsey, L; Bullen, D; Wood, L; Li, Y; Almada, A. Effects of ingesting supplements designed to promote lean tissue accretion on body composition during resistance training. *Int J Sport Nutr*., 1996, 6(3), 234-46.

[134] Volek, JS; Duncan, ND; Mazzetti, SA; Staron, RS; Putukian, M; Gomez, AL; Pearson, DR; Fink, WJ; Kraemer, WJ. Performance and muscle fibre adaptations to creatine supplementation and heavy resistance training. *Med Sci Sports Exerc*., 1999, 31(8), 1147-56.

[135] Mujika, I; Padilla, S; Ibanez, J; Izquierdo, M; Gorostiaga, E. Creatine supplementation and sprint performance in soccer players. *Med Sci Sports Exerc*., 2000, 32(2), 518-25.

[136] Juhn, MS; O'Kane, JW; Vinci, DM. Oral creatine supplementation in male collegiate athletes: a survey of dosing habits and side effects. *J Am Diet Assoc*., 1999, 99(5), 593-5.

[137] Rawson, ES; Volek, JS. Effects of creatine supplementation and resistance training on muscle strength and weightlifting performance. *J Stength Cond Res*., 2003, 17(4), 822-31.

[138] Gilreath, E; Judge, LW; Bellar, D. Petersen. Creatine Monohydrate: Safe and Effective? *Indiana AHPERD J*., 2011, 40(3), 14-20.

[139] Volek, JS; Ratamess, NA; Rubin, MR; Gómez, AL; French, DN; McGuigan, MM; Scheett, TP; Sharman, MJ; Häkkinen, K; Kraemer, WJ. The effects of creatine supplementation on muscular performance and body composition responses to short-term resistance training overreaching. *Eur J Appl Physiol.*, 2004, 91(5-6), 628-37.

[140] Pearson, DR; Hamby, DG; Russel, W; Harris, T. Long-term effects of creatine monohydrate on strength and power. *J Stength Cond Res*., 1999, 13(3), 187-92.

[141] Theodorou, AS; Cooke, CB; King, RF; Hood, C; Denison, T; Wainwright, BG; Havenetidis, K. The effect of longer-term creatine supplementation on elite swimming performance after an acute creatine loading. *J Sports Sci*., 1999, 17(11), 853-9.

[142] Skare, OC; Skadberg Wisnes, AR. Creatine supplementation improves sprint performance in male sprinters. *Scand J Med Sci Sports*, 2001, 11(2), 96-102.

[143] Kreider RB. Effects of creatine supplementation on performance and training adaptations. *Mol Cell Biochem*., 2003, 244(1-2), 89-94.

[144] Volek, JS; Kraemer, WJ; Bush, JA; Boetes, M; Incledon, T; Clark, KL; Lynch, JM., Creatine supplementation enhances muscular performance during high-intensity resistance exercise. *J Am Diet Assoc*., 1997, 97(7), 765-70.

[145] Kreider, RB; Melton, C; Rasmussen, C; Greenwood, M; Lancaster, S; Cantler, E; Milnor, P; Almada, A. Long-term creatine supplementation does not significantly affect clinical markers of health in athletes. *Mol Cell Biochem*., 2003, 40, 95-104.

[146] Greenwood, M; Kreider, R; Melton, C; Rasmussen, C; Lundberg, J; Stroud, T; Cantler, E; Milnor, P; Almada, AL. Short- and long-term creatine supplementation does not affect hematological markers of health. *J Strength Cond Res*., 2000, 14(3), 362-3.

[147] Schilling, BK; Stone, MH; Utter, A; Kearney, JT; Johnson, M; Coglianese, R; Smith, L; O'Bryant, HS; Fry, AC; Starks, M; Keith, R; Stone, ME. Creatine supplementation and health variables: a retrospective study. *Med Sci Sports Exerc*., 2001, 33(2), 183-8.

[148] Stone, MH; Schilling, BK; Fry, AC; Johnson, M; Keith, RE; Kearney, JT; Coglianese, RH; Stone, ME; Utter, A; Smith, L; O'Bryant, HS., A retrospective study of long-term creatine supplementation on blood markers of health. *J Strength Cond Res*., 1999, 13, 433.

[149] Sheppard, HL; Raichada, SM; Kouri, KM; Stenson-Bar-Maor, L; Branch, JD. Use of creatine and other supplements by members of civilian and military health clubs: Across-sectional survey. *Int J Sport Nutr Exerc Metab*., 2000, 10(3), 245-59.

[150] Kim, HJ; Kim, CK; Carpentier, A; Poortmans, JR. Studies on the safety of creatine supplementation. *Amino Acids*, 2011, 40(5), 1409-18.

[151] Chrusch, MJ; Chilibeck, PD; Chad, KE; Davison, KS; Burke, DG. Creatine supplementation combined with resistance training in older men. *Med Sci Sports Exerc*., 2001, 33(12), 2111-7.

[152] Tarnopolsky, MA. Potential benefits of creatine monohydrate supplementation in the elderly. *Curr Opin Clin Nutr Metab Care* 2000, 3(6), 497-502.

[153] Stöckler, S; Schutz, PW; Salomons, GS. Cerebral creatine deficiency syndromes. Clinical aspects, treatment and pathophysiology. *Subcell Biochem*., 2007, 46, 149-66.

[154] Schulze, A. Creatine deficiency syndromes. *Mol Cell Biochem*., 2003, 244(1-2), 143-50.

[155] Braissant, O; Henry, H; Béard, E; Uldry, J. Creatine deficiency syndromes and the importance of creatine synthesis in the brain. *Amino Acids*, 2011, 40(5), 1315-24.

[156] Stockler, S; Hanefeld F. Guanidinoacetate methyltransferase deficiency: A newly recognized inborn error of creatine biosynthesis. *Wien Klin Wochenschr*., 1997, 109(3), 86-8.

[157] Degrauw, TJ; Cecil, KM; Byars, AW; Salomons, GS; Ball, WS; Jakobs, C. The clinical syndrome of creatine transporter deficiency. *Mol Cel. Biochem*., 2003, 244(1–2), 45-8.

[158] Rosenberg, EH; Almeida, LS; Kleefstra, T; de Grauw, RS; Yntema, HG; Bahi, N; Moraine, C; Ropers, HH; Fryns, JP; deGrauw, TJ; Jakobs, C; Salomons, GS. High prevalence of SLC6A8 deficiency in X-linked mental retardation. *Am J Hum Genet*., 2004, 75(1), 97-105.

[159] Vorgerd, M; Grehl, T; Jager, M; Muller, K; Freitag, G; Patzold, T; Bruns, N; Fabian, K; Tegenthoff, M; Mortier, W; Luttmann, A; Zange, J; Malin, JP. Creatine therapy in myophosphorylase deficiency (McArdle disease): a placebo-controlled crossover trial. *Arch Neurol*., 2000, 57(7), 956-63.

[160] Stöckler, S; Marescau, B; DeDeyn, PP; Trijbels, JMF; Hanefeld, F. Guanidino compounds in guanidinoacetate methyltransferase deficiency, a new inborn error of creatine synthesis. *Metabolism*, 1997, 46, 1189-93.

[161] Tarnopolsky, M; Martin, J. Creatine monohydrate increases strength in patients with neuromuscular disease. *Neurology*, 1999, 52(4)854-7.

[162] Tarnopolsky, MA; Parise, G. Direct measurement of high-energy phosphate compounds in patients with neuromuscular disease. *Muscle Nerve*, 1999, 22, 1228-33.

[163] Hespel, P; Op't, Eijnde, B; Van, Leemputte, M; Urso, B; Greenhaff, PL; Labarque, V; Dymarkowski, S; Van Hecke, P; Richter, EA. Oral creatine supplementation facilitates the rehabilitation of disuse atrophy and alters expression of muscle myogenic factors in humans. *J Physiol. (Lond)*, 2001, 536, 625-33.

[164] Gualano, B; Artioli, GG; Poortmans, JR; Lancha Junior, AH, Exploring the therapeutic role of creatine supplementation. *Amino Acids*, 2010, 38(1), 31–44.

[165] Poo-Arguelles, P; Arias, A; Vilaseca, MA; Ribes, A; Artuch, R; Sans-Fito, A; Jakobs, C; Salomons, G. X-Linked creatine transporter deficiency in two patients with severe mental retardation and autism. *J Inherit Metab Dis*., 2006, 29(1), 220-3.

[166] Bender, A; Samtleben, W; Elstner, M; Klopstock, T. Long-term creatine supplementation is safe in aged patients with Parkinson disease. *Nutr Res.*, 2008, 28(3), 172-8.

[167] Lin, YS; Cheng, TH; Chang, CP; Chen, HM; Chern, Y. Enhancement of brain-type creatine kinase activity ameliorates neuronal deficits in Huntington's disease. *Biochim Biophys Acta.*, 2013, 1832(6), 742-53.

[168] Brosnan, JT; Brosnan, ME. Creatine: endogenous metabolite, dietary, and therapeutic supplement. *Annu Rev Nutr*., 2007, 27, 241-61.

[169] Faurion, AT; Passirani, C; Béjaud, J; Dézard, S; Valayannopoulos, V; Taran, F; de Lonlay, P; Benoit, JP; Mabondzo, A. Dodecyl creatine ester and lipid nanocapsule: a double strategy for the treatment of creatine transporter deficiency. *Nanomedicine*, 2015, 10(2), 185-91.

[170] Faurion, AT; Dézard, S; Taran, F; Valayannopoulos, V; de Lonlay, P; Mabondzo, A. Synthesis and Biological Evaluation of New Creatine Fatty Esters Revealed Dodecyl Creatine Ester as a Promising Drug Candidate for the Treatment of the Creatine Transporter Deficiency. *J Med Chem*., 2013, 56, 5173-81.

[171] Yar, RA; Akbar, A; Iqbal, F. Creatine monohydrate supplementation for 10 weeks mediates neuroprotection and improves learning/ memory following neonatal hypoxia ischemia encephalopathy in female albino mice. *Brain Res*., 2015, 1595, 92-100.

[172] Jensen, LH. The Crystal Structure of Creatine Monohydrate. *Acta Cryst*., 1955, 6, 237.

[173] Mendel, H; Hodgkin, DC. The Crystal Structure of Creatine Monohydrate. *Acta Cryst*., 1954, 7, 443.

[174] Kato, Y; Haimoto, Y; Sakurai, K. A Refinement of Crystal Structure of Creatine Monohydrate. *B Cheml Soc Jpn*., 1979, 52(1), 233-4.

[175] Goswami, S; Jana, S; Hazra, A; Fun, HK; Anjumc, S; Rahman, A. Recognition of creatinine by weak aromatic acids in solid phase along with their supramolecular network. *Cryst Eng Comm*., 2006, 8, 712-8.

[176] Dash, AK; Mo, Y; Pyne, A. Solid-State Properties of Cretaine Monohydrate. *J Pharm Sci*., 2002, 91(3), 708-18.

[177] Eadie, GS; Hunter, A. The Apparent dissociation Constants of Creatine and Creatinine. *J Biol Chem*., 1926, 67, 237-44.

[178] Gufford, BT; Sriraghavan, K; Miller, NJ; Miller, DW; Gu, X; Vennerstrom, JL; Robinson, DH. Physicochemical characterization of creatine N-methylguanidinium salts. *J Diet Suppl*., 2010, 7(3), 240-52.

[179] Diamond, BJ. Temperature and Ph Dependence of the Cyclization of Creatine: A Study Via Mass Spectrometry. Marshall Univers., 2005, 1-56.

[180] Wang; X; Yin, Q. *J Chem Eng Chin Univ*., 2003, 17(5), 569-574.

[181] Howard, AN; Harris, RC. Compositions Containing Creatine. United States Patent 1999, 1-6.

[182] Witkowska, A. Kinetics of in vitro conversion of creatine to creatinine. *Acta Alimentaria Polonica*., 1985, 9(2), 263-9.

[183] Uzzan, M; Nechrebeki, J; Zhou, P; Labuza, TP. Effect of water activity and temperature on the stability of creatine during storage. *Drug Dev Ind Pharm*., 2009, 35(8), 1003-8.

[184] Vennerstrom, JL; Miller, DW. Creatine Ester PronutrientCompounds and Formulations, United States Patent Application Publication., 2003, 1-4.

[185] Gufford, BT; Ezell, EL; Robinson, DH; Miller, DW; Miller, NJ; Gu, X; Vennerstrom, JL. pH-Dependent Stability of Creatine Ethyl Ester: Relevance to Oral Absorption. *J Diet Suppl*., 2013, 10(3), 241-251.

[186] Katseres, NS; Reading, DW; Shayya, L; DiCesare, JC; Purser, GH. Non-enzymatic hydrolysis of creatine ethyl ester. *Biochem Biophys Res Commun*., 2009, 386(2), 363-367.

[187] Giese, MW; Lecher, CS. Qualitative in vitro NMR analysis of creatine ethyl ester pronutrient in human plasma. *Int J Sports Med*., 2009, 30(10), 766-770.

[188] Giese, MW; Lecher, CS. Non-enzymatic cyclization of creatine ethyl ester to creatinine. *Biochem Biophys Res Commun*., 2009, 388(2), 252-255.

[189] Velema, MS; de Ronde, W. Elevated plasma creatinine due to creatine ethyl ester use. *Neth J Med.*, 2011, 69(2), 79-81.

[190] Spillane, M; Schoch, R; Cooke, M; Harvey, T; Greenwood, M; Kreider, R; Willoughby, DS. The effects of creatine ethyl ester supplementation combined with heavy resistance training on body composition, muscle performance, and serum and muscle creatine levels. *J Int Soc Sports Nutr*., 2009, 6, 6.

[191] Golini, JM. Oral Creatine Supplement and Method for Making Same. United States Patent 2002, 1-3.

[192] Selsby, JT; DiSilvestro, RA; Devor, ST. $Mg2^{+}$-creatin chelate and a lo-dose creatine supplementation regimen improveexercise performance. *J Strength Cond Res*., 2004, 18(2), 311-315.

[193] Brilla, LR; Giroux, MS; Taylor, A; Knutzen, KM. Magnesium-creatine supplementation effects on body water. *Metabolism*, 2003, 52(9), 1136-40.

[194] Miyazaki, S; Oshiba, M; Nadai, T. Precaution on use of hydrochloride salts in pharmaceutical formulation. *J Pharm Sci.*, 1981, 70(6), 594-6.

[195] Miller, DW; Vennerstrom, JL; Faulkner, MC. Creatine oral supplementation using creatine hydrochloride salt. United States Patent Application Publication. 2011, 1-9.

[196] Dhar, NR; Ghosh, GP. Complex compounds of acid, base, and salts with nitrogenous and other organic substances. *Proc Natl Acad Sci India*, 1961, 31A, 74-77.

[197] Arnold, MJ. Pyruvate savvaride ketals. United States Patent., 2001, 1-4.

[198] Qian, H; Ye, F; Huang, Z. Method for synthesizing dicreatine malate. Chinese Patent CN 1683327 A,PR China: Jiangyin South Pole Star Bioproducts Co., Ltd., 2005.

[199] Thomson, JK. Dicreatine citrate and tricreatine citrate and method of making same. United States Patent., 2001, 1-4.

[200] Heuer, M; Molino, M. Creatine hydroxycitric acids salts and methods for their production and use in individuals. United States Patent., 2010, 1-7.

[201] Jäger, R; Harris, RC; Purpura, M; Francaux, M. Comparison of new forms of creatine in raising plasma creatine levels. *J Int Soc Sports Nutr*., 2007, 4, 17.

[202] Kalman, D; Colker, CM; Wilets, I; Roufs, JB; Antonio, J. The effects of pyruvate supplementation on body composition in overweight individuals. *Nutrition.*, 1999, 15(5), 337-40.

[203] Stanko, RT; Robertson, RJ; Galbreath, RW; Reilly, JJ; Jr. Greenawalt, KD; Goss, FL. Enhanced leg exercise endurance with a high-carbohydrate diet and dihydroxyacetone and pyruvate. *J Appl Physiol*., 1990, 69(5), 1651-6.

[204] Oöpik, V; Saaremets, I; Medijainen, L; Karelson, K; Janson, T; Timpmann, S. Effects of sodium citrate ingestion before exercise on endurance performance in well trained college runners. *Br J Sports Med.*, 2003, 37(6), 485-9.

[205] Van, Schuylenbergh, R; Van Leemputte, M; Hespel, P. Effects of oral creatine-pyruvate supplementation in cycling performance. *Int J Sports Med.*, 2003, 24(2), 144-50.

[206] Nuuttilla S. Edustusmelojat testasivat kreatiinipyruvaatin. *Suomen urheilulehti*, 2000, 23(Supplement), 4.

[207] Eckerson, JM; Stout, JR; Moore, GA; Stone, NJ; Nishimura, K; Tamura, K. Effect of two and five days of creatine loading on anaerobic working capacity in women. *J Strength Cond Res.*, 2004, 18(1), 168-73.

[208] Smith, AE; Walter, AA; Herda, TJ; Ryan, RD; Moon, JR; Cramer, JT; Stout, JR. Effects of creatine loading on electromyographic fatigue threshold during cycle ergometry in college-aged women. *J Int Soc Sports Nutr*, 2007, 4, 20.

[209] Jäger, R; Metzger, J; Lautmann, K; Shushakov, V; Purpura, M; Geiss, KR; Maassen, N. The effects of creatine pyruvate and creatine citrate on performance during high intensity exercise. *J Int Soc Sports Nutr*., 2008, 5, 4.

[210] Stone, MH; Sanborn, K; Smith, LL; O'Bryant, HS; Hoke, T; Utter, AC; Johnson, RL; Boros, R; hruby, J; Stone, ME; Garner, B. Effects of in-season (5 weeks) creatine and pyruvate supplementation on anaerobic performance and body composition in American football players. *Int J Sport Nutr*., 1999, 9(2), 146-65.

[211] http: //krealkalyn.com/

[212] http: //krealkalyn.com/index.php?option=com_content&view=article& id=83&Itemid =153

[213] Jagim, AR; Oliver, JM; Sanchez, A; Galvan, E; Fluckey, J; Riechman, S; Greenwood, M; Kelly, K; Meininger, C; Rasmussen, C; Kreider, RB. A buffered form of creatine does not promote greater changes in muscle creatine content, body composition, or training adaptations than creatine monohydrate. *J Int Soc Sports Nutr*., 2012, 9(1), 43.

[214] Seddon, KR. Ionic Liquids A taste of the future. *Nat Mater*., 2003, 2, 363-5.

[215] Rogers, RD; Seddon, K. Ionic Liquids-Solvents of the Future? *Science*, 2003, 302(5646), 792-3.

[216] Vraneš, M; Papović, S; Tot, A; Zec, N; Gadžurić, S. Density, excess properties, electrical conductivity and viscosity of 1-butyl-3-methylimidazolium bis(trifluoromethylsulfonyl)imide + γ-butyrolactone binary mixtures. *J Chem Thermodyn*., 2014, 76, 161-71.

[217] Vraneš, M; Tot, A; Zec, N; Papović, S; Gadžurić, S. Volumetric Properties of Binary Mixtures of 1-Butyl-3-Methylimidazolium Tris(pentafluoroethyl)trifluorophosphate with N-Methylformamide, N-Ethylformamide, N,N-Dimethylformamide, N,N-Dibutylformamide, and N,N-Dimethylacetamide from (293.15 to 323.15) K. *J Chem Eng Data*., 2014, 59(11), 3372-9.

[218] Gadžurić, S; Tot, A; Zec, N; Papović, S; Vraneš, M. Volumetric Properties of Binary Mixtures of 1-Butyl-1-Methylpyrrolidinium Tris(pentafluoroethyl)trifluorophosphate with N-Methylformamide, N-Ethylformamide, N,N-Dimethylformamide, N,N-Dibutylformamide, and N,N-Dimethylacetamide from (293.15 to 323.15) K. *J Chem Eng Data*., 2014, 59(4), 1225-31.

[219] Vraneš, M; Zec, N; Tot, A; Papović, S; Dožić, S; Gadžurić, S. Density, electrical conductivity, viscosity and excess properties of 1-butyl-3-methylimidazolium bis(trifluoromethylsulfonyl) imide + propylene carbonate binary mixtures. *J Chem Thermodyn*., 2014, 68, 98-108.

[220] Vraneš, M; Tot, A; Papović, S; Zec, N; Gadžurić, S. Ideal and non-ideal behaviour of {1-butyl-1-methylpyrrolydinium bis(trifluoromethylsulfonyl) imide + γ-butyrolactone} binary mixtures. *J Chem Thermodyn*., 2014, 81, 66-7.

[221] Vraneš, M; Armaković, S; Tot, A; Papović, S; Zec, N; Armaković, S; Gadžurić, S. Understanding solvation in the [bmim][Sal] Third Generation of Ionic Liquids: Experimental and Computational Study. *Unpublished Manuscript* 2015.

[222] Vraneš, M; Dožić, S; Đerić, V; Gadžurić, S. Physicochemical Characterization of 1-Butyl-3-methylimidazolium and 1-Butyl-1-methylpyrrolidinium Bis(trifluoromethylsulfonyl)imide. *J Chem Eng Data*., 2012, 57(4), 1072-7.

In: Human Health and Nutrition
Editor: Sergej M. Ostojic
ISBN: 978-1-63482-823-9

Chapter VII

The Impact of Human Intervention Studies on the Evaluation of Medicinal Plant Antioxidant and Anti-Inflammatory Activities

Diana Ivanova*, Milka Nashar, Yoana Kiselova-Kaneva, Oskan Tasinov, Deyana Vankova and Neshe Nazifova-Tasinova
Department of Biochemistry, Molecular Medicine and Nutrigenomics,
Medical University "Prof. Dr. Paraskev Stoyanov", Varna, Bulgaria

Abstract

Inflammation as an integral part of immune response involves the cytokine production and the subsequent activation of redox sensitive gene expression. Thus many diseases are accompanied by nonspecific inflammation and increased free radical oxidation. Even obesity and related metabolic disturbances involve low-grade inflammation associated with oxidative stress. Scientific data reveal that many of the beneficial effects reported by the traditional medicines are based on anti-inflammatory and antioxidant properties of medicinal plants. On the other hand, given the multifactorial nature of many diseases, especially metabolic diseases, the need for multi-targeted therapies rises while drug discovery research develops more single-target synthetic drugs. In this respect synergic multicomponent herbal remedies appear as the good new old alternative for pharmaceutical research. Identification of appropriate biomarkers and standardized intervention studies are needed to ensure comparability and reliability of results. For these reasons analysis of available human intervention studies is needed. The aim of the current investigation was to explore the applicability of different biomarkers for the assessment of antioxidative and anti-inflammatory plant potential. Results from three pilot intervention studies evaluating multi-target biological activities of two medicinal plants are presented. *Sambucus ebilus and Agrimonia eupatoria* tea effects

* Corresponding author: Email: divanova@mu-varna.bg.

were tested on healthy volunteers in the period of 2011-2014. Despite the fact that human subjects have a strong phenotypic flexibility and capacity to maintain homeostasis, biomarkers, such as plasma total antioxidant capacity that showed consistently response to altered diet could be identified.

Keywords: Human intervention studies; Medicinal plants; Biomarkers; Inflammation; Antioxidative potential

Introduction

Traditions of folk medicine in many countries around the world have preserved the millennial knowledge of the beneficial effects of herbs on health. Herbs, also called medicinal plants or medicinal herbs, comprise a large group of plants that are used in medical and veterinary practice for the prevention and treatment of diseases. In many societies, nutrition and health care are inextricably linked, and many plant species are used, in addition to treatment, also as food [1]. According to the World Health Organization today 80% of the world's population relies on traditional medicine treatment [2]. About 40% of all medicines are plant-derived and medicinal plant species used for their preparation are more than 20 000. A quarter of all drugs prescribed in USA, Canada and Europe contain active ingredients derived from plants (a report from 2003 of BCC, American marketing research agency). However, despite their wide use, only 5 to 15% of the world's known 250,000 vascular plants are examined for the presence of bioactive ingredients and biological effects (http://www.bccresearch.com/report/BIO022C.html). Understanding the links between existing empirical data from folk medicine and the mechanism of therapeutic action of plants is facilitated by the increased capacity of modern biomedical research and clinical trials. The need for accurate science-based information on the operation of medicinal plants by pharmaceutical industry is becoming more acute in relation to the risks of their incompetent use [3]. There are various methods of extrapolating data from *in vivo* animal models to humans, the allometric approach being the best described technique for predicting human pharmacokinetics from *in vivo* preclinical data. In recent years even parameters like "No adverse effects level (NOAEL)", defined in preclinical studies by the most sensitive and relevant animal model, and "The minimum anticipated biological effect level (MABEL)" (Guideline on strategies to identify and mitigate risks for first-in-human clinical trials with investigational medicinal products. CHMP/SWP/28367/07) were introduced. Animal models are in the focus of studies for prediction of drug response in humans. However, they have limitations due to the specificities of human and animal metabolisms. These specificities are based on the genetic variations in the human genome (including single nucleotide polymorphisms, SNPs and the presence of multiple copies of the same allele, CNVs), the effects of the deletions and insertions of genetic material, as well as the epigenetic changes during ontogeny. This imposes the necessity to study antioxidant action and biological effects of medicinal plants also in healthy volunteers. In folk medicine, various parts of the plants, prepared in the form of tea or other kinds of infusions and extracts are used in the treatment and prophylaxis of a variety of diseases. An analysis of the use of Bulgarian herbs makes apparent that many of these diseases are associated with inflammatory processes of different etiology [4]. Numerous *in vitro* and *in vivo* studies have been associated with antioxidant

properties of various plant products and extracts. Recently systematic studies of antioxidant properties of large groups of plants used in countries known for their traditions in folk medicine have been reported [5-12].

Various biologically active substances with different chemical nature were identified in plants. These include phenolic compounds, alkaloids, terpenes, saponins, xanthophylls, phytosterols, vitamins and others. The biological effects of the different plant extracts are attributed in particular to the content of such ingredients. Of these groups undoubtedly one of the most numerous is the group of polyphenols, gained initial popularity with anti-oxidant properties.

Inflammation as an integral part of immune response involves the cytokine production and the subsequent activation of redox sensitive gene expression. Thus many diseases are accompanied by nonspecific inflammation and increased free radical oxidation. Oxidative stress is associated with diseases and conditions such as obesity and related diabetes and metabolic syndrome [13-15]. There are several possible causes of oxidative stress in obesity – hyperglycemia [16], enhanced muscle work for the movement of larger body mass [17, 18], increased tissue lipid levels [18,19], inferior antioxidant protection [20-22], chronic inflammation [23-25], overproduction of reactive oxygen species (ROS) in the endothelium [26-28] hyperleptinemia [29] and others. All these factors are not mutually exclusive and systemic oxidative stress accompanying obesity may involve some or all of the above [30]. Scientific data reveal that many of the beneficial effects reported by the traditional medicines are based on anti-inflammatory and antioxidant properties of medicinal plants. On the other hand, given the multifactorial nature of many diseases, especially metabolic diseases, the need for multi-targeted therapies rises, while drug discovery research develops more single-target synthetic drugs. In this respect synergic multicomponent herbal remedies appear as the good new old alternative for pharmaceutical research. Identification of appropriate biomarkers and standardized intervention studies are needed to ensure comparability and reliability of results. For these reasons analysis of available human intervention studies is needed.

Methods

Study Description

Three pilot intervention studies on the evaluation of two medicinal plants' multi-target biological activities are presented with the aim to explore the applicability of different biomarkers for the assessment of antioxidative and anti-inflammatory plant potential. *Sambucus ebulus* and *Agrimonia eupatoria* tea effects were tested on healthy volunteers in the period of 2011-2014.

Healthy volunteers intervention procedures were in accordance with international standards (Ethics for researchers, Facilitating Research Excellence in FP7, 2013) and were approved by the Ethics Committee for Scientific Research of Medical University "Prof. Dr. Paraskev Stoyanov" – Varna. The approvals for the interventions were released prior to studies start as follows: Protocols/Decisions No. 6/23.07.2009 and No. 13/24.03.2011 for the first *Agrimonia eupatoria* study and Protocol/Decision No. 27/21.02.2013 for the second

Agrimonia eupatoria study; Protocol/Decision No. 6/23.07.2009 for *Sambucus ebulus* study. The studies were supported by the National Scientific Fund, COST Action BM0602, "Medical Science Fund" of Medical University Varna.

Participants and Study Design

Each of the study participants completed informed consent and health history questionnaire. Exclusion criteria were: smoking, daily alcohol consumption, pregnancy, chronic diseases, liver or kidneys dysfunction, the use of drugs and/or dietary supplements in the course of the study. Subjects were obliged to maintain their normal lifestyle and dietary regimes through the duration of the study. Upon the initial recruitment 19 healthy volunteers met the criteria to take part in the first *Agrimonia eupatoria* study (AE1) carried out in a period of 30 days (28.03.2011 to 26.04.2011). The participants aged 20-55 years, the ratio of women-to-men was 14:5. The second *Agrimonia eupatoria* study (AE2) was designed as a follow up study to involve 40 healthy volunteers, divided in two groups, according to their BMI (normal weight group with BMI < 25 and overweight group with BMI ≥ 25) and aimed at exploring the effect of the herbal tea on biomarkers of obesity related oxidative stress and low grade inflammation. It was carried out in a period of 25 days (from 25.04.2013 to 20.05.2013). The participants aged 19-60, the women: men ratio was 37:3. The study of the effect of *Sambucus ebulus* tea (SE) was carried out in a period of 30 days (17.04.2012 to 17.05.2012) and involved 22 healthy volunteers, 16 women and 6 men aged between 20 and 59 years. Blood samples were collected at the start (day 0) and at the end (day 25/30, respectively) of the studies after an overnight fast. Subjects consumed 200 ml infusion per day (SE and AE2 study) and 2 x 200 ml infusion per day (AE1 study) at one and the same day time (9 a.m. for SE and AE2 interventions and at 9 a.m. and 4 p.m. for AE1 intervention).

Plant Material

The *Agrimonia eupatoria* tea tested was a commercial product of Selibum Ltd., Varna, Bulgaria. *Sambucus ebulus* fruits were supplied by EkoHerb® Ltd., Suhindol, Bulgaria. Plant material is standardized according to the European Pharmacopoeia. The technology of the commercial preparation of the dried plant product included botanical identification prior to plant processing.

Intervention

Aerial parts of *A. eupatoria* and rape fruits of *S. ebulus* were used for infusion preparations. The recipes were adopted from Bulgarian folk medicine as follows: (a) AE intervention No 1 (AE1): 200 ml boiled water was added to 1.0 g dried plant material and was incubated for 10 minutes as described earlier [7]; (b) AE intervention No 2 (AE2): 2.5 g of dried plant material were incubated in 200 ml boiling water for 10 minutes; (c) SE intervention: the recipe for SE infusion preparation was according to the manufacturer's

prescription - 2.5 g dried fruit material to 300 ml boiling water. After 10 min of boiling and 30 minutes of incubation the infusion was filtered and volunteers were given 200 ml infusion each. Daily intake of antioxidants (mg/day) was calculated as follows: daily intake = concentration in the infusion * MW of the respective calibrator (UA, Q or CG) *volume infusion per day, where UA is uric acid, Q is quercetin and CQ is cyanidin-3-glucoside. The plant material was weighed on electron scales. The infusions were freshly prepared in ewers, daily, at one and the same time, by one and the same person, just before the scheduled time on spot at the Department of Biochemistry, Molecular Medicine and Nutrigenomics. No sugar or other sweeteners were added. The volunteers were asked to be present earlier and each received a cup with one and the same content (from one pot), which was consumed immediately on spot. Total antioxidant capacity (TAC) and polyphenol content of herbal infusions were determined immediately prior to the survey.

Experimental Protocol

Venous blood samples were collected at the beginning (day 0) and at the end (day 25/30) of the study between 8 and 9 a.m. after an overnight fast. Plasma/serum samples were separated and used for biochemical analyses. Serum that was not used immediately was divided in aliquots and was stored at -80º C until analyses. Height, weight, waist and hip circumferences and arterial blood pressure were measured using standardized protocols and validated scales. BMI was calculated as kg/m^2 and waist/hip ratio was evaluated. TAC in blood samples and infusions was measured applying the spectrophotometric method of Re et al. [31] based on the ability of the antioxidants to reduce preformed ABTS cation radical [2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)]. Values were expressed as mM uric acid equivalents (UAE). ABTS was purchased from Sigma-Aldrich Chemie, Steinheim, Germany, uric acid and potassium persulfate - from Aldrich Chemical Company, Inc., Milwaukee, USA. Ferric reduction potential of plasma (FRAP) was measured by the method of Benzie and Strain [32]. The change in absorbance is directly related to the total reducing power of the electron donating antioxidants present in the reaction mixture. Values were expressed as mM $FeSO_4$. 2.4.6-tri(2-pyridyl)-s-triazine was purchased from Sigma Aldrich Chemie, Steinheim Germany and $FeSO_4$ – from Merck, Darmstadt, Germany. Total polyphenol content (TPC) was measured using the Folin-Ciocalteu reagent as described by Singleton and Rossi [33]. Polyphenol content was expressed as mmol/L quercetin equivalents (QE). Total anthocyanin content (AC) was evaluated by pH-differential method as described by Giusti and Wrolstad [34]. Data are presented as mg/L cyanidin-3-glucoside equivalents (CGE). Total thiols (TT) concentration in serum in AE1 and SE study, and glutathione (GSH) in erythrocyte lysates in SE study were quantified using Ellman's reagent (5,5'-dithiobis-(2-nitrobenzoic acid), DTNB), (Sigma-Aldrich, Germany), as previously described [35]. For preparation of erythrocyte lysates 500 µL whole blood was centrifuged at 2500 g for 5 min at 4º C. After removal of plasma pellets were lysed in 1 mL 5% metaphospforic acid. Vortexed samples were centrifuged at 3000 rpm for 10 min at 4º C and the resulting lysates were used for further analysis. Values were expressed as reduced glutathione equivalents (GSHE). In AE2 intervention commercially available kit from Percipio Biosciences, OxisResearch, USA was used to measure both total thiols and GSH. The measurement is based on the interaction between the mercaptans (RSH) with the chromogenic agent 4-chloro-1-methyl-7-

triflurомethyl-quinolinium methylsulfate. The formed chromophoric thiones and thioethers are spectrophotometrically measured to determine the concentration of total thiols and GSH. All measurements were performed in triplicate. Serum CRP levels were measured using commercially available ELISA kit (DIAsource Immunoassay S.A., Nivelles, Belgium and LUCIO, Nal von minden GmbH, Germany) and tumor necrosis factor alpha (TNF-alpha) and interleukin 6 (IL-6) levels by ELISA kits (Gen-Probe Diaclone SAS, Besancon Cedex, France). For IL-6 measurements in AE2 intervention the used ELISA kit was purchased from Ani Biotech Oy, Orgenium Laboratories Business Unit, Finland. Serum adiponectin was measured using ELISA kits from DIAsource Immunoassay S.A., Nivelles, Belgium and Invitrogen, Life Technologies, USA. Leptin was measured using ELISA kit from BioVendor R&D, Karasek, Czech Republic.

Statistical Analyses

The data If not specified otherwise, data are presented as mean ± standard error of mean (SEM). Statistical analyses were performed by *t*-test (95% confidence interval) using GraphPadPrism v.5.00 statistical program. Results with P values < 0.05 were considered to be statistically significant.

Results

Anthropometric and Demographic Data

All of the enrolled participants completed the interventions. One subject complained from gastrointestinal discomfort and slight diarrhea in the first *Agrimonia eupatoria* intervention study. Anthropometric and demographic data are presented in Table 1.

Table 1. Anthropometric measurements and demographic data of study subjects

	Day 0	**Day 25/30**	**Change (%)**	***P* value**
Sambucus ebulus intervention				
Gender (male/female)	6/15			
Age (years)	25.19 (20-58)			
Height (m)	1.68 (1.55-1.85)			
Body weight (kg)	65.22 ± 9.58	64.68 ± 8.82	-0.83	ns
BMI (kg/m^2)	23.12 ± 6.01	22.93 ± 5.91	-0.83	ns
WHR	0.78 ± 0.07	0.81 ± 0.13	3.77	ns
Agrimonia euparoria (1) intervention				
Gender (male/female)	5/14			
Age (years)	27.79 (19-58)			
Height (m)	1.69 (1.62-1.86)			
Body weight (kg)	64.08 ± 3.43	64.69 ± 3.73	1.08	ns
BMI (kg/m^2)	22.15 ± 0.87	22.37 ± 0.85	1.08	ns

	Day 0	**Day 25/30**	**Change (%)**	***P* value**
WHR	0.75 ± 0.09	0.76 ± 0.06	1.33	ns
Agrimonia euparoria (2) intervention				
Gender (male/female)				
BMI < 25	1/22			
BMI ≥ 25	2/15			
Age (years)				
BMI < 25	26.43 (19-54)			
BMI ≥ 25	34.29 (20-60)			
Height (m)				
BMI < 25	1.65 (1.50-1.82)			
BMI ≥ 25	1.65 (1.55-1.78)			
Body weight (kg)				
BMI < 25	57.28 ± 1.48	56.44 ± 1.44	-1.47	<0.0001
BMI ≥ 25	78.03 ± 4.17	77.39 ± 4.22	-0.82	<0.05
BMI (kg/m^2)				
BMI < 25	21.07 ± 0.37	20.76 ± 0.36	-1.46	<0.0001
BMI ≥ 25	28.60 ± 1.23	28.37 ± 1.26	-0.82	<0.05
WHR				
BMI < 25	0.71 ± 0.01	0.68 ± 0.01	-3.81	<0.001
BMI ≥ 25	0.75 ± 0.02	0.75 ± 0.02	-0.25	ns

Data are presented as mean ± SD. BMI = body mass index; WHR = waist/hip ratio.

In AE1 and SE studies plant infusion consumption for a period of 30 days did not result in significant reduction in body weight and related BMI, as well as waist/hip ratio and blood pressure. At the end of the intervention period in AE2 study significantly reduced body weight and BMI were established for both groups. In addition, waist/hip ratio decreased in normal weight group.

Table 2. Total antioxidant capacity, total polyphenol and anthocyanin concentration and content in *Sambucus ebulus* L and *Agrimonia eupatoria* infusions, designated for the intervention studies

	Total antioxidant capacity	**Total polyphenols**	**Total anthocyanins**
Sambucus ebulus	7.23±0.03 mM UAE	0.75±0.04 mM QE	18.31±0.13 mg/L CGE
Agrimonia euparoria (1)	3.76±0.05 mM UAE	0.70±0.00 mM QE	NA
Agrimonia euparoria (2)	6.61±0.09 mM UAE	1.58±0.06 mM QE	NA
Daily intake with the infusion (mg/day)			
Sambucus ebulus	243.09±1.01 UAE	50.73±2.71 QE	3.66±0.03
Agrimonia eupatoria (1)	252.84±3.36 UAE	94.71±0.00 QE	NA
Agrimonia eupatoria (2)	222.24±3.03 UAE	106.94±4.05 QE	NA

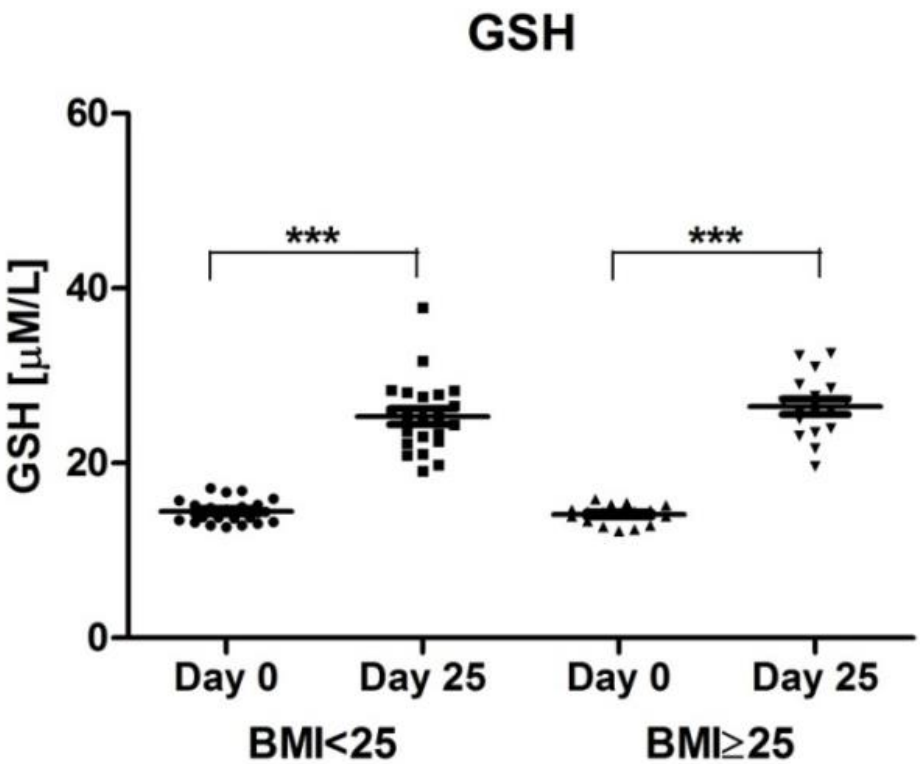
Data are presented as mean ± SD. QE - quercetin equivalents; CGE - cyanidin-3-glucoside equivalents.

Antioxidant Capacity of the Infusions

TAC and TPC of AE and SE herb infusions and AC of SE fruit infusion designated for intervention studies, as well as their calculated daily intake are presented in Table 2. AC was calculated to represent about 8% of the infusion's TPC (Table 2).

Blood Antioxidant Parameters

Table 3 presents parameters of blood antioxidant capacity form studies AE1, AE2 and SE. All of the three interventions resulted in significantly increased TAC in blood samples. TT levels increased in SE study, whereas in both AE1 and AE2 interventions their levels were significantly lowered at the end of the investigation period. At the same time, plasma GSH levels were significantly increased in AE2 intervention ($p<0.001$) (Figure 1). GSH levels in erythrocyte lysates in SE study remained unchanged (1047.12±65.75 μM vs. 1052.24±59.42 μM at day 0). In AE1 study FRAP analysis of blood samples was also performed and no significant changes were established (0.95±0.04 mM at day 30 vs. 0.92±0.02 mM at day 0).



Data are presented as mean ± SEM, *** $P < 0.001$.

Figure 1. Glutathione (GSH) levels in plasma samples of AE1 volunteers.

Table 3. Blood total antioxidant capacity and total thiols content in SE and AE1 and AE2 intervention studies

	Day 0	Day 25/30	Change (%)	*P* value
Sambucus ebulus intervention				
Total antioxidant capacity (mM UAE)	1.66 ± 0.02	2.09 ± 0.02	26.08	<0.0001
Total thiols (μM/g protein)	7.13 ± 0.13	7.45 ± 0.13	4.51	<0.05
Agrimonia euparoria (1) intervention				
Total antioxidant capacity (mM UAE)	2.09 ± 0.02	2.18 ± 0.01	5.05 ± 1.09	<0.001
Total thiols (μM)*	456.9 ± 11.2	428.14 ± 11.5	-79 ± 2.63	<0.05
Agrimonia euparoria (2) *intervention*				
Total antioxidant capacity (mM UAE)				
BMI < 25	2.43 ± 0.04	2.6 ± 0.03	6.51	<0.05
BMI ≥ 25	2.42 ± 0.03	2.67 ± 0.02	10.04	<0.001
Total thiols (μM/g protein)				
BMI < 25	45.27 ± 0.81	33.62 ± 0,26	-25.73	<0.0001
BMI ≥ 25	43.41 ± 0.96	31.48 ± 0.5	-27.48	<0.0001

Data are presented as mean ± SEM. UAE – uric acid equivalents. *Values are presented as concentration, as total protein was not measured.

Proinflammatory Cytokines

Proinflammatory cytokines as measured in the three studies are presented in Table 4. Plasma IL-6 levels significantly decreased in AE1 study, whereas those in the other two interventions remained unchanged. On the other hand, SE intervention significantly affected levels of CRP and IL-β. At the end of the study levels of these parameters were found to be significantly lower (Table 4, Figure. 2). TNF-alpha levels in SE intervention did not change significantly (10.79 ± 2.67 pg/ml at day 30 vs. 21.13 ± 10.73 pg/ml at day 0). In AE1 intervention plasma TNF-alpha levels were below the detection limit.

Adipokines

Adipokines blood levels as measured in the three studies are presented in Table 5. Consumption of SE and AE2 infusion resulted in significantly reduced adiponectin levels. While the levels of leptin decreased in the SE study, the concentration of the adipokine was higher at the end of AE2 study in normal weight group. Both adipokine levels did not change in AE1 intervention.

Table 4. Interleukin 6 (IL-6), C-reactive protein (CRP), and tumor necrosis factor alpha (TNF-alpha) blood content in SE and AE1 and AE2 intervention studies

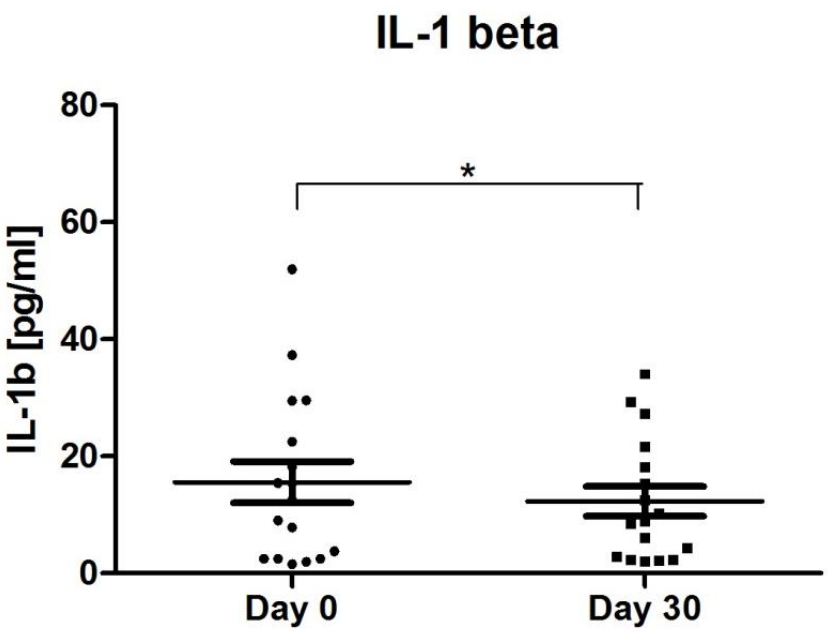
	Day 0	**Day 25/30**	**Change (%)**	***P* value**
Sambucus ebulus intervention				
IL-6 (pg/mL)	18.33 ± 4.10	15.09 ± 3.04	-17.72	ns
CRP (mg/L)	1.25 ± 0.41	0.84 ± 0.32	-32.83	<0.05
Agrimonia euparoria (1) intervention				
IL-6 (pg/ml)	9.75 ± 1.73	5.2 ± 0.55	-18.12	<0.05
CRP (mg/L)	0.64 ± 0.23	0.47 ± 0.1	-26.62	ns
Agrimonia euparoria (2) *intervention*				
IL-6 (pg/ml)				
BMI < 25	26.9 ± 9.18	28.74 ± 10.03	6.85	ns
CRP (mg/L)				
BMI < 25	0.82 ± 0.23	0.89 ± 0.23	9.61	ns
BMI ≥ 25	1.95 ± 0.36	2.45 ± 0.45	25.94	ns

Data are presented as mean ± SEM.

Table 5. Leptin and adiponectin blood content in plasma/serum samples from volunteers in SE and AE1 and AE2 intervention studies

	Day 0	**Day 25/30**	**Change (%)**	***P* value**
Sambucus ebulus intervention				
Leptin (ng/mL)	8.68 ± 1.73	6.08 ± 1.31	-29.94	<0.01
Adiponectin (µg/mL)	19.11 ± 2.16	13.95 ± 1.56	-26.98	<0.0001
Agrimonia euparoria (1) intervention				
Leptin (ng/mL)	4.65 ± 0.9	2.64 ± 0.7	-43.3	ns
Adiponectin (µg/mL)	9.5 ± 1.03	10.1 ± 0.9	11.3 ± 6.2	ns
Agrimonia euparoria (2) *intervention*				
Leptin (ng/ml)				
BMI < 25	11.28 ± 1.71	14.09 ± 2.13	24.85	<0.05
BMI ≥ 25	23.05 ± 3.67	27.03 ± 4.02	17.25	ns
Adiponectin (ng/ml)				
BMI < 25	19.83 ± 1.72	16.54 ± 1.82	-16.63	<0.0001
BMI ≥ 25	15.56 ± 1.78	12.44 ± 1.52	-20.04	0.059

Data are presented as mean ± SEM.



Data are presented as mean ± SEM, * $P < 0.05$.

Figure 2. Interleukin 1 beta (IL-1beta) levels in subjects from SE intervention.

Discussion

Results obtained in the three pilot interventions indicated changes in selected markers for antioxidative and anti-inflammatory activity of two medicinal plants, which had been not well examined scientifically, although traditional medicine reported high healing potential. *S. ebulus* L. fruits are used in folk medicine for treatment of different inflammatory disorders [3, 36-38], for stimulation of the immune system against respiratory infectious diseases [3, 36, 37, 39]. They are known also for their diuretic, antiseptic and laxative activity and jam or juices prepared from fruits are used for amelioration of some gastrointestinal inflammatory disorders (colitis, hemorrhoids, etc.) [39, 40]. Traditional medicine points out the usage of *A. eupatoria* in cases of variety of inflammatory and metabolic diseases. For example, the herb is widely used for treatment of liver and gall bladder diseases, mild diarrhea, pulmonary, gastrointestinal inflammatory diseases, edemas and kidney diseases and even in diabetes or obesity. Thanks to its diuretic properties the herb is applied against atony of the bladder and disuria. Many other applications of agrimony infusions by Bulgarian folk medicine include cases of rheumatism, hemorrhoids, bleeding gums, varicose ulcers, laryngitis, pulmonary and cutaneous tuberculosis. The extracts could be used also externally as compresses or gargle [41].

The results obtained presented increased serum/plasma TAC at the end of the AE1, AE2 and SE interventions. SE induced serum TAC levels could be due to the good anthocyanin bioavailability. Plasma FARP levels did not change in the AE1 intervention, probably due to the assays' sensitivity to the different antioxidants present in serum.

In SE intervention the daily intake of polyphenols, including anthocyanins was 50.73 ± 2.71 mg/day QE. Earlier study compared SE fruits with other anthocyanin containing fruits establishing highest TAC and TPC of the aqueous and aqueous-methanolic SE fruit extracts [42]. It has been found that SE fruits are rich in phenolic acids, including caffeic acid and derivatives, chlorogenic acid and p-coumaric acid [43-45]. In addition, SE aqueous and aqueous-ethanolic fruit extracts were found to be rich in anthocyanins [45, 46]. Cyanidin-3-Osambubioside, the most abundant anthocyanin in elderberry fruits accounted for more than a half of all anthocyanins identified in the berries [47]. Recent studies established that TAC of SE fruit extracts correlated with TP and AC content [46]. Unlike many other polyphenols, anthocyanins can be absorbed in their glycosylated, as well as acetylated form in humans [48, 49]. Moreover, a correlation between serum TAC and anthocyanin levels has been established [48]. Serum antioxidant activity has been found to increase in rats and human subjects supplemented with polyphenol rich teas and anthocyanins [50, 51]. Additionally, Cao et al. [52] also reported that consumption of strawberries or red wine increased serum TAC measured with different methods. Grape extracts increased plasma antioxidant capacity by 20–25% in rats in absence and also in oxidative stimulation after 10-d consumption [53].

Agrimony is a poorly studied herb and a lot of its properties are not well examined yet. Daily intake of total polyphenols in AE1 and AE2 intervention studies was 94.71 mg/day QE and 106.94±4.05 mg/day QE, respectively. Although bioavailability of polyphenols is limited, the products of their intestinal metabolism overcome the intestinal barrier and reach the tissues, where they may exert biological activity [54]. Our study demonstrates for the first time the effect of agrimony tea consumption on antioxidant potential in humans in accordance with experimental data reported for animal models and in cell cultures. Similarly, green tea and green tea polyphenols intake increases plasma antioxidant capacity [55, 56]. Strong evidence about antioxidant potential of agrimony have been obtained using *in vivo* model of hepatic oxidative stress [57]. *In vitro* studies show that the antioxidant properties of the herb exert neuroprotective effects on in a model of induced oxidative neurotoxicity in HT22 hippocampal cells [58]. A single animal study [57] reports a beneficial effect of agrimony on antioxidant enzyme activity and hepatic glutathione levels.

There are some investigations on the phytochemical composition of different alcoholic and aqueous-alcoholic extracts of the herb [58-62]. Literature reports are related mainly to its antioxidant capacity [7, 61, 63-65]. The potential of agrimony plant extracts and fractions to scavenge ROS *in vitro* has been documented [7, 61, 63, 65] following different extraction procedures. Polyphenol composition of *A. eupatoria* extracts was studied in an attempt to explain biological effects of the plant [60, 63] and high amount of tannins, flavonoids and phenolic acids was established [59]. High correlation between polyphenolic content and total antioxidant activity (TOA) has been demonstrated in aqueous-alcoholic agrimony extracts applying various methods [60, 61, 63, 65].

Serum TT levels were increased at the end of the SE study. Similarly, serum TT concentrations have been established to increase significantly in Type 2 diabetic patients supplemented with fruit extracts [66, 67]. Polyphenols, which could act as free radical scavengers and reducing agents [68], along with other absorbed from the infusion constituents with antioxidant properties, most likely contribute to maintain the reduced state of thiol groups reported by this study. Unexpectedly, both AE interventions resulted in a significant decrease of plasma TT levels. It could be suggested that some AE extract constituents may have specific effect on plasma thiol balance. On the other hand, GSH levels significantly

increased both in normal and overweight subjects in the AE2 study. More studies are needed to elucidate the mechanism by which plant constituents could have an impact on plasma thiol and glutathione balance. In a previous study we found that AE extract stimulated GSH synthesis in cultured cells (3T3-L1 preadopocytes) [69]. GSH levels in erythrocyte lysates did not change in SE study (1047.12±65.75 μM/L at day 30 vs. 1052.24±59.42 μM/L at day 30).

UA is one of the major contributors to serum antioxidant capacity [70]. We did not establish any increase in serum/plasma UA levels after 25/30 d of AE and SE infusion intake (data not shown). This observation, together with the strongly increased plasma GSH levels in the AE2 intervention, suggest that plant antioxidants may interfere with plasma/serum TAC via mechanisms involving direct antioxidant activity of absorbed plant constituents and their metabolites, as well as induction of glutathione biosynthesis.

Whereas neither TT, nor GSH or UA levels were comparably and reliably changed, we could consistently identify response in all three interventions by measuring significant increase in blood TAC levels in the groups. Thus the established small but clearly detectable TAC changes allow us to suggest it as a biomarker for antioxidant capacity applicable in food intervention studies with healthy subjects who flexibly respond to changes in the environment resuming their metabolic balance.

Polyphenols are present in a variety of plants utilized as important components of both human and animal diets [71-73]. In addition to their well-known antioxidant effects, they also have been reported to possess anti-inflammatory, anti-carcinogenic, anti-viral, anti-ulcer, anti-apoptotic properties, and even lipid lowering activity [74, 75].

S. ebulus and *A. eupatoria* are well known as folk remedies for many inflammatory conditions [41, 76, 77]. The anti-inflammatory activity of the herbs has been demonstrated by their inhibitory effects on proinflammatory cytokines production such as TNF α, and IL-6 in cell cultures models of inflammation [4, 45, 78].

Cytokines such as TNFα, IL-1β and IL-6 play an important role in triggering and transmission of inflammatory response. Furthermore, they are reported to induce synthesis of C-reactive protein (CRP), an acute-phase protein produced during infections and inflammation, both *in vitro* and *in vivo* [79]. The results from the presented pilot studies demonstrated that everyday consumption of SE and AE infusion contributed to reduced concentration of the measured proinflammatory factors at the end of the interventions. This result is in accordance with recent epidemiologic study reporting that dietary intake of antioxidants was inversely associated with IL-1β and TNF-α levels [80]. To our knowledge, these results are the first scientific evidence for anti-inflammatory properties of these plants in humans, in support folk medicine data.

Dietary and medicinal plants contain multifunctional compounds with polyphenolic nature including anthocyanins, also recognized to suppress inflammation thus efficiently protecting vascular function, and preventing CVD and metabolic diseases [74, 81-83]. The beneficial anti-inflammatory effects of polyphenols have been studied both *in vitro* and *in vivo*. Their potential to inhibit expression of TNF α, IL-6 and other mediators of inflammation has been demonstrated in different cell types, as well as in rodent models of inflammation [84-88]. In various interventional studies it has been found that the diet enriched with polyphenols is inversely associated with blood inflammation markers such as CRP, IL-6 and TNFα [89-91].

The polyphenolic profile of AE aqueous-alcoholic extract includes compounds such as procyanidins, kaempferol and quercetin glycosides that are recognized to possess anti-

inflammatory properties [60]. Several studies reported that anthocyanins, which are the major group of polyphenolic compounds in SE fruits, modulate the inflammatory responses in cell cultures and animal models by inhibiting the secretion of pro-inflammatory cytokines such as IL-8, MCP-1, IL-1β, IL-6, and TNFα [81, 92-96]. Consumption of SE fruit infusion in Intervention 1 resulted in significantly reduced serum CRP (Table 4). Similar results have been reported after intervention with diet rich in anthocyanins in healthy subjects [97], while agrimony tea did not affect significantly CRP concentrations (Intervention 2 and 3). Seemingly, SE along with pro-inflammatory cytokines affects also the production of acute-phase proteins. One explanation to this could be the different phytochemical composition of the two plants, specifically to anthocyanins contained in SE tea. Higher anthocyanin intake is associated with lower hs-CRP concentrations [98].

The main driver of CRP release from hepatocytes is believed to be IL-6 [99, 100]. In SE intervention IL-6 levels did not change significantly and this was accompanied by a decrease in CRP. In AE interventions (teas differed in preparation and in composition) IL-6 was either decreased (intervention 2) or not changed (intervention 3) and CRP remained within the reference values in both interventions. These effects could be explained by human metabolic flexibility which allows to maintain body's homeostasis exhibiting buffer-like capacities to respond to environmental stress, including response to food intake. As the volunteers involved in all three studies were healthy subjects it was not unexpected that significant effect could be measured in these acute-phase proinflammatory factors. Their values stayed within the reference ranges throughout the whole intervention, also for the group with BMI>25. At the same time, cultured preadipocyte and macrophage cells treated solely with SE or AE extracts responded with an increased IL-6 and TNF alpha production, which was decreased by the pretreatment with the herbal extracts followed by proinflammatory stimuli, such as LPS, conditioned medium or ethanol [101]. Upon 30 days of agrimony or dwarf elder tea consumption, no specific decrease of the TNFα concentrations was observed either, except for the fact that at the end of the interventions subjects had values below the detection level of the assay serum TNFα levels. The controversial effects on cytokine levels could be explained by the immunomodulatory activity of the plant polyphenols as earlier reported for various extracts, fractions or biologically active plant components, which could increase baseline cytokine levels in a healthy immune system and inhibit their activity in case of induced inflammation [102-106].

Folk medicine from different countries reports strong anti-inflammatory potential of *A. eupatoria* and *S. ebulus* preparations and various homeopathic remedies based on this knowledge are produced. The above discussed effects are the first scientific evidence received from human studies in support to traditional medicine usage of agrimony and dwarf elder fruits for prevention or treatment of inflammation.

In addition to the above effects, IL-6 is known to antagonize the secretion of adiponectin [107], and the pro-inflammatory TNFα factor blocks the synthesis of adiponectin and interferes with insulin signaling [108]. Adiponectin known for its high anti-inflammatory power, is known also to inhibit production of ROS [109]. High adiponectin levels are associated with higher insulin sensitivity [110]. High plasma levels of leptin have been positively correlated with obesity and metabolic syndrome [111] and administration of leptin was associated with increased CRP levels indicating an inflammatory effect [112]. Leptin is secreted mainly by adipocytes proportionally to the fat tissue mass and in inflammation acts directly on macrophages to increase phagocytic activity and ROS production.

AE1 and SE interventions did not cause significant reduction in body weight and related BMI. Although that leptin secretion is known to correlate with fat mass, SE tea consumption resulted in a decrease in both adiponectin and leptin levels at the end of the intervention. Second AE intervention resulted in significant decrease in BMI, while leptin levels increased in the group of BMI < 25 and adiponectin was reduced significantly. AE1 did not affect adipokine levels significantly. In accordance to our finding from SE intervention study Wang et al. [113] in their review on effects of dietary polyphenols reported similar decrease in these two adipokines. Other plant substances may also contribute to this effect. Yang et al. [114] have shown that treatment with berberine (an isoquinoline derivative alkaloid isolated from various kinds of medicinal herbs), resulted in a major inhibition of human preadipocyte differentiation and leptin and adiponectin secretion accompanied by downregulation of PPARγ2, C/EBPα, adiponectin, and leptin mRNA expression [114]. These results suggest that SE tea possibly improves insulin sensitivity by inhibiting fat store and adjusting adipokine profile in humans. In support to this earlier published data [115] report improved lipid profile in healthy volunteers upon SE fruit infusion consumption: significant decrease in triglycerides, total cholesterol and LDL-cholesterol. Changes in body weight not necessarily relate to changes in fat mass. Evaluation of fat tissue mass in such intervention studies could provide more evidence on the mechanisms of leptin metabolism. The different chemical composition, as well as the good health status of the participants could be the prerequisites that determine the moderate effect of agrimony on adiponectin and leptin levels.

Conclusion

The results in these human intervention studies with *A. eupatoria* and *S. ebulus* indicate that these plants have a potential to improve plasma antioxidant capacity, as well as to modulate inflammatory cytokines and adipokines levels. Although some of the effects of both plants on cytokine and adipokine were not definite, all of the three interventions resulted in strongly increased plasma antioxidant capacity. According to our knowledge these are the first scientific data received from human studies revealing mechanisms probably involved in the healing power of these plants. Thus we may conclude that supplementation with agrimony and elderberry teas could be an option to combat oxidative stress related conditions, including low-grade inflammation and metabolic disturbances.

References

[1] Stoyanov, N; Kitanov, B. *Divi polezni rasteniya v Bulgaria*. Bulgarian Academy of Sciences, Sofia, 1960.

[2] Gurib-Fakim, A. Medicinal plants: Traditions of yesterday and drugs of tomorrow. *Mol Aspects Med*., 2006, 27, 1-93.

[3] Nikolov, S. *Specializirana enciklopediya na lechebnite rasteniya. Bulgaria*. Trud, Sofia, 2007.

[4] Kiselova-Kaneva, Y. *Biologichni efekti na oksidativniya stres i bilkite kato sredstvo za protivodeystvie*. Antida, Varna, 2013.

[5] Cai, Y; Luo, Q; Sun, M; Corke, H. Antioxidant activity and phenolic compounds of 112 traditional Chinese medicinal plants associated with anticancer. *Life Sci.*, 2004, 74, 2157-84.

[6] Miliauskas, G; Venskutonis, P; van, Beek, T. Screening of radical scavenging activity of some medicinal and aromatic plant extracts. *Food Chem*., 2004, 85, 231-7.

[7] Ivanova, D; Gerova, D; Chervenkov, T; Yankova, T. Polyphenols and antioxidant capacity of Bulgarian medicinal plants. *J Ethnopharmacol*. 2005, 96, 145-50.

[8] Kiselova, Y; Ivanova, D; Chervenkov, T; Gerova, D; Galunska, B; Yankova, T. Correlation between the in vitro antioxidant activity and polyphenol content of aqueous extracts from Bulgarian herbs. *Phytother Res*. 2006, 20, 961-5.

[9] Surveswaran, S; Cai, Y; Corke, H; Sun, M. Systematic evaluation of natural phenolic antioxidants from 133 Indian medicinal plants. *Food Chem*., 2007, 102(3), 938-53.

[10] Kim, M; Park, J; Lim, S. Antioxidant activity and cell toxicity of pressurised liquid extracts from 20 selected plant species in Jeju, Korea. *Food Chem.*, 2010, 122, 546-52.

[11] Asgharpour, F; Pouramir, M; Khalilpour, A; Alamdar, SA; Rezaei, M. Antioxidant Activity and Glucose Diffusion Relationship of Traditional Medicinal Antihyperglycemic Plant Extracts. *Int J Mol Cell Med*., 2013, 2(4), 169-76.

[12] Shaikh, R; Pund, M; Dawane, A; Iliyas, S. Evaluation of Anticancer, Antioxidant, and Possible Anti-inflammatory Properties of Selected Medicinal Plants Used in Indian Traditional Medication. *J Tradit Complement Med*., 2014, 4(4), 253-7.

[13] Keaney, J; Larson, M; Vasan, R; Wilson, P; Lipinska, I; Corey, D; Massaro, J; Sutherland, P; Vita, J; Benjamin, E. Obesity and systemic oxidative stress: clinical correlates of oxidative stress in the Framingham study. *Atheroscler Thromb Vasc Biol.*, 2003, 23, 434-9.

[14] Fujita, K; Nishizawa, H; Funahashi, T; Shimomura, I; Shimabukuro, M. Systemic oxidative stress is associated with visceral fat accumulation and the metabolic syndrome. *Circ J*., 2006, 70, 1437-42.

[15] Grattagliano, I; Palmieri, V; Portincasa, P; Moschetta, A; Palasciano, G. Oxidative stress-induced risc factors associated with the metabolic syndrome: a unifying hypothesis. *J Nutr Biochem.*, 2008, 19, 491-504.

[16] Aronson, D; Rayfield, E. How hyperglycemia promotes atherosclerosis: molecular mechanisms. *Cardiovasc Diabetol.*, 2002, 1, 1-10.

[17] Salvadori, A; Fanari, P; Fontana, M; Buontempi, L; Saezza, A; Baudo, S; Miserocchi, G; Longhini, E. Oxygen uptake and cardiac performance in obese and normal subjects during exercise. *Respiration*., 1999, 66, 25-33.

[18] Vincent, H; Powers, S; Dirks, A; Scarpace, P. Mechanism for obesity-induced increase in myocardial lipid peroxidation. *Int J Obes Relat Metab Disord*., 2001, 25, 378-88.

[19] Beltowski, J; Wojcicka, G; Gorny, D; Marciniak, A. The effect of dietary-induced obesity on lipid peroxidation, antioxidant enzymes and total plasma antioxidant capacity. *J Physiol Pharmacol*., 2000, 51(2), 883-96.

[20] Moor, de, Burgos, A; Wartanowicz, M; Ziemlanski, S. Blood vitamin and lipid levels in overweight and obese women. *Eur J Clin Nutr*., 1992, 46, 803-8.

[21] Ohrvall, M; Tengblad, S; Vessby, B. Lower tocopherol serum levels in subjects with abdominal adiposity. *J Intern Med*., 1993, 234, 53-60.

[22] Wallstrom, P; Wirfalt, E; Lahmann, P; Gullberg, B; Janzon, L; Berglund, G. Serum concentrations of beta-carotene and alphatocopherol are associated with diet, smoking, and general and central adiposity. *Am J Clinic Nutr*., 2001, 73(4), 777-85.

[23] Davi, G; Guagnano, M; Ciabattoni, G; Basili, S; Falco, A; Marinopiccoli, M; Nutini, M; Sensi, S; Patrono, C. Platelet activation in obese women: role of inflammation and oxidant stress. *J Am Med Assoc*., 2002, 288, 2008-14.

[24] Fernandez-Real, J; Broch, M; Vendrell, J; Ricart, W. Insulin resistance, inflammation, and serum fatty acid composition. *Diabetes Care*., 2003, 26, 1362-8.

[25] Saito, I; Yonemasu, K; Inami, F. Association of body mass index, body fat, and weight gain with inflammation markers among rural residents in Japan. *Circ J*., 2003, 67, 323-9.

[26] Egan, B; Greene, E; Goodfriend, T. Insulin resistance and cardiovascular disease. *Am J Hypertens*. 2001, 14, 116S-25S.

[27] Wheatcroft, S; Williams, I; Shah, A; Kearney, M. Pathophysiological implications of insulin resistance on vascular endothelial function. *Diabet Med*., 2003, 20, 255-68.

[28] Bekyarova, G; Ivanova, D; Madjova, V. Molecular mechanisms associating oxidative stress with endothelial disfunction in the development of various vascular complications in diabetes mellitus. *Folia Med*., 2007, XLIX(3&4), 13-9.

[29] Bouloumie, A; Marumo, T; Lafontan, M; Busse, R. Leptin induces oxidative stress in human endothelial cells. *FASEB J*., 1999, 13, 1231-8.

[30] Vincent, H; Taylor, A. Biomarkers and potential mechanisms of obesity-induced oxidant stress in humans. *Int J Obes*., 2006, 30, 400-18.

[31] Re, R; Pelegrini, N; Proteggente, A; Pannala, A; Yang, M; Rice-Evans, C. Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radic Biol Med*., 1999, 26, 1231-7.

[32] Benzie, F; Strain, J. Ferric Reducing/Antioxidant Power Assay: Direct Measure of Total antioxidant Activity of Biological Fluids and Modified Version for Simultaneous Measurement of Total Antioxidant Power and Ascorbic Acid Concentration. *Methods Enzymol*., 1999, 299, 15-23.

[33] Singleton, V; Rossi, J. Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. *Am J Enol Vitic*., 1965, 16, 144-58.

[34] Giusti, MM; Wrolstad, RE. Characterization and measurement of anthocyanins by UV-visible spectroscopy. *Curr Protocols Food Analyt Chem*., 2001, F1.2.1-F1.2.13.

[35] Kiselova, Y; Chervenkov, T; Ivanova, D; Galunska, B; Gerova, D; Yankova, T. Oxidative stress assessment by determination of the amount of total thiols and lipid hydroperoxides in serum. *Sixth International Symposium Technomat Infotel*., 2004, 1, 99-106.

[36] Kultur, S. Medicinal plants used in Kırklareli Province (Turkey). *J Ethnopharmacol*., 2007, 111, 341-64.

[37] El Beyrouthy, M; Arnold, N; Delelis-Dusollier, A; Dupont, F. Plants used as remedies antirheumatic and antineuralgic in the traditional medicine of Lebanon. *J Ethnopharmacol*., 2008, 120, 315-34.

[38] Saric-Kundalic, B; Dobes, C; Klatte-Asselmeyer, V; Saukel, J. Ethnobotanical study on medicinal use of wild and cultivated plants in middle, south and west Bosnia and Herzegovina. *J Ethnopharmacol*., 2010, 131, 33-55.

[39] Petkov, V. *Savremenna fitoterapiya*. Sofia, Medicine, 1982.

[40] Dimkov, P. *Balgarska narodna medicina*. Bulgarian Academy of Sciences, Sofia, 1977.
[41] Pamukov, D; Ahtardziev, H. *Prirodna apteka*. Zemizdat, Sofia, 1989.
[42] Kiselova, Y; Marinova, S; Ivanova, D; Gerova, D; Galunska, B; Chervenkov, T; Yankova, T. Investigation of the antioxidative potential of edible wild Bulgarian fruits. *Proceedings of the Balkan Scientific Conference of Biology in Plovdiv (Bulgaria)*., 2005, 233-9.
[43] Ebrahimzadeh, MA; Pourmorad, F; Bekhradnia, AR. Iron chelating activity, phenol and flavonoid content of some medicinal plants from Iran. *Afr J Biotechnol*., 2008, 7, 3188-92.
[44] Ebrahimzadeh, MA; Nabavi, SF; Nabavi, SM; Pourmorad, F. Nitric oxide radical scavenging potential of some Elburz medicinal plants. *Afr J Biotechnol*., 2010, 9, 5212-7.
[45] Shokrzadeh, M; Saeedi, Saravi, S. The chemistry, pharmacology and clinical properties of *Sambucus ebulus*: A review. *J Med Plants Res*., 2010, 4(2), 95-103.
[46] Tasinov, O; Kiselova-Kaneva, Y; Ivanova, D. Antioxidant activity, total polyphenol content and anthocyanins content of *Sambucus ebulus* L. aqueous and aqueous-ethanolic extracts depend on the type and concentration of extragent. *Sci Technol*., 2012, 2, 37-41.
[47] Anton, AM; Pintea, AM; Rugina, DO; Sconta, ZM; Hanganu, D; Vlase, L; Benedec, D. Preliminary studies on the chemical characterization and antioxidant capacity of polyphenols from Sambucus sp. *Dig J Nanomater Bios*., 2013, 8, 972-80.
[48] Mazza, G; Kay, C; Cottrell, T; Holub, B. Absorption of anthocyanins from blueberries and serum antioxidant status in human subjects. *J Agric Food Che*., 2002, 50, 7731-7.
[49] Manach, C; Scalbert, A; Morand, C; Rémésy, C; Jiménez, L. Polyphenols: food sources and bioavailability. *Am J Clin Nutr*., 2004, 79, 727-47.
[50] Talavera, S; Felgines, C; Texier, O; Besson, C; Mazur, A; Lamaison, JL; Remesy, C. Bioavailability of a bilberry anthocyaninextract and its impact on plasma antioxidant capacityin rats. *J Sci Food Agric*., 2006, 86, 90-7.
[51] Mertens-Talcott, SU; Rios, J; Jilma-Stohlawetz, P; Pacheco-Palencia, LA; Meibohm, B; Talcott, ST; Derendorf, H. Pharmacokinetics of anthocyanins and antioxidant effects after the consumption of anthocyanin-rich acai juice and pulp (Euterpe oleracea Mart.) in human healthy volunteers. *J Agric Food Chem*., 2008, 56, 7796-802.
[52] Cao, G; Russell, RM; Lischner, N; Prior, RL. Serum antioxidant capacity is increased by consumption of strawberries, spinach, red wine or vitamin C in elderly women. *J Nutr*., 1998, 128, 2383-90.
[53] Lionetto, MG; Giordano, ME; Calisi, A; Erroi, E; De, Nuccio, F; Schettino, T. Effect of the daily ingestion of a purified anthocyanin extract from grape skin on rat serum antioxidant capacity. *Physiol Res*., 2011, 60, 637-45.
[54] Pandey, K; Rizvi, S. Plant polyphenols as dietary antioxidants in human health and disease. *Oxid Med Cell Longev*., 2009, 2(5), 270-8.
[55] Mejia, EG; Ramirez-Mares, MV; Puangpraphant, S. Bioactive components of tea: Cancer, inflammation and behavior. *Brain Behav Immun*., 2009, 23, 721-31.
[56] Bogdanski, P; Suliburska, J; Szulinska, M; Stepien, M; Pupek-Musialik, D; Jablecka, A. Green tea extract reduces blood pressure, inflammatory biomarkers, and oxidative stress and improves parameters associated with insulin resistance in obese, hypertensive patients. *Nutr Res*., 2012, 32(6), 421-7.

[57] Gião, M; Pestana, D; Faria, A; Guimarães, J; Pintado, M; Calhau, C; Azevedo, I; Malcata, F. Effects of extracts of selected medicinal plants upon hepatic oxidative stress. *J Med Food*., 2010, 13, 131-6.

[58] Lee, K; Hwang, L; Jeong, E; Kim, S; Kim, Y; Sung, S. Effect of neuroprotective flavonoids of *Agrimonia eupatoria* on glutamate-induced oxidative injury to HT22 Hippocampal cells. *Biosci Biotechnol Biochem*., 2010, 74(8), 1704-6.

[59] Shabana, M; Weglarz, Z; Geszprych, A. Phenolic constituents of agrimony (*Agrimonia eupatoria* L.) herb. *Herb Pol Y*., 2003, 49, 24-8.

[60] Correia, H; González-Paramás, A; Amaral, M; Santos-Buelga, C; Batista, M. Polyphenolic profile characterization of *Agrimonia eupatoria* L. by HPLC with different detection devices. *Biomed Chromatogr*., 2006, 20, 88-94.

[61] Venskutonis, P; Skemaite, M; Sivik, B. Assessment of radical scavenging capacity of Agrimonia extracts isolated by supercritical carbon dioxide. *J Supercritical Fluids*., 2008, 45, 231-7.

[62] Zhang, J; Chen, Y. Studies on the lowering blood sugar substances from Agrimony. *J Chinese Med Materials*., 2009, 32, 1537-9.

[63] Kiselova, Y; Galunska, B; Ivanova, D; Yankova, T. Total antioxidant capacity and polyfenol content correlation in aqueous-alcoholic plant extracts used in phytotherapy. *Scr Sci Med*., 2004, 36, 11-3.

[64] Correia, H; Batista, M; Dinis, T. The activity of an extract and fraction of *Agrimonia eupatoria* L. against reactive species. *Biofactors*., 2007, 29, 91-104.

[65] Venskutonis, P; Škėmaitė, M; Ragažinskienė, O. Radical scavenging capacity of *Agrimonia eupatoria* and *Agrimonia procera*. *Fitoter*., 2007, 78, 166-68.

[66] Bonina, FP; Leotta, C; Scalia, G; Puglia, C; Trombetta, D; Tringali, G; Roccazzello, AM. Evaluation of oxidative stress in diabetic patients after supplementation with a standardised red orange extract. *Diabetes Nutr Metab*., 2002, 15, 14-9.

[67] Rosenblat, M; Hayek, T; Aviram, M. Anti-oxidative effects of pomegranate juice (PJ) consumption by diabetic patients on serum and on macrophages. *Atheroscler*., 2006, 187, 363-71.

[68] Bräunlich, M; Slimestad, R; Wangensteen, H; Brede, C; Malterud, KE; Barsett, H. Extracts, anthocyanins and procyanidins from Aronia melanocarpa as radical scavengers and enzyme inhibitors. *Nutrients*., 2013, 5, 663-78.

[69] Ivanova, D; Tasinov, O; Vankova, D; Kiselova-Kaneva, Y. *Agrimonia eupatoria* L. extract modulates glutamate-cysteine ligase and glutathione peroxidase expression in 3T3-L1 cells. *Bulg J Agric Sci*., 2013, 2(19), 171-4.

[70] Maxwell, SR; Thomason, H; Sandler, D; Leguen, C; Baxter, MA; Thorpe, GH; Jones, AF; Barnett, AH. Antioxidant status in patients with uncomplicated insulin-dependent and non-insulin-dependent diabetes mellitus. *Eur J Clin Invest*., 1997, 27, 484-90.

[71] Bravo, L. Polyphenols: Chemistry, dietary sources, metabolism, and nutritional significance. *Nutr Rev*., 1998, 56, 317-33.

[72] Chung, KT; Wong, TY; Wei, CI; Huang, YW; Lin, Y. Tannins and human health: A review. *Crit Rev Food Sci Nutr*., 1998, 38, 421-64.

[73] Crozier, A; Burns, J; Aziz, AA; Stewart, AJ; Rabiasz, HS; Jenkins, GI; Edwards, CA; Lean, ME. Antioxidant flavones from fruits, vegetables and beverages: measurements and bioavailability. *Biol Res*., 2000, 33, 79-88.

[74] Raj, Narayana, K; Sripal, Reddy, M; Chaluvadi, M; Krishna, D. Bioflavonoids classification, pharmacological, biochemical effects and therapeutic potential. *Ind J Pharmacol*., 2001, 33, 2-16.

[75] Panickar, KS; Anderson, RA. Effect of polyphenols on oxidative stress and mitochondrial dysfunction in neuronal death and brain edema in cerebral ischemia. *Int J Mol Sci*., 2011, 12(11), 8181-207.

[76] Fabricant, D; Farnsworth, N. The Value of Plants Used in Traditional Medicine for Drug Discovery. *Environ Health Perspect*., 2001, 109(1), 69-75.

[77] Cakilcioglu, U; Turkoglu, I. An ethnobotanical survey of medicinal plants in Sivrice (Elazığ-Turkey). *J Ethnopharmacol*., 2010, 132, 165-75.

[78] Bae, H; Kim, HJ; Shin, M; Lee, H; Yin, CS; Ra, J; Kim, J. Inhibitory effect of Agrimoniae Herba on lipopolysaccharide-induced nitric oxide and proinflammatory cytokine production in BV2 microglial cells. *Neurol Res*., 2010, 1, 53-7.

[79] Sheldon, J; Riches, P; Gooding, R; Soni, N; Hobbs, JR. C-Reactive Protein and Its Cytokine Mediators in Intensive-Care Patients. *Clin. Chem*., 1993, 39(1), 147-50.

[80] Luu, HN; Wen, W; Li, H; Dai, Q; Yang, G; Cai, Q; Xiang, YB; Gao, YT; Zheng, W; Shu, XO. Are Dietary Antioxidant Intake Indices Correlated to Oxidative Stress and Inflammatory Marker Levels? *Antioxid Redox Signal*., 2015. [Epub ahead of print].

[81] Youdim, KA; McDonald, J; Kalt, W; Joseph, JA. Potential role of dietary flavonoids in reducing microvascular endothelium vulnerability to oxidative and inflammatory insults (small star, filled). *J Nutr Biochem*., 2002, 13, 282-8.

[82] Russell, JA. Vasopressin in septic shock. *Crit Care Med*., 2007, 35, 609-15.

[83] González-Gallego, J; García-Mediavilla, MV; Sánchez-Campos, S; Tuñón, MJ. Fruit polyphenols, immunity and inflammation. *Br J Nutr*., 2010, 104, S15-S27.

[84] Varilek, GW; Yang, F; Lee, YE; William, JS; Zhong, ZO; Westberry, FK; McClain, CJ. Green tea plyphenol extracts ayyenuates inflammation in interleukin-2-deficient mice, a model of autoimmunity. *J Nutr*., 2001, 131, 2034-9.

[85] Xia, J; Song, X; Bi, Z; Chu, W; Wan, Y. UV-induced NF-kappaB activation and expression of IL-6 is attenuated by (–)-epigallocatechin-3-gallate in cultured human keratinocytes in vitro. *Int J Mol Med*., 2005, 16, 943-50.

[86] Ebrahimzadeh, MA; Mahmoudi, M; Pourmorad, F; Saeidnia, S; Salimi, E. Anti-inflammatory and anti-nociceptive properties of fractionated extracts in different parts of *Sambucus ebulus*. *J Mazandaran Uni Med Sci*., 2006, 16(54), 35-42.

[87] Kim, SH; Chang-Duk, J; Suk, K; Choi, BJ; Lim, H; Park, S; Lee, SH; Shin, HY; Kim, DK; Shink, TY. Gallic Acid Inhibits Histamine Release and Pro-inflammatory Cytokine Production in Mast Cells. *Toxicolog Sci*., 2006, 91(1), 123-31.

[88] Ebrahimzadeh, MA; Mahmoudi, M; Karami, M; Saeedi, S; Ahmadi, AH; Salimi, E. Separation of active and toxic portions in *Sambucus ebulus*. *Pak J Biol Sci*., 2007, 10, 4171-3.

[89] Nanri, A; Yoshida, D; Yamaji, T; Mizoue, T; Takayanagi, P; Kono, S. Dietary patterns and C-reactive protein in Japanese men and women. *Am J Clin Nutr*., 2008, 87, 1488-96.

[90] Salas-Salvadó, J; Garcia-Arellano, A; Estruch, R; Marquez-Sandoval, F; Corella, D; Fiol, M; Gómez-Gracia, E; Viñoles, E; Arós, F; Herrera, C; Lahoz, C; Lapetra, J; Perona, JS; Muñoz-Aguado, D; Martínez-González, MA; Ros, E. Components of the

Mediterranean-type food pattern and serum inflammatory markers among patients at high risk for cardiovascular disease. *Eur J Clin Nutr*., 2008, 62, 651-9.

[91] Holt, EM; Steffen, LM; Moran, A; Basu, S; Steinberger, J; Ross, JA; Hong, CP; Sinaiko, AR. Fruit and vegetable consumption and its relation to markers of inflammation and oxidative stress in adolescents. *J AmDiet Assoc*., 2009, 109(3), 414-21.

[92] Tsuda, T; Horio, F; Osawa, T. Cyanidin 3-O-beta-D-glucoside suppresses nitric oxide production during a zymosan treatment in rats. *J Nutr Sci Vitaminol (Tokyo)*., 2002, 48, 305-10.

[93] Atalay, M; Gordillo, G; Roy, S; Rovin, B; Bagchi, D; Bagchi, M; Sen, CK. Anti-angiogenic property of edible berry in a model of hemangioma. *FEBS Lett*., 2003, 544, 252-7.

[94] Wang, J; Mazza, G. Effects of anthocyanins and other phenolic compounds on the production of tumor necrosis factor alpha in LPS/IFN-gamma-activated RAW 264.7 macrophages. *J Agric Food Chem*., 2002, 50, 4183-9.

[95] Herath, HM; Takano-Ishikawa, Y; Yamaki, K. Inhibitory effect of some flavonoids on tumor necrosis factor-alpha production in lipopolysaccharide-stimulated mouse macrophage cell line J774.1. *J Med Food*., 2003, 6, 365-70.

[96] Cimino, F; Ambra, R; Canali, R; Saija, A; Virgili, F. Effect of cyanidin-3-O-glucoside on UVB-induced response in human keratinocytes. *J Agric Food Chem*., 2006, 54, 4041-7.

[97] Kelley, DS; Rasooly, R; Jacob, RA; Kader, AA; Mackey, BE. Consumption of Bing sweet cherries lowers circulating concentrations of inflammation markers in healthy men and women. *J Nutr*., 2006, 136, 981-6.

[98] Jennings, A; Welch, AA; Spector, T; Macgregor, A; Cassidy, A. Intakes of anthocyanins and flavones are associated with biomarkers of insulin resistance and inflammation in women. *J Nutr*., 2014, 144(2), 202-8.

[99] Heinrich, PC; Castell, JV; Andus, T. Interleucin-6 and the acute phase response. *Biochem J*., 1990, 265, 621-36.

[100] Ridker, PM; Hennekens, CH; Buring, JE; Rifai, N. C-reactive protein and other markers of inflammation in the prediction of cardiovascular disease in women. *N Engl J Med*., 2000, 342(12), 836-43.

[101] Ivanova, D. *Antioksidantna aktivnost na bulgarski lechebni rasteniya i svarzani s neya biologichni efekti*. DSc Thesis. MU, Varna, 2013.

[102] Kim, KH; Lee, YS; Jung, IS; Park, SY; Chung, HY; Lee, IR; Yun, YS. Acidic polysaccharide from Panax ginseng, ginsan, induces Th1 cell and macrophage cytokines and generates LAK cells in synergy with rIL-2. *Planta Med*., 1998, 64(2), 110-5.

[103] Hsu, HY; Hua, KF; Lin, CC; Lin, CH; Hsu, J; Wong, CH. Extract of Reishi polysaccharides induces cytokine expression via TLR4-modulated protein kinase signaling pathways. *J Immunol*., 2004, 173(10), 5989-99.

[104] Spelman, K; Burns, J; Nichols, D; Winters, N; Ottersberg, S; Tenborg, M. Modulation of cytokine expression by traditional medicines: a review of herbal immunomodulators. *Altern Med Rev*., 2006, 11(2), 128-50.

[105] Sullivan, AM; Laba, JG; Moore, JA; Lee, TD. Echinacea-induced macrophage activation. *Immunopharmacol Immunotoxicol*., 2008, 30(3), 553-74.

[106] Ghildyal, P; Grønhaug, TE; Rusten, A; Skogsrud, M; Rolstad, B; Diallo, D; Michaelsen, TE; Inngjerdingen, M; Paulsen, BS. Chemical composition and immunological activities of polysaccharides isolated from the Malian medicinal plant Syzygium guineense. *J Pharmacol Phytother*., 2010, 2(6), 76-85.

[107] Fonseca-Alaniz, MH; Takada, J,Alonso-Vale, MI; Lima, FB. Adipose tissue as an endocrine organ, From theory to practice. *J Pediatr*., 2007, 83(5), 192-203.

[108] Lastra, G; Manrique, CM; Hayden, MR. The role of beta-cell dysfunction in the cardiometabolic syndrome. *J Cardiometab Syndr*., 2006, 1, 41-6.

[109] Sánchez, F; García, R; Alarcón, F; Cruz, M. Adipocytokines, adipose tissue and its relationships with immune system cells. *Méd Méx*., 2005, 141, 505-12.

[110] Qiao, L; Shao, J. SIRT1 regulates adiponectin gene expression through Foxo1-C/enhancerbinding protein alpha transcriptional complex. *J Biol Chem*., 2006, 281, 39915-24.

[111] Ntyintyane, L; Panz, V; Raal, FJ; Gill, G. Leptin, adiponectin, and high sensitivity C-reactive protein in relation to the metabolic syndrome in urban South African blacks with and without coronary artery disease. *Metab Syndr Relat Disord*., 2009, 7, 243-8.

[112] Steffes, M; Gross, M; Lee, D; Schreiner, P; Jacobs, D. Adiponectin, visceral fat, oxidative stress and early macrovascular disease: The coronary artery risk development in young adults study. *Obes*., 2006,14, 319-26.

[113] Wang, S; Moustaid-Moussa, N; Chen, L; Mo, H; Shastri, A; Sua, R; Bapat, P; Kwun, I; Shen, C. Novel insights of dietary polyphenols and obesity. *J Nutr Biochem*., 2014, 25, 1-18.

[114] Yang, J; Yin, J; Gao, H; Xu, L; Wang, Y; Xu, L; Li, M. Berberine Improves Insulin Sensitivity by Inhibiting Fat Store and Adjusting Adipokines Profile in Human Preadipocytes and Metabolic Syndrome Patients. *J Evid Based Complementary Altern Med*., 2012, 1-9.

[115] Ivanova, D; Tasinov, O; Kiselova-Kaneva, Y. Improved lipid profile and increased serum antioxidant capacity in healthy volunteers after *Sambucus ebulus* L. fruit infusion consumption. *Int J Food Sci Nutr*., 2014, 65(6), 740-4.

In: Human Health and Nutrition
Editor: Sergej M. Ostojic
ISBN: 978-1-63482-823-9

Chapter VIII

The Role of Edible Wild Plants in Human Nutrition: Ethnobotanical Uses and Nutritional Properties. The Case Study of Umbria, Central Italy

***Mara Bodesmo*[*1], *Angela Maurizi*[2], *Isabella Di Leonardo*[1] *and Aldo Ranfa*[1]**
[1]Dipartimento di Ingegneria Civile ed Ambientale,
University of Perugia, Italy
[2]Dipartimento di Scienze farmaceutiche, University of Perugia, Italy

Abstract

This chapter deals the importance of edible wild plants as regards not only the ethnobotanical uses but also their value in human nutrition. Particularly in the past, these species played an important supporting role in daily nutrition and provided a balanced intake of oligoelements, vitamins and minerals. However, aspects that have attracted recent interest are the nutraceutical properties of these species and the health benefits that derive from their habitual consumption. Data on the use of 60 species were collected through informed consent ad hoc semi-structured interviews with local informants. Furthermore, the nutraceutical analysis centered on some of the commonly used wild edible plants demonstrates how these species contain many of the so-called minor nutrients, such as antioxidant vitamins and polyphenols.

Keywords: Edible Wild Plants; Ethnobotanical analysis; Safeguarding of biodiversity; Antioxidant properties; Traditional use

[*] Corresponding Author address: Email: bodesmo@gmail.com

Introduction

In recent years, wild plants have been the objects of study worldwide, as many possess new and unusual therapeutic and nutritional properties [1-3]. In some rural areas of Southern Europe the use of numerous edible wild plants has survived, as they have always been seen as a source of simple, healthy food. Indeed the beneficial effects of the Mediterranean diet on human health are well documented and various authors have studied the nutritional properties of edible wild plants, such as high fiber content, vitamins with an antioxidant function, total polyphenols, vitamins and minerals [4-10]. Defining the content of these components is essential because of their antioxidant functions [11, 12], indeed at present, oxidant stress is known to be responsible for some forms of cancer [13], as well as for degenerative pathologies such as those affecting the cardio-circulatory system (hypertension, atherosclerosis, heart attack and stroke) [14, 15], and the autoimmune system [16], with repercussions on the central nervous system thus leading to Alzheimer's and Parkinson's disease [17, 18].

Figure 1. The study area in central Italy. Local markets, thematic excursions and exhibitions aimed at promoting knowledge of edible wild plants.

A renewed interest in the use of edible wild plants is closely linked to the rediscovery of local traditions [19], food habits of the past and the role that these species have played in different cultures or ethnic groups [20, 21]. To this end, cultural and economic indices have been drawn up to estimate the importance of wild plants in a given society [22-26]. Recently they have become the subject of local markets, thematic excursions and exhibitions to satisfy the curiosity of the general public. However, up until today very few studies have been made on how edible wild plants were and are still used, and in particular on the nutraceutical

properties attributed to them [27-29]. In Italy the first ethnobotanical studies were carried out in the second half of the twentieth century. From the 1970s several research were done in various regions of Italy, focalizing the attention on the use of wild plants in folk medicine, in human nutrition, in magic rituals and ethno-veterinary practices [30-38]. In Umbria few authors have looked into these questions, and those who have done so have dealt mainly with the rediscovery of local traditional medicinal and nutritional uses of some wild plants [3, 9, 39-47]. This study takes the point of view that the rediscovery of traditional uses of edible wild plants is an important facet of the reappraisal of one's own origins and underlines how a renewed interest in their nutritional uses has arisen, given their high nutraceutical properties. Indeed, the main objective was to demonstrate the importance of edible wild plants both as a reserve of genetic resources capable of satisfying both present and future biotechnological and agroindustry needs as well as of improving daily diet and preventing degenerative processes thanks to their high antioxidant nutrient content.

The study was conducted in Umbria, Central Italy, a region characterized by numerous natural and semi-natural areas and by a high level of floristic and vegetational diversity (over 2,000 vascular species) [48]. In this territory where many edible wild plants can be found, and where, in small rural communities, traditional uses of these species are still very much alive, local markets, thematic excursions and exhibitions are organized (Figure 1).

Methods

The study began with an ethnobotanical analysis by means of an ad hoc semi-structured interview involving 150 females and 70 males (average age 57 years) living locally. They were asked to provide information on local names, alimentary use, parts used, and popular uses and remedies. The nutraceutical research centred on four of the most commonly used wild edible species in Umbria: *Bellis perennis* L., *Bunias erucago* L., *Chondrilla juncea* L., and *Sanguisorba minor* Scop (Figure 2).

These four species were chosen because they belong to the Compositae, Cruciferae and Rosaceae families that are among the most representative and well known in traditional folk recipes. [9, 45-47]. The data obtained were compared with the INRAN table of food composition [49]. Various samples of the four species were collected, which were analyzed with a *Stereomicroscope* SX45, and were determined according to Checklist of Flora Vascolare Italiana[50,51].

All the *exsiccata* of the aforementioned species are preserved in the 'Erbario PERU' of the Università degli Studi di Perugia.

To determine the nutraceutical aspects, the various determinations were carried out on pools of fresh samples of the four species collected in the spring in Umbria, using the traditional methods described in literature, the Official Methods of Analysis of Association of Official Analytical Chemists [49, 52, 53] and the most recent analytical techniques [11, 12, 54]. In this way, apart from the classical chemical percentage composition, other, so-called minor components were determined which have been difficult to define until recently.

Particular attention was paid to defining the principal components with antioxidant functions and Total Antioxidant Capacity using the ORAC method (Oxygen Radical Absorbance Capacity) [55, 56]. The mineral content was determined by means of

Spectrophotometry and Atomic Absorption. All data were processed using the Star version 4.10 software package and where necessary some integration corrections were made manually. The statistical analysis of the data was performed using the Statistical Analysis System (release 8.1, SAS Institute Inc).

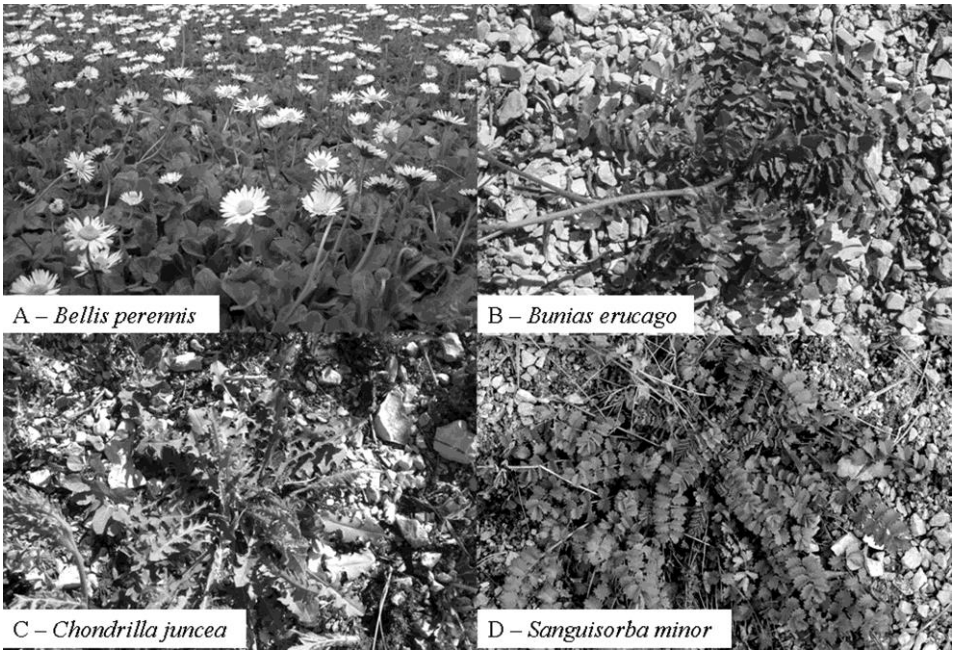


Figure 2. The four edible wild plants analyzed in the nutraceutical analysis: *Bellis perennis L.* (A), *Bunias erucago L.* (B), *Chondrilla juncea L.* (C), and *Sanguisorba minor Scop* (D).

Results

Ethnobotanical Analysis

The analysis revealed that the most well-known and widely used edible wild plants in Umbria belong mainly to the *Compositae* family, followed by the *Cruciferae* and *Umbelliferae* families. The main use of wild plants was as a food (73%), followed by percentages of medicinal or popular uses and remedies (12%) and veterinary use (2%) in accordance with local habits and customs handed down from one generation to the next.

The plants were mainly used in raw salads (45%), boiled (38%), as side dishes or in ravioli fillings (7%), fried with or without eggs (6%) or in vegetable soups (4%). In both cases it was uncommon to find the use of a single species, as a mixture was preferred to balance the different flavors. The data collected during the interviews provided information on the uses of 60 among the most common edible wild plants in Umbria, as well as on local names (See Table 1).

Table 1. Information on 60 species of edible wild plants

Scientific names	Botanical family	Local names	Part(s) used	Preparation	Citation	Medicinal properties / Popular uses and remedies
Alliaria petiolata (M. Bieb.) Cavara & Grande	Cruciferae	Alliaria comune, erba alliaria	Leaves, young and seed	Raw-salad, boiled	52	
Allium neapolitanum Cirillo	Liliaceae	Aglio napoletano	Leaves, bulbs	Raw-salad	58	
Allium triquetrum Ten.	Liliaceae	Aglio angolare, aglio trigono, aglio selvatico	Leaves, bulbs	Raw-salad, boiled	53	
Arctium lappa L.	Compositae	Bardana maggiore, lappa bardana, lappola	Leaves, young shoots, roots	Raw-salad, boiled	54	Diuretic, astringent, emollient
Asparagus acutifolius s L.	Liliaceae	Asparago, asparago dei boschi	Young shoots	Fried in fat, without or with beaten eggs ("Frittata"), boiled	36	
*Bellis perennis*L.	Compositae	Margheritina, pratolina	Young leaves and flowers		37	
Borago officinalis L.	Boraginaceae	Borragine, boragine	Leaves	Raw, in salads, fried in fat, without or with beaten eggs ("Frittata"), ravioli filling, boiled	30	Diuretic to refresh the kidneys and very effective treatment for rheumatism
*Bunias erucago*L.	Cruciferae	Cascellora, cascellore comune, cotecacchie, navone selvatico	Leaves, young shoots, flowers	Raw-salad, boiled	2	Anti-inflammatory, antiseptic, emollient
Campanula rapunculus L.	Campanulaceae	Raponzolo, ramponzolo, raperonzolo, rapunzoli	Leaves, roots or rootstocks, shaft, young shoots, whole aerial parts	Raw in salads, boiled	13	
Calamintha nepeta (L.) Savi s.l.	Labiatae	Calamento, mentucciacomune, nepitellaselvatica	Young leaves and flowers	Raw in salads, boiled	53	
Calendula arvensis (Vaill.) L.	Compositae	Calendula dei campi, fiorrancio selvatico, primofiore	Leaves, young shoots, flowers	Raw in salads, boiled	30	Anti-inflammatory, antiseptic
*Cardamine hirsuta*L.	Cruciferae	Billeri primaticcio, cardamine	Young leaves	Raw in salad, vegetable soup	53	
Capsella bursa-pastoris (L.) Medik. subsp. *bursa-pastoris*	Cruciferae	Borsa di pastore, borsacchina, capsella	Leaves, whole aerial parts, young shoots	Raw in salads, boiled, vegetable soup	36	
Centranthus ruber(L.) DC. subsp. *ruber*	Valerianaceae	Valeriana rossa	Young leaves	Raw in salad, boiled	52	

Table 1. (Continued)

Scientific names	Botanical family	Local names	Part(s) used	Preparation	Citation	Medicinal properties / Popular uses and remedies
*Chondrilla juncea*L.	Compositae	Mastrici, pioletta, piole	Leaves, young shoots	Raw in salads	43	
Cichorium intybus L. s.l.	Compositae	Cicoria, cicorietta, radicchio selvatico, radici amare	Leaves, whole aerial parts, leaves stalks, young shoots	Boiled, raw in salads, ravioli filling	6	Digestive
Clematis vitalba L.	Ranunculaceae	Clematide, vitabbie	Young shoots	Fried in fat, without or with beaten eggs ("Frittata")	53	
Crepis sancta (L.) Babc. subsp. *sancta*	Compositae	Crepide, dolcetta, radicchiella di terrasanta	Young leaves	Raw in salads, boiled	52	
*Crepis vesicaria*L. s.l.	Compositae	Cota, crepide vescicosa, radicchi ella vescicosa, radicchio scoltellato	Young leaves	Raw in salads, boiled	53	Detoxifying
*Daucus carota*L. s.l.	Umbelliferae	Carota selvatica	Whole aerial parts, roots	Raw in salads, boiled	52	
Diplotaxis erucoides (L.) DC. subsp. erucoides	Cruciferae	Maraiuole, ruchetta violacea, ruchettone	Young leaves	Raw in salads, boiled	53	
Diplotaxis tenuifolia (L.) DC.	Cruciferae	Rucola, rucoletta, rucoletta di campo, ruchetta selvatica	Leaves	Raw in salads, vegetable soup	52	
Helminthotheca echioides (L.) Holub	Compositae	Aspraggine volgare, erba brusca, erba bruscia, spraggine	Leaves	Boiled	58	
*Hyoseris radiata*L. subsp. radiata	Compositae	Trenette, trinciatella, radicchio selvatico	Young leaves	Raw in salads, boiled	58	
*Hypochaeris achyrophorus*L.	Compositae	Costolina annuale	Young leaves	Raw in salads, boiled	54	
*Hypochaeris radicata*L.	Compositae	Costoled'asino, costolina giuncolina, ingrassaporci, piattello	Young leaves	Raw in salads, boiled	36	
Lactuca muralis (L.) Gaertn.	Compositae	Lattuga dei boschi	Young leaves	Raw in salads, vegetable soup	52	
Lactuca perennis L. subsp. *perennis*	Compositae	Lattuga perenne, lattuga rupestre	Young leaves	Raw in salads, boiled	58	
*Lactuca serriola*L.	Compositae	Lattuga selvatica	Leaves, leaves stalks	Raw in salads	36	

Scientific names	Botanical family	Local names	Part(s) used	Preparation	Citation	Medicinal properties / Popular uses and remedies
Lactuca viminea (L.) Presl.	Compositae	Lattuga alata	Leaves	Raw in salads	36	
Leopoldia comosa(L.) Parl.	Liliaceae	Cipollaccio, lampagione, lampascione	Bulb	Boiled, sott'olio o sott'aceto	50	Diuretic, emollient
*Malva sylvestris*L. subsp. *sylvestris*	Malvaceae	Malva	Leaves, young shoots, flowers	Raw in salad, vegetable soup, ravioli filling, infusions, decoctions	53	Infusions for the relief of heartburn and indigestion
Nasturtium officinale R. Br. Subsp. *officinale*	Cruciferae	Crescione d'acqua, crescione delle fontane, crescione di sorgente	Leaves	Raw in salads	58	
Papaver rhoeas L. subsp. *rhoeas*	Papaveraceae	Papavero comune, rosolaccio	Young leaves, seed	Vegetable soup, ravioli filling, raw in salads, seed in bread and cookies	36	
*Parietaria officinalis*L.	Urticaceae	Vetriolacomune	Young leaves, young shoots	Boiled, vegetable soup	30	
Pastinaca sativa L.	Umbelliferae	Pastinaca comune	Root, young leaves	Boiled, raw in salads	54	Fruits and roots are diuretic and emollient
Picris echioides L.	Compositae	Aspraggine volgare, raspraggine, spraggine	Leaves	Boiled	6	Astringent
Picris hieracioides L. s.l.	Compositae	Aspraggine comune, erba brusca	Young leaves	Boiled	54	
*Plantago lanceolata*L.	Plantaginaceae	Plantago, lanciuola, lingua di cane	Leaves, whole aerial parts	Boiled, raw in salads	30	
*Portulaca oleracea*L. s.l.	Portulaceaceae	Porcacchia, porcellana		Boiled, in salads	52	
Rhagadiolus stellatus (L.) Gaertn.	Compositae	Erba cornetta, radicchio lirato, ragaggiolo, raggiolo	Young leaves	Boiled, raw in salads	58	
*Rhaphanus raphanistrum*L.	Cruciferae	Rafano, ramolaccio selvatico, rapastrello, ravanello selvatico	Young leaves, roots	Boiled	36	Diuretic
Reichardia picroides (L.) Roth	Compositae	Caccialepre, scaccialepre, grattalingua	Leaves, whole aerial parts, flowers, roots	Raw in salads, boiled	35	
*Rumex acetosa*L. subsp. *acetosa*	Polygonaceae	Acetosa, romice acetosa	Young leaves	Vegetable soup	53	
Rumex acetosella L. s.l.	Polygonaceae	Acetosella, romice acetosella	Young leaves	Raw in salads, boiled, vegetable soup, ravioli filling	53	

Table 1. (Continued)

Scientific names	Botanical family	Local names	Part(s) used	Preparation	Citation	Medicinal properties / Popular uses and remedies
Salvia pratensis L.	Labiatae	Chiarella, salvia dei prati, salvia pratense	Young leaves	Vegetable soup, fried with eggs (frittata)	36	
Sanguisorba minor Scop. S.l.	Rosaceae	Pimpinella, pimpinellone	Leaves, whole aerial parts, young shoots	Raw in salads, boiled	1	Digestive properties
Scandix pecten-veneris L.	Umbelliferae	Acicula comune, erba spilletta	Leaves, young shoots	Raw in salad	53	Digestive, diuretic
Silene vulgaris (Moench) Garckes.l.	Caryophyllaceae	Strigoli, stricoli	Whole aerial parts, young shoots	Risotto, fried with eggs (Frittata)	6	
Sonchus asper (L.) Hill s.l.	Compositae	Crespigno spinoso, crespignola	Young leaves	Raw in salad, boiled	6	Insect bites or as a remedy for mouth ulcers
Sonchus oleraceus L.	Compositae	Gruspigno, cruspino, crispigno, cruspigno, grespigno, crespigno	Leaves, whole aerial parts, young shoots, shaft	Boiled, Raw in salads	37	Remedy for mouth ulcers
Stellaria media (L.) Vill.	Caryophyllaceae	Centocchio comune, erba gallinella	Young leaves	Raw in salad, boiled	30	Diuretic, astringent,
Taraxacum officinale (group)	Compositae	Pisciacane, piscialletto, soffione, dente di leone, dendelion	Leaves, whole aerial parts, young shoots, shaft, flowers	Boiled, raw in salads	16	Depurative and diuretic properties
Thymus serpyllum S.L.	Labiatae	Pepolino, serpillo, serpollino	Leaves	Per aromatizzare i cibi	34	Anti-inflammatory
Tordylium apulum L.	Umbelliferae	Pimpinellone, pimpinella vellutata, zampa d'oca, saporitella	Leaves, whole aerial parts	Raw in salads	13	
Tragopogon pratensis L. s.l.	Compositae	Baciapreti, barba di beccocomune, barba di prete, salsefica	Roots, young leaves, shaft	Raw in salads, boiled	52	
Tragopogon porrifolius L. s.l.	Compositae	Barba di becco violetta, raperonzolos elvatico, salsefica	Roots, young leaves	Raw in salads, boiled	53	
Urospermum dalechampii(L.) F.W. Schmidt	Compositae	Cicoriamatta, grugno, grugnoamaro, grugnola	Leaves, whole aerial parts	Boiled, raw in salads	2	
Urtica dioica L. subsp. *dioica*	Urticaceae	Ortica	Leaves, young shoots, roots, whole aerial parts, roots	Boiled, ravioli filling, risotti, fried with eggs (frittata)	30	Excellent remedy for dysmenorrhoea
Viola odorata L.	Violaceae	Viola mammola	Flowers	Raw in salad	36	Emollient

The following is a description of some of the most common uses: in salads the herbs such as ‘grespigni’- sow thistle (*Sonchus* L. spl.), ‘piantaggine’ - plantain (*Plantago lanceolata*L.) and ‘borragine’ - borage (*Borago officinalis* L.), mitigate the bitterness of ‘cicoria’ - chickory, (*Cichorium intybus* L. s.l.), ‘pisciacane’ - dandelion (*Taraxacum officinale* group), costole d'asino, costolina giuncolina [*Hypochaeris radicata* L.] and ‘erba brusca’ - bristly ox-tongue [*Helminthotheca echioides*(L.) Holub]. In raw salads there is a mixture of ‘pimpinella’ - salad burnet (*Sanguisorba minor* Scop. s.l.), ‘raponzolo’ - bellflower (*Campanula rapunculus* L.), ‘pimpinellone’ (*Tordylium apulum* L.), ‘caccialepre’ [*Reichardia picroides* (L.) Roth], ‘carota selvatica’ - wild carrot (*Daucus carota* L. s.l.), ‘erba bussola’ - prickly lettuce (*Lactuca serriola* L.), ‘costolina annuale’ (*Hypochaeris achyrophorus* L.), ‘borsa del pastore’ - shepherd’s purse [*Capsella bursa-pastoris*(L.)Medik.subsp. *bursa-pastoris*], and ‘calendula dei campi’, ‘fiorrancio selvatico’ [*Calendula arvensis* (Vaill.) L], .

Many species are used as condiments in pasta and rice dishes, such as ‘ortica’ - stinging nettles (*Urtica dioica*L. subsp. *dioica*), ‘strigoli’ - bladder campion [*Silene vulgaris* (Moench) Garckes.l.] and ‘asparago selvatico’ - wild asparagus (*Asparagus acutifolius* L.). These species are also used to give a distinctive flavour to omelettes and soups (Table 1).

Some wild plants have medicinal properties. Some folk remedies recorded are as follows: borage (*Borago officinalis* L.) is a diuretic, it refreshes the kidneys and is a very effective treatment for rheumatism; infusions of mallow (*Malva sylvestris* L. subsp. sylvestris) relieves heartburn and indigestion; grispigni (*Sonchus oleraceus* L.) is a remedy for mouth ulcers and stinging nettle (*Urtica dioica* L. subsp. dioica) is an excellent remedy for dysmenorrhoea.

Table 2. Comparison of chemical composition and mineral content (mg/100 g per edible parts) values in edible plants with those of cultivated species [49]

Names	Chemical composition					Mineral content				
	Water	Protein	Lipids	Carbohydrates	Dietary fibers	Fe	Ca	P	Na	Mg
Bunias erucago	83.1	2.2	0.4	3.0	8.2	2.4	425	18.2	6.1	216
Bellis perennis	84.2	1.4	0.4	1.0	7.6	4.8	444	18.3	56	123
Chondrilla juncea	87.8	1.9	0.5	2.0	5.8	4.9	159	12.7	3.8	100
Sanguisorba minor	76.2	3.8	0.8	6.0	10.5	5.1	283	21.9	21	282
Potato	78.5	2.1	1	17.9	1.6	0.8	10	54	7	28
Raw artichoke (*Cynara cardunculus* L. subsp. scolymus (L.) Hayek)	91.3	2.7	0.2	2.5	5.5	1	86	67	133	45
Raw spinach (*Spinacia oleracea* L)	90.1	3.4	0.7	2.9	1.9	2.9	78	62	100	60
Ripe tomatoes (*Solanum lycopersicum* L.)	94	1	0.2	3.5	2	0.3	9	25	6	10
Salad tomatoes (*Solanum lycopersicum* L.)	94.2	1	0.2	3.5	2	0.4	11	26	3	10
Raw zucchini (*Cucurbita pepo* L.)	93.6	1.3	0.1	1.4	1.2	0.5	21	65	22	25
Lettuce (*Lactuca sativa* L.)	94.3	1.8	0.4	2.2	1.5	1.2	45	31	9	-
Raw carrot (*Daucus carota* L.)	91.6	1.1	0.2	7.6	3.1	0.7	44	37	95	11
Pepper (*Capsicum annuum* L.)	92.3	0.9	0.3	4.2	1.9	0.7	17	28	2	18
Eggplant (Solanum melongena L.)	92.7	1.1	0.4	2.6	2.6	0.3	14	33	26	-

NutraceuticalAnalysis

All four species analyzed showed the presence of all the dietary energetic principles, although in different concentrations. Chemical composition and energy content were compared with those of some cultivated species [49]. The total fat content is very low in all four species, always below 1.0%. It is interesting to note mineral content. It was found that *B.perennis* show higher Iron and Calcium values than all the cultivated species with which they were compared. *S. minor* showed high protein and iron content similar to that of raw spinach (Table 2).

Table 3 shows, among antioxidant vitamins, high concentrations of β-carotene (pro-vitamin A), vitamin A in *B. erucago*, and significant vitamin A values, similar to raw carrot. *S. minor* shows high value of Vitamin E. Total polyphenol content varies greatly from rather low in *C. juncea*, to quite high in the other species, particularly in *S. minor* (258 mg/100 of edible parts), especially when the high water content of these types of samples is taken into consideration, followed by *B. erucago* and *C. juncea* (Table 3).

Table 3. Antioxidant vitamins, total polyphenols and ORAC in edible plants *vs.* cultivated species

Names	β-carotene (μg/100 g)	Vitamin A (μg Ret. Eq./100 g)	Vitamin E (μg/100 g)	Total polyphenols (mg/100 g)	ORAC (μmol TE/100 g)
Bunias erucago	5774	962	2942	86	529
Bellis perennis	2537	423	3101	49	221
Chondrilla juncea	2134	356	2728	23	427
Sanguisorba minor	3339	556	5226	258	904
Potato	-	3	-	-	-
Raw artichoke (*Cynara cardunculus* L. subsp. scolymus (L.) Hayek)	-	18	-	-	-
Raw spinach (*Spinacia oleracea* L.)	-	485	-	-	-
Ripe tomatoes (*Solanum lycopersicum* L.)	-	610	-	-	-
Salad tomatoes (*Solanum lycopersicum* L.)	-	42	-	-	-
Raw zucchini (*Cucurbita pepo* L.)	-	6	-	-	-
Lettuce (*Lactuca sativa* L.)	-	229	-	-	-
Raw carrot (*Daucus carota* L.)	-	1148	-	-	-
Pepper (*Capsicum annuum* L.)	-	139	-	-	-
Eggplant (Solanum melongena L.)	-	-	-	-	-

Conclusion

The present study shows that gathering and consuming edible wild plants is still very much alive in Umbria Region, although in most cases their nutritional value is unknown. Local names, parts used, folk medicinal properties and variations in culinary use linked to local traditions are still very important, while conventions, exhibitions and themed courses provide information and promote these species as an environmental resource. It was found that the quality and quantity of the various components of the four species under examination could make an excellent contribution to balancing and rationalizing diet and preventing metabolic pathologies. This research demonstrates that edible wild plants contain many of the so-called minor nutrients (because they are found in small quantities), such as polyphenols and antioxidant vitamins, that can further improve a diet which is already balanced, thus offering protection against degenerative processes. A new, interesting factor is represented by the presence of adequate antioxidant components that can combat the effect of free radicals. As at present the amount necessary to maintain this equilibrium is estimated to be about 5000 ORAC units, and considering that health authorities recommend the consumption at least five portions of fruit and vegetables a day [49] the wild plants which were dealt with in this study could make a notable contribution towards this objective. Many of the macro and micronutrients contained in these wild plants merit more attention, but the lack of an adequate national and regional nutrient database limits available knowledge, which at present is much more limited than that relevant to cultivated species [57]. More extensive knowledge could lead to improved diet in many areas of the Mediterranean area and the Developing World, and in general facilitate the use of these species. Thanks to their antioxidant properties, it would certainly be worthwhile to foster further study of edible wild plants and to promote commercialization campaigns, particularly in view of the growing demand s by the food industry for natural antioxidant. Human health in general would benefit greatly from a reconsideration of these plants because they represent a naturally-occurring, easy to obtain source of powerful vegetable antioxidants. Furthermore a renewed interest in edible wild plants could be a stimulus to further encourage the study of local flora and disseminate knowledge so as to preserve local cultural customs and traditions. The words of Breda sum up the fundamental importance of these species in safeguarding and preserving biodiversity (2001) [58]: *'When, together with the plants, we shall also have preserved the flavors, the memories, the words, and the love associated with them, and we shall have been able to communicate all this to future generations, only then we shall have a right to claim that we have really preserved biodiversity'*.

Acknowledgments

The authors thank the FondazioneCassa di Risparmio of Perugia for making this research possible.

References

[1] Etkin NL, Ross PJ. Food as medicine and medicine as food. An adaptive framework for the interpretation of plant utilisation among the Hansa of Northern Nigeria. *Soc .Med.* 1982;161559-73.

[2] Etkin N. The cull of the wild. In: Etkin NL, editor. *The pharmacologic ecologic, and social implications of using noncultigens*, Tuscon: University of Arizona Press; 1994, p. 1-21.

[3] Moreno-Black G, Somnasang P, Thamathawan S. Cultwatingcontinmty and creating change: women's home garden practices in northeastern Thailand. *Agricult. Hum. Values.* 1996;13:3-11.

[4] Guerrero Guil JL, Campra Madrid P, ToruaIsasa ME. Mineral elements determination in wild edible plants. *Ecol. Food Nutr.* 1999;38:209-22.

[5] Grivetti LE, Ogle BM. Value of traditional foods in meeting macro and micronutrient needs: the wild plant connection. *Nutr. Res. Rev.* 2000;13:31-46.

[6] Cao G, Alessio HM, Culter RG. Oxygen-radical absorbance capacity assay for antioxidants. *Free Radic. Biol. Med.* 1993;14:303-11.

[7] Simopoulos AP. Omega-3 fatty acids and antioxidants in edible wild plants. *Biol. Res.* 2004;37:263-77.

[8] Schaffer S, Schmitt-Schillig S, Müller WE, Eckert GP. Antioxidant properties of mediterranean food plant extracts: geographical differences. *J. Physiol. Pharmacol.* 2005;56:115-124.

[9] Ranfa A, Bodesmo M, Cappelli C, Quaglia M, Falistocco E, Burini G, *et al. Aspettifitoecologici e nutrizionali di alcune specie vegetalispontanee in Umbria per la conoscenza, recupero e valorizzazione di risorseambientali*. Perugia: TipografiaGrifo; 2011.

[10] Vanzani P, Rossetto M, De Marco V, Sacchetti LE, Paoletti MG, Rigo A. Wild Mediterranean plants as traditional food: a valuable source of antioxidants. *J. Food Sci*. 2011;76:C46-C51.

[11] Luterotti S, Franko M, Bicanic D. Ultrasensitive determination of β-carotene in fish oil-based supplementary drugs by HPLC-TLS. *J. Pharm. Biomed. Anal.* 1998;21:901-9.

[12] Redi R. *Determinazionesimultaneadeitocoferoli, del β-carotene e del retinolonel latte per HPLC* [dissertation]. Perugia: UniversitàdegliStudi di Perugia; 1999.

[13] Cohen JH, Kristal AR, Stanford JL. Fruit and vegetables intakes and prostate cancer risk. *J. Natl. Cancer Inst.* 2000;92:61-8.

[14] Polidori MC, Frei B, Cherubini A. Increased plasma levels of lipid hydroperoxides in patients with ischemic stroke. *Free Radic. Biol. Med*. 1998;25:561-7.

[15] Yang T, Devaraj S, Jialal I. Stress ossidativo e aterosclerosi. *J. Clin. Ligand Assay.* 2001;24:13-24.

[16] Iborra A, Palacio Martinez JR. Oxidative stress and autoimmune response in the infertile woman. *Chem. Immunol. Allergy.* 2005;88:150-62.

[17] Linert W, Jameson GN. Redox reactions of neurotransmitters possibly involved in the progression of Parkinson's Disease. *J. Inorg. Biochem*. 2000;79:319-26.

[18] Sudha K, Rao A, Rao S, Rao A. Free radical toxicity and antioxidants in Parkinson's disease. *Neurol. India.* 2003;51:60-2.

[19] Hadjichambis ACH, Paraskeva-Hadjichambi D, Della A, Giusti M, De Pasquale C, Lenzarini C, Censorii E, Gonzales-Tejero MR, Sanchez-Rojas CP, Ramiro-Gutierrez J, Skoula M, Johnson CH, Sarpakia A, Hmomouchi M, Jorhi S, El-Demerdash M, El-Zayat M, Pieroni A. Wild and semi-domesticated food plant consumption in seven circum-Mediterranean areas. *Int. J. Food Sci. Nutr*. 2008;59:383-414.

[20] Ladio AH, Lozada M. Comparison of wild edible plant diversity and foraging strategies in two aboriginal communities of northwestern Patagonia. *Biodiv. Conserv*. 2003;12:937-51.

[21] Leonti M, Nebel S, Rivera D, Heinrich M. Wild gathered food plants in the European Mediterranean: A comparative analysis. *Econ. Bot.* 2006;60:130-42.

[22] Turner NJ. The importance of a rose: evaluating the cultural significance of plants in Thompson and Lillooet Interior Salish. *Am. Anthropol.* 1988;90:272-90.

[23] Stoffle RW, Halmo DB, Evans MJ, Olmsted JE. Calculating the cultural significance of American Indian plants: Paiute and Shoshone ethnobotany at Yucca Mountain, Nevada. *Am. Anthropol.* 1990;92:416-32.

[24] High C, Shackleton CM. The comparative value of wild and domestic plants in home gardens of a South African rural village. *Agrofor. Sys.* 2000;48:141–56.

[25] Pieroni A. Evaluation of the cultural significance of wild food botanicals consumed in northwestern Tuscany, Italy. *J. Ethnobiol.* 2001;21:89-104.

[26] Reyes-García V, Huanca T, Vadez V, Leonard W, Wilkie D. Cultural, practical, and economic value of wild plants: A quantitative study in the Bolivian Amazon. *Econ Bot.* 2006;60:62-74.

[27] Pieroni A. Gathered wild food plants in the upper valley of the Serchioriver (Garfagnana), Central Italy. *Econ. Bot.* 1999;53:327-41.

[28] Vitalini S, Grande S, Visioli F, Agradi E, Fico G, Tome F. Antioxidant activity of wild plants CollectedinValsesia, an Alpine region of Northern Italy. *Phytother. Res*. 2006;20:576–80.

[29] Pardo de Santayana M, Tardío J, Blanco E, Carvalho AM, Lastra JJ, San Miguel E, Morales R. Traditional knowledge of wild edible plants used in the northwest of the Iberian Peninsula (Spain and Portugal): a comparative study. *J. Ethnobiol. Ethnomed.* 2007;3:1-11.

[30] Caneva G, Pontrandolfi MA, Fascetti S. *Le piantealimentarispontaneedella Basilicata*. Villa D'Agri: ArsGrafica; 1997

[31] Caneva G, Pieroni A, Guarrera PM. *Etnobotanica. Conservazione di unpatrimonioculturale come risorsa per unosvilupposostenibile*. Bari: Edipugliasrl, 2013.

[32] Guarrera PM, Forti G, Marignoli S. Ethnobotanical and ethnomedicinal uses of plants in the district of Acquapendente (Latium, Central Italy). *J. Ethnopharmacol*. 2005;96:429-44.

[33] Guarrera PM, Salerno G, Caneva G. Food, flavouring and feed plant traditions in the Tyrrhenian sector of Basilicata, Italy. *J. Ethnobiol. Ethnomed.* 2006;2:37.

[34] Guarrera PM, Leporatti ML. Ethnobotanical remarks on Central and Southern Italy. *J Ethnobiol Ethnomed.* 2007;3:23.

[35] Ghirardini MP, Carli M, Del Vecchio N, Rovati A, Cova O, Valigi F *et al.* The importance of a taste. A comparative study on wild food plant consumption in twenty-one local communities in Italy. *J. Ethnobiol. Ethnomed.* 2007;3:22.

[36] Camangi F, Stefani A, Sebastiani L. *Etnobotanica in val di Vara, l'usodellepiantenellatradizionepopolare*. Firenze: Press Service; 2009.
[37] Cornara L, La Rocca A, Marsili S, Mariotti MG. Traditional uses of plants in the Eastern Riviera (Liguria, Italy). *J. Ethnopharmacol.* 2009;125:16–30.
[38] Guarrera PM, Lucchese F, Medori S. *Contributoallaconoscenzadella Flora d'Italia. L'usotradizionaledellepiantenell'alto Molise*. Firenze: ArtiGrafiche; 2009.
[39] Menghini A, Mincigrucci G, Bencivenga M. I Pascoli del monte La Pelosa (Appenninoumbro-reatino). In: Maria degliAngeli S, editor. *EstrattodagliannalidellaFacoltàd'Agrariadell'Università di Perugia*. *Vol. XXX*, Perugia: Tipografia Porziuncola:901-9; 1975.
[40] Leporatti ML, Posocco E, Pavesi A. Phytotherapy in the Valnerina, Marche (Central Italy). *J. Ethnopharmacol.* 1985;14:53-63.
[41] Nardelli GM. *Cultura e tradizione. Demomedicina nell'alta Umbria*. Perugia: Provincia di Perugia; 1987
[42] Pezzotta R. *Saperinaturalisticilocali*. Perugia: Centro EducazioneAmbientale di Cerreto di Spoleto, Centro per la Documentazione e la RicercaAntropologica in Valnerina, Centro StampaRegionale; 1994.
[43] DallaRagione I, DallaRagione L. *Archeologiaarborea*. Perugia: Città di Castello (PG); 2003.
[44] Parziani A, Sforna S, Nieri C. *La reginadelleerbette*. Perugia: Undonodeglianziani di Spelloallacomunità, Comune di Spello, DimensioneGrafica; 2005.
[45] Ranfa A. *Pianteamiche e nemichedell'uomo*. Perugia: Ali&noeditrice; 2004.
[46] Ranfa A. *Unpatrimonioculturale da salvaguardare: le piantespontaneecommestibili del Parco del Monte Subasio*. *La Regina delleerbette, Comune di Spello.*Spello: DimensioneGrafica; 2005.
[47] Ranfa A. *Pianteamiche e nemichedell'uomo.* Perugia: Ali&noeditrice; 2014.
[48] Orsomando E, Ragni B, Segatori R. *SitiNatura 2000 in Umbria*. Regionedell'Umbria; 2004.
[49] INRAN IstitutoNazionale di Ricerca per gliAlimenti e la Nutrizione. *Lineeguida per unasanaalimentazioneItaliana*. Roma; 1993. Available at http://www.inran.it/.
[50] Conti F, Abbate G, Alessandrini A, Blasi C. *An annotated checklist of the italian vascular flora*. Roma: Palombi e Partner Srl; 2005.
[51] Conti S, Alessandrini F, Bacchetta A, Banfi G, Barberis E, Bartolucci G *et al.*Integrazionialla checklist della flora vascolareitaliana. *Nat Vicentina.* 2007;10:5-74.
[52] AOAC. *Official methods of analysis of the Association of Official Analytical Chemists*. 15th edition. Washington: Association of Official Analytical Chemists; 1990.
[53] AOAC. *Methods 953.01, 975.03. official Methods of Analysis*. 18th ed. Gaithesburg: International; 2006.
[54] Burini G, Coli R. *Determinazione di componentifenolici e dellacapacitàantiossidantetotaledella farina di castagne*. Parma: CongressoNazionale di ChimicadegliAlimenti; 2003.
[55] USDA. *Database for the Oxygen Radical Absorbance Capacity (ORAC) for selected foods, Release 2*. Nutrient Data Laboratory, Beltsville Human Nutrition Research Center (BHNRC), Agricultural Research Service (ARS); 2014. Available from: http://www.nal.usda.gov/fnic/foodcomp

[56] Ou B, Hampsch-Woodill M, Prior RL. Development and validation of an improved oxygen radical absorbance capacity assay using fluorescein as the fluorescent probe. *J. Agric. Food Chem.* 2001;49:4619-26.

[57] Vincetti B, Eyzaguirre P, Johns T. The nutritional role of forest plant foods for rural communities. In: Coler CJP. *Human health and forests: a global overview of issues practice and policy*, Earthscan: London; 2008, p. 63-96.

[58] Breda N. *La biodiversità e la suaconservazione dal punto di vista antropologico*. Roma: Notiziario ERSA; 2001.

In: Human Health and Nutrition
Editor: Sergej M. Ostojic
ISBN: 978-1-63482-823-9

Chapter IX

Factors influencing Fruit and Vegetable Intake in Adolescents

Hala Hazam Al-Otaibi*
Department of Food Sciences and Nutrition,
Community Nutrition, College of Agriculture and Food Science,
King Faisal University, Hofuf, Saudi Arabia

Abstract

Fruit and vegetable intake (FVI) is an important factor in the preservation of health and the prevention of disease. Many dietary habits established during adolescence continue into adulthood, such as FVI. According to the World Health Organization, the daily FVI of adolescents was below the recommended values worldwide, despite the long-term health benefits associated with FVI. In this chapter, we updated and expanded previous research about factors influencing FVI during adolescence. Due to inductive thematic analysis based on Social Ecological Theory and Social Cognitive Theory, we identified three key factors that influence FVI: (a) individual factors (e.g., gender, age, knowledge, self-efficacy, taste preference and liking of FV, outcome expectations/expectancies, skill in preparing fruit and vegetable); (b) social factors (e.g., parents intake and modeling, parents and family support, family meals, peers influence); and (c) environmental factors (e.g., income, parents occupational status, parents education, household availability, school availability, neighborhood, television viewing). Development strategies and effective intervention programs aimed to increase FVI and to promote adolescents' healthy dietary behaviors could be achieved by understanding the relationship between FVI and above factors.

Keywords: Fruit, Vegetable, Intake, Adolescence, Individual, Social, Environmental, School

* Corresponding Author address Email: hhalotaibi@kfu.edu.sa

Fruit and Vegetable Global Outlook

Fruit and vegetable intake (FVI) is an important factor in the preservation of health and the prevention of disease. A variety of fruits and vegetables provides several essential nutrients that we need for optimal growth and repair, such as water, dietary fibers, vitamins, minerals, phytochemicals, with most fruits and vegetables are low fat foods needed in a healthy diet [1]. According to the World Health Organization (WHO) recommendations, and most national and international dietary guidelines around the world, all individuals should consume 400 grams of fruit and vegetables daily, or five servings of fruit and vegetables per day as a minimal amount [2-4]. Starting at childhood, high FVI seems to decrease prevalence of many chronic diseases, such as coronary heart disease (up to 31%), cancer (12-20%), stroke (up to 19%), while 2.7 million deaths per year are attributable to diets low in fruits and vegetables worldwide [2, 5]. A review from 52 low- and middle-income countries showed that 77.6% of men and 78.4% of women consumed less than the minimum recommended daily servings of fruits and vegetables [6]. Another study in five Asian countries showed that 63.5% of men and 57.5% of women had inadequate FVI [7]. In developed countries like USA and Australia, authors reported similar low FVI [8, 9]. Furthermore, studies conducted among adolescents in developing and developed countries showed insufficient FVI as reported in adults [8, 10]. Recent study in Curitiba [11] estimated frequency and adequacy of FVI among 341 Brazilian adolescents. Authors shown that only 3.5% of Brazilian adolescents have adequate FVI. Another cross sectional study among 402 adolescents in Tabriz, Iran reported only one third of adolescents had the optimal FVI, with 30.3% and 34.6% adolescents consumed recommended daily servings of fruits and vegetables, respectively [12]. Furthermore, data from five Southeast Asian countries (16,084 adolescents aged 13-15 years) shown that 76.3% of adolescents had FVI less than five servings per day [7]. Developed countries had similar results concerning FVI in adolescents. De-Bourdeaudhuij and colleagues [13] investigated predictors of daily FVI in nine European countries that, with 13305 adolescents recruited. Only 43.2% and 46.1% eat fruit and vegetables daily, respectively. EAT-2010 study (Eating and Activity among Teens) that examined physical activity patterns, eating behaviors and weight among 2,793 adolescents in United States, found the mean daily intake of fruit and vegetable of 2.7 servings per day only [14]. Authors concluded that developing strategies and intervention programs aimed to increase FVI and promoting adolescents' healthy dietary behaviors can be achieved by exploring the relationship between FVI behaviors and variables such as individual, social and environmental factors.

Adolescents and Diet

WHO defines adolescents as people who are 10-19 years of age [15], while other extended range from 10 to 20 years of age [16]. Adolescence is characterized by rapid physical growth, with hormonal, cognitive, and emotional changes. Adolescence is divided into three stages, with each stage has different characteristics. Early adolescence (10-13 years of age) is characterized by the start of puberty and increased cognitive development. Middle adolescence (14-16 years of age) characterizes increased independence and experimentation.

Late adolescence (17-20 years of age) is a time for making important personal decisions to start adulthood [16-18]. Many lifestyle habits that are established during adolescence have been reported to have a significant influence on the social and behavioral aspects of life for this age group [19]. At the same time, adolescence is an intense anabolic period where adolescents require a high quality diet, with adequate amount of energy, vitamins and minerals to support their physical growth. In addition, unhealthy eating behaviors during adolescence can negatively affect health and contribute to chronic diseases later in life, and are difficult to change once established [20]. FVI during adolescence influences many different perspectives. Several authors proposed comprehensive theoretical models of eating behavior among adolescents, with FVI affected by multiple interacting factors [21, 22]. The researchers adopted a theoretical framework based on Social Cognitive Theory (SCT) and the Social Ecological theory (SET) to explain adolescent's FVI [21-23]. SET has been employed through individual, social and environmental context [22, 23], while SCT explains behavior by the interaction of person's behavior, personal factors, and the environment factors [24]. The following sections highlight the effects of these factors for adolescents FVI.

Individual Factors

Age

Dietary behavior seems to change as children become adolescents. FVI often declines when entering adolescence, and energy-dense foods and beverages intake increases [22, 25]. Research reported negative correlation between age and FVI during the adolescent period. In addition, parental influence declines as adolescents get older while level of self-efficacy for choosing their own foods influenced by their peers increases [26]. Previous studies including quantitative research showed FVI decreased with age in 60% of studies reviewed while 40% of studies reported no correlation between age and FVI [22]. Al-Hazzaa and colleagues found FVI decreased with age in Saudi adolescents [27]. Similar results were found in Ghana, where younger adolescents (12-15 years old) had higher FVI than older adolescents (16-18 years old) [28]. A recent study conducted in Iran found that adolescents over 14 years old have low FVI; older adolescents had more authority to select and consume foods they preferred [12].

Gender

Gender differences in FVI are widely documented in many studies, with strong correlation found between FVI and gender [12]. Most studies reported that girls consume more fruits and vegetables than boys [21, 29, 30]. They are more concerned with health and body image, have greater knowledge, outcome expectations/expectancies, self-efficacy and role models [31]. On other hand, review of 98 papers by Rasmussen and colleagues [22] found that girls have a higher or more frequent intake of fruit and vegetables than boys in 27 studies, but 18 studies found no gender differences and only four papers report opposite result were boys had higher or more frequent intake than girls. Similar results were reported among Kuwaiti adolescents, where more boys regularly consumed vegetables (26.0% vs. 22.1%) and

fruits (17.5% vs. 11.8%) as compared to girls [32]. Youth Risk Behavior Survey with US adolescents found a large percentage of girls at risk for inadequate intakes of fruits and vegetables [33]. Resent study [34] explored eating habits, physical activity, and sedentary behaviors among Iraqi adolescents in Mosul City (723 adolescents, 350 boys and 373 girls). Authors reported significantly higher FVI among girls than boys. Similar results were reported among Mexican adolescents. Authors reported significant gender difference in FVI, with 15.2% of girls ate three or more fruits and vegetables per day as compared to 6.7% of boys [30]. Gender difference found might reflect the fact that boys have more independence in their food choices in some communities (e.g., eating away from home) than girls, and that can be a risk factor for poor dietary habits [21].

Knowledge

Nutrition knowledge can influence our food behavior as how and why we eat healthy food [21]. School based intervention study on 3878 American adolescents from Minnesota reported knowledge as important predictor for FVI [35]. Authors investigated the meanings of "healthy" and "unhealthy" eating and the importance of healthy eating among twenty-five structured focus groups of 203 adolescent girls and boys. It seems that adolescents had a significant amount of knowledge regarding healthy foods, and they believed that healthy eating involves moderation, balance, and variety, where fruits and vegetables were the most commonly mentioned healthy foods [36]. In addition, knowledge was positive predictor of daily FVI among adolescents in five European countries out of nine (Austria, Belgium, Portugal, Spain and Sweden) [13]. Furthermore, more girls (61%) than boys (55%) knew the daily fruit recommendations, and almost a similar percentage of girls and boys (24%, 23%; respectively) knew the daily vegetable recommendations [37]. Similarly, a cross-sectional study conducted among Saudi adolescent's girls reported that knowledge was potential determinant of FVI [38]. American Children and adolescents (aged 8 to 15 years) who participated in ten week youth gardening programs identified the health benefits of eating fruit and vegetable, and were more willing to eat nutritious food at the end of program [39]. However, knowledge seems not to be isolated factor for healthy food choices since several studies didn't find any association between FVI and knowledge [40-42].

Self-Efficacy

Self-efficacy is defined as "the power to produce desired changes by one's actions" [43]. It has been recognized as an important factor that enables a change in the individual attitude towards healthy eating in adolescents, and the most important predictor for eating behavior such as FVI [21, 44, 45]. Fitzgerald and colleagues [44] examined the relationship between self-efficacy, parent and peer support for healthy and unhealthy eating, and food intake patterns among 483 Irish adolescents aged 13–18 years. Authors reported that higher self-efficacy was associated with healthy food intake such as FVI. On the other hand, in a sample of 1321 adolescents from Denmark, authors reported the same self-efficacy related to FVI [46]. Similar results were reported among Saudi adolescents [38]. Granner and Evans [47] found that FVI was significantly correlated with self-efficacy among American adolescents

aged 11-15 years who participated in cross-sectional study aimed to evaluate scales that measure constructs related to Social Cognitive Theory related to FVI.

Taste Preference for Fruit and Vegetable

Taste preference or liking has been found to be an important factor that influences FVI among adolescents. Several studies reported smell, shape, deliciousness, diversity, color, and good or bad experiences after eating fruits and vegetables as important factors for FVI. Recent reviews [22, 48] found taste preference or liking as strong factors associated with an increased FVI. A review of longitudinal and cross-sectional FVI studies in adolescents [29] reported preference being the most consistent influences on FVI. De-Bourdeaudhuij et al. [13] reported that preferences and liking have been shown to be good predictors of FVI in adolescents in most European countries (seven out of nine). In a Pro Children project, twelve focus groups with school children aged 10-11 years old from Rotterdam (the Netherlands) and Ghent (Belgium-Flanders) identified taste preferences and taste as personal factors related to FVI [49]. In addition, FVI tends to decline during the transition from adolescence to young adulthood, where most of the young adults had low FVI. Larson and colleagues [50] identified longitudinal correlates of FVI in early young adults followed-up for 5 years (1495 adolescent at baseline in 1998-1999 and follow-up in 2003-2004). Results showed that, after adjusting for baseline intake only, tastes preferences were identified as correlates of FVI during young adulthood across gender. Sometimes adolescents' FVI were associated with unpleasant and negative taste for vegetables or salads intake, or previous experiences. Many adolescents perceived green vegetables as healthy foods but they disliked it due to their bland or unpleasant taste [41]. In the school environment, adolescents sometimes demonstrated negative experiences as fruits being squashed in their bags, and adolescents not preferring to take it to the school [49, 51]. Usually adolescents have ingrained tastes which is a big challenge. For this matter, we should always convince them to change their eating behavior through offering adolescents different fruit and vegetable choices and/or options.

Outcome Expectations/Expectancies

Outcome expectations/expectancies describes expected positive or negative set of beliefs about the outcome of behavior [43]. Granner and Evans [47] found that health or positive outcome expectations of FVI (grow bigger muscles, become better in sports, be healthier, have energy to run, play, and think) were significantly correlated with FVI among American middle school students (aged 11-15 years old). Another study explained dietary behaviors among 357 adolescent girls from low-income communities in Australia. Authors measured the outcome expectations/expectancies referring to the benefits/values placed on anticipated outcomes of healthy eating (e.g., eating at least three servings of fruit and four servings of vegetables each day, choosing foods low in fat and added sugar, and monitoring portion sizes). The authors found outcome expectancies were more strongly associated with behavior than outcome expectations; they suggested that girls may recognize the benefits of healthy eating considering those benefits to be values [52]. Recent study reported similar results among American youth (aged 8-18 years old), where positive outcome expectations had

direct effects on youth diet quality [53]. A qualitative research [54] among Tehranian adolescents (aged 11-14 years old) found positive outcome expectations/expectancies among adolescents, as eating fruits made them feel good due to the taste. Eating vegetables was reported as delightful and made the food tasty, while FVI was indicated as useful for health, being energetic, better vision, enhancing body resistance, and better learning. On the other hand, McClain and co-workers [55] conducted a literature review from 16 countries, to understand the correlation between effective dietary intake and promotion of healthy dietary behaviors among children (age <13 years) and adolescents (age > 13-18). Thirty-five studies tested for outcome expectation correlates for fruit, juice, and vegetable consumption did not show consistent relationships between outcome expectations and dietary outcomes.

Skill in Preparing Fruit and Vegetable

Possessing skills to prepare healthful foods among adolescents would promote improvements in diet quality. In general, when adolescents helping their parents in preparing meals, more nutrient-rich eating patterns and healthier food choices were demonstrated [56-59]. Rakhshanderou et al. [54] found that girls who had skills in preparing fruits and vegetables (e.g., cleaning, washing correctly, peel, cut, and slicing) ate more fruit and vegetable, while some girls cited preparing skill as barriers of FVI. Similarly, a family-based newsletter intervention aimed to increase FVI in adolescents through two newsletter packs (recipes and tip sheets) over a one-month period encouraged adolescents on trying new fruit and vegetables. By preparing and shopping fruits and vegetables with their parents, authors reported improved use of fruit and vegetables at home as meals and snacks [23]. In contrast to above findings, Nago and colleagues [60] found preparing skills as barriers to FVI in Beninese adolescents aged 13 - 19 years. Adolescents preferred fruits to vegetables because of its uncomplicated use (e.g., no need to cook fruits). In addition, girls consumed more vegetables during weekends because there is more time for cooking. Moreover, some studies reported gender differences in preparing skills between girls and boys. Girls were more involved in preparation and cooking while boys helped in preparation tasks only; older adolescents who were living with one parent were more involved in preparing meals on a regular basis than younger adolescents living with two parents [61, 62].

Social Factors

Parental Intake and Modeling

Parental FVI and modeling are important social factors, and have been found to be strongly associated with adolescent FVI and food preferences. Adolescents usually live with their parents, share FV that are available in the home, with adolescent usually have the same culture of eating as their parents. Many studies conducted in developed and developing countries well documented this association [13,22-24,48,49]. Studies used different methods to assess parental FVI and modeling, such as parent's report of modeling, adolescent's report of parental role modeling, or utilized both parents and adolescents report. All studies reported

positive association between parental intake and adolescent FVI no matter of methodology used. A recent study conducted in the United States utilized both parents and adolescents' reports of parental role modeling. Authors found significant association for fruit and green salad at dinner only, with participants were more likely to meet FVI recommendations [63]. Similar results were reported among African American and Caucasian adolescents where parental modeling was significantly correlated with FVI [47]. Pedersen and colleagues [46] found Denmark adolescents are influenced by parents when it comes to FVI more than their friends. Intervention study conducted among adolescents and their parents found that parental FVI is a significant predictor of adolescent fruit FVI when parents increase their FVI and provide more FV to be accessible to their children at home [24]. Tehranian adolescents learned how to eat FV from their parents, with positive dietary patterns found for the entire family [54]. Unfortunately, sometimes parental role modeling it not always the easiest thing to utilize, which was reported in a qualitative study conducted among Ecuadorian adolescents. Some parents reported that they are not always good role models for their children, having constant struggle to encourage their children to eat healthily and trying to be good role models especially for FVI [51].

Parents and Family Support

In general, parents and family support play a principal role to encourage their children to consume more FV by supervising adolescents' nutritional behaviors and establishment rules about household nutrition. A literature review from 16 countries conducted by McClain and colleagues [55] found parents' support correlated with FVI among adolescents. Also, active parental encouragement was found to be related to FVI among adolescents from nine European countries [13]. Furthermore, Rasmussen and colleagues [22] reported in their review that three out from three studies found positive association between FVI and parents support. Similar results were documented in intervention and longitudinal studies [23, 64]. In the HOME Plus study, authors confirmed the associations between parent's role for FVI at snacks and dinner in 160 parents and their children (aged 8-12 years). Encouragement of parental role modeling of FVI at snacks and salad at dinner is warranted and may increase healthful dietary habits among children [63]. Shokrvash et al. [12] reported that practical and emotional support were the most important factors of family support associated with adolescents FVI among Iranian adolescents, with low emotional support for boys and low practical support for girls were found to be significant predictors of low FVI. Irish parents' restricted certain unhealthy foods within the home to limit their consumption in aim to encourage their children to use healthy foods as fruits and vegetables [42].

Family Meals

Eating family meals has been found to have a positive impact on adolescents' dietary quality and better nutritional intake including increased FVI [21]. Furthermore, family meal frequency is inversely associated with engagement in use of drugs and alcohol, disordered eating behaviors, depression, and suicide [65, 66].

Gillman and colleagues conducted a study to examine the association between frequency (e.g., most nights, infrequently) of eating family meals (dinner), and the dietary intake patterns of adolescents [67].

The authors found that adolescents who consumed family dinners more frequently consumed 0.8 more servings of fruits and vegetables as compared to those who never or rarely ate family dinners. Another longitudinal study examined the FVI and family meals relation among 18,177 adolescents [69]. Results showed that adolescents who ate four or five family meals per week 22% less likely reported poor fruit intake, 19% less likely reported poor vegetable intake, and 19% less likely reported poor dairy intake, and infrequently skipped breakfast. This positive effect was highlighted as the number of family meals (six or seven) increased per week. A qualitative study examined the differences between Irish children and adolescents perceptions of factors influencing their food choices. Focus group discussions were conducted with 29 young people aged between 9-18 years. The authors concluded that participation in family meals is important in influencing their food choices including FVI [42]. Another study conducted among 520 Saudi adolescent girls (aged 13-19 years) found positive associations between family meal frequency and increasing three major meal intakes, also a significant increase in the FVI, dairy products, grain and bread products, meat and fish, and legumes [68]. Longitudinal study [64] determined if family meal frequency during adolescence is associated with diet quality during young adulthood. Authors reported that family meal frequency during adolescence predicted higher intakes of fruit, vegetables, dark green and orange vegetables during young adulthood, and increased social eating in young adulthood which is an important time for adolescents to interact with family and friends.

Peer Influence

Peer influence or friends influence is an important social factor and challenging issue during adolescence [26]. Researchers found correlation between adolescents and their friend eating behaviors, with understanding similarities and differences between friends in their eating habits can helps dietitians and health professionals to design a diet and lifestyle interventions. EAT-2010 (Eating and Activity among Teens) trial [14] examined the association between adolescents and their friends healthy eating behaviors, specifically concerning breakfast, fruit, vegetable, whole-grain, and dairy food intake. Authors found significant correlation between adolescents and their best friends vegetable intake, also for whole-grain and dairy food intakes, but no associations were seen among friends for fruit intake.

Granner and Evans [47] reported similar results among middle school adolescents in United States, where peer influence is significantly correlated to FVI. Sometimes friends have negative influence for healthy eating by encouraging adolescents to consume unhealthy foods, with Irish adolescents reported significant peer support for unhealthy eating [44]. Similar results were found among 757 Denmark adolescents, with population FVI was less influenced by friends than by parents [46].

Environmental Factors

Income

Several studies reported income as the major determinate of dietary intake, with families with higher income having the affordability to purchase more expensive food such as FV comparable to lower income families [70, 71]. In Sao Paulo, Brazil, it has been found that 52% of adolescents who had a household income equal to or lower than $ 75.00, consumed less than one FV serving/daily, while adolescents who had a higher income consumed between 140 g and 460 g of FV per day [72]. Positive association between FVI and family income was reported in several studies among American [73], Canadian [74] and Norwegian adolescents [75].

Recent study reported positive association between income and FVI in the international Health Behavior in School-Aged Children study, research collaboration aimed to describe young people's well being, health behaviors and their social context within the WHO and 44 countries across Europe and North America. Among the low-income Norwegian adolescents who participated in this study, they were more likely to eat low FVI than the higher income adolescents [76]. In addition, Denmark adolescents with low income have low FVI combined with high fast food outlet exposure, and low exposure to supermarkets to buy FV [77]. In two years follow-up study conducted in Australia, families who spend more money use more FV in their diet [78]. On the other hand, FV are cheap and available in Ghana during the whole year, especially in harvest seasons from June to August, and all adolescents from different socioeconomic level had frequent daily FVI [28].

Parental Occupational Status

It has also been shown that parental occupational status has a strong relationship with adolescents FVI. In an early review of family correlates for FVI, all the studies included in this review found parent's occupational status was positively correlated with fruit intake [23]. Another review study found the same positive association with FVI [22].

The parental occupational status might affect three major meal intakes and parental supervision of adolescents' eating habits, which causes unhealthy eating patterns among adolescents [21].

Father occupational status is determinant of healthy eating; if father had high occupation status, the adolescents were eating healthier food while mother occupational status is not always associated with eating healthier [79]. Traditionally, mother is responsible for grocery shopping and meal preparation, and because of her work schedule there is a possibility for insufficient time for meal preparation and FV, which can further decrease food availability and parental support for healthy eating habits as FVI [80]. Iranian adolescents whose mother is employed mother had lower FVI, while adolescents with an unemployed mother had higher FVI [12].

Parental Education

The level of parents' education influence adolescents FVI. Recent study in Brazil reported that the head of the household (father, mother) who had a higher education had a better knowledge about healthier food choices, especially for FVI [72]. Increased level of education is usually combined with higher income that leads to purchasing expensive foods as FV, and enhanced knowledge about the benefits of FVI. A review of studies examining the association between parental education and FVI reported positively association between two factors [23]. In longitudinal Norwegian study conducted in 896 adolescents (mean age 12.5 years) parents' education level was positively related to FVI among adolescents [75]. In Ghana, level of mother's education predicted FVI among adolescents, while no correlation was found between father educational level and FVI [28]. Lawrence and colleagues [81] reported that mothers with low education level had more barriers and less knowledge for healthy eating. Furthermore, these mothers often bought cheapest food that usually not includes FV. Canadian cross-sectional study with 18,524 adolescents identified the impact of socio-demographic factors on FVI pattern. It seems that parental education positively impacts adolescent FVI. PRO-GREENS project that took place in ten European countries found parents with higher educational level as significant mediators for more frequent daily FVI in children, although correlation was modest or absent in some countries [82].

Household Availability

Household availability is defined as how plentiful and visible FV is in the house [83]. Most studies showed strongly correlation between household availability and FVI [61,84]. Fruit and vegetable availability at home depends on many factors, such as income, parental education, time, benefit of FV from parents view. Early studies found home availability as the strongest correlate to adolescent FVI. One of the largest studies conducted on 4,746 adolescents in USA (Project EAT-I) reported that home availability significantly affected adolescents FVI [84]. Another qualitative study identified personal, behavioral, and environmental influences on FVI among low-income black American adolescents from Mississippi. Authors found household availability as a barrier to FVI by some of the subjects, who identified other relatives' homes (especially grandmother's house) and restaurants as a source of vegetables than fruits [61]. A European study examined the determinants of FVI in normal weight compared to overweight boys; authors found that availability of FV at home was related to increased FVI consumption in overweight boys [13]. Recent review reported an association between vegetable home availability and vegetables intake in adolescents from sixteen countries where parent and adolescents report household availability [85]. Adolescents reported availability more likely to be associated with vegetable intake as compared to parent report. In the PRO-GREENS project, authors reported availability as a significant mediator for fruit intake in Finland, and for vegetable intake in Finland, Germany and Iceland [82]. Furthermore, household availability for fruit and vegetable was related to parental education level in Norway [75]. A qualitative study examining availability of fruits and vegetables at home among Iranian adolescents found fruit household availability was associated with more frequent intake as compared to vegetables [54].

School Food Availability

Adolescents spend most of their time in the school, with school environment has a large and powerful influence (positive or negative) on adolescents eating behavior, especially FVI [26, 86]. Previous cross-sectional study that included 598 adolescents in 165 American schools examined the association between the dietary behaviors and the availability of school vending machines *a la carte* (e.g., candy, chips, and cookies) programs, and fried potatoes begin served at school lunch. Results indicated *la Carte* availability was positively associated with total and saturated fat intake and inversely associated with FVI. Vending machines were negatively correlated with fruit consumption. Serving fried potatoes' at school lunch was positively associated with FVI [87]. In Australian longitudinal study, researchers found that adolescents who managed to avoid purchasing food or drink from vending machines at school frequently consumed fruit [78]. Authors concluded that adolescents may prefer the taste of fruit over high energy foods, and adolescents should be encouraged to take home-prepared school lunches and snacks, while schools should remove vending machines from their campuses [78].

Another study conducted in USA found the availability of FV at school has a positive impact on low-income adolescents, who had higher FVI if they ate school food, but not for the high income adolescents who had lower FVI if they ate school food. This is mitigated via income related disparities in adolescent FVI, and this mitigation is beneficial for low-income students [88]. Sometimes, school availability was not related to adolescents FVI. European group recommended more research to study how the school environment can affect FVI, through health education aspects and availability of healthy/unhealthy snacks in school environment [13].

Neighborhood Environments

Few studies demonstrated that neighborhood environment might influence dietary intake in adolescents [89, 90]. The neighborhood has been defined as "the area around one's place of residence, as stores within walking distance from home and restaurants within county boundaries" [90]. The findings concerning relationship between neighborhood environment and adolescent FVI have been mixed. Some studies found that greater access to neighborhood convenience stores, restaurants, and fast food facilities, has been associated with low FVI, obesity and low diet quality [90-92].

Riediger and colleagues [74] found that low FVI among Canadian adolescents from low-income families might be related to living in neighborhoods with fewer grocery stores, and not offer healthful foods with reasonable price. Other studies have shown an appositive association between FVI available in convenience stores that are closer to residential households, and higher FVI in adolescents [93]. Recent study among American adolescents showed minimal significant association between the neighborhood environment and healthful dietary intake as FVI, and lower BMI in adolescents [94].

Television Watching

Many adolescents in the world found television (TV) watching as the most popular leisure-time activity. A growing number of studies found TV watching has obesogenic effect in adolescents, as well as decreases FVI, fiber intake, physical activity and increases energy-dense foods and beverages intake. [95-97]. Moreover, during TV watching adolescents are exposed to food commercials, with products with lower nutritional value and energy-dense products more often available in home, with this phenomena might affect children health [98]. A review study identified negative association between FVI and TV watching, as a sedentary lifestyle behavior among adolescents [99]. TV watching in adolescents was associated with decreased fruit juice intake [50]. Furthermore, in large school-based cross-sectional study conducted among 2908 Saudi adolescents aged 14-19 years, authors reported that TV watching was inversely correlated with FVI and breakfast intake [27]. Also, TV watching was positively associated with home availability of energy-dense snacks among Australian adolescents but not with healthy food as fruits and vegetables [100]. However, little is known about the potential mechanisms of TV watching and healthy or unhealthy eating behaviors among adolescents [95, 96].

Conclusion

Fruit and vegetable intake is found to be less than the minimum recommended (five daily servings) among adolescents from the developed and developing countries. Several factors (individual, social and environmental) affect FVI in adolescents. Taste preference and liking fruits and vegetables (individual factors), parental intake and family meals frequency (social factors), and household income and availability (environmental factors) are the most important factors that affect adolescent FVI. Adolescence is a critical period in the development of healthy dietary behaviors that prolongs into adulthood. A combination of individual, social and environmental factors is related to FVI, and the relationship between these factors is complex. There is an increasing need for better understanding of how these factors affect FVI in adolescents in aim to develop multilevel intervention strategies based on social and ecological theories and models, to help adolescents to adopt healthy choices as FVI especially in developing countries.

References

[1] Pajk T, Rezar V, Levart A, Salobir J. Efficiency of apples, strawberries, and tomatoes for reduction of oxidative stress in pigs as a model for humans. *Nutrition.* 2006;22(4):376–84.

[2] WHO/FAO. Diet, nutrition and the prevention of chronic diseases. Report of a Joint WHO/FAO Expert Consultation. 2003.

[3] US Department of Agriculture and US Department of Health and Human Services. (USDA/DHHS). Dietary Guidelines for Americans (7th ed). Washington: US

Government Printing Office, 2010. Retrieved from: http://www.cnpp.usda.gov /DGAs2010-PolicyDocument.htm.

[4] US Department of Agriculture. *Food groups- fruits. Why is it important to eat fruit?* Retrieved from: http://www.mypyramid.gov/ pyramid/fruits_why_print.html.

[5] Lock K, Pomerleau J, Causer I, Ezzai M, Lopez AD, Rodgers A, Murray CJI. Low fruit and vegetable consumption. Comparative quantification of health risks: global and regional burden of disease attributable to selected major risk factors. Geneva: WHO. 2004.

[6] Hall JN, Moore S, Harper SB, Lynch JW. Global variability in fruit and vegetable consumption. *Am. J. Prev. Med*. 2009;36(5):402-9.

[7] Peltzer K, Pengpid S. Fruits and vegetables vonsumption and associated factors among in-school adolescents in five Southeast Asian countries. *Int. J. Environ. Res. Public Health*. 2012;9(10):3575-87.

[8] Kimmons J, Gillespie C, Seymour J, Serdula M, Blanck HM. Fruit and vegetable intake among adolescents and adults in the United States: percentage meeting individualized recommendations. *Medscape J. Med*. 2009;11(1):26.

[9] National Health & Medical Research Council. Dietary Guidelines for Children and Adolescents in Australia. 2003. Retrieved from: http://www.nhmrc.gov.au/ _files_nhmrc/file/publications/synopses/n30_pamphlet.pdf

[10] Centers for Disease Control and Prevention. Youth Online: High School YRBS 2010. Retrieved from http://www.cdc.gov/healthyyouth/ yrbs/cdcreports.htm.

[11] Monticelli DB, de Souza MP, de Souza SB. Adolescent students consumption of fruit, greens and vegetables. *J. Hum. Growth Dev*. 2013;23(3):331-7.

[12] Shokrvash B, Majlessi F, Montazeri A, Nedjat S, Shojaeezadeh D, Rahimi A, Djazayeri A, Saghafi-Asl M. Fruit and vegetables consumption among adolescents: A study from a developing country. *World Appl. Sci. J*. 2013;21(10):1502-11.

[13] De Bourdeaudhuij I, te Velde SJ, Brug J, Brug J, Due P, Wind M, Sandvik C, Maes L, Wolf A, Perez Rodrigo C, Yngve A, Thorsdottir I, Rasmussen M, Elmadfa I, Franchini B, Klepp KI. Personal, social and environmental predictors of daily fruit and vegetable intake in 11-year-old children in nine European countries. *Eur. J. Clin. Nutr*. 2007;62(7):834-41.

[14] Bruening M, Eisenberg M, MacLehose R, Nanney MS, Story M, Neumark-Sztainer, D. Relationship between adolescents' and their friends' eating behaviors. Breakfast, fruit, vegetable, whole-grain, and dairy intake. *J. Acad. Nutr. Diet*. 2012;112(10):1608–13.

[15] WHO. Nutrition throughout the life cycle. The 4th report on the nutrition situation. Geneva: WHO. 2000.

[16] Kliegman R, Nelson WE. Nelson textbook of pediatrics (18th ed). Philadelphia: Saunders. 2007.

[17] WHO. Adolescent health. Geneva: WHO. 2011. Retrieved from: http://www.who.int /topics/adolescent_health/en

[18] Dixon SD, Stein MT. *Encounters With Children: Pediatric Behavior and Development* (3rd ed). St. Louis: Mosby Inc. 2000.

[19] Hetherington MM. Eating disorders. *New Engl J Med*. 2000;15:295-303.

[20] Evers S, Taylor J, Manske S, Midgett C. Eating and smoking behaviors of school children in Southwestern Ontario and Charlottetown, PEI. *Can. J. of Public Health*. 2001;92(6):433-6.

[21] Story M, Neumark-Sztainer D, French S. Individual and environmental influences on adolescent eating behaviors. *J. Am. Diet Assoc*. 2002;102(3):S40-S51.

[22] Rasmussen M, Krolner R, Klepp I, Lytle L, Brug J, Bere E. Determinants of fruit and vegetable consumption among children and adolescents. A review of the literature. Part I. Quantitative studies. *Int. J. Behav. Nutr. Phys. Act*. 2006;3: 22.

[23] Pearson N, Biddle SJ, Gorely T. Family correlates of fruit and vegetable consumption in children and adolescents. A systematic review. *Public Health Nutr*. 2009;12(2):267–83.

[24] Burke LE, Froelich RA, Zheng Y, Glanz K. Current theoretical bases for nutrition intervention and their uses. In: Nutrition in the Prevention and Treatment of Disease, 2nd ed. (Eds.: Coulston AM, Boushey C, Ferruzzi M). Burlington: Elsevier Academic Press. 2008. pp. 141–156.

[25] Perez A, Hoelscher D, Brown HS, Kelder SH. Differences in food consumption and meal patterns in Texas school children by grade. *Prev. Chronic Dis.* 2007;4(2):A23.

[26] Gifford-Smith M, Dodge KA, Dishion TJ, McCord J. Peer influence in children and adolescents: Crossing the bridge from development to intervention science. *J. Abnorm. Child Psych*. 2005;33(3):255-65.

[27] Al-Hazzaa HM, Abahussain N, Al-Sobayel H, Qahwaji D, Musaiger AO. Physical activity, sedentary behaviors and dietary habits among saudi adolescents relative to age, gender and region. *Int. J. Behav. Nutr. Phys. Act*. 2011;8:140.

[28] Doku D, Koivusilta L, Raisamo S, Rimpela A. Socio-economic differences in adolescents' breakfast eating, fruit and vegetable consumption and physical activity in Ghana. *Public Health Nutr*. 2013;16(5):864-72.

[29] Geller KS, Dzewaltowski DA. Longitudinal and cross-sectional influences on youth fruit and vegetable consumption. *Nutr. Rev*. 2009;67(2):65-76.

[30] Perez-Lizaur AB, Kaufer-Horwitz M, Plazas M. Environmental and personal correlates of fruit and vegetable consumption in low income, urban Mexican children. *J. Hum. Nutr. Diet*. 2008;21(1):63-71.

[31] Bere E, Brug J, Klepp KI. Why do boys eat less fruit and vegetables than girls? *Publ. Health Nutr*. 2008;11(3):321–5.

[32] Allafi A, Al-Haifi AR, Al-Fayez MA, Al-Athari BI, Al-Ajmi FA, Al-Hazzaa HM, Musaiger AO, Ahmed F. Physical activity, sedentary behaviours and dietary habits among Kuwaiti adolescents: gender differences. *Public Health Nutr*. 2014;17(9):2045-52.

[33] Pesa J, Turner L. Fruit and vegetable intake and weightcontrol behaviors among U.S. youth. *Am. J. Health Behav*. 2001;25(1):3–9.

[34] Musaiger AO, Al-Mufty BA, Al-Hazzaa HM Eating habits, inactivity, and sedentary behavior among adolescents in Iraq: Sex differences in the hidden risks of noncommunicable diseases. *Food Nutr. Bul*. 2014; 35(1):12-9.

[35] Lytle LA, Varnell S, Murray DM, Story M, Perry C, Birnbaum AS, Kubik MY. Predicting adolescents' intake of fruits and vegetables. *J. Nutr. Educ. Behav.* 2003;35(4):170-5.

[36] Croll JK, Neumark-Sztainer D, Story M. Healthy eating: what does it mean to adolescents? *J. Nutr. Educ*. 2001;33(4):193–8.

[37] Sandvik C, De Bourdeaudhuij I, Due P, Brug J, Wind M, Bere E, Pérez-Rodrigo C, Wolf A, Elmadfa I, Thórsdóttir I, Vaz de Almeida MD, Yngve A, Klepp KI. Personal,

social and environmental factors regarding fruit and vegetable intake among schoolchildren in nine European countries. *Ann. Nutr. Metab*. 2005;49(4):255–66.

[38] Hussein R. Can knowledge alone predict vegetable and fruit consumption among adolescents? A transtheoretical model perspective. *J. Egypt Public Health Assoc*. 2011; 86(5-6):95–103

[39] Lautenschlager L, Smith C. Beliefs, knowledge, and values held by innercity youth about gardening, nutrition, and cooking. *Agric Human Values*. 2007;24(2):245-58.

[40] Resnicow K, Davis-Hearn M, Smith M, Baranowksi T, Lin LS, BaranowskinJ, Doyle C, Wang DT. Social-cognitive predictors of fruit and vegetable intake in children. *Health Psychol.* 1997;16(3):272-6.

[41] Stevenson C, Doherty G, Barnett J, Muldoon OT, TrewK. Adolescents' views of food and eating: identifying barriers to healthy eating. *J. Adolesc*. 2007;30(3):417–34.

[42] Fitzgerald A, Heary C, Nixon E, Kelly C. Factors influencing the food choices of Irish children and adolescents: a qualitative investigation. *Health Promot. Int.* 2010;25(3):289–98.

[43] Bandura A. Health promotion by social cognitive means. *Health Educ. Behav*. 2004;31(2):143–64.

[44] Fitzgerald A, Heary C, Kelly C, Nixo, E, Shevlin M. Self-efficacy for healthy eating and peer support for unhealthy eating are associated with adolescents' food intake patterns. *Appetite*. 2013;63:48–58.

[45] Young EM, Fors SW, Hayes DM. Associations between perceived parent behaviors and middle school student fruit and vegetable consumption. *J. Nutr. Educ. Behav*. 2004;36(1):2–12.

[46] Pedersen S, Grønhøj A, Thøgersen J. Following family or friends. Social norms in adolescent healthy eating. *Appetite*. 2015;;86:54-60.

[47] Granner ML, Evans AE. Measurement properties of psychosocial and environmental measures associated with fruit and vegetable intake among middle school adolescents. *J. Nutr. Educ. Behav*. 2012;44(1):2-11.

[48] Krolner R, Rasmussen M, Brug J, Klepp KI, Wind M, Due P. Determinants of fruit and vegetable consumption among children and adolescents: a review of the literature. Part II: qualitative studies. *Int. J. Behav. Nutr. Phys. Act*. 2011;8:112.

[49] Wind M, Bobelijn K, De Bourdeaudhuij I, Klepp KI, Brug J. A qualitative exploration of determinants of fruit and vegetable intake among 10- and 11-year-old schoolchildren in the low sountries. *Ann. Nutr. Metab*. 2005;49(4):228-35.

[50] Larson NI, Neumark-Sztainer DR, Harnack LJ, Wall MM, Story MT, Eisenberg ME. Fruit and vegetable intake correlates during the transition to young adulthood. *Am. J. Prev. Med.* 2008;35(1):33–7.

[51] Verstraeten R, Van Royen K, Ochoa-Avile A, Penafiel D, Holdsworth M, Donoso S, Maes L, Kolsteren P. A conceptual framework for healthy eating behavior in ecuadorian adolescents: a qualitative study. *PLoS One*. 2014;9(1):e87183.

[52] Lubans DR, Plotnikoff RC, Morgan PJ, Dewar D, Costigan S, Collins CE. Explaining dietary intake in adolescent girls from disadvantaged secondary schools. A test of social cognitive theory. *Appetite*. 2012;58(2):517–24.

[53] Nansel TR, Haynie DL, Lipsky LM, Wang J, Mehta SN, Laffel L. Relationships among parent and youth healthful eating attitudes and youth dietary intake in a cross-sectional study of youth with type 1 diabetes. *Int. J. Behav. Nutr. Phys. Act*. 2013;10:125.

[54] Rakhshanderou S, Ramezankhani A, Mehrabi Y, Ghaffari M. Determinants of fruit and vegetable consumption among Tehranian adolescents: A qualitative research. *J. Res. Med. Sci*. 2014;19(6):482-9.

[55] McClain AD, Chappuis C, Nguyen-Rodriguez ST, Yaroch AL, Spruijt-Metz D. Psychosocial correlates of eating behavior in children and adolescents: a review. *Int. J. Behav. Nutr. Phys. Act.* 2009;6:54.

[56] Wayland J, Coe B. The purchasing role of the adolescent in the family unit: implications for the 1990s. *J. Food Prod. Market.* 1993;1:49-60.

[57] Watt R, Sheiham A. Dietary patterns and changes in inner city adolescents. *J. Hum. Nutr. Diet.* 1996;9:451- 61.

[58] Hebert K, Jacobson A. Adolescent evening meal practices and attitudes toward the maternal role in evening meal preparation. *J. Consum Stud. Home Econ.* 1991;15:249-59.

[59] Hinton M, Chadderton H, Eppright E, Wolins L. Influences on girls' eating behavior. *J. Home Econ.* 1962;54:842-6.

[60] Nago ES, Verstraeten R, Lachat CK, Dossa RA, Kolsteren PW. Food safety is a key determinant of fruit and vegetable consumption in urban Beninese adolescents. *J. Nutr. Educ. Behav*. 2012;44(6):548-55.

[61] Molaison EF, Connell CL, Stuff JE, Yadrick MK, Bogle M. Influences on fruit and vegetable consumption by low-income black American adolescents. *J. Nutr. Educ. Behav*. 2005;37(5):246-251.

[62] Hill L, Casswell S, Maskill C, Jones S, Wyllie A. Fruit and vegetables as adolescent food choices in New Zealand. *Health Promot. Int*. 1998;13:55-65.

[63] Draxten M. Fulkerson JA, Friend S, Flattum CF, Schow R. Parental role modeling of fruits and vegetables at meals and snacks is associated with children's adequate consumption. *Appetite*. 2014;78:1–7.

[64] Larson N, Nuemark-Sztainer D, Hannan P, Story M. Family meals during adolescence are associated with higher diet quality and healthful meal patterns during young adulthood. *J. Am. Diet. Assoc*. 2007;107(9):1502-10.

[65] Neumark-Sztainer D, Wall M, Story M, Fulkerson JA. Are family meal patterns associated with disordered eating behaviors among adolescents? *J. Adolesc. Health.* 2004;35(5):350-9.

[66] Eisenberg M, Olson RE, Neumark-Sztainer D, Story M, Bearinger L. Correlations between family meals and psychosocial well-being among adolescents. *Arch. Pediatr Adolesc. Med.* 2004;158(8):792-6.

[67] Gillman MW, Rifas-Shiman SL, Frazier AL, Rockett HR, Camargo CA,Jr, Field AE, Berkey CS, Colditz GA. Family dinner and diet quality among older children and adolescents. *Arch. Fam. Med*. 2000;9(3):235-40.

[68] AL-Oboudi LM, AL-Khudhayri DA. The difference between meal frequencies and some specific food frequencies with and without family among saudi adolescent female. *Int. J. Food Nutr. Public Health*. 2013;6(1):37-49.

[69] Videon TM, Manning CK. Influences on adolescent eating patterns: the importance of family meals. *J. Adolesc. Health*. 2003;32(5):365-73.

[70] Nilsen SM, Krokstad S, Holmen TL, Westin S. Adolescents' health-related dietary patterns by parental socio-economic position, The Nord-Trøndelag Health Study (HUNT). *Eur. J. Public Health.* 2010;20(3):299-305.

[71] French SA, Story M, Neumark-Sztainer D, Fulkerson JA, Hannan P. Fast food restaurant use among adolescents: associations with nutrient intake, food choices and behavioral and psychosocial variables. *Int. J. Obes. Realt. Metab. Dosord.* 2001;25(12):1823-33.

[72] Bigio RS, Verly Junior E, Castro MA, Cesar CL, Fisberg RM, Marchioni DM. Determinants of fruit and vegetable intake in adolescents using quantile regression. *Rev. Saùde Pùblica*. 2011;45(3):448-56.

[73] Ding D, Sallis JF, Norman GJ, Saelens BE, Harris SK, Kerr J, Rosenberg D, Durant N, Glanz K. Community food environment, home food environment, and fruit and vegetable intake of children and adolescents. *J. Nutr. Educ. Behav.* 2012;44(6):634–8.

[74] Riediger ND, Shooshtari S, Moghadasian MH. The influence of sociodemographic factors on patterns of fruit and vegetable consumption in Canadian adolescents. *J. Am. Diet Assoc*. 2007;107(9):1511–8.

[75] Bere E, van Lenthe F, Klepp KI, Brug J. Why do parents' education level and income affect the amount of fruits and vegetables adolescents eat? *Eur. J. Public Health*. 2008;18(6):611–5.

[76] Fismen A, Smith OR, Torsheim T, Samdal O. School based study of time trends in food habits and their relation to socio-economic status among Norwegian adolescents, 2001–2009. *Int. J. Behav. Nutr. Phys. Act*. 2014;11(1):115.

[77] Svastisalee CM, Holstein BE, Due P. Fruit and vegetable intake in adolescents: association with socioeconomic status and exposure to supermarkets and fast food outlets. *J. Nutr. Metab*. 2012;2012:185484.

[78] Stephens LD, McNaughton SA, Crawford D, Ball K. Longitudinal predictors of frequent vegetable and fruit consumption among socio-economically disadvantaged Australian adolescents. *Appetite*. 2014;78:165–71.

[79] Fahlman MF, McCaughtry N, Martin J, Shen B. Racial and socioeconomic disparities in nutrition behaviours: targeted interventions needed. *J. Nutr. Educ. Behav.* 2010; 42(1):10-6.

[80] Reinaerts EJ, de Nooijer J, Candel M, deVries N. Explaining school children's fruit and vegetable consumption: the contributions of availability, accessibility, exposure, parental consumption and habit in addition to psychosocial factor. *Appetite*. 2007; 48(2):248-58.

[81] Lawrence W, Skinner C, Haslam C, Robinson S, Inskip H, Barker D, Cooper C, Jackson A, Barker M. Why women of lower educational attainment struggle to make healthier food choices: the importance of psychological and social factors. *Psychol. Health*. 2009; 24(9):1003–20.

[82] Letho E, Ray C, TeVelde S, Petrova S, Duleva V, Krawinkel M, Behrendt I, Papadaki A, Kristijansdottir A, Thorsdotti I, Yngve A, Lien N, Lynch C, Ehrenblad B, Vaz de Almeida MD, Ribic CH, Simcic I, Roos E. Mediation of parental education level on fruit and vegetable intake among schoolchildren in ten European countries. *Public Health Nutr*, 2015;18(1):89-99.

[83] Cullen K, Baranowski T, Owens E, Marsh T, Rittenberry L, de Moor C. Availability, accessibility, and preferences for fruit, 100% fruit juice, and vegetable influence children's dietary behavior. *Health Edu. Behav*. 2003;30(5):615-26.

[84] Neumark-Sztainer D, Wall M, Perry C, Story M. Correlates of fruit and vegetable intake among adolescents. Findings from Project EAT. *Prev. Med*. 2003;37(3):198-208.

[85] Cook LT, O'Reilly GA, Derosa CJ, Rohrbach LA, Spruijt-Metz D. Association between home availability and vegetable consumption in youth: a review. *Public Health Nutr*. 2015; DOI: http://dx.doi.org/10.1017/S1368980014000664

[86] Wechsler H, Brener NC, Kuester S, Miller C. Food service and foods and beverages available at school: results from the School Health Policies and Programs Study 2000. *J. Sch. Health.* 2001;71(7):313–24.

[87] Kubik MY, Lytle LA, Hannan PJ, Perry CL, Story M. The association of the school food environment with dietary behaviors of young adolescents. *Am. J. Public Health*. 2003; 93(7):1168-73.

[88] Longacre MR, Drake KM, Titus LJ, Peterson KE, Beach ML, Langeloh G, Hendricks K, Dalton MA. School food reduces household income disparities in adolescents' frequency of fruit and vegetable intake. *Prev. Med*. 2014;69C:202–7.

[89] Cummins S. Commentary: investigating neighborhood effects on health – avoiding the 'local trap'. *Int. J. Epidemiol*. 2007;36(2):355–7.

[90] Larson NI, Story MT, Nelson MC. Neighborhood environments: disparities in access to healthy foods in the US. *Am. J. Prev. Med*. 2009;36(1):74–81.

[91] Bodor J, Rose D, Farley T, Swalm C, Scott S. Neighborhood fruit and vegetable availability and consumption: the role of small foods to resin an urban environment. *Public Health Nutr*. 2008;11(4):413–20.

[92] Laska MN, Hearst MO, Forsyth A, Pasch KE, Lytie L. Neighborhood food environments: are they associated with adolescent dietary intake, food purchases and weight status? *Public Health Nutr*. 2010;13(11):1757–63.

[93] Jago R, Baranowski T, Baranowski JC, Cullen KW, Thompson D. Distance to food stores & adolescent male fruit and vegetable consumption: mediation effects. *Int. J. Behav. Nutr. Phys. Act*. 2007;4:35.

[94] Berge JM, Wall M, Larson N, Forsyth A, Bauer KW, Neumark-Sztainer D. Youth dietary intake and weight status: healthful neighborhood food environments enhance the protective role of supportive family home environments. *Health Place*. 2014;26:69–77.

[95] Jackson DM, Djafarian K, Stewart J, Speakman JR. Increased television viewing is associated with elevated body fatness but not with lower total energy expenditure in children. *Am. J. Clin. Nutr*. 2009;89(4):1031–6.

[96] Manios Y, Kourlaba G, Kondaki K, Grammatikaki E, Anastasiadou A, Roma-Giannikou E. Obesity and television watching in preschoolers in Greece: the GENESIS study. *Obesity*. 2009;17(11):2047–53.

[97] Vereecken CA, Todd J, Roberts C, Mulvihill C, Maes L. Television viewing behavior and associations with food habits in different countries. *Public Health Nutr*. 2006;9(2): 244–50.

[98] Wiecha JL, Peterson KE, Ludwig DS, Kim J, Sobol A, Gortmaker SL. When children eat what they watch: impact of television viewing on dietary intake in youth. *Arch. Pediatr Adolesc. Med*. 2006;160(4):436–42.

[99] Pearson N, Biddle SJ. Sedentary behavior and dietary intake in children, adolescents, and adults. A systematic review. *Am. J. Prev. Med*. 2011;41(2):178–88.

[100] Pearson N, Biddle SJ, Williams L, Worsley A, Crawford D, Ball K. Adolescent television viewing and unhealthy snack food consumption: the mediating role of home availability of unhealthy snack foods. *Public Health Nutr*.2012; 17(2): 317–323.

In: Human Health and Nutrition
Editor: Sergej M. Ostojic
ISBN: 978-1-63482-823-9

Chapter X

A New Perspective in Physical Inactivity Related Mechanisms of Muscle Mass Loss

Boštjan Šimunič* and Rado Pišot
Science and Research Centre,
Institute for Kinesiology Research
University of Primorska Koper,
Slovenia

Abstract

Physical inactivity continues to be a major public health concern and is on the path to become the highest risk factor for global health. Sustained periods of physical inactivity are more common in older people, and when superimposed on the natural aging process, can cause significant declines in physiological and cognitive function and a loss of independence.

Furthermore, statistics show that hospitalization rates increase exponentially with age. Physical inactivity, together with unhealthy and unbalanced diet, represents an aggravating factor for chronic non-contagious diseases, muscle mass and strength decreases. Due to these epidemiological health concerns, the importance of developing interventions that reverse the decline associated with physical inactivity and/or aging is essential.

To remain independent and healthy, an important factor to consider is the maintenance of skeletal muscle, as the elderly become prone to a progressive age-related skeletal muscle wasting (sarcopenia and dynapenia). In this chapter we advanced the use of tensiomiography (TMG) as a novel approach to evaluate and manage sarcopenic muscle. TMG reliability, non-invasiveness and simplicity seem to enable researchers to investigate new strategies for preventing physical inactivity-related skeletal muscle adaptations.

* Corresponding Author address: Email: bostjan.simunic@zrs.upr.si

Keywords: Skeletal muscle; Tensiomyography; Physical inactivity; Bed rest; Aging

Introduction

The number of people in Europe over the age of 65 years is expected to almost double over the next 50 years. Additionally, physical inactivity continues to be a major public health concern and is progressively becoming the ultimate risk factor for global death. Even more, sustained periods of physical inactivity are more common in older people, and when superimposed on the natural aging process, can cause significant declines in physiological and cognitive function and a loss of independence [1,2]. Not only in elderly, physical inactivity is implicated in the recent worldwide epidemic of obesity and indicated as a major risk factor for morbidity and mortality also in adults as well as an independent risk factor for metabolic morbidity in children and adolescents [3–5]. Due to these epidemiological health concerns, the importance of developing interventions that reverse the negative consequences associated with physical inactivity and/or aging is essential. To remain independent and healthy, an important factor to consider is the maintenance of skeletal muscle.

The lifelong maintenance of muscle mass is an important health issue where physical activity with exercise and nutrition plays an important role. Regimes leading to maintaining muscle mass not only improves muscle mass but also bone mineral density, connective tissue, balance, blood-sugar control, sleep, mental health, decrease fall risk, ease arthritis pain, and increase energy demand for more efficient body weight control and finally improve mechanisms against widespread syndrome of frailty.

Sarcopenia and Dynapenia

The age related loss of muscle mass (sarcopenia) [6] and strength (dynapenia) [7] are a major causes of disability and frailty in the elderly. Both could be a consequence of normal aging that does not require a disease to occur, although muscle loss can be accelerated by chronic illness [8]. Important is to say that introducing a dynapenia as a new term for a parallel process to sarcopenia, suggests different mechanisms for either processes or at least the processes are not directly linked.

There are many candidate mechanisms leading to sarcopenia, including age-related declines in alpha-motor neurons, growth hormone production, sex steroid levels, protein intake, changes in muscle fibers and physical inactivity. Furthermore, fat gain, increased production of catabolic cytokines, and inadequate energy and protein intake are also potentially important causes of sarcopenia. The observation that strength greatly reduces the association between muscle mass and functional decline [9] and also to early death [10] to a statistically non-significant level suggests that the contribution of muscle mass on certain outcomes may be primarily due to its association with strength. Thus it is important to establish mechanisms of dynapenia, a strength loss due to aging.

Several mechanisms of dynapenia arise from neural and muscular factors [7]. However, subclinical deficits in the structure and function of the nervous system and/or impairments in the intrinsic force-generating properties of skeletal muscle are potential antecedents to

dynapenia [11]. Sarcopenia is an important muscular factor; however its level of association to dynapenia is blurred as cross sectional studies reported up to 35% of explained variance [12] but longitudinal studies < 5 % of explained variance [13]. Other muscular mechanisms are: muscle architecture changes, muscle specific tension decline, while neural mechanisms are: α-motoneuron excitation decline and presence of neuropathic processes.

Assessing Muscle Mass and Strength in Aging Studies

Currently, there is no consensus on the assessing of sarcopenia, although there are five methods to assess muscle mass and function in aging studies. We could summarize them in three approaches [14]: (a) comparing standardized (to body height, mass, fat) muscle mass using dual-energy X-ray absorptiometry or bio-impedance analysis and compare the individual values to younger reference populations; (b) compare individual values of muscle function to younger reference populations; and (c) the combination of both – muscle size and function. Altogether seven methods have been developed (Table 1) for diagnosing sarcopenia but little evidence is on their interexchange reliability. Even more, only 0.2% of all participants were recognized as sarcopenic consistently by all five methods [14]. That leads to a conclusion that the prevalence of sarcopenia is strongly dependent on the method being used and therefore, these consensus definitions are not clinically applicable. Therefore, if sarcopenia classification is performed a method should be described in details. As age itself is no longer considered a causal factor of disease [15], there is a substantial need for a new consensus definition or a method in order to make studies comparable and for implementation in clinical care.

Table 1. Seven methods for diagnosing sarcopenia

Measure	Reference
ALM/BH^2	[16–18]
Residuals of linear regression of ALM with BH and FM	[17]
100% × SLM/BM	[19]
SLM/BH^2	[20]
Optimal cut-point for grip strength, identified in the ROC curve, predicting walking slower than 0.8 m/s	[21]

Abbreviations: ALM – appendicular lean mass; SLM – skeletal lean mass; BH – body height; BM – body mass; FM – fat mass

Tensiomyography – A New Measure for Sarcopenia, Dynapenia or Both?

Validation

In 1990 a new method was introduced – named tensiomyography (TMG) as another mechanomyographic method to assess skeletal muscle contractile properties [22]. From a time response, four contractile parameters are calculated as presented in Figure 1. Firstly, it was introduced to assess contractile properties of denervated muscle [22] and later to noninvasively distinguish between muscles with high and low proportion of type I fibers [23, 24]. After many reliability studies [25–27] a solid evidence exists that method has high inter-rater, within- and between-day repeatability in all except one extracted parameters (ICC > 0.88) with only half-relaxation time being less reliable (95% CI ICC [0.78, 0.96]) [27].

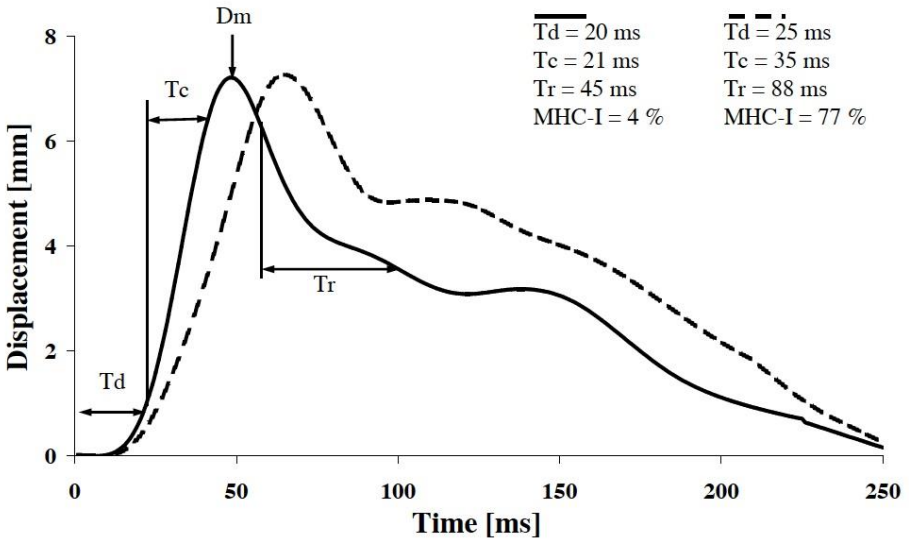


Figure 1. Vastus lateralis tensiomyographic response of muscle with low proportion of myosin heavy chain type I (full line) and with high proportion of myosin heavy chain type I (dotted line). Four contractile parameters are presented, where: Dm is maximal amplitude; Td is defined from electrical impulse to 10% of Dm; Tc is time from 10% to 90 % of Dm; and Tr is half relaxation time from 90% to 50 % Dm. Adapted from [28].

From our opinion three breaking points exist in the TMG method development:

First is a high correlation between TMG contraction time (Tc) and myosin heavy chain I (MHC-I) proportion in vastus lateralis muscle ($r = 0.87$; $MHC\text{-}I = 3.93 \cdot Tc - 158.2$; $p < 0.001$) [28]. Furthermore, the same authors found even higher multivariate correlation between three contractile parameter (delay time - Td, Tc, and half-relaxation time - Tr) estimated from TMG with MHC-I proportion ($r = 0.93$; $MHC\text{-}I = 2.829 \cdot Td + 2.98 \cdot Tc + .127 \cdot Tr - 121.023$; $p < 0.001$) [28]. That clearly indicates that TMG is highly sensitive for a non-invasive assessment

of muscle composition changes due to aging, disease, training, inactivity, etc. However, such a relation is confirmed only in vastus lateralis muscle and not in other muscles.

Second is a relation between skeletal muscle mechanical response detected by TMG and with standard twitch torque assessment method. Skeletal muscle mechanical response detected by both methods is related only in Tr ($r = 0.61$; $p = 0.025$) but not in Td ($r = 0.08$) and Tc ($r = 0.23$) [29, 30]. Important is also to know that TMG Tc is 46.7 % shorter than when estimated from torque twitch response [30]. That indicates that TMG introduces new information about mechanical muscle output that is quick and not detected by standard twitch torque detection method (probably filtered through its propagation through connective tissue and dumping effect of joint mechanics, muscle mass bulk movement, etc.).

Thirdly, there is a negative correlation between the TMG amplitude increase and muscle belly thickness decrease (measured with ultrasonography) after 35-days of bed rest ($r = -0.70$; $P < 0.01$) in gastrocnemius medialis muscle of young participants [31]. This finding came to us as a surprise, as we found that higher the atrophy, the higher the TMG amplitude. Later on, we demonstrated the same findings in 12 muscles after 35-days of bed rest, where after 14-days of recovery almost in all muscles TMG amplitude recovered to baseline values, or at least there was a trend towards recovery [32]. Beside a confirmed relation between TMG amplitude and muscle belly thickness changes, authors later also reported that TMG amplitude increases without muscle volume change [33] and attribute this to muscle tone (or stiffness) decrease, where increased TMG amplitude reflects loss of muscle tone. However, to proof this, several experiments have been performed, as presented below.

Skeletal Muscle Atrophy Dynamics

Immediate response of human body when going from upright to horizontal posture is decrease in leg and increase in trunk muscle size [34, 35]. Fluid movement from vasculature to interstitium is likely the cause in early muscle volume alterations. The authors refer to this phenomenon as redistribution of the body fluids or fluid shift as a consequence of gravity-dependent alterations in hydrostatic pressure.

Previous studies have documented that skeletal muscle mass and strength are reduced with as little as seven days of spaceflight [36, 37] and continue to decline with the length of exposure [38]. But there is little research on anatomical muscle atrophy rate or function loss within first seven days of inactivity exposure even though, short-term inactivity exposures are much more frequent than long-term, and therefore more research is needed to investigate early muscle atrophy dynamics and processes related to it.

To demonstrate the usefulness of TMG in detecting early processes of muscle atrophy and function loss due to physical inactivity we put 10 young participants in 35-day 6 degrees head down tilt bed rest and assessed muscle thickness using ultrasound, and contractile properties change using TMG in three different muscles [33]. Results for vastus medialis obliques and biceps femoris are presented in Figure 2.

We found that vastus medialis obliques and biceps femoris thickness declined after 7^{th} and 16^{th} day of bed rest, respectively. Findings in vastus medialis obliques are in agreement with other studies reporting muscle atrophy after 7 days of spaceflight [36, 37] while little data is available for biceps femoris, as a non-postural muscle. However, this is in agreement with our data that shows higher thickness loss in postural vastus medialis obliques (-21%)

than in non-postural biceps femoris (-13.5%) after 35-day of bed rest, as it was also presented by others [39]. However, TMG amplitude increased markedly already after the 1st and 7th day of bed rest for vastus medialis obliques and biceps femoris, respectively. Therefore, initial TMG amplitude increase preceded thickness decline and provides us an important insight in early processes that could not be detected by clinical assessment, but are evident from TMG response. Later on TMG amplitude increased even more and followed the trend of thickness change. TMG amplitude increase is related to thickness loss and also to muscle stiffness loss [31]. The latter is a consequence of muscle tone loss [40]. The exact mechanisms of TMG amplitude increase are still under investigations; however it seems that abovementioned two factors explained most of its variance.

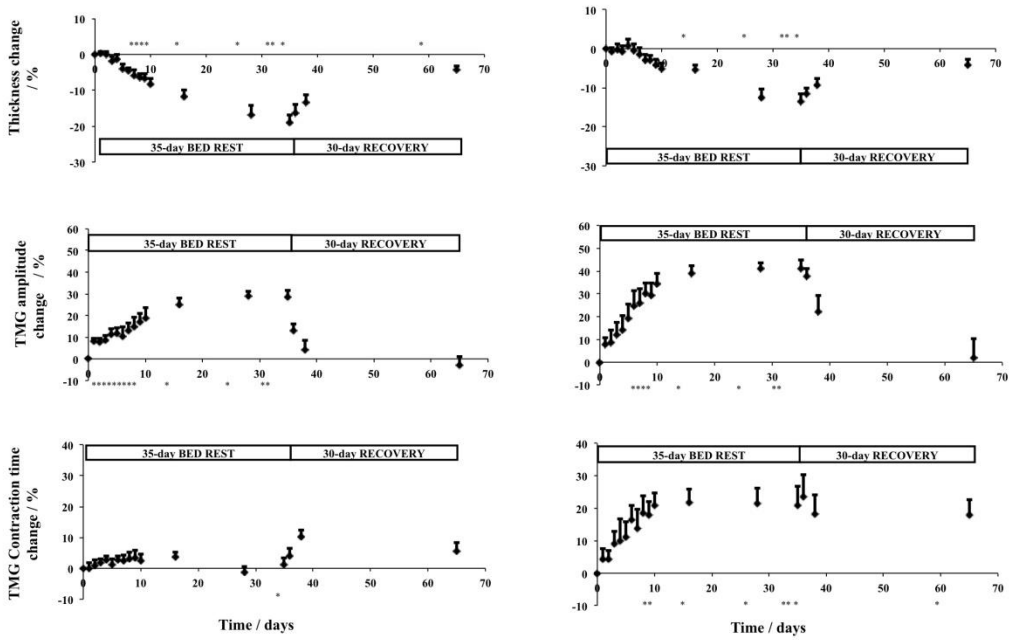


Figure 2. Mean (with standard error) of thickness (upper row), tensiomyographic (TMG) maximal amplitude (middle row) and contraction time (bottom row) changes during 35-day bed rest and followed 30-day recovery in vastus medialis obliques (left) and biceps femoris (right) muscles. * Different from baseline at $P < 0.05$. Adapted from [33].

TMG Tc did not change much in vastus medialis obliques, except on 2nd day of recovery when increased, probably due to reported delayed muscle soreness reported by most of participants, which was triggered by post bed rest evaluation tests. However, Tc increased markedly in biceps femoris already after 9th day of bed rest and remained increased also after 30-day of recovery. This provides us an important clinical finding that Tc changes are muscle specific and even more important is that in non-postural biceps femoris bed rest provoked increase is irreversible for at least 30 day afterwards.

This study provides us an important insight into atrophic, early atrophic and atrophy-related modifications of skeletal muscle geometry, stiffness and composition.

We clearly demonstrated that we could, by using non-invasive investigation, obtain for each muscle of interest an early modification of its tone/stiffness that might lead towards

muscle atrophy. Furthermore, even though our study participants were young and fit healthy males, we have demonstrated irreversible increase of Tc in non-postural muscle that has a huge role for our knee health and sport performance. Both findings are of major importance when designing interventions or rehabilitation protocols in future physical inactivity studies or real life settings (sport and rehabilitation).

Skeletal Muscle Atrophy in Young and Old

Only three studies evaluated physical decline in older participants after bouts of physical inactivity [41–43]. However, only [43] directly compared young and older male participants after 14 day of unilateral leg immobilization. In 2012/13 we conducted a 14-day bed rest study with 7 younger (18-30 years) and 16 older (55-65 years) participants (article in press). Knee extensor volume change for both age groups is presented in Figure 3. Interestingly we found higher volume decline in older group (P = 0.031) after 14-day bed rest.

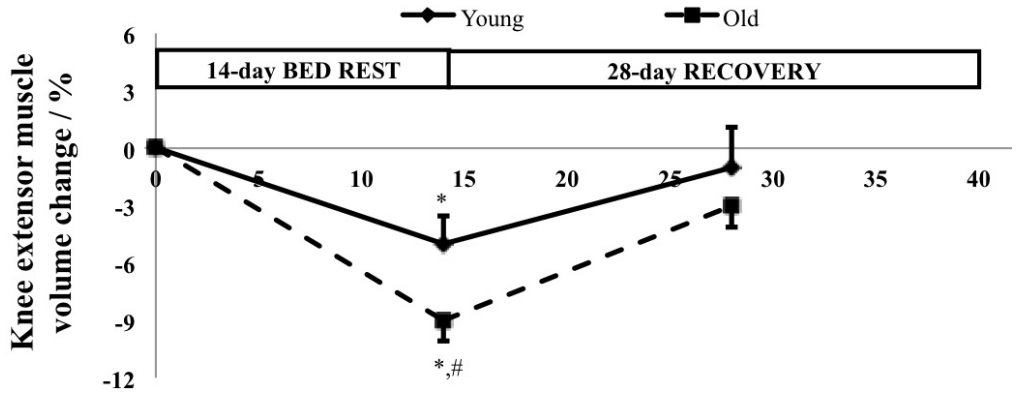


Figure 3. Knee extensor muscle volume change after 14-day bed rest in young and old age groups. Data is presented also after 14-day of recovery. * Different from baseline at P < 0.05. # Different from both groups after controlling baseline differences at P < 0.05.

When we compared TMG data of both age groups (Figure 4) we found significant TMG amplitude increase in both groups after 14-day bed rest, higher but not significantly in older participants (P = 0.112). After 14 days of recovery TMG amplitude recovered to baseline values in both groups. Changes in young are in agreement with our previous findings (Figure 2); however surprisingly after 14-day bed rest in older participants TMG amplitude tends to increase even more than in young, even though older participants have 36% higher TMG amplitude already at baseline. The same trend but even more pronounced was found in TMG Tc. After 14-day bed rest Tc increased in the group of older participants and remained increased after 14-day of recovery with very small slope of recovery. In young no significant changes were observed which is in agreement with our previous findings for postural muscle (Figure 2) [33].

Our study is the first to compare TMG assessed skeletal muscle contractile properties in the group of young and older participants submitted to disuse. Our results clearly demonstrate more pronounced responses after bout of physical inactivity in the group of old. This is in agreement with Deschenes et al. [44] who calculated rate of twitch force development (from

0–30 milliseconds) and found the same responses after 7-day immobilization in rats as we did in TMG Tc. Furthermore, from only one study performed in rats [44] examined time to torque twitch peak in both age groups, and found unchanged values; however, time to torque peak is not related to TMG Tc [30].

The mechanisms of our results could be explained by muscle fiber cross sectional area (CSA) changes after 14-day of immobilization [45]. They reported significant decrease in type I and Type IIa CSA in younger and only in Type IIa in older participants. Assuming mathematical model developed to describe intrinsic (not measured at distal limb) torque twitch responses of muscles with various fiber type composition [46], this fully explained our results. Even more, after 28-day of recovery both fiber types in young recover fully, while in older participants Type IIa did not fully recover [45]. That is in agreement with our data (Figure 4) where TMG Tc was increased after 14-day of recovery in older group and we could extrapolate that TMG Tc full recovery might be longer than 28-days or even irreversible.

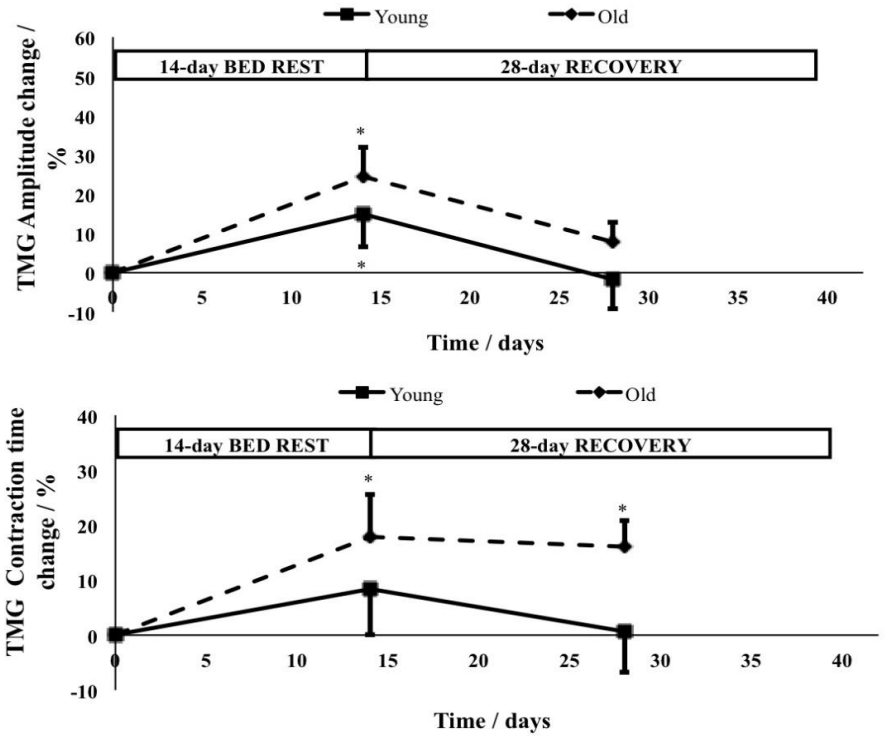


Figure 4. Mean (with standard error) of tensiomyographic (TMG) maximal amplitude (left) and contraction time (right) changes after 14-day bed rest and during 28-day recovery (on 14th day) in vastus lateralis. * Different from baseline at $P < 0.05$

Skeletal Muscle Atrophy Related to Food Intake

Factors that have been implicated for sarcopenia are: (a) decreased physical activity level [41]; (b) declining androgen concentrations [46]; (c) specific nutritional deficiencies (dietary

protein and vitamin D) [48]; (d) chronic inflammation [49, 50]; (e) insulin resistance [51]; and a number of other factors.

Importantly, decreased physical activity that occurs with aging may contribute to age-related sarcopenia, where atrophy results in decreased rate of muscle protein synthesis, while sarcopenia results in alteration of both: decreased rate of muscle protein synthesis and increased rate of muscle protein degradation. Therefore, the rate of muscle loss is accelerated even more when an older person undergoes a period of bed rest.

In individuals from early bed rest studies [52, 53] and from space-flown missions [54] it was shown that decreased protein imbalance could be achieved by using orally or intravenously administered isotopically labeled amino acids.

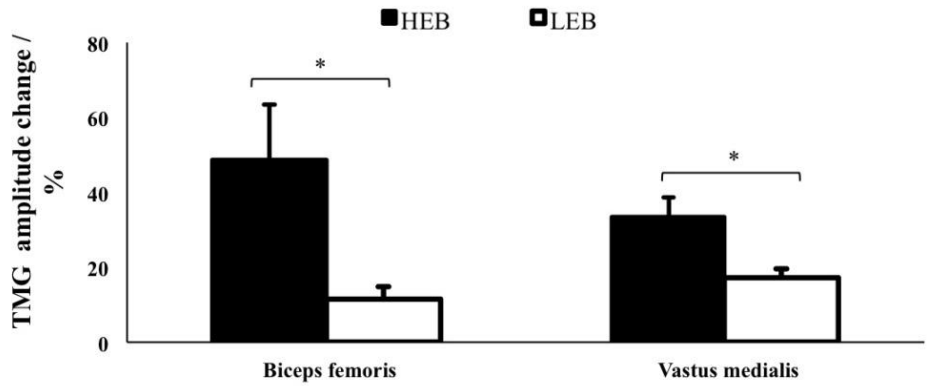


Figure 5. TMG amplitude change after 35-day bed rest for two muscles in high (HEB) and low (LEB) energy groups. * Different between groups at $P < 0.05$.

This data suggest that physical inactivity is a partly responsible for muscle wasting and protein intake have an important prophylactic role. Not only additional protein intake also neutral energy balance must be achieved during periods of inactivity to prevent muscle wasting [55] as it was clearly demonstrated that by adjusting neutral (lower) energy balance in one group of participants (N = 5; LEB) while allow other to achieve high energy balance (N = 5, HEB) during 35-day bed rest of healthy young males, muscle thickness was only diminished in HEB group. TMG results were never evaluated to the food intake, regardless the close relation between muscle mass and food intake [52–55]. Therefore, we post-study compared TMG data from the same participants and methods used as proposed by [55] and for both groups (HEB and LEB) confirm higher TMG amplitude increase after 35-day bed rest while no changes in TMG Tc. However, in Figure 5 higher TMG amplitude increase is observed in HEB comparing to LEB for both muscles. Physical inactivity is regularly associated to muscle atrophy; however, in healthy humans, physical inactivity is frequently associated also to positive energy balance leading to increased fat mass. Those who gain more fat, increase plasma leptin concentrations and suffer with greatest loss of fat-free mass, muscle mass specifically [55]. Positive energy balance and fat-free mass loss are associated with increased systemic inflammation and free radical production as well as activation of the glutathione system in erythrocytes. Additionally decreased muscle mass as a result of interaction between physical inactivity and HEB resulted in higher increase of TMG

amplitude of vastus medialis and biceps femoris (Figure 5), which was previously explained by alterations in muscle tone and thickness [31].

Conclusion

After the first introduction of TMG in 1990, as a noninvasive method for the measurement of the skeletal muscle contractile properties, TMG continues to provide important insights in skeletal muscle adaptation during physical inactivity and rehabilitation. In particular, use of tensiomyography might be applicable novel approach to evaluate and manage sarcopenic muscle; the procedure is reliable, inexpensive and simple to use, and sensitive enough to detect early atrophic changes.

References

[1] Davis HP, Trussell LH, Klebe KJ. A ten-year longitudinal examination of repetition priming, incidental recall, free recall, and recognition in young and elderly. *Brain Cogn.* 2001;46(1-2):99–104.

[2] Nybo H, Petersen HC, Gaist D, Jeune B, Andersen K, McGue M, Vau-pel JW, Christensen K. Predictors of mortality in 2,249 nonagenarians - the Danish 1905-Cohort Survey. *J. Am. Geriatr. Soc.* 2003;51:1365–73.

[3] Jakičič JM, Clark K, Coleman E, Donnelly JE, Foreyt J, Melanson E, Volek J, Volpe SL. Appropriate intervention strategies for weight loss and prevention of weight regain for adults. *Med. Sci. Sports Exerc.* 2001;33(12):2145–56.

[4] Caballero B. The global epidemic of obesity: An overview. *Epidemiol Rev.* 2007;29:1–5.

[5] Strong WB, Malina RM, Blimkie CJ, Daniels SR, Dishman RK, Gutin B, Hergenroeder AC, Must A, Nixon PA, Pivarnik JM, Rowland T, Trost S, Trudeau F. Evidence based physical activity for school-age youth. *J Pediatr.* 2005;146:732–7.

[6] Rosenberg IH. Summary comments. *Am. J. Clin. Nutr.* 1989;50:1231–3.

[7] Clark BC, Manini TM. Sarcopenia ≠ Dynapenia. *J. Gerontol.* 2008;63A8):829–34.

[8] Roubenoff R. Sarcopenia: a major modifiable cause of frailty in the elderly. *J. Nutr. Health Aging.* 2000;4(3):140–2.

[9] Visser M, Goodpaster BH, Kritchevsky SB, Newman AB, Nevitt M, Rubin SM, Maughan RJ, Watson JS, Weir J. Strength and cross-sectional area of human skeletal muscle. *J. Physiol.* 1983;338:37–49.

[10] Newman AB, Kupelian V, Visser M, Simonsick EM, Goodpaster BH, Kritchevsky SB, Tylavsky FA, Rubin SM, Harris TB. Strength, but not muscle mass, is associated with mortality in the Health, Aging and Body Composition Study cohort. *J. Gerontol. A Biol. Sci. Med. Sci.* 2006;61:72–7.

[11] Clark BC, Manini TM. What is dynapenia? *Nutrition.* 2012;28(5):495–503.

[12] Maughan RJ, Watson JS, Weir J. Strength and cross-sectional area of human skeletal muscle. *J. Physiol.* 1983;338:37–49.

[13] Hughes VA, Frontera WR, Wood M, Evans WJ, Dallal GE, Roubenoff R, Fiatarone Singh MA. Longitudinal muscle strength changes in older adults: influence of muscle mass, physical activity, and health. *J. Gerontol. Biol. Sci.* 2001;56A:B209–17.

[14] Bijlsma AY, Meskers CGM, Ling CHY, Narici MV, Kurrle SE, Cameron ID, Westendorp RGJ, Maier AB. Defining sarcopenia: the impact of different diagnostic criteria on the prevalence of sarcopenia in a large middle aged cohort. *Age.* 2013;35:871–81.

[15] Holliday R. Ageing in the 21st century. *Lancet.* 1990;354(Suppl):SIV4.

[16] Baumgartner RN, Koehler KM, Gallagher D, Romero L, Heymsfield SB, Ross RR, Garry PJ, Lindeman RD. Epidemiology of sarcopenia among the elderly in New Mexico. *Am. J. Epidemiol.* 1998;147:755–63.

[17] Delmonico MJ, Harris TB, Lee JS, Visser M, Nevitt M, Kritchevsky SB, Tylavsky FA, Newman AB. Health, Aging and Body Composition Study. Alternative definitions of sarcopenia, lower extremity performance, and functional impairment with aging in older men and women. *J. Am. Geriatr. Soc.* 2007;55:769–74.

[18] Kelly TL, Wilson KE, Heymsfield SB. Dual energy X-ray absorptiometry body composition reference values from NHANES. *PLoS One.* 2009;4(9):e7038.

[19] Janssen I, Heymsfield SB, Ross R. Low relative skeletal muscle mass (sarcopenia) in older persons is associated with functional impairment and physical disability. *J. Am. Geriatr. Soc.* 2002;50:889–96.

[20] Janssen I, Baumgartner RN, Ross R, Rosenberg IH, Roubenoff R. Skeletal muscle cutpoints associated with elevated physical disability risk in older men and women. *Am. J. Epidemiol.* 2004;159:413–21.

[21] Lauretani F, Russo CR, Bandinelli S, Bartali B, Cavazzini C, Di Iorio A, Corsi AM, Rantanen T, Guralnik JM, Ferrucci L. Age-associated changes in skeletal muscles and their effect on mobility: an operational diagnosis of sarcopenia. *J. Appl. Physiol.* 2003;95:1851–60.

[22] Valenčič V. Direct measurement of the skeletal muscle tonus. In Popović D., editor. Advances in external control of human extremities. *Beograd: Nauka*. 1990:102–8.

[23] Dahmane R, Valenčič V, Knez, N, Eržen I. Evaluation of the ability to make noninvasive estimation of muscle contractile properties on the basis of the muscle belly response. *Med Biol Eng Comput.* 2001;38:51–5.

[24] Dahmane R, Djordjevič S, Šimunič B, Valenčič V. Spatial fiber type distribution in normal human muscle histochemical and tensiomyographical evaluation. *J. Biomech.* 2005;38:2451–9.

[25] Križaj D, Šimunič B, Žagar T. Short-term repeatability of parameters extracted from radial displacement of muscle belly. *J. Electromyogr Kinesiol.* 2008;18(4):645–51.

[26] Tous-Fajardo J, Moras G, Rodríguez-Jiménez S, Usach R, Doutres DM, Maffiuletti NA. Inter-rater reliability of muscle contractile property measurements using non-invasive tensiomyography. *J. Electromyogr. Kinesiol.* 2010;20(4):761–6.

[27] Šimunič B. Between-day reliability of a method for non-invasive estimation of muscle composition. *J. Electromyogr. Kinesiol.* 2012;22(4):527–30.

[28] Šimunič B, Degens H, Rittweger J, Narici MV, Mekjavić IB, Pišot R. Noninvasive estimation of myosin heavy chain composition in human skeletal muscle. *Med. Sci. Sports Exer.* 2011;43(9):1619–25.

[29] Šimunič B, Križaj D, Narici MV, Pišot R. Twitch parameters in transversal and longitudinal biceps brachii response. *Annales Kinesiologiae.* 2010;1(1):61–80.
[30] Koren K, Šimunič B, Rejc E, Lazzer S, Pišot R. Differences between skeletal muscle contractile parameters estimated from transversal tensiomyographic and longitudinal torque twitch response. *Kinesiol.* 2015;in press.
[31] Pišot R, Narici MV, Šimunič B, De Boer M, Seynnes O, Jurdana M, Biolo G, Mekjavić IB. Whole muscle contractile parameters and thickness loss during 35-day bed rest. *Eur. J. Appl. Physiol.* 2008;104(2):409–14.
[32] Šimunič B, Rittweger J, Cankar G, Jurdana M, Volmut T, Šetina T, Mekjavić IB, Pišot R. Changes in body composition, muscle stiffness and postural stability occurring in healthy young men submitted to a 35-day bed rest. *Slovenian Journal of Public Health.* 2008;47(2):60–71.
[33] Šimunič B, Degens H, Rittweger J, Narici MV, Pišot V, Mekjavić IB, Pišot R. Tensiomyographic measurement of atrophy related processes during bed rest and recovery. *Proceedings of Life in Space for Life on Earth*, Editors: Ouwehand L, ESA Communications, 2012.
[34] Berg HE, Tedner B, Tesch PA. Changes in lower limb muscle cross-sectional area and tissue fluid volume after transition from standing to supine. *Acta. Physiologica Scandinavica.* 1993;148(4):379–85.
[35] Conley MS, Foley JM, Ploutz-Snyder LL, Meyer RA, Dudley GA. Effect of acute head-down tilt on skeletal muscle cross-sectional area and proton transverse relaxation time. *J. Appl. Physiol.* 1996; 81(4):1572–7.
[36] LeBlanc A, Rowe R, Schneider V, Evans H, Hedrick T. Regional muscle loss after duration spaceflight. *Aviat. Space Environ. Med.* 1995;66:1151–4.
[37] Grigoryeva LS, Kozlovskaya IB. Effect of weightlessness and hypokinesis on velocity and strength properties of human muscles. *Kosmicheskaya Biologiya I Aviakosmicheskaya Meditsina.* 1987;21:27–30.
[38] Adams GR, Caiozzo VJ, Baldwin KM. Skeletal muscle unweighting: spaceflight and ground-based models. *J. Appl. Physiol.* 2003;95:2185–201.
[39] De Boer MD, Seynnes OR, di Prampero PE, Pišot R, Mekjavić IB, Biolo G, Narici MV. Effect of 5 weeks horizontal bed rest on human muscle thickness and architecture of weight bearing and non-weight bearing muscles. *Eur. J. Appl. Physiol.* 2008;04(2):401–7.
[40] Abe T, Kawakami Y, Suzuki Y, Gunji A, Fukunaga T. Effects of 20 days bed rest on muscle morphology. *J. Gravit. Physiol.* 1997; 4(1):S10–4.
[41] Kortebein P, Ferrando A, Lombeida J, Wolfe R, Evans WJ. Effect of 10 days of bed rest on skeletal muscle in healthy older adults. [Letter Research Support, N.I.H., Extramural]. *JAMA.* 2007;297(16):1772–4.
[42] Drummond MJ, Timmerman KL, Markofski MM, Walker DK, Dickinson JM, Jamaluddin M, Brasier AR, Rasmussen BB, Volpi E. Short-term bed rest increases TLR4 and IL-6 expression in skeletal muscle of older adults. *Am. J. Physiol Regul. Integr. Comp Physiol.* 2013;305(3):R216–23.
[43] Suetta C, Hvid LG, Justesen L, Christensen U, Neergaard K, Simonsen L, Ortenblad N, Magnusson SP, Kjaer M, Aagaard P. Effects of aging on human skeletal muscle after immobilization and retraining. [Comparative Study Research Support, Non-U.S. Gov't]. *J. Appl. Physiol.* 2009;107(4):1172–80.

[44] Deschenes MR, Britt AA, Chandler WC. A comparison of the effects of unloading in young adult and aged skeletal muscle. *Med. Sci. Sports Exerc.* 2001;33(9):1477–83.

[45] Hvid L, Aagaard P, Justesen L, Bayer ML, Andersen JL, Ortenblad N, Kjaer M, Suetta C. Effects of aging on muscle mechanical function and muscle fiber morphology during short-term immobilization and subsequent retraining. [Comparative Study Research Support, Non-U.S. Gov't]. *J. Appl. Physiol.* 2010;109(6):1628–34.

[46] Savelberg HHCM. Rise and relaxation times of twitches and tetani in submaximally recruited, mixed muscle: a computer model. In: *Skeletal Muscle Mechanics: From Mechanism to Function*. Herzog, W. (editor), John Wiley & Sons, Ltd., Chichester, England, 2000.

[47] Morley JE. Hormones and the aging process. *J. Am. Geriatr Soc.* 2003;51:S333–7.

[48] Visser M, Deeg DJ, Lips P. Low vitamin D and high parathyroid hormone levels as determinants of loss of muscle strength and muscle mass (sarcopenia): the Longitudinal Aging Study Amsterdam. *J. Clin. Endocrinol Metab.* 2003;88:5766–72.

[49] Cesari M, Kritchevsky SB, Baumgartner RN, Atkinson HH, Penninx BW, Lenchik L, Palla SL, Ambrosius WT, Tracy RP, Pahor M. Sarcopenia, obesity, and inflammation—results from the Trial of Angiotensin Converting Enzyme Inhibition and Novel Cardiovascular Risk Factors study. *Am. J. Clin. Nutr.* 2005;82:428–34.

[50] Cesari M, Penninx BW, Pahor M, Lauretani F, Corsi AM, Rhys Williams G, Guralnik JM, Ferrucci L. Inflammatory markers and physical performance in older persons: the InCHIANTI study. *J. Gerontol. A Biol. Sci. Med. Sci.* 2004;59:242–8.

[51] Evans WJ, Farrell PA. The aging pancreas: the effects of aging on insulin secretion and action. In: Jefferson JS, Cherrington AD, eds. *The Handbook of Physiology*. Oxford, United Kingdom: Oxford University Press, 2001:969–99.

[52] Shangraw RE, Stuart CA, Prince MJ, Peters EJ, Wolfe RR. Insulin responsiveness of protein metabolism in vivo following bedrest in humans. *Am. J. Physiol.* 1988 Oct;255:E548–58.

[53] Stuart CA, Shangraw RE, Prince MJ, Peters EJ, Wolfe RR. Bed-rest-induced insulin resistance occurs primarily in muscle. *Metabolism.* 1988;37(8):802–6.

[54] Stein TP, Gaprindashvili T. Spaceflight and protein metabolism, with special reference to humans. *Am. J. Clin. Nutr.* 1994;60:806S–19S.

[55] Biolo G, Agostini F, Šimunič B, Sturma M, Torelli L, Preiser JC, Deby-Dupont G, Magni P, Strollo F, di Prampero P, Guarnieri G, Mekjavić IB, Pišot R, Narici MV. Positive energy balance is associated with accelerated muscle atrophy and increased erythrocyte glutathione turnover during 5 wk of bed rest. *Am. J. Clin. Nutr.* 2008;88(4):950–8.

Index

B

C

D

E

F

G

H

I

J

K

L

M

N

O

P

Q

R

S

T

U

V

W

X

Y

Z