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Vitamin A

Edited by Leila Queiroz Zepka,
Veridiana Vera de Rosso and Eduardo Jacob-Lopes

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Scope of the Series

Biochemistry, the study of chemical transformations occurring within living organisms, impacts all of life sciences, from molecular crystallography and genetics, to ecology, medicine and population biology. Biochemistry studies macromolecules - proteins, nucleic acids, carbohydrates and lipids –their building blocks, structures, functions and interactions. Much of biochemistry is devoted to enzymes, proteins that catalyze chemical reactions, enzyme structures, mechanisms of action and their roles within cells. Biochemistry also studies small signaling molecules, co-enzymes, inhibitors, vitamins and hormones, which play roles in the life process. Biochemical experimentation, besides coopting the methods of classical chemistry, e.g., chromatography, adopted new techniques, e.g., X-ray diffraction, electron microscopy, NMR, radioisotopes, and developed sophisticated microbial genetic tools, e.g., auxotroph mutants and their revertants, fermentation etc. More recently, biochemistry embraced the 'big data' omics systems.

Initial biochemical studies have been exclusively analytic: dissecting, purifying and examining individual components of a biological system; in exemplary words of Efraim Racker, (1913 –1991) "Don't waste clean thinking on dirty enzymes." Today however, biochemistry is becoming more agglomerative and comprehensive, setting out to integrate and describe fully a particular biological system. The 'big data' metabolomics can define the complement of small molecules, e.g., in a soil or biofilm sample; proteomics can distinguish all the proteins comprising e.g., serum; metagenomics can identify all the genes in a complex environment e.g., bovine rumen. This Biochemistry Series will address both the current research on biomolecules, and the emerging trends with great promise.

Contents

Preface

This book presents a global perspective on the structure, key sources, metabolism, functions, deficiency, and fortification strategies of vitamin A. Divided into six chapters, the book is intended to amplify the understanding of vitamin A through aspects related to either basic science or applied technology. This book is primarily dedicated to scientists, academicians, industrial representatives, innovative technology representatives, and also any nonspecialist reader willing to learn about the dynamic field of vitamins and their association with foods and health.

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Chapter 1

Introductory Chapter: A Global Perspective on Vitamin A

Tatiele Casagrande do Nascimento, Eduardo Jacob-Lopes, Veridiana Vera de Rosso and Leila Queiroz Zepka

1. General aspects of vitamin A

Vitamin A is a globally essential nutrient belonging to the group of fat-soluble vitamins that was first described in 1913 in a study of animals fed with ethereal egg or butter extract [1]. In the same year, Osborne and Mendel [2] made the first association of this vitamin with growth. Later, observations made by Steenbock [3] concluded their association with foods of yellow pigmentation (now known as β-carotene). The importance of vitamin A in vision health has been considered since ancient Egypt (1500 BC), where people suffering from night blindness were treated with a topical extract of hepatic liver extract (recognized today as a rich source of vitamin A) [3–6].

Its deficiency has typically been associated with continued malnutrition and childhood blindness; it is estimated that 254 million people suffer from vitamin A lack or related ocular disease [7]. It is now known that its benefits go beyond its role in vision yet include numerous essential metabolic and systemic functions [8].

To supply their metabolic functions and to avoid deficiency or overdosage, daily intake requirements were established according to their activity. This activity can be expressed as international units (IU) or retinol equivalents (RE): 1 IU is equivalent to 0.3 μg of total trans retinol or 0.6 μg of total all-*trans*-β-carotene, whereas 1 RE is equivalent to 1 μg of all-*trans*-retinol, 6 μg of all-*trans*-β-carotene, or 12 μg of another provitamin A carotenoids [9].

The recommended daily requirement for adult men and women is 900 and 700 μg RE/day, respectively, while 300 and 250 μg RE/day are the minimum intake limits. During pregnancy and lactation, recommendations are 700 and 950 μg RE/day, respectively [8, 9]. In the case of children in populations considered vitamin A deficient, doses of 60,000 μg RE/day are distributed twice a year [10]. According to Stephensen et al. [11], acute toxic reactions are uncommon at the dosages below 30,000 μg RE/day.

2. Basic chemistry and potential sources

Vitamin A is a lipophilic molecule; its structure was first elucidated by Paul Karrer in 1931 from fish liver oils, for which he won the Nobel [12]. It is now known that the term "vitamin A" is a generic term for retinol and its active metabolites, such as the retinal and retinoic acid [13]. Retinyl esters and carotenoids are also considered vitamin A forms; however, they are oxidized to active forms as soon as they enter the digestive tract of mammals [14].

In general, a retinoid is C_20 compound formally constituted of a β-ionone nucleus attached to four isoprenoid units and a functional group at the end of the acyclic chain [7]. As can be seen in **Figure 1**, retinol (all-*trans*-retinol) has an

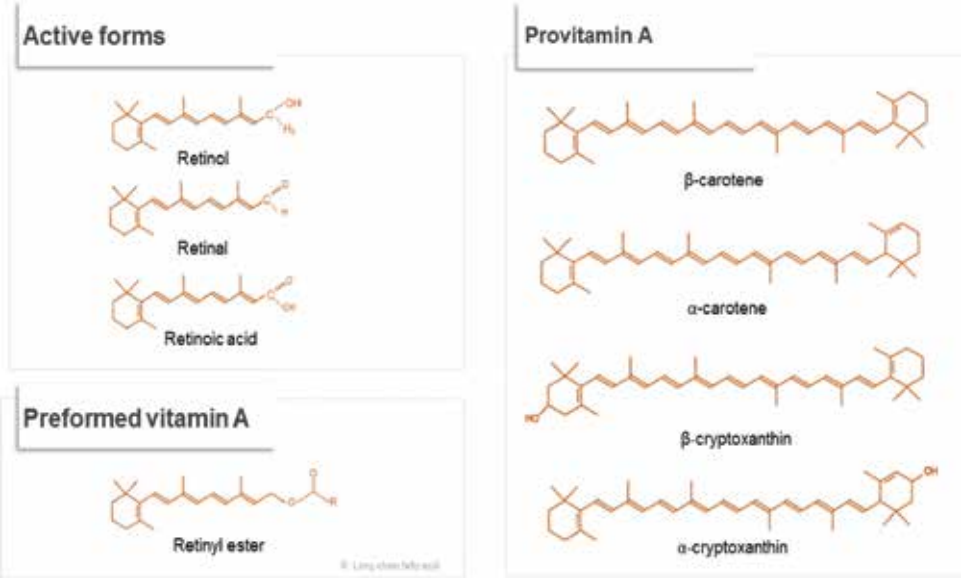

Figure 1.
Chemical structure of the different forms of vitamin A.

alcoholic end group, where oxidation of this group gives rise to an aldehyde group which characterizes the retinal (all-*trans*-retinal) structure, which may be further converted to all-*trans*-retinoic acid [15, 16].

These structures are essential for life in mammals; however, they cannot be de novo synthesized, and their supply depends on dietary intake. They are supplied to the human body in different forms, such as carotenoids provitamin A (mainly β-carotene) or preformed vitamin A (retinyl esters) [13, 17–19].

Carotenoids of provitamin A are present in both plant (via de novo synthesis) and animal products (via dietary intake) [7]. Those having provitamin A activity have at least one β-ring unsubstituted with an 11-carbon polyene chain, which undergoes enzymatic cleavage (in humans and animals) to produce at least one molecule of retinol [20–22]. It is estimated that 1178 natural carotenoids were characterized correctly and reported in the literature [23, 24]. Of these, about 60 have provitamin A activity [20]. However, only some of these carotenoids are commonly found in foods, such as β-carotene, α-carotene, β-cryptoxanthin, and α-cryptoxanthin, with β-carotene being the only one with 100% activity [25].

On the other hand, preformed vitamin A, predominantly retinyl palmitate, is obtained only from sources of animal origin, the main related foods are milk, meat, and eggs [7]. Also, for infants, breast milk is the primary source of vitamin A, aimed at meeting the daily needs and the formation of hepatic reserve of this vitamin [26].

3. Metabolism and biological functions

Regardless of the source of origin, retinol can be stored in various tissues (predominantly in the liver) to maintain adequate serum levels for extended periods [8]. Among the three states of oxidation (retinol, retinal, and retinoic acid), retinol is the most active form, whereas retinoic acid is the only one that is not stored [27].

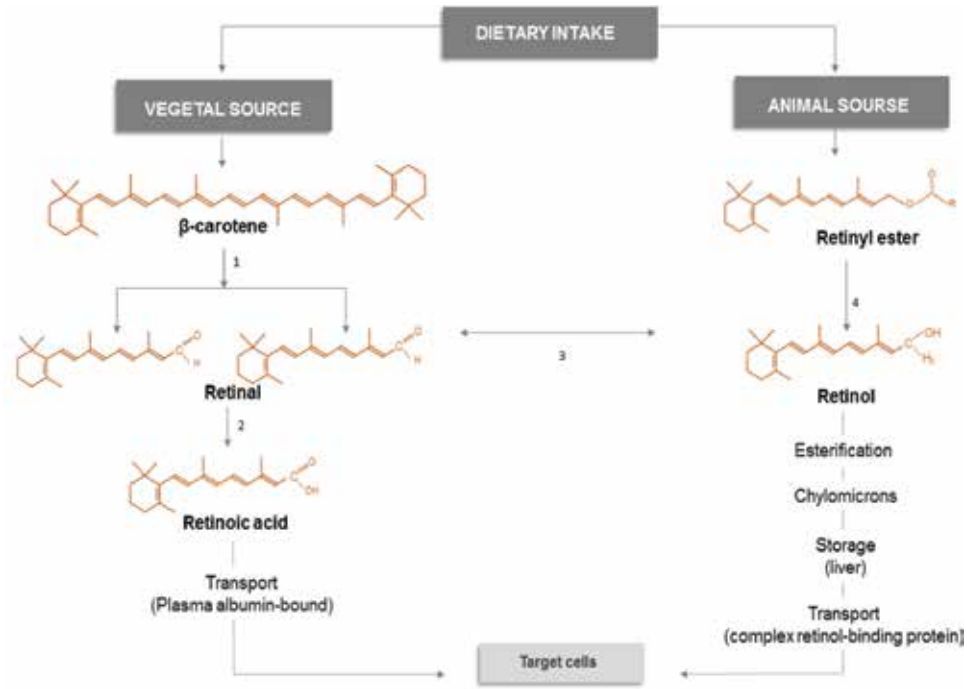

Figure 2.
Metabolic fate of vitamin A. (1) β-carotene dioxygenase, (2) retinol dehydrogenase, (3) retinal reductase, and (4) ester hydrolases of retinyl.

Figure 2 summarizes the metabolic fate of vitamin A. The different forms of diet enter the digestive tract and are predominantly absorbed in the proximal part of the small intestine [8].

Pro-vitamin A carotenoids are cleaved (via β-carotene dioxygenase) to retinal [28]. This retinal can be reversibly converted to retinol (via retinal reductase) or irreversibly to retinoic acid (via retinol dehydrogenase); such interconversion occurs in the gut of all mammals [29] On the other hand, retinyl esters of animal origin are converted to retinol via ester hydrolases of retinyl (REHs) [30].

The retinol formed is esterified to long-chain saturated fatty acids (mainly palmitic acid), packaged in chylomicrons, secreted into the lymphatic system, and stored in the liver (80% of the body supply) [31]. Minor amounts are distributed and stored in extrahepatic tissues and organs (eye, lung, adipose tissue, kidneys, small intestine, adrenal gland, testis, uterus, bone marrow, thymus, skin, and spleen) [32, 33].

When the retinoids reach the hepatic system, they are hydrolyzed and complexed with the retinol-binding protein to be transported the target cells as required. In contrast, retinoic acid is carried in plasma bound to albumin [34].

The most common benefit of this vitamin relates to vision, due to the high demand for this supply by the retina and maintenance of the cornea [8]. However, they are still associated with several functions such as the maintenance of healthy epithelium, cell differentiation, reproduction, immunity, and growth [16].

According to Engelking [29], retinol, retinal, and retinoic acid bind to nuclear proteins, where they are most likely involved in the control of gene expression. Recent research has shown that vitamin A supplementation can positively regulate the expression levels of proteins that improve the intestinal barrier [35]. Mucosal healing was significantly higher in the vitamin A-supplemented group in cases of ulcerative colitis [36].

4. Deficiency of vitamin A and strategies of fortification

Some physiological implications about the intake of high doses of vitamin A have been reported; however, it is their deficiency that causes catastrophic damage. Depletion of this nutrient has become a global problem affecting millions of people worldwide, especially those in developing countries [37]. It harms the health of approximately 190 million children and 19 million pregnant women worldwide [37].

In developing countries, it is the leading cause of childhood blindness and further contributes significantly to the morbidity and mortality of common childhood infections such as continuous malnutrition [38]. Additionally, it causes potential changes in the epithelial barrier of vital organs and tissues [39]. It further reduces the synthesis of specific glycoproteins in the intestinal mucosa and liver, as well as the gene expression of glycosyltransferases, fibronectin, and transglutaminases, disrupting macrophage function, blood clotting, and adhesion. Also, it disrupts normal bone growth as it is essential for the activity of cells in the epiphyseal cartilage [29]. Studies with animals also indicate that in the deficiency of this nutrient, the spermatogenesis is blocked causing infertility [29]. Problems in the regulation of vitamin D receptors may also be affected by the lack of vitamin A [40].

Because of all these controversial effects, highlighting the high morbidity and mortality, government agencies have recognized the problem as a public calamity situation and since then have been developing and supporting strategies to combat vitamin A deficiency [41].

Typically, these approaches are based on the fortification of basic foods, food ready for use, condiments, and mostly milk [42]. Among them are microencapsulation techniques and genetic crosses (biofortification) [18, 43, 44].

Microencapsulation has emerged as an alternative to increasing the stability and bioavailability of labile compounds such as vitamin A, and for this reason, it is believed that this process may be a strategy in food fortification to treat vitamin A deficiency [16, 45]. It is based in the encapsulation of the nutrient into microparticles of polymeric material with the variable diameter [46].

In contrast, the biofortification is an integrated approach of agriculture and nutrition, which uses traditional breeding or genetic engineering techniques [47]. In this case, species of foods containing high β-carotene content are used to obtain hybrid species adapted to places where vitamin deficiency remains a severe problem [48]. For example, genetic crosses made from yellow maize rich in β-carotene gave rise to other tropically adapted maize species [43, 44]. Besides, hybridization of sweet potatoes has also been extensively explored, especially in sub-Saharan Africa [44]. "Golden rice" is another successful example in several vitamin A-deficient countries [49]. According to Tanumihardjo [50], cassava is also included in the basic crops targeted for biofortification.

The chapters presented in this book are intended to help provide a deeper understanding and insight processes of perception and challenges for vitamin A, contributing substantially to the role of future vitamin A effects on human health.

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Chapter 2

Vitamin A in Health and Disease

Mohd Fairulnizal Md Noh, Rathi Devi Nair Gunasegavan and Suraiami Mustar

Abstract

Vitamin A refers to a group of related compounds with all-trans retinol biological activity and includes retinol, retinal, retinoic acid as well as the retinyl esters. Dietary source of vitamin A ranges from animal-based or plant-based foods, fortified food products and supplements. The vital biological roles of vitamin A compounds include normal cell growth, cell differentiation, vision and immunology. Vitamin A status is monitored to prevent occurrence of both subclinical deficiency and toxicity. Vitamin deficiency or excess is determined through the measure of vitamin A status. Prolonged vitamin A intake at high doses is shown to be toxic, which leads to various health symptoms. Xerophthalmia, a dry eye condition is the most severe clinical effects known to be caused by vitamin A deficiency. The resulting deleterious effects on human health led to efforts of supplementation, food fortification and dietary diversification in combating vitamin A deficiency. In brief, this chapter covers on vitamin A, with focus on its general information, dietary recommendations, biological roles, vitamin A status assessment, deficiency or excess effects to human health as well as the prevention measures.

Keywords: vitamin A, biological roles, deficiency, excess, human health

1. Introduction

Vitamin A in health and disease chapter intends to introduce general information of vitamin A with specific focus mainly on its dietary recommendations and its importance to human health. In line with this, continuous monitoring of vitamin A status that determines deficiency or toxicity state that could significantly affect human health along with prevention efforts is also described.

Vitamin A is a fat-soluble vitamin and also comprises of a group of unsaturated nutritional organic compounds. These compounds include preformed vitamin A that exist in the form of retinol (alcohol), retinal (aldehyde), retinoic acid (irreversibly oxidized form of retinol) and several pro-vitamin A carotenoids (mainly β–carotene). The preformed vitamin A can only be obtained from the diet in food of animal origin and is the most abundant form of vitamin A in the human body. Retinol is a yellow fat-soluble substance, an absorbable form of vitamin A present in animal food sources. This chemical structure makes it poorly soluble in water but easily transferable through membrane lipid bilayers. Retinol is an alcohol and is known to be unstable. Vitamin A is mainly found in human tissues in the form of retinyl esters, which explains why the vitamin is commercially produced and administered as esters of retinyl acetate or palmitate. Retinyl esters will subsequently be converted into retinols in the small intestine [1, 2]. The pro-vitamin A comes from plant-derived

foods primarily in oils, fruits and vegetables. β-Carotene is the major source of vitamin A precursor from plants and is represented as two connected retinyl groups. The molecules contribute to the body's total vitamin A level. All forms of vitamin A have a β-ionone ring, which is attached to an isoprenoid chain (retinyl group). Both of these structural moieties are essential for the vitamin to exert biological activity. The β-ionone ring containing carotenoids include α-carotene, β-carotene and the xanthophyll β-cryptoxanthin [2].

Vitamin A can be found in a variety of foods. The bioavailability of carotenoids in food is variable because the efficacy of metabolic processes that convert carotene into retinol varies from one person to another. **Table 1** shows important dietary sources of vitamin A. Foods rich in retinol include meat, butter, retinol-enriched margarine, dairy products and eggs, while foods rich in β-carotene include vegetables and fruits (e.g. sweet potatoes, carrots, dark-green leafy vegetables, sweet red peppers, mangoes, melons). Several processed foods have been fortified with vitamin A and are good sources of the vitamin, such as cornflakes, malted milk powder and milk powder [2–4]. Foods containing pro-vitamin A carotenoids tend to have less biologically available vitamin A but are more affordable than animal products especially in the diets of economically deprived populations.

Retinol, in the form of retinyl esters, and pro-vitamin A carotenoids enter the human body as a component of nascent chylomicrons secreted into the lymphatic system. Most dietary retinol (in chylomicrons and chylomicron remnants) is taken up by the liver, which is the major site of retinol metabolism and storage. Once circulating retinol is absorbed from the intestine, it will bind primarily to a protein

Food category	Vitamin A (μg retinol equivalent/100g)
Meat/poultry/fish	
Liver (ox/beef, chicken)	9000–16,000
Egg, whole (duck, hen)	208–304
Chicken, duck (thigh)	50–69
Fish, mackerel, Indian/Spanish	8
Vegetables	
Carrot, raw	835
Sweet potato	709
Spinach	469
Broccoli	31
Fruits	
Mango	54–214
Papaya	55–193
Apricot	96
Watermelon	28–68
Processed Food	
Butter	200–684
Cheddar cheese	117–265
Full cream milk powder	400
Malted milk powder	711

Table 1.
Dietary sources of vitamin A and retinol activity equivalences (adapted from [5, 6]).

called retinol-binding protein (RBP). The RBP will enter and leave the liver several times daily due to its lipophilic properties in a process known as retinol recycling. The retinol will bind to a cellular RBP (CRBP-I or CRBP-II) and can then be esterified by enzyme lecithin: retinol acyltransferase (LRAT), which enables the vitamins to be interconvertible, i.e. the stored ester and circulating retinol form. The storage efficiency and retinol catabolism are dependent on vitamin A status. Low retinol stores are associated with reduced storage efficiency and decrease the absolute catabolic rate [2].

To express the vitamin A activity of carotenoids in diets on a common basis, a concept of the retinol equivalent (RE) was introduced [7]. Based on this concept, the relationships among food sources of vitamin A were established as shown below:

1 μg retinol	=	1 μg RE
1 μg β-carotene	=	0.167 μg RE
1 μg other pro-vitamin A	=	0.084 μg RE

A new term, retinol activity equivalent (RAE), was introduced in order to express the activity of carotenoids after taking into account new research on vitamin A activity of carotenoids [8, 9]. Specific carotenoids/retinol equivalence ratios are defined for pro-vitamin A carotenoids, which account for the less efficient absorption of carotenoids and their bioconversion to retinol. Recent work has shown that the absorption of carotenoids, the vitamin A precursors, is only half of as much as that previously considered. Institute of Medicine established the following conversion factor equivalents:

1 μg retinol	=	1 μg RAE
1 μg β-carotene in oil	=	0.5 μg RAE
1 μg β-carotene in mixed foods	=	0.083 μg RAE
1 μg β-carotene and other pro-vitamin A carotenoids in mixed foods	=	0.042 μg RAE

The use of SI units (weight and molar) is strongly recommended to replace the use of IU in many databases to decrease confusion and overcome limitations in the nonequivalence of the IU values for retinol and β-carotenes. The conversion factors to be used are as follows:

1 IU retinol	=	0.3 μg retinol
1 IU β-carotene	=	0.6 μg β-carotene
I IU retinol	=	3.0 μg β-carotene

2. Biological roles of vitamin A

Vitamin A is an essential micronutrient required in small amounts by human throughout the life cycle to perform multiple metabolic functions. It is important for growth and development, the maintenance of immune function and maintenance of epithelial cell integrity, good vision, reproduction as well as lipid metabolism. Vitamin A is also an important antioxidant, a property shared with vitamins

E and C, respectively [3]. New biological functions of vitamin A such as lipid metabolism, insulin response, energy balance and the nervous system are continuously being discovered.

2.1 Vitamin A and health importance

Vitamin A has long been known to play a critical role in vision. Night blindness or reduced vision ability under dim light is a very early and purely subjective symptom of vitamin A deficiency (VAD). In the eye, the 11-cis retinal binds to protein, term opsins, to form both the rhodopsins (rods) and iodopsins (cones) visual pigments [10]. Light that enters the eyes will isomerise the bound 11-cis retinal to all-trans form which initiates excitation of the photoreceptor cell. This isomerisation reaction will trigger nervous signal and passes along the cranial 'optic nerve' destined for the visual centre of the cerebral cortex that translates into a picture [11, 12]. A vitamin A metabolite, retinoic acid (RA), is essential for the normal functioning of the immune system [13]. Retinol and its derivatives function as an immune enhancer that potentiates the antibody response; at the same time it maintains and restores the integration of all mucosal cells and their functions. Retinols are also required for the development of leukocytes that play a major role in mounting an immune system. The major site of vitamin A action in the immune response is thought to be the T helper cell and T lymphocytes cell. The retinol derivative '4-hydroxyretinoic acid' rather than retinoic acid is important in this aspect [14].

Along with its role in vision and immune system, vitamin A has also been shown to be actively involved in the production of red blood cells, which are derived from stem cells that depend upon retinoid for their proper differentiation. Vitamin A also appears to facilitate the mobilisation of iron stores to the developing erythrocytes where it is incorporated into haemoglobin, the oxygen carrier complex protein [15]. In addition, vitamin A (retinol, retinoic acid, all-trans retinal) is an important signalling molecule that affects gene expression and is called 'retinoid-controlled genes' which are involved in the differentiation and development of foetal and adult tissues, stem cell differentiation, apoptosis, support of reproductive and immune functions and regulation of lipid metabolism and energy homeostasis [16]. Retinol and retinoic acid also play a vital part in the development of human embryo and differentiation of three germ layers and propagation of the signalling process in the formation of the neural tube, organogenesis and development of limbs during embryogenesis. There are two main types of high-affinity receptor for trans- and cis-retinoic acid isomers within the nucleus cells of vertebrates including mammals. Each set of these receptors has six different domains which are involved in gene expression [17].

In terms of skin health, the isoform retinoic acid will switch on genes that differentiate immature skin cells into mature epidermal cells. Vitamin A and its metabolites have also been shown to improve photo-aged and chronologically aged skin pathologies. They promote the deposition of new collagen fibres and prevent degradation occurring in such skin types [11]. Growth hormone is a peptide hormone that stimulates growth (anabolic metabolite), cell reproduction and cell regeneration in humans and other animals. Growth hormone is a 191-amino acid, single-chain polypeptide that is synthesised, stored and secreted by the somatotropic cells within the lateral wings of the anterior pituitary gland. The availability of vitamin A is necessary for expression of many genes including those human growth hormones [11].

Studies showed that vitamin A in the form of retinol is required for maintenance of adult mammalian spermatogenesis. Spermatogenesis is the production and development of sperm. It is a process which sperm cells undergo a series of cellular changes and divisions in order to fully develop. The cell begins as a spermatogonium and the undeveloped diploid sperm cell and ends as four spermatids. These

spermatids form fully developed sperm cells that comprise semen. Retinoid acid is an essential regulator of gametogenesis both in male and female gametes, such that they can enter meiosis [18]. Antioxidant activity is another identified vital role, where the presence of vitamin A or β-carotene in small doses showed anticancer effect. It appears to stem from its ability to scavenge for reactive oxygen species (ROS) and can improve immune function in addition to eliciting an anti-proliferative effect through the retinoic acid receptor (RAR) and retinoid X receptor (RXR). ROS are the most important free radical in biological system and harmful by-products generated during the normal cellular functions. In this way, they can block certain carcinogenic processes and thus inhibit tumour cell growth [11, 19].

3. Assessing vitamin A status

Vitamin A status of a specific population is important to better understand health status of the community in that particular area. VAD can lead to many health consequences, with children, pregnant and lactating women known to be the prominent groups suffering from VAD in many low-income countries [20]. Its prevalence of deficiency in a population is assessed by specific indicators/biomarkers [21].

3.1 Indicators to assess vitamin A status

There are several indicators/biomarkers to detect VAD. The 'gold standard' method to assess vitamin A status is through the direct measurement of liver reserves of vitamin A through biopsy, since in human, vitamin A is stored abundantly (>90%) in the liver [22]. A study in average-weight individuals for 4 months had shown that an estimated cut-off at 0.07 μmol/g liver was able to protect them from any clinical signs of VAD [23]. Unfortunately, this method is not feasible for population evaluation [24, 25]. Therefore, other various methods are being proposed to assess and monitor VAD based on their different aspects. The two different ways include biological (clinical, functional, histological) and biochemical indicators [26]. In 2010, liver reserves of vitamin A were plotted against the commonly used indicators to define the range of liver reserves associated with the specific indicators. It was later updated in 2015 as presented in **Figure 1** [27].

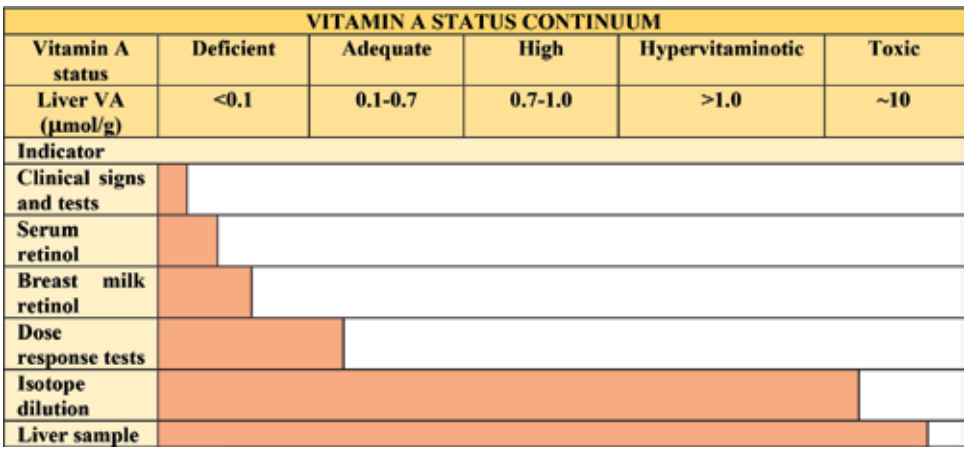

Figure 1.
The definition of vitamin A status assessed by using vitamin A indicators associated with vitamin A concentration in the liver. In 2010, 0.7–1 μmol/g was considered adequate, but this range is considered high (updated in 2015) until more biologically meaningful data are generated.

3.1.1 Biological indicators

Biological indicators consist of clinical, functional and histological components. The clinical indicators are xerophthalmia where it consists of two words, 'xeros' (dry) and 'ophthalmia' (eye), which refers to specific eye diseases caused by VAD [28]. It is classified into several groups with night blindness being the earliest ocular sign of VAD. The xerophthalmia classifications and its associated criteria of public health problems by WHO are highlighted in **Table 2**.

Xerophthalmia classifications	Symbol	WHO criteria	Epidemiological aspect	Method of assessment
Night blindness	XN	>1%	Difficult for children below 2 years, highly specific, less sensitive	Survey/questionnaire
Conjunctival xerosis	X1A	Not applicable	Not a reliable indicator of prevalence	Clinical examinations
Bitot's spot	X1B	>0.05%	Common in men, mostly occur in preschool children. Usually associated with history of X1A and night blindness	
Corneal xerosis	X2	>0.01%	A mild superficial haze due to obvious corneal change	
Corneal ulceration/ keratomalacia (<1/3 corneal surface)	X3A	>0.01%	Rapidly induced by VAD and measles infection	
Corneal ulceration/ keratomalacia (≥1/3 corneal surface)	X3B	>0.1%	Irreversible stage even with supplementation	
Corneal scar	XS	>0.05%	Bilateral with onset before 5 years	
Xerophthalmic fundus	XF	Not applicable	Rare manifestation of VAD	

Table 2.
Xerophthalmia classifications and its associated criteria of public health problem as per WHO (adapted from [57]).

3.1.1.1 Functional indicators

Night blindness (XN) or poor adaptation to the dark is a functional indicator of VAD, which is assessed by taking a history from mothers and both pregnant and lactating women. The cut-off point to indicate the deficiency in mothers and children (age 24–71 months) is ≥1% report history of night blindness [29]. Night blindness occurs if vitamin A is seriously depleted since it is responsible for vision under very low illumination [30].

3.1.1.2 Clinical indicators

Signs of chronic, long-standing VAD of xerophthalmia are conjunctival xerosis (X1A) and Bitot's spots with conjunctival xerosis (X1B). In general, a very bright torchlight in natural light is used to examine the eyes [29]. Conjunctival xerosis or drying can occur in both eyes where eyes turn dry and non-wettable with wrinkle presence at the temporal conjunctiva [31, 32]. Bitot's spots are the accumulation of fine white foamy cheesy material comprising keratin, on the conjunctival surface [28].

Signs of acute, sudden onset of VAD are corneal xerosis (X2), corneal ulceration with xerosis (X3A), keratomalacia (X3B) and xerophthalmia fundus (XF). Corneal xerosis (X2) is drying of the cornea due to the lack of mucus and tears (wetting agent) because glands in the conjunctiva are no more functioning normally [33]. Lesions on the cornea become denser, and stromal oedema starts to develop during corneal xerosis. The cornea appears to be granular, rough and blurry when examined using a hand light [32]. At this stage, treatment with vitamin A will heal the eyes within 1 to 2 weeks without leaving any scars. Corneal ulceration with xerosis (X3A) is permanent destruction of all or some parts of the corneal stroma which are prominent. Ulcers may be shallow but usually become deep if it penetrates into the cornea. Vitamin A therapy can cure superficial ulcer, leaving small scars, while deeper ulcers and perforations form dense scars [28].

Keratomalacia (X3B) means softening of the cornea, and it is a rare stage of xerophthalmia. The cornea may become thickened and melt away due to a progression of necrosis or death of tissue, affecting the collagen in the cornea [32]. Blindness is usually inevitable, although other eyes and the lives of children can be instantly saved by vitamin A therapy. Keratomalacia is also usually associated with secondary eye infections but can be treated with an antibiotic [28]. Xerophthalmia fundus (XF) is the appearance of small yellowish lesions on the fundus of the eye, which occurs due to the loss of pigment from the retinal pigment epithelium caused by VAD. The lesions are sometimes accompanied by blind spots or scotomas, congruent with their distribution on the retina [34]. The healing or end result of corneal ulceration and keratomalacia is corneal scars (XS). Scars are left on the cornea with varying densities, known as staphyloma (permanent bulging of the damaged cornea) or phthisis bulbi (shrunken globe), whereby the contents of the intraocular are gone and can lead to blindness [28].

3.1.1.3 Histological indicators

The morphological changes of epithelial cells from the conjunctiva surface can be assessed using a piece of filter paper. Normal conjunctiva cells show an abundance of mucin-secreting goblet cells and small epithelial cells. However, if there is a deficiency in vitamin A, the goblet cells and mucin droplets will reduce, and the epithelial cells become enlarged, separated and flattened [29]. Histological indicators include conjunctival impression cytology (CIC) and impression cytology with transfer (ICT). Assessing VAD using both techniques requires standard pore size filter paper, slides and a simple light microscope. The method involves gently applying a filter paper on the surface of the conjunctiva for 2–3 seconds, and after removal, it is placed in fixative and stained to differentiate the goblet cells from the endothelial cells. The eye is classified as normal or abnormal based on the number of goblet cells, which is counted under a microscope [35]. The differences between the two techniques are ICT only require single staining while CIC include extra processing steps for fixing, staining and mounting specimens. Comparatively, the CIC technique is more efficient in transferring cells of high quality from filter paper to slide [29].

3.1.2 Biochemical indicators

Biochemical indicators include serum and breast milk retinol concentrations, relative dose response (RDR) test, modified relative dose response (MRDR) test and isotope dilution (ID) assay.

3.1.2.1 Serum retinol concentrations

Serum retinol concentrations are among the most common method used to identify populations at risk of VAD [36]. They are determined using high-performance liquid chromatography (HPLC). The current cut-offs for VAD are <0.70 μmol/L, while severe VAD is classified below 0.35 μmol/L [21]. However, this indicator is affected by infections [37], inflammation and inadequate intakes of protein, zinc or energy, which are needed for retinol-binding synthesis [38]. Therefore, before using serum retinol concentration to assess VAD in a population, these factors should also be taken into consideration. In addition, serum retinol concentrations are homeostatically controlled over a broad range of body store and only decline when the liver reserves are very low [39]. Serum retinol concentrations should be used in conjunction with another biological indicator or when four or more of the following risk factors are detected in the population being assessed [40]. These risk factors include:

a. Infant mortality rate and under 5 years old mortality rate are >75 of 1000 and >100 of 1000 live births, respectively.

b. Less than 50% of children of 12–23 months old have full immunisation coverage.

c. The prevalence of breastfeeding in 6-month-old infants are <50%.

d. Among 75% of children (1–6 years old) have median dietary intakes <50% of the recommended safe levels of intake.

e. The prevalence of 2-week period of diarrhoea is ≥20%.

f. Fatality rate of measles cases is ≥1%.

g. More than 50% of women (15–44 years old) have no formal schooling.

h. Less than 50% of households has a safe water source (e.g. boiled, treated, filtered, properly stored).

3.1.2.2 Breast milk retinol concentrations

Breast milk retinol concentration is a unique indicator in lactating women. It has also been proposed as a measure of the population status of vitamin A, since the probability of infant and children at risk of VAD is very high if the lactating women are of a community with marginal vitamin A status [41]. Vitamin A deficiency is considered a moderate public health problem if the prevalence of inadequate milk retinol concentrations (≤1.05 mmol/L or ≤8 mg/g milk fat) is ≥10–<25% [29]. The breast milk samples are easier to obtain, and the concentration of retinol in milk can be determined after saponification by HPLC, similar to those used to determine serum retinol [42].

3.1.2.3 Relative dose response test (RDR)

The test principle of the RDR is on the basis that when vitamin A undergoes depletion, apo-retinol-binding protein (apo-RBP) accumulates in the liver. In this test, a challenge dose of retinyl ester is given to the subject, and blood samples are withdrawn prior to dosing (baseline) and 5 hours after dosing. The retinol from retinyl ester will bind to the excess RBP and is released into serum as holo-retinol/retinol-binding protein complex (holo-RBP-retinol complex). A percentage change is measured as per Eq. 1 where RDR ≥ 20% indicates VAD [35].

$$RDR\,(\%) = \frac{[A5] - [A0]}{[A5]} \times 100 \quad (1)$$

where, [A5] is the serum retinol concentration at 5-hr post-dosing; and [A0] is the serum retinol concentration just before dosing (baseline).

3.1.2.4 Modified relative dose response test (MRDR)

MRDR is a modified test of RDR using 3, 4-didehydroretinyl acetate (DRA) as the challenge dose, followed by a high-fat snack to ensure adequate absorption. In this method, a single blood sample is taken after 4 to 7 hours dosing [43]. In parallel to retinyl esters, DRA is hydrolysed to 3, 4-didehydroretinol (DR) within small intestine, taken up by enterocytes and esterified to form various didehydroretinyl esters. The esters are de-esterified to form DR in the liver, which can bind to apo-RBP and be released into serum or can be re-esterified and stored in stellate cells. The only difference between DR and retinol is the presence of a double bond located in the 3–4 position on β-ionone ring of DR. This structural difference can be separated using HPLC due to their difference in polarity. The MRDR value, which is used to indicate liver reserves, is the ratio of DR to retinol in serum [27]. The ratio of 3, 4-didehydroretinol (DR) to retinol is calculated, and the value of ≥0.06 indicates VAD in children [44]. The MRDR test has been widely used to diagnose a subclinical vitamin A status.

3.1.2.5 Isotope dilution (ID) assay

Of all the indicators available, the most accurate method to indirectly measure the vitamin A storage in the liver known till now is the isotope dilution assay [45–47]. Isotope dilution assay could detect a full range of vitamin A content in the body from deficient state up to the toxic level [48]. This test involves blood sample collection before and after the administration of a stable isotope tracer (deuterated or ^{13}C-labelled retinyl acetate) at an appropriate equilibration period. The variations in the equation and assumptions used in the calculation are dependent on the study design based on the population assessed. The method of mass spectrometry used, the dosage size given to the subjects and the time allowed for equilibration were also taken into consideration when calculating the total body reserve in the ID test [41]. The ID assay is determined as shown in Eq. 2.

$$(F_a \times a) + (F_b \times b) = (F_c \times c) \quad (2)$$

where:
a is the amount of dose absorbed and stored (dose × absorption rate).
b is the baseline total body reserves of vitamin A.

c is the total body reserve in μmol after the dose (c = a + b).

$$F = \frac{R}{R+1} \quad \text{and R is} \, {}^{13}C/{}^{12}C \tag{3}$$

where F_a, F_b and F_c are the abundance of isotopes [^{13}C/total C; At %/100; R/(R + 1)] from dose, baseline serum and serum after the dose.

4. Effects of vitamin A deficiency or toxicity on human health

Routine monitoring of vitamin A status serves as an important measure in the determination of toxicity due to excessive intake or deficiency in a population. Under circumstances where dietary consumption does not meet the recommended criteria, this could lead to vitamin deficiency or toxicity depending on whether the vitamin consumption is insufficient or in excess, respectively. Various health implications have been reported as a consequence of both vitamin deficiency and excess, as discussed below.

4.1 Vitamin A deficiency (VAD)

Dietary source of vitamin A is generally available in various forms, of which the preformed retinol from animal-based source (eggs, liver, dairy) is the most bioavailable form of vitamin A. Plant-based food sources are rich in pro-vitamin A; however, populations that are dependent solely on these sources are at higher risk of VAD since its absorption is reliant on various factors [49, 50]. VAD is commonly associated with decreased immunity and higher risk of night blindness [51]. It is worthwhile to note that this deficiency is highly prevalent in countries with an alarming increase of diabetes especially among those of lower income group in United States as well as Asian developing countries [51, 52].

Vulnerability to VAD differs according to specific life stages that include infancy, childhood and pregnancy. VAD in neonates is highly related to insufficient vitamin A in breast milk or formula milk. Apart from dietary shortage, VAD could have also been triggered by reduced intestinal absorption of vitamin A. Prolonged deprivation of body requirements for vitamin A leads to vitamin A deficiency disorders (VADDs) that affects gastrointestinal, renal, musco-skeletal organ systems as well as harming growth and development [53]. Xerophthalmia and anaemia are two most common examples of VADDs. In line with vitamin A roles as immunity enhancer, its deficiency is often associated with an increased risk of infections [54, 55]. Respiratory tract infections and diarrhoeal diseases are the most common form of infections with high incidence of mortality along with marked susceptibility to severe measles infection [55–57]. The representation of VADD association to risk of mortality is presented in **Figure 2** below.

4.1.1 Vitamin A deficiency and xerophthalmia

Xerophthalmia refers to a spectrum of ocular manifestations due to VAD and varies according to its severity and age. It is characterised by pathological dryness of the conjunctiva and cornea that turns out as a leading cause of childhood corneal blindness, especially in nutritionally deprived populations [58]. All of such signs encompass those involving impaired retinal sensitivity to light (night blindness) and epithelial disruptions of the cornea and conjunctiva (conjunctival xerosis, Bitot's spot, corneal xerosis and keratomalacia) [59, 60]. The classifications of xerophthalmia stages in order of severity based on WHO criteria are shown in **Table 2** (Section 3.1.1).

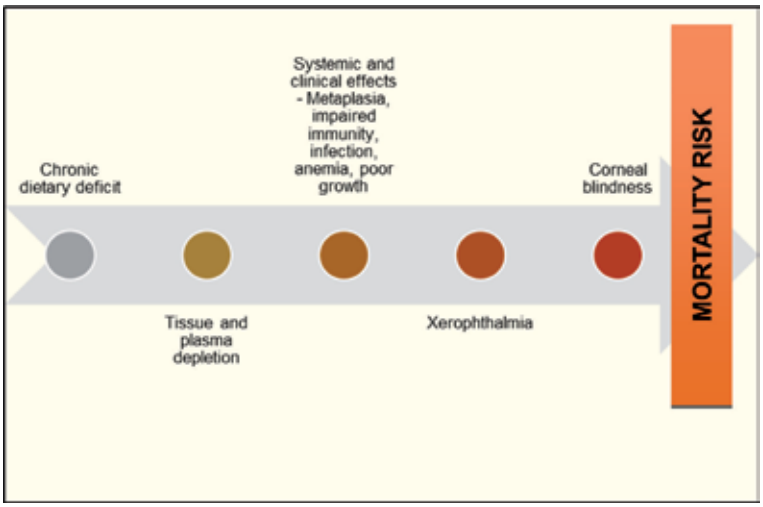

Figure 2.
Representation of VADDs in relation to risk of mortality (adapted from [53]).

Xerophthalmia can occur in any age group with higher possibilities in preschool-age children, adolescents and pregnant women. In line with greater requirements for growth, children are more prone to VAD and xerophthalmia [61]. The initial symptoms of VAD are characterised by impaired adaptation to dark, which starts when the serum retinol concentration falls below 1.0 μmol/L and becomes more often when it falls lower than 0.7 μmol/L. A further drop in serum retinol concentration level below 0.35 μmol/L leads to more frequent and severe xerophthalmia condition [62, 63]. The incidence of xerophthalmia is often associated with higher risk of mortality [57].

Night blindness is generally the earliest manifestation, and it is indicated by vision limitation under dim light and is both a sensitive and specific indicators for low serum retinol levels [63, 64]. Vitamin A in the form of retinal within the eyes combines with opsin to form rhodopsin, which is the photosensitive visual pigment of rods. Rhodopsin level decreases when vitamin A is deficient, and this impairs the rod function causing night blindness [61]. Bitot's spot is the representation of opaque whitish deposits on the scleral conjunctiva, which is the most characteristic sign of problems related to VAD. Conjunctival xerosis is already present at this stage, with the conjunctiva appearing dry and dull. Under conditions where VAD persists, corneal xerosis (hazy cornea) occurs, followed by keratomalacia (liquefaction of part or all cornea) [61].

Several risk factors have been associated with onset of VAD and xerophthalmia based on epidemiological findings. These include demographic, geographic, childhood, parents and household factors. The mechanism of these factor effects on the prevalence of xerophthalmia is summarised in **Table 3**.

4.2 Vitamin A toxicity

On another note, the increase in supply and consumption of fortified foods and supplements led to intake of preformed vitamin A at higher than the recommended level [75]. The side effects of vitamin A excess could occur in two forms, known as hypervitaminosis A and hypercarotenemia [76].

4.2.1 Hypervitaminosis A

Hypervitaminosis A can occur due to both acute and chronic intoxications that generally result from excessive intake of vitamin A from nutritional supplements

Risk factors	Epidemiological findings	References
Demographic	Higher prevalence is observed in neonates, preschool children and pregnant women as they are more vulnerable to be deficient	[57, 65]
Geographic	VAD and xerophthalmia are generally more prevalent in rural areas due to variations of vitamin A-rich food sources, supplementation efforts, limited access and climate changes	[57, 66–68]
Childhood	Breastfed children are at minimal risk of infections and xerophthalmia compared to the non-breastfed children	[57, 69]
Parental	Education literacy is important since it is highly protective against xerophthalmia development and VAD in preschool children	[57, 70]
Household	Poor hygiene, inefficient water supply and cultural and behavioural practices of a family can increase the risk of xerophthalmia. Its prevalence is higher in lower socioeconomic status areas	[57, 70–74]

Table 3.
Risk factors associated with prevalence of VAD and xerophthalmia.

and foods rich in vitamin A [76]. Acute toxicity occurs when adults and children ingest more than their respective recommended dietary allowance within few hours or days, while chronic toxicity results from prolonged consumption of preformed vitamin A over the months or years. However, acute conditions create minimal consequences to human health compared to those under chronic toxicity [77, 78]. Vitamin A, being an essential fat-soluble micronutrient, is quickly absorbed upon ingestion, although it is only cleared slowly from the body. Under such conditions, toxicity could arise either from high-dose exposure or low intake over short or prolonged duration, respectively [79]. Chronic hypervitaminosis A leads to various clinical manifestations that include xerosis, epistaxis, alopecia, weakness and fatigue, bone and joint pain, insomnia, drowsiness, anorexia, bulging fontanelle in infants as well as psychiatric symptoms [76].

Previous research findings have shown that elevated serum concentrations of vitamin A is highly associated with risk of hip fracture [80, 81]. This association is supported by evidence of rat-based experimental studies that demonstrated excessive intake of vitamin A leads to increased bone resorption and less formation at the outer surface that results in bone narrowing [82, 83]. In contrast, the mechanism takes place in opposing effect on the bone marrow surface, where an increase in vitamin A intake reduces bone resorption while increasing its formation. This contradictory effect takes place by the action of vitamin A or its metabolites on osteoblasts at the outer surface together with indirect effect on bone marrow surface [83, 84].

4.2.2 Hypercarotenemia

Hypercarotenemia, which is also referred to as carotenemia or carotenodermia, is a benign phenomenon characterised by pigmentation of the skin. The yellow-orange pigmentation is a result of carotene deposition at the stratum corneum, which is the outermost layer of epidermis [76]. Hyperlipidaemia, consumption of excessive carotene or failure of converting carotenes into vitamin A are conditions that lead to the onset of carotenemia. In view that there is a direct relationship between β-carotene and β-lipoprotein, other medical conditions that are associated with hyperlipidaemia also could lead to this pigmentation. Those conditions include diabetes mellitus, nephrotic syndrome and hypothyroidism. Apart from these, patients suffering from liver disease are also at higher risk of carotenemia due to the

impaired conversion of β-carotene into vitamin A [76]. In contrast to hypervitaminosis A, there are no clear indications of carotenemia to health, and the pigmentation could disappear within weeks to months along with a steady decrease in β-carotene concentration [76, 85].

4.3 Prevention of vitamin A deficiency in nutritionally vulnerable populations

Dietary factors are highly correlated with VAD, especially with increasing requirements at different stages of life. Apart from these, sociocultural factors (intra-household distribution, gender preference) and other economic constraints to achieve adequate dietary requirements for well-being are where a high prevalence of deficiency occurs that leads to prevention efforts. The undertaken prevention efforts should also cater to reduce infectious disease apart from improving vitamin A levels [53]. The prevention approach includes dietary diversification, fortification as well as supplementation. The feasibility of applying each preventive strategy concurrently is somehow dependent on deficiency prevalence and severity as well as infrastructure, financial capacity, potential benefits and safety [86]. In addition, it is also necessary to understand that the success of each preventive programme is interrelated to all levels, inclusive of family, community, district, national and global [53].

4.3.1 Dietary diversification

Dietary diversification refers to efforts of increasing vitamin A intake from commonly accessible and easily available food sources. This approach is deemed feasible provided there is diverse, affordable and continuous supply of vitamin A-enriched dietary sources. Extended breastfeeding is also regarded an important dietary intervention measure, especially as a first-line defence and protection for infants and young children against xerophthalmia. A combined approach of weaning with a routine provision of vitamin A-enriched sources (fruits, vegetables, eggs and others) has proven effective in increasing serum levels of retinols among children [87]. However, under circumstances that the dietary supply is inadequate, home or community gardening will be a good alternative in ensuring food security. In addition to food security, this effort is viable for income generation as well as providing nutritional education to the community. The attempt to involve community-level participation is vital for behavioural adaptation that could considerably improve vitamin A status [53].

4.3.2 Fortification

Fortified foods have been a common intervention globally in combating multiple nutrient deficiencies. The effectiveness of fortification-based intervention is highly dependent on few factors. These include the fortified food vehicle that are widely consumed by high-risk groups, incurs minimal cost and is of a high quality along with centralised processing and distribution [53]. Comparatively, preventive measure via food fortification is much more beneficial and effective than either dietary diversification or vitamin A capsule distribution. Numerous food sources have been subjected to fortification, and these range from oils, flours, cereals, rice, infant formula and also beverages. As such, fortification relates to exploitation of current fortified food consumption patterns towards enhancing vitamin A status [53].

4.3.3 Supplementation

Vitamin A supplementation at high dosage is the most widely practiced prevention measures throughout the world. The supplementation is channelled on

an interval basis with a designated duration. This mode of prevention comprises community involvement and efforts to provide vitamin A supplements to nutritionally vulnerable groups, especially preschool children and mothers. The rationale for high-dose supplementation of vitamin A is based on the assumption that this fat-soluble compound will be stored in the liver and is released together with the transport proteins as per body tissue requirement [53].

5. Conclusion

Vitamin A is one of the fat-soluble vitamins that are vital for various biological roles in the human body, as it is essential for embryogenesis up to adulthood. It can be sourced from both animal-based (preformed vitamin A) and plant-based (provitamin A) foods. The evaluation of whether a population is vitamin A deficient or excess is determined by status monitoring. Biological and biochemical indicators are the most widely applied parameters in assessment of vitamin A status. Vitamin A deficiency or toxicity state arises under conditions where the dietary intake does not comply with recommended levels. It is crucial to note that both conditions could lead to various health complications with VAD leading to mainly xerophthalmia, increased infection risk and anaemia, while toxicity could result in chronic hypervitaminosis and hypercarotenemia. In line with this, prevention efforts that could improve vitamin A status are widely explored. Dietary diversification, fortification and supplementation are the three main approaches that are widely applied for this purpose. These continuous efforts are believed to be able in improving vitamin A status among the vitamin A-deficient populations.

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Conflict of interest

It is declared that there is no conflict of interest involved in the publication of this book chapter.

Abbreviations

apo-RBP	apo-retinol-binding protein
CIC	conjunctival impression cytology
CRBP	cellular retinol-binding protein
DR	3, 4-didehydroretinol
DRA	3, 4-didehydroretinyl acetate
Holo-RBP-retinol complex	holo-retinol/retinol-binding protein complex
HPLC	high-performance liquid chromatography
ICT	impression cytology with transfer
ID	isotope dilution
LRAT	lecithin: retinol acyltransferase

MRDR	modified relative dose response
RA	retinoic acid
RAE	retinol activity equivalent
RAR	retinoic acid receptor
RBP	retinol-binding protein
RDR	relative dose response
RE	retinol equivalent
ROS	reactive oxygen species
RXR	retinoic X receptor
VAD	vitamin A deficiency
VADDs	vitamin A deficiency disorders
XF	xerophthalmia fundus
XN	night blindness
XS	corneal scars
X1A	conjunctival xerosis
X1B	Bitot's spots with conjunctival xerosis
X2	corneal xerosis
X3A	corneal ulceration with xerosis
X3B	keratomalacia

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Chapter 3

The Role of Vitamin A-Storing Cells (Stellate Cells) in Inflammation and Tumorigenesis

Isao Okayasu, Mutsunori Fujiwara and Tsutomu Yoshida

Abstract

Characteristic localization and distribution of vitamin A-storing cells (stellate cells) were demonstrated as hepatic stellate cells in the hepatic lobule and as subepithelial myofibroblasts in the colonic crypt. The stem cell-stem cell niche is maintained by stellate cells in the periportal area and crypt base. Periportal vitamin A-rich stellate cells decrease in patients with chronic hepatitis C who are habitual smokers. Mice fed a vitamin A-supplemented diet show reduced severity of dextran sulfate sodium (DSS)-induced colitis and development of subsequent colonic neoplasia in a model of the ulcerative colitis-dysplasia-carcinoma sequence, compared with mice fed a vitamin A-deficient diet. Decreased colonic subepithelial myofibroblasts and IgA/IgG-positive cells, and increased CD11c-positive dendritic cells in the colonic mucosa, in the vitamin A-deficient state suggest dysfunction of the stem cell niche at the colonic crypt base and colonic immunity. Accordingly, vitamin A deficiency may worsen inflammation and subsequent tumor development, indicating the possibility that vitamin A supplementation might be effective against chronic inflammation and cancer development.

Keywords: vitamin A-storing cells, stellate cells, subepithelial myofibroblasts, stem cell niche, chronic hepatitis, DSS colitis, ulcerative colitis, colonic tumorigenesis

1. Introduction

In mammals, vitamin A is mostly stored in the liver, particularly in perisinusoidal stellate cells. It is also detectable in the lung, kidney, and intestine. Storage of total retinol is increased considerably in the lungs, kidneys, and intestines of rats fed a vitamin A-rich diet [1]. Vitamin A-rich lipids can be identified in the cells using electron microscopy [2]. Vitamin A-storing cells (stellate cells) correspond to subepithelial myofibroblasts in the lung and intestine [3]. Stellate cells are necessary for the differentiation of epithelial cells, known as the stem cell niche [4–7].

It is thought that deficiency of vitamin A worsens inflammation and accelerates tumorigenesis, possibly due to local immunity and stem cell niche dysfunction [3, 8–11]. Data presented and discussed in this chapter show (1) the distribution, localization, and function of stellate cells in the liver and intestine, and (2) the role of stellate cells in inflammation and tumorigenesis.

2. Characteristic distribution, localization, and function of stellate cells

2.1 Hepatic stellate cells

Vitamin A is mostly (≈80%) stored in hepatic stellate cells, which are located in the perisinusoidal (Disse) space [1, 2]. Lipid droplets containing vitamin A can be assessed by electron microscopy. Vitamin A-rich (containing ≥10 vitamin A lipid droplets) (**Figure 1A**) and vitamin A-poor stellate cells (<10 vitamin A lipid droplets) can be identified by counting the numbers of lipid droplets in the cytoplasm. In humans and experimental animals, vitamin A-rich stellate cells change to vitamin A-poor stellate cells or myofibroblasts, which induce fibrosis by collagen formation in chronic hepatitis or cirrhosis (**Figure 1B**).

Portal fibrosis is induced by collagen produced mainly by stellate cells but not by hepatocytes. Collagen-producing stellate cells change into myofibroblast-like cells, which show a decrease in the number of vitamin A granules in the cytoplasm, and produce collagen around the cytoplasm. Lastly, vitamin A granules disappear completely from the cytoplasm, and stellate cells change their phenotype to that of myofibroblasts and fibrocytes, which are immunohistochemically positive for anti-αSM-actin antibody [12–14].

The periportal area is a microenvironment with a high concentration of vitamin A due to an abundance of vitamin A-rich stellate cells, and the complex of retinol-retinol binding protein is paracrine-transferred from hepatic parenchymal cells to stellate cells, leading to direct secretion of the complex from stellate cells into the plasma [15–17]. Since hepatic stem cells are localized around the periportal area, namely the canal of Hering, the differentiation and maturation of these cells may be impaired due to vitamin A depletion in this area [18–20]. Hepatic stellate cells require vitamin A-rich lipids to maintain their niche function. Accordingly, the hepatic stem cell-stem cell niche relation is maintained in the periportal area [21, 22] (**Figure 2A**).

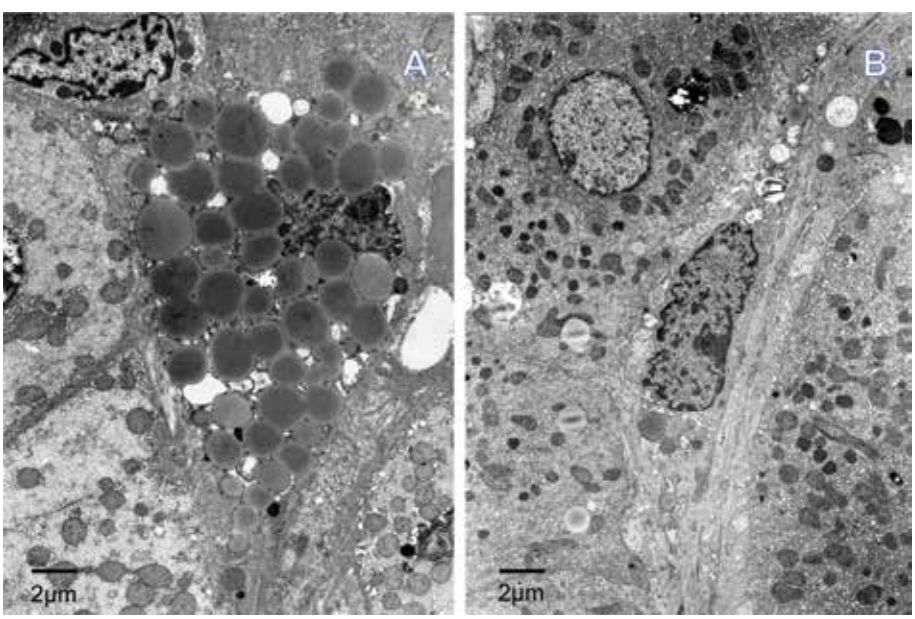

Figure 1.
(A) A periportal vitamin A-rich stellate cell containing many lipid droplets and exerting pressure on the nucleus (human liver). (B) A periportal vitamin A-poor stellate cell containing a few lipid droplets (arrow) and well-developed endoplasmic reticulum (human liver).

2.2 Subepithelial myofibroblasts as colonic stellate cells

In the colonic mucosa, subepithelial myofibroblasts correspond to hepatic stellate cells, although they are usually vitamin A-poor in the cytoplasm, suggesting a different endotype from hepatic stellate cells (**Figure 3**). Subepithelial myofibroblasts are localized more at the crypt base than at other regions (**Figure 2B**) [3]. Subepithelial myofibroblasts express αSM-actin, NCAM, cytoglobin, and HSP47, indicating multipotential roles [3, 11, 23] (**Figure 4**). Because stem cells are localized at the crypt base, subepithelial myofibroblasts around the crypt base are considered a stem cell niche, which has been shown by experimental and histopathological studies [3, 5, 23–25]. Accordingly, mucosal stem cells require subepithelial myofibroblasts for their differentiation. Critical gene expression patterns were shown from the colon basal crypts to the colon tops, including bone morphologic protein (BMP) antagonists, gremlin 1 (*GREM1*), *GREM2, CHRDL1*, and active Wnt signaling using human colon microarray analysis [5]. Along the colon crypt axis, Wnt signaling and Notch signaling expression were activated at the crypt base, while BMP signaling was activated at the top. Wnt signaling and Notch signaling by subepithelial myofibroblasts of the crypt base and smooth muscle cells of the muscularis mucosa regulate epithelial cell positioning and proliferation, and BMP induces epithelial differentiation. Further, isolated human colonic crypt epithelial cells expressing musashi-1, β1-integrin, BerEP4, and CD133 have been shown to adhere to colonic myofibroblasts in cell incubation experiments, indicating an intimate interaction with each [26, 27].

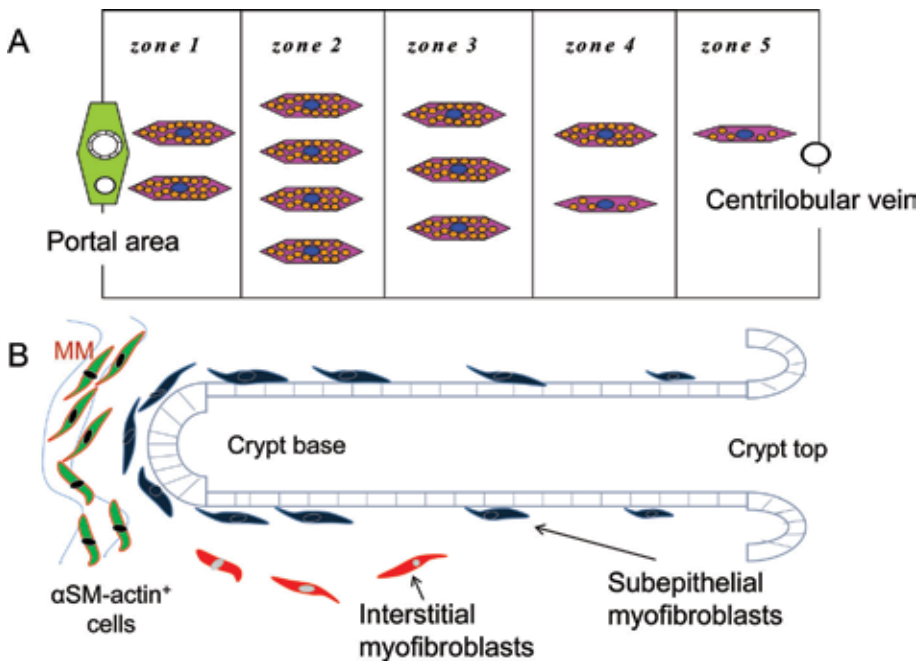

Figure 2.
(A) Characteristic distribution of periportal vitamin A-rich stellate cells in the liver. Hepatic stem cells located in zone 2, where vitamin A-rich stellate cells are collected, form a stem cell niche. Periportal hepatic cells differentiate and maturate toward zone 5, close to the centrilobular vein [22]. (B) Localization and distribution of subepithelial myofibroblasts in the colonic mucosa. Many subepithelial myofibroblasts can be seen around the crypt base forming a stem cell niche, where colonic stem cells are located. Epithelial cells at the crypt base differentiate and maturate toward the crypt top [3, 11]. The characteristic localization and maintenance of stem cell-stem cell niche are similar to those of the hepatic lobule. MM: Muscularis mucosa.

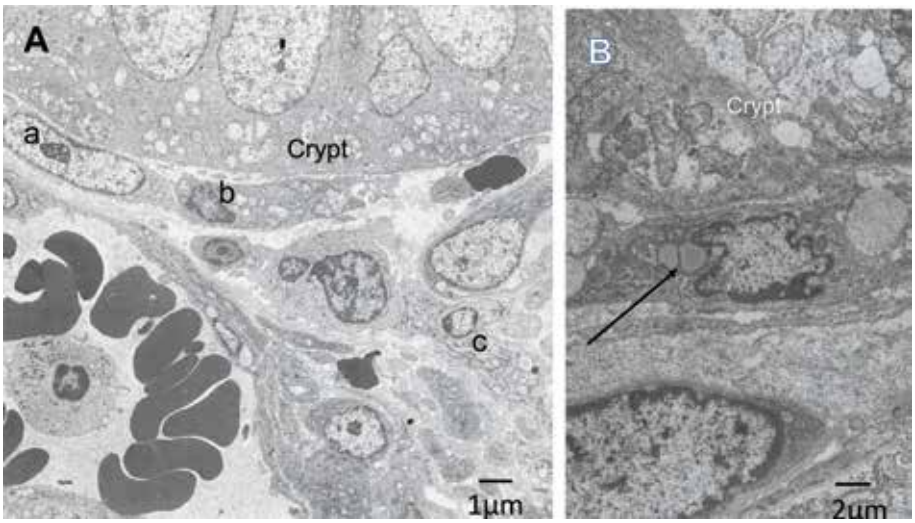

Figure 3.
(A) Localization of subepithelial myofibroblasts in human colonic mucosa. Many subepithelial myofibroblasts, vitamin A-absent (a) or vitamin A-poor (b), are located around the crypt base. An interstitial myofibroblast is also seen (c). (B) A vitamin A-poor (arrow) subepithelial myofibroblast adjacent to the crypt.

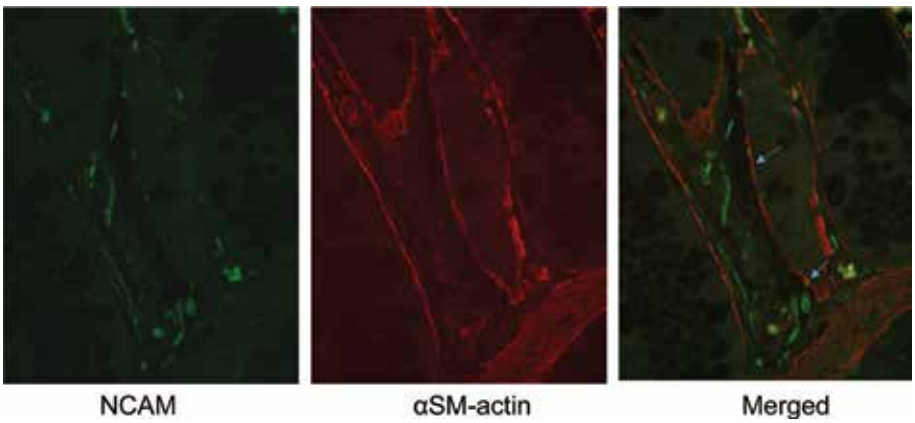

Figure 4.
Expression of αSM-actin and NCAM by subepithelial myofibroblasts in human colonic mucosa. Double immunofluorescence staining shows expression of both αSM-actin (red arrows) and NCAM (green arrows).

Thus, the localization and relation of the stem cell-stem cell niche is the same in the liver and intestine.

3. Vitamin A and stellate cells in inflammation and tumorigenesis

3.1 Decrease of vitamin A-rich stellate cells in chronic hepatitis C

With respect to liver disease, the number of periportal vitamin A-rich stellate cells is decreased in chronic hepatitis C patients, which is associated with aggravation of hepatitis, as indicated by elevated serum alanine aminotransferase (ALT) levels [28] (**Figure 5A**). This tendency was demonstrated in both vitamin A-rich and vitamin A-poor stellate cells. Additionally, the number of periportal vitamin A-rich stellate cells or of both vitamin A-rich and vitamin A-poor stellate cells combined is

significantly decreased during the progression of chronic hepatitis C (from chronic persistent hepatitis, CPH or chronic active hepatitis, CAH2A to CAH2B) [29] (**Figure 5B**). Since vitamin A-absent stellate cells, that is, fibrotic myofibroblasts, were not counted in these studies, the change of vitamin A-storing stellate cells to myofibroblasts in chronic hepatitis was not assessed. Hepatic stellate cells in the normal human liver express both cellular retinol-binding protein-1 (CRBP-1) and αSM-actin, while myofibroblasts express only αSM-actin in fibrotic or cirrhotic liver, suggesting a change of stellate cells to myofibroblasts due to chronic inflammation [30]. This fact indicates that the decrease of vitamin A-rich and vitamin A-poor stellate cells correlates with the severity and progression of chronic hepatitis C is in line with the chronic hepatitis-liver cirrhosis-hepatocellular carcinoma (HCC) sequence proposed epidemiologically and clinically [31–33].

Furthermore, chronic hepatitis C patients who are habitual smokers show decreased numbers of vitamin A-rich stellate cells or vitamin A-rich and vitamin A-poor stellate cells combined, compared with those who are non-smokers [29] (**Figure 5C**). The smoking prevalence in the CAH2B group was 54.1%, which was

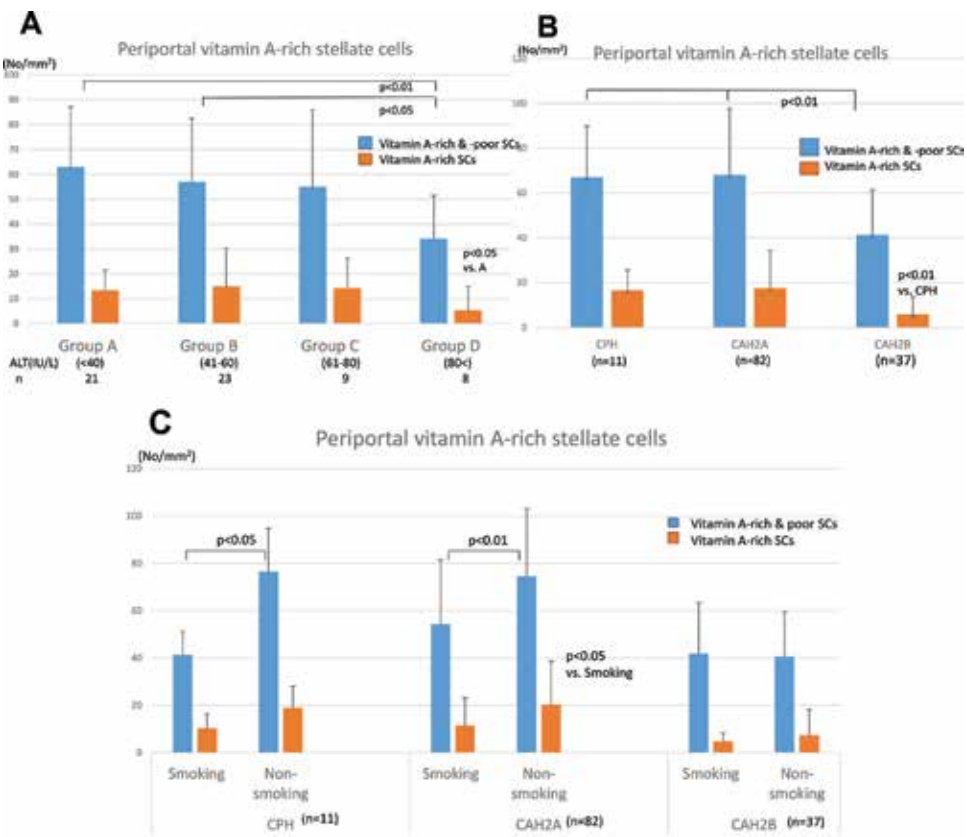

Figure 5.
Relationship between the number of periportal vitamin A-rich stellate cells (SCs) and chronic hepatitis C. (A) Chronic hepatitis C monitored by serum ALT level. Both vitamin A-rich (≥10 lipid droplets in a cytoplasm) and vitamin A-poor (<10 lipid droplets) or vitamin A-rich stellate cells in the periportal area are significantly decreased in Group D (ALT >80 IU/L), compared with the groups with lower ALT level [28]. (B) Chronic hepatitis C. Both vitamin A-rich and vitamin A-poor stellate cells or vitamin A-rich stellate cells in the periportal area are significantly decreased in the CAH2B group compared with the CPH and CAH2A groups [29]. (C) Habitual smoking and the number of periportal vitamin A-rich stellate cells in patients with chronic hepatitis C. Smokers with chronic hepatitis C show low numbers of both vitamin A-rich and vitamin A-poor cells or vitamin A-rich stellate cells in the periportal area compared with non-smoking patients with chronic hepatitis C [29].

approximately 1.8 times that of the CPH and CAH2A groups in this study. Habitual smoking is encountered in 59.4% of patients with HCC, according to a report by the Liver Cancer Study Group of Japan [34]. This fact suggests that habitual smoking in CPH and CAH2A may cause the clinicopathological progression of chronic hepatitis C, in line with the results of other studies reporting that smoking is a risk factor for HCC development [35, 36]. It is possible that the decrease in the number of periportal vitamin A-rich stellate cells causes a decrease in local vitamin A content and a reduction in the anti-inflammatory effect of vitamin A. It has not yet been established whether the decrease of vitamin A-rich stellate cells results from harmful chemicals such as specific nitrosamines or benzopyrenes associated with smoking [37, 38]. The effects of the chemicals remain to be examined in stellate cell cultures to determine whether or not they result in hypoplasia of vitamin A-rich stellate cells.

In summary, habitual smoking can be a risk factor for acceleration of chronic hepatitis C, possibly due to a decrease or hypoplasia of vitamin A-rich stellate cells, resulting in the development of HCC.

3.2 Inhibition of dextran sulfate sodium (DSS) colitis by vitamin A supplementation

As a model of ulcerative colitis, DSS colitis was induced in mice fed a vitamin A deficient-diet or a vitamin A-supplemented diet [39, 40]. Subepithelial myofibroblasts in the colonic mucosa showed significant presence of cytoplasmic vitamin A lipid in the vitamin A-supplemented mice, in addition to the presence of vitamin A-rich hepatic stellate cells (**Figure 6**). Further, α smooth muscle (SM)-actin-positive subepithelial myofibroblasts increased in vitamin A-supplemented mice compared with vitamin A-deficient mice (**Figures 7**, **8A**). In addition, CD11c-positive macrophages in the colonic mucosa decreased in vitamin A-supplemented mice compared with vitamin A-deficient mice (**Figure 8B**). IgA-positive cells and the ratio of IgA-positive/IgG-positive cells increased in vitamin A-supplemented mice compared with vitamin A-deficient mice (**Figure 8C**). Experimental DSS colitis, as a murine model of ulcerative colitis, showed significantly higher severity of colitis and colonic ulcer, and shorter colon length in vitamin A-deficient mice compared with vitamin A-supplemented mice (**Figure 9**). In addition, recovery after DSS colitis was delayed in vitamin A-deficient mice compared with vitamin A-supplemented mice. The severity was greater in vitamin A-deficient mice than in vitamin A-supplemented mice with repeated bouts of DSS colitis.

Dietary vitamin A is required as a precursor of retinol in tissues. Tissue retinol plays an important role in immunity and cell differentiation. In immunity, excess Th1 and insufficient Th2 function occur in vitamin A deficiency, resulting in helper T cell imbalance. Further, α4β7-positive memory/activated T cell generation is reduced in vitamin A-deficiency [41]. Supported by retinoic acid, a vitamin A metabolite, γδ T cells produce IL-22, leading to improvement of DSS-induced colitis [42]. CD11c-positive dendritic cells in the colonic mucosa of vitamin A-deficient mice are increased, in line with results shown in a study of vitamin A-deficient rats [9]. The increase of CD11c-positive dendritic cells may represent a compensatory response to vitamin A deficiency, which induces maturation of dendritic cells [43, 44], since vitamin A deficiency causes dendritic cell dysfunction in the activation of T lymphocytes. Gut-homing IgA-secreting B cells are generated by intestinal dendritic cells in the sufficient vitamin A state [41]. Accordingly, a decrease in IgA^+ cells or the ratio of IgA^+ cells/IgG^+ in the colonic mucosa is thought to be indicative of disorganized mucosal immunity in vitamin A deficiency [41, 45, 46]. Additionally,

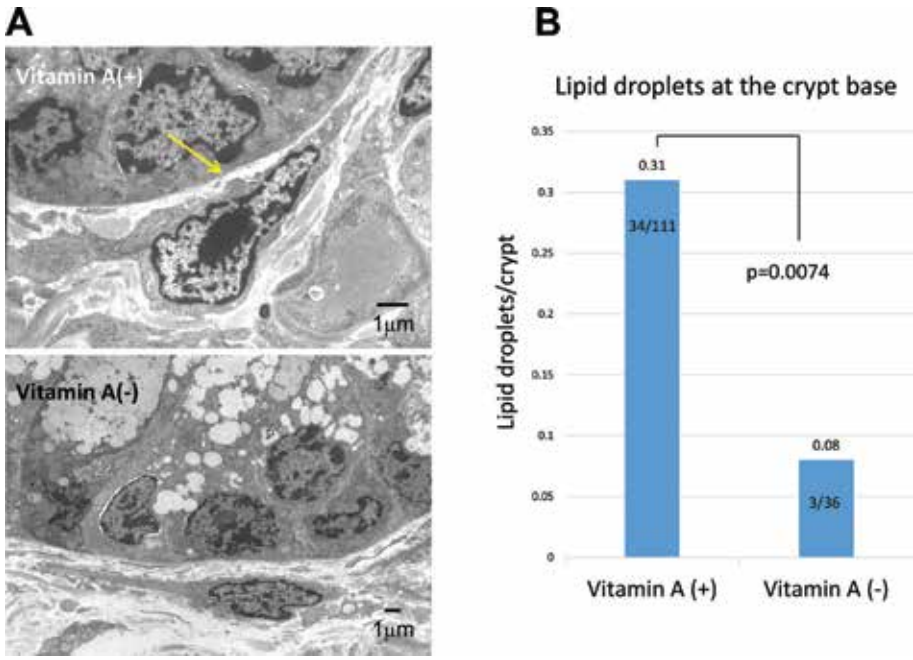

Figure 6.
Subepithelial myofibroblasts of colonic mucosa in mice fed a vitamin A-deficient or vitamin A-supplemented diet. (A) A subepithelial myofibroblast from a vitamin A-supplemented mouse contains a few lipid droplets (arrow, upper panel). Conversely, a fibroblast-like subepithelial myofibroblast from a vitamin A-deficient mouse has no lipid droplets (lower panel). (B) Lipid droplets at the crypt base of vitamin A-supplemented mice were significantly more than in vitamin A-deficient mice [40].

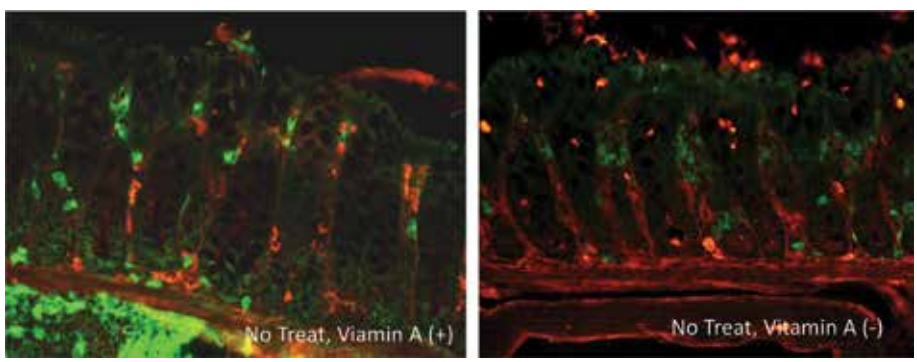

Figure 7.
Localization and distribution of CD11c-positive dendritic cells (green) and αSM-actin (red) in the colonic mucosa of vitamin A-deficient (B, vitamin A (−)) and vitamin A-supplemented mice (A, vitamin A (+)) [40].

intestinal epithelial expression of retinaldehyde dehydrogenase 1 (RALDH1) differs among mouse strains. BALB/c mice, which have high RALDH1, show an increased activity for induction of IgA class switching from B cells [42]. The severity of DSS colitis might depend on RALDH1 expression, suggesting the possibility of differences in susceptibility to ulcerative colitis in humans. Further studies should be conducted to clarify this possibility.

Thus, possible dysfunction of mucosal immunity and poor epithelial cell differentiation by malfunction of colonic subepithelial myofibroblasts in vitamin A-deficient mice are presumed to accelerate DSS colitis.

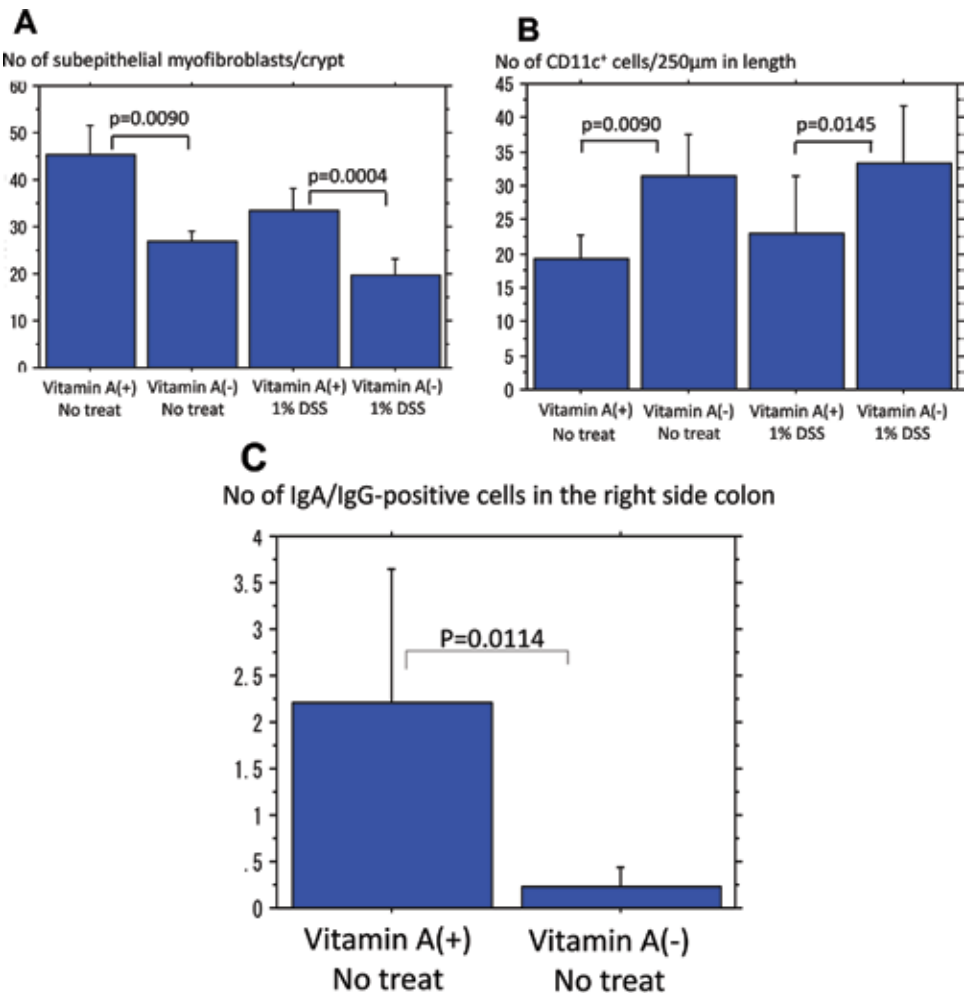

Figure 8.
(A, B) Decreased αSM-actin-positive subepithelial myofibroblasts (a) and increased CD11c-positive dendritic cells (B) are shown in vitamin A-deficient mice (vitamin A (−)) compared with vitamin A-supplemented mice (vitamin A (+)), both in the non-treated and DSS colitis-induced groups [40]. (C) Comparison of IgA/IgG-positive cells in the right side colonic mucosa between vitamin A-deficient and vitamin A-supplemented mice. IgA/IgG-positive cells increased in vitamin A-supplemented mice (vitamin A (+)), compared with vitamin A-deficient mice (vitamin A (−)) [40].

3.3 Inhibition of development of colonic tumors by vitamin A supplementation in a DSS colitis model of the ulcerative colitis-carcinoma sequence

A combination of azoxymethane (AZM) preinjection followed by induction of DSS colitis is a well-known experimental murine model of the ulcerative colitis-dysplasia-adenocarcinoma sequence [47]. Vitamin A-deficient mice developed more dysplasia and adenocarcinoma than vitamin A-supplemented mice, as well as more severe colitis (**Figures 10**, **11**) [40]. These results demonstrate that a vitamin A-supplemented diet inhibited DSS colitis and the subsequent development of dysplasia-carcinoma seen with a vitamin A-deficient diet.

Cytoplasmic vitamin A lipids decreased in subepithelial myofibroblasts at the colonic crypt base of vitamin A-deficient mice compared with vitamin A-supplemented mice. Furthermore, a decrease in αSM-actin-positive subepithelial myofibroblasts was also found, suggesting dysfunction of niche regulation for the protection and differentiation of mucosal stem cells or progenitor cells [3, 23, 25].

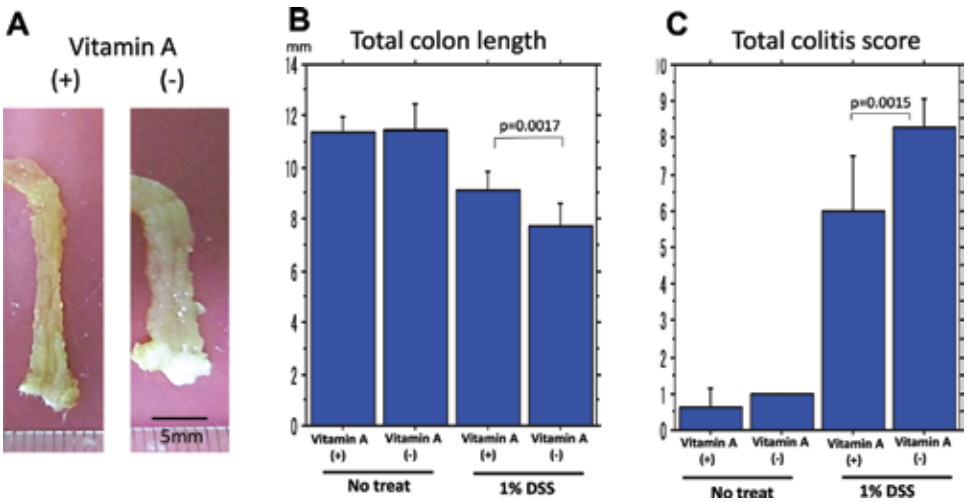

Figure 9.
Comparison of DSS colitis between vitamin A-deficient and vitamin A-supplemented mice. Vitamin A-supplemented mice showed improvement of colon length (A, B) and colitis score (C) assessed by erosion, loss of crypts, and inflammatory cell infiltration [40].

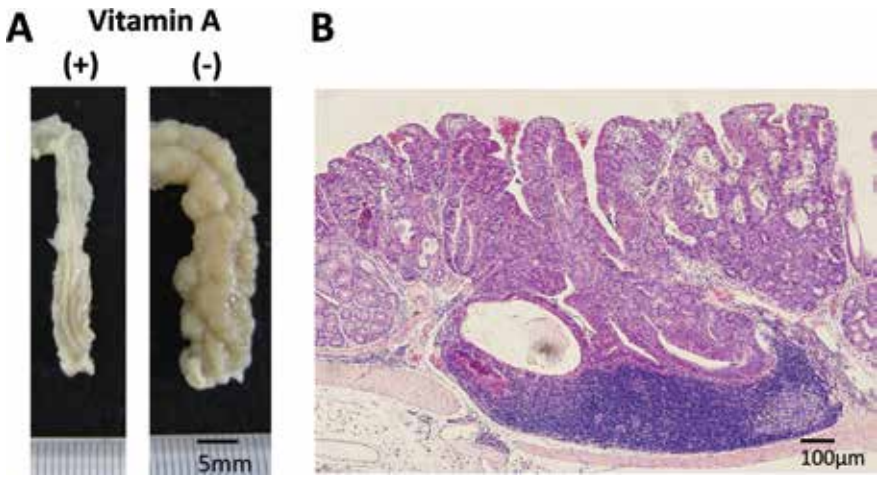

Figure 10.
Colon cancer in vitamin A-deficient mouse induced by a combination of azoxymethane (AZM) injection and DSS colitis [40].

These findings might indicate that dysfunction of stem cell niche regulation in subepithelial myofibroblasts causes accelerated DSS colitis, resulting in the development of colorectal neoplasia.

Vitamin A and its metabolites, retinoids, play an important role in cell differentiation [25, 48]. It is well known and clinically accepted that retinoids have chemopreventive effects against cancers, particularly with differentiation therapy for acute promyelocytic leukemia [49]. In addition, there are many clinical and experimental reports that vitamin A deficiency promotes cancer development and progression [50–55]. The CYP26A1 gene, which encodes for the cytochrome P450 enzyme involved in metabolic inactivation of retinoic acid, was highly expressed in cancers of various organs and is related to cancer progression. This may suggest a link between intracellular retinoic acid status and tumorigenesis [56–58]. Furthermore, prolonged recovery from severe DSS colitis and the subsequent development of

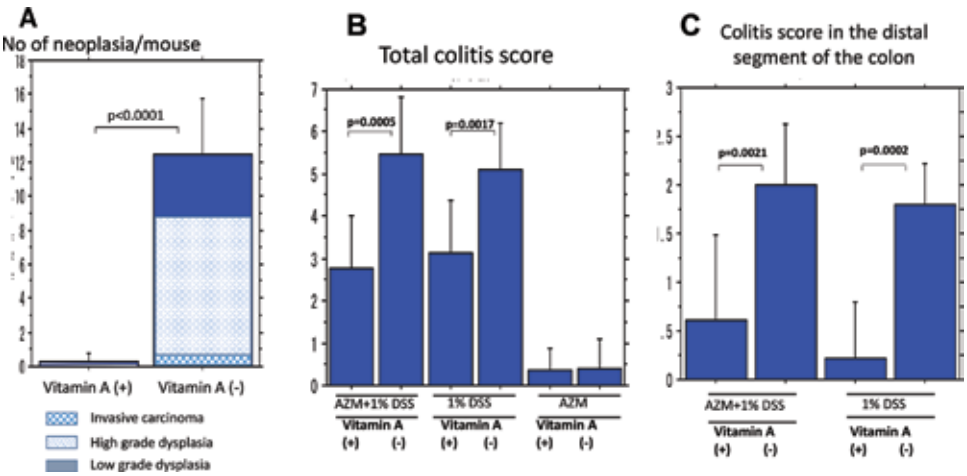

Figure 11.
Comparison of induced colon neoplasia and DSS colitis between vitamin A-deficient and vitamin A-supplemented mice. Vitamin A-supplemented mice showed significant inhibition of neoplasia development (A) and reduction of colon shortness (B) and colitis (C) compared with vitamin A-deficient mice [40].

colonic tumors in vitamin A-deficient mice were significantly improved by vitamin A supplementation, suggesting a cause-effect relationship between local vitamin A status and the development and progression of cancer.

It has been shown that gut microbiota have a possible influence on colitis and the development of colorectal neoplasia. Particularly, correction of microbiota-induced retinoic acid deficiency stimulates protective $CD8^+$ T cell-mediated immunity, resulting in inhibition of colitis and its associated colorectal tumorigenesis in mice [59]. Since malnutrition including vitamin A insufficiency accelerates inflammatory bowel disease in children [60, 61], it is thought that the difference in microbiota in response to a vitamin A-deficient versus a vitamin A-supplemented diet may have a substantial effect on colitis and the development of colonic neoplasia [62–64]. Further study is needed to address this possibility.

There are no definite clinical indications for vitamin A administration to protect against inflammation and tumor development, although it has been proposed that dietary vitamin A is closely related to exacerbation and continuity of inflammation, particularly in chronic hepatitis C [65, 66] and inflammatory bowel disease [67, 68]. The results described herein raise the possibility that vitamin A administration inhibits chronic hepatitis and colitis, and the subsequent development of cancer. Further studies are needed to identify the possible mechanisms for inhibition of chronic inflammation and subsequent neoplasia induced by vitamin A supplementation.

4. Conclusions

Vitamin A is stored in stellate cells, mainly in the liver and to a lesser extent in the lung and intestine, and plays important roles in immunity, cell differentiation, and the stem cell niche. In the liver, decreased vitamin A-rich stellate cells, or decreased vitamin A-rich and vitamin A-poor stellate cells combined, relates to the severity of chronic hepatitis C and habitual smoking. In the colon, a vitamin A-supplemented diet inhibits DSS colitis and subsequent colonic tumor development in vitamin A-deficient diet mice, an experimental mouse model of ulcerative colitis. Vitamin A administration could be effective to treat and/or prevent liver

disease and ulcerative colitis, although the clinical effects of vitamin A administration in this context have not been thoroughly characterized to date. Further study is needed to identify the effect of vitamin A administration on chronic inflammation and tumorigenesis.

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Chapter 4

Retinoic Acid in Ocular Growth Regulation

Jody A. Summers

Abstract

All-*trans*-retinoic acid (atRA) is a metabolite of vitamin A (retinol) and is required for growth and development of a variety of organ systems in all higher animals from fish to humans. Evidence is accumulating to suggest that atRA may also be an important molecular signal in the postnatal control of eye size. Choroidal synthesis of atRA is modulated during periods of visually-induced changes in ocular growth and has pronounced effects on eye growth and refraction in several animal models of myopia. Choroidal atRA synthesis is exclusively regulated by expression of the enzyme, retinaldehyde dehydrogenase 2 (RALDH2). In chicks and humans, RALDH2 is synthesized by a unique population of uncharacterized extravascular stromal cells concentrated in the proximal choroid. The identification of choroidal atRA and RALDH2 as visually induced ocular growth regulators provides the potential for new therapeutic targets for the treatment of childhood myopia. The objective of this chapter is to discuss what is presently known about atRA biosynthesis and transport in the eye during visually guided eye growth and how this research can contribute to a better understanding of the mechanisms underlying the development of myopia.

Keywords: retinoic acid, choroid, myopia, sclera, RALDH2, emmetropization

1. Introduction

All-*trans*-retinoic acid (atRA) is the transcriptionally active derivative of vitamin A. atRA is an essential signaling molecule for developmental processes of numerous organ systems including those of the brain, limbs, lungs, pancreas, heart, and eye in many organisms from fish to humans [1, 2]. With the advent of increasingly sensitive methods to measure endogenous concentrations of atRA, data is accumulating to suggest that atRA is also important in the growth and maintenance of a number of organ systems during postnatal and adult life [3–7]. Within the postnatal eye, atRA has been detected in the retina, where its synthesis was shown to be mediated by oxidation of the chromophore all-*trans*-retinaldehyde, released from bleached rhodopsin in the photoreceptor outer segments following exposure to light [8]. More recently, the choroid (the highly vascular layer between the retina and the sclera) has been shown to synthesize and accumulate high levels of atRA [9, 10]. A number of studies in several animal models suggest that choroidal atRA may be an important molecular signal for the control of postnatal ocular growth [9, 11–13]. We and others have demonstrated that in response to visual stimuli, ocular atRA synthesis is regulated exclusively via choroidal expression of the atRA synthe sizing enzyme, retinaldehyde dehydrogenase 2 (RALDH2) by a unique population of cells [10, 14]. Furthermore, choroidally derived retinoic acid is transported to the

sclera (the outer connective tissue shell of the eye) by apolipoprotein A-1 (ApoA-1) which functions as a specific extracellular atRA-binding and transport protein in the eye [15, 16]. Once delivered to the sclera, we speculate that atRA regulates the transcription of many genes in the sclera to effect changes in scleral extracellular matrix remodeling, ocular size, and refraction.

This chapter therefore focuses primarily on the potential role of atRA on the control of postnatal growth of the eye, and implications for the development of new therapies for the control of myopia in children.

2. Retinoic acid is a vitamin A derivative

atRA is synthesized in two steps from vitamin A (all-*trans*-retinol). The first step produces all-*trans*-retinaldehyde from all-*trans*-retinol through the action of cytosolic or membrane bound alcohol dehydrogenases (ADH). The second, irreversible, step in atRA synthesis involves the oxidization of all-*trans*-retinaldehyde to atRA through the actions of the cytosolic retinaldehyde dehydrogenases (RALDH1, RALDH2, RALDH3; a.k.a. Aldh1α1, Aldh1α2, Aldh1α3) [16, 17]. Tissue concentrations of atRA are regulated by the activities of these synthesizing enzymes, as well as the atRA-metabolizing enzyme CYP26, a member of the cytochrome P450 family [17]. Cyp1B1 may also contribute to atRA synthesis in the chick embryo [18] via synthesis of all-*trans*-retinaldehyde and atRA from all-*trans*-retinol. Furthermore, the rate of these reactions is regulated by the availability of the substrates, the accessibility of the enzymes to their substrates, and the catalytic activity of the enzymes [19]. Once synthesized, atRA can act within its own cell of synthesis (autocrine signaling) or be transported to nearby cells (paracrine signaling) and bind with nuclear retinoic acid receptor complexes to directly control the transcriptional activity of more than 100 target genes [20].

3. Visual regulation of intraocular retinoic acid synthesis

3.1 Emmetropization: vision-dependent ocular growth regulation

Clinical and experimental evidence have indicated that postnatal eye growth is regulated, at least in part, by a vision-dependent "emmetropization" mechanism that acts to minimize refractive error through the coordinated regulation of the growth of the ocular tissues [21, 22]. Interruption of emmetropization in animal models, such as the chick, primate, and guinea pig, through the application of translucent occluders (form deprivation) causes a distortion in visual quality, which results in ocular growth and myopia through changes in the regulation of scleral extracellular matrix (ECM) remodeling [23–27]. Form deprivation-induced myopia is reversible; removal of the occluder and subsequent detection of myopic defocus results in a rapid cessation of axial growth and the eventual reestablishment of emmetropia (recovery) [24]. Even stronger evidence for the presence of an emmetropization mechanism comes from studies in which animals are fitted with either concave (minus) lenses or convex (plus) lenses that shifts the focal plane behind (hyperopic defocus) or in front of (myopic defocus) the retinal photoreceptors, respectively. In animals with functional emmetropization, the axial length of the lens-treated eye will increase or decrease until the retinal location has shifted to match that of the new focal plane [28–31]. The emmetropization mechanism does not require the central nervous system and appears to be regulated by locally produced chemical signals within the eye itself. When visual form deprivation is restricted to nasal or temporal visual

fields, excessive growth of the sclera is limited to that portion corresponding to the visually deprived part of the retina [32, 33]. Furthermore experimental myopia can be induced animals lacking a functional optic nerve [34–36], suggesting that the central nervous system is not required for the development of myopia. It is now generally accepted that visually guided eye growth is regulated by a series of locally generated chemical events that begin in the retina in response to specific visual stimuli and terminate in the sclera where they result in scleral extracellular matrix (ECM) remodeling, changes in ocular length and refractive status [37–42]. Therefore the elucidation of the chemical events responsible for visually-induced changes in ocular growth is of great interest as it may provide new avenues for the development of therapies to slow or prevent the progression of myopia.

3.2 Choroidal retinoic acid: a potential ocular growth regulator

Several studies in a variety of animal models indicate that all-*trans*-retinoic acid (atRA) may be one of the chemical signals required for the regulation of eye growth during emmetropization [9, 11–13]. Mertz and Wallman [9] were the first to show that choroidal synthesis of atRA was increased in chick eyes during recovery from form deprivation myopia and following application of positive lenses (imposed myopic defocus), two visual conditions that cause a deceleration in ocular growth rates. Moreover, atRA was shown to be decreased in eyes undergoing form deprivation myopia and compensation for hyperopic defocus compared with the fellow control eye, conditions that stimulate ocular elongation. It was therefore suggested that choroidal atRA could act as a locally produced (within the eye) scleral growth modulator during visually guided ocular growth. atRA is an attractive candidate for a visually regulated ocular growth regulator because it is readily diffusible, has pronounced effects on scleral extracellular matrix metabolism, and exerts its effects through highly regulated, locally controlled synthesis and degradation.

Studies by Simon et al. [43] and Rada et al. [10] identified transcriptional changes in choroidal *RALDH2* in response to imposed defocus or recovery from in induced myopia. RALDH2 mRNA concentration was found to decrease in the choroid following treatment with negative lenses and to increase with positive lenses or during recovery from induced myopia. No changes were observed in the expression of the atRA metabolizing enzymes, *RALDH3*, *RDH10*, *CYP1B1*, *CYP26*, and transcript levels of choroidal *RALDH1* were undetectable [10]. Additionally, changes in choroidal RALDH2 protein concentrations and enzymatic activity in recovering eyes were reflective of the transcriptional changes in choroidal RALDH2 [14] suggesting that, in response to myopic defocus or recovery from induced myopia, the concentration of choroidal RALDH2 increases which, in turn, results in increased production of atRA. No RALDH activity was detected in the sclera or retina/RPE of control or treated chick eyes, indicating that the choroid is responsible for the majority of atRA synthesized in the chick eye [14].

3.3 Choroidal RALDH2+ cells: a novel cell type

In chicks and humans, RALDH2 is synthesized by a population of extravascular choroidal stromal cells, some of which are closely associated with blood vessels (**Figure 1**) [10, 14, 44]. In chicks, RALDH2+ cells increase in number markedly over 1–7 days of recovery due, in part, to cellular proliferation (**Figure 1F** and **G**) and become concentrated on the proximal (RPE) side of the choroid [14]. Immunohistochemical analyses of chick choroids indicate that many of RALDH2+ express pro-collagen type IA (**Figure 1B** and **C**), similar to activated pericytes (a.k.a. perivascular stromal cells) within the CNS perivascular space [45]. Additionally RALDH2 is expressed in the chick

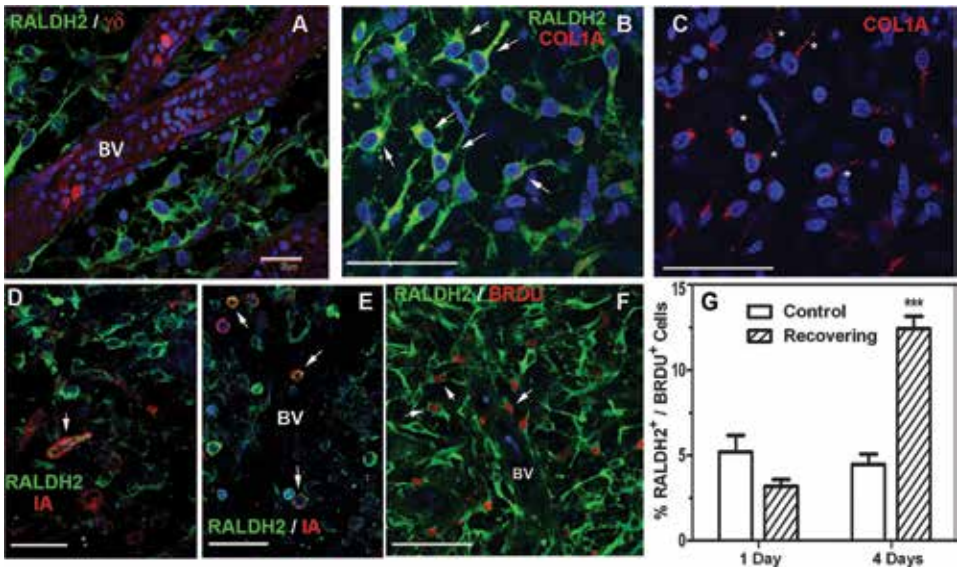

Figure 1.
*Choroidal RALDH2 positive (+) cells are heterogeneous. (A–E) RALDH2+ cells (green) are identified following labeling of 4 day recovering choroids with anti-RALDH2, together with anti-TCRγδ (γδ), pro-collagen IA (COL1A), or Ia antigen (IA) (red). Double-labeled cells are indicated by arrows. Asterisks in (C) indicate RALDH2+ cells that co-express Col1A. (F) Proliferating RALDH2+ cells were labeled with BrdU and identified with anti-BRDU (red). (G) Percentage of RALDH2+/BRDU+ cells is ≈ 3× higher in 4 day recovering choroids, suggesting that RALDH2 activity is partially controlled by proliferation of RALDH2+ cells. Bar = 20 μM in A, 40 μM in B–F. ***Student's t test, p < 0.0001. Error bars = SEM. Nuclei in A–F are stained with DAPI. BV, blood vessel.*

choroid by a small population of round cells that are positive for the Ia antigen [46, 47], indicating similarities with thymic macrophages/dendritic cells (**Figure 1D** and **E**), but are negative for the macrophage markers KuL01, MHC-II, and IgY [48]. A sub-population of RALDH2+ cells also express α-smooth muscle actin (αSMA) [10, 14], but are negative for the smooth muscle/myofibroblast proteins, smoothelin, desmin and myocardin. RALDH2+ cells do not co-localize with CD-45 [14], TCRδγ (**Figure 1A**), CD5, or GRL(2) positive cells [49, 50], indicating they are not of hematopoietic origin. RALDH2+ cells also do not co-localize with neuron-specific beta III tubulin, NOS (pan), or tyrosine hydroxylase, indicating they are not of neuronal origin. Negative results were also obtained using anti-NG2 (a pericyte marker), vimentin, and PECAM-1 (an endothelial marker). Similarly, RALDH2+ cells in the human choroid were negative for the endothelial cell marker, CD31, the pericyte markers, NG2 and CD146, α-smooth muscle actin, the macrophage markers CD68 and LYVE1, IBA1 (microglia) and the pan-neuronal marker PGP9.5 [51]. Unlike results in the chick, some RALDH2+ cells in the human choroid co-localized with vimentin, suggesting a mesenchymal origin [51]. Based on the markers used in these studies, RALDH2+ cells seem to represent an independent cell-population. Studies are in progress using additional markers as well as transcriptome analyses on RALDH2+ cells isolated from chick and human choroids to further classify this new cell-population as this cell type may represent a potential target for therapies to slow or prevent myopia in children.

4. Retinoic acid on scleral proteoglycan synthesis

The retina, choroid and sclera are three possible tissue targets for choroidally generated atRA within the eye. Of these three targets, the sclera is a leading candidate. Based on results using a specific inhibitor of proteoglycan synthesis

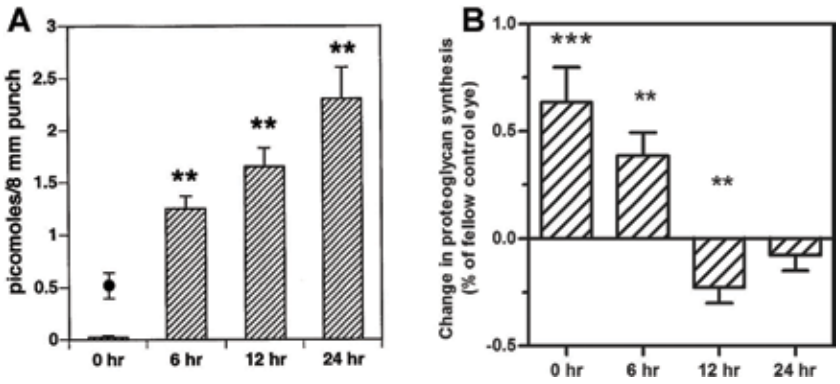

Figure 2.
Choroidal retinoic acid synthesis and scleral proteoglycan synthesis during recovery from form deprivation myopia. (A) Changes in choroidal all-trans-retinoic acid (atRA) synthesis during recovery from experimental myopia. (B) Changes in scleral proteoglycan synthesis in during recovery from experimental myopia. A from: Mertz and Wallman [9]. B adapted from: Summers and Hollaway [53]. Reproduced with permission © Elsevier.

(*p*-nitrophenyl-beta-D-xylopyranoside), we previously demonstrated that the rate of proteoglycan synthesis in the cartilaginous layer of the chick sclera is largely responsible for visually guided changes in eye size [27, 52]. Moreover, proteoglycan synthesis is rapidly upregulated in the sclera in response to visual form deprivation and is rapidly downregulated in the posterior sclera to levels significantly below those of fellow controls within 12 hours upon restoration of unrestricted vision (=recovery from induced myopia) [53, 54]. Interestingly, the time course of the increase in choroidal atRA synthesis during recovery from induced myopia [9] was remarkably similar to that of the decrease in rate of sclera proteoglycan synthesis observed in the early phase of recovery from induced myopia [53] suggesting a causal relationship between choroidal atRA synthesis and scleral proteoglycan synthesis (**Figure 2**). Moreover, *RALDH2* mRNA pools in the chick choroid were shown to increase and decrease in a manner that inversely correlated with changes in scleral proteoglycan synthesis in recovering eyes [10], suggesting that RALDH2 gene expression in the choroid regulate choroidal atRA synthesis during visually induced ocular growth. It is well known that atRA is a potent inhibitor of proteoglycan biosynthesis by chondrocytes [55, 56] and that it facilitates cartilage catabolism through the increased synthesis of matrix-degrading enzymes [55, 57, 58]. atRA inhibits scleral proteoglycan synthesis in a dose-dependent manner with an IC_{50} of 8×10^{-9} M, which is similar to the measured endogenous levels of atRA in choroid organ cultures (4×10^{-9} to 7×10^{-9} M) [10]. At this concentration, atRA would be able to regulate scleral growth matrix remodeling through the stimulation or repression of transcription factors, extracellular matrix constituents, and MMPs or TIMPs.

5. Identification of apolipoprotein A-1 as a retinoic acid binding protein

Due to its hydrophobicity, atRA cannot diffuse freely in the hydrophilic extracellular microenvironment. Therefore, the requirement for carrier proteins capable of forming a soluble complex with atRA and transporting atRA to target cells is necessary to achieve high efficiency and specificity while avoiding toxicity associated with random diffusion. Mertz and Wallman [9] and our lab [15] identified a secreted protein of *M*r = 27,000 that was the major atRA binding protein present in choroid and sclera conditioned medium. This *M*r 27,000 protein did not correspond in size to any of the previously identified atRA binding proteins [59]. We subsequently determined that the *M*r 27,000 protein was apolipoprotein a-1 (ApoA-1) [15] (**Figure 3**).

We have also shown that choroidal expression of ApoA-1 is transcriptionally regulated by atRA, *and* choroidal ApoA-1 mRNA and protein synthesis are upregulated during recovery from induced myopia, suggesting the presence of a regulatory feedback mechanism to regulate atRA transport and activity [15]. We postulate that ApoA-1 functions to transport atRA from its site of synthesis by RALDH2+ cells in the proximal choroid to the sclera for the regulation of scleral ECM remodeling. This idea is supported by our observation that the chick sclera (which is avascular) also releases significant amounts of ApoA-1 into culture medium, despite undetectable *de novo* protein synthesis [15]. These data provide further evidence that choroidally derived ApoA-1 accumulates in the sclera, presumably as a consequence of retinoid transport.

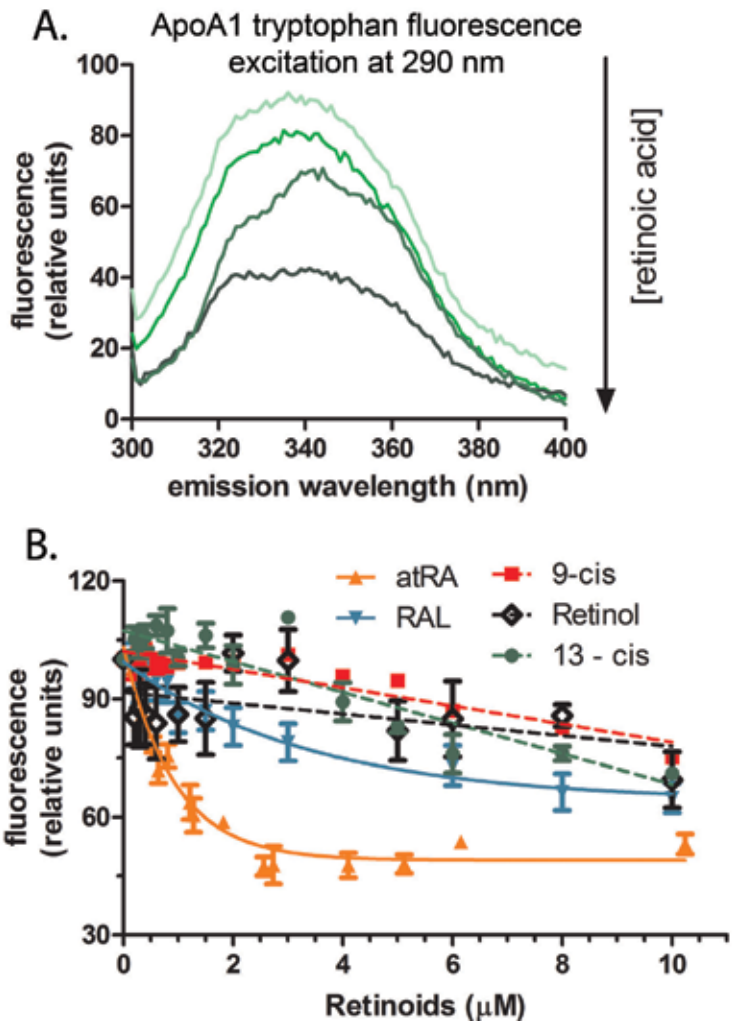

Figure 3.
ApoA-1 is a specific atRA-binding protein. (A) Retinoic acid (atRA) lacks intrinsic fluorescence (not shown), but can quench intrinsic protein fluorescence excited at 290 nm due to energy transfer from tryptophan residues on ApoA-1. Increasing atRA concentrations cause decreased fluorescence emission following excitation at 290 nm. (B) Titration of ApoA-1 with various retinoids by measuring quenching of protein fluorescence (emission = 340 nm). Significant quenching of protein fluorescence was observed only for atRA, indicating that ApoA-1 is a specific atRA-binding protein. atRA, all-trans-retinoic acid; RAL, all-trans-retinaldehyde; 9-cis, 9-cis-retinoic acid; 13-cis, 13-cis-retinoic acid. This research was originally published in the Journal of Biological Chemistry [70]. © The American Society for Biochemistry and Molecular Biology.

6. Role of retinoic acid in postnatal ocular growth

To elucidate the role of atRA in the regulation of postnatal ocular growth, several studies have been carried out in which either atRA or non-specific atRA synthesis inhibitors (i.e., citral, disulfiram) were administered either systemically or locally in several animals undergoing visually induced changes in eye growth [12, 60, 61]. Results of studies using chicks and mammals to examine the role of atRA in emmetropization, myopia development and postnatal ocular growth are difficult to interpret due to species differences in the processes of scleral remodeling and in the mechanisms by which ocular length and refraction are modulated by visual stimuli [62]. Moreover, these studies are further complicated by the multiple targets of atRA within the eye and pleiotropic cellular responses to retinoid signaling [63]. The mammalian sclera consists of a single fibrous layer that undergoes scleral thinning, and increased distensibility during periods of ocular elongation and myopia development. Scleral thinning during myopia development in mammals is the consequence of decreased sulfated glycosaminoglycan and collagen synthesis [11, 64, 65]. In contrast, the chick sclera consists of both cartilaginous and fibrous scleral layers. Ocular elongation during induced myopia in chicks is the result of growth of the cartilaginous sclera, with increases in sulfated glycosaminoglycan synthesis, increased protein synthesis, and increased total scleral mass [27, 66–68]. In chicks, increased choroidal synthesis of atRA during recovery from form deprivation myopia results in inhibition of scleral proteoglycan synthesis and slowing of the rate of ocular elongation. In primates [11] and guinea pigs [12], choroidal atRA synthesis is increased in treated eyes following induced myopia, a condition that is also associated with decreased proteoglycan synthesis in the posterior sclera but, in contrast to chicks, results in increased ocular elongation and myopia due to weakening of the fibrous sclera and localized ectasia at the posterior ocular pole. Considering the negative effect of atRA on scleral proteoglycan synthesis in animals containing either a single fibrous sclera (i.e., guinea pigs, primates) as well as chicks that contain both cartilaginous and fibrous scleral layers [9, 11], choroidally derived atRA represents a mechanism to regulate ocular length and refraction common to multiple species.

Furthermore, interpretation of experiments in which atRA agonists and atRA synthesis inhibitors are delivered either systemically or intraocularly is complicated by the widespread multicellular effects of atRA. Eye growth is increased following dietary delivery of atRA to chicks and is decreased after oral delivery of citral, a non-specific inhibitor of atRA synthesis [61]. Similarly, intraocular delivery of the non-specific atRA synthesis inhibitor, disulfiram, inhibited the development of form-deprivation myopia in chicks [60], a result generally opposite of what would be predicated if atRA acted to inhibit ocular elongation in chicks. It is likely that untargeted administration of atRA or use of non-specific atRA synthesis inhibitors that also inhibit other aldehyde dehydrogenases lead to multicellular effects that may differ from those mediated by endogenous atRA. We have recently developed a small molecule inhibitor, dichloro-all-*trans*-retinone (DAR) that is an irreversible inhibitor of RALDH1, 2, and 3 that effectively inhibits RALDH1, 2, and 3 in the nanomolar range but has no inhibitory activity against mitochondrial ALDH2 [69]. It is hoped that DAR, or similar compounds can be used to modulate endogenous concentrations of atRA through specific inhibition of the RALDH isoenzymes within the eye for future experimental and clinical studies to elucidate the role of atRA on postnatal ocular growth and myopia development.

7. Conclusions

Although the cause of myopia in humans is complex, clinical and experimental studies indicate that failure of the emmetropization process often leads to the development of myopia. It has been well-established that visually induced changes in ocular length are the result of altered extracellular matrix remodeling of the scleral shell. However no therapeutic targets have been identified and no pharmaceutical or optometric approaches have proven effective for the treatment of high myopia. The increasing prevalence of myopia and earlier age of onset emphasize the need for the development of an effective therapy. The identification of choroidal atRA, RALDH2, and the choroidal cells responsible for atRA synthesis, may provide new targets for the development of effective myopia therapies. Moreover the development of small molecule inhibitors specifically targeting RALDH2 would greatly expand our basic understanding atRA's role in postnatal growth and development as well as provide potential new therapies to slow or prevent the progression of myopia.

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Conflict of interest

The author certifies that she has no conflicts of interest and no affiliations with or involvement in any organization or entity with any financial interest.

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Chapter 5

From Neglected and Underutilized Crops to Powerful Sources of Vitamin A: Three Case Studies of Mozambican Cultivated *Tacca leontopetaloides*, Cowpea, and Cassava

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Abstract

About 1 billion people are currently suffering from chronic hunger, malnutrition, and vitamin A deficiency, while it is predicted that world food production needs to increase by 70% by 2050 to satisfy 9.9 billion predicted population in the world, relying on a natural resource base that is reaching its limits and with climate change adding further pressures on agriculture and acting as the main driver of crop diversity loss. The main goal of this chapter was to discuss the role of neglected crops (arrowroot, cassava, and cowpea) as potential sources of vitamin A with case studies of Mozambique country where the current population (30.5 million—mid-2018) is predicted to more than double by mid-2050 (67.4 million) while vitamin A deficiency and food insecurity are serious issues. Crops have an important role in rural communities and are nutrient dense and can be used in diet diversification and vitamin A alleviation. They are highly adapted to agroecological niches and marginal areas. The current research reinforces that neglected crops are potential sources of vitamin A with an extra extensive phytochemical composition that together are important in alleviating vitamin A deficiency. Their production promotion must be reinforced and incorporated in crop diversification.

Keywords: sources of vitamin A, orphan crops, famine foods, *Vigna unguiculata* L. Walp, *Tacca leontopetaloides* L. Kuntze, *Manihot esculenta* Crantz

1. Introduction

1.1 Status of vitamin A deficiency in the world and in Mozambique

Recent statistics estimate that 821 million people in the world are undernourished and the world hunger continues to rise in recent years [1]. Undernourishment and severe food insecurity are increasing in almost all regions of Africa, as well as in South America. Actually, 237 million people are undernourished in sub-Saharan Africa (SSA) [1]. The increase in hunger and food insecurity indicates that there is considerable work to be done against malnutrition and vitamin A deficiency globally.

Vitamin A (also called retinol) is one of the fat-soluble vitamins necessary for good health. It has an important role as an antioxidant by helping to prevent free radicals from causing cellular damage and for proper function of the immune, skeletal, respiratory, reproductive, and integumentary (skin) systems and decrease the risk of certain cancers, heart attacks, and strokes [2]. It is also essential for the proper function of the retina, where it can act to prevent night blindness, as well as lower the odds of getting age-related macular degeneration [2]. As reported by Han et al. [3], vitamin A deficiency (VAD) is known to cause ocular changes, including corneal ulcers and xerophthalmia, ocular globe modifications, and loss of palpebral and pupillary reflexes.

According to GEM [4], the recommended dietary dose (RDA) for vitamin A is 1.0 mg/day for the adult man and 0.8 mg/day for the adult woman. Vitamin A can be supplied entirely via β-carotene (6 mg of β-carotene is considered to be the equivalent of 1 mg of vitamin A [4]). The deficiency of vitamin A occurs when the chronic failure to eat sufficient amounts of vitamin A or β-carotene results in levels of blood serum vitamin A that are below a defined range [4]. Globally, Low [5] reported that 190 million preschool children and 19 million pregnant women are affected by vitamin A deficiency. In sub-Saharan Africa (SSA), more than 40% of children under five suffer from VAD.

In Mozambique, 44% of children under the age of five are stunted, 4% are wasted, and 18% are underweight. The statistics indicate that 15% of infants are born with a low birth weight. The under-5 mortality rate has been estimated to be 138 per 1000 live births, and globally the ranking of stunting prevalence is the 19th highest out of 136 countries [6, 7]. Annually, Mozambique loses US$116 million to vitamin and mineral deficiencies [6, 7]. As reported by Aguayo [8], an estimated 2.3 million children below the age of 5 years are vitamin A deficient. In the absence of appropriate policy and program action, VAD is the attributable cause of over 30 000 deaths annually, representing 34.8% of all-cause mortality in this age group. Historically, Mozambique was devastated by a postindependence civil war from 1977 to 1992 which destroyed infrastructures, ruined livelihoods, and severely hampered agricultural production and economic development [9]. There is a problem of physical access in rural areas, where the majority of the population lives. Contrarily, economic access to food is a major issue, especially in times of soaring food prices in urban areas. Natural shocks such as floods and drought regularly affect agricultural production [9]. The Mozambican diet is mainly composed of cassava, maize, beans, and imported wheat. There is a low supply of micronutrient-rich foods. Eighty percent of the dietary energy supply is provided by cereals and starchy roots; this very poor level of dietary diversification has not improved for the last 40 years and is currently the lowest in the region.

Mozambique's population of almost 30 million people (30,804,450 current total population) remains one of the most vulnerable in the world, ranking 180 out of 188 countries in the United Nations Development Programme. Forty-four

percent of the woman at reproductive age are anemic, and 69% of children of 6–24 months old are vitamin A deficient [10]. One-third of the population is chronically food insecure. Undernutrition in children remains at 44% while the prevalence of vitamin A and iron deficiencies at 69 and 74%, respectively. Micronutrient malnutrition, also known as hidden hunger, leads to high social and public costs, reduced work capacity, and the tragic loss of human potential. Mozambican urban dwellers are increasing at 1.5% each year, due to the migration trend from rural to urban settings. Malnutrition is affecting low-income consumers living.

VAD is a public health problem in more than half of all countries, especially in Africa and Southeast Asia, hitting hardest young children and pregnant women in low-income countries. According to the World Health Organization [11], an estimated 250 million preschool children are vitamin A deficient, and it is likely that in VAD areas a substantial proportion of pregnant women is vitamin A deficient. According to the same source, 250,000 to 500,000 vitamin A-deficient children become blind every year, half of them dying within 12 months of losing their sight. VAD is the major cause of preventable blindness in children and increases the risk of disease and death from severe infections. VAD also causes night blindness in pregnant woman and may increase the risk of maternal mortality. Supplying adequate vitamin A in high-risk areas can significantly reduce mortality and is crucial for maternal and child survival. The absence of this vitamin causes a high risk of disease and death.

According to the WHO [11], an intensified action to end and eradicate worldwide the VAD and ensure universal access to healthier and more sustainable diets for all people wherever they live is the main goals of the United Nations Decade of Action on Nutrition 2016–2025. All countries are claimed to undertake activities and nutrition actions to reduce malnutrition. Such actions will require large commitment from all institutions and communities. Such commitment implies that new interventions must be created, sustained, and strengthened over time. One of the solutions is the promotion of orphan crops in the diet of small farmer agriculture. The main goals of the chapter are to discuss the use of orphan or underutilized crops (arrowroot, cassava, and cowpea) as potential sources of vitamin A with key examples of Mozambican situation.

1.2 Global population growth: challenges in food production versus climate changes

The world population is projected to reach 9.9 billion by 2050, representing up to 2.3 billion or 29% from an estimated 7.6 billion people now, according to projections by the Population Reference Bureau (PRB) included in the 2018 World Population Data Sheet [12]. The PRB projects that Africa's populations will more than double to 2.6 billion by 2050 and account for 58% of the global population increase by that date. Particularly, the Mozambican population will increase to 36.9 million by 2050 (**Table 1**). The number of people in Asia, America, and Oceania is projected to increase about 717 million to 5.3 billion, from 1 billion now to 1.2 billion, and from 41 million to 64 million, respectively, while in Europe will decline from 746 million to 730 million.

The Global Agricultural Productivity report [13] points out that in India, national production will only meet 59% of the country's food demand in 2030. In East Asia, only 67% of food demand up to 2030 will be supported within the region. In sub-Saharan Africa, projections indicate that only 15% of food demand will be reached by 2030, which would require significant imports or food assistance or the opening of new areas for development, which may not be suitable for sustainable production [13]. The need for increased production is facing important constraints.

Region**	Current population (mid-2018, millions)	Predicted population by mid-2050 (millions)
World (total)	**7621 (≈100%)**	**9852**
Asia	4536 (59.52%)	5253
Africa	**1284 (16.85%)**	**2586**
Sub-Saharan Africa	1049 (13.76%)	22
Mozambique	**30.5 (0.4%)**	**67.4**
Americas	1014 (13.31%)	122
Europe	746 (9.79%)	730
Oceania	41 (0.53%)	64

***Current population (2018 world data sheet) and predicted growth by 2050 with focus in Africa and Mozambique Special attention is also given for sub-Saharan Africa (SSA) region and Mozambique country.*

Table 1.
Current population (mid-2018) and the predicted growth by 2050 in the world and for each region in particular.

Among them are the depletion and reduction in the productivity growth rates of the main crops, the dependence of phosphorus derived from rock, a nonrenewable resource, and climate change [14].

On the other hand, the increase in production alone is not enough to achieve economic, environmental, and socially sustainable food systems. Food and agricultural systems are vulnerable to a variety of risks, including extreme weather events and climate change, market volatility, and political instability. Climate change affects the availability, access, use, and stability of food supply, as well as work, capital, and the choice of crops intended for food production [15, 16]. Studies show that the average global yields of rice, maize, and wheat are projected to decrease between 3 and 10% by a heating degree above historical levels [17]. Climate change poses a major challenge to food security, which is very vulnerable to changes in climate patterns [18]. Food production in developing countries is negatively affected by climate change, especially in countries that are already vulnerable to climate effects (drought or floods) and that have low yields and high indices of hunger and poverty. Vulnerable populations are the most affected by climate change, and threats to these groups can interfere with their food, nutrition, access to water, and income, exacerbating conflicts [19].

There is a need for new approaches to ensure food and nutrition security. These should be sustainable, resilient, and practical solutions. Given this context, biodiversity, especially neglected crops, is essential to addressing the impacts of climate change [20]. They are nutrient-rich crops that demonstrate the potential for adaptation and production in several continents, reinforcing the importance of diversity in the face of climate change and which must be the priority in future research [21]. In addition, small farmers need support to improve agricultural production, diversifying crops such as the use of neglected or underutilized species. This chapter reinforces and calls attention for the use of neglected crops to face climate changes and as potential sources of vitamin A while combating malnutrition and vitamin A deficiency.

1.3 Neglected crops: basic concept and nutritional potential

Staple foods are facing major challenges and will continue in the near future due to climate changes. In this regard, diversification of crops including neglected crops is important if the world needs to achieve the goal of food security. Neglected

crops are crop species traditionally used with local communities with great potential to contribute with food security and vitamin A deficiency issues [22].

According to the Food and Agriculture Organization (FAO) [23], neglected, underutilized, or orphan crops (NUCs) are plant species which for social, agronomic, or biological reasons have lost their importance over the 500 years. The original function of some neglected crops or their potential uses have been marginalized along the time; others have practically been forgotten. They constitute plant species which played a fundamental role in the agriculture and food supply of indigenous peoples and local communities. Their neglect was in many cases the result of the deliberate suppression of self-sufficient ways of life which characterized traditional cultures [23].

Currently, agriculture needs to explore nonconventional pathways such as underutilized crops (NUCs) as possible future crops. This is premised on reports that NUCs are adapted to a range of agroecologies and may be nutrient dense and offer better prospects in marginal production areas. Neglected crops are drought and heat stress tolerant, resistant to pests and diseases, and adapted to semiarid and arid environments and could be useful in diversifying diets and addressing micronutrient deficiencies in poor rural communities [24, 25].

Neglected crops are integral part of local culture, are present in traditional food preparations, and are the focus of current trends to revive culinary traditions; they have comparative advantages over staple crops because they have been selected to stressful conditions and can be cultivated using low input and biological techniques. These crops are generally ignored by policymakers and excluded from research and development agendas, and if we need to survive future climate changes and food production challenge, special efforts must be needed to improve their cultivation, management techniques, and harvesting and post-harvesting processes, and we need to better research the nutritional status and create secure policies and legal frameworks to regulate their use [22]. Neglected crops are represented by ecotypes or landraces which require some genetic improvement. Due to dependence of modern crops and industrialization, unfortunately, neglected crops are suffering rapid erosion of traditional knowledge [24]. These crops are represented in ex situ gene banks, but efforts are needed to rescue and conserve the genetic diversity of these underutilized species. Without better characterization and evaluation of these species, they will remain poorly understood [25]. A combination of ex situ with in situ (on-farm) conservation efforts must be implemented [24]. Another approach to promote the use of neglected crops is conservation through use. Neglected crops are also characterized by having fragile seed supply systems, and efforts need to be made to provide planting material to farmers in order to make the cultivation of underutilized species more feasible and sustainable over time [25].

Nowadays, only four main crops, i.e., maize, potato, rice, and wheat, supply more than 60% of the human's energy intake. The projected increase in the world's population has driven up the need for food and increased food demand [26]. It has become clear that the lack of diversity due to concentrating on fewer crops can have negative consequences for the human diet, which may cause malnutrition and diet-related diseases [26]. Since neglected crops are also rich in nutrients and health-promoting compounds with preventive effects against malnutrition and some chronic diseases and can survive in marginal or stressful conditions, they have a great potential in improving nutrition in local communities [26]. Diversifying the food chain by including these neglected species could be an effective tool to improve overall human nutrition and health [26].

In Africa and Mozambique to address vitamin A deficiency and malnutrition, focus must be concentrated not only on staple foods but also on diet-relevant neglected crops [27]. Currently, sub-Saharan Africa accounts for 9% of the global

population and is characterized by high prevalence of food and nutrition insecurity, which is partly due to a lack of crop diversification [27]. Apart from playing an important role in the African diet, neglected crops can also contribute to the local economy and are part of traditional medicine as leaves of certain crops are used both as a food and a medical source. Most of the plants used grow indigenously in the wild or are cultivated on a very small scale [27]. Hence, production, as well as availability, is limited [27].

1.4 *Vigna unguiculata* L. (Walp): production, nutritional composition, and potential source of vitamin A

Vigna unguiculata L. Walp is an annual herbaceous plant species of the leguminous family (Fabaceae) and subfamily Papilionoideae (Faboideae). Historically, the origin and domestication of the cowpea occurred in Africa, near Ethiopia, and is now cultivated in more than 100 countries [28]. It is cultivated mainly by small farmers in many parts of the world. Cowpea is rich in nutrients and is well adapted to different edaphoclimatic regions and of vital importance for the livelihood of millions of people in Central, West, and South Africa, mainly due to its tolerance to heat and drought [28]. All these factors are particularly important because cowpea is more consumed by nutritionally vulnerable populations. In addition, it is of growing interest due to its vegetable proteins [28].

The latest productive data of updated cowpea provided by the United Nations Food and Agriculture Organization (FAO) refer to the 2016 harvest. The area cultivated worldwide was 12.3 million hectares and the production of 6.9 million tons [29]. Among all countries, the largest producers are Nigeria (3 million tons), Niger (1.9 million tons), and Burkina Faso (603 mil tons) [29]. Cowpea presents countless forms of use, being used mainly as dry grains, pods, and green grains in natura for human consumption and may vary in size, color, shape, and texture, as well as in its nutritional composition. Also, cowpea is a rich source of bioactive compounds, such as peptides, resistant starch, dietary fiber, phytochemicals, and antioxidants, as well as certain types of vitamins and minerals, important for health. The string bean contains a complex and unique protein profile, including globulins (8.2%), albumins (11.9%), glutelins (14.4–15.6%), and prolamins (2.3–5.0%) [30–32]. The protein is composed mainly of the globulin fraction (50–70%). The quality and singularity of the protein depend on the composition of amino acids; according to Gupta et al. [33], the maximum and minimum total contents of essential amino acids were 33.43 and 27.50 g/100 g of protein, respectively.

Phenolic compounds and their mechanisms of action, both in raw cowpea and cooked, have been reported in many studies. Adjei-Fremah et al. [34] observed phenolic compounds, condensed tannins, and antioxidant capacity of the seed extracts of several cowpea varieties. The folic acid and ferulic acid have been claimed to be the most abundant phenolic acids in cowpea seed, while in the seed shell, the main phenolic acid is the gallic acid, followed by the protocatechuic, P-hydroxybenzoic, and coumaric acids [35].

The cowpea contains a high amount of resistant starch and dietary fiber and can be considered a food of low glycemic index [36]. Cowpea resistant starch has been thoroughly studied by Eashwarage et al. [37] and Chen et al. [38], while total dietary fiber in cowpea was reported by Kirse and Karklina [39], Eashwarage et al. [37], and Khan et al. [40]. According to Gonçalves et al. [41], cowpea flour can be used as a supplement to provide additional vitamin A activity and zinc in cereal-based weaning foods. Carotenoids such as lutein, β-carotene, γ-carotene, and cryptoxanthin, which are precursor substances of vitamin A, are also present in the grains, pods, and leaves of cowpea [42–45].

The main limiting factors of cowpea consumption include low digestibility, deficiency of amino acids containing sulfur, and the presence of antinutritional factors. The presence of some types of phenolic compounds, such as proanthocyanidins [46], phytic acid [47], tannins [48], haemagglutinins [49], cyanogenic glycosides, oxalic acid [20], dihydroxyphenylalanine, and saponins, may be nutritionally disadvantageous for humans. In addition, enzymatic inhibitors of cowpea, such as protease inhibitors, are also considered antinutritional compounds [50]. On the other hand, α-amylase and β-glucosidase inhibitors in cowpea may be extremely beneficial for human health, as they may reduce the rate of glucose release during digestion. Ojwang et al. [43] reported that the proanthocyanidin content of cowpea varies from 2.2 to 6.3 mg/g, which is similar to other legumes, such as peas, lentils, and combs [43]. However, proper processing methods can be used to destroy these antinutritional factors and improve the levels of bioavailability, especially when used as food for infants and children [28].

In addition to phenolic compounds, proteins, peptides, and protease inhibitors, the string bean presents other functional properties responsible for the improvement in the lipid profile, control of blood glucose level, and arterial pressure and also for helping in cancer prevention. Moreover, being more than an individual compound, reports indicate that cowpea exerts positive effects on disease prevention, indicating a probability of synergistic interactions among the compounds present in the species. However, in vitro data on anticancer and anti-inflammatory properties of cowpea are inconclusive and require further studies [28].

Considering the characteristics of the species as to the nutritional aspects, a product with high social and economic value is observed. Thus, cowpea cultivation can be considered an opportunity for producers, although there are still countless challenges to be overcome in terms of research and investments in the transfer of technologies to increase the productive potential of species.

1.5 *Manihot esculenta* Crantz: production, nutritional composition, and potential source of vitamin A

Cassava belongs to the genus *Manihot* that comprises 98 species and is one of the widely cultivated species. It is a dicotyledonous, perennial, arbustive that can reach up to 4 m of height belonging to the family Euphorbiaceae [51]. Cassava is considered one of the most important cultures in the tropics and subtropics around the world being widely cultivated by possessing tuberous roots. It is the fifth most important basic culture globally after maize, rice, wheat, and potato in relation to production and caloric intake [52, 53]. Currently, cassava is produced in 103 countries, with a total global production of 270 million tons, in approximately 25 million hectares worldwide. Thirty countries located in Africa, Latin America, and Asia are considered large global cassava producers, together producing more than 50 million tons annually [54]. Nigeria, Thailand, Indonesia, Brazil, and Congo dominate 60% of the world's cassava production [55].

Estimates suggest that cassava is a staple food for 800 million people living in South America, the Caribbean, Africa, and Asia [56]. It is cultivated by poor farmers, many of them women, often in marginal lands. The main product of the cassava plant is its amylaceous roots; however, the leaves are consumed in at least 60% of the countries of sub-Saharan Africa, providing an important source of proteins, vitamins, and micronutrients [57]. The nutritional content of cassava can vary depending on the part of the plant that is consumed (leaves or root), variety, age of the plant, place of cultivation, and environmental conditions. The root of cassava is considered a great source of energy due to a large amount of carbohydrates present. The carbohydrate content varies from 32 to 35% of its fresh mass and from

80 to 90% of its dry mass [58]. Eighty percent of the carbohydrates produced is starch, mainly in the form of amylopectin (83%) and amylose (17%) [59]. The fiber content is approximately 1.5%, and this value may vary according to the variety and stage of root development. The lipid (0.1–0.3% root dry mass) and protein (1–3% root dry mass) contents are considerably lower than those shown by other crops, such as corn and sorghum [60].

The essential amino acids lysine, cysteine, leucine, methionine, threonine, and tryptophan are present in low amounts. On the other hand, arginine, aspartic acid, and glutamic acid are the amino acids that appear in larger amounts in the cassava roots [61]. The calcium, iron, potassium, magnesium, copper, zinc, and manganese contents are compared to those found in many legumes. The calcium content is relatively high compared to other basic crops and may reach approximately 360 mg/100 g of root [60].

In the regions where cassava is cultivated, the roots are mainly of white pulp possessing low levels of vitamins including vitamin A [62]. The vitamin C content is relatively high, ranging from 15 to 45 mg/100 g of root [61]. On the other hand, cassava varieties with yellow pulp root have a higher content of β-carotene; this carotenoid is important in human food because it is a precursor of vitamin A [53]. Breeding programs are focused on the development of biofortified varieties with the presence of carotenoids as β-carotene in the roots of yellow coloration [63]. The new varieties of root cassava with yellow pulp have the potential to provide up to 25% of the vitamin A needed daily by children and women [64]. The cassava roots of biofortified yellow pulp have starch and flour with physicochemical characteristics and functional properties similar to those found in the roots of cassava of white pulp; however, the values of provitamin A are higher [53]. Cassava is the staple foods of millions of people in the world; the consumption of biofortified cassava with β-carotene can help fight vitamin A deficiency which is a serious public health problem in many parts of the world [65]. This plant plays a key role in food security and income generation.

1.6 Case study 1: *Tacca leontopetaloides* as source of vitamin A in Mozambique

Hunger and malnutrition have serious ramification in humans, and an example is the increase of dietary-related diseases globally. *Tacca leontopetaloides* tubers are known to be a staple food in Mozambique, mainly in Inhassunge District, Zambézia Province (center region of Mozambique). Previous research has demonstrated that most rural dwellers depend largely on *Tacca* to meet up with shortages in nutrients like minerals, proteins, lipids, carbohydrates, and vitamins [66, 67]. Despite the nutritional content, it is also known to contain high levels of antinutritional factors which could be toxic to the body. Therefore, knowledge of the nutritional status and toxic levels is imperative in order to encourage its cultivation and consumption. Regarding its vitamin A contents, there is a lack of information in the literature. The principal amino acids present in the protein are arginine, glutamic and aspartic acids, leucine, lysine, and valine. In the study of Bosha [68], the presence of reducing sugars, tannins, flavonoids, steroids, glycosides, and hydrogen cyanide was observed. The presence of potassium, sodium, magnesium, selenium, manganese, vanadium, and some heavy metals like lead, aluminum, arsenic, and mercury was also reported including vitamins A, B1, B2, B3, C, and E. The proximate analysis showed moisture, ash, fats, fiber, crude protein, and carbohydrates.

According to USDA National Nutrient Database for Standard Reference Release 1 Basic Report November 21 [69], arrowroot contains vitamins in (mg/100 g) such as vitamin C (0.143), riboflavin (0.059), niacin (1.693), vitamin B-6 (0.266), folate

(338 μg/100 g), and vitamin A—RAE (1 μg/100 g). In a study of Upkabi et al. [67], *Tacca* presented dry matter in average of 29%, starch content in average of 26%, ascorbic acid and proteins (1.1%), ash (2.7%), 0.5% fiber, 0.1% fat, 95% total carbohydrates, 10% of starch moisture content, starch with water absorbing content around 5.6 g/g, and oil absorbing capacity around 7%.

The results of Vu [70] indicated that high total phenolic content and total flavonoid contents were presented in leaves of *Tacca*. The chemical compositions of *Tacca* flour showed 0.66% total of nitrogen, 0.91% lipid, 0.05% ash, and 85.7% starch content on dried weight. The extract of peels showed to possess potential antimicrobial activity against different microorganisms. *Tacca* is a promising crop for food and pharmaceutical excipient industries. Jagtap and Satpute [71] while studying the flavonoid fingerprinting of *Tacca* revealed the presence of diosmin, rutin, epigenin, saponin, hesperidin, phenolic acid, chlorogenic acid, quercetin, and isoquercetin with strong medicinal value. Despite the antinutrional content, its starch can also be explored in the pharmaceutical industry.

1.7 Case study 2: cassava as source of vitamin A in Mozambique

In sub-Saharan Africa, the major cassava-producing countries include Nigeria (53 million mt in 2013), Democratic Republic of Congo (16 million mt), Angola (16.4 million mt), Ghana (15.9 million mt), and Mozambique (10 million mt [72]). Mozambique has a world share of 3.3% [73]. The Mozambican diet is mainly composed of cassava—a staple with low protein content. With the exception of green leafy vegetables which often accompany the staples, the supply of micronutrient-rich foods (other vegetables, fruit, and foods of animal origin) is dramatically low [74]. The consequences of malnutrition should be a significant concern for policymakers in Mozambique where chronic malnutrition (stunting or low height for age) affects more than 2 million children under 5 years (43%—[75]). Food insecurity together with poor diet quality is among the main problems in Mozambique, resulting in insufficient micronutrient intake. In the rural areas of the northern part of the country, households consume mostly maize and green leafy vegetables consumed as infrequently as 2–3 days per week. Due to poor diet, there are high levels of micronutrient deficiencies, such as anemia, which affects 69% of children under 5 years and 54% of women of reproductive age [75]. Efforts are being made; for example, in 2017, Mozambique created the National Council for Nutrition and Food Security (CONSAN) with the aim of having a high-level, institutionalized coordination structure for nutrition and food security to support the reduction of food insecurity and chronic malnutrition and to promote the effective implementation of nutrition and food security policies [75].

Poor nutrition contributes also to high rates of childhood mortality in Mozambique. Those who are nutritionally deficient are more susceptible to diseases, which further complicate the situation [76]. Forty-four percent of children in Mozambique under five are stunted due to poor diet and suffer chronic illness. Iron, iodine, and vitamin A deficiencies are among the main perpetrators at the microlevel. Deficiency in iron in Mozambique affects 75% of the children who grow anemic and are apathetic, anorexic, and energyless. Iodine deficiency has mental and physical repercussions [76]. Vitamin A deficiency weakens the body's immunity to infections by 69% of children. It also affects 11% of mothers, who find it hard to breastfeed their children because they are also undernourished [76].

Vitamin A deficiency is a major challenge of public health in Mozambique, and on the other hand, yellow cassava or provitamin A-rich cassava has great potential to alleviate vitamin A deficiency and can be used as a complementary approach to

other interventions [77]. Considering the high prevalence of vitamin A deficiency, supplementation of this nutrient by neglected crops even in small quantities is likely to result in major public health gains [77].

Many cassava varieties cultivated in Africa have white roots with virtually no provitamin A. New developed yellow cultivars are rich of carotenoids and have provitamin A activity. These yellow varieties have been crossbred with African cassava varieties, by using conventional techniques, to increase provitamin A content [77]. Yellow cassava contains provitamin A carotenoids primarily as beta-carotene, which humans absorb and convert to retinol (vitamin A). Cassava is a nutty flavored, starch tuber in the spurge family (Euphorbiaceae) of plants. Cassava is one of the highest value calorie food for any tropical starch-rich tubers and roots. It has been argued that 100 g root provides 160 calories. Their calorie value mainly comes from sucrose which accounts for more than 69% of total sugars and 16–17% amylose, another major source of complex carbohydrates. Cassava has more protein than other tropical root tubers and is free of gluten. The leaves are also a good source of dietary proteins and vitamin K as they are consumed in Africa, Asia, and Latin America. Cassava presents also B-complex group of vitamins such as folates, thiamin, pyridoxine (vitamin B-6), riboflavin, and pantothenic acid and minerals such as zinc, magnesium, copper, iron, manganese, and potassium [78]. According to an in-depth analysis of nutrients, cassava root, raw, has the following nutrients per 100 g [75]: energy value (160 Kcal), carbohydrates (38.06 g), protein (1.36 g), fat (0.28 g), and fibers (1.8 g). According to this in-depth analysis, cassava presents also vitamins such as folates; niacin; pyridoxine; riboflavin; thiamin; vitamins A, C, E, and K; electrolytes such as sodium and potassium; and minerals (calcium, iron, magnesium, manganese, phosphorus, and zinc).

Globally, between 250,000 and 500,000 vitamin A-deficient children become blind every year; half of them are reported to die within 12 months of losing their sight. Rather than fortifying the cassava after it is grown, the cassava naturally grows with high levels of vitamin A [79]. Annually, 150 000 children die of vitamin A deficiency because it makes them more susceptible to infections [80]. A promotion of neglected crops such as yellow cassava in Mozambican environment can help reduce the problem of vitamin A deficiency.

1.8 Case study 3: *Vigna unguiculata* as source of vitamin A in Mozambique

Mozambique lies along the southeastern coast of Africa with an extensive coastline of 2470 km and an area of 801,590 km^2. It has about 36 million hectares of arable land, suitable for agriculture. At present, approximately 3.9 million hectares, which make about 10% of the arable land, are under cultivation with 97% cultivated by smallholder farmers [81, 82]. Maize, cassava, and cowpeas are the most common food crops, cultivated by 79, 73, and 50% of the farmers, respectively [82]. Currently, crop diversification has been promoted through different strategies such as capacity building and practical demonstrations at school garden and community levels. Seventeen percent of total legume area (752,000) in Mozambique is destined to cowpea [83].

Cowpea is widely grown in Mozambique, and currently 63,000 million tonnes are produced annually on about 126,000 ha. Consumers in Mozambique eat the green grain (pods), dried grains, and tender leaves. Farmers generally grow spreading varieties, which are photosensitive and low grain yielding but have high biomass that serves as vegetable produce over a long period. It is due to the importance that farmers give to the leaves for their household consumption as well as for the market. Higher importance is given to leaves than the grain in different regions of the country [83–85].

According to Chiulele [85], cowpea is one the most widely grown food crops in Africa. It is estimated that more than 90% of the world cowpea grain production of 5.7 million tonnes is produced in about 10 million hectares in Africa. The crop is most important in the semiarid and hot areas of Africa where other crops may fail due to poor adaptation to heat, drought, and low soil fertility conditions. Cowpea is an important crop in Mozambique where the grain and leaves are major sources of food and family income, particularly for resource-poor households. The crop has a high protein content of about 25% in the grain (dry weight basis) and serves as a cheap source of protein, vitamins, and minerals. The crop enhances the quality of the cereal-based diets when its high lysine content is combined with the high content of methionine and cysteine of cereals. In addition, the crop improves the cropping systems and soil fertility by reducing soil erosion, suppressing the weeds, and fixing atmospheric nitrogen which contributes to increased yields of nitrogen-demanding crops [85].

According to Gerrano [86], in southern Africa, cowpea can be used as a food for humans as well as for fodder production and for weed control in forestry plantations. The seeds contain small amounts of β-carotene (precursor of vitamin A), thiamin, riboflavin, niacin, folic acid, and ascorbic acid. It is a major source of inexpensive protein in human diets with grains containing about 23–25% protein, 1.8% fat, and 60.3% carbohydrates, and it is a rich source of calcium and iron [86].

Globally, cowpea is cultivated on about 12 million hectares worldwide out of which more than 98% is located in Africa [87]. Africa contributes to 96.4% of the world production followed by 2% Asia, 1.2% Americas, and 0.4% Europe. At African level, East and West Africa together contribute 94.2% in terms of harvested area [86]. Currently, the top ten world producers are Nigeria (3,027,596 tonnes), Burkina Faso (1,987,100 tonnes), Cameroon (603,635 tonnes), Tanzania, Sudan, Kenya, Mali, and Myanmar and Mozambique (82,931 tonnes) [87].

Cowpea (*Vigna unguiculata* L. Walp) has shown several agronomic, environmental, and economic advantages, contributing to further improve the diets and incomes of peasant farming across Africa, Asia, and South America. Cowpea can grow in semiarid regions with low input requirements. Due to its high protein and low fat content, cowpea is considered to be a multipurpose crop [71, 88]. According to USDA Food Composition Database [81], cowpea is a powerful source of vitamin A. For example, leaf tips contain 36 μg/100 g of vitamin A, young pods with seeds (68 μg/100 g), mature seeds (2 μg/100 g), mature seeds boiled or cooked (1 μg/100 g), and leaf tips cooked or boiled (29 μg/100 g) of vitamin A. As reported by [88, 89], cooking and sprouting of legumes greatly influence nutritional quality by increasing bioavailability of nutrients as well as enhancing digestibility and utilization of nutrients. As demonstrated in its multipurpose functions, cowpea can be promoted and used as in food diversification to supply vitamin A and contribute fighting the malnutrition in Mozambique.

2. Conclusions and future outlook

Extensive literature available on neglected crops and in situ/in vivo experience with rural communities until today prompt us to claim that neglected crops have great potential not only as a source of vitamin A but by their ability to adapt to different environments and marginal areas. Despite the extensive works in neglected crop promotion, more real and practical actions have to be taken if we want to halt the continuous rise in vitamin A deficiency in the world and food insecurity. Scientists and policymakers are urged to recognize the potential of neglected crops and create real alternatives and technologies to promote the sustainable use of neglected crops

while contributing to fight hunger, malnutrition, and food insecurity. Cassava, cowpea, and arrowroot are crops of the future; their use must be maximized to help eradicate vitamin A problems and food insecurity in Mozambique, in Africa, and in the world.

Orphan crops have been overlooked by research, extension services, and policy-makers; governments rarely allocate resources for their promotion and development which results in small farmers planting them less often and due to reduced access to high-quality seeds, with consequences in loss of traditional knowledge. Currently, the world uses a mere 30 species to feed the world from 30,000 available. Yet these neglected and underutilized crops can help to increase the diversification of food production, adding new species to our diets that can result in a better supply of particular nutrients. Neglected and underutilized crops can also provide economic and environmental benefits as farmers can use them as part of crop rotation systems or interplant them with other crops, protecting and enhancing agro-biodiversity at the field level.

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Chapter 6

Golden Rice: To Combat Vitamin A Deficiency for Public Health

Adrian Dubock

Abstract

Vitamin A deficiency (VAD) has been recognised as a significant public health problem continuously for more than 30 years, despite current interventions. The problem is particularly severe in populations where rice is the staple food and diversity of diet is limited, as white rice contains no micronutrients. Golden Rice is a public-sector product designed as an additional intervention for VAD. There will be no charge for the nutritional trait, which has been donated by its inventors for use in public-sector rice varieties to assist the resource poor, and no limitations on what small farmers can do with the crop—saving and replanting seed, selling seed and selling grain are all possible. Because Golden Rice had to be created by introducing two new genes—one from maize and the other from a very commonly ingested soil bacterium—it has taken a long time to get from the laboratory to the field. Now it has been formally registered as safe as food, feed, or in processed form by four industrialised counties, and applications are pending in developing countries. The data are summarised here, and criticisms addressed, for a public health professional audience: is it needed, will it work, is it safe and is it economic? Adoption of Golden Rice, the next step after in-country registration, requires strategic and tactical cooperation across professions, non-governmental organisations (NGOs) and government departments often not used to working together. Public health professionals need to play a prominent role.

Keywords: Golden Rice, VAD, biofortification, β-carotene, micronutrients, estimated average requirement (EAR), recommended daily allowance (RDA), novel proteins, allergenicity, substantial equivalence, hidden hunger

1. Introduction

Research was initiated in the early 1990s which led in 2000 to the publication of the technology behind what came to be known as Golden Rice [1, 2]. From the outset, the intention was to create a source of vitamin A in the endosperm of rice, as an additional intervention for vitamin A deficiency. Philanthropy and the public sector funded the research [1]. In 2001, the inventors, Professor Ingo Potrykus and Dr. (now Professor) Peter Beyer, assigned their patents to Syngenta for commercial exploitation as part of a transaction which obliged the company to assist the inventors' humanitarian and altruistic objectives [1, 3, 4]. At the same time, the nutritional technology was donated by its inventors for use in developing countries [3, 4]. The inventors licenced a network of Asian government-owned rice research institutes to deliver their objectives. Product development was initiated through the International Rice Research Institute (IRRI) and the network. The whole network,

including IRRI, worked to a common set of goals defined in licences each institution signed with the inventors. The terms included that there would be no charge for the nutritional technology and it would only be introduced to publicly owned rice varieties. Improvements were made to the technology by Syngenta scientists [5]. In 2005 and 2006, pursuant to Syngenta's legal obligations entered into with the inventors in 2001, Syngenta provided selected transformation events of the improvements to the Golden Rice Humanitarian Board. The Humanitarian Board, via Syngenta and IRRI, made these new versions available to the Golden Rice licensee network [4, 6]. In 2004 Syngenta ceased its commercial interest in Golden Rice [7]. From 2004 development was again only funded by philanthropy and the public sector; the national budgets of Bangladesh, China, India, Indonesia, Philippines and Vietnam; as well as the US National Institutes of Health together with the Rockefeller and Bill & Melinda Gates Foundations and USAID. Golden Rice is a not-for-profit project: no individual, nor organisation involved with its development, has any financial interest in the outcome.

To date the Golden Rice project has principally engaged plant scientists. Activist opposition to Golden Rice has been led principally by non-scientists, who have been very successful in developing a narrative about Golden Rice and gmo crops which serves the activist's purpose[1] but is fundamentally inaccurate [8]. Further background to the development of Golden Rice, including the political dimensions, is detailed elsewhere [6, 9, 10].

A few years ago, at Tufts University, USA, I gave a presentation about Golden Rice. The symposium was organised by the Friedman School of Nutrition Science and Policy whose strategic aims today include 'Reduce nutrition-related health inequities' and 'Promote food systems that increase agricultural sustainability while improving human health' [11]. I was dismayed to learn that the anti-gmo and anti-Golden Rice activists' narrative was widely accepted by the participants—all of whom were studying or working in nutrition and well aware of nutritional inequities in public health.

Without adoption, that is, regular growth and consumption of Golden Rice by populations in countries where rice is the staple and VAD is problematic, Golden Rice cannot deliver any public health and welfare benefits. Adoption requires cooperative working by different specialists, including medical, nutritional and public health specialists [12]. This chapter is designed to answer anticipated questions from such specialists, to facilitate adoption of Golden Rice as an additional intervention for vitamin A deficiency.

2. Rice, diet and deficiency

Rice is the most important staple crop [6]: more than half of the global population eats it every day. In some countries, 70–80% of an individual's calorie intake is from consumption of rice [13, 14].

For storage without becoming rancid, the husk and the aleurone layer of rice have to be removed. What remains after polishing-white rice, the endosperm-contains small amounts of fat and is an excellent source of carbohydrate for energy but contains no micronutrients. Yet humans require both macronutrients (carbohydrates, proteins, fats) and micronutrients (minerals and vitamins) for a healthy life. Like all plants, rice obtains its minerals from the soil. Vitamins are synthesised by plants and/or animals, including humans.

[1] For example: https://www.heartland.org/_template-assets/documents/12-3-18%20Analysis%20of%20Greenpeace%20Business%20Model.pdf

Human health is best served by a 'balanced diet' that is varied, containing both macronutrients and micronutrients, including animal products and, as sources of provitamin A, coloured fruits and vegetables. Micronutrient sources are insufficiently represented in the diets of many people in countries where rice is the staple. The reasons often include poverty: such dietary components are expensive compared to the cost of rice [15]. In countries where rice is the staple, the average consumption is 75.20 kg/capita/year. Of those countries where micronutrient deficiencies are common, consumption increases to 150 kg/capita/year [16]. In such populations micronutrient deficiencies, like poverty itself, often occur as part of an intergenerational cycle [17].

For the past 15 years, 800 million people—more than 10% of the global population—are hungry every day. These chronically hungry individuals lack sufficient calories in their daily diet [18–20]; indeed over the past 3 years, the trend is upward [20]. Even more alarming is that 2 billion people—almost 25% of global population—are micronutrient deficient; they suffer from 'hidden hunger', with important associated morbidity and mortality [17] and related economic impact [6, 17]. **Figure 1** shows that over the 20-year period 1990–2010, the rate of reduction of chronic hunger (that is, macronutrient—carbohydrate, proteins and fats—dietary insufficiency) has been faster than the rate of reduction for hidden hunger (that is, dietary insufficiency of minerals and vitamins) [21] Dr. Matin Qaim, member of the Golden Rice Humanitarian Board and one of the authors of the paper from which **Figure 1** is extracted, has commented: 'In the future the hidden hunger [e.g. micronutrient deficiency] burden will be larger, [than chronic hunger – principally carbohydrate deficiency] unless targeted efforts to reduce micronutrient malnutrition are implemented at larger scale' (pers comm: Dr. M Qaim).

Interventions for micronutrient deficiencies include *supplementation* (with pills, syrups or capsules containing micronutrients [22]) and *fortification* (adding micronutrients to processed food). Both interventions require some level of manufacturing and/or distribution infrastructure.

With the creation of Golden Rice in 1999 [2]—the first purposefully created biofortified crop—a new term was required: 'biofortification'. The word was first used in 2002 [23] and first defined in 2004 [24]: "biofortification" is a word coined

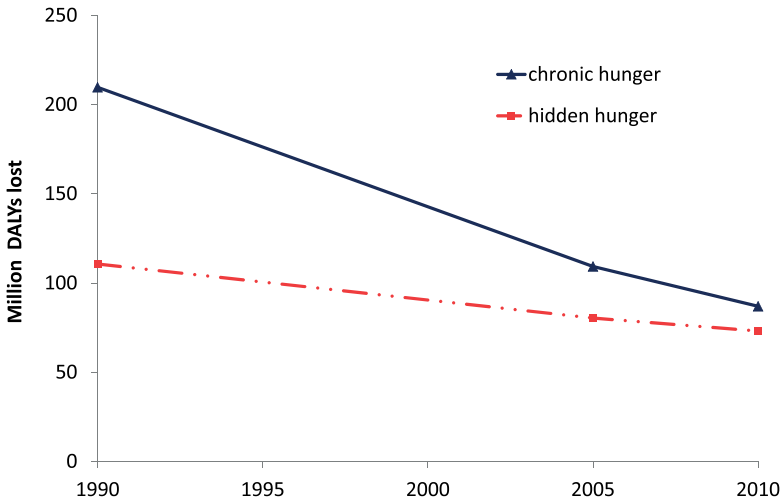

Figure 1.
*Disability-adjusted life years (DALYs) lost due to chronic hunger and hidden hunger between 1990 and 2010. Please refer to text for further explanation (***Figure 1** *here is part of* **Figure 2** *from Ref. [21]).*

to refer to increasing the bioavailable micronutrient content of food crops through genetic selection via plant breeding.' In 2003 'Harvest Plus' a not-for-profit public-sector programme started to biofortify staple crops by conventional plant breeding, to benefit the poor, and progress with biofortification through conventional plant breeding was rewarded by the World Food Prize in 2016 [25].

The intention of biofortification is to deliver public health benefits to populations which are micronutrient deficient, through consumption of the staple crop including the extra nutrition within the edible part of the crop. In this way minimal cultural change is required to food—production, processing or consumption—systems. For the most marginal members of the population, this biofortification approach overcomes the inherent access, cost and non-sustainability difficulties of supplementation and fortification. In 2017 the World Bank recommended that biofortified staple crops should be the norm rather than the exception: 'conventionally' bred biofortified crops and also genetically engineered crops—gmo crops—were both recommended with Golden Rice specifically mentioned [26].

For Golden Rice to deliver benefits, it has to be grown and consumed within target countries where VAD remains problematic despite significant progress with other interventions, notably vitamin A capsules, which have undoubtedly saved millions of lives and will save more, since they were introduced (accompanied by controversy) in the 1990s [15, 22]. And success or failure with Golden Rice will directly affect future adoption also of high zinc, high iron and high folate rice and their impact on public health for hundreds of millions of people. All these traits, introduced to the endosperm of rice, necessitated using gmo techniques [16, 27], and all cost no more than white rice to the grower or consumer. Eventually, as the end point of product development, it is planned to include all these nutritional traits together in multi-micronutrient-Golden Rice.

Adoption of Golden Rice requires public health professionals as well as agricultural and other professionals, to work together in each country [12]. Any scepticism created by the past 18 years of negative activist influence will prevent success, if not positively addressed by all involved. For billions of people, the stakes could not be higher.

3. The questions and answers

3.1 Is Golden Rice needed?

For more than a quarter of a century, vitamin A deficiency (VAD) has been recognised by the United Nations as a significant public health problem. Key milestones included the:

1990 United Nations (UN) World Summit for Children, where 50 heads of government and senior government officials committed their governments to the virtual elimination of VAD by the year 2000 [28].

1992 UN International Conference on Nutrition, which concluded that

- VAD control is the most cost-effective child health/survival strategy governments can pursue.
- All sectors of society should support the virtual elimination of VAD.
- Strategies should include promoting breast-feeding, dietary diversification, vitamin A supplementation and food fortification.
- Locally available food-based strategies are the first priority. Vitamin A capsule supplementation is only an interim measure [29].

2004 United Nations International Children's Emergency Fund (UNICEF) and the Micronutrient Initiative Report 'Vitamin and Mineral Deficiency', which concluded that 'controlling vitamin and mineral deficiency is an affordable opportunity to improve the lives of two billion people and strengthen the pulse of economic development' and that 'probably no other technology available today offers as large an opportunity to improve lives and accelerate development at such low cost' [30].

Nevertheless, vitamin A deficiency (VAD) remains a major public health problem, in more than half of all countries, especially in Africa and south-east Asia (**Figure 2**), hitting hardest young children and pregnant women [31] especially in countries where rice is the staple food. Food sources that are most valuable in terms of micronutrients—for vitamin A, animal products including milk, eggs, butter, liver and fish—are usually more expensive and 'beyond the reach of poor families' [15]. Food security staple crops such as rice are cheaper and therefore make up most of the diet.

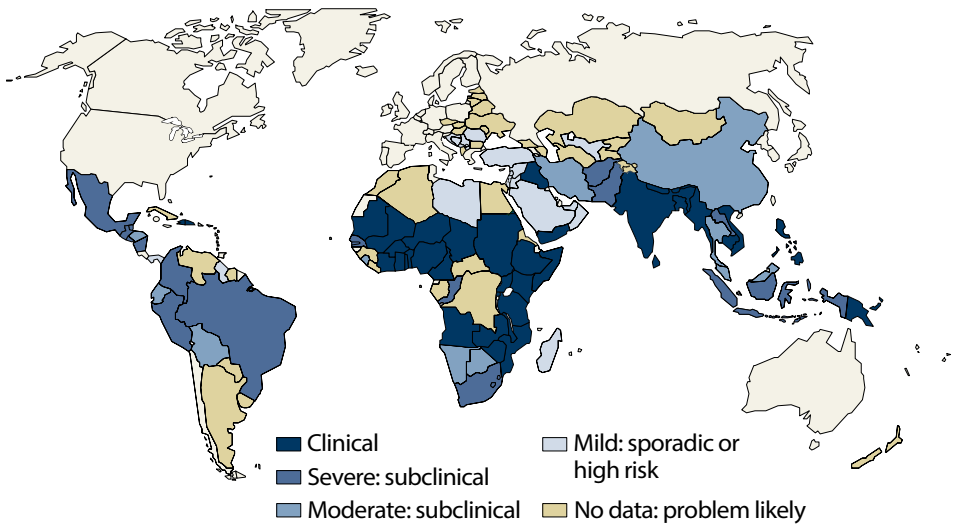

Figure 2.
Public health importance for vitamin A deficiency, by country. Source [32] (the original WHO map has been redrawn and is provided here by courtesy of Banson, a publisher).

The problem of VAD is exacerbated by the limited bioavailability of vitamin A from fruit and vegetables [33]. It has been estimated that young children between ages 1 and 3 years would need to eat eight servings of dark green leafy vegetables per day in order to meet the recommended dietary allowance ('RDA') for vitamin A. These facts have resulted in the conclusion of 'the virtual impossibility for most poor, young children to meet their vitamin A requirements through vegetable and fruit intake alone' [15].

VAD is the principal cause of irreversible blindness in children [34]. Another morbidity of VAD is related to impairment of the immune system [15]: most children and mothers who die as a result of VAD do not become blind first but die of common childhood diseases. VAD is a *nutritionally acquired immune deficiency syndrome* [15]. Increased susceptibility to disease as a result of VAD results in the majority of the millions of preventable deaths annually, mainly of children less than 5 years old (<5 years) [22]. Meta-analyses have shown that 23–34% of global mortality of children <5 years can be prevented by a universally available source of vitamin A [22, 35, 36] and up to 50% for measles sufferers [31]. As the UN regularly

Global mortality (millions)	2010[a]	2014[a]	2016/2017
Vitamin A deficiency	1.9–2.8	1.4–2.1	1.3–1.9 (2016)[b]
HIV/AIDS	1.8	1.2	0.94 (2017)[c]
Tuberculosis (TB)	1.4	1.1	1.6 (2017)[d]
Malaria	0.7	0.6	0.45 (2016)[e]

[a]*Source: [6]*

[b]*Source: 23–34%—see text—of 5.6 months <5 years children in 2016 [37]*

[c]*Source: http://www.unaids.org/en/resources/fact-sheet [Accessed: January 10, 2019]*

[d]*Source: https://www.who.int/news-room/fact-sheets/detail/tuberculosis [Accessed: January 10, 2019]*

[e]*Source: https://reliefweb.int/report/world/world-malaria-report-2017 [Accessed: January 10, 2019]*

Table 1.
Annual mortality from different public health diseases (VAD deaths exclude significant maternal mortality).

measures and publishes global all-cause child mortality, the importance of VAD mortality can be stated compared with other public health mortality causes, also reported regularly by the UN (**Table 1**).

In 2016, 26 years after the first UN commitment to *virtually eliminate VAD by the year 2000* [28], and despite existing knowledge and interventions, 1.3–1.9 million, mostly children less than 5 years old, and many mothers, died from this preventable vitamin deficiency (**Table 1**).

3.2 Will Golden Rice work?

There is not one type of Golden Rice. The 'genetic modification' part of the process used to create Golden Rice occurred only once, in about 2004 [5]. The preferred 'transformation event GR2E' was selected in late 2013 [6, 9] and subsequently introduced by 'conventional plant breeding' into more than a dozen cultivars of the *Oryza sativa indica* rice variety agronomically adapted to and preferred by the farming and rice-consuming populations of India and Asia. These cultivars can be grown directly and harvested and the polished Golden Rice sold and consumed, or the Golden Rice seed can be used by rice breeders as 'parents' to introduce the trait into any locally adapted and preferred rice variety, of which there are over 20,000.

The agronomy of Golden Rice—how it grows, its resistance to pests and diseases, its water requirements and days to maturity and plant and grain morphologies—and yield are the same as the variety into which the nutritional trait has been introduced. An avoidable human error was made in an earlier selection of 'a lead transformation event: GR2R', which led to plants in open fields falling over when subject to wind and rain, and a small yield loss of about 2% was the result [9, 38]. GR2R was dropped from development in late 2013. The current lead transformation event, GR2E, was selected in the same year. GR2E has been, and will be, registered for use and has no problems associated with it [6].

In his wonderful book *The Vitamin A Story: Lifting the Shadow of Death* [15], the author Dr. Semba wrote (p. 159): 'From a public health standpoint, for food fortification to be effective in reducing a population's micronutrient deficiency, the food to be fortified must be a dietary staple eaten daily with little or no variation. Further, the fortified food should reach the entire population. Of course, the fortification process must be economically feasible and have minimal effect on the cost of the food treated. The micronutrient with which the staple is treated must be chemically stable and undetectable by persons consuming it. Finally, to enable observation and measurement of results, location or processing and distribution must be finite and constant'. The book was published in 2012, when biofortification was known, but

not sufficiently established to have any practical history. For whatever reason, Dr. Semba does not mention biofortification, nor Golden Rice, in his book.

Nevertheless, for Golden Rice 'from a public health standpoint, for food fortification to be effective', all the characteristics listed by Dr. Semba are satisfied, except when it comes to 'undetectable by persons consuming it'. The Golden Rice colour is caused by the β-carotene content, a source of vitamin A for humans, which in Golden Rice is about 80–90% of all carotenoids [5]. It is the same β-carotene which colours mangos, papaya, squash and carrots, all of which consumers readily accept, and there is no taste associated with the β-carotene content. In Golden Rice, the intensity of the colour is proportional to the β-carotene content. The colour is obvious and cannot be ignored (**Figure 3**).

Figure 3.
Polished white and Golden Rice and (a different cultivar, after 2 months of postharvest storage) after cooking.

In 2009 MBA students at the Asian Institute of Management conducted qualitative attitudinal surveys of small farmers and consumers in four different representative island locations in the Philippines. Neither the colour nor the way it was created was considered a block to trying Golden Rice, so long as it was expected to assist their family's health and was affordable. The solid colour of Golden Rice was recognisably distinct from the rather blotchy yellow colour of poorly stored white rice, which is sometimes offered cheaply by governments to assist poor people.

From several perspectives the colour of Golden Rice is positive. Consumers have a choice about whether to select it for cooking and whether to consume it or not. Such consumer choice is denied and therefore only made by governments or plant breeders, when the biofortified trait is 'undetectable by persons consuming it' [15], as in the case of invisible biofortificants such as iron or zinc introduced into biofortified grain crops or used in fortification of processed food. The colour of Golden Rice makes the consumers' choice clear, even in populations with a variety of languages and dialects or where individuals are illiterate: each grain of Golden Rice is individually labelled, by its colour. No labelling is required on any packaging, and preference can be beneficially affected by communication of its lack of any adverse associations, and anticipated health benefits, from consumption.

Eighty percent—about 380 million tonnes—of global rice production is produced on small farms for family consumption, usually unprocessed except for polishing [38]. It is probably not stored for long, as rice is produced, usually, in two or three growth cycles annually, and storage facilities are limited. Data have shown that degradation of the β-carotene is minimal 2 months after harvest and samples of Golden Rice stored in ambient temperatures for 4.5 years remain noticeably yellow, indicating continued presence of β-carotene [39].

In early 2001, a year after the seminal paper describing the 'proof of concept' technology [2], Greenpeace made a press release: 'Genetically modified "Golden Rice" containing provitamin A will not solve the problem of malnutrition in developing countries,... Greenpeace calculations show... , that an adult would have to eat

at least 3.7 kilos of dry weight rice, i.e. around 9 kilos of cooked rice, to satisfy their daily need of vitamin A from "Golden Rice" ...' [40].

It is unclear how Greenpeace came to their conclusion. At the time, it was known that the bioavailability of carotenoids is influenced by nine different factors [41]. But no one knew how efficiently the β-carotene in Golden Rice was converted to circulating vitamin A, retinol, by human adults or children. And nutritionists agreed that animal models would not be helpful because animals metabolise carotenoids differently than humans. Research was needed to determine how efficiently the β-carotene in Golden Rice is converted to circulating retinol, in children in developing countries where rice is the staple, the population segment which suffers most from VAD.

A February 2002 grant application to the US governments National Institutes of Health (NIH) for a project, which is entitled 'Retinol Equivalents of Plant Carotenoids in Chinese Children', states 'This project is to determine the vitamin A value (equivalence) of dietary provitamin A carotenes from spinach, Golden Rice, and pure β-carotene (β-c) in oil. These experiments will be conducted in children (ages 6–8) with/without adequate vitamin A nutrition'.

On February 10, 2004, Tufts University Institutional Review Board (IRB) approved the research Protocol for 'Retinol Equivalents of Plant carotenoids in Chinese Children' and noted that 'The Zhejiang Academy of Medical Sciences [China] approval is on file'.

On March 11, 2008, the Tufts IRB reviewed and on May 10, 2008, approved the study 'Vitamin A Value of Plant Carotenoids (Spinach and Golden Rice in Children)' based on the Protocol 'Retinol equivalents of plant carotenoids in Chinese children'. Both Protocols referenced 'NIH grant proposal 1R01 DK060021'.

On March 30, 2008, with respect to 'Retinol Equivalents of Plant carotenoids in Chinese Children' and 'NIH Grant 1R01 DK060021-01': The Ethical Review Committee of Zhejiang Academy of Medical Sciences confirmed that they had 'reviewed the proposed use of human subject identified on June 27, 2003' and certified that 'the approval notice is still valid'.

Although the Chinese children research was planned in 2003, various practical setbacks in the production[2] of the deuterium-labelled Golden Rice [9] meant that the field work in China was not completed until mid-June 2008 and, due to the complexity of analysis combined with limited analytical resources, publication not until 2012.

In the meantime, similar research was approved and conducted with adult volunteers in the USA. Data confirmed that 3.8 molecules of β-carotene derived by consumption of a single meal of Golden Rice converted to one molecule of circulating retinol [42]; this 3.8:1 bioconversion compared very favourably with conversion ratios established using other plant sources [33]. When the Chinese children research were published online on August 8, 2012, the authors reported a bioconversion ratio of 2.3:1.0, later adjusted to 2.1:1.0, and neither ratio significantly different, statistically, from the 2.0:1.0 of β-carotene in oil, another treatment in the same research. A third treatment, spinach, showed a 7.5:1.0 conversion. In each case the sophisticated research design measured the efficiency of conversion of β-carotene to circulating retinol following a single meal containing the β-carotene source. The publication noted that 'In summary, the high bioconversion efficiency of Golden Rice β-carotene to vitamin A shows that this rice can be used as a source of vitamin A. Golden Rice may be as useful as a source of preformed vitamin A from vitamin A capsules, eggs or milk to overcome VAD in rice-consuming populations' [4, 6].

These results were clearly very different from Greenpeace's 2001 prediction. Instead of welcoming the excellent news of a potentially useful additional VAD intervention, Greenpeace, on August 29, 2012, issued a further press release in

[2] At Baylor College of Medicine, Children's Nutrition Research Center, Houston, USA

China from their Netherlands HQ: 'Greenpeace alarmed at US-backed GE food trial on Chinese children'..'It is incredibly disturbing to think that an American research body used Chinese children as guinea pigs for genetically engineered food,... The relevance of this study is questionable,...Nor does high conversion rate solve all the technical, environmental and ethical issues around Golden Rice' [6, 10]. Greenpeace claimed that the Chinese authorities agreed to halt the research before it started[3] but were unable to substantiate their claim to an independent journalist. The press release created hysteria in China and, 4 years after the field research had been completed, caused the parents of the subject children consternation.

Tufts University IRB carried out an investigation and concluded that there were 'no concerns related to the integrity of the study data, the accuracy of the research results or the safety of the research subjects. In fact, the study indicated that a single serving of the test product, Golden Rice, could provide greater than 50 percent of the recommended daily intake of vitamin A in these children, which could significantly improve health outcomes if adopted as a dietary regimen'. Tufts also noted that 'the research itself was found not to have been conducted in full compliance with IRB policy or federal regulations' [43].

Eventually following this Greenpeace Press release, Tang et al. (2012) was retracted by the American Society of Clinical Nutrition in 2015 for procedural reasons. The full details of this and other impediments to Golden Rice's development are given elsewhere [6, 9, 10, 43].

Separately, the Chair of the Tufts IRB, a computer scientist, in complaint to the publisher of one critical review of the case [10], wrote: 'There was no research ethics committee or IRB review and approval in effect for the study when it was conducted in 2008'. This gross error of fact, with reference to the NIH grant and related IRB authorisations quoted above, itself calls into question the professionalism or objectivity of the 2012 Tufts IRB review which led to the retraction. (The research sophistication and quality of the retracted paper can be reviewed online [44]).

Henry Miller, a physician, molecular biologist and the founding director of the US Food and Drug Administration (FDA), commented in 2015 on the retracted paper: 'A 2012 article in the nutrition literature might have been the most momentous contribution to public health worldwide since Dr. Jonas Salk's announcement of the polio vaccine. The operative phrase is might have been, because intimidation, politics and the dishonest, anti-science efforts of NGOs to impugn the research have delayed the translation of its findings to life-saving interventions for millions of children. Why do anti-genetic engineering activists want to save the whales but let children go blind and die?' [45].

The data generated by the above-mentioned research allow determination of the proportion of the estimated average requirement (EAR) the β-carotene content of Golden Rice can provide to children and adults (**Table 2**). If Golden Rice was the sole source of β-carotene in the diet, 50% of the EAR is sufficient to combat VAD [46]. Many nutritionists consider that supply of 30–40% of the EAR will be sufficient to combat VAD because the biofortified staple crop is seldom the only source of β-carotene. (The recommended daily allowance—RDA—which implies maintenance of 3-months liver stores of vitamin A, is not required to combat VAD.) The calculations (**Table 2**) use the β-carotene levels observed in different Golden Rice cultivars (e.g. RC82, BR29, IR36, IR64) of Golden Rice GR2E 2 months after harvest, when degradation has stabilised. A 6% loss of β-carotene in cooking Golden Rice, or 25% loss of β-carotene when a Golden Rice meal is parboiled first, and then reheated, has not been taken into account.

[3] http://www.greenpeace.org/eastasia/news/blog/24-children-used-as-guinea-pigs-in-geneticall/blog/41956/

Amount of β-carotene in Golden Rice µg/g	Rice consumption per day (g of dry rice before cooking)	Percentage of EAR provided
β-carotene to circulating retinol bioconversion rate: 2.1:1 (e.g. children)		**To a child[a]**
4.0	40	36%
4.0	100	91%
6.0	40	54%
6.0	100	136%
11.2	40	102%
11.2	100	254%
β-carotene to circulating retinol bioconversion rate: 3.8:1 (e.g. adults)		**To an adult**
4.0	40	20%
4.0	100	50%
6.0	40	30%
6.0	100	75%
11.2	40	56%
11.2	100	140%

[a]For 1- to 3-year-old child, 100% of EAR is 210 µg RAE/day. An EAR that does not ensure adequate stores but is enough for normal dark adaptation is set at 112 µg ~50% EAR [46]

Table 2.
The potential for Golden Rice to deliver the estimate average requirement of β-carotene, as a source of vitamin A, to 1–3-year-old children and adults.

3.3 Is Golden Rice safe?

Golden Rice differs from white rice only in that it contains β-carotene, that is, provitamin A, which the human body converts to vitamin A. Golden Rice contains no vitamin A itself. So the question about safety relates principally to β-carotene, which is anyway ubiquitous in a balanced human diet and the environment.

At the levels found in food, β-carotene is a safe source of vitamin A, and classed as 'generally recognised as safe' (GRAS), by the United States Food and Drug Administration (US FDA) [47, 48]. At these physiological doses, consumption of β-carotene over several years has no adverse health effects [49–52]. The human body only converts to vitamin A, in the form of circulating retinol, the amount of β-carotene necessary, with the rest being excreted or stored unchanged in body tissues (e.g. fat, liver, etc.). It is impossible to induce vitamin A toxicity by consuming β-carotene (pers. comm. Dr. R Russell).

In all β-carotene-containing crops, immediately after harvest the level of β-carotene reduces. For Golden Rice carotenoid degradation mechanisms have been thoroughly investigated[4] and the products of degradation quantitated. Additionally, 102 plant food items from Philippine markets, together with orange- or yellow-coloured soft drinks, as well as non-gmo field grown, in all cases, orange maize cobs and yellow cassava storage roots from Zambia, and orange-fleshed sweet potato tubers from Uganda, were analysed for the cleavage products of β-carotene, apocarotenoids [53]. The potential risks arising from 'aberrant plant carotenoid synthesis' [54] in genetically modified plants, including Golden Rice, or from non-gmo crops biofortified with pro-vitamin A, have been thoroughly investigated, the authors

[4] Golden Rice cv. Kaybonnet was investigated because it was available [5] and has high degradation potential. Kaybonnet is not a cultivar that will be used anywhere.

reporting that 'Our analysis and quantification of β-carotene derived cleavage products across biofortified and non-biofortified crop plant tissues combined with the calculation of potential exposure document no reason for concern' [53].

For the formal regulatory approvals for the use of a gmo crop in food, as animal feed or in food or feed processing, on a country by country basis, detailed data sets have to be submitted. For permission to grow a gmo crop in a country, additional data have to be generated[5] and submitted showing environmental safety.[6] The 'food, feed and processing' data package developed for Golden Rice GR2E is extensive (42 megabytes of data). It is available without cost to all Golden Rice licensee countries consistent with long-standing Golden Rice Humanitarian Board policy. Here are the key summaries of the regulatory data submission made in the Philippines:

"PROPOSAL FOR DIRECT USE AS FOOD AND FEED, OR FOR PROCESSING

Provitamin A Biofortified GR2E Rice

Description of the Regulated Article for Direct Use

Rice event GR2E (IR-ØØGR2E-5) was developed using recombinant-DNA techniques to express elevated levels of provitamin A (mainly β-carotene) in the rice endosperm, which is converted in the body to vitamin A. GR2E rice was produced by Agrobacterium tumefaciens-mediated transformation of embryogenic rice calli with plasmid pSYN12424 resulting in the introduction of the phytoene synthase (psy1) gene from Zea mays (Zmpsy1), the carotene desaturase I (crtI) gene from Pantoea ananatis,[7] and the phospho-mannose isomerase (pmi) gene from Escherichia coli as a selectable marker.

GR2E rice is intended to complement existing efforts to mitigate vitamin A deficiency by supplying consumers in societies whose diet is primarily rice-based with a portion of the estimated average requirement for vitamin A.

Summary of Potential Effects on Human and Animal Health

The safety assessment of GR2E rice evaluated information on the history of safe use of rice as a crop, the source of donor genes introduced into GR2E rice, the molecular characterisation of the modified plant, the stability of the inserted genetic elements, characterisation of new proteins produced in the modified plant and their expression levels, the potential allergenicity and potential toxicity of the newly expressed proteins, and the nutrient composition of GR2E rice compared to conventional rice.

Molecular characterisation of the introduced DNA within event GR2E confirmed the presence at a single insertion site of one copy of the inserted DNA that was stably inherited over multiple generations as a single genetic locus per Mendelian rules of inheritance. Expression of the ZmPSY1 and CRTI proteins was limited to the rice endosperm with maximum concentrations in mature grain of approximately 0.245 and 0.03 ppm, respectively. The PMI protein was expressed in all rice tissues measured and accumulated to maximum concentrations of 1.89 and 0.796 ppm in mature grain and straw, respectively.

A tiered "weight-of-evidence" approach was followed in assessing the safety of the ZmPSY1, CRTI, and PMI proteins expressed in GR2E rice. The ZmPSY1 and CRTI proteins did not display significant amino acid sequence similarity with known allergens nor were there any primary sequence structural alerts for potential toxicity based on similarity searches against a database of known and putative protein toxins. Both ZmPSY1 and CRTI were rapidly and completely digested in the presence of simulated gastric fluid containing pepsin, and the enzymatic activity of both proteins was destroyed following treatment at temperatures well below those used during cooking.

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[5] Agronomic/phenotypic data and related studies for GR2E Golden Rice can be found at: http://www.agbios.org/?page_id=767

[6] The regulations exist and have to be complied with. Nevertheless, many disagree that they are justified [6, 10, 59, 73–76].

[7] This is the same organism as *Erwinia uredovora* [2, 5]. The name was changed.

Due to the non-food source of the crtI gene, acute oral toxicity testing of CRTI protein in mice was conducted as a further assurance of safety and demonstrated a lack of any observable adverse effects at a dose of 100 mg/kg body weight, which represents at least a 115,000-fold margin of exposure relative to any realistically conceivable human dietary intake from GR2E rice.

Based on its presence in a wide range of food and feedstuffs derived from genetically engineered maize lines, and on the extensive history of prior regulatory reviews in the Philippines, additional characterisation of the PMI protein was unnecessary. Previously submitted safety studies reviewed in the context of other genetically engineered plant events are directly applicable to the safety assessment of PMI protein expressed in GR2E rice.

The genetic modification resulting in GR2E rice was only intended to increase levels of provitamin A (primarily β-carotene) in the rice endosperm. To confirm the intended effect and the lack of any meaningful unintended consequences of the genetic modification, compositional parameters were compared between GR2E rice and control, unmodified, rice. Compositional analyses were performed on samples of rice grain and straw obtained from PSB Rc82 rice containing event GR2E and near-isogenic control PSB Rc82 rice that was grown at four separate sites in the Philippines during 2015 and again in 2016. The compositional assessment included analyses for proximates, fibre, and minerals in samples of straw, and analyses for proximates, minerals, vitamins, amino acids, fatty acids, vitamins, and key anti-nutrients in grain samples. Samples of processed bran derived from GR2E and control rice were also analysed for proximates, fibre, and minerals.

Among the 69 compositional components that were tested for in samples of GR2E and control PSB Rc82 rice grain, and 10 components that were assessed in derived bran and straw samples, the only statistically significant difference observed from the multi-year combined-site analysis was for stearic (C18:0) acid, a minor fatty acid component, measured in grain samples (not including the intended difference in provitamin A levels). Except for β-carotene and related carotenoids, the compositional parameters measured in samples of GR2E rice, including stearic acid, were within or similar to the range of natural variability of those components in conventional rice varieties with a history of safe consumption. Overall, no consistent patterns emerged to suggest that biologically meaningful changes in composition or nutritive value of the grain or straw had occurred as an unexpected, unintended consequence of the genetic modification.

Collectively, the studies performed for GR2E rice have not identified potential health and safety concerns, and support the conclusion that food and/or livestock animal feed derived from provitamin A biofortified GR2E rice is as safe and nutritious as food or feed derived from conventional rice varieties."

Although it is hard to imagine that such golden grains of polished Golden Rice could be included in commercial shipments of white rice by accident, in the modern world, any such inclusion could be damaging to international trade. To prevent even such an unlikely situation, the Golden Rice regulatory data have been submitted to regulatory authorities in countries which import rice, where VAD is not a public health issue. As a result of these data submissions, Golden Rice GR2E has been confirmed as safe for use as food, in feed, and for processing by the government's regulatory authorities in Australia, Canada, New Zealand and USA. The regulatory deliberations and decisions are publicly available: Australia and New Zealand,[8] Canada[9] and the USA.[10]

[8] http://www.foodstandards.gov.au/code/applications/Pages/A1138GMriceGR2E.aspx

[9] https://www.canada.ca/en/health-canada/services/food-nutrition/genetically-modified-foods-other-novel-foods/approved-products/golden-rice-gr2e.html

[10] https://www.accessdata.fda.gov/scripts/fdcc/?set=Biocon&id=IR-00GR2E-5

Because in these industrialised countries rice forms only a tiny proportion of standard diets which already contain ample sources of vitamin A, the amounts of β-carotene in Golden Rice would have no significant additional nutritional benefit there. Comments to this effect by the US regulatory authorities were implied by anti-gmo crop opponents to be applicable also in developing countries where the dietary situation is completely different. Such implication has been rebutted by the US FDA [55]. The regulators in these industrialised countries concurred with Tufts University's statement issued after their investigation of the 'Chinese children' research: '... Golden Rice, ...could significantly improve health outcomes if adopted as a dietary regimen' [43].

Further regulatory submissions have been made, and registrations are expected, in countries where VAD is a public health problem [56]. In the Philippines the process is not yet complete; nevertheless various government departments have already expressed their support.[11]

Gmo crops have been vilified by activist groups since the 1990s. 'Frankenstein foods' were used in a letter in the *New York Times* on June 16, 1992. *The Daily Mail*, a UK newspaper, headlined the same phrase in February 1998 and subsequently and extensively used 'Frankenfoods' [57]. The 'anti-gmo groups', in various guises, have been critical of Golden Rice, a gmo crop, since 2001 [6, 10, 40].

Notwithstanding this opposition, all independent scientific institutions globally have determined, for many years, that there is no inherent danger to crop plants, or the human use of crops plants, or the environment from transferring genes from one organism to another, to create gmo crops, also known as genetically engineered (GE) crops, including transfer of genes between species which cannot sexually reproduce to transfer the genes 'naturally' [6, 58, 59].

Norero [60] provides a list of more than 240 independent science institutions from all over the globe which have commented on the safety of the techniques of genetic modification. A particularly clear reference comes from the heart of the geography politically most opposed to gmo technology, the European Commission of the European Union:

'The main conclusion to be drawn from the efforts of more than 130 research projects, covering a period of more than 25 years of research and involving more than 500 independent research groups, is that biotechnology, and in particular GMOs, are not per se more risky than, for example, conventional plant breeding technologies' [61].

At the time of writing, 141 Nobel Laureates, of about 290 living, have signed an open letter dated June 29, 2016, addressed to the leaders of Greenpeace, the United Nations and governments around the world calling for the campaign against Golden Rice specifically, and crops and foods improved through biotechnology in general, to cease 'Opposition based on emotion and dogma contradicted by data must be stopped' [8]. The letter also has the support of more than 13,000 other scientists and citizens.

3.4 Is Golden Rice economic?

Golden Rice seed and regulatory data packages are available—without cost—to public-sector rice-breeding institutions in less developed countries where rice is the staple and vitamin A deficiency endemic. Supply is subject only to national and international regulations and simple and free agreements [4]. The licences

[11] http://www.agbios.org:8085/wp-content/uploads/2018/07/Consolidated-Report-gr2e-rice_revised-1.pdf

ensure that the inventor's, Professors Potrykus and Beyer, objectives for their donated technology cannot be frustrated: only publicly owned rice varieties can be used, and the nutritional trait cannot be 'stacked' with any other gmo trait, unless the latter is also under the control of the public sector. There will be no charge to growers or consumers for the nutritional trait: Golden Rice will cost the same as white rice. Golden Rice homozygous seed, which breeds true generation to generation, will be provided by public-sector rice breeders. All small-holder family farmers—responsible for 80% of global rice production [38]—will eventually have access to it, with (except for commercial export—not a resource-poor farmer activity) no limitations on planting or replanting, harvest or sale of seed or grain.

Addressing micronutrient malnutrition, including VAD, is consistently ranked by the Copenhagen Consensus process, as the first, or at least within the top 5, most cost-effective investments with the potential to address the world's 30 most intractable problems [62–64]. Investing in alleviating malnutrition would repay $45 for each dollar invested compared with $36 from fighting malaria and $10 from combatting HIV [65].

Compared with the World Bank standard, or the full cost of provision of vitamin A capsules, a common dietary supplement intervention for VAD since the early 1990s [15, 22], the cost of Golden Rice to save each disability-adjusted life year (DALY) is expected to be very low, perhaps US$0.5 [9, 66, 67].

Costs (US$ of 2006)	Highest efficiency		Lowest efficiency	
World Bank cost-effective standard[a]	$200		$200	
Providing vitamin A capsules[a]	$134		$599	
Vitamin A fortification of food[a]	$84		$98	
Golden Rice, India @ 12:1[a]	$3.10		$19.4	
Golden Rice, Bangladesh 6:1[b] & 12:1[c]		$4.0[b]		$54.0[c]
Golden Rice, above[a,b,c] adjusted 2.1:1[d]	$0.5	$1.4	$3.4	$9.5

The earlier studies occurred before these bioconversion ratios had been elucidated
[a]*Source: [66]*
[b]*Source: [67]*
[c]*Source: [67]*
[d]*Source: From the bioconversion efficiency 2.1:1* **Table 2**

Table 3.
Relative costs of saving one disability-adjusted life year using different sources of vitamin A and, for Golden Rice, different bioconversion ratios of β-carotene to circulating vitamin A.

Economists have calculated that conservative adoption of Golden Rice would benefit the gross domestic product (GDP) of Asian countries by US$6.4 billion (value in US$ of 2005) annually through increased productivity enabled by reduced vitamin A deficiency-induced sickness, and improved eyesight, and ~US$17.4 billion (value in US$ of 2005) if Golden Rice adoption encouraged adoption of other nutritional traits in rice [68]. Recently, HarvestPlus has exceeded target levels of iron and zinc in rice, which they were unable to achieve by conventional breeding, using gmo techniques [16]. Genetic modification has also been used to introduce folate into rice endosperm [27, 69]. The delay to the introduction of Golden Rice in India has been calculated to have cost Indian GDP US$199 million per annum for the decade from 2002 [70, 71], in total about US$1.7 billion (value in US$ of 2014).

Goal #	Goal	Potential impact of biofortification
1	No poverty	Micronutrients in staple crops reduce effects
2	Zero hunger	Whole populations will be micronutrient sufficient
3	Good health	Provitamin A, Fe, Zn, Folate: less morbidity and mortality
4	Quality education	Pupils learn when adequately fed: Fe important
5	Gender equality	Biofortified staples available to whole population
7	Decent work and economic growth	Increased productivity from biofortified rice alone will add US$17.4 (in US$ of 2005) to Asian GDP

Table 4.
Biofortification and some Sustainable Development Goals 2015–2030.

Adoption of biofortified crops, including Golden Rice, will facilitate attainment of six of the most important Sustainable Development Goals 2015–2030 (**Table 4**). The standard costs used by the economists referenced in **Tables 3** and **4** [62–64, 66, 67] refer to the costs of supplementation with vitamin A capsules. As when using Golden Rice, the vitamin A source has zero cost to the grower or consumer; the cost benefit of Golden Rice will be very significantly better than using vitamin A capsules.

4. Conclusions

Vitamin A deficiency remains a huge public health problem despite existing interventions. Biofortification of staple foods is a new policy priority internationally. Golden Rice is safe. There is excellent human evidence that it will work. It is expected to be extremely cost-effective.

For successful adoption of Golden Rice as an additional intervention for vitamin A deficiency, the support of public health professionals is critical.

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Dr. Robert Russell chaired the 'panel on micronutrients' the output of which, published in 2001 [72], created the US Governments' dietary reference intakes for 14 micronutrients, including vitamin A. Also, in 2001, he joined the Golden Rice Humanitarian Board. I am grateful for his nutritional advice and instruction over the intervening years and for checking my calculations in connection with **Table 2** and the surrounding text. I have known Dr. Guangwen Tang almost as long and thank her for providing, years ago, copies of the original documents which allowed me to construct with confidence the chronology of the IRB permissions 2003 and 2008 in the USA and China, all referring to the same NIH grant. Since 2015, the project has benefited from another specialist professional, Dr. Donald MacKenzie, who, aside from managing the GR2E regulatory data package generation, compilation and submissions, has also provided the web-links, which I have listed as footnotes in the 'Safety' section of this chapter. Thank you, Don, for your critically important work.

Conflict of interest

The author declares no conflict of interest.

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