CHAPTER 1

INTRODUCTION
1.1 Vitamins

Vitamins are defined as a group of naturally occurring organic compounds whose presence in the diet ensures wellbeing and normal metabolism of man and animals. Deficiency of vitamins in the diet results in drastic effects on human health. Many of the deficiency diseases such as xerophthalmia, scurvy, beri-beri and pellagra are common all over the world particularly in the developing countries. The dietary vitamin requirements are necessary to prevent these deficiency disorders.

1.1.1 Vitamin A

Vitamin A was first recognised as an essential nutritional factor by Elmer McCollum in 1915 and isolated from fish liver oils. Vitamin A alcohol (retinol) is an unsaturated alcohol with the empirical formula C_{20}H_{30}O. The structure of vitamin A alcohol (Fig. 1.1) consists of cyclohexene ring linked to a polyunsaturated chain which terminates in an alcoholic group.

![Fig. 1.1 Structure of Retinol](image)

1.1.2 Retinoids

The term vitamin A designates a group of retinoid compounds with the biologic activity of all-trans-retinol. The five conjugated double bonds in retinol are
an easy point of attack for oxygen. Vitamin A can exist in different isomeric forms with different biological activities. Biological active retinoid compounds are vitamin A aldehyde (retinal), vitamin A acid (retinoic acid) and naturally occurring retinyl esters having distinct and divergent effects. The basic structure of retinoids consist of a β-ionone ring, a polyene side chain and a polar end group such as hydroxyl group in retinols, an aldehyde in retinals and a carboxylic group in retinoic acid. The structure of all-trans retinal, retinoic acid and 3,4-didehydroretinol is given in Fig.1.2.

![Structures of retinoids](image)

(a) All-trans Retinal  
(b) Retinoic acid  
(c) 3,4-Didehydroretinol

Vitamin A₂ (3, 4-didehydroretinol) differs from vitamin A₁ in which there is an additional double bond at carbon atoms 3 and 4. Naturally occurring vitamin A consists of predominantly the all trans isomers which have the highest vitamin A activity.
1.1.3 Sources of vitamin A

All-trans-retinol is the abundant form of vitamin A and occurs naturally in the form of fatty acid esters. Preformed vitamin A is found in egg yolks, liver, fish oil, whole milk and butter. Fruits and vegetables are the major sources of provitamin A carotenoids such as β-carotene, α-carotene and β-cryptoxanthin. Conversion of provitamin A carotenoids to retinol occurs in the human body. There are more than 600 types of carotenoids with red, yellow and orange in colour.

1.1.4 Physiological functions of vitamin A and its mechanism

Vitamin A has several physiological functions in the body. The roles of vitamin A in relation to certain physiological functions are discussed below.

Vitamin A and vision

Vitamin A has got an essential role in vision. Night blindness is considered as the earliest manifestation of Vitamin A Deficiency (VAD) in human beings. The retina of the vertebrates contain two distinct types of photoreceptor cells (a) cones that are for colour and distinct vision in bright light and (b) rods that are for visual activity in dim. If the blood does not contain sufficient vitamin A, the time required for rhodopsin synthesis may not reach optimal quantities. The following Fig 1.1 (Travis et al. 2007) shows the various steps involved in the visual processes.

Fig. 1.3 The vision cycle
The visual cycle scheme was studied over the years and different reactions and enzymes for catalyzing these reactions were identified. Recently Kiser et al. (2014) reviewed mainly focussing the involvement of retinoids in vision. Photon absorption causes the isomerization of 11-cis retinal to all-trans retinal. This transformation results in the transmission of signals to the brain. All-trans retinal is then reduced to all-trans retinol by all-trans retinol dehydrogenase (All-trans RDH) in a NADPH dependent reaction. All-trans retinol then diffuses into the retinal pigment epithelium (RPE). In RPE this alcohol is esterified with fatty acids by an enzyme Lecithin retinol acyl transferase (LRAT). The next step involves two reactions that led to 11-cis retinol catalyzed by retinal pigment epithelium-specific protein-65kDa (RPE65). Since this reaction catalyzed by RPE65 involves two steps i.e. cleavage of ester bond and isomerisation at the alkene bond so, RPE65 is referred as isomerohydrolase. 11-cis retinol is then complexed with cellular retinaldehyde binding protein (CRALBP) followed by the oxidation to 11-cis retinal by 11-cis retinol dehydrogenase (11-cis RDH) using NAD as a cofactor. The 11-cis retinal is then sent again to rod outer segment (ROS) and combines with opsin to reform rhodopsin (a photoactive visual pigment).

**Retinoids and Cancer**

Many reviewers from time to time reported that both natural and synthetic derivatives of retinol had a potential for use in the treatment and prevention of cancer (Niles 2000, Altucci and Gronemeyer 2001, Okuno et al. 2004, Goodman et al. 2008, Tang and Gudas 2011, Connolly et al. 2013). All-trans retinoic acid (ATRA) and its isomer 9-cis retinoic acid are considered as chemotherapeutic agents which inhibits the development of number of tumours. ATRA is used to treat acute
promyelocytic leukaemia. Expression of retinoic acid receptor β (RARβ) is selectively lost in premalignant oral lesions, breast cancer, oesophagus cancer and renal cancer. RARβ levels were significantly reduced in premalignant and tumour tissue but other retinoid receptors are expressed in both normal and tumour cells (Pavan et al. 2006). Restoration of RARβ expression by retinoic acid treatment suggest that RARβ acts both as a mediator of retinoic acid response and as a biological marker in chemoprevention trials. Therefore an agent which induces RARβ expression is used in the treatment of cancer. The use of retinoids alone to prevent cancer may not be optimal if the retinoid signaling expression has already occurred. The combination therapy using retinoids along with other chemopreventive agents is considered as an important strategy. Retinoic acid receptors had a decreased tendency of expression in epithelial gastric cancer and RARα is considered as an indicator of positive prognosis (Hu et al. 2012). Further Kropotova et al. (2013) suggested a sharp reduction in the expression of genes encoding the enzymes which convert retinol and retinaldehyde to retinoic acid resulting in the decrease of retinoic acid which leads to dysregulation of cell proliferation and initiate the development of gastric cancer. Many retinoids either alone or in combination with selective estrogen modifiers are used in the treatment or prevention of breast cancer. Koay et al. (2010) reported that combination therapy shows strong synergistic inhibition of proliferation and is found to be effective in both ER-positive and ER-negative human breast cancer cells. Growth inhibition of breast cancer cells by retinoic acid is associated with expression of RARβ. However, Garattini et al. (2012) proposed that approaches which are based on combinations between RARα agonists and RARγ antagonists may be more effective clinical
strategy to prevent the development of breast cancer cells. A new RXR-selective retinoid known as rexinoids has also been studied as chemopreventive agent. Preclinical study in mice (Wu et al. 2002) has demonstrated that this is able to maintain the chemopreventive effect also in ER-negative setting with minor toxicity.

**Retinoids as Antioxidant**

Vitamin A and its analogs have been found to act efficiently *in vitro* as antioxidants and radical scavengers. A study conducted by (Das 1989) using *in vitro* peroxidation system and ranked antioxidant activities as retinol > retinal > retinyl palmitate > retinoic acid. Gujardo et al. (1999) examined the effects of α-tocopherol, all-trans retinol and retinyl palmitate on the non-enzymatic lipid peroxidation induced by ascorbate-Fe$^{2+}$ of rod-outer segment membrane isolated from bovine retinal and reported all-trans retinol to be 10 times higher in inhibiting lipid peroxidation than that observed for α-tocopherol and retinyl palmitate. Palacios et al. (1996) studied the effect of vitamin A administration on *in vitro* peroxidation and the results showed that rat liver microsomal and mitochondrial membranes are protected by vitamin A when subjected to non-enzymatic lipid peroxidation. Oztay et al. (2013) reported all-trans retinoic acid to exhibit antioxidant effect in mice brain under hyperoxia induced oxidative stress.

**Vitamin A in embryonic development and during pregnancy**

After the absorption and arrival into circulation, inside the cell retinol is stored either as retinyl esters or undergo oxidative metabolism to all-trans retinaldehyde and further to retinoic acid (RA). The first step involved in the formation of retinoic acid is by the action of cystolic alcohol dehydrogenases and
microsomal retinol dehydrogenases to give all-\textit{trans} retinaldehyde. Several retinal dehydrogenases (RALDH 1, 2 and 3) act as a catalyst in the irreversible oxidation of all-\textit{trans} retinaldehyde to all-\textit{trans} retinoic acid. Metabolism of RA at C4 and C18 positions by cytochrome P450 enzymes of the CYP26 family (A1, B1 and C1) give oxidative metabolites like 4-hydroxy-RA, 18-hydroxy-RA, 4-oxo-RA. Retinol and its metabolites are lipophilic compounds which are generally associated with serum and cellular binding proteins. The main families of retinoid-binding proteins are retinol-binding protein (RBP), cellular retinol binding protein (CRBP I, II and III) and cellular retinoic acid binding protein (CRABP I and II). It is known that RBP carries the majority of retinol in circulation. RA acts as a ligand which binds to nuclear retinoid receptors protein of which there are two classes, RARs and RXRs, each of which has three subtypes (\(\alpha, \beta\) and \(\gamma\)). RARs recognize both ATRA and 9-\textit{cis} retinoic acid, while RXRs recognize only 9-\textit{cis} retinoic acid. RAR dimerizes with RXR to form a heterodimer, which then initiate gene transcription by binding to the retinoic acid response element (RARE).

Many literature data regarding the role of RA on embryonic development have been reviewed from time to time by many reviewers (Ross \textit{et al.} 2000, Zile 1998, Clagett-Dame and Knutson 2011, Rhinn and Dolle 2012, Kam \textit{et al.} 2012). The authors have pointed out that RA regulates body axis formation, neurogenesis, cardiogenesis and the development of pancreas, kidney and eye.

The roles of CRBPs and CRABPs have been studied in null mutant mice which reports that some of them plays role in embryonic development (Moise \textit{et al.} 2007). The RA metabolic enzymes (RALDHs and cytochrome P450 enzymes of the CYP26 family) show distinct differential expression pattern which are widely
expressed in the developing embryo. Many genetic experiments reported that RAR is important in mediating the actions of RA in developing embryos (Lohnes et al. 1995, Mark et al. 2006, Mark et al. 2009). These genetic studies provide clear evidence that RA acts through a RAR signaling mechanism in support of normal development of the embryo. Different approaches are used to study the essential function of vitamin A during embryonic development such as exposure to excess retinoid, retinoid receptor knock out studies, retinoid ligand knock out models.

The RALDH2 among the three dehydrogenases is essential for normal embryonic development and its knockout results in complete failure of embryo survival. CYP26 enzymes display differential expression patterns which are complementary to the RALDH expression domains. RALDHs and CYP26 enzymes participate in development of central development system by maintaining a gradient of ATRA along the axis (White and Schilling 2008) and are also reviewed in detail by Kam et al. 2012. RARα and RARγ play an important role at early stages of hindbrain patterning. RALDH2 null mutant embryos do not develop dorsal pancreatic bud (Martin et al. 2005) and CYP26A1 is reported to play a role in setting the anterior limit of the pancreas (Kinkel et al. 2009). RALDHs show a very dynamic role in the development of eye. A number of eye defects in RALDH3 and RALDH1/3 compound mutants (Matt et al. 2005, Molotkov et al. 2006) have been reported. RARα is expressed in the layers of the developing neural retina, whereas RARβ is expressed in the inner nuclear layer. RARs are expressed throughout lung development. Studies with RARβ and RARα null mutant mice show left lung agenesis and hypoplasia (Ghyselinck et al. 1997). CYP26A1 also enables more distal branching (Malpel et al. 2000). RA plays important role in kidney
development. Inactivation of both RARα and RARβ in mice embryos resulted in renal malformations (Mendelsohn et al. 1994). Lelievre et al. (1998) carried out studies with mild vitamin A deficient rodents and reported that it can result in 20% reduction in the number of nephrons.

Vitamin A is required for both male and female reproduction. Research in this field carried out by Snyder et al. (2010), Hogarth and Griswold (2010) have also been reviewed by Clagett-Dame and Knutson (2011) and suggested that retinoic acid is needed for adult male spermatogonial differentiation and the entrance into meiosis.

Clagett-Dame and DeLuca (2002) reviewed which focuses the essential role of vitamin A in female reproduction and points out that VAD effect in female is dependent upon the time when deficiency is observed and its severity. Clagett-Dame and Knutson (2011) also pointed out in the review that if there is severe VAD prior to mating, reproduction fails. In case of less severe VA deficient rats, fertilization and implantation occurs but results in embryonic death in mid gestation. Maternal vitamin A also plays a role in placental development. Deficiency of this vitamin in females can lead to either failure of reproduction prior to implantation or foetal malformation. Therefore, vitamin A status of the female both at the time of conception and throughout pregnancy is very critical.

Shah and Rajlakshmi (1984) and Shah et al. (1987) also reported that maternal VAD also affects the foetal development like preterm birth, reduced intrauterine growth and development and decreased birth weight. VAD in pregnant women are found to be at the risk of developing frequent bacterial infection in vagina (Christian et al. 1998), pre-eclampsia (Mikhail et al. 1994, Ziari et al. 1996),
preterm rupture of foetal membranes (Barrett et al. 1994) as well as risk of anaemia (Dreyfuss et al. 2000). Christian et al. (2011) reported that weekly supplementation of vitamin A to vitamin A deficient women reduces the risk of bacterial vaginosis during postpartum and pregnancy period. A study conducted in Nepal (Christian et al. 2000) reported that supplementation of vitamin A or β-carotene reduces the postpartum prevalence of loose stools and night blindness. It also reports that vitamin A supplementation reduces the illness symptoms during pregnancy.

Other functions of Vitamin A

VAD in children increases the risk of mortality and morbidity caused by measles and other infections and mortality in women during pregnancy. Many of these effects are found to be due to the immunological function of vitamin A. Healthy epithelial tissues are barriers to infection but in VAD, these cells are damaged and invasion by pathogens become easier. Natural Killer (NK) cells are important against tumour and viral infection. Zhao et al. (1994) carried out a study in rats and reported that number of circulating NK cells is reduced during VAD. Stephensen (2001) on the basis of results from in vitro experiments and animal studies have proposed that VAD induces shift in the immune response towards Th1-cell-mediated activity and VA supplementation tend to boost Th2 type response. Villamor and Fauzi (2005) reviewed the available literature on the impact of VA supplementation as a preventive and therapeutic interaction on immunity. The authors suggested that the positive effects of VA supplementation among children with severe measles and diarrhoea could be mediated by short term increase in antibody production through increased lymphocyte proliferation and due to its role in restoring and maintaining gut mucosal integrity. Studies conducted with vitamin
A deficient or retinoid receptor deficient animal models reveal the role of retinoic acid (metabolite of vitamin A) in immunity. During VAD or retinoid receptor deficiency, impaired or dysregulated T cell response have been observed (Stephensen et al. 2004, Dzhagalov et al. 2007, Hall et al. 2011a). Retinoic acid is found to be involved in multiple T cell effector response by binding to RAR (Hall et al. 2011a, Hall et al. 2011b) which shows that retinoic acid plays an important role in development of both T helper and Treg cells. Antigen presenting cells provides retinoic acid to antigen T cells thereby promoting the development of Treg cells (Iwata 2009). Retinoic acid is also reported to be critical in formation of immunoglobulin A (IgA) secreting B cells which helps in mucosal immunity (Mora et al. 2006).

Supplementation of vitamin A in children reduces the number of episodes of severe diarrhoea causing deaths and also reduces intestinal permeability in children in developing countries (Chen et al. 2003, Vieira et al. 2008). It supports the role of vitamin A in maintaining the intestinal barrier function.

Supplementing vitamin A among children and pregnant women is found to have beneficial effects on iron deficiency anaemia. It is found that the combination of vitamin A and iron is better to reduce anaemia than either iron or vitamin A alone. The mechanisms between vitamin A and iron status is not known clearly but some hypothesis is proposed which includes that vitamin A is necessary in metabolizing iron stores from the liver and increasing iron absorption (West et al. 2007, Bloem 1995). Some studies reported VAD as a challenge in the eradication of anaemia among infants and pregnant women (Tanumihardgo 2002, Van
Stuijvenberg et al. 1997). Dreyfuss et al. (2000) in a study observed that low serum retinol was most strongly associated with mild anaemia.

In a recent study conducted with rats, it was concluded that deficiency of vitamin A alters the subunit composition of collagen IV and laminin and the lung’s proteolytic potential in rats which are found to be partially reverted by retinoic acid. These alterations may contribute to impaired lung function and may result in pulmonary disease (Esteban-Pretel et al. 2013).

1.1.5 Metabolism of Vitamin A

Preformed retinol (retinyl esters) and provitamin A carotenoids are the dietary sources of vitamin A. Retinyl esters from food are hydrolyzed to retinol in the intestinal lumen. It is then trapped intracellularly by re-esterification and is incorporated in chylomicron. Re-esterified retinol is transported into the bloodstream through the thoracic duct. Retinyl esters are then hydrolysed and taken up by parenchymal liver cells. If retinol is not required immediately, it is re-esterified and retained in the stellate cells. Otherwise retinol combines with a plasma-specific transport protein known as retinol-binding protein (RBP). The RBP-retinol complex (holo-RBP) after secretion into the blood associates with another protein known as transthyretin. The transthyretin-RBP-retinol complex circulates in the blood and delivers retinol to tissues. Retinol and retinyl esters in the RBP and chylomicrons are taken up by the hepatocytes bound to cellular retinol binding protein (CRBP). The retinol may be stored as retinyl esters or as discussed earlier, it may be oxidized to retinal and to retinoic acid by retinol dehydrogenase (ROLDH) and retinal dehydrogenase (RALDH). Retinoic acid undergoes degradation by cytochrome P450 enzymes (CYP26). Biologically active forms of retinol are associated with
nuclear receptors. Transcriptions of several hundreds of genes are modulated by retinoids. In case of excess vitamin A, retinol moves to stellate cells and undergoes reesterification either by ARAT (acyl-CoA retinol acyltransferase) or LRAT (lecithin retinol acyltransferase). In case of VAD, the retinyl esters stored in the stellate cells are hydrolysed by retinyl ester hydrolase (REH) to free retinol which again moves back to hepatocytes. The following figure (Noy, 2000) shows the pathway involved in the metabolism of retinol in any animal body including human being.

Fig.1.4 Metabolism of vitamin A
1.1.6 Assessment of vitamin A status

Different indicators have been developed to diagnose different degrees of vitamin A status. This has been reviewed (Tanumihardjo, 2011) and grouped into two broad categories as follows:

1) Biological, functional and histologic indicators

2) Biochemical indicators

**Biological**

Individual with ocular histologic indicators are considered as severely vitamin A deficient. Xerophthalmia has different degrees of severity. Bitot’s spots are reversible with vitamin A treatment. On the other hand, scarring of the cornea may lead to irreversible blindness.

**Functional**

Night blindness because of VAD is found to be reversible with increase intake of vitamin A rich foods or supplements. Night blindness is used as a population indicator of vitamin A status. Impaired dark adaptation is used to evaluate intervention studies carried out with pregnant Nepali women (Haskell *et al.* 2005, Graham *et al.* 2007).

**Histologic**

Integrity of epithelial cells is compromised in VAD. A normal impression of conjunctival cells will reveal sheets of small epithelial cells and an abundance of mucin-secreting goblet cells but when there is VAD, the epithelial cells are flattened and enlarged and there is also a reduction of goblet cells. In this method a small
circle of filter paper is quickly touched to the surface of the eye followed by staining with hematoxylin and eosinophil. After counting the number of goblet cells, the eye is classified as normal or abnormal.

**Biochemical Indicators**

**Serum retinol concentrations**

Serum retinol concentrations are used extensively to identify populations at risk of VAD. In addition to analysis with HPLC, carrier protein retinol-binding protein (RBP) are also analyzed using either serum or blood spots. Measuring serum retinol concentrations has some disadvantage because serum retinol concentration reflects the body vitamin A only when the liver retinol stores are depleted. But measuring serum retinol is still used to identify populations at risk.

**Retinoyl β-glucuronide hydrolysis test and RBP: prealbumin measurements**

Retinoyl β-glucuronide (RAG) hydrolysis test (Barua et al. 1998, Goswami et al. 2003, Sarma et al. 2009) and RBP: prealbumin measurements (Rosales and Ross 1998, Rosales et al. 2002) are the other two biochemical methods that have also been widely used.

In the case of RAG hydrolysis test, RAG dose is given and the change in the concentration of retinoic acid is measured in the serum. The retinoic acid in serum was found to be greater in individual with VAD. Doses of 25, 50, and 75 mg RAG were administered to VAD children of 3–18 years old by Sarma et al. (2009) in Guwahati, Assam. The authors reported that in normal volunteers RAG remains in the body tissue. On the contrary, when RAG was administered to VAD volunteers (serum retinol<20µg/dl), RAG was hydrolysed to retinoic acid which appears in the
serum as metabolite. Barua et al. in 1998 carried out a study of conversion of RAG to retinoic acid in rats of different vitamin A status where the authors reported that the ratio of retinoic acid to RAG in vitamin A deficient rats is 1.3 to 12.5 fold higher than those of vitamin A sufficient rats on oral administration of RAG. Goswami et al. (2003) synthesized radioactive 15-[\(^{14}\)C]-retinoyl β-glucuronide and fed to rats of different vitamin A status. The blood sample was analysed after 5 to 24 hours and reported the presence of radioactivity in retinoic acid in the blood samples of VAD rats. RBP: prealbumin have been done in Zambian children with measles (Rosales and Ross 1998) and Bangladeshi surgical patients (Rosales et al. 2002) i.e. with humans having high degree of infections or inflammation.

**Breast-milk retinol concentrations**

Measurement of breast milk retinol concentration is considered as a unique indicator of vitamin A status in lactating women. Mother’s milk retinol concentration can predict the retinol concentration of breastfed infants. The children of a community are found to be at a high risk of VAD if the lactating women have marginal vitamin A status. Breast milk retinol concentration is regarded as an alternative measure of retinol in lactating women. Dancheck et al. (2005a) studied the effect of acute phase response and inflammation on breast milk retinol concentration and reported that women with or without inflammation had similar retinol levels which indicates that breast milk retinol concentration is not affected by inflammation.
Dose-response tests

A small dose of retinyl ester is given in case of the relative dose response (RDR) test and blood sample is collected at 0 and 5 hours after the dose. The principle of this method is that during the depletion of vitamin A, apo-RBP accumulates in the liver. Therefore, when retinyl ester dose is administered, the retinol binds to this accumulated RBP and is released into the serum. The RDR value is expressed in percentage and calculated as

\[ \frac{(A_5-A_0)}{A_5} \times 100 \]

Where \( A_5 \) is the serum retinol concentration at 5 h after retinyl ester is given and \( A_0 \) is the serum retinol concentration at 0 hour. The individual is likely to have deficient liver reserves, if the percentage difference is >20%. In this test 2 blood samples are to be collected from the same individual. This method was then modified by using an analog of retinol, 3,4-didehydroretinol and termed as the modified relative dose response (MRDR) test. The molar ratio of 3,4-didehydroretinol to retinol in the serum is calculated 4-6 hours after dosing which is referred as the MRDR value. Initially Tanumihardgo developed the MRDR test in rats (Tanumihardgo et al. 1987, Tanumihardgo and Olson 1988) and later on it was used in humans in the United States (Tanumihardgo et al. 1990a) and Indonesia (Tanumihardgo et al. 1990b). The low liver reserves of retinol are significant if the MRDR value ≥0.060.

\[ \text{MRDR} = \frac{\text{Serum Didehydroretinol Concentration}}{\text{Serum Retinol Concentration}} \]

In both of these tests retinol and didehydroretinol appears in the serum in significant amounts above borderline levels only when endogenous liver retinol concentration are inadequate.
**Isotope dilution assays**

In this type of analysis newly absorbed vitamin A labelled with stable isotope is mixed with body pools of vitamin A. Deuterated retinyl acetate as the tracer is mostly used in isotope test. $^{13}$C-retinyl acetate has been used as the tracer in rats. Conventional gas chromatography–mass spectrometry (GC-MS) are used in case of deuterated retinol test and gas chromatography-combustion-isotope ratio mass spectrometry (GCCIRMS) is used in $^{13}$C-retinol test.

**Liver biopsy or autopsy**

Distribution of vitamin A across the liver is not uniform (Olson *et al.* 1979). Biopsy samples are found to reflect the vitamin A status of the individual but it cannot be widely used as it is practically not possible. Furr *et al.* in 1989 carried out liver biopsies and reported that estimated liver vitamin A concentration from the isotope dilution assay agrees with the measured liver vitamin A concentration by biopsy.

**1.1.7 Carotenoids**

Carotenoids are red, yellow or orange natural pigments synthesised by plants, algae, fungi, yeasts and bacteria. These are C$_{40}$ tetraterpenoids formed from eight C$_{5}$ isoprenoid units which are joined in a head to tail linkage except at the centre where a tail to tail linkage reverses the order which results in a symmetrical molecule. All carotenoids are derived from hydrogenation, dehydrogenation, cyclization, oxidation, or combinations of these processes of the acyclic C$_{40}$H$_{56}$. Though, there are more than 600 carotenoids in the nature, but the most prevalent dietary carotenoids are $\alpha$-carotene, $\beta$-carotene, lycopene, lutein, zeaxanthin, and $\beta$-cryptoxanthin. $\alpha$-carotene, $\beta$-carotene and $\beta$-cryptoxanthin are the precursors of
vitamin A whereas lutein, zeaxanthin and lycopene cannot be converted to vitamin A. Humans cannot synthesize these carotenoids but can modify the carotenoids by enzymatic or oxygen interaction. Carotenoids containing only carbon and hydrogen (e.g. β-carotene, α-carotene, lycopene) are termed as carotene while which contains only hydroxyl groups (lutein, zeaxanthin, β-cryptoxanthin) or keto groups (canthaxanthin) are called as xanthophylls. Structures of some of the carotenoid are given in Fig.1.5.

Fig. 1.5 Structures of common carotenoids (a) β-Carotene (b) α-Carotene
(c) Lycopene (d) Lutein (e) Zeaxanthin (f) β-Cryptoxanthin
(g) Canthaxanthin
1.1.8 Functions of Carotenoids

Carotenoids play an important role in biology and medicine because of its provitamin A activity, antioxidant activity, immune function enhancement, UV skin protection. For high conjugation, flexibility and easily oxidising nature, carotenoids can either quench singlet oxygen or interact with radicals which have also been nicely mentioned recently (Fiedor and Burda 2014).

Singlet oxygen \((^1\text{O}_2)\) is formed by electronic transfer from the excited state of the sensitizer \((^3\text{SENS})\) to oxygen resulting \(^1\text{O}_2\), which may result in the detrimental effects including DNA damage and lipid peroxidation.

\[ ^3\text{SENS} + ^3\text{O}_2 \rightarrow ^3\text{SENS} + ^1\text{O}_2 \]

In the presence of carotenoid, transfer of excitation energy from \(^1\text{O}_2\) to carotenoid takes place to give ground state oxygen \(^3\text{O}_2\) and triplet state carotenoid \(^3\text{CAR}\).

\[ ^1\text{O}_2 + \text{CAR} \rightarrow ^3\text{O}_2 + ^3\text{CAR} \]

The triplet excited carotenoid \(^3\text{CAR}\) can now come to the ground state either by dissipating energy as heat or can be quenched via intersystem crossing by \(^3\text{O}_2\).

Electron transfer, addition reaction and hydrogen abstraction are the reactions involving between carotenoids and free radicals.

Oxidising radicals react with the carotenoid to give either carotenoid radical cation (\(\text{CAR}^+\)) or carotenoid radical anion (\(\text{CAR}^-\)).

\[ \text{R}^- + \text{CAR} \rightarrow \text{R}^- + \text{CAR}^+ \]
\[ \text{R}^- + \text{CAR} \rightarrow \text{R}^+ + \text{CAR}^- \]
Carotenoid adduct radicals are formed by the addition reaction of carotenoid and radical which again reacts with radical to give a non radical product.

\[ R^\cdot + \text{CAR} \rightarrow \text{RCAR}^\cdot \]

\[ \text{RCAR}^\cdot + R^\cdot \rightarrow \text{R-CAR-R} \]

Neutral carotenoid radical are also formed by the hydrogen abstraction from the carotenoid.

\[ R^\cdot + \text{CAR (H)} \rightarrow \text{RH} + \text{CAR}^\cdot \]

Carotenoids have anti-tumour effects both \textit{in vitro} and \textit{in vivo}. Tanaka and his co-workers (2012) have proposed different mechanisms by which carotenoids suppress carcinogenesis. Some of these are hormone and growth factor signalling, regulatory mechanisms of cell cycle progression, anti-oxidative effects, anti-inflammation, immune modulation, induction of cell differentiation etc. There is good number of data in literature which indicates that dietary carotenoids are cancer preventative. Donaldson (2004) in his review pointed out that overall intake of a mixed carotenoid is more protective than a high intake of a single carotenoid. Of the major dietary carotenoids, lycopene and \(\beta\)-carotene have been extensively investigated for their anti-carcinogenic activity. Lycopene is found to be very protective in prostrate cancer. Lycopene is known to be involved in anti-inflammatory activity and also modulates the signal transmission between cells and expression of genes which controls cell growth and division. Many observational and epidemiological studies demonstrated that lycopene rich foods could reduce the risk of developing prostrate cancer (Giovannucci 2005, Bommareddy \textit{et al.} 2013, Ilic 2014). Chen \textit{et al.} (2001) reported that prostrate cancer patients who consumed
dishes based on tomato sauce for three weeks (30 mg of lycopene per day) exhibited a significant decrease in oxidative DNA damage in the prostate tissues and also decreases the serum Prostate-Specific Antigen (PSA) levels. Recently Zu et al. (2014) conducted a prospective study with 49,898 males and concluded that dietary intake of lycopene was associated with reduced risk of prostrate cancer and also reduces the formation of new blood vessels that encourage the cancer growth. β-Carotene has also been linked to possible protective effect against various types of cancer. Observational and clinical studies have reported that β-carotene can decrease the incidence of cancer of the mouth and throat (pharynx), larynx and the esophagus. In a retrospective study conducted by Jung et al. (2013) demonstrated that β-carotene suppresses the risk of colorectal adenoma in combination with other carotenoids. Ge et al. (2013) conducted a meta-analysis to evaluate the association between carotenoid intake and esophageal cancer. The author suggested that β-carotene significantly reduces the risk of adenocarcinoma and α-carotene, lycopene and β-cryptoxanthin reduces the squamous cell cancer of esophagus. Prevention of lung cancer in high risk groups i.e. subjects with heavy smokers and asbestos workers have established the fact that long term consumption of β-carotene in large amounts increases the risk of lung cancer (Omenn et al. 1996). Goralczyk (2009) has explained the above observation that tobacco smoking or exposure to asbestos cause oxidative environment in the lungs which results in the abnormal decomposition of β-carotene thereby increases the risk of cancer. Many studies so far have reported the association between carotenoids and breast cancer (Toniolo et al. 2001, Tamini et al. 2005). Recently another study conducted by Wang et al. (2014) among chinese women found an inverse association between consumption of
α-carotene, β-carotene, β-cryptoxanthin and lutein and zeaxanthin and risk of breast cancer except lycopene. These associations were found to be more evident among pre-menopausal women.

Carotenoids are known to reduce the risk of cardiovascular diseases (CVD) by reducing the oxidation of LDL (low density lipoprotein) and oxidative stress. A study also reported that associations between serum carotenoids and inflammatory cytokines help to explain the protective effects of carotenoids on atherosclerosis (Xu et al. 2012). Results of many studies regarding the association between carotenoids and the risk of heart disease have been summarised by Voutilainen et al. (2006). The authors further pointed out that the high intake of fruits and vegetables reduces the risk of CVD. Ciccone et al. (2013) reviewed many literature data regarding the association between dietary carotenoid supplementation and cardiovascular risk reduction reported that though some of the studies have contradictory results but many data supports the anti-inflammatory action of carotenoids which supports its protective role in cardiac health. A recent study suggested that low β-carotene concentrations in serum may be associated with an increased risk of congestive heart failure (Karppi et al. 2013a).

A study regarding the relationship between serum lycopene, oxidative stress and bone resorption parameters in postmenopausal women was investigated. The results of the study reveal that increase in serum lycopene reduces the risk of osteoporosis (Rao et al. 2007). Sahni et al. (2009) reported inverse associations between carotenoids (except for β-cryptoxanthin and α-carotene) and loss in bone mineral density (BMD) in men and bone loss at the lumbar spine in women which supports its protective role for BMD in older men and women. Carotenoids may
protect by reducing oxidative stress as Schreck et al. in 1991 and Sen and Packer in 1996 in their study suggested that oxidative stress may increase bone resorption through activation of nuclear factor-kappa B protein which is a mediator of tumor necrosis factor-α and osteoclastogenetic activity.

Carotenoids also support human reproductive system. Gupta and Kumar in 2002 reported a significant improvement in both sperm concentration and motility when 2000 µg of lycopene, twice a day for three months was administered to a group of infertile men. This study supports the role of oral lycopene in the improvement of semen parameters. Another study demonstrates lower serum carotenoid concentrations to be positively correlated with sperm motility, morphology and concentration (Benedetti et al. 2012). A study conducted with smoking women undergoing IVF (in vitro fertilization) reported lower fertilization rate in women with lower carotenoids in plasma and follicular fluid (Palan et al. 1995).

Low levels of β-carotene, lycopene and canthaxanthin in maternal serum and placentas of pre-eclamptic women is reported which suggests that dietary antioxidant may influence pre-eclampsia (Palan et al. 2001). Sharma et al. (2003) reported that lycopene reduces the development of pre-eclampsia and intrauterine growth retardation in primigravida women.

Lutein, zeaxanthin and meso-zeaxanthin (a non-dietary carotenoid derived from lutein) are abundant in the macula of the eye and is therefore known as macular pigment. Age-related macular degeneration (AMD) is the leading cause of blindness in the developed world. Tissue damage caused by reactive oxygen intermediates and retinal damage by blue light are the causes of AMD (Beatty et al.
Lutein and zeaxanthin have also been identified in the human crystalline lens (Yeum et al. 1995). These xanthophylls are thought to protect against UV-induced oxidative damage which is observed in cataract. Data obtained from epidemiological, clinical and interventional studies have been reviewed and pointed out that these carotenoids are effective in reducing the risk of both AMD and cataract (Koushan et al. 2013, Abdel-Aal et al. 2013).

Liu et al. (2014) evaluated the relationship between blood lutein and zeaxanthin concentration and the risk of age-related cataract in which one cohort study and seven cross-sectional studies were included. The results of which suggested that high blood lutein and zeaxanthin are significantly associated with a decrease in the risk of nuclear cataract. Rubin et al. (2012) reported that risk for progressive retinopathy of prematurity (ROP) increases with low level of lutein and zeaxanthin in serum of preterm infants.

Studies also reported that in individuals with mild cognitive impairment and Alzheimer's disease have low lutein and zeaxanthin levels (Rinaldi et al. 2003). A study carried out in 44 counties in northern Georgia with old persons by Johnson et al. (2013) reported that serum lutein, zeaxanthin, and β-carotene concentrations were most consistently related to better cognition. Recently Dias et al. (2014) in a study reported HDL (High Density Lipoprotein) concentration to be significantly lower in subjects with Alzheimer's disease accompanied by cardiovascular diseases compared to those in subjects with only Alzheimer's disease and control subjects. This study also demonstrated lutein, lycopene and zeaxanthin concentrations to be significantly lower in subjects with Alzheimer's disease accompanied by cardiovascular comorbidities.
1.1.9 Absorption and metabolism of Carotenoids

The fig 1.6 shows the pathway involved in the absorption, metabolism and transport of carotenoids (Yeum and Russell 2002).

Fig. 1.6 Absorption, metabolism and transport of carotenoids

Carotenoids are released into the gastrointestinal tract after their release from the food matrix. Solubilisation into lipid globules takes place followed by transformation into smaller lipid emulsion. These lipid emulsions are then transported from the stomach to the duodenum of the small intestine. Xanthophyll esters are cleaved in the lumen of the small intestine. Some of the absorbed β-carotene and other provitamin A carotenoids are converted to vitamin A by the enzyme β-carotene-15, 15′ dioxygenase which is again re-esterified. Carotenoids and retinoids in the small intestine are packaged with triacylglycerol rich chylomicrons. These are then secreted into lymph where the chylomicrons are degraded to chylomicron remnants by lipoprotein lipase. Some carotenoids are taken up by the extrahepatic tissues. Most of the chylomicron remnants deliver carotenoids to the liver and are stored or resecreted into the bloodstream in Very Low Density Lipoproteins (VLDL).
Lipoproteins contain variable proportion of four major constituents i.e. cholesterol, triglycerides, phospholipids and apoproteins. The core of the lipoproteins contains cholesterol ester and triglycerides which are nonpolar and hydrophobic. The outer layer of the lipoprotein particle contain free cholesterol, phospholipid and apolipoprotein which are polar thereby helps the lipoprotein particle to be transported in the circulation. The lipoproteins are classified as chylomicrons, VLDL, IDL, LDL, and HDL. Each lipoprotein varies in density and the proportion and composition of lipids. The Table 1.1 depicts the lipoprotein classification showing the density, percentage of lipid and protein.

Table 1.1: Classification of lipoproteins along with the density, percentage of lipid and protein

<table>
<thead>
<tr>
<th>Lipoprotein</th>
<th>Density</th>
<th>Percentage of lipids</th>
<th>Percentage of protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chylomicrons</td>
<td>&lt;1.006</td>
<td>98</td>
<td>2</td>
</tr>
<tr>
<td>VLDL</td>
<td>0.950-1.006</td>
<td>90</td>
<td>10</td>
</tr>
<tr>
<td>IDL</td>
<td>1.006-1.019</td>
<td>88</td>
<td>12</td>
</tr>
<tr>
<td>LDL</td>
<td>1.019-1.063</td>
<td>75</td>
<td>25</td>
</tr>
<tr>
<td>HDL</td>
<td>1.063-1.210</td>
<td>45</td>
<td>55</td>
</tr>
</tbody>
</table>

The carotenoid content of individual lipoprotein groups is not uniform. The distribution of carotenoids among the different lipoprotein fractions depends largely on the physical properties of the carotenoid and also on the type of lipoprotein. Studies carried out by Deming and Erdman (1999), Zaripheh and Erdman (2002) reported that xanthophylls such as lutein, zeaxanthin and β-cryptoxanthin are surface
oriented whereas non-polar hydrocarbon carotenes like lycopene, α-carotene and β-carotene are localized in the core of lipoproteins. In many studies (Krinsky et al. 1958, Goulinet and Chapman 1997, Oshima et al. 1997), it is reported that carotenes are carried predominantly by LDL while the xanthophylls are evenly distributed between HDL and LDL and to a lesser extent VLDL. This is because xanthophylls are preferentially solubilized in the phospholipid surface and hydrocarbon carotenes in the triglyceride core of the lipoprotein.

1.1.10 Bioavailability of Carotenoids

The bioavailability of carotenoid is poor and only about 10-20% of the carotenoids in meals is absorbed into circulation. A variety of dietary and non-dietary factors affect carotenoid bioavailability. Many reviewers have published reviews that focused on various factors affecting the bioavailability of carotenoids (Castenmiller and West 1998, Parker et al. 1999, Yeum and Russell 2002, Rodriguez-Amaya 2010, Tanumihardjo et al. 2010, Fernandez-Garcia et al. 2012, Borel 2012, Abourashed 2013). The term ‘SLAMENGLHI’ summarized the main factors: Species of carotenoid, Molecular Linkage, Amount of carotenoid consumed in a meal, Matrix in which the carotenoid is incorporated, Effectors of absorption and bioconversion, Nutrient status of the individual, Genetic factors, Host related factors, Mathematical Interactions. Food matrix, original concentration of carotenoids in the food sources, methods of preparation are the most investigated factors. Apart from the effect of food composition and processing recently Goltz et al. (2013) studied the effect of meal pattern on carotenoid bioavailability and suggested that absorption of carotenoid is greatest when recommended vegetables are consumed in one meal compared to smaller meals. A study (Biehler et al. 2011)
studied the impact of different minerals on the carotenoid bioavailability from spinach. High levels of Na increases β-carotene and decreases lutein and zeaxanthin micellarization. Calcium and magnesium had an inhibitory effect. In many bioavailability studies, the processed foods mainly soups, tomato paste, carrots and spinach have been the subjects. The results of these studies reveal that mild heating enhanced the bioavailability and excessive heating decreases the bioavailability by increasing the formation of isomers or oxidized products.

1.2 Analysis of Carotenoids and Retinol

Earlier TLC and open-column chromatography played important roles in analysis of retinoids and carotenoids. These earlier methods were eventually replaced by more efficient HPLC techniques because of enhanced resolution, lower limits of detection and ease of quantitation. However TLC is still used as it involves rapid analysis with different mobile phases in less time than is required for re-equilibration of HPLC columns in HPLC. Many indirect methods such as RDR, MRDR, tracer dilution technique are more sensitive for assessing vitamin A status but these tests cannot be widely used as they are expensive. Thus, serum retinol concentration continues to be widely used to assess vitamin A status. In recent years, HPLC has become the predominant method for the analysis of retinoids and carotenoids in biological tissues. Carotenoids and retinoids absorb light because of the conjugated polyene systems in the visible or ultraviolet region (i.e. around 450nm for carotenoids and 325-380nm for retinoids). Absorbance spectroscopy is therefore important in quantitation of retinoids and carotenoids and absorbance detectors have been used for the quantitative analysis of carotenoids and retinoids in HPLC. With the increasing use of more sensitive photodiode-array detectors (PDA)
for HPLC, it is possible not only to separate retinoids and carotenoids but also to identify the compound in each peak by its UV-visible absorption spectrum. HPLC is divided as normal phase and reversed phase chromatography. Both NP-HPLC (normal phase) (adsorption) and RP-HPLC (reversed phase) (partition) have been used but RP-HPLC has become more popular. The stationary phase in the NP-HPLC is a polar phase such as silica, alumina or silica modified with polar groups such as CN or NH$_2$. The mobile phase used in this is a non polar solvent like hexane. In RP-HPLC stationary phases of various polarities are available, such as C18, C8, phenyl, and cyano derivatives but the C18 phase is the most popular. Both normal phase and reversed phase HPLC are used for the separation of carotenoids and retinoids. Vitamin A and carotenoid related compounds and its chromatographic analysis are described in details by Rahiman et al. (2013) and Furr (2004). Barua and Furr (1998a), Britton et al. (1995), Barua et al. (2000) reviewed in detail sample preparation techniques and HPLC of retinoids and carotenoids. Nuclear Magnetic Resonance (NMR) spectroscopy and infrared spectroscopy (IR) are used for structural determination of organic compounds. Proton and $^{13}$C NMR spectra are useful in characterizing cis-trans isomers and Mass Spectrometry (MS) is useful in determining the molecular weight and structure. Gas Chromatography is little used for analysis because carotenoids and retinoids are more labile to destruction and isomerizes readily at high temperature and moreover has limited volatility. However, coupling analytical techniques like LC-MS, GC-MS or HPLC with NMR and mass spectroscopy, HPLC-NMR-MS are also applied successfully in separation, identification and quantitation. Other techniques such as immunoassays,
supercritical fluid chromatography and capillary electrophoreses have also been useful.

1.3 Aims and objectives

Since the roles played by vitamin A and carotenoids are particularly critical during periods of pregnancy, infancy and early childhood so, the supply of this vitamin must be closely regulated. An extensive literature survey was carried out to know the concentration of these nutrients in pregnant women and children less than 2 years old but no such data for the North Eastern Region of India was found available to the best of our knowledge. As such, we felt necessary to carry out a study on this aspect amongst some of the randomly selected women and children residing in and around Guwahati city, Assam, India. This thesis presents the finding on the following aspects.

1) Serum retinol is determined in pregnant women in third trimester and in cord blood.

2) Retinol and carotenoids concentrations are determined in the colostrum and matured milk.

3) Blood serum carotenoids and retinol of children less than 2 years of age are studied.

4) Carotenoids i.e. lutein and β-carotene are identified and estimated in serum of adult volunteers and in the lipoprotein fractions.

The results of this type of study may be useful in planning, designing and targeting interventions. This epidemiological data will help to design supplementation strategies for vitamin A in deficient pregnant women and improve
their vitamin A status by applying some of the simple programmes like nutrition education, nutritional feeding programmes and administration of massive doses of vitamin A. Finally the advantages of breast feeding, which is beneficial for reducing the problem of vitamin A and carotenoid deficiencies in infants would be further substantiated amongst the masses.