UNIVERSITY OF SOUTHAMPTON.

Vitamin A metabolism, in healthy adults and patients with Cystic Fibrosis.

BY

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A THESIS SUBMITTED FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

INSTITUTE OF HUMAN NUTRITION
FACULTY OF MEDICINE, HEALTH AND BIOLOGICAL SCIENCES.

MARCH 2003

This thesis was submitted for examination in March 2003.

It represents the final form of the thesis as deposited in the University after examination.

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ABSTRACT

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Vitamin A plays a pivotal role in mucus production, non-specific barrier function of the respiratory and gastrointestinal tract and specific immune function. Although low circulating retinol concentrations are frequently observed in patients with CF, the clinical significance of such observations and what constitutes the most appropriate clinical management are unclear. Current guidelines simplistically assume that low retinol concentrations arise from either poor intake and / or poor availability of vitamin A. It is recommended that patients with CF receive prophylactic supplements of vitamin A (3-5times RNI). However, there is little evidence to support such guidelines and there is concern that high intakes over prolonged periods could cause harm. Safe and effective clinical management requires that we determine whether low circulating retinol concentrations are related to the clinical course and the extent to which they are associated with elevated faecal losses, dietary supply, or impaired mobilisation from the liver.

An audit of clinical notes was used in conjunction with stable isotope tracer methodology to examine the processes that affect plasma concentrations of retinol in patients with CF.

Over 40% of the adult patients with CF had plasma retinol concentrations below the lower limits observed in the reference population, despite supplementation. Patients with the lowest concentrations have the poorest clinical course and raised markers of inflammation. Low concentrations of retinol were not associated with supply of the vitamin, and modest losses of vitamin A in stool could be easily overcome by increased intake, in that patients with CF absorb more retinol than controls. Results suggest that the pool of vitamin A stored in the liver is large due to a constraint in mobilisation.

These observations support the view that there is a need to reconsider current guidelines in CF. Current practice should be reviewed in order to prevent potential harm of high intakes.

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MAIN ABBREVIATIONS USED IN THE TEXT.

ACT	α_1-antichymotrypsin
AGP	α_1-acid glycoprotein
APO-RBP	Retinol free - not bound
ARAT	Acyl-CoA:retinol acyltransferase
BAL	Bronchoalveolar lavage
CI	Confidence intervals
CIC	Conjuntival Impression Cytology
CF	Cystic Fibrosis
CFTR	Cystic Fibrosis transmembrane conductance regulator
CM	Chylomicron
CMr	Chylomicron Remnant
CRBP	Cellular Retinol Binding Protein
CRP	C- reactive protein
CV	Coefficient of variation
DRV	Dietary Reference Values
ER	Endoplasmic reticulum
GCMS	Gas chromatography mass spectrometry
GCCIRMS	Gas chromatography isotope ration mass spectrometry
GI	Gastrointestinal
HDL	High density lipoprotein
HOLO-RBP	Major form of circulating vitamin A, retinol bound to binding protein
HPLC	High performance liquid chromatography
I.U.	International Units
LCFA	Long chain fatty acids
LDL	Low density lipoprotein
LPL	Lipoprotein Lipase
LRAT	Lecithin:retinol acyltransferase
LRP	LDL receptor mediated protein
MRDR	Modified relative dose response test

NHANES	National Health and Nutrition Examination Surveys
NK	Natural Killer
PEM	Protein energy malnutrition
PERT	Pancreatic enzyme replacement therapy
PI	Pancreatic Insufficient
PS	Pancreatic Sufficient
PUFA	Poly-unsaturated fatty acid
RA	Retinoic acid
RBP	Retinol binding protein
RDI	Recommended dietary intake
RDR	Relative dose response
R.E.	Retinol Equivalents
SPE	Solid phase extraction
TAG	Tri-acyl glycerol
TB	Tuberculosis
Th-1	T-helper type-1
Th-2	T-helper type-2
TRL	Triglyceride rich lipoproteins
TTR	Transthyretin
QC	Quality control
VLDL	Very low-density lipoprotein

PUBLICATIONS

Unless otherwise indicated by acknowledgments or references to published literature, the work contained in this thesis is the work of the author. The findings reported in this thesis have been reported, in part, in the following publications.

Cawood,A.L., Murphy,J.L., Gregory,K., West,R., Carroll,M.P., Wootton,S.A. **An audit of plasma vitamin A in adults with Cystic Fibrosis**. (2001) *Journal of Cystic Fibrosis Abstracts.*

Murphy J.L., Badaloo,V.A., Cawood A.L., Chambers B., Forrester T.E., Wootton, S.A., Jackson A.A. (2001) **Gastrointestinal handling of vitamin A in severely malnourished children at admission and following treatment.** *Proc. Nut. Soc.* : **60** : 173A.

Cawood.AL, Murphy,J.L. Wootton,S.A. (2001) **Postprandial concentrations of retinyl palmitate following ingestion of oil soluble and water miscible preparations of retinyl palmitate in healthy adults.** *Proc. Nut. Soc.* : **60** : 172A.

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Cawood A.L., Murphy J.L., Carroll, M.P., Connett G., Afolabi P., Jackson J.J., Wootton S.A. (2003) **Altered availability and mobilisation of vitamin A in CF.** In press; *Journal of Cystic Fibrosis.*

Gavin J., Murphy J.L., Connett G., Carroll, M.P., Cawood A.L., Wootton S.A. (2003). **Vitamin A supplements in children with CF – are we giving too much?** In press; *Journal of Cystic Fibrosis.*

ACKNOWLEDGEMENTS.

The completion of this thesis has only been possible due to everyone involved in my last three years of studying, and my gratitude goes to all of you. In particular I would like to take this opportunity to express my thanks to a few people without whom this research would not have been possible.

Firstly I would like to thank my supervisor Dr. Steve Wootton, who has guided me along the often rocky path and to Professor Alan Jackson as Director of the Institute of Human Nutrition. I would also like to thank the CF trust and Rank Prize funds who provided funding for my research.

A big thank you to Dr Jane Murphy for her guidance, encouragement and support and to Dr John Jackson and Dr Graham Burdge for their scientific and technical expertise, and their fantastic ability to humour me and my mathematical capabilities! To Dr Mary Carroll, Dr Gary Connett and all the CF team at Southampton for their valued support and amazing ability to provide willing subjects. To all the staff at the Wellcome Trust Clinical research facility who assisted on study days, along with Dr Gary Constable who provided clinical cover and Becky for her welcomed help and fantastic timing at providing tea and chocolate after the early starts. To my fellow PhD student Ang and to Angela and Christiaan who made the months in the lab fly by. To Dr Paul Afolabi who provided a valued contribution to the laboratory analysis and validation of the stool work. To Janice for her kind support and endless words of encouragement and to all my willing subjects whom without which this research would not have bee possible.

I would also like to thank everyone who has provided support outside of the Institute of Human Nutrition. Specifically I would like to thank my family, particularly, my Mum and Dad who have always been there to provide love and support to enable me to fulfil my ambition, and to my two sisters Jemma and Hollie. To all my friends, in particular Paula who always knew when to pop round with a bottle of wine. Undoubtedly the biggest thank you goes to Dan who has long suffered my commitment to higher education and without his encouragement, love, understanding, never ending patience, and fantastic IT expertise I would never have reached the end. All those weekends working seem worthwhile now!

CHAPTER 1

INTRODUCTION.

1.1 BACKGROUND TO RESEARCH

Vitamin A deficiency has long been identified as a problem of public health proportions and has been associated with xerophthalmia and increased rates of morbidity and mortality within the developing world. The World Health Organization estimates that at least 250 million pre-school children are subclinically deficient and about 3 million have clinical xerophthalmia. For these reasons vitamin A has classically been explored in the context of a constrained dietary intake and a body of evidence has shown that improving vitamin A nutrition can reduce all-cause mortality in children. Currently in place are global health initiatives relating to the supplementation of vitamin A in instances of vitamin A deficiency. Although evidence supports the importance of vitamin A in survival, mechanisms are not clear and an understanding of how to accurately identify and correct vitamin A deficiency is still required. This will prove important information when assessing the implications of applying supplementation regimens to industrialized countries, especially with respect to the increasing evidence suggesting that mechanisms underlying vitamin A deficiency are more complex than a simple lack of the nutrient.

Vitamin A plays many different roles within the body, one of the most important is as a regulator of the human immune system. No one nutritional deficiency is more consistently synergistic with the immune system and infectious disease than that of vitamin A. Increased susceptibility to infection was one of the first features of vitamin A deficiency that was recognised, it is now widely believed that vitamin A plays an important role in many aspects of immune function [Semba, 2002]. The intimate relationship between nutrition and the immune system is of particular importance with relation to vitamin A, in that under-nutrition can impair immunity and infections in turn can lead to under-nutrition.

The relationship between frank vitamin A deficiency and types of sub-clinical deficiencies more apparent in industrialised countries are not well understood. The extent, to which knowledge gained from managing the public health issues of vitamin A deficiency applies to clinical management of individual patients within hospital settings, is not accurately known. Many different patient groups exhibit apparently low retinol concentrations, which may or may not arise from a simple dietary deficiency and may or may not contribute to the ongoing disease process. In order to manage these patients we need to understand how the disease process may alter the handling of vitamin A thereby enabling the most appropriate therapeutic approaches to be implemented. Cystic Fibrosis (CF) is one disease in which patients commonly exhibit apparently low plasma concentrations of retinol. CF is a multi organ disease in which a number of factors may be acting independently, or synergistically, to cause effects on retinol concentrations. The biochemical deficiency may not simply be a consequence of a poor nutrient intake, but may also be associated with other factors including availability of the vitamin, mobilisation of the vitamin, demand, direct loss, and the influence of recurrent infections, which are central in the pathogenesis and progression of CF. The underlying biological processes resulting in this biochemical deficiency within the functional pools are not well understood, however it may potentially affect barrier function and alter the immune response in CF, potentially worsening the clinical course and increasing morbidity and mortality. The complexity of the disease and a lack of understanding of both the mechanisms at work and the consequences of low retinol concentrations confound the ability to make informed clinical decisions with regard to therapy. An increased knowledge of the processing of micronutrients in the disease CF will enhance clinical care by informing clinical practice from evidence based science.

In conclusion, vitamin A deficiency is a large public health problem in the developing world and an interrelationship between vitamin A deficiency and immune function is widely accepted. In this context the use of supplements may be required in order to correct the nutrient deficiency. However vitamin A deficiency and large-scale supplementation regimens of this kind are distinct from the potential nutritional therapy required to correct the biochemical

deficiency of low retinol concentrations seen in clinical settings within the developed world.

The mechanisms and metabolic processes, at the whole body level, that result in low retinol concentrations require further investigation. It still remains unclear what mechanisms, other than dietary supply, mediate changes in retinol concentrations, and if biochemically low retinol concentrations are important in affecting disease progression and clinical outcomes. The present understanding is constrained by a lack of knowledge of how vitamin A is handled and transported within the body. A better understanding of the way in which vitamin A enters the body from the diet and is transported through the system (as retinol and retinyl esters) in both health and disease is required to act as a first step to begin to address questions associated with vitamin A, immune function and supplementation. This information will enable the development of a conceptual framework in which it will be possible to determine how the processing of dietary vitamin A may act to affect retinol concentrations and the possible resultant effects.

The aim of the present research was to develop an understanding of the processing of vitamin A. As a first step the metabolism of vitamin A in both healthy individuals and patients with CF will be investigated, in order to better understand the mechanisms resulting in low retinol concentrations and the clinical significance of this.

The overall issues to be investigated within this thesis include:

- The prevalence of low concentrations of retinol in patients with CF compared to healthy adults.
- Factors which are associated with low concentrations of retinol.
- The mechanisms and processes that act to affect retinol concentrations.
- The consequences to an individual of exhibiting a low retinol concentration.

1.2 THESIS OUTLINE

This thesis is divided into chapters beginning with an introduction to the field of research (chapter 1). The next section (chapter 2) reviews the current literature, concentrating on the handling of vitamin A, the disease Cystic Fibrosis, and previous work examining the processing of vitamin A, highlighting limitations of existing knowledge. Methods used during the research and validation work to justify the use of the methods are presented in chapter 3. This is followed by chapters describing the results (chapters 4-8) and finally a general discussion (chapter 9). Additional information is shown in the appendices (chapter 10).

CHAPTER 2

REVIEW OF LITERATURE.

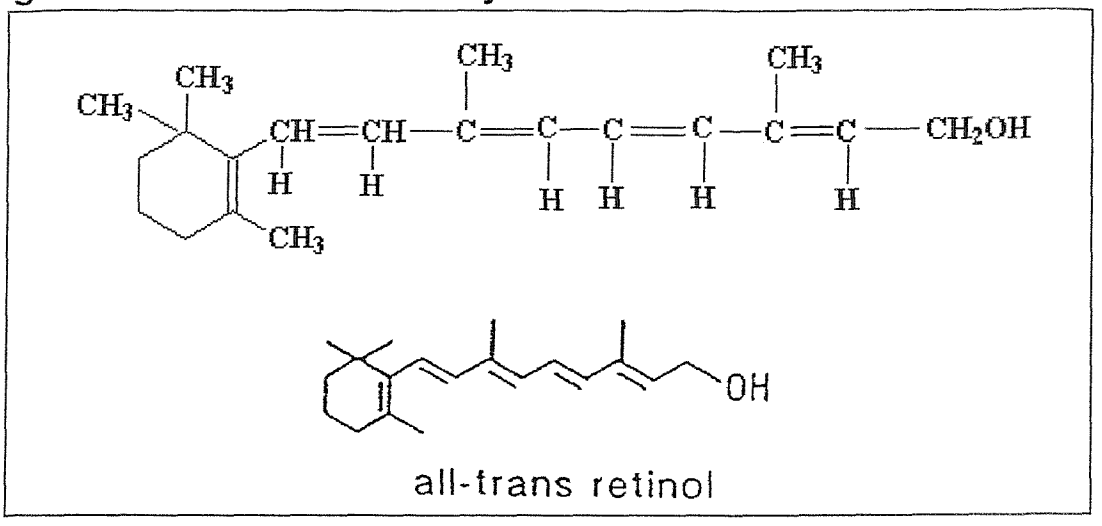
2.0 INTRODUCTION.

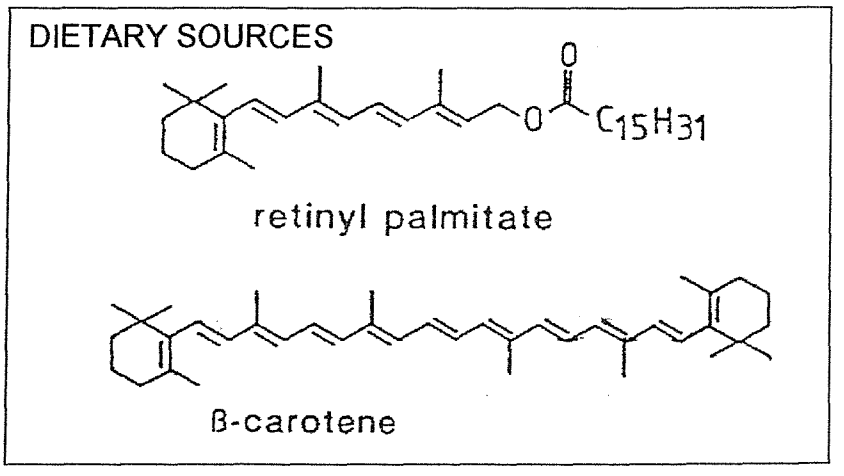
The purpose of this chapter is to introduce the area of research presented in the thesis, and to identify areas of our understanding that remain unclear. The review of literature starts by introducing the topic of vitamin A, including descriptions of both the functions and metabolic fates of the vitamin. The following section reviews the normal metabolic processing of the vitamin within the body. The next section addresses issues related to measuring vitamin A status, and the final section describes how processing of vitamin A can be altered during disease, particularly Cystic Fibrosis. The review ends with an overall summary, aims of the present research and hypotheses.

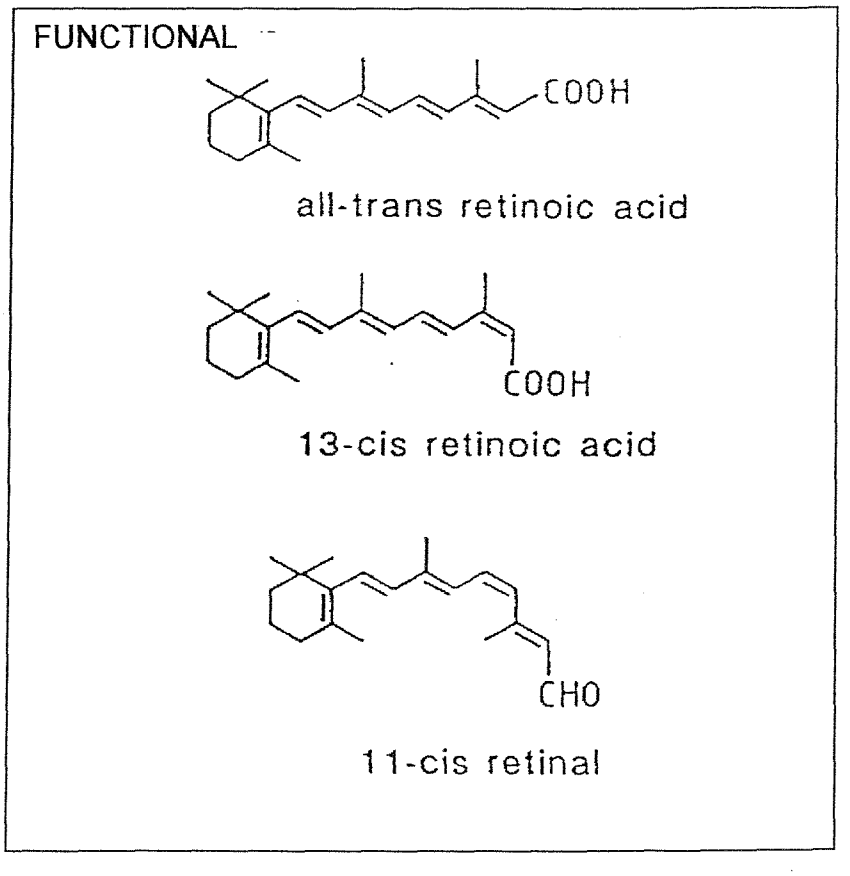
2.1 VITAMIN A - GENERAL

Vitamin A is the nutritional term that describes a family of essential, lipid-soluble dietary compounds, which include retinol, retinyl ester, retinal, retinoic acid (RA) and the provitamin A carotenoids. All retinoids are isoprenoid compounds, having in common an 11-carbon polyene chain attached to a substituted cyclohexenyl ring [Furr, 1999]. Preformed vitamin A, (retinol and retinyl esters) are the primary focus of this thesis. The molecule retinol comprises a β-ionone ring, a tetraene side chain, and a primary hydroxyl group at C-15. The system of five conjugated double bonds confers spectral properties that are used for detection, identification and quantification (see chapter 3; methods). The functions of the main retinoids will be discussed in this section and the structures are presented in figure 2.1. Retinoid metabolism occurs in many organs such as the liver, intestine, kidney, and skin [Ross, 1999]. There are three metabolic fates of retinol (figure 2.2), it can either be, 1) esterified and stored as retinyl esters, 2) converted to active metabolites such as retinoic acid or retinal, or 3) metabolised to a form (glucuronides) which are excreted from the body mainly in bile and urine [Norum & Blomhoff 1992].

Figure 2.1 Structure of the major retinoids.







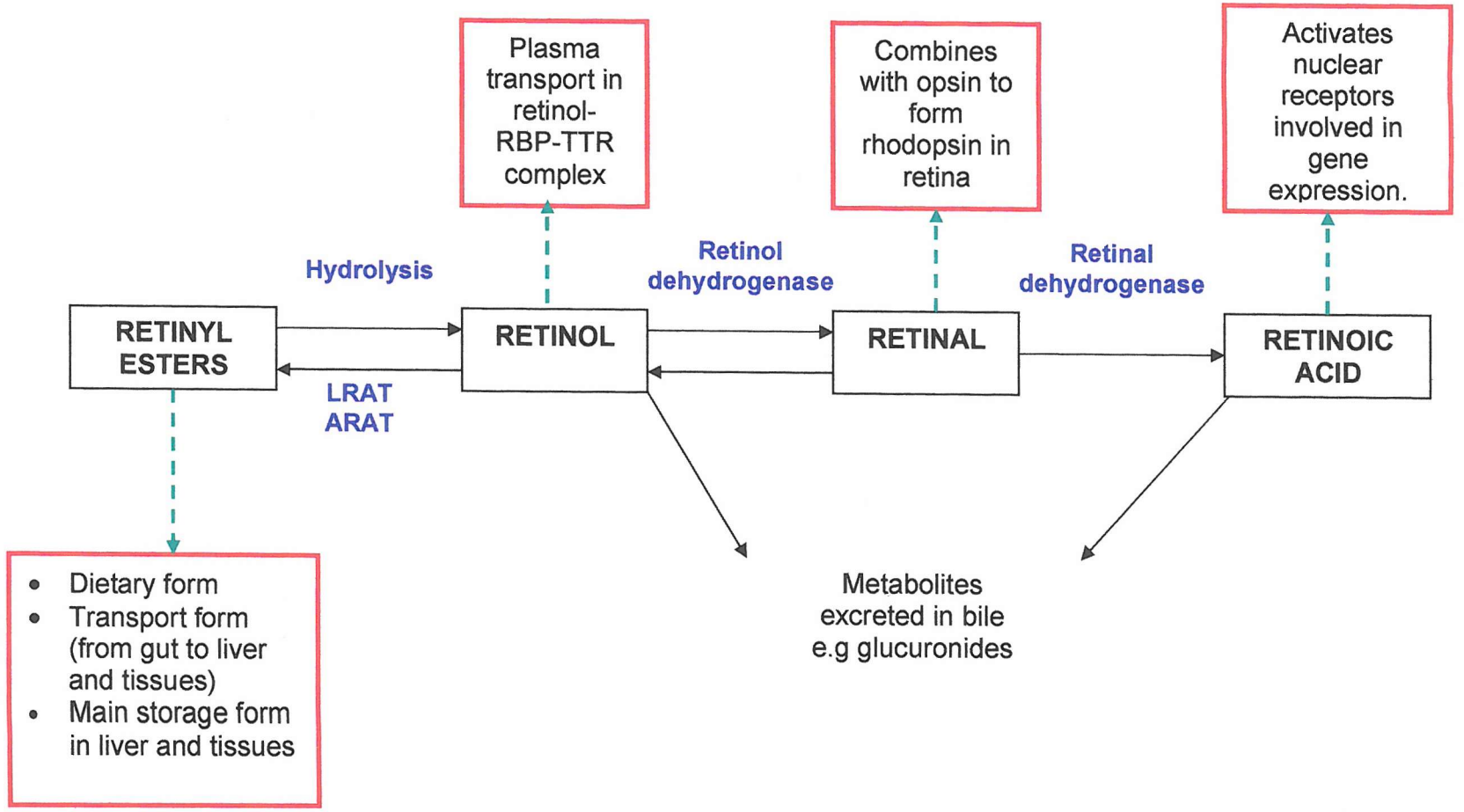


Figure 2.2: Metabolic fates of vitamin A. (Main functions of retinoids presented in red boxes)

2.1.1 FUNCTIONS OF VITAMIN A.

The main functions of vitamin A within the body (as retinol, retinal and retinoic acid), include, vision, cell differentiation, maintenance of epithelial tissue, gene expression, normal growth and development, embryogenesis, reproduction and immunity. A brief summary of these functions are presented in the following section, more in depth information that has particular relevance to the thesis is presented in other sections of the review.

Vision.

Two metabolites of vitamin A are required for vision, 11-*cis*-retinal in the retina allows the transduction of light into neural signals necessary for vision, and RA maintains the normal differentiation of cells in the conjunctival membranes, cornea and other ocular structures [Ross, 1999]. Inadequate supply of vitamin A manifests itself as visual disturbances such as impaired dark adaptation, night blindness and xerophthalmia [Sklan, 1987].

Cellular differentiation and gene expression.

Vitamin A is required for cell differentiation of tissues. Wolbach and Howe [1925], examined tissues of vitamin A deficient rats and found that the epithelia which is normally composed of columnar or cuboidal mucus secreting cells were instead, flat, dry and keratinised. The more recent discovery of two RA receptors, RAR and RXR, has clarified the molecular action of vitamin A in gene expression. The receptors are able to bind RA and through their activation can regulate the expression of a large number of genes [Ross, 1999].

Reproduction and Embryonic Development.

Vitamin A is essential for reproductive functions in both males and females. Animal models of vitamin A deficiency have shown, in males, testicular degeneration of germinal epithelium and impaired spermatogenesis, and in females, alterations in the reproductive tract, incomplete pregnancies, and fetal malformations [Gerster, 1997].

Both retinol and RA are essential for embryonic development. RA has been implicated in the expression of hox genes, which determine the sequential development of various parts of an organism, and retinoids are required in the development of the central nervous system, limbs, cardiovascular system and eyes.

Immune Function.

In the 1920's and 1930's there were many reports linking infections to a deficiency of vitamin A, and vitamin A became known as the "anti-inflammatory vitamin" [Green, 1928]. It has become widely recognized that vitamin A plays an important role in many different aspects of immune function as vitamin A modulates several of the normal immunological protection mechanisms [Semba, 2002]. Vitamin A deficiency impairs innate immunity by impeding normal regeneration of mucosal barriers damaged by infection and by diminishing the function of neutrophils, macrophages, and natural killer cells [Stephensen, 2001]. It is also required for adaptive immunity and plays a role in the development of both T helper cells and B cells. Specifically vitamin A deficiency affects, keratinisation, mucin production, apoptosis, neutrophil function, natural killer (NK) cell function, macrophage function, both T and B lymphocyte function, immunoglobulin production, TNF-α production, and interleukin 1,2,3,&4 production [Semba, 1998]. The role of vitamin A in immune function will be discussed in greater depth in other sections of this review.

Whilst it is self evident from the literature that vitamin A plays many important functions within the body, the functions that are the primary focus of the rest of the thesis include cell differentiation, barrier function and immune function.

2.2 HOW IS VITAMIN A TYPICALLY PROCESSED WITHIN THE BODY?

An overall summary of the processing of vitamin A under normal conditions is shown in figure 2.3. This section will review the evidence of how vitamin A from the diet, as retinyl palmitate, is handled within the body. The events of digestion, absorption, transport, storage and mobilisation of vitamin A will be discussed. At the end of the section the processing of vitamin A will be compared to that of lipid.

2.2.1 DIETARY INTAKE OF VITAMIN A.

Vitamin A is an essential nutrient, which is required in the diet. Vitamin A and carotenoids can be quantified in several different units, but it is preferable to use the international system of units (SI) such as μmol; 1μmol of retinol is equivalent to 286μg of retinol [IVACG]. Vitamin A activity can be expressed in units of weight, as Retinol Equivalents (RE); 1μg RE corresponds to 1μg of retinol, and 1.83μg of retinyl palmitate. The previously used definition of International Units (IU) corresponds to 0.3μg of retinol.

Vitamin A activity can be obtained from two primary forms; preformed vitamin A and provitamin A carotenoids. Preformed vitamin A, mainly as esters of longer chain fatty acids, occurs naturally in foods of animal origin. The richest sources of preformed vitamin A are liver (3000-15000μg retinol/100g), milk (30-70μg/dL), eggs (100-300μg/100g), and other dairy produce [Olson, 1987]. Nutritionally active carotenoids expressed as β-carotene equivalents, are found in abundance in carrots (2000-7000μg/100g; ~333-1166μg/100g retinol), and in dark green leafy vegetables, such as spinach (2000-3000μg/100g) [Olson, 1987]. A major pathway for the conversion of *all-trans* β-carotene and other provitamin A carotenoids to retinol by animals, including humans, is oxidative cleavage of the central 15,15' double bond [Olson, 1996]. Bioavailability of carotenoids can vary widely; nonetheless as a rough general guide, most national and international committees have accepted by convention that 6μg of *all-trans* β-carotene or 12μg of other *all-trans* provitamin A carotenoids in food are equivalent nutritionally to 1μg of retinol [Olson, 1996].

Vitamin A from preformed sources is the main dietary form ingested within the UK, only a quarter to a third of dietary vitamin A in the U.K. is derived from carotenoids [Department of Health report 41]. Although the consumption of carotenoids plays an important role in vitamin A metabolism, as the majority of vitamin A is consumed as retinyl esters, intake as preformed vitamin A will be the main focus of the thesis.

Assessing habitual intake of vitamin A is not easy, primarily due to wide ranges of sources available within the diet. Some foods have extremely high vitamin A content, such as liver (fried calf liver; 39.8mg of retinol/100g), whereas others have lower levels (whole milk; 52μg of retinol/100g) [MAFF, 1991]. For this reason intake on a single day may not adequately reflect an individual's typical intake and a number of days of dietary record may be required to overcome the variance. Nelson and co-workers found that 20 days of recorded food intake was required in order to assess the intake of retinol in adults [Nelson *et al* 1989]. Despite this, when investigating populations, those atypical, but important effects are balanced out. National surveys estimate intakes of whole populations with the principle objective of assessing dietary patterns and making policy and program decisions. The intake of retinol within the U.K. population has been assessed as part of the National Dietary and Nutritional Survey [NDNS] of British adults [Gregory *et al* 1990]. Men had an average daily intake of retinol from all sources, of 1.28mg (median; (range) 0.62mg; (0.194-6.7mg)); compared to an average value for women of 1.13mg (median; (range) 0.49mg; (0.135-5.8mg)) of retinol. On average food supplements were not a major source of preformed retinol; they raised the average intakes for men and women above that from food sources alone by 4% and 7% respectively. There was a wide range of pre-formed retinol intakes within the sample and there was a marked skewness in distribution. For example in men the typical (median) intake was ~0.6mg of retinol but the average (1.28mg) was skewed by individuals with high intakes. These increased intakes may have occurred from the use of supplements or the intake of foods with high vitamin A content. The requirements of vitamin A are discussed later in section 2.2.10.

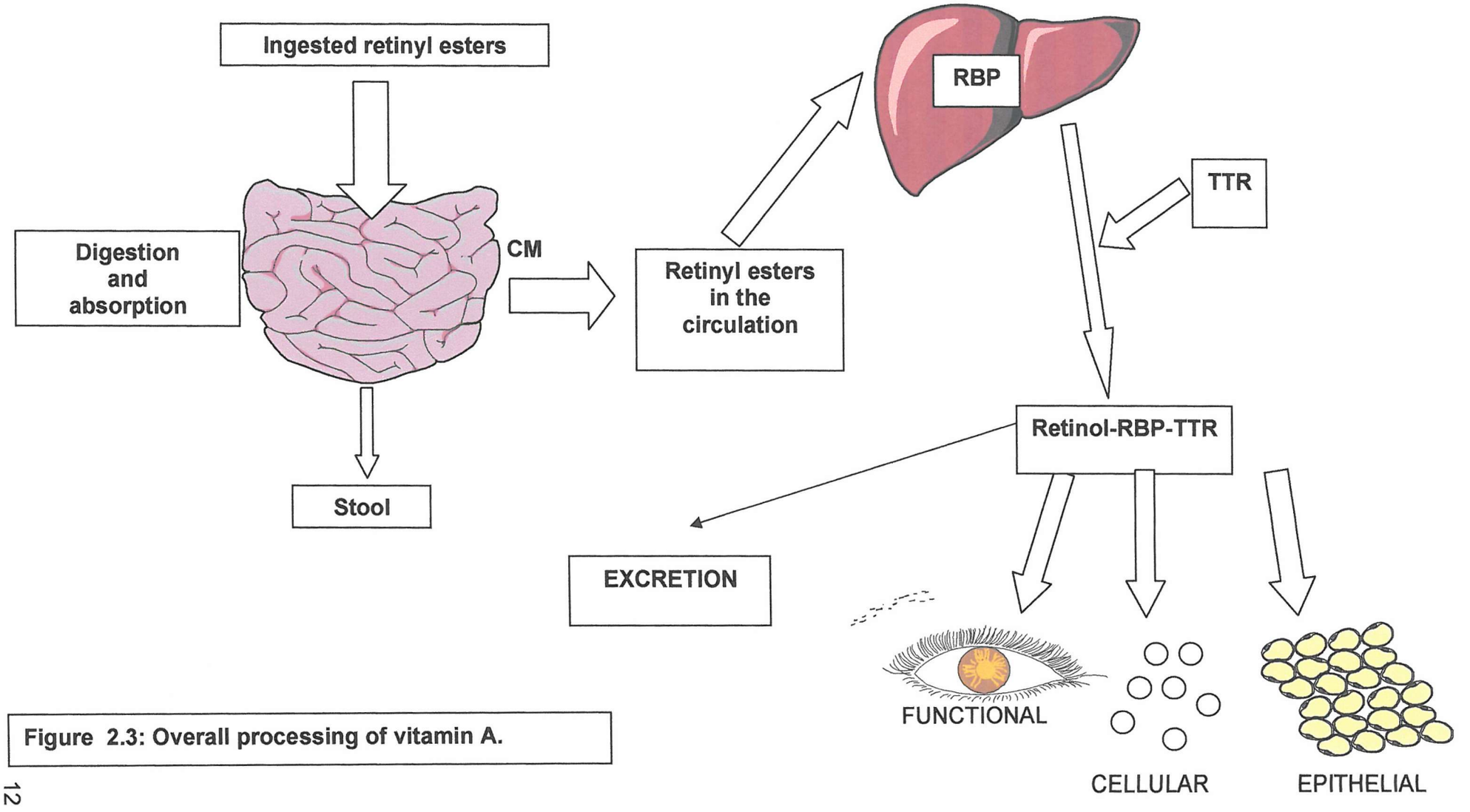


Figure 2.3: Overall processing of vitamin A.

2.2.2 LUMINAL PROCESSING OF RETINYL ESTERS.

Luminal processing describes the action of digestion, emulsification, solubilisation and hydrolysis. The processing of ingested retinyl esters occurs in the lumen of the small intestine. Retinyl esters must be hydrolysed to retinol, solubilised by bile acids, and dispersed into micelles, prior to absorption. There are two different ideas relating to the enzyme that initiates hydrolysis. The first is that hydrolysis occurs by the action of pancreatic esterase, which is activated by bile salts, and the second suggests that the pancreatic enzyme is pancreatic triglyceride lipase, and the intestinal brush border enzyme is phospholipase B [Harrison & Hussein 2001].

As fat-soluble vitamins are insoluble in aqueous environments, such as that of the intestinal lumen, solubilisation of vitamin A occurs by the action bile acids, and retinol is dispersed into mixed micelles. These micellar particles, present the polar ends of the bile acids towards the aqueous environment and the non-polar portion toward the interior of the particles. Micelles interact with the brush border of the epithelial cells in the intestinal mucosa and retinol is transported into the enterocyte [Olson, 1996].

2.2.3 ABSORPTION OF RETINOL.

Retinol is absorbed across the enterocyte. Absorption of physiological concentrations is assumed to occur by facilitated diffusion (a protein carrier mediated process), however when retinol is present in pharmacological amounts absorption occurs by simple passive diffusion [Hollander, 1981]. Once in the enterocyte retinol is released from its micelle and enters the cytoplasm of the cell [Olson, 1996].

Lipid is thought to modulate the availability of vitamin A across the GI tract [Borel *et al* 1997]. One review states that the absorption efficiency of dietary retinol in healthy individuals who ingest significant amounts of lipid (>10g/day) is >80% [Olson, 1996], however there appears to no direct evidence to support this. Two studies [Reddy & Srikantia 1966; Jappesen *et al* 1995] found that increased dietary lipid improved absorption of retinol, however others have found no beneficial role [Figueira *et al* 1969; Lewis *et al* 1947; Borel *et al* 1997].

There have been no studies that directly titrate the amount of lipid in the diet against the apparent availability of the vitamin across the GI tract.

It is stated in the literature, that under normal physiological conditions retinol is efficiently absorbed (i.e. >90%). Measuring losses of the vitamin in stool is the technique used to estimate apparent absorption of retinol across the GI tract (intake minus excretion). This data with information of postprandial excursions provides a way to assess availability of vitamin A from the GI tract. Measuring stool losses, either traditionally by gross balance or by the use of radioactive labels has been adopted by a number of investigators. Losses have been estimated by assessing, 1) loss of habitual intake [Ahmed, 1990; Halford, 1992], 2) loss of an oral bolus dose of retinol or retinyl esters [Barnes, 1950; Lewis, 1947], or 3) loss of an oral bolus dose of labelled retinol or retinyl esters [Silvakumar, 1972; Reddy, 1960; Sauberlich, 1974]. Before reviewing evidence from the literature, limitations of these methods should be considered. For example the gross balance technique makes the assumption that all vitamin A excreted in stool is derived from the diet, it is a crude measure of absorption, which is unable to differentiate between endogenous and exogenous losses of vitamin A. Radioactive tracer studies overcome this as they label the diet directly, however there are issues relating to radiation hazards and safe use in human studies. Evidence obtained from these methods (shown in table 2.1) suggests that apparent absorption of retinol ranges from 62-100%. The majority of investigators found mean apparent absorption of retinol to be greater than 85% [Silvakumar *et al* 1972, Ahmed *et al* 1990, Barnes *et al* 1950, Lewis *et al* 1947, Reddy *et al* 1960, Halford *et al* 1993]. Only two studies suggested that absorption maybe less complete, 62% [Lewis *et al* 1947], and 66.8% [Reddy *et al* 1960].

There are a number of factors that may have contributed to variability in these data. Firstly, the form in which vitamin A is presented to the GI tract may affect subsequent availability of vitamin A. Lewis and colleagues [1947], administered retinyl palmitate in an oil preparation and retinol as a water miscible preparation, to healthy individuals. Excretion of retinol in stool was equivalent to 38% (11-60%) of the intake following the oil preparation and only 7% (2-17%) following

the water preparation. This can be interpreted in two ways. Firstly, retinol in the water miscible preparation was more available to the body than retinyl palmitate in oil. Or, as the water miscible form of vitamin A was presented as retinol, not as the ester, it may have been more effectively absorbed. Barnes and co-workers [1950], also found less vitamin A excreted in stool following the water miscible preparation, compared to the oil solution (32.3% v 56.6%), however, once again retinyl esters were the base of the oil soluble preparation, and retinol the form in the water miscible preparation. This phenomenon has also been observed in the circulation. Johnson and colleagues [1992b] found retinol in the water miscible preparation produced a greater mean peak rise in retinyl esters than retinyl palmitate in oil and interpreted this as the water miscible preparation being more available across the GI tract (less lost in stool, more in circulation). One study investigated postprandial responses of water miscible and fat-soluble preparations both presented as retinyl palmitate [Silva, 2001]. Retinyl palmitate in the water miscible preparation was still more available than the fat-soluble preparation [Silva, 2001]. This may therefore reflect differences in the processing of the two preparations and may contribute to the lower apparent absorption rates seen in the literature in studies that use oil preparations. The matrix in which vitamin A is presented to the GI tract may also affect availability. Buss and colleagues [1994], administered retinyl palmitate in a supplement and compared this to an equivalent amount of retinyl palmitate in liver. A greater (almost double) peak rise in retinyl esters and area under the curve was apparent following retinyl palmitate in the supplement.

Secondly the dose of administered vitamin A may affect the subsequent availability of vitamin A. For example Reddy and co-workers compared a physiological dose (3000IU retinyl palmitate; 1.65mg retinyl palmitate; 0.9mg retinol), with a pharmacological (200000IU retinyl palmitate; 110mg retinyl palmitate; 60mg retinol) of retinyl palmitate in oil [Reddy *et al* 1960]. Apparent absorption of retinol was lower following the pharmacological dose compared to the physiological dose (66.8% v 95.9%). It is however important to consider these results in terms of net availability. Despite apparently incomplete absorption of the pharmacological dose, on average 47% of the dose was retained (equivalent to 106,000IU), compared to 2677IU of the lower dose. This

is also reflected in studies of postprandial excursions, where the physiological dose of retinyl palmitate has lower postprandial responses than the pharmacological doses of retinyl palmitate [Johnson *et al* 1992a; Johnson *et al* 1992b]. Therefore although the apparent absorption capacity of the pharmacological dose is lower, there appears to be no constraint in net availability.

In summary, the availability of vitamin A across the GI tract has been investigated by both gross balance and postprandial excursions of retinyl esters in the circulation. It would appear that in healthy individuals apparent absorption of retinol is high (~85%), although factors associated with the study designs (dose and form of administered vitamin A), and limitations in the methodological techniques, may contribute to the variability seen in the results obtained.

2.2.4 THE ENTEROCYTE: ESTERIFICATION OF RETINOL

Following digestion and absorption, retinol becomes bound to cellular retinol binding protein ($CRBP_2$), which is localised solely in the small intestine, and is transported to the endoplasmic reticulum (ER) for esterification. $CRBP_2$ presents retinol to enzymes for re-esterification mainly with long chain fatty acids (LCFA). Greater than 80% of retinol that leaves the intestine within the chylomicron (CM) is esterified with LCFA [Blomhoff *et al* 1991]. It appears unclear as to the fate of the retinol that is not esterified with LCFA. Studies have demonstrated that the pattern and proportion of fatty acids in these synthesised retinyl esters is remarkably consistent even when dietary fatty acids are altered [Goodman *et al* 1966]. This suggests that the source of these fatty acids maybe from a restricted pool and is probably not the same as that used by enterocytes for TAG synthesis [Ong, 1994]. The patterns are similar in both rat and man, with palmitate, stearate, oleate, and linoleate accounting for the majority of ester fatty acids observed, in the approximate relative proportions of 8:4:2:1. Two enzymes are important for esterification, acyl-CoA: retinol acyltransferase (ARAT) and lecithin: retinol acyltransferase (LRAT). LRAT is the main intestinal enzyme that esterifies retinol under normal conditions and normal loads of retinol, and ARAT functions to esterify excess when $CRBP_2$ is saturated.

Table 2.1: Summary of the apparent absorption of retinol in healthy individuals.

N.B. Vitamin A loss in stool is presented in most investigations, in these cases apparent absorption (highlighted) is an estimate, derived from the data presented in the publication.

Reference	Apparent absorption %	Loss in stool % intake	Approach	Intake	Number of subjects
Barnes *et al* 1950	95.2%	4.8% (0.2-4.4%)	Gross balance.	Retinol in water miscible preparation	12
Barnes *et al* 1950	87.8%	12.2% (0.3-49.5%)	Gross balance.	Retinyl palmitate in oil	12
Ahmed *et al* 1990	98.2%	1.8% (0.8-9.8%)	Gross balance	Habitual intake. Median 632μg/d (122-2730μg/d)	12 aged 8-21yrs
Lewis *et al* 1947	62%	38% (11-60%)	Gross balance	12500 or 35000 units vitamin A as retinyl esters in fish oil	7 aged 1-3 months
Lewis *et al* 1947	93%	7% (2-17%)	Gross balance	12500 or 35000 units vitamin A as retinol in a watery solution of glycerin	7 aged 1-3 months
Halford *et al* 1992	100%	0%	Gross balance	582.9μg/d retinol habitual intake from 7d diary.	10 aged 3-16yrs
Sivakumar & Reddy 1972	99.2%		Label	4-5μCi [11,12-$^{3}H_2$] retinyl acetate in oil with 1000μg non-radioactive R-acetate	5 aged 2-10yrs
Reddy & Sivakumar 1960	95.9 ± 2%	<5%	Label	Radioactive labelled retinyl palmitate in oil and 3000IU non-radioactive retinyl palmitate	11 aged 2-10 yrs
Reddy & Sivakumar 1960	66.8 ± 3.5%		Label	Radioactive labelled retinyl palmitate in oil plus 200000IU non-radioactive R-palmitate	11 aged 2-10 yrs
Sauberlich *et al* 1974	82.2 - 62.7%	17.8-37.3%	Label	Radioactive labelled retinyl-15-^{14}C acetate.	8 male subjects

2.2.5 POST-PRANDIAL TRANSPORT – CIRCULATING VITAMIN A AS RETINYL ESTERS.

The CM containing retinyl esters are transported from the GI tract to the liver via the lymphatic system. Over 90% of vitamin A in lymph is recoverable as retinyl ester in rats [Huang & Goodman, 1965], and 86-88% in humans [Goodman *et al*, 1966]. Under normal conditions it is presumed that retinyl esters are transported solely in the CM, the nature of the portal transport mechanism for vitamin A has not yet been clarified [Olson, 1996]. It appears that the predominant form of retinyl ester in the circulation following absorption is retinyl palmitate. Following an oral dose, one study reported, retinyl palmitate to be the predominant retinyl ester present in the circulation (C16:0) (69.7%), followed by retinyl stearate (C18:0) (24.8%), and retinyl oleate (C18:1) (5.6%) [Reinersdorf *et al* 1996].

Following secretion into the circulation, the CM undergoes lipolysis catalysed by lipoprotein lipase (LPL), an enzyme found on the luminal surface of capillary endothelial cells (especially in adipose tissue and skeletal muscle). The resulting delipidated chylomicron remnants (CMr) are rich in cholesterol esters, retinyl esters, carotenoids [West, 1998] and contain apo-E and apo-B. The literature suggests that retinyl esters are relatively non-transferable components of the CM. Evidence to support this arises from one main study in humans [Wilson *et al* 1983]. They state <5% of absorbed retinyl esters appear in the low-density lipoprotein (LDL) fraction and therefore the majority of retinyl esters are still associated with the CMr and cleared by the liver. This evidence also supports the theory that LPL is a specific enzyme for the hydrolysis of TAG. Along with this, data from studies in several species indicate that from <50% to 86% of labelled CM retinyl esters are recovered in the liver as a result of CMr uptake [Blomhoff *et al* 1991], thus some diet derived vitamin A is presumably taken up by extra hepatic tissues. Extra hepatic clearance of CM retinyl esters may occur during the interaction of CM with LPL, during this process the CM constituents could partition into cell membranes. Goodman and colleagues injected retinyl ester labelled CM into rats [Goodman *et al* 1965.]. At times up to 3 hours, measurable amounts of radioactivity were recovered in adipose tissue (3-10%), skeletal muscle (4-7%), kidneys (0.4-4%), and the rest of the carcass

(0-10%), compared to (70%) within the liver. However, quantitative importance of extra hepatic uptake of diet-derived vitamin A has not been investigated in humans [Blomhoff *et al* 1991]. It is not known whether extra hepatic uptake of retinyl esters is an important route of delivery to tissues when normal processing within the liver is impaired.

2.2.6 ARE VITAMIN A AND LIPID PROCESSED IN A SIMILAR WAY?

It is commonly stated that there is an intimate relationship between the processing of lipid and vitamin A. The following section will describe the processing of lipid with respect to the processing of vitamin A in order to discern similarities and differences in the two processes.

2.2.6.1 LUMINAL PROCESSING OF LIPID.

The digestion and luminal processing of lipid is similar to that of retinyl esters. Hydrolysis of TAG is initiated in the stomach by gastric lipase, and continues in the duodenum by the action of pancreatic lipase, which acts on positions *Sn*-1 and *Sn*-3 of the TAG molecule. TAG is hydrolysed to monoglycerides (MAG) and free fatty acids [Gurr *et al* 1989]. Bile acids and salts mix with the fatty acids to produce mixed micelles, which are absorbed across the brush border membranes.

2.2.6.2 ABSORPTION OF LIPID

Lipid absorption in man occurs predominantly in the jejunum and is similar to that of vitamin A [Gurr *et al* 1989]. Micelles formed from lipid digestion diffuse among the microvilli of the enterocyte, which allows the large surface area of the brush border membrane to participate in lipid absorption. Unabsorbed lipid is excreted in stool; in healthy adults 5-6 g/day is lost via this route, equivalent to approximately 5% of lipid intake [Wollaeger *et al* 1947; Wrong *et al* 1981; Murphy *et al* 1991]. It is however clear in lipid metabolism that fatty acids of chain length less than 12 carbon atoms are absorbed directly into the portal blood, are metabolised chiefly by β-oxidation in the liver and do not contribute to plasma lipids or adipose tissue stores [Sickenger, 1975].

2.2.6.3 ESTERIFICATION OF LIPID

Within the enterocyte, fatty acids and monoacylglycerols (MAG) are re-esterified to form new TAG molecules, this occurs mainly by the monoacylglycerol esterification pathway (MAG – DAG – TAG). The smooth ER generates a CM from TAG, phospholipids, cholesterol and cholesterol esters along with apolipoprotein B. These large vesicles are secreted from the cell by exocytosis and enter the lymphatic system.

2.2.6.4 POSTPRANDIAL TRANSPORT - CIRCULATING LIPID AS TAG

TAG is hydrolysed from the CM by the action of LPL [Frayn *et al* 1995]. In contrast to retinyl esters, peripheral tissues can clear resulting fatty acids. Fatty acids can be entrapped by adipose tissue and re-esterified to TAG for storage, or taken up by the muscle for oxidation, or taken up by the liver for re-esterification [Frayn *et al* 1995]. The residual delipidated particle remnants (CMr) are internalised by the liver for further degradation. Free fatty acids in the liver have three fates; they can be esterified, oxidised or used for synthesis. The liver, like the gut, makes and secretes TAG rich lipoproteins; very low-density lipoproteins (VLDL). The major apolipoprotein of VLDL, apoB100, is synthesised in ribosomes and transferred to the Golgi where it combines with TAG [Grundy, 1996]. The metabolism of VLDL in the circulation resembles that of CM.

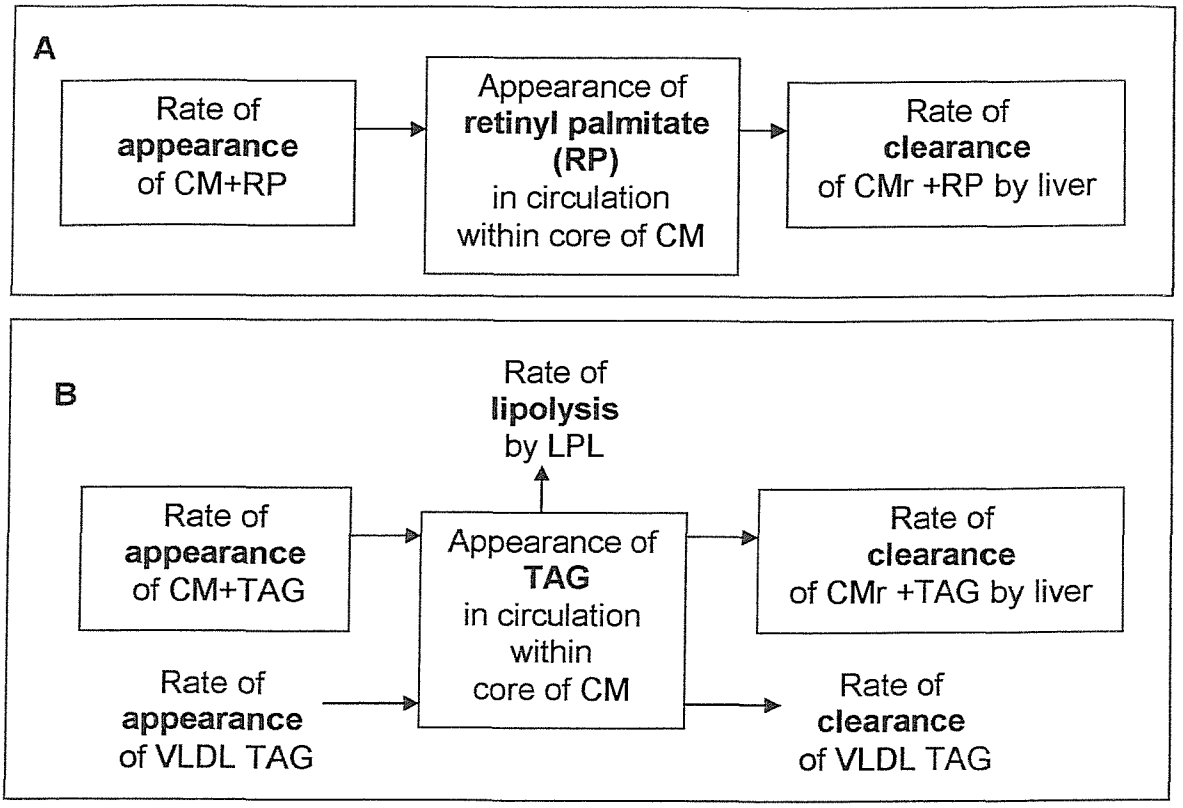
Vitamin A and TAG are assumed to be processed in a similar way, despite differing processes governing their appearance and clearance from the circulation. Figure 2.4 demonstrates that the appearance of retinyl palmitate into the circulation is solely dependant upon the appearance and clearance of CM, whereas more additional factors affect the appearance of TAG. Most evidence relating to the processing of lipid and vitamin A has been obtained from studies that investigate CM partitioning [Lovegrove *et al* 1999]. In these studies retinyl esters have been used as markers of CM clearance, with the assumption that retinyl esters remain with the CMr after delipidation, are cleared by the liver, and not re-secreted. Evidence from these studies suggests that TAG and retinyl esters may not be handled in the same way, however this may simply be a consequence of the form in which retinyl ester is consumed. For example after

dosing with both retinyl palmitate and lipid, postprandial increases in lipid appear sooner than the rise in plasma retinyl esters (1-3 hours v 6 hours) [Krasinski *et al* 1990; Cohn *et al* 1993]. However delayed appearance of retinyl esters compared to TAG is not a consistent finding, [Lovegrove *et al* 1999], and the form of vitamin A used to label the CM has been considered a major cause for this apparent discrepancy. Karpe and colleagues suggested that delayed absorption of vitamin A in oily preparations causes delayed appearance of retinyl esters in plasma [Karpe *et al* 1995]. However as previously discussed (section 2.2.3), the form of vitamin A varies between studies, making comparisons hard. For instance retinol in the water miscible preparation can be taken up directly, compared to retinyl palmitate in the oil preparation, which must be hydrolysed to retinol before uptake by the enterocyte. Only one study has directly compared the use of retinyl palmitate as either a water miscible or oil-soluble preparation on the appearance of retinyl esters and TAG into the circulation. Silva and colleagues found a delayed appearance of retinyl esters following the oil soluble preparation compared to the water miscible preparation (327 minutes v 117 minutes) [Silva *et al* 2001], although the time of TAG appearance was similar to that seen following vitamin A in the water miscible preparation (182±18SE minutes v 117±12SE minutes).

In summary the assumption that vitamin A and lipid are processed in the same manner within the GI tract may not be entirely true. The appearance into the circulation of retinyl esters, which are presented in oil soluble preparations, seems to be delayed when compared to TAG. However the appearance of retinyl esters and TAG may be similar when retinyl esters are presented to the GI tract in a water miscible preparation. Despite this information, it still remains unclear as to the extent to which lipid is required in the diet for effective absorption of retinol, and the extent to which the movement of lipid and vitamin A are associated. There is a need to effectively trace both retinyl esters and lipid presented in a meal, to better understand the relationship between their handling.

Figure 2.4:

Factors that influence the appearance of both retinyl palmitate (a) and TAG (b) in the circulation.



2.2.7 PROCESSING OF VITAMIN A IN THE LIVER.

During the processing of vitamin A the main functions of the liver are to, clear CMr containing retinyl esters from the circulation, store vitamin A and release retinol bound to retinol binding protein (RBP) (which is synthesised within the liver) (figure 2.5).

2.2.7.1 UPTAKE OF RETINYL ESTERS BY PARENCHYMAL CELLS.

Parenchymal cells account for 90-95% of the total liver cells [Blomhoff *et al* 1991]. They are responsible for the uptake and processing of CMr and synthesis and release RBP. The liver takes up CMr by receptor-mediated endocytosis. This process requires participation of cell surface receptors that recognise the apolipoprotein components (apoB & apoE) of the CMr [Blaner *et al* 1994]. During lysosomal degradation of the CMr, retinyl esters are hydrolysed and free retinol is transferred to CRBP [Olson,1986]. Retinol-CRBP is

transported to the ER, where it binds to RBP. This binding is believed to initiate a translocation of retinol-RBP from the ER to the Golgi complex, which is followed by secretion of retinol-RBP into the circulation [Blomhoff, 1994].

Rat studies suggest that parenchymal cells do not incorporate retinyl esters together with TAG in the core of VLDL [Norum & Blomhoff, 1992]. However Cohn and co-workers [1993] detected 25% of retinyl esters in the apoB100 triglyceride rich lipoprotein (TRL), 12 hours after ingestion of retinyl palmitate and lipid in humans. As apoB-100 is assumed to be of hepatic origin, the rise in retinyl esters in this fraction may be due to postprandial synthesis and release of retinyl esters in the apoB-100 TRL (VLDL). Possible explanations for these findings are that; 1) retinyl esters are secreted from the intestine within CM containing apoB-100, 2) retinyl esters are secreted from the liver in VLDL containing apoB-100, or 3) retinyl esters are transferred to apoB-100 TRL in the circulation [Cohn *et al* 1993]. It is not accurately known if retinyl esters are mobilised from the liver in VLDL. However, if true, this may be a mechanism for releasing vitamin A from the liver during instances of reduced RBP, but it is not known if tissues can directly take up retinyl esters or if retinyl esters in VLDL are still processed by the liver.

2.2.7.2 STORAGE OF RETINYL ESTERS IN LIVER STELLATE CELLS

Retinol that is not released into the circulation is transferred (bound to $CRBP_1$) to the stellate cells, where it is re-esterified and stored primarily as retinyl palmitate [West, 1998]. Stellate cells are smaller and less abundant than parenchymal cells accounting for 5-15% of total liver cells. This transfer is thought to be influenced by the nutritional state of the individual, for instance in vitamin A deficiency, retinyl esters do not accumulate in stellate cells, but during vitamin A sufficiency vitamin A is stored. It has been hypothesised that CRBP concentrations are the rate-limiting factor for retinol uptake by stellate cells. When retinol concentrations are low, the amount of CRBP is reduced and therefore, retinol uptake by stellate cells, esterification, and storage are inhibited, resulting in a net increase in mobilization of retinol. In contrast when vitamin A intake is increased an increase in CRBP occurs which transfers retinol to LRAT for esterification and storage [Blomhoff *et al* 1991].

It is well accepted that large amounts of retinol, in excess of requirements, can increase liver stores and cause harm, particularly in terms of liver and bone damage. Symptoms of chronic vitamin A toxicity include dry thickening skin, itching, cracking lips, brittle nails, bone and joint pain, reduced bone mineral density, and hepatotoxicity [Expert Group On Vitamins And Minerals, 2002]. Hepatotoxicity, due to chronic vitamin A ingestion is characteristically associated with peripheral signs of vitamin A toxicity, high plasma retinol concentrations and daily ingestion of at least 90000IU (27mg of retinol) for more than 1 year. Despite this, one case report, observed massive amounts of vitamin A in the liver (19000IU/g; 5.75mg retinol/g) of an individual who had ingested between 6-15mg of retinol per day for 7 years, exhibited no clinical signs, and had below normal retinol concentrations [Weber *et al* 1982]. The potential risk of liver disease caused by prolonged ingestion of vitamin A has originated mainly from single case reports [Di Benedetto, 1967; Rubin *et al* 1970; Lippel *et al* 1981]. A larger study investigating liver damage caused by therapeutic vitamin A administration found that the smallest continuous daily consumption leading to cirrhosis was 25000IU over 6 years (7.5mg retinol), whereas higher doses (>100000IU; 30mg retinol) taken over 2.5 years resulted in similar lesions [Geubel *et al* 1991]. This study highlights that retinol concentrations in the plasma do not closely reflect stores of vitamin A within the liver. Expert committees in Europe and USA have repeatedly stressed that single doses of 300mg in adults or 100mg in children are harmful, but toxicity usually arises from chronic ingestion of retinol or retinyl esters, not necessarily in large amounts but significant over a period of time to build up stocks that exceed the livers ability to destroy and store them. Chronic hypervitaminosis A has been generally attributed to a total daily intake of 7.5-15mg over a period of weeks, month or years. However one trial has revealed mild symptoms in individuals receiving supplements of 3mg per day for 6 months [Wald *et al* 1985]. Government recommendations are that regular intakes should not exceed 7.5mg/day in adult women, and 9mg/day in adult men [Department of Health Report 41]. As children are more sensitive, intakes should not exceed; 0.9mg in infants; 1.8mg from 1-3 years of age; 3mg from 4-6 years; 4.5mg from 6-12years old or 6mg in adolescents [Department of Health, 1991].

2.2.7.3 MOBILISATION OF RETINOL FROM THE LIVER

Transport of retinol from stores in the liver to target tissues is accomplished exclusively by means of a specific transport protein, retinol-binding protein (RBP). RBP is a transport protein that acts by solubilizing and protecting retinol in aqueous spaces. Once in the circulation retinol-RBP forms a complex with transthyretin (TTR) (formally known as prealbumin), this complex reduces glomerular filtration of retinol [Blomhoff, 1994], to reduce loss in urine. The complex retinol-RBP-TTR, serves to transport retinol in the circulation and deliver it to target tissues. Reference values of RBP and TTR in the serum of adults are 30–60 mg/L (1.43-2.86μmol/L) and 200-400mg/L (3.70-7.4μmol/L) respectively [Dati, 1996].

RBP has one binding site for retinol that consists of a hydrophobic pocket designed to bind and protect retinol [Blomhoff, 1994], therefore retinol circulates in an 1:1 molar complex with RBP. The RBP molecule is made up of an N-terminal coil, a β-sheet core, and α-helix and a C-terminal coil. RBP is synthesised as a precursor protein (MW 24000) that is rapidly processed by the ER to the mature protein (MW 21000) [Sivaprasadarao *et al* 1994], the mature protein is then transferred to the Golgi for secretion. Synthesis and processing of pre-RBP does not appear to depend on the vitamin A status of the animal [Sivaprasadarao *et al* 1994]. Factors that regulate RBP secretion from the liver still remain to be fully elucidated however one review suggests that secretion of RBP from the liver is tightly regulated by availability of retinol [Noy, 2000], however the question of how tightly vitamin A regulates RBP secretion has not been resolved.

Vitamin A deficiency specifically inhibits secretion of RBP from the liver, RBP accumulates in the liver to levels which are 4-10 times higher than those observed in control livers [Blaner, 1989]. Retinol deficiency largely prevents movement of newly synthesised RBP from the ER to the Golgi apparatus [Ronne *et al* 1983] and apo-RBP accumulates in the ER. Upon repletion of vitamin A, secretion of RBP from the liver is stimulated [Blaner, 1989]. Studies using cultured primary parenchymal cells from retinol deficient rats found the addition of retinol to the culture had a rapid and specific effect on secretion of

RBP and authors reported that deficient heptocytes still secreted low levels of RBP into the medium [Dixon *et al* 1987]. These data provide evidence that the secretion of retinol from the liver is influenced by availability of RBP, however mechanisms underlying the production and release of RBP are not known. For example is RBP continually produced and degraded if it is not required? and can RBP circulate in the plasma unbound to retinol?. It may be possible for some apo-RBP to escape from the liver into the circulation by non-specific bulk flow. The presence of apo-RBP in plasma and the demonstration that apo- to holo-RBP ratio increases in vitamin A deficiency provides circumstantial evidence for this argument [Sivaprasadarao *et al* 1994]. Whether extra hepatic tissues make any contribution to plasma RBP and if so by what mechanism, is not clear at present.

In summary, during the processing of vitamin A, the liver functions as a storage organ from which retinol can be mobilised into the circulation, yet little is known about the factors that regulate hepatic processing and secretion of retinol.

2.2.8 UPTAKE OF RETINOL TO PERIPHERAL TISSUES

The specific mechanism of retinol uptake by peripheral tissues has not been fully elucidated. Retinol uptake by cells appears to involve one or more of the following processes: 1) the small amount of retinol in equilibrium with retinol-RBP in plasma may partition into plasma membranes, 2) retinol might enter the cell as a result of fluid phase endocytosis of retinol-RBP-TTR and retinol-RBP, or, 3) it may be taken up by receptor mediated transport [Blomhoff *et al* 1991]. It is well known that once in the cell, retinol is metabolised to active intermediates (e.g. retinal, retinoic acid) that bind to appropriate effector proteins (e.g. rhodopsin, retinoic acid receptor), which in turn influence the physiological and metabolic events of cells.

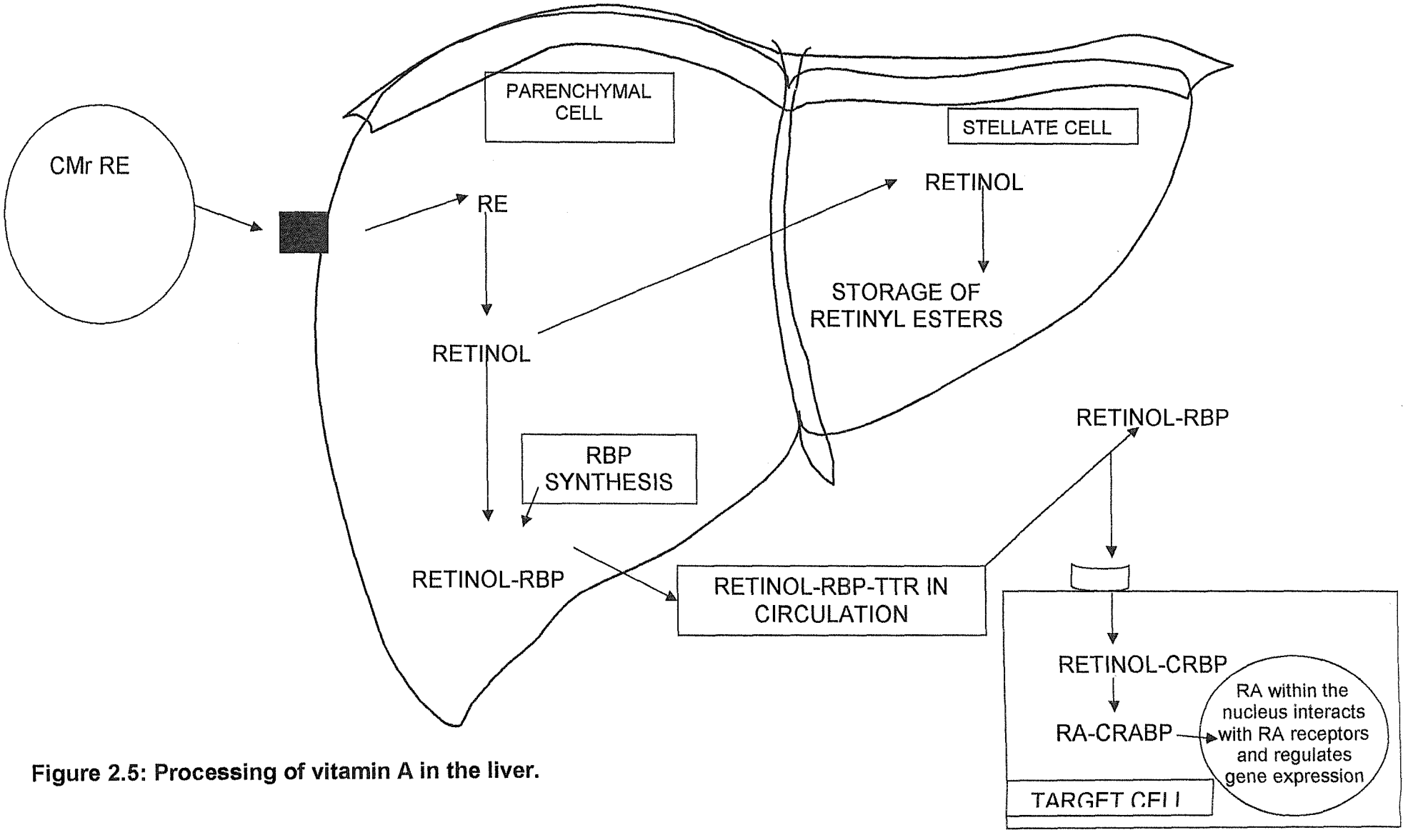


Figure 2.5: Processing of vitamin A in the liver.

2.2.9 EXCRETION OF VITAMIN A.

Under normal conditions, in adults, vitamin A is excreted from the body, in only trace amounts. The main excretory route of vitamin A is in the form of glucuronides (figure 2.6). Vitamin A is primarily metabolised at positions C4 in the tri-methyl-cyclohexene ring and C15 at the end of the side chain [Olson, 1996]. Retinol is excreted in urine and bile as metabolites of either retinol or RA [Olson, 1969]. RA and retinol are stored in the liver as retinyl esters, and in the presence of uridine diphosphoglucuronic acid, are able to form retinoyl β-glucuronides and retinyl β-glucosiduronates respectively [Lippel *et al* 1968]. It is in these forms that retinol is excreted from the body. Retinoid β-glucuronides released as endogenous components of bile are reabsorbed from the intestine and recycled in an entero-hepatic circulation back to the liver [Olson, 1996]. It has been estimated, from the rate of excretion of radioactivity in one depletion study, that the total body stores lost per day is roughly 0.5% in adults ingesting a vitamin A free diet [Olson, 1987]. Direct loss of retinol in urine occurs only in negligible amounts, 0.0016μmol/d of retinol [Stephensen *et al* 1994], 0.00±0.001μmol/d of retinol, [Alvarez *et al* 1995], although loss via this route maybe increased during infection (see section 2.4.1.4). Excretion of vitamin A via faeces, other than glucuronides, does not appear to be a primary excretory route.

In summary, the component parts relating to the excretion of vitamin A have been described, although the mechanisms of integration and control are not clear and estimates of actual losses of vitamin A from an individual still remain unclear.

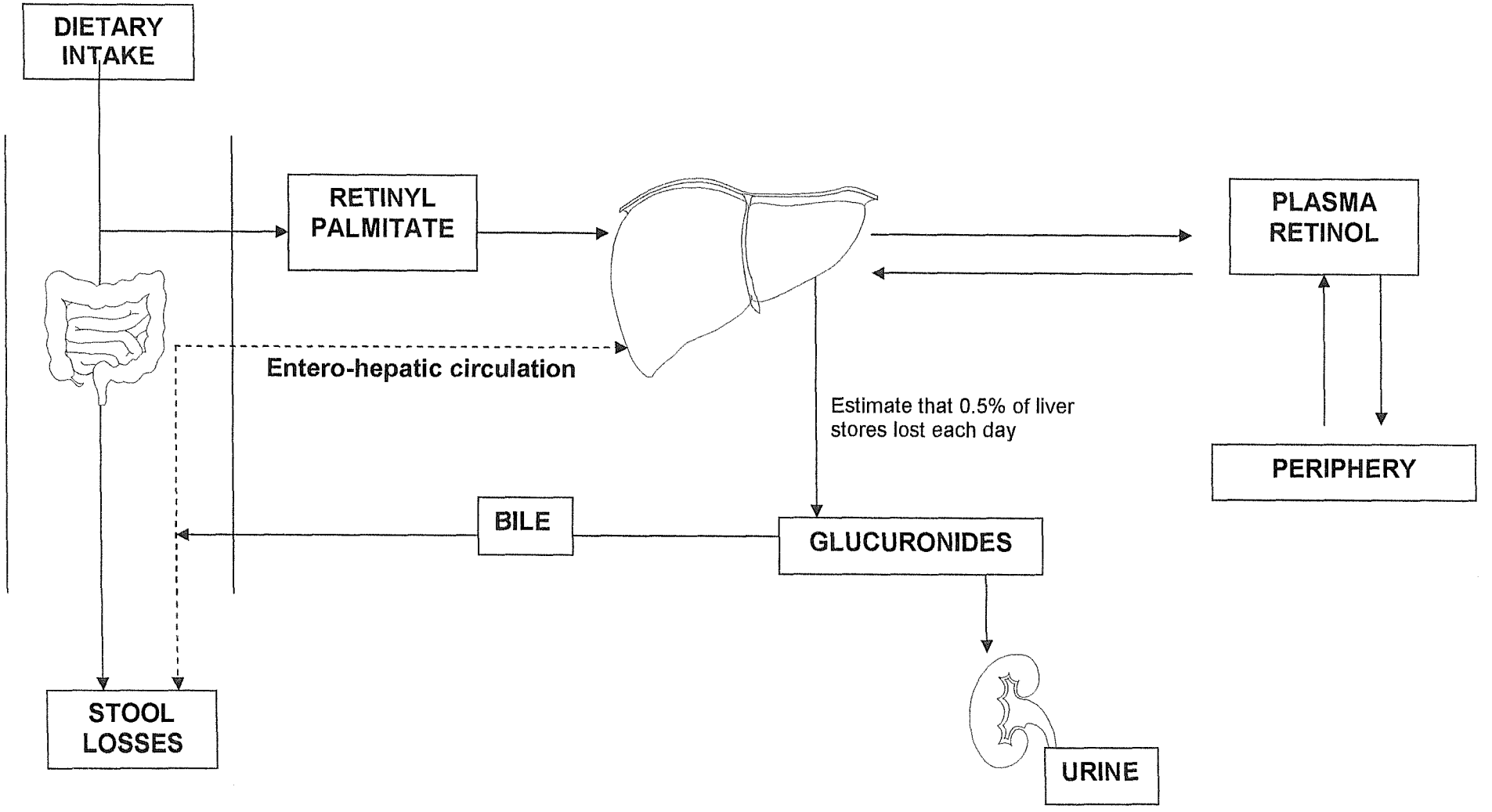


Figure 2.6 : Excretory routes of vitamin A.

2.2.10 REQUIREMENTS OF VITAMIN A.

Requirements for a nutrient differ from one individual to another, but it has been assumed that in a group of individuals requirement is normally distributed. Classically, requirement of an individual for a nutrient has been the amount of that nutrient required to prevent clinical signs of deficiency. While this is important, populations should expect more than the basic need to avoid deficiency, and allowance should be made for periods of low intake or high demand without detriment to health [Department of Health, 1991].

Only a small number of experiments have been designed and conducted to determine the adult human requirement of vitamin A, these studies have been reviewed by Rodriguez [1972]. Of these, two studies, based on repletion of vitamin A depleted volunteers, are considered the most extensive data set available [Hume *et al* 1949; Sauberlich *et al* 1974]. Hume and co-workers [1949] concluded that 390μg of retinol daily was the minimum protective dose, however they recommended that 750μg of retinol should be accepted as the requirement. Sauberlich and colleagues found that dark adaptation was readily corrected by retinol supplements of 150μg/d, and that 300μg/d partially corrected abnormal electroretinograms. Depletion / repletion studies however tend to overestimate requirements as deficiency signs often take some time to improve and there is a reluctance to keep volunteers on low intakes for long periods.

In the absence of other experimental studies an alternative approach was adopted to make the WHO recommendations [1988], and the Dietary Reference Values [1991]. Adequate vitamin A status can be defined in terms of an adequate body pool, based on the amount of vitamin A in the liver. Determination of an adequate liver reserve should reflect a concentration of vitamin A in the liver that prevents deficiency, provides a suitable reserve for periods of low intake and is consistent with good health. A liver concentration of 20μg of vitamin A expressed as retinol per gram of wet weight meets the adequacy criteria, and was used as the basis of the WHO recommendations [Olson, 1987; Department of Health Report, 1991]. Sauberlich [1974] reviewed data on liver concentrations from North American populations. Mean

concentrations of liver vitamin A were 149±132μg/g. Retinol values were relatively constant (10-25μg/g) compared to the greater variation seen in retinyl ester concentrations (18-1353μg/g).

In the attempt to estimate requirements, an assessment of the fractional catabolic rate of the vitamin was required. As part of the depletion studies of Sauberlich, radioactive vitamin A was administered to volunteers and the first order depletion rate of vitamin A from the body was calculated from the rate of excretion of radioactivity. The mean half-life for depletion of body reserves was reported to be 154 days (75-240 d) [Sauberlich *et al* 1974; Olson, 1987]. Total body stores lost per day was roughly 0.5% in adults ingesting a vitamin A free diet [Olson, 1987], and it is presumed that this will not alter on addition of vitamin A to the diet. The data does not indicate if this rate occurs in all individuals, or what the ranges of catabolic rates are likely to be in a population. However using this rate (0.5%), and making the assumptions that; i) the liver weight:body weight ratio is 0.03:1; ii) the liver reserves represent 90% of the total body vitamin A, and iii) efficiency of storage in the liver of an ingested dose of vitamin A is 50%; the mean dietary daily intake needed to maintain liver retinol concentrations of 20μg/g could be calculated [Department of Health, 1991]. Although these calculations rest on assumptions that are somewhat arbitrary [Olson, 1987], the derived requirements are consistent with the effects of deficiency signs observed in depletion repletion studies. Most importantly they provide some indication of what the range of individual requirements might be [Department of Health Report, 1991].

The dietary reference values for vitamin A in adults (μg retinol equivalents/day), along with the intake of vitamin A from all sources (μg of retinol equivalents/day) are shown in table 2.2. Both the mean and median values of intake of vitamin A from all sources are in excess of the RNI. From the table it should be noted that ~2.5% of the population have an intake <LRNI, the typical intake (median) is greater than the RNI, and ~2.5% of the population have an intake of >6mg retinol/day. It would appear that within the population of adults in the UK the probability of having a dietary deficiency of vitamin A is low. However some individuals have intakes over ten times the RNI, some of which may be moving towards potentially toxic intakes (7.5mg/d women; 9mg/d men).

In summary dietary guidelines are based on an understanding of requirement and it would appear that we know relatively little about the true requirement of vitamin A. This is mainly due to the poor knowledge base on which to make a statement. The Department of Health recommendations have been based on a number of assumptions, some of which have been obtained from a limited evidence base.

Table 2.2 :

Table illustrating the typical intakes of vitamin A within the UK adult population and the Dietary Reference values set by the DoH (μg/RE/day).

INTAKE OF VITAMIN A, UK [Gregory, 1990]	MEN	WOMEN
Mean	1679μg	1488μg
Median	1033μg	849μg
Lower 2.5 Percentile	300μg	250μg
Upper 2.5 Percentile	7042μg	6326μg
DIETARY REFERENCE VALUES [DoH, 1991]		
Lower reference nutrient intake (LRNI)	300μg	250μg
Estimated average requirement (EAR)	500μg	400μg
Reference nutrient intake (RNI)	700μg	600μg

2.3 IS IT POSSIBLE TO ASSESS VITAMIN A STATUS?

The term vitamin A status is commonly used within the literature, to represent total body stores of vitamin A that are present within the liver. Vitamin A status reflects the adequacy of intake to maintain liver stores, for instance, if dietary supply is adequate to maintain liver stores in a range that is satisfactory to meet functional requirements. It is well accepted that a low vitamin A intake can lead to low body stores (poor vitamin A status), and a high vitamin A intake can lead to high body stores (toxicity), both of which are associated with health risks. The terminology becomes confusing when vitamin A status is classified indirectly particularly, biochemically by the use of plasma vitamin A concentrations. The assessment of vitamin A status is not straightforward. The following section will review how vitamin A status is assessed and the limitations of these methods.

2.3.1 HOW IS VITAMIN A STATUS MEASURED?

Vitamin A status can be assessed at various levels. Both dietary, biochemical, functional and clinical tests are used for the classification of individuals (table 2.3). However clear relationships between measures of vitamin A status, clinical symptoms and measures of morbidity and mortality are only seen in studies performed in areas where vitamin A deficiency remains a general health problem such as in developing countries. The sensitivity and specificity of the tests for assessment of vitamin A status in the sub-optimal and / or marginal range appears more difficult [Van den Berg, 1996].

Table 2.3

Methods to assess adequacy of vitamin A intake and status.

[Van den Berg 1996]

Most cut-off values indicated are intended for use at the population level.

METHOD	ADEQUATE RANGE*
DIETARY ASSESSMENT	RDA
BIOCHEMICAL ASSESSMENT	
Liver content	>20μg/g (0.07μmol/g)
Plasma retinol	>0.7μmol/L
Relative dose response test (RDR)	<20%
Modified RDR (MRDR)	<0.06
FUNCTIONAL ASSESSMENT	
Conjuctival impression cytology (CIC)	Normal range
Dark adaptation	Normal range
CLINICAL SYMPTOMS AND SIGNS	
Conjuctival and Corneal xerosis	No symptoms
Keratomalacia	
Night Blindness	
Corneal Scar	

2.3.1.1 ASSESSMENT OF BODY STORES.

Estimation of the principal body store (liver vitamin A content) is the best tool for assessment of vitamin A status. However direct measurement by biopsy is an invasive measure and is only carried out in exceptional circumstances, therefore more indirect approaches have been adopted, such as isotope dilution using a labelled tracer or using the relative dose response test (RDR). Information relating to liver stores of vitamin A has been discussed previously in sections 2.2.7.2 and 2.2.10.

Isotope dilution.

The isotope dilution method quantitatively estimates total body reserves. The technique is a method of chemical analysis of total body vitamin A based on administration of a known amount of labelled vitamin A (commonly deuterated retinol) followed by the measurement of the ratio of labelled to unlabelled vitamin A in an isolated plasma specimen after appropriate equilibration [Clifford *et al* 1990]. Total body reserves of vitamin A are estimated based on principles of isotope dilution, a set of assumptions regarding; 1) retention of the dose of labelled vitamin A, 2) the ratio of specific activities of vitamin A in plasma to that in liver and 3) the irreversible loss of vitamin A over time [Furr, 1989]. Total body stores = $F \times dose \times (S \times a \times [(1/D{:}H)-1])$; where F is a factor for the efficiency of storage of an orally administered dose of vitamin A, which is estimated to be 0.5 on the basis of previous work [Bausch *et al* 1977]; the *dose* is the amount of isotope administered, expressed as μmol retinol equivalents; S is the assumed ratio of $[^2H_3]$ retinol to retinol in plasma to that in liver, taken as 0.65; a is the fraction of absorbed dose of deuterated retinol remaining in the liver reserves at the time of sampling; $D{:}H$ is the isotopic ratio of $[^2H_3]$ retinol to retinol measured in plasma; and -1 corrects for the contribution of the administered dose to the total body pool. The liver vitamin A concentration expressed as μmol/retinol/g liver was calculated by assuming liver weight is 0.024 of total body weight in non-obese subjects [Furr, 1989].

Limitations in these studies were first reported in early studies. Although the technique provides a good correlation between analysed hepatic reserves and calculated hepatic reserves in rats with low vitamin A stores, results are more

variable in rats with adequate stores [Huque, 1981]. The method is also limited by the assumptions of isotopic dilution, and the issues relating to equilibration. Labelled vitamin A can not truly equilibrate with body stores as unlabelled vitamin A is continually consumed within the diet and the time to reach equilibration may differ from individual to individual. This technique appears to provide a good estimate of hepatic reserves in populations however there is a wide prediction interval when used to assess reserves in individuals [Haskell *et al* 1997].

Relative dose response test.

The relative dose response test (RDR) was first described by Loerch and co-workers in rats [1979], and was proposed to be an indirect indicator of liver stores. The test is based on the findings that RBP accumulates in the livers of vitamin A deficient animals, and synthesis continues at a rate that exceeds secretion as holo-RBP. Vitamin A administration to a person with depleted stores results in an immediate release of retinol-RBP into the circulation, with a maximum increase in plasma retinol after about 5 hours. In the test, plasma is sampled at baseline, a dose of retinol is given orally in oil, and plasma is collected again five hours later. When liver stores are adequate (>20μg/g) no increase in retinol is observed. An increase in retinol in excess of 20% of baseline is considered a positive result, and indicates that vitamin A reserves are not adequate [Ross, 1999]. A modified RDR test, which only requires 1 blood specimen has been developed using 3,4, didehydroretinol as the test dose, however the limitation of the MRDR is that the specific retinol derivative is not commercially available.

The RDR test has been successfully applied in regions with a high incidence of poor vitamin A status; however the reliability in vitamin A replete populations is poor. It has also been observed that the test has poor within-subject reproducibility [Solomons *et al* 1990], and is compromised in states of PEM [Van den Berg, 1996] and infection. For instance the RDR test was useful in assessing vitamin A status of children recovering from pneumonia when CRP concentrations were <10mg/L but not when CRP concentrations were higher [Stephensen *et al* 2002]. In summary, these indirect measures appear to be

most accurate in assessing poor vitamin A status in populations, but have limitations when addressed vitamin A status in more replete individuals.

2.3.1.2 FUNCTIONAL AND PHYSIOLOGICAL MEASURES.

The ocular signs of abnormal dark adaptation are the basis of clinical assessment. Functionally rhodopsin is generated when the protein opsin in the rods of the retina combines with the cis-isomer of retinol. Clinically, measurements (electroretinograms) are available to measure directly the level of rhodopsin and its rate of regeneration [Underwood, 1990]. The problem with using this approach is that it simply measures deficiency in relation to clinical signs. By the time clinical signs manifest deficiency will have been undetected for a long period of time. It would be more beneficial to detect deficiency and prevent clinical manifestations.

Conjuctival impression cytology.

Conjunctival impression cytology (CIC) evaluates microscopically the number of globlet cells present and the morphological characteristics of epithelial cells, in the conjunctiva of vitamin A deficient individuals [Underwood, 1990]. Cells are transferred from the conjunctiva to filter paper with gentle pressure, stained, examined microscopically, and scored.

2.3.1.3 PLASMA RETINOL CONCENTRATIONS.

Plasma concentrations are the most common biochemical parameters used to assess vitamin A status of individuals and population groups. Limitations are well recognised for instance concentrations are under homeostatic regulation and it is assumed that plasma values only adequately reflect status (liver stores) at extremely low or high vitamin A intakes (figure 2.7). It is well documented that concentrations may be altered in the presence of sub-clinical infection [Stephensen, 2000]. One question therefore arises: in healthy (replete) individuals can plasma retinol concentrations be used to assess an individual's status (liver stores)?

Vitamin A status has been classified into categories with relation to retinol concentrations (table 2.4), critical cut-off points have been set at <0.7μmol/L,

generally accepted as indicating likely inadequate vitamin A status, and <1.05μmol/L, interpreted as probably responsive to greater intake of vitamin A [Ballew *et al* 2001]. Hypervitaminosis and vitamin A toxicity have been associated with increased fasting retinyl ester concentrations. Under normal dietary conditions retinyl ester concentrations are <5% of circulating vitamin A, but >10% in chronic vitamin A toxicity [Underwood, 1984].

Figure 2.7:

Relationship between liver stores and plasma retinol.
(Graph adapted from Ross *et al* 1999.)

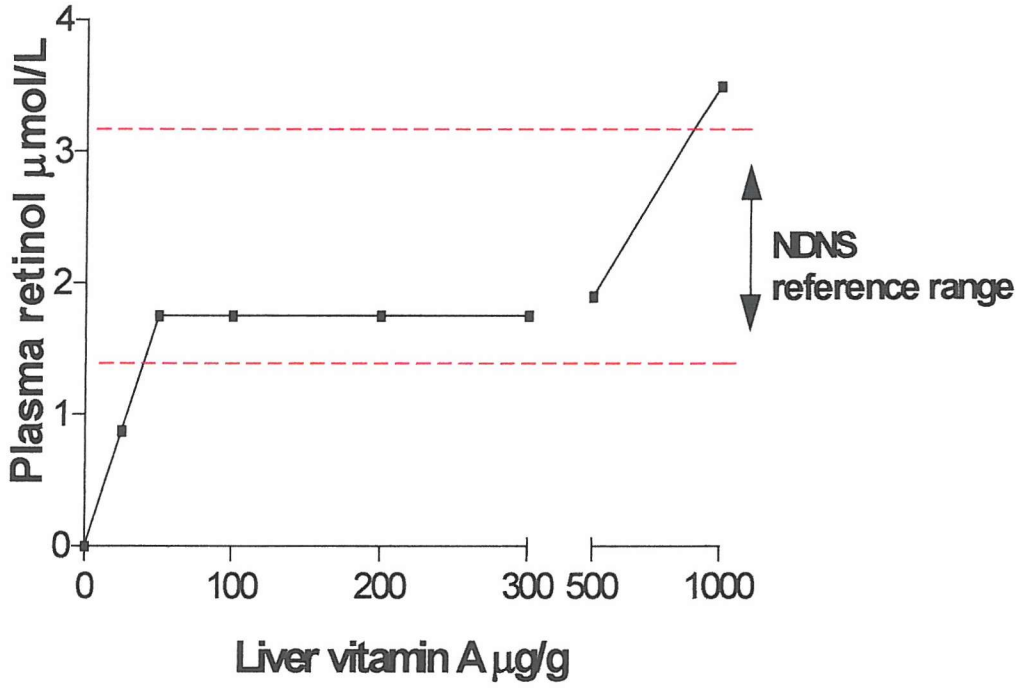


Table 2.4 :

Classification of Status

A: Adapted from Ross 1999.

	Plasma retinol concentrations µmol/l	**Liver stores µg/g**	**Clinical signs.**
DEFICIENCY	<0.35	Severely depleted (<5)	Ocular manifestations.
MARGINAL	0.35 – 0.7	Severely depleted (5-20)	None.
ADEQUATE	>0.7 – 3	~20-300	None.
EXCESSIVE	High normal – >3	High (>300)	Not apparent or mild.
TOXIC	>3.5	Very high in liver and increased in peripheral tissues.	Headache, bone pain, elevated liver enzymes in plasma and clinical signs of liver disease.

B: WHO classifications [WHO 1982].

Classification	**Plasma retinol concentration**
DEFICIENT	<0.35µmol/L
LOW	0.35-0.7µmol/L
ADEQUATE	>0.7µmol/L

The mean plasma retinol concentration seen in the population of UK adults was 2.2µmol/L, with 95% of the population ranging from 1.4-3.2µmol/L [NDNS; Gregory *et al* 1990]. The mean value for children aged 4-18years was 1.29µmol/L with 95% of the population ranging from 0.61-1.97µmol/L [NDNS; Gregory *et al* 2000]. The U.S. National Health and Nutrition Examination Surveys (NHANES) (1988-1994) reported plasma retinol concentrations ranging from 0.87-3.42µmol/L. Table 2.5, summarises plasma retinol concentrations of controls that have been reported in the literature. When considering results presented in the table, the mean plasma retinol reported in children were 1.33µmol/L (1.22-1.58µmol/L), which was significantly lower than that reported in adults (mean 2.06µmol/L; range 1.50-2.56µmol/L).

Two factors that may influence the concentration of circulating retinol are gender and age. The U.S. National Health and Nutrition Examination Surveys (NHANES) found that retinol concentrations are similar for preschool males and females, but a gender difference arises during the adolescent period and continues until approximately 65 years. During this period males have higher circulating retinol concentrations than females [Garry *et al* 1987; Green & Green 1994; Krasinski *et al* 1989]. A secondary observation, from a large population study, is that mean retinol concentrations reach a plateau (2.09umol/L). For men this occurs between the ages of 30-40 years and for females later at approximately 60 years [Garry *et al* 1987]. The British Survey of Adults (NDNS) found that men showed little variation in retinol concentrations with age and women had similar mean values across the age ranges, until 50-64 years when there was a significant increase. Across all age groups women had lower concentrations than those observed in men [Gregory *et al* 1990]. In conclusion it would appear that retinol concentrations increase significantly with age, and women have lower concentrations than men during early adulthood and middle age [Stephensen *et al* 2000]. The effect of infection on concentrations of retinol is discussed later in 2.4.1.5.

Table 2.5 : Plasma retinol concentrations reported in the literature.

Paper	Mean Plasma retinol μmol/L	
Garry et al 1987	2.12+/-0.46	51 males 60-93y
Garry et al 1987	2.07+-/0.43	58 females 60-93y
Garry et al 1987	2.12+-/ 0.38	40 males 20-45y
Garry et al 1987	1.76+/-0.35	54 females 20-45y
NHANES 1 report from Garry et al 1987	2.10	18-74y males
NHANES 1 report from Garry et al 1987	1.80	18-74 years females
NHANES 1 report from Garry et al 1987	1.23	Preschool children
Krasinski 1989 Supplement users	Mean 2.56+/-0.08 Median =2.51	65 males 60-98y
Krasinski 1989 Non supp users	Mean 2.45+/-0.04 Median = 2.35	211 females 60-98y
Krasinski 1989 Supplement users	Mean 2.50+/-0.05 Median = 2.44	170 females 60-98y
Krasinski 1989 Non supp users	Mean 2.50+-/0.11 Median = 2.37	57 males 19-59y
Krasinski 1989 Supplement users	Mean 2.33+/-0.13 Median =2.14	39 males 19-59y
Krasinski 1989 Non supp users	Mean 2.08+/-0.08 Median = 1.95	52 females 19-59y
Krasinski 1989 Supplement users	Mean 2.22+/-0.09 Median = 2.01	46 females 19-59y
Johnson et al 1992b	1.79 +/-0.07	50 subjects 23-74y
Johnson et al 1992a	1.8+/-0.1	17males 35-39y
Johnson et al 1992a	1.5+/-0.1	11 females 30-36y
Johnson et al 1992a	2.0+/-0.2	11 males 66-70y
Johnson et al 1992a	1.8+/-0.1	20 females 62-66y
Zaman 1993	2.28 (1.26-2.99)	20 subjects 23-55y
Zaman 1993	2.13 (0.94-3.99)	20 subjects 77-89y
Reinersdorff et al 1996	1.88 +/- 0.39	11 males 24-40y
Olson 1996 In :Sight and Life Manual McLaren D.S.	1.28 (0.73-2.0)	725 males 689 females 3-5y
Olson 1996 In :Sight and Life Manual McLaren D.S.	1.31 (0.84-1.9)	930 males 927 female 6-11y
Olson 1996 In :Sight and Life Manual McLaren D.S.	1.58 (1.0-2.3)	1026 males 1009 females 12-17y
Olson 1996 In :Sight and Life Manual McLaren D.S.	1.94 (1.2-2.9)	2164 males 4871 females 18-44y
Olson 1996 In :Sight and Life Manual McLaren D.S.	2.20 (1.3-3.3)	2911 males 3200 females 45-74y
Olmedilla B et al 2001	2.15 (0.7-3.55)	175 males 25-45y
Olmedilla B et al 2001	1.91 (1.07-3.3)	174 females 25-45y

Can plasma retinol concentrations be improved with supplements?

Wald and colleagues conducted a randomised double blind trial to investigate if vitamin A supplementation increases concentrations of retinol [Wald *et al* 1985]. A total of 376 people were studied and allocated to one of 7 regimens, covering doses from 0 – 36000IU of retinyl palmitate daily (0-10.8mg retinol). They found a small but statistical increase in retinol concentrations associated with supplementation. On average for every 10,000 IU of retinyl palmitate per day retinol concentrations increased by 13μg/L (0.05μmol/L) after 3 months (2% increase) and 12μg/L (0.04μmol/L) after 6 months (2% increase) [Wald *et al* 1985]. They noted that concentrations showed the greatest increase in subjects with the lowest retinol concentration at the start of the trial. Although retinol concentrations increase significantly changes were modest, and may simply reflect those patients with low retinol concentrations have responded well due to an initial dietary deficiency. Willet and colleagues [1983], compared retinol concentrations in 15 supplemented subjects taking 25,000IU of retinyl palmitate per day (7.5mg retinol) with 15 control subjects who took placebo for 16 weeks. Mean retinol concentrations increased by 23μg/L (0.08μmol/L) after 8 weeks of supplementation, it was concluded that supplements had no appreciable effect on plasma retinol concentrations [Willet *et al* 1983]. In summary it would appear that supplements have modest effects on plasma retinol concentrations and the greatest rises are seen in patients with low concentrations.

A number of studies have found greater increases in plasma retinol concentrations in patients with initially low concentrations. Willet and co-workers demonstrated an increase in mean retinol levels of 45μg/L (0.15μmol/L) in 16 subjects who took 10,000IU of vitamin A per day for 4 weeks compared to 19 patients who took placebo. Supplementation was most effective in subjects with initially low serum retinol concentrations [Willett *et al* 1984]. One trial that assessed supplementation (5.5mg retinyl palmitate) in well-fed women with lower than usual blood concentrations of retinol found plasma retinol concentrations were 9% higher in the supplemented group compared to the placebo group [Willett *et al* 1984]. Aside from using supplements to improve vitamin A status the potential toxic effects of increased intake should be considered. Wald and co-workers found that 5% of subjects allocated to

regimens of 22500IU retinyl palmitate or more (>6.27mg retinol), reported symptoms of toxicity, skin dryness and itching compared to only 0.6% in subjects taking less vitamin A [Wald *et al* 1985].

In summary, data relating to the use of supplements is limited and confusing and results may reflect other factors as well as that of supplement use. Some reports describe no effect of vitamin A supplementation on retinol concentrations, while others report increases in retinol concentrations in subjects with initially low concentrations and one report describes signs of toxicity after dosing over 6mg of retinol. However when dietary supply of vitamin A is poor there appears to be benefit of supplementation and the use of intervention programmes to prevent vitamin A deficiency are well recognised as being beneficial in the developing world.

In conclusion it would appear that it is hard to accurately measure vitamin A status, particularly as the only direct measure is of liver stores. First, inaccuracy arises from inappropriate use of terms, for instance the term vitamin A status is often used to reflect that liver stores are adequate to maintain function. However low concentrations of retinol are often interpreted to reflect poor vitamin A status, which in some cases may be true but this may not always be the case. It would appear that methods used to indirectly assess status are most accurate when assessing vitamin A status in deficient populations, but the accuracy of assessing vitamin A status in replete individuals is less clear.

2.4 HOW ARE THE PROCESSES BY WHICH VITAMIN A IS HANDLED ALTERED BY DISEASE?

This section will review how the normal biological processing of vitamin A could be altered in situations of disease and infection. The section is divided into two main parts; the first part will discuss in general terms how disease and infection may influence the processing of vitamin A, and the second part will focus specifically on the disease CF, a multi-factorial disease in which a number of factors may effect the processing of vitamin A.

2.4.1 ALTERED PROCESSING: GENERAL OVERVIEW

A number processes and organs systems are involved in the whole body handing of vitamin A, it would therefore follow that a number of diseases may effect the processing of vitamin A, for example, malnutrition, GI disease, liver disease, renal disease and infection. This section will review the literature with respect the effect of these factors on the processing of the vitamin.

2.4.1.1 DIETARY SUPPLY.

It is well documented that individuals with a poor dietary supply of vitamin A (vitamin A deficiency), suffer from night blindness and other visual consequences. Vitamin A deficiency associated with malnutrition also affects other functions of vitamin A, for instance the immune response. Children with vitamin A deficiency are at an increased risk of respiratory disease, and more vulnerable to diarrhoea and severe measles [Calder & Jackson, 2000].

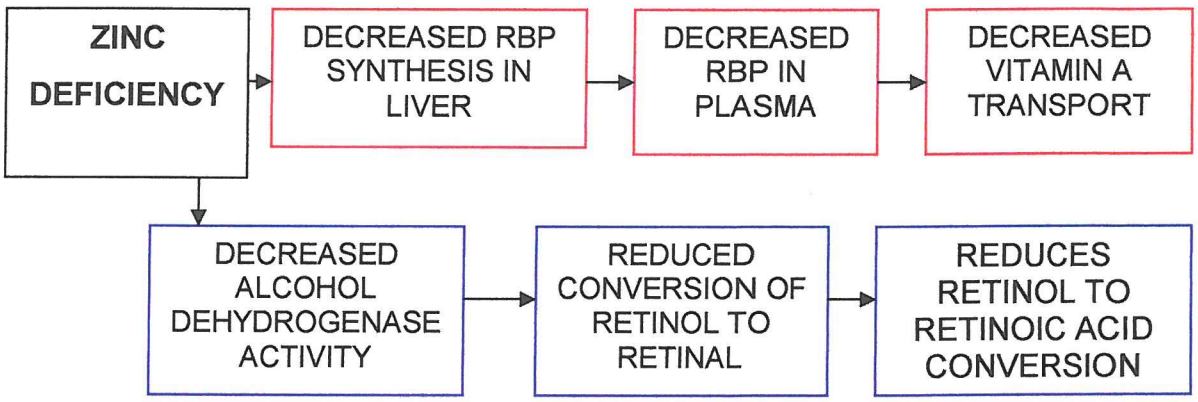
Along with an adequate supply of vitamin A, there are two main components of the diet that may affect the vitamin A status of an individual, protein and zinc. Protein is required for synthesis of RBP, in order that retinol can be mobilised from the liver. Vitamin A metabolism is dependant on zinc, as zinc is required for; 1) the oxidative conversion of retinol to retinal which requires the action of zinc dependent retinol dehydrogenase enzyme and 2) for the hepatic synthesis of RBP [Smith *et al* 1972]. Increased concentrations of vitamin A have been found in the livers of zinc deficient rats fed a vitamin A supplemented

or adequate diet, the addition of zinc to these diets allowed the mobilisation of retinol from the liver stores [Solomons *et al* 1980]. A lack of zinc can therefore effect vitamin A processing at two levels, the liver and functional cell (figure 2.8).

The beneficial effects of increased dietary supply, by supplementation initiatives, in malnourished populations are well accepted. Vitamin A supplementation has been shown to decrease morbidity and mortality in children with vitamin A deficiency [Sommer *et al* 1986]. It is not clear if increased dietary supply in instances of adequate intake is beneficial and requires further investigation.

Figure 2.8:

The two hypotheses that relate zinc deficiency with vitamin a metabolism.



2.4.1.2 AVAILABILITY OF VITAMIN A ACROSS THE GI TRACT.

It has previously been discussed that retinol appears to be efficiently absorbed under normal physiological conditions, however evidence suggests that disease and infection may affect the availability of the vitamin. A number of methods have been used to assess availability of vitamin A during disease and infection. These include, the vitamin A absorption test, gross balance studies of an oral bolus dose, gross balance studies using radioactive tracer, and postprandial excursions of retinyl palmitate following an oral dose. Table 2.6 presents a summary of the current literature on availability of vitamin A in disease, and table 2.7 summaries availability of vitamin A during infection. Evidence suggests that in diseases associated with impaired lipid handling, availability of vitamin A

from the GI tract is, to some extent, disturbed. For example studies that employed the vitamin A absorption test found impaired availability in patients with celiac disease, and CF. This is also supported by data from gross balance studies which suggest that apparent absorption is impaired during steatorrhoea, and CF, although results are highly variable and one study found essentially complete absorption of retinol in patients with CF [Halford, 1993], this data will be discussed in more detail in section 2.4.3.3. These apparently increased losses in stool and decreased apparent absorption of retinol in diseases of impaired lipid handling are also associated with lower postprandial excursions of retinyl esters. In one study investigating the postprandial response to an oral bolus dose of retinyl palmitate in patients with GI disease, Johnson and co-workers [1992b], found that patients with GI disease without lipid malabsorption (peak rise 349 ± 122nmol/L; AUC 855 ± 318nmol/h/L) and patients with GI disease and lipid malabsorption (peak rise 171 ± 40nmol/L; AUC 421 ± 116nmol/h/L) both had lower peak rises and AUC of retinyl esters than the controls (peak rise 787 ± 40nmol/L; AUC 2015 ± 124nmol/h/L).

The reports within the literature (table 2.7) conclude that patients with infection have an impaired availability of vitamin A, irrespective of steatorrhoea. One study that employed the vitamin A absorption test [Katsampes *et al* 1944] found impaired absorption during infection with Giardiasis, caused by a parasitic protozoan in the small intestine. Studies that used gross balance, found variable results, ranging from 70.2% - 99.7% apparent absorption [Sivakumar *et al* 1973; Ahmed *et al* 1992; Silvakumar *et al* 1975; Reddy *et al* 1960]. However when this literature is compared to the absorption of retinol in healthy individuals as determined by gross balance (table 2.1 mean range 66.8-100%) it is not dis-similar (mean range 70.2-99.7%). It could therefore be interpreted that infection does not affect the apparent absorption of retinol.

2.4.1.3 LIVER PROCESSING OF VITAMIN A.

As previously discussed the liver is the main storage organ for vitamin A, and retinol-RBP is mobilised from the liver to make vitamin A available to meet the needs of cells and tissues. Low concentrations of retinol seen in patients with CF, and primary biliary cirrhosis (discussed in section 2.4.1.5), have been

attributed to in part disturbed liver processing. Low concentrations of retinol could result from two potential factors, 1) a failure to mobilise retinol from the liver and / or 2) low stores of vitamin A in the liver. A failure to mobilise retinol from the liver has been described in protein malnutrition [Smith *et al* 1973]. Availability of protein for RBP synthesis has been considered a possible factor for poor mobilisation, for instance if dietary intake is low or absorption poor; there will be a reduced availability of protein for RBP synthesis [Shetty *et al* 1979]. Studies have described decreased RBP concentrations in the acute stage of Kwashiorkor, which rise after appropriate re-feeding [Ingenbleek *et al* 1975]. Low liver stores of vitamin A have been reported in alcoholic liver disease and in children with cholestasis [Leo *et al* 1982; Amedee-Manesme *et al* 1985]. However patients with primary biliary cirrhosis have a tendency towards increased hepatic vitamin A concentrations compared to controls [Nyberg *et al* 1988]. Nyberg and colleagues concluded that the apparent inverse relationship between liver vitamin A and serum RBP concentrations suggests a disease-associated block in the release mechanism of vitamin A. It has been recognised that infection may play a role in the apparent constraint in mobilisation of vitamin A. For instance activation of the acute phase response may cause a shift in liver priorities to produce proteins required to fight infection over those required for transport e.g. RBP. These observations raise questions concerning recommended prophylactic supplement and the potential of toxicity [Nyberg *et al* 1988].

When considering vitamin A toxicity, rat studies have shown that vitamin A status of the liver plays an important role in liver fibrogenesis. While dietary vitamin A shortage does not promote liver fibrogenesis, high levels of vitamin A have the potential to increase systemic and hepatic toxicity, and liver fibrosis patients may have enhanced susceptibility to adverse effects [Vollmar *et al* 2002]. The majority of data in vitamin A toxicity is from case studies. One case of stellate cell hyperplasia, fibrosis, and portal hypertension has been reported in a patient with renal failure who was receiving therapeutic doses of vitamin A (4000IU day) [Doyle *et al* 2000]. Other instances have been reported in individuals taking high dose supplements. For example a 62-year-old male presented with protein malnutrition and abnormal liver function had ingested

40000-50000IU vitamin A per day for 7 years [Weber *et al* 1982]. The subject had massive amounts of vitamin A in liver tissue (19999IU/g), although plasma retinol and RBP concentrations were below normal, and serum RBP was not saturated. One other case reported an adult presenting with respiratory symptoms caused by hepatic hydrothorax secondary to vitamin A intoxication (270000IU/d) [Miksad *et al* 2002].

Table 2.6:

Vitamin A availability in disease: a summary of the literature.

DISEASE	APPARENT ABSORPTION %	APPROACH & DOSE	REF
Celiac disease	Impaired compared to controls	Vitamin A absorption test.	Chesney *et al* 1934
Celiac disease	Impaired compared to controls	Vitamin A absorption test. 7000 U.S.P units /kg body wt.	Breese *et al* 1939
Celiac disease	Poor compared to controls	Vitamin A absorption test. 7000 U.S.P units /kg body wt.	Katsampes *et al* 1944
Celiac disease	Impaired compared to controls	Vitamin A absorption test. Retinyl palmitate in oil.	Rassmusen *et al* 1986
Cystic Fibrosis	Impaired compared to controls	Vitamin A absorption test. Retinyl palmitate in oil.	Rassmusen *et al* 1986
Steatorrhoea	67.7% (85.7-29%)	Gross balance. 37000 U.S.P units retinol in water miscible preparation.	Barnes *et al* 1950
Steatorrhoea	43.4% (68.9-19.8%)	Gross balance 50000 U.S.P units R-palmitate in oil preparation.	Barnes *et al* 1950
Cystic Fibrosis	60% (91.3-5%)	Gross balance.	Ahmed *et al* 1990
Cystic Fibrosis	98.7% (99.8-83%)	Gross balance. Habitual intake mean 2023μg/d	Halford *et al* 1993
GI disease & lipid malabsorption	Patients had lower rise in plasma retinyl esters compared to controls	Postprandial excursions following 3.4mg retinyl palmitate in oil.	Johnson *et al* 1992b
GI disease without lipid malabsorption	Patients had lower rise in plasma retinyl esters compared to controls	Postprandial excursions following 3.4mg retinyl palmitate in oil.	Johnson *et al* 1992b
Pancreatic insufficiency	Patients had lower rise in plasma retinyl esters compared to controls	Postprandial excursions following 3.4mg retinyl palmitate in oil.	Johnson *et al* 1992b

Table 2.7:

Vitamin A availability during infection: a summary of the literature.

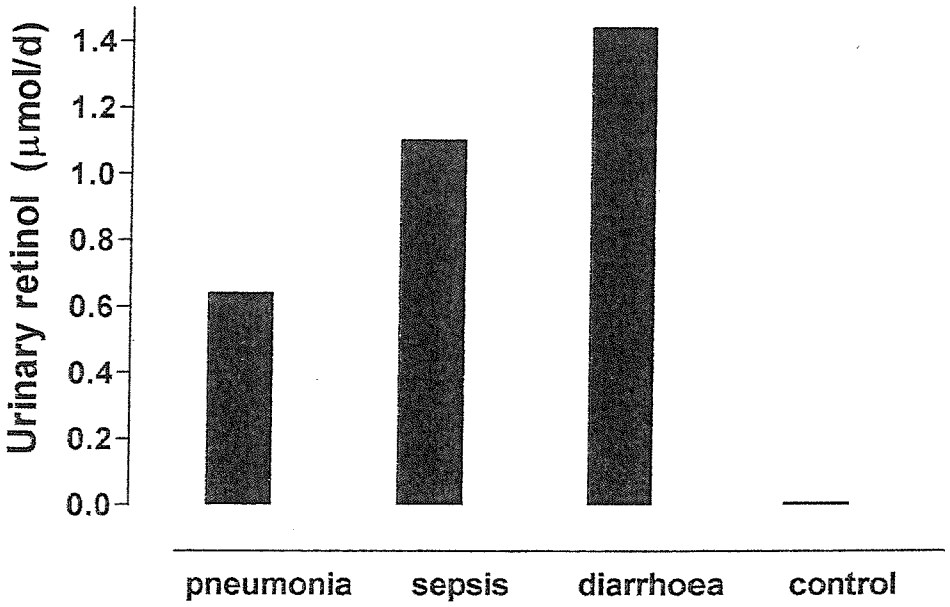
DISEASE	APPARENT ABSORPTION %	APPROACH & DOSE	REFERENCE
Infection	74.3±6.83%	Gross balance. Labelled vitamin A, 4μCi of 11,12—$^{3}H_2$ retinyl acetate	Sivakumar et al 1973
Gastroenteritis with acute diarrhoea	70.2%	Gross balance. Labelled vitamin A, 4μCi of 11,12—$^{3}H_2$ retinyl acetate	Sivakumar et al 1973
Ascariasis	99.7%	Gross balance. 12mg retinyl palmitate.	Ahmed et al 1992
Ascariasis	80.1±0.46%	Gross balance. 4μCi of 11,12—$^{3}H_2$ retinyl acetate	Sivakumar et al 1975
Respiratory infection	74.3±6.83	Gross balance. Labelled retinol acetate and 3000IU unlabelled retinol acetate.	Reddy & Sivakumar 1972
Diarrhoea	70.2±5.92	Gross balance. Labelled retinol acetate and 3000IU unlabelled retinol acetate	Reddy & Sivakumar 1972
Giardiasis	Impaired compared to controls	Vitamin A absorption test. 7000 U.S.P units of vitamin A in oil per kg body weight.	Katsampes et al 1944

2.4.1.4 EXCRETION OF VITAMIN A.

Two studies have investigated the excretion of retinol:RBP in urine during infection. Data (figure 2.9) indicates that patients with pneumonia, sepsis and diarrhoea excrete variable but significant amounts of retinol in urine (geometric mean 0.64μmol/d; 1.10μmol/d; 1.44μmol/d retinol respectively), compared to trace amounts in healthy controls (0.0016μmol/d; 0.00±0.001μmol/d retinol) [Stephensen *et al* 1994; Alvarez *et al* 1995]. Results from 29 patients with pneumonia or sepsis, found 24% of patients excreted greater than 3.49μmol/retinol/day and 34% of patients excreted greater than 1.75μmol/retinol/day [Stephensen *et al* 1994]. The lowest excretion of retinol was approximately 1μmol/d. Assuming a habitual daily intake of 1mg of retinol (3.49μmol/d) these losses could represent 100%, 50% and 28.6% loss of daily

intake respectively. These studies did not detect RBP in the urine of controls but did detect RBP (geometric mean 2.8μmol/d) in the urine of 59% of the infected patients [Stephensen *et al* 1994]. The only study that used labelling techniques [Reddy & Sivakumar 1972] found that 23.3±2.96% and 16±3.24 % of absorbed labelled retinol was excreted in the urine of patients with respiratory infection and diarrhoea respectively. The contribution of the excretion of retinol during infection could therefore be a major loss.

Figure 2.9:
Graph depicting the loss of retinol in urine during infection.
(adapted from data of [Stephensen *et al* 1994; Alvarez *et al* 1995).



2.4.1.5 PLASMA CONCENTRATIONS OF RETINOL.

Evidence from the current literature suggests that plasma concentrations of retinol are lower in diseases that involve defective lipid absorption or transport, in pyrexia, during infection, and liver disease, when compared to healthy individuals. For example, retinol concentrations have been reported to be lower in patients with CF, crohn's disease, pancreatic insufficiency, alcoholic induced pancreatitis, HIV, primary biliary cirrhosis, and liver disease [Underwood 1971; Main *et al* 1983; Dutta *et al* 1982; Marotta *et al* 1994; Semba *et al* 1993; Phillips *et al* 2001; Nyberg *et al* 1988; Ukleja et al 2002]. If low retinol concentrations reflect an inadequate availability of retinol for the functional cell, low

concentrations will have functional consequences. For instance vitamin A deficiency is known to affect functions of vision, cellular differentiation, gene expression, and immune competence. In particular impaired cellular differentiation alters epithelial integrity and can affect all epithelial surfaces including the eye, skin, salivary gland, respiratory and GI tract [McCullough *et al* 1999].

Low concentrations of retinol are often interpreted as poor vitamin A status, in that inadequate vitamin A is available to meet functional needs, however this may not be accurate during inflammation and infection. Several researchers have documented the effect of the acute phase inflammatory response on retinol concentrations in subjects without dietary deficiency. Studies have shown a negative association between acute phase proteins (e.g. C-reactive protein (CRP), α_1-antichymotrypsin (ACT) and α_1-acid glycoprotein (AGP)) and retinol concentrations [Semba *et al* 2000; Filteau *et al* 1993; Christian *et al* 1998b; Filteau *et al* 1995 Stephenson *et al* 2000; Rosales *et al* 2000; Paracha *et al* 2000]. In particular several reports have observed a transient decrease in plasma retinol in response to infection that returns to normal concentrations on recovery [Duggan et al 1996; Frieden *et al* 1992; Mitra *et al* 1998]. The presence of infection could therefore lead to the misclassification of an individual's retinol concentration. The actual mechanism related to this phenomenon is unclear, but both increased metabolic requirements and an acute phase shift in hepatic protein synthesis are presumed to play a role. Whether this mechanism is of benefit or detriment to the host or if episodes of acute infection deplete liver stores of vitamin A has not been established.

One question therefore arises; is it beneficial to supplement vitamin A to patients with low concentrations of plasma retinol? Low retinol concentrations have been shown to have detrimental effects during the measles infection. Children with low concentrations of vitamin A have more severe forms of measles, with higher fever, prolonged fever episodes and an increased rate of hospitalisation and supplementation of vitamin A reduces measles mortality and morbidity significantly [Gerster, 1997]. However vitamin A is known to drive the immune system towards a Th-2 dominated immune response, and a lack of

retinol switches the system towards a Th-1 dominated immune response. It is not accurately known which response is beneficial to the host. The decrease in plasma retinol seen during infection, driving a Th-1 type response, maybe beneficial to the host in respiratory infections. Observations report that high dose vitamin A supplementation in the face of respiratory infections and pneumonia in children is not beneficial and actually produces adverse effects [Stephensen *et al* 1998; Bresee *et al* 1996]. Adverse effects included longer duration of clinical signs, longer hospital stays, and a greater need for supplemental oxygen. These adverse effects may be caused by the high dose vitamin A supplements enhancing some aspects of immunity mediated by Th-2 cells, potentially increasing the severity of lung inflammation. Or a second possibility is that the vitamin A regimen produces toxic side effects, by damaging endothelial cells, resulting in increased effusion of fluids into the alveoli, thus impairing oxygen diffusion into the blood.

2.4.2 CYSTIC FIBROSIS.

CF represents a condition in which pancreatic insufficiency, impaired lipid handling, recurrent pulmonary infections and liver disease may all affect the biological processing of vitamin A. This section will first briefly introduce the disease CF, and secondly describe the evidence from the literature associated with the handling of vitamin A in CF.

2.4.2.1 CF GENE

CF is the most common fatal inherited autosomal recessive disease. It affects approximately 1 in 2000 live births and 1 in 25 individuals carry the CF gene. The disease is characterised by abnormalities in fluid and electrolyte transport in exocrine epithelia, and results in the accumulation of sticky tenacious mucus in relation to the epithelial surfaces in many organs particularly the lungs, GI tract, pancreas and liver [Sheppard & Nicholson 2000].

In 1985 the CF defect was identified on chromosome 7 [Knowlton *et al* 1985], and the CF gene was later identified and named the CF transmembrane conductance regulator (CFTR) [Riordan *et al* 1989]. The primary physiological defect related to CFTR, is that of reduced chloride conductance at the apical

surface of the epithelia [Quinton, 1986]. The CFTR gene encompasses about 250kb of genomic DNA. The most common mutation, delta F 508 (ΔF508), found in about 68% of CF chromosomes, is a deletion of three base pairs that corresponds to the loss of a phenylalanine residue at position 508 of the protein [Davis *et al* 1996]. Since the discovery of the ΔF508 mutation, more than 400 other mutations have been identified [Davis *et al* 1996].

2.4.2.2 CLINICAL IMPLICATIONS

There are a number of clinical implications of the defective CFTR gene in patients with CF; these include alterations in normal functions of the intestine, respiratory tract, pancreas and liver. These will be discussed in more depth in the following section.

PANCREAS

The importance of the involvement of the pancreas has been long known in patients with CF [Anderson, 1939]. Patients suffering from pancreatic insufficiency secrete inadequate amounts of lipolytic enzymes (lipase), proteolytic enzymes (trypsin, chymotrypsin), estrerases and bicarbonate [Davidson, 2000]. This in turn affects digestion in the GI tract, as acid in the stomach is not adequately neutralized and pancreatic enzymes are unable to act optimally. Therapy of pancreatic insufficiency in CF patients is based on oral pancreatic enzyme replacement therapy (PERT).

GASTROINTESTINAL TRACT

In the GI tract the gene defect, related to abnormal ion transport, is associated with deceased intestinal fluid and electrolyte transport, thick, viscoid secretions and decreased duodenal pH [Davidson, 2000]. Alterations in the functions of the GI tract can result in meconium ileus at birth and intestinal obstruction later in life [Dalzell *et al* 1990a]. Other observed functional defects in the intestinal tract include abnormalities of absorption, permeability [Dalzell *et al* 1990b], gastric emptying [Cucchiara *et al* 1996], transit time [Bali *et al* 1983] and motility. Further factors that influence the function of the gut are the interaction of other pathologies for instance the degree of pancreatic insufficiency affects the ability of the GI tract to digest and absorb intestinal contents. Evidence has found that

despite PERT patients with CF continue, to some extent, to maldigest and malabsorb lipid, and lipid losses remain elevated [Murphy *et al* 1991].

LIVER

The involvement of the liver in CF has been recognised for over 50 years [Anderson, 1938]. CFTR is localised to the epithelial cells lining the biliary ducts in the liver. These cells in healthy individuals secrete chloride and water follows passively allowing bile acids, and proteins in the bile to stay soluble. In the absence of a functional CFTR these ducts become plugged with secretions which can lead to obstructive cirrhosis [Davis *et al* 1996]. Data collected prospectively has shown that chronic liver disease is negatively correlated with survival and is an important disease predictor outcome in CF [Hayllar *et al* 1997].

AIRWAYS AND LUNG

Respiratory disease is the cause of most mortality in patients with CF, however mechanisms underlying the initiation of lung disease and respiratory morbidity in CF are poorly understood. The CF lung is regarded as being essentially normal in-utero however airway inflammation is present in early life, even before the onset of infection. Inflammatory cells such as neutrophils and IL8 have been detected in the bronchoalveolar lavage (BAL) during the first few months of life [Khan *et al* 1995]. The progression of respiratory disease appears in part to be due to recurrent infections that elicit an immune response which becomes persistent and self sustaining.

IMMUNOLOGY

CF is a chronic infective disease in which the immune system appears to be ineffective in regaining sterility after an infective episode [Doring *et al* 2000]. The basic defect in CF predisposes the individuals to chronic bacterial airway infections particularly with *Staphylococcus aureus*, *Haemophilus influenzae*, *Pseudomonas aeruginosa* and *Burkholderia cepacia*. Each infective episode compromises lung function of the individual and adds another cycle of injury to the host defence mechanisms [Doring *et al* 2000]. Pulmonary infection and

neutrophil dominated inflammation are the main causes of morbidity and mortality in CF [Konstan *et al* 1994].

The high concentrations of immune complexes produced as a result of the immune response have both beneficial effects, in achieving a host–parasite equilibrium, but can also be harmful and contribute to lung pathology [Doring *et al* 2000]. High immune cell concentrations have been correlated with poor clinical status in patients with CF [Van Bever *et al* 1988; Dakin *et al* 2002]. In particular several studies have revealed that high concentrations of subclasses IgG_2 and IgG_3 correlate with poor lung function [Pressler *et al* 1988; Pressler *et al* 1990]. Neutrophils rather than macrophages dominate the inflammatory cell infiltrate in inflamed CF airways. Neutrophil activation is an important part of the immune response to infection however in CF this may be harmful to the host. The reactive oxygen species that are produced from respiratory burst are toxic to the host, as reduced free radical trapping, and altered antioxidant status have been reported in CF patients [Portal *et al* 1995; Langley *et al* 1993]. Along with this, neutrophils normally die by apoptosis and the failure of this in the CF airway may result in necrotic cell death with the release of the intracellular material of the cell. Investigators have detected neutrophil enzymes in the BAL and the respiratory epithelia lining fluid of CF patients [Birrer *et al* 1994; Fink *et al* 1984]. Cytokine expression is also increased during the activation of the inflammatory system in patients with CF. Some studies show elevated cytokine concentrations, particularly TNFα, IL1, IL6 and IL8, in the serum and BAL of CF patients with infection [Rosenfeld *et al* 2001; Bonfield *et al* 1995; Wolter *et al* 1999; Norman *et al* 1991; Greally *et al* 1993] along with decreased concentrations of the pro-inflammatory cytokine IL10 [Bonfield *et al* 1995]. Consequences to the host may also occur if the inflammatory system remains chronically activated with these high cytokine concentrations.

In summary, CF is a multi-organ genetic disease in which the gene defect CFTR plays a major role in the patho-physiology of the disease. CF is characterized by abnormalities in fluid and electrolyte transport in exocrine epithelia. This has clinical implications and affects the normal functions of the pancreas, GI tract, liver and respiratory tract. As CF is a chronic infective

disease, individuals are predisposed to recurrent airway infections, and respiratory disease is the greatest cause of mortality in these patients. One model found that forced expiratory volume (FEV), short stature, high white cell count and chronic liver disease in CF are all negativity correlated with survival [Hayllar *et al* 1997].

2.4.3 CF - EFFECT ON PROCESSING OF VITAMIN A

2.4.3.1 DIETARY INTAKE.

Before the development of enteric-coated enzymes in the mid 1980s patients with CF were advised to follow a low fat diet to minimise steatorrhea [Dowsett, 2000]. It may therefore follow, at this time that restricted low fat diets were associated with diets low in vitamin A. Current advice however is that patients with CF are recommended to consume a diet high in energy, and fat, providing 120-150% of the recommended energy intake [CF trust consensus report, 2002]. In a group of 30 patients with CF reported energy intakes were 34% greater than that reported by controls when energy intakes were expressed relative to body weight [Ellis *et al* 1992]. However the reported mean energy intake of the CF patients was 92% of the RDA and only three patients reported intakes in excess of 120% RDA [Ellis *et al* 1992].

As already discussed (section 2.2.10) there is little knowledge relating to the actual requirements of vitamin A in healthy individuals, and even less information relating to requirements in patients with CF. Losses of the vitamin are not well documented and although it may be rational that CF patients have increased demands of the vitamin and therefore increased requirements there is no evidence to support this. Despite a poor evidence base, it currently is recommended that all patients with CF who are pancreatic insufficient require supplementation of vitamin A [Ramsey *et al* 1992; Congdon *et al* 1981; Littlewood & Wolfe 2000; Anderson *et al* 1939; Peters & Rolles 1993], and vitamin A (as plasma retinol) should be reviewed annually to monitor the risk of vitamin A deficiency and adjust supplementation accordingly [Ramsey *et al* 1992]. This routine prophylactic supplementation of vitamin A is used in an attempt to increase plasma retinol concentrations, and overcome the widely held belief that availability of vitamin A is compromised [Durie *et al* 1989; Eid *et*

al 1990]. However the true requirement for vitamin A has not been established within this group of patients and there are considerable differences in the doses recommended and prescribed in clinical practice [Peters *et al* 1993].

2.4.3.2 EFFECT ON LUMINAL PROCESSING OF RETINYL ESTERS

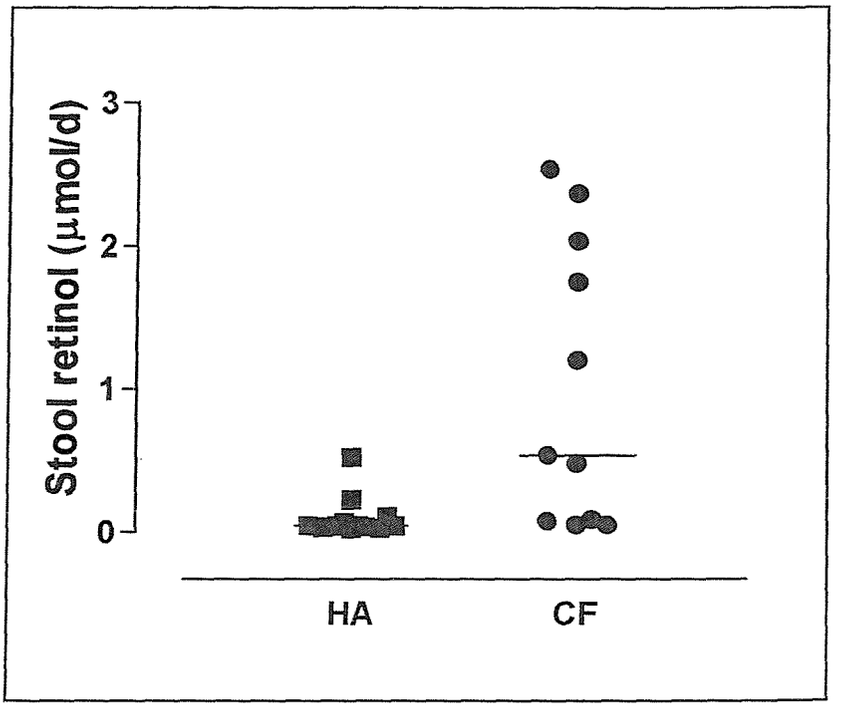
As described earlier (section 2.2.2) the luminal processing of retinyl esters requires adequate digestion, emulsification and solubilisation of retinyl esters, however in CF these processes may be compromised. A large number of patients suffer from pancreatic insufficiency causing a decrease in the secretory capacity of digestive enzymes, bicarbonate, and bile. A lack of digestive enzymes can lead to maldigestion and malabsorption of lipid. It is a well-held belief that patients with CF have reduced concentrations of retinol due to impaired processing within the GI tract related to a reduction in pancreatic digestive enzyme secretion [Rausmussen *et al* 1986]. In CF, insufficient amounts of bile and pancreatic enzymes may interfere with the process of retinyl ester digestion within the GI tract.

2.4.3.3 AVAILABILITY OF VITAMIN A ACROSS THE GI TRACT.

Despite the belief that availability of vitamin A is limited in CF, there is only a small amount of literature which has assessed availability across the GI tract. Two studies have used gross balance techniques to estimate the mean apparent absorption of retinol as 60% (91.3%-5%) and 98.7% (99.8%-83%) [Ahmed *et al* 1990, Halford *et al* 1993]. From these studies it would appear that some but not all, patients exhibit elevated losses of total retinol (median 0.54μmol/d; range 0.05-2.53μmol/L) in the faeces compared to that seen in healthy controls (median 0.04μmol/d; range 0.02-0.23μmol/d) [Ahmed *et al* 1990] (figure 2.10). These studies are small, limited in patient numbers and confounded by the limitations associated with gross balance techniques. Further studies are required to investigate the amount of vitamin A lost in the stool specifically from dietary supply and whether losses are associated with the maldigestion or malabsorption of vitamin A.

Figure 2.10:

Graph depicting the loss of retinol in the stool (μmol/L) in both control subjects and patients with CF. (Ahmed et al 1990).



Only one study in CF has investigated the postprandial excursions of retinyl esters [James *et al* 1992]. On average patients with CF had lower AUC (median 203nmol/L/h; range 67-625nmol/L/h) when compared to controls (median 1102nmol/L/h; range 650-1681nmol/L/h).

2.4.3.4 LIVER – STORAGE AND MOBILISATION.

From measurements of liver vitamin A at autopsy, retinyl ester concentrations were 2.5 times higher (156μg/g) in the livers of children with CF when compared to control subjects (57μg/g) [Underwood, 1972]. Liver stores appear to be increased but plasma retinol concentrations appear low (2.5 times lower) in comparison with controls and may suggest a defect in mobilisation or transport of the vitamin from the liver in CF [Underwood, 1971]. It remains unclear as to the effect that infection may have on the storage of retinyl esters in the liver in CF. This information raises the question as to the appropriateness of prophylactic vitamin A supplementation in this group of patients and the

potential risk of toxicity. There are no studies that directly investigate the process of mobilisation of retinol from the liver in CF.

Patients with CF, supplemented with oral vitamin A adequate to maintain hepatic stores, still have significantly lower plasma concentrations of retinol, and RBP when compared to controls [Smith *et al* 1972]. During infection, defective mobilisation of retinol from the liver has been suggested to be due to an acute phase shift in hepatic protein synthesis of RBP [Duggan *et al* 1996]. As a consequence retinol-RBP is unable to be mobilized from the liver and retinol accumulates in the liver and concentrations fall in the plasma [Underwood *et al* 1972]. A number of investigators have reported retinol and RBP concentrations in the plasma of CF patients (table 2.8). Some of the studies report low retinol and RBP concentrations compared to controls, however caution should be taken during interpretation as limited markers of inflammation have been measured. Only one investigator has measured CRP concentrations concurrently with plasma retinol concentrations in CF. They reported patients with low CRP concentrations had normal retinol concentrations in plasma [Huet *et al* 1997].

In summary the mechanism of mobilisation of vitamin A from liver stores requires further investigation, especially with respect to the effect of the acute phase response. This data would provide an evidence base to better inform clinical practice with relation to correcting apparently low concentrations. Plasma retinol concentrations and factors that may be acting to affect them in disease will be discussed in greater depth in section 2.4.3.5.

2.4.3.5 CIRCULATING RETINOL CONCENTRATIONS.

It is well accepted that plasma retinol concentrations are often low in patients with CF, but the mechanisms that contribute to this and the clinical outcomes of low retinol concentrations are less well understood. One study reports that low concentrations of retinol continue to be reported even in patients who eat well, take vitamin A supplements, and have established and effective PERT to limit malabsorption [Lancelloti *et al* 1996]. Table 2.8 summarises the data on

circulating retinol and RBP concentrations in patients with CF. From the table it can be seen that concentrations of plasma retinol range from 0.45-2.8μmol/L. When these results are compared with the NDNS reference range for a population of free-living individuals (1.4-3.2μmol/L) not all patients with CF have concentrations below that seen in the population. However these results are hard to accurately interpret due to the lack of measured inflammatory markers.

One study in CF has previously investigated the relationship between infection and concentrations of retinol [Duggan *et al* 1996]. Results show that on admission to hospital with an exacerbation, plasma retinol concentrations were lower than that measured at discharge (1.14μmol/L v 1.7μmol/L). During hospitalisation, in the period from admission to discharge (exacerbation to no-exacerbation) mean plasma RBP increased (1.46-2.24μmol/L) and mean CRP concentrations decreased (25.7-9.8mg/L).

Low concentrations of retinol in the functional pool may reflect a limited delivery of retinol to extra hepatic tissues and this could result in functional consequences. Investigating functional consequences of low circulating retinol concentrations is not straightforward. A handful of studies in patients with CF have investigated visual consequences of low retinol concentrations. Results have found that patients with CF can develop night blindness, conjunctival xerosis, mild xerophthalmia [Raynor *et al* 1989, Leguire *et al* 1992, Tsinopoulos *et al* 2000] and abnormal dark adaptation [Huet *et al* 1997]. Recovery from these reductions in visual function following vitamin A therapy has also been reported [Huet *et al* 1997, Raynor *et al* 1989]. One study however reports conflicting evidence [Ansari *et al* 1999], in that dark adaptation was normal in all patients with CF. Despite these studies investigating the potentially visual consequences of a low retinol, there is no data in CF that investigates other functional consequences, for example effects on barrier function, and the ability to mount an appropriate immune response. Studies in rats have shown that vitamin A deficiency alters the structure of the respiratory epithelium by replacing mucus-secreting ciliated epithelium with squamous epithelium. Even mild vitamin A deficiency increases the number of secreting cells and decreases ciliated cells leading to impairment of the cilia, which normally mediates the

cleaning action of the lung [Carr, 2000], this could have an important impact upon the pathology of CF.

Only two preliminary studies have specifically assessed if low retinol concentrations affect the clinical outcomes of the patient. One study found that low concentrations of retinol were associated with poor lung function [Carr *et al* 1996], even when allowing for the influence of infection. The other study found patients with low retinol concentrations had significantly lower Shwachman scores than patients with a concentration in the normal range. It was concluded that patients with low retinol concentrations might be more susceptible to pulmonary infection and rapid progression of lung disease [Rayner *et al* 1992]. One other study whose primary aim was to assess vitamin A status in patients with CF also found concentrations of vitamin A were lowest in patients with the poorest clinical grading [Congdon *et al* 1981].

This section has reviewed how the disease CF may affect the processing of vitamin A. In particular, low retinol concentrations could be attributed to one or a number of factors; these include poor supply, poor availability (pancreatic insufficiency, malabsorption, maldigestion) of the vitamin, poor mobilisation of retinol from the liver (lack of RBP), and infection. Other factors that may have an effect are compliance to vitamin supplements and the form in which the vitamin is administered. Compliance of vitamin therapy in patients where vitamin A availability may be limiting is an issue. It has been documented that in patients with CF, compliance is one factor that may affect circulating concentrations of retinol. Compliance is defined as the degree of patient adherence to medical advice and treatment regimens that may be an important factor in the successful management of the disease [Abbott *et al* 1994]. In a study of 67 patients 45 were prescribed vitamins of these 21 (47%) always took them 16 usually did, 7 occasionally did and 1 never did [Abbott *et al* 1994], therefore 82% of patients were assessed as being compliant to vitamin therapy (always and occasionally).

There is no one form or dose used to supplement vitamin A in patients with CF [Peters *et al* 1993]. Recommendations of supplements within the literature

range from 0.36mg-3mg of retinol, dosed primary as oil based preparations [Peters *et al* 1993]. However limited data suggests that the form of administered vitamin A may effect retinol concentrations. CF patients have problems associated with the luminal processing of the retinyl ester, it may therefore be beneficial to administer vitamin A in a form that requires least luminal processing, potentially making it more available to the body. Patients with CF were given a small (4000IU) dose of water miscible retinyl acetate for two weeks. Blood was taken before supplementation and after the last vitamin dose, mean retinol concentrations rose from 1.68-3.09 μmol/L [Congdon *et al* 1981]. One study in CF has compared the effect of administering vitamin A in an oil soluble and water miscible preparation. Patients were given 250,000 units of vitamin A (833mg retinol) either as retinol in water or retinyl palmitate in oil. Following the water preparation, rises in plasma retinol were noted, in contrast to no change following the use of the oily preparation [Lewis *et al* 1947]. Both the effect of the water preparation, and the fact that vitamin A was administered as retinol may have played a role in this investigation. However experiments carried out in rats, guinea pigs and children indicate that the superiority of aqueous preparations is not due to the presence of retinol, but rather the dispersion of vitamin A in an aqueous vehicle. Water miscible preparations may be better absorbed than oil based preparations, but are not routinely available [Congdon *et al* 1981, Harries & Muller 1971, Raynor *et al* 1989]. However it may be important to consider, firstly if low retinol concentrations require correction and secondly the extent to which luminal processing is the limiting factor resulting in low concentrations of retinol before water miscible preparations are routinely prescribed.

2.4.7 SUMMARY

Both disease and infection are factors that impact upon the processing of vitamin A within the body. In particular, the relationship between the disease CF and vitamin A has been known for over 60 years. From the limited literature available on this topic, it would appear, when compared to healthy individuals, patients with CF have impaired luminal processing of retinyl esters due to their inherent pancreatic insufficiency. Along with this stool losses of vitamin A on average are increased but variable. To summarise this data would appear to

support the hypothesis that impaired GI handling of vitamin A contributes to low retinol concentrations. However evidence from a single study found significantly higher concentrations of vitamin A in the livers of patients with CF when compared to controls. This data in conjunction with evidence of depressed RBP and retinol concentrations in CF may possibly reflect some defect in the mobilisation of retinol from the liver but there is a poor evidence base to support this.

Current evidence in the literature remains limited only to a few studies investigating some aspects relating to the processing of vitamin A in CF. Research is required to assess the absorption, metabolism and losses of fat-soluble vitamins amongst today's CF population [Leonard & Knox 1997]. The inherent relationship between the pathologies of the disease CF and the processing of vitamin A requires further investigation. By investigating these relationships it may be possible to determine the specific mechanisms within the process which lead to the biochemical marker of low plasma retinol concentrations and the consequences that this may have to the patient. In conclusion the literature within this area is limited and confusing. It is hard to draw any conclusive evidence to how disease effects the processing of vitamin A, particularly as the processing in healthy individuals still remains unclear.

Table 2.8: Current literature on plasma retinol concentrations in patients with CF.

REFERENCE	PERT	VITAMIN A SUPPLEMENT	DIET	SUBJECTS	PLASMA RETINOL µmol/L (Range 1.4-3.2µmol/L)	PLASMA RBP µmol/L (Range 1.43-2.86µmol/L)
Bennett et *al* 1967	Yes	No info	No info	45 CF children	0.44µmol/L no range	
Smith *et al* 1972	Yes	Daily supplement	No info	43 CF mean 12.3y	0.82µmol/L	1.15µmol/L
Underwood *et al* 1972	Yes	Daily multivitamin	High fat	48 CF 6m-22y	0.94µmol/L (0.24-2.69)	
Jacob *et al* 1978	16 subjects	Daily multivitamin	No info	17 CF 6-17y	1.07µmol/L (0.48-1.67) calc from S.D.	1.35µmol/L
Palin et *al* 1979	No info	No info	No info	36 CF 15y ±4.9y	0.81µmol/L (0.21-1.41) calc from S.D.	
Congdon et *al* 1981	32 subjects	27 subjects	Low fat	36 CF 10m-16y	1.98µmol/L (0.5-4.5) from graph	1.62µmol/L
Fulton *et al* 1982	No info	No info	No info	34 CF 4-34y	0.73µmol/L no range	
Raynor *et al 1989*	41 subjects	2 multivitamins daily	No info	43 CF 8-44y Median 16y	34 normal dark adaptation - 1.1µmol/L (0.5-2.3) 9 abnormal dark adaptation - 0.53µmol/L (0.2-1.4)	34 normal dark adaptation 1.24µmol/L (0.21-2.19) 9 abnormal dark adaptation 0.36µmol/L (0.05-1.48)
James *et al* 1992	8 subjects	Yes	No info	9 CF median 22y	1.61µmol/L (1.14-2.39)	
Winklhofer *et al* 1995	Yes	12 subjects only	No info	35 CF 11.5±7.5y	1.05µmol/L (0.33-1.77)	
Duggan *et al* 1996	34 subjects	23 subjects only	No info	35 CF (CRP 9.8mg/L)	1.70µmol/L	2.24µmol/L
Huet *et al* 1997	Yes	Yes	No info	10 CF mean 15.2y	1.47µmol/L (0.87-2.80)	1.05µmol/L
Leonard *et al* 1998	Yes	Yes ADEK	No info	58 CF 19 children (7-15y) 35 adults (17-36y)	7-10y; 1.40µmol/L (0.7-2.0) 11-15y; 1.85µmol/L (1.2-2.2) 17-36y; 2.00µmol/L (0.7-3.0)	
Ansari *et al* 1999	Yes	Yes	No info	28 CF Median 20y (12-33y)	1.68µmol/L (1.08-2.80)	1.71µmol/L (0.61-3.86)

2.5 SUMMARY OF LITERATURE AND AIMS OF RESEARCH

This review of literature has attempted to provide an overview of vitamin A processing within a healthy population before discussing the effect that disease may have on the handling of vitamin A. The review has highlighted that although the basic physiology behind of the processing of vitamin A is well accepted the factors that influence metabolic control are less well understood. Investigating the whole body processing of vitamin A in healthy individuals will provide a conceptual framework in which to address how the process may be disrupted in states of disease and infection.

Cystic Fibrosis patients commonly exhibit low concentrations of plasma retinol. A biochemical deficiency of this kind within the functional pools could potentially affect many of the organ systems. In particular they may act to alter the immune response, potentially worsening the clinical course and increasing morbidity and mortality. However the clinical significance of low retinol concentrations and what constitutes the most appropriate approach to management is unclear. Current prophylactic supplementation of all pancreatic insufficient patients and all patients who exhibit low retinol concentrations is routine, and often around four times the RNI. It would appear that these guidelines are simply based on the assumptions of either poor intake and / or impaired availability of the vitamin associated with lipid maldigestion and malabsorption. However these guidelines are based on little evidence, and it has been reported that low concentrations still occur in patients who eat well, take vitamin A supplements and have effective PERT regimens. There is a need to reflect whether such an approach is justified, and reconsider the current management, especially given evidence that has accumulated over recent years. Firstly, the initial reports of low retinol concentrations were observed in patients on restricted fat diets, however practices are now based on high energy, high fat and therefore increased vitamin A diets. Secondly, it is well accepted that retinol concentrations fall in the face of infection, thus it maybe inappropriate to supplement on observations of a biochemical deficiency. Thirdly, even though losses of vitamin A may be elevated in these patients, whether such high intakes are required to

compensate these losses maybe unlikely, and raises the potential of hepatotoxicity within this patient group.

In conclusion our present understanding of the role that vitamin A plays in the disease CF is constrained. There is a need to address a number of issues in order to establish a conceptual framework of how vitamin A is handled within the body and the processes that may be altered in patients with CF. It is clear from the literature that the following questions need to be addressed:

- What is the prevalence of low plasma retinol concentrations in patients with CF on established therapy?
- Does the concentration of plasma retinol relate to the patients clinical course?
- What factors are associated with a low plasma retinol concentration?
- What levels of dietary intake are observed in these patients and how much extra vitamin A is consumed as supplements and in what form?
- Are faecal vitamin A losses increased in patients with CF on their established PERT and habitual supplementation?
- Do faecal vitamin A losses reflect dietary residue or excretory losses of retinol from within the body?
- Are faecal vitamin A losses related to faecal lipid losses?
- Once absorbed from the gut are the processes that influence the way in which vitamin A (as retinyl palmitate) is handled in the circulation and removed by the liver altered in CF?
- To what extent do raised excretory losses in urine (as retinyl glucuronides) increase the metabolic demands for vitamin A in patients with CF?
- Does retinol accumulate in the liver of patients with CF?
- Is there a constraint on RBP (and TTR) synthesis in patients with CF?
- How are the processes that determine retinol metabolism altered in patients during an acute phase exacerbation?

There are two general paradigms in which these issues can be considered. The first is of a simple dietary deficiency, where low retinol concentrations simply reflect an inadequate effective supply, which is unable to meet demands, and if left unresolved by supplementation, will affect clinical course (figure 2.11;paradigm 1). The second is that low retinol concentrations are not simply a consequence of a poor effective supply but of poor mobilisation of the vitamin from the liver (figure 2.11; paradigm 2), and low retinol concentrations affect the clinical outcomes of the patient. Within the context of these paradigms three hypotheses, to be tested within this thesis, have been generated:

Hypothesis One:

Patients with CF have lower plasma retinol concentrations compared to healthy adults that affect or are affected by their clinical outcomes.

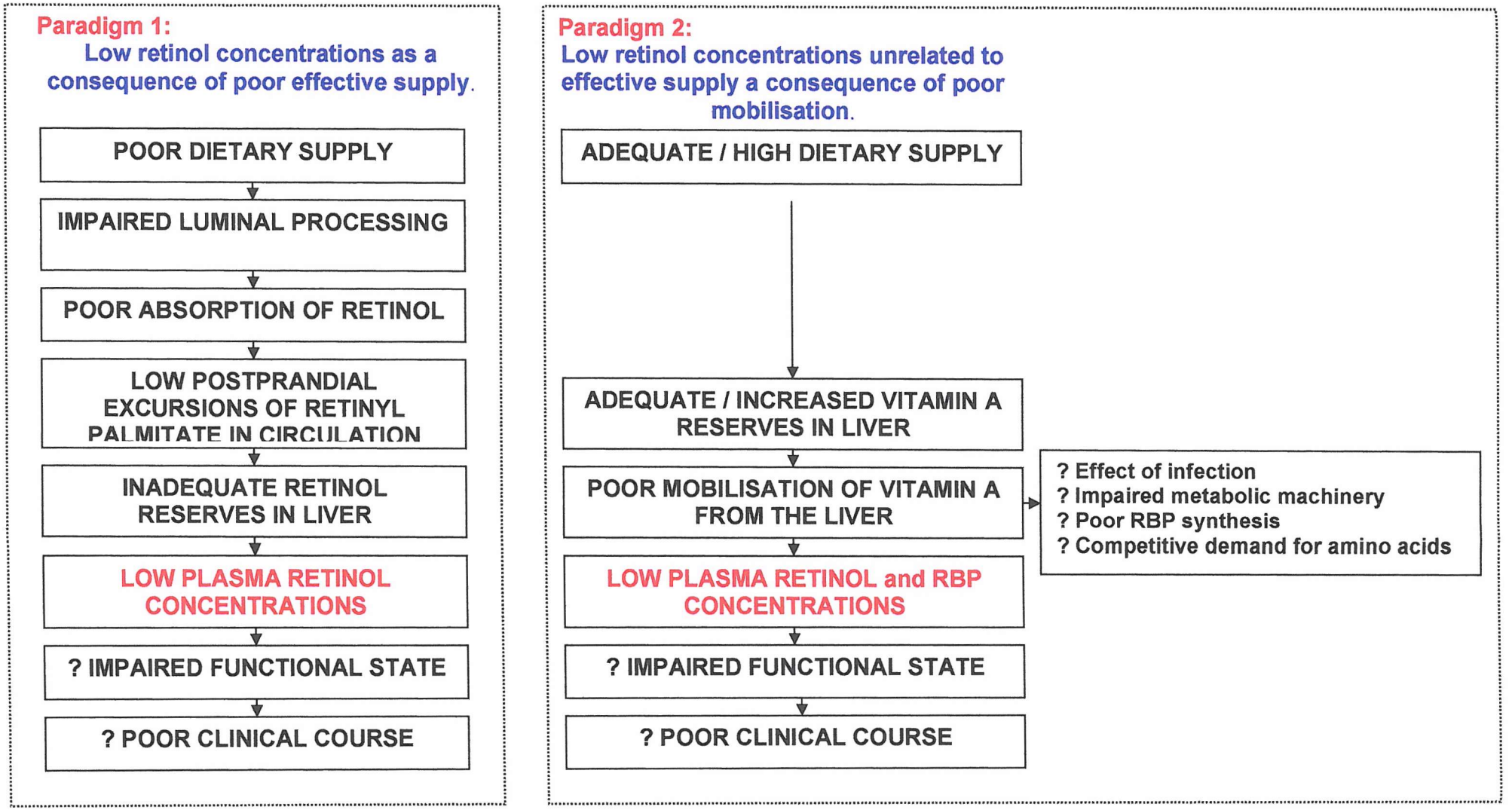
Hypothesis Two:

Patients with CF have lower concentrations of retinol in their circulation due poor dietary supply, and / or impaired GI handling and availability of the vitamin.

Hypothesis Three:

Patients with CF have lower concentrations of retinol in their circulation that are unrelated to supply, but are a consequence of poor mobilisation of retinol from the liver.

Figure 2.11:


Paradigm 1:
Low retinol concentrations as a consequence of poor effective supply.
POOR DIETARY SUPPLY
IMPAIRED LUMINAL PROCESSING
POOR ABSORPTION OF RETINOL
LOW POSTPRANDIAL EXCURSIONS OF RETINYL PALMITATE IN CIRCULATION
INADEQUATE RETINOL RESERVES IN LIVER
LOW PLASMA RETINOL CONCENTRATIONS
? IMPAIRED FUNCTIONAL STATE
? POOR CLINICAL COURSE
Paradigm 2:
Low retinol concentrations unrelated to effective supply a consequence of poor mobilisation.
ADEQUATE / HIGH DIETARY SUPPLY
ADEQUATE / INCREASED VITAMIN A RESERVES IN LIVER
POOR MOBILISATION OF VITAMIN A FROM THE LIVER
? Effect of infection
? Impaired metabolic machinery
? Poor RBP synthesis
? Competitive demand for amino acids
LOW PLASMA RETINOL and RBP CONCENTRATIONS
? IMPAIRED FUNCTIONAL STATE
? POOR CLINICAL COURSE

The specific aims of the research include:

1) To development and validate methods.
- Method for the measurement of retinol and retinyl esters in plasma
- Method for the measurement of retinol and retinyl esters in stool
- Method for the measurement of isotopically labelled lipid and vitamin A in stool and plasma.

2) The examine circulating plasma retinol and RBP concentrations in a group of CF patients and a group of healthy adults, in order to determine the prevalence of low circulating retinol and RBP concentrations in patients with CF.

3) To investigate if reductions in the functional pool of vitamin A can affect clinical outcomes.

4) To investigative the whole body processing of vitamin A in healthy adults and patients with CF.

5) To investigate the factors that may be associated with low circulating concentrations of retinol in patients with CF. These include:

- Examining the relationship between the processing of lipid and vitamin A
- Examining the exogenous losses of vitamin A in stool (availability).
- Investigating the appearance of vitamin A as retinyl esters in the circulation (availability).
- Investigating the effects of an acute pulmonary exacerbation.
- Investigating mobilisation of retinol from the liver.

CHAPTER 3.

METHODOLOGY.

3.0 INTRODUCTION.

This chapter describes the analytical methods used in studies presented in this thesis. In brief, to address the research questions defined at the end of the review, studies were set up using stable isotope tracers to characterise the whole body processing of exogenous vitamin A. Samples of both stool and blood were collected for the analysis of unlabeled and labelled vitamin A and lipid. The methods used in the studies allow the measurement of total concentrations of:

- Plasma retinol by High Performance Liquid Chromatography (HPLC).
- Plasma retinyl palmitate by HPLC
- Serum Retinol Binding Protein (RBP) by Nephelometry
- Serum Transthyretin (TTR) by Nephelometry
- Stool retinol by HPLC
- Stool retinyl palmitate by HPLC
- Plasma TAG by enzymatic assay or Gas Chromatography Combustion Isotope Ratio Mass Spectrometry (GC-C-IRMS)

In the studies that involve the use of stable isotope tracers (chapters 7 & 8), methods for the analysis of plasma and stool samples allow the measures of:

- Plasma $d_4$retinol by gas chromatography mass spectrometry (GCMS).
- Plasma d_4-retinyl palmitate by GCMS
- Plasma ^{13}C-palmitic acid by GC-C-IRMS
- Stool d_4-retinol by GCMS
- Stool d_4-retinyl palmitate by GCMS

This chapter is divided into four main sections. The first section (3.1), introduces the general principles of the methods used in this thesis. The section provides an overview of the methods of chromatography and mass spectrometry, and a summary of the use of stable isotope tracers to study metabolism in humans. Finally, the section highlights methods previously used to analyse vitamin A in both plasma and stool. The second section (3.2) briefly summarises the development of a new method to simultaneously analyse retinol and retinyl palmitate in plasma and the third section (3.3) describes the specific methods used in the studies presented in this thesis. The methods include sample preparation and storage, preparation and composition of the test meal, and analysis of vitamin A and lipid in stool and in plasma. The final section (3.4) describes the validation of the new method developed specifically for the studies. Appendix 3.1 lists the reagents and equipment used in the methods described in this chapter.

3.1 GENERAL PRINCIPLES.

In order to address questions relating to the processing of vitamin A (as retinol and retinyl esters) and lipid within the body, there is a need to measure natural and isotopically enriched retinol, retinyl palmitate and lipid. Studies presented in this thesis used a number of difference methods of chromatography. HPLC was used to measure total vitamin A metabolites, GC-C-IRMS was used to measure labelled lipid, and GCMS was used to measure labelled vitamin A. This section will briefly outline; a) the principles of chromatography, including both liquid and gas chromatography; b) the principles of mass spectrometry c) the principles of stable isotope tracers, and d) methods that have previously been used to analyse vitamin A in plasma and stool. Details of the specific methods will be discussed in section 3.3.

3.1.1 CHROMATOGRAPHY.

The technique of chromatography is widely used for the separation, identification, and determination of chemical components in a complex mixture. Chromatography is a physical method of separation in which the components to be separated are distributed between two phases, one of which is stationary

while the other moves in a specific direction. Separation is achieved by the distribution of substances between the mobile phase (liquid or gas) and the stationary phase that is physically linked to the column. Substances are eluted from the column in order of their distribution coefficients with respect to the stationary phase. Choices of materials for the phases allow enormous versatility for separating substances. Thus, the separation of molecules by chromatography depends on exploiting subtle differences in physical properties of the molecules.

A suitable detector can monitor the elution of samples from the column and the series of peaks produced in known as a chromatogram. The chromatogram is useful for both qualitative and quantitative analysis. Each peak represents a discrete chemical compound, or a mixture of compounds. The time required for each component to emerge from the column is characteristic of the compound and is known as the retention time. The area under the peak is proportional to the amount of the component in the sample. Data for qualitative and quantitative analysis can be derived from the positions and areas of the peaks.

3.1.1.1 Liquid Chromatography.

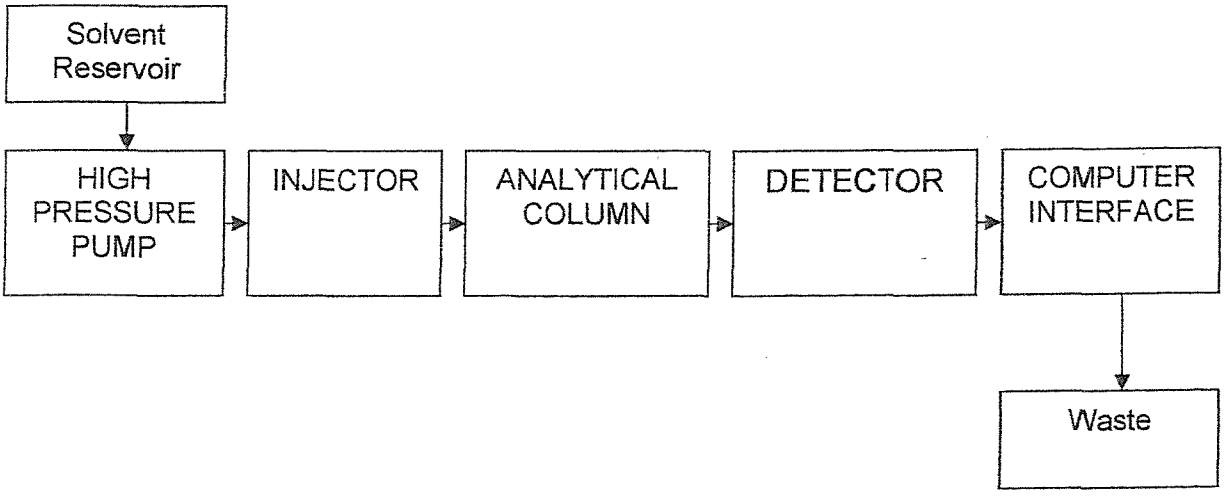
Liquid chromatography uses a liquid (solvent) as the mobile phase. As molecules pass through the column containing solid particles they interact with both phases. Separation occurs according to the relative partitioning of the soluble components between the mobile and solid phases. This is principally related to the polarity of the component relative to the polarities of the mobile and stationary phase.

Many different types of stationary phases can be used including, normal phase, reverse phase, ion exchange and chiral-specific phases. A normal phase packing uses a polar stationary phase and a less polar mobile phase. This causes non-polar (hydrophobic) compounds to elute more quickly than polar (hydrophilic) compounds. A reverse phase packing is non-polar and consists of a silica solid support with covalently bound *n*-alkyl chains, for example a C-18 or ODS has an octadecyl ligand in the matrix. The more non-polar the ligand, the greater the tendency of the column to retain non-polar molecules. Thus for

reverse phase, polar compounds elute more quickly than non-polar compounds. Further discussion will be limited to reverse phase packings that have been commonly used to analyze vitamin A.

Efficiency of separation can be optimised by the use of very small particles of stationary phase (3-5micron). This requires a high-pressure system to pump the mobile phase. Such a system is called high performance (pressure) liquid chromatography (HPLC) and the typical components of the system are illustrated in figure 3.1. The detector for an HPLC emits a response proportional to the concentrations of the eluting sample component. There are many types of detectors that can be used with HPLC. Some of the more common detectors include: spectrophotometric (ultra violet (UV) and visible), fluorescence, radiochemical, and electrochemical. For the analysis of retinol and retinyl esters in the studies presented in this thesis a UV detector was used. More details of the specific HPLC system used within this thesis are presented later in this chapter.

Figure 3.1:
Components of the high-pressure liquid chromatography system (HPLC).



3.1.1.2 Gas Chromatography.

Gas chromatography uses gas as the mobile phase. The sample of gas or volatile liquid to be analysed is injected into the inlet. The components move through with the mobile phase (usually hydrogen, helium, or argon) at rates influenced by their interaction with the stationary phase. The temperature, nature of the stationary phase, and column length can be varied to improve separation. The gas stream from the end of the column passes into a detector (mass spectrometer, flame ionisation detector, electron capture), which records the magnitude of the response. Components that are normally insufficiently volatile maybe analysed following derivatisation to a more volatile product.

3.1.2 MASS SPECTROMETRY.

Mass spectrometry is an analytical technique by which chemical substances are identified by inducing fragmentation of the molecule and sorting the resulting ions by mass using magnetic fields. The technique can identify unknown compounds, quantify known compounds, and be used to elucidate the structure and chemical properties of molecules. The technique is widely used to measure masses and relative abundances of different isotopes, and also as a detector to analyse products of a separation by liquid or gas chromatography. In the methods presented in this chapter mass spectrometry is used as a detector following gas chromatography.

During mass spectroscopy the molecule is ionized, fragmented and passed through a mass analyser in which the ion mixture is separated according to the mass to charge ratio (m/z), and the current due to each ion is recorded. When used as a detector with gas chromatography the two main methods of ionisation are: a) electron impact; where an electron current or beam is used to ionize the sample migrating off the column; and b) chemical ionization, which is a less aggressive method, and uses an ionised gas to transfer charge to the compounds eluting from the column. Chemical ionisation was used in the GCMS system in methods presented in this thesis.

The use of stable isotope methodologies to trace metabolic processes in vivo leads to individual chemical components with multiple isotopic compositions.

Different isotopes can be individually quantified by mass spectrometry by monitoring ions characteristic of each isotope (selective ion monitoring). The technique of GCMS is widely used to separate chemical components chromatographically so that each can be analysed isotopically in the mass spectrometer. Chemical ionisation is particularly suitable for this application. The gentle ionisation used results in little fragmentation of the molecule and a high yield of the molecular ion or a very closely related fragment. The background ionisation is low so that a high signal to noise ratio is obtained and allows a precise measurement of ion ratios.

The technique of GC-C-IRMS is an even more sensitive method to measure the isotope abundance of carbon containing compounds. GC-C-IRMS can measure isotopic composition at very low enrichment levels [Meier-Augenstein, 1999]. This means that minute variations of the heavier isotope are detected in the presence of large amounts of the lighter isotope [Meier-Augenstein, 1999]. In this method the effluent from the GC is combusted to carbon dioxide before entering the mass spectrometer. The mass spectrometer is tuned to identify the ions, 44($^{12}CO_2$) and 45($^{13}CO_2$). Table 3.1 illustrates the features of both the GCMS and GC-C-IRMS.

Table 3.1

Typical features and specifications of mass spectrometry systems used for stable isotope analysis. [Meier-Augenstein 1999].

	GC-MS	GC-C-IRMS
Sample introduction	Injection of liquid or gaseous sample matrix onto GC column	Injection of liquid or gaseous sample matrix onto GC column
Sample separation	By gas chromatography	By gas chromatography
Sample manipulation before mass spectroscopic analysis	None	Combustion/reduction of compounds into CO_2 N_2 in the interface
Interface	Heated transfer capillary directly connected to ion source	Capillary, incorporating wide bore combustion/reduction furnaces
Mass analyser	Quadrapole	Magnet
Detector	One electron multiplier	Triple Faraday cup collector
Mode of charged mass detection.	SIM, switching between e.g M+ and [M+1]. Ratios (M+1 / M+) are calculated on the basis of measured ion current	Simultaneous detection of particles with three adjacent masses. For ^{13}C the Faraday cups are tuned to masses 44, 45, and 46, which correspond to $^{12}C(^{16}O)_2$, $^{13}C(^{16}O)_2$ and $^{14}C(^{16}O)_2$.
Measurable enrichment range In [APE]	+0.5-100	0-0.1-+2.0
Samples size requirement	<1 pmol	0.1-5nmol

3.1.3 STABLE ISOTOPE TRACERS.

Over the past 20 years the use of stable isotope tracers in metabolic studies have become increasingly popular. Increased availability and variety of substrates labelled with stable isotopes, improvements in analytical procedures and increasing awareness of the potential hazards of using radioactive tracers in humans, have all contributed to this increased use.

Radioactive isotopes have been used for many years in biomedical research to act as tracers to follow metabolism and cellular processes. However the use of radioactivity in human studies of this nature has come under scrutiny due to radiation hazards. The use of stable isotope tracers has allowed safer and more acceptable methods. The advantages of stable isotope methodology are the non–toxic levels required in clinical studies, allowing a greater range of subjects to be studied, and the possibility of multiple stable isotope labelling of different or identical nutrients which enables the simultaneous investigation of nutrients within various body compartments.

Stable isotopically labelled tracers were first used to measure metabolic processes in the 1930's [Keston *et al* 1937]. The isotopic tracer behaves chemically and metabolically in an identical way to the tracee, and separation of the administered isotope from the natural isotope allows the exogenous processing of the ingested isotope to be investigated. In studies presented within this thesis the form in which the stable isotope tracer is presented to the body is important, and should reflect the form in which the tracee is habitually ingested. Lipids and lipid soluble compounds such as vitamin A in the diet are generally consumed as emulsions. A glucose:sucrose:casein:lipid emulsion has been developed from that described by Emken *et al* [1993], to act as a vehicle to deliver the tracer to the body. This emulsion was administered with a standard meal. The test meal used in the studies presented in this thesis was designed as a breakfast to reflect approximately one third of the typical composition of an average UK daily diet (40% of total energy from dietary lipid, 45% from carbohydrate, and 15% from protein). The protocols for the test meal and emulsion are described in section 3.3.3.

3.1.3.1 ^{13}C labelled substrates.

The tracer [1,1,1-^{13}C] Tripalmitin (TP), 10mg/kg body weight, was used in one of the studies reported in this thesis (chapter 8), to trace the metabolism of dietary TAG in the test meal. Each fatty acid in the TAG is labelled at the carboxyl end of the chain. The labelled TAG was enriched to 99 APE, indicating 99% of the carbon atoms in the carboxyl position would be ^{13}C. In this instance, the method of analysis to determine ^{13}C enrichment in biological specimens is GC-C-IRMS. The method, described previously, utilises the different masses of the two isotopes to differentiate between ^{12}C and ^{13}C and determine the ratio between the two. The concentration of ^{13}C when expressed as a percentage of total carbon is known as the abundance in units of atoms percent (atom %).

3.1.3.2 d_4 labelled substrates.

The tracer [10,19,19,19] ^{2}H-retinyl palmitate (d_4RP), 300μg/kg body weight, was used in two of the studies reported in this thesis (Chapter 7 & 8), to trace the metabolism of dietary retinyl palmitate. Retinyl palmitate was chosen as the labelled substrate as it is the predominant retinyl ester present in the UK diet. HPLC was used to quantify retinol and retinyl palmitate and a fraction collector allowed recovery of the separated components. The fractions containing d_4-label were saponified. Following derivatisation, the isotopic composition of retinol was analysed by GCMS by comparison with standards of known isotopic composition. Concentrations of natural and labelled forms were calculated from the total concentration measured by HPLC.

3.1.4 ANALYSIS OF VITAMIN A IN BIOLOGICAL SPECIMENS.

The original method for the measurement of vitamin A was published in 1926 [Carr & Price 1926], and was based on the reaction between vitamin A and antimony trichloride. However the reagents and reaction products were unstable and the method did not produce reproducible results. A spectrophotometric method using trifluoroacetic acid (TFA) [Neeld & Pearson 1963] had fewer problems but was still non-specific and measured total vitamin A. In recent years analysis of vitamin A, as retinol and other metabolites, has been almost exclusively by HPLC, coupled with detection by UV absorption.

3.1.4.1 Analysis of vitamin A in plasma.

A number of published methods that measure plasma vitamin A by HPLC are presented in appendix 3.2, however a lack of detailed descriptions in analytical journals makes it difficult to reproduce these methods. It would seem that there is no one common routine method for the measurement of both retinol and retinyl palmitate from the same plasma specimen. Methods differ in 1) the solvent used for lipid extraction, 2) the overall HPLC system, 3) the solvents used as the mobile phase, and 4) the type of internal standard. The majority of methods address the analysis of retinol in plasma without the analysis of retinyl esters, however these methods were specifically set up to analyse only retinol. Commonalties in the methods to quantity plasma retinol concentrations are a hexane extraction followed by reverse phase HPLC with relatively non polar mobile phases (mixtures of water, and methanol or acetonitrile). It would appear that the measurement of retinyl esters is more difficult. Investigators have reported unresolved retinyl esters [De Ruyter *et al* 1978; Bhat et al 1983], instances where retinyl esters co-elute [Banksom *et al* 1986; Eckhoff *et al* 1991], and instances where the retinol peak elutes close to the void volume [Reinersdorff *et al* 1996].

In the studies reported in this thesis it was important to be able to, 1) accurately quantify retinol and retinyl palmitate by HPLC, and 2) use the HPLC system to fractionate the peaks of interest for collection and further analysis of isotopically labelled substances. Previous reported methods use HPLC for quantification but and not as a method of fraction collection of retinol and retinyl esters. It was important to adequately resolve the retinol and retinyl palmitate peaks to allow exclusive collection. Consequently, it was necessary to develop a technique that would extract and quantify both retinol and retinyl palmitate from plasma.

3.1.4.2 Analysis of vitamin A in stool.

There are few reports from the literature that describe the measurement of vitamin A in stool. Five of the seven studies that investigated excretion of vitamin A were undertaken in the mid 1900's before the development of HPLC methods [Lewis *et al* 1947; Barnes *et al* 1950; Reddy *et al* 1960; Sivakumar *et al* 1972; Sauberlich *et al* 1974]. Only the two more recent studies used HPLC

analysis [Ahmed *et al* 1990; Halford *et al* 1992]. These were crude gross balance studies that measured total vitamin A in stool, as retinol. These methods were not able to differentiate between retinol and retinyl esters or differentiate between exogenous and endogenous vitamin A. For these reasons, reported stool losses might have represented losses other than that from the diet. These published methods do not allow the quantification of both retinol and retinyl palmitate, or separation of these molecules for fraction collection. Previously described methods therefore did not allow the measurements necessary to answer the questions addressed in this thesis. There was a need to develop and validate a novel method for the measurement of retinol and retinyl esters in stool following ingestion of an isotopically labelled tracer of retinyl palmitate. The use of an isotopic tracer makes it possible to a) differentiate dietary residue that has not been digested and absorbed from endogenous secretions and b) follow the partitioning of absorbed retinyl palmitate in the circulation.

3.2 DEVELOPMENT OF METHODS.

This section summarises development of a method for the simultaneous analysis of retinol and retinyl palmitate in plasma specimens. The required method was designed to measure the concentration of retinol and retinyl palmitate by HPLC, and allow the collection of each fraction for the subsequent analysis of isotopic composition by GCMS. The procedure involves, the extraction of retinol and retinyl palmitate from plasma along with separation and quantification by HPLC. The final method used for analysis of plasma retinol and retinyl palmitate is described in detail in section (3.3.4), and the details of the method validation are presented in section (3.4.1).

Based on the method developed to analyse vitamin A in plasma samples, Dr. Paul Afolabi validated a method for the simultaneous analysis of retinol and retinyl palmitate in stool samples. The method is described in section 3.3.5 and validation data presented in section 3.4.2.

3.2.1 SOLVENT EXTRACTION

The function of solvent extraction is to extract vitamin A, as retinol and retinyl esters, from components of the plasma sample that would interfere with the HPLC analysis. Retinol is a non-polar compound soluble in organic solvents such as methanol and chloroform but practically insoluble in water. Retinyl palmitate is less polar (more non-polar) than retinol. A number of solvent systems were assessed for their efficiency in extracting both retinol and retinyl esters from plasma. A plasma pool was used, half the plasma was left native and half was spiked with standards of retinol, retinyl acetate and retinyl palmitate in chloroform methanol and allowed to stabilise. Each sample was extracted twice, and each combination of plasma and solvent was processed in triplicate. The solvent systems initially examined included, 100% hexane, methanol, ethanol, and propan-2-ol. Recoveries of each standard spike were determined by subtracting the peak area of the non-spiked sample from the peak area of the spiked samples and comparing this to the peak area of the standard processed without addition to plasma. All solvents tested extracted retinol with high efficiency but gave low recoveries of retinyl esters. Following these initial experiments several combinations of solvents were examined. Chloroform:methanol, a modified Folch extraction [Folch *et al* 1957], is used to extract lipids from plasma and stool specimens routinely within the nutrition laboratory, at the Institute of Human Nutrition, Southampton. Chloroform:methanol (2:1 v/v) is less polar than methanol and extracted ~100% of retinol, retinyl acetate and retinyl palmitate from plasma. Further validation of the chloroform methanol lipid extraction method to extract retinol and its esters found no difference in the results obtained following extraction of 500μL or 1ml of plasma ($P>0.05$). The full method for the extraction of vitamin A from plasma is described later in this chapter.

3.2.2. QUANTIFICATION BY HPLC

The aim of the HPLC was to adequately resolve the retinol, retinyl acetate and retinyl palmitate peaks, to both quantify and collect the peak fractions for subsequent analysis by GCMS. The details of the HPLC equipment used are described in section 3.3.4.3 and appendix 3.1.

During preliminary method development an isocratic solvent was used. A mobile phase of 100% methanol at 1ml/min allowed good resolution of the three molecules of interest with mean (SD) retention times of 5.4(±0.015) minutes, 6.7(±0.05) minutes, & 37.2(±0.383) minutes for retinol, retinyl acetate, and retinyl palmitate respectively. A number of different sensitivities of the UV detector were tested to optimise the system and allow the detection of small amounts of vitamin A. It was shown that peak height and area were linearly related to the photometer sensitivity and that, in the range of interest, increased sensitivity did not impair the signal to noise ratio. The optimal sensitivity that allowed measurement of both the lowest and highest concentrations of vitamin A as retinol and retinyl palmitate without losing peak integrity was determined to be 0.025ABU.

The subsequent availability and use of a gradient solvent system allowed both optimisation of the resolution of the retinol and retinyl acetate peaks with a shorter retention time for retinyl palmitate. Optimising separation was achieved by manipulation of the solvent. By using methanol:water (95:5) as the initial solvent (solvent A) the polarity was increased along with the binding capacity for retinol and retinyl acetate to the C18 column. Increased polarity caused greater retention times for retinol and retinyl acetate that allowed sufficient separation for satisfactory operation of the fraction collector to collect retinol exclusively of retinyl acetate. The use of methanol:isopropanol (1:1) for solvent B, introduced after elution of retinyl acetate provided a less polar mobile phase that reduced the retinyl palmitate retention time and reduced peak width. This enhanced quantification, improved fraction collection and reduced the run time. The final conditions were 100% solvent A 1ml/min (5% water in methanol) for 10 minutes, then over 1 min changing to 100% solvent B 1ml/min (1:1 methanol: isopropanol) for 10 minutes. The column was re-equilibrated to solvent A, by pumping 100% solvent A for 15mins at 2ml/min. Total run time was 35 minutes and the elution times for the three peaks of interest were approximately, retinol 7.9 minutes, retinyl acetate 11.4 minutes, and retinyl palmitate 22.9 minutes. This gradient system allowed good separation of all three peaks of interest. The complete method is described in section 3.3.4.

3.3 METHOD PROTOCOLS

The methods used within the studies reported in this thesis are described in the following section. The study protocols are presented first, along with the general methods of sample collection, and test meal and emulsion preparation. This is followed by descriptions of the specific analytical methods; these include methods to quantify lipid and vitamin A in plasma and stool.

3.3.1 STUDY PROTOCOLS.

A general study protocol (figure 3.2) was followed in the studies investigating the whole body processing of vitamin A and lipid (chapter 7 and 8). The studies had the approval of the Southampton Hospitals and South West Hampshire Health Authority Ethical Committee. Subjects (healthy adults and patients with CF) were recruited to the study, and attended the Wellcome Trust Clinical Research Facility, Southampton General Hospital, on the study day. Firstly, informed consent was obtained from the subject, which was followed by cannulation and baseline blood collection. The start of the study was recorded as the time of isotopic label administration. Subjects only drank water between sampling and were provided with a standard lunch at 5 hours, low in vitamin A.

In the study presented in chapter 7 one isotopic label was administered, [10,19,19,19]^{2}H retinyl palmitate. Post-dose bloods were drawn throughout the studies, along with a 3-day stool collection. Plasma and stool samples were analysed for total concentrations of retinol and retinyl palmitate along with concentrations of deuterated retinol and retinyl palmitate.

In the study presented in chapter 8, two isotopic labels were administered, [10,19,19,19]^{2}H retinyl palmitate and [1,1,1,^{13}C] tripalmitin. Post-dose bloods were drawn throughout the study period. Plasma samples were analysed for total lipid, retinol and retinyl palmitate along with deuterated retinol and retinyl palmitate and ^{13}C lipid. Summary flow diagrams for the measurement of both vitamin A and lipid from stool and plasma specimens are illustrated in figures 3.3 and 3.4.

Figure 3.2 General study protocol.

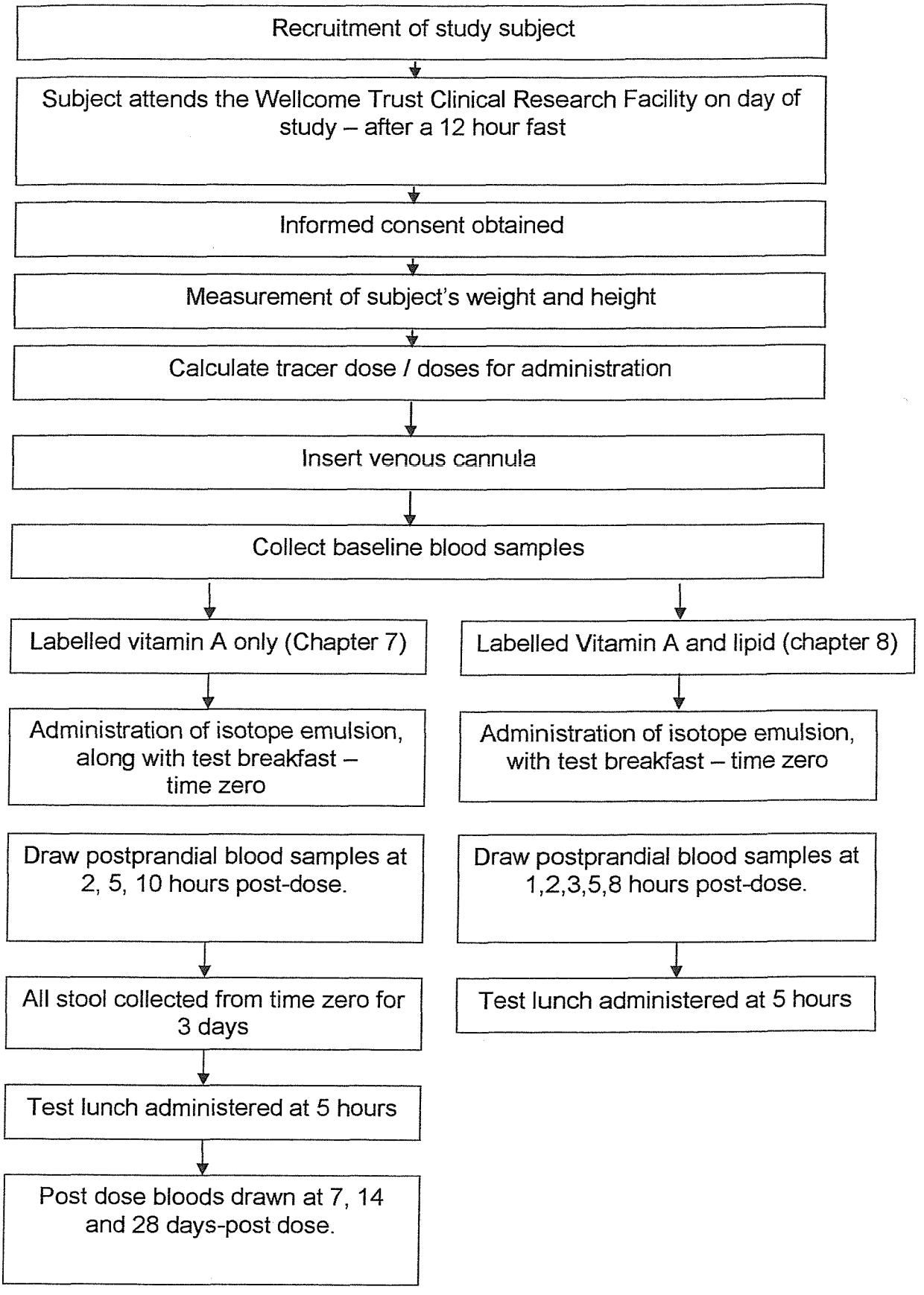


Figure 3.3

Flow diagram illustrating the steps in the analysis of vitamin A (retinol and retinyl palmitate) from stool and plasma specimens.

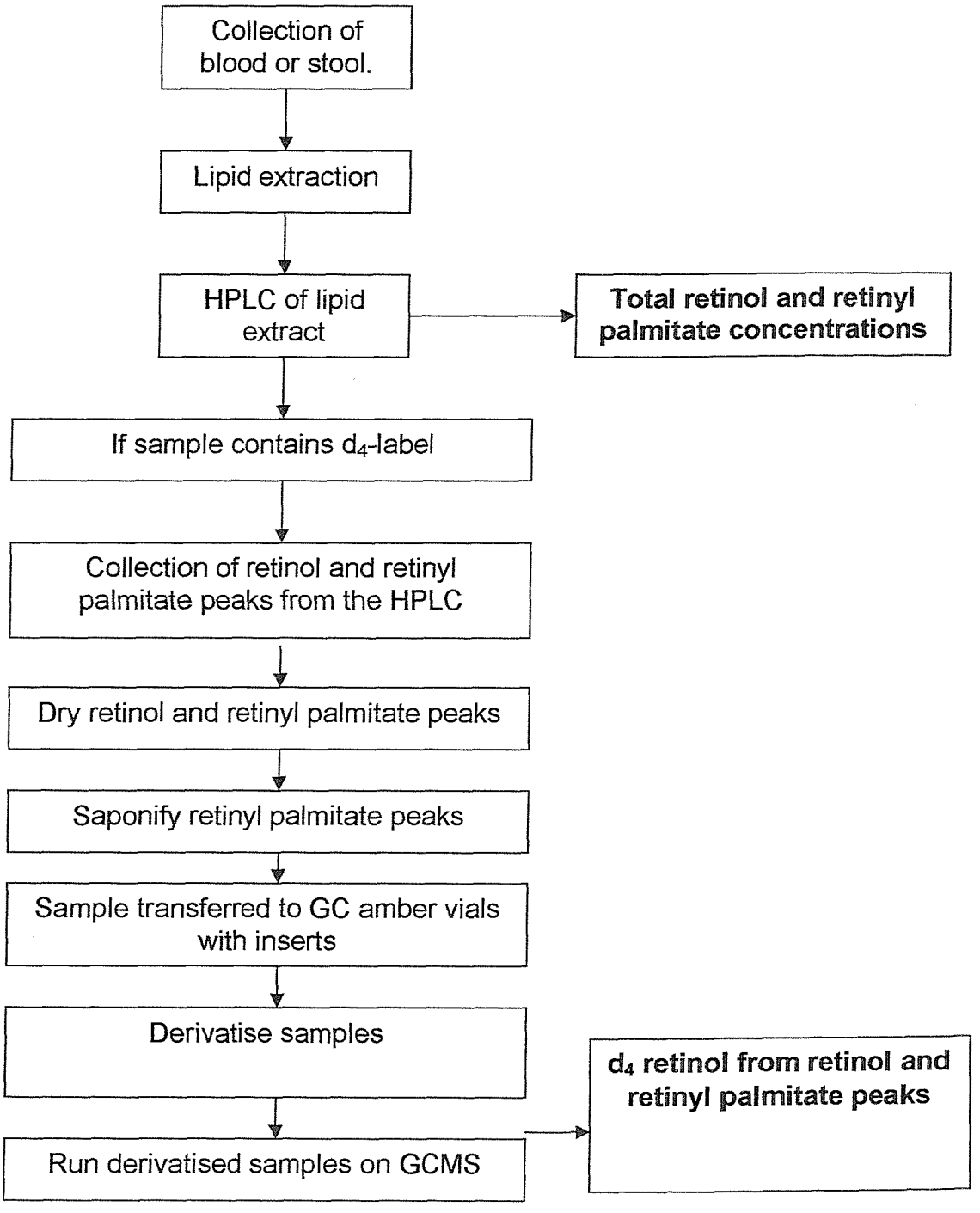
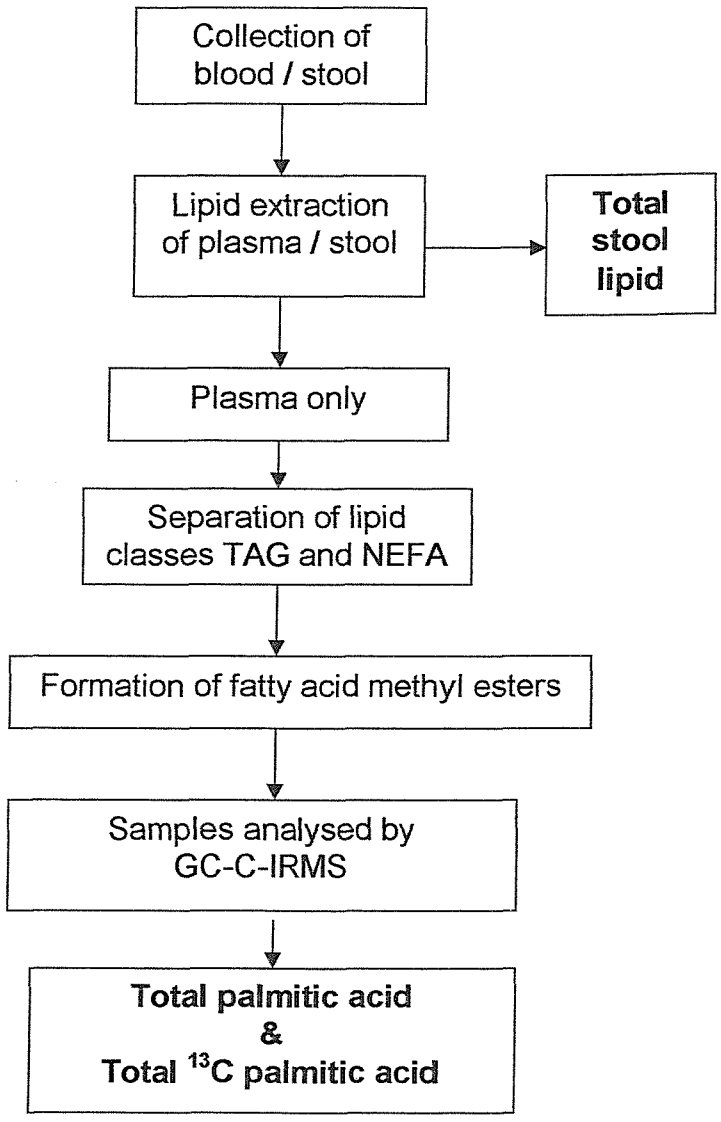


Figure 3.4

Flow diagram illustrating the steps in the analysis of lipid from stool and plasma specimens.



3.3.2 SAMPLE COLLECTION AND STORAGE.

Figure 3.5 illustrates the procedures of sample collection and storage described below.

3.3.2.1 Plasma vitamin A.

All samples were handled in dark or subdued lighting. In studies that required one sample of blood for the analysis of plasma retinol, blood specimens were collected into 10ml 1% EDTA tubes, using the Vacutainer system. In studies that investigate postprandial changes in plasma retinyl esters sequential blood specimens were collected throughout the day. An indwelling cannula was inserted into the forearm vein, permitting repeated sampling of blood with no added discomfort to the subjects. The cannula was inserted prior to collection of baseline blood specimens. Blood specimens were taken at each time point into a syringe and aliquoted into Vacutainer tubes. Cannulas were flushed with saline between sampling. Blood was kept away from direct light and centrifuged within 10 minutes of collection at 1620g for 15 minutes at 4°C. Plasma was divided into 1.5ml aliquots in 5ml glass tubes covered in aluminium foil to protect from direct light and immediately frozen and stored at -70°C until analysis.

3.3.2.2 Serum RBP and TTR.

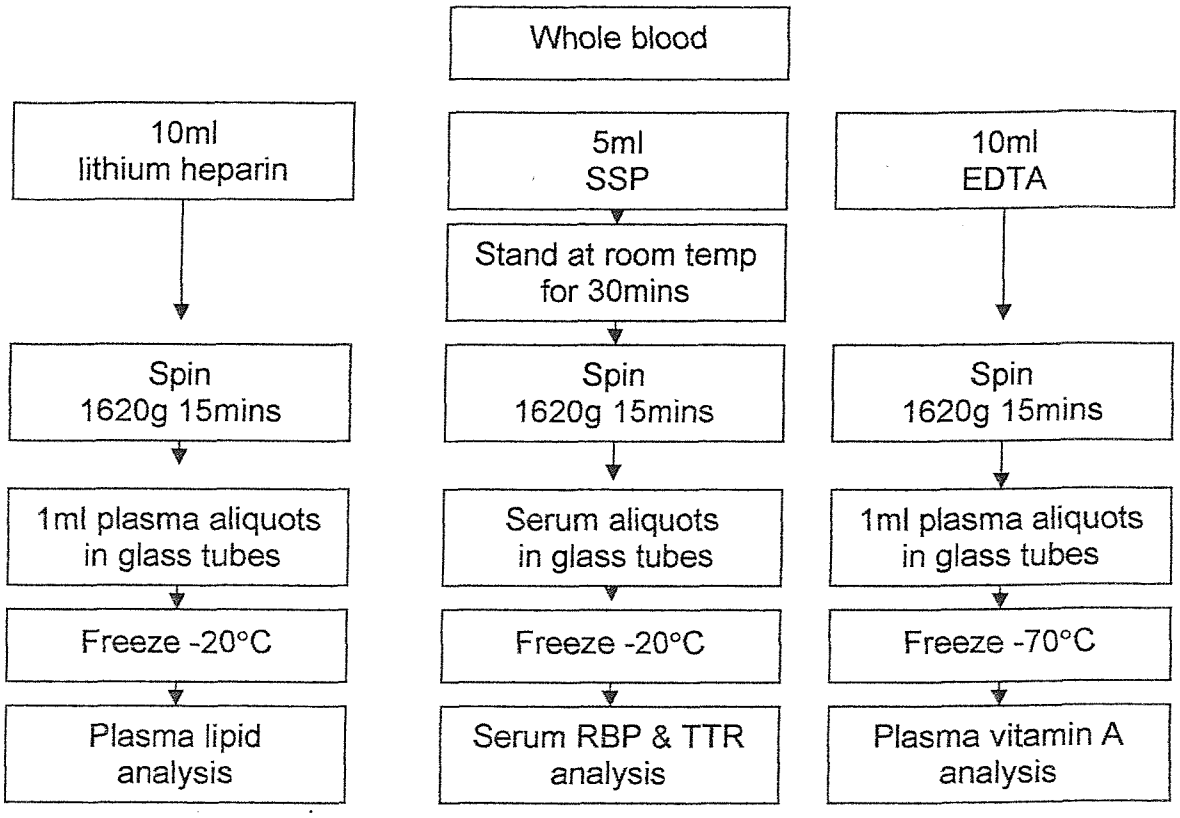
For the measurement of serum retinol binding protein (RBP) and transthyretin (TTR) blood was collected, (either using the vacutainer system or from the cannula), into 5ml serum separating tubes (SST). Tubes were covered in aluminium foil, and allowed to clot for 30 minutes before centrifugation at 1620g for 15 minutes at 4°C. Serum was aliquoted and stored at -20°C until analysis.

3.3.2.3 Plasma lipid.

In studies that investigated the postprandial changes in plasma TAG sequential blood specimens were collected throughout the study from the indwelling cannula, as described. Blood specimens were collected into 10ml lithium heparin tubes and centrifuged at 1620g for 15 minutes at 4°C. Plasma was divided into 1.5ml aliquots in 5ml glass tubes and immediately stored at -20°C until analysis.

Figure 3.5

The processes involved in the separation of whole blood specimens to plasma for analysis of vitamin A, lipid, RBP and TTR.



3.3.2.4 Stool for vitamin A and lipid analysis.

Stools were collected in the study described in chapter 7. All stools passed over the three-day period following the test meal were collected into individual polythene bags, labelled with name, date and time, and frozen immediately on dry ice. All stools were transferred to -70° C freezers within 12 hours of collection. All stools passed on an individual day were pooled (total weight recorded) and homogenised with distilled water (total weight recorded).

3.3.3 TEST MEAL AND EMULSION. (Appendix 3.3)

Studies (chapter 7 & 8), that investigated losses of vitamin A and lipid in stool along with the postprandial excursions in plasma concentrations required administration of isotopically labelled tracers. Tracers were administered in an emulsion at the same time as a standardised test breakfast. Diets were controlled over the study period and all subjects received the same diet. At 5

hours post dose a test lunch, low in vitamin A, was administered. The computerised program Foodbase determined the compositions of the diets. The components of the emulsion are shown in Table 3.2 and the compositions of the test breakfast, and test lunch are shown in Tables 3.3 and 3.4, respectively. Test meals were designed to provide subjects with approximately one third of their daily energy requirement. The study presented in Chapter 7 used a single emulsion of [10,19,19,19]^{2}H retinyl palmitate and the study presented in Chapter 8 used two separate emulsions of [10,19,19,19]^{2}H retinyl palmitate and [1,1,1,^{13}C]tripalmitin.

Table 3.2: Composition of the emulsion.

Casein	0.65g
Beet Sugar	0.25g
Glucose	0.5g
Chocolate Nesquick powder	0.6g
Sunflower oil	1ml
Water	6ml
1 stable isotope tracer per emulsion of either:	
[1,1,1,^{13}C]tripalmitin	10mg/kg body weight
[10,19,19,19]^{2}H retinyl palmitate	300μg/kg body weight

Table 3.3: Composition of the test breakfast.

Food Item	Portion Size (g)	Protein (g)	Fat (g)	CHO (g)	Energy (kJ)	Retinol (μg)	Carotene (μg)
Rice Krispies	40	2.44	0.36	35.88	628.8	0	0
Whole Milk	150	4.8	5.85	7.2	412.5	78	31.5
Orange juice	200	1	0.2	17.6	306	0	34
White Toast	80	7.44	1.28	7.04	903.2	0	0
Butter	10	0.05	8.17	0	303.1	81.5	43
Jam	20	0.12	0	15	111.4	0	0
TOTAL		**15.85**	**15.86**	**82.72**	**2665**	**159.5**	**108.5**

Table 3.4: Composition of the test lunch.

Food Item	Portion Size (g)	Protein (g)	Fat (g)	CHO (g)	Energy (kJ)	Retinol (μg)	Carotene (μg)
White bread	80	6.08	1.04	37.44	740.8	0	0
Ham	30	4.32	7.08	0.42	342	0	0
Salad Cream	15	0.255	4.65	2.505	171	1.35	2.55
Lettuce	20	0.14	0.2	0.38	10.6	0	10
Tomato	40	0.28	0.12	1.24	29.2	0	256
Crisps	25	1.4	9.4	12.33	568.75	0	0.5
1 Twix	58	3.25	14.21	36.66	1167.5	0	4.06
1 Apple	160	0.64	0.16	18.88	190.4	0	28.8
TOTAL		**16.37**	**36.86**	**109.8**	**3220.2**	**1.35**	**301.91**

3.3.4 ANALYSIS OF RETINOL AND RETINYL PALMITATE IN PLASMA

3.3.4.1 Standardisation.

The internal standard used for the analysis of both retinol and retinyl palmitate in plasma was retinyl acetate. Internal standard was made up in aliquots of 10μg/ml on a regular basis and concentrations were determined by spectrophotometry using the molar extinction coefficient of 51090 absorbance units/ cm ($E^{1\%}_{1cm}$ 1550). Standards were stored in amber vials at -70°C until required. On the day of analysis standards were re-constituted with 1ml of chloroform-methanol (2:1) and added to the plasma sample.

3.3.4.2 Extraction.

Plasma (500μL) was dispensed into a 10ml glass tube and retinyl acetate internal standard (0.5μg) was added in chloroform:methanol (10μL; 2:1, v/v). The plasma containing internal standard was vortexed. Chloroform methanol (5.0ml; 2:1 v/v) was then added. The preparation was vortexed, shaken and sodium chloride (NaCl; 1M; 1.0ml) added. Solvent and aqueous phases were separated by centrifugation (720g; 10 minutes; 4°C). Vitamin A and lipid

separate into the lower solvent phase under the protein plug. The upper aqueous phase was removed and the organic phase collected by aspiration. Sodium chloride (NaCl; 0.9%; 1.0ml) was added to the interfacial protein disc and vortexed. The disk was re-extracted with chloroform-methanol (5.0ml; 2:1, v/v), mixed and shaken (15 minutes), sodium chloride (NaCl; 1M; 1.0 ml) was added, mixed and centrifuged (720g; 10 minutes; 4°C). The lower phase collected was combined with the initial extract, dried under nitrogen and transferred to amber vials with inserts.

3.3.4.3 Analysis.

Retinol and retinyl palmitate concentrations were measured by reverse phase high performance liquid chromatography (HPLC). The HPLC hardware consists of a Beckman System Gold 508 Auto sampler, Beckman System Gold 125 Solvent pump, Spectraphysics UV detector set at 325nm range 0.025 and Beckman 32 karet software and computer (more details in appendix 3.1). The dried residue from the extraction was reconstituted in chloroform:methanol (50μL; 2:1, v/v) and 10μL was applied to the C18 ODS 30cm reverse phase column. A 2cm guard cartridge packed with Perisorb 18 preceded the analytical column. For elution of retinol, retinyl acetate and retinyl palmitate, 100% solvent A (HPLC grade water/HPLC methanol, 5:95,v:v) was pumped for 10 minutes, then 100% solvent B (isopropanol:methanol, 1:1, v:v) for 10 minutes at a flow rate of 1.0 ml/min. The column was re-equilibrated by pumping 100% solvent A for 15 minutes at 2 ml/min between runs. Under these conditions the retention times for retinol, retinyl acetate and retinyl palmitate were approximately 7.9 minutes, 11.4 minutes and 22.9 minutes, respectively. Figure 3.6 illustrates a typical HPLC chromatogram from an extracted plasma sample.

3.3.4.4 Calculation of results.

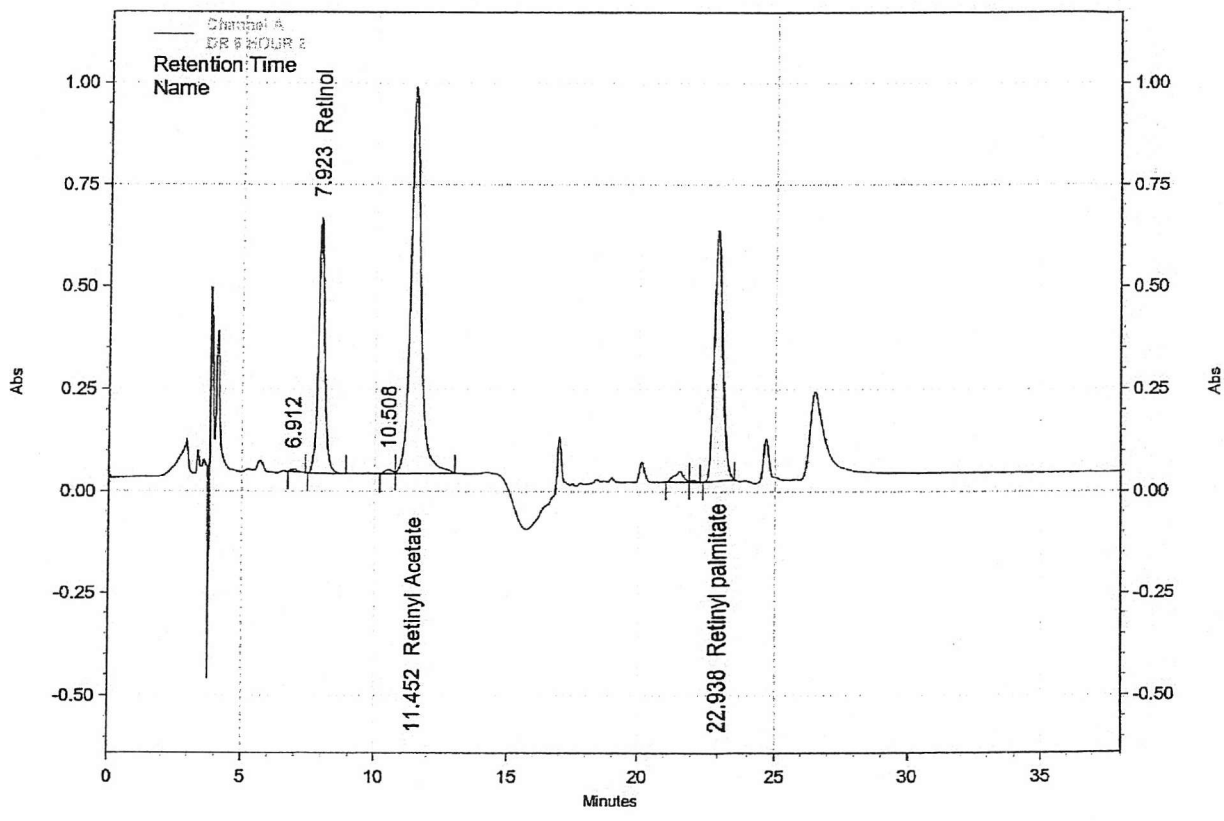
Plasma retinol and retinyl palmitate concentrations were calculated by comparison of the chromatographic peak areas with the area of the retinyl acetate internal standard of known concentration, which had been verified by spectrophotometry on the day of the assay. The calculations are summarised in appendix 3.4. The accuracy and precision of the analytical method were assessed using pooled plasma samples obtained as part of the UK external

quality assurance scheme and by using a human serum standard quality control reference material verified by the National Institute of Standards and Technology.

Figure 3.6

Typical HPLC chromatogram obtained from an extracted postprandial plasma sample.

(Chromatogram illustrates retinol, retinyl acetate and retinyl palmitate peaks.)



Detector 1 Results

Name	Retention Time	Area	Height
Retinol	7.923	101984	6246
Retinyl Acetate	11.452	248088	9468
Retinyl palmitate	22.938	121808	6120
Totals		471880	21834

3.3.5 ANALYSIS OF RETINOL AND RETINYL PALMITATE IN STOOL.

All procedures involving retinol, retinyl palmitate and retinyl acetate were performed in dark or subdued lighting. All stock preparations of retinol, retinyl palmitate or retinyl acetate were standardised by spectrophotometry using molar extinction coefficients of 52481, 50840 and 51090 absorbance units/ cm respectively.

3.3.5.1 Extraction

For each stool specimen, 4 aliquots of 0.5g (exact weight noted) homogenised stool were weighed into 10ml labelled glass tubes. Two aliquots were extracted as below (unspiked samples). To the other two samples retinol and retinyl palmitate standards in chloroform:methanol (0.5μg; 2:1 v/v) were added. All samples were thoroughly mixed. To the stool chloroform methanol (4ml; 2:1 v/v including 50mg/L of BHT) and retinyl acetate (0.5μg) were added. Samples were vortexed, shaken (15 minutes) and centrifuged (1620g; 10minutes; 4 degrees) to separate the particulate matter from the solvent. Using a glass Pasteur pipette the solvent (top layer) was removed into a clean pre-weighed 10ml glass tube. The complete process was repeated by re-extracting the particulate matter (dry stool) and combining the solvents. The combined solvents were washed with calcium chloride ($CaCl_2$; 1.76ml ; 0.04%w/v) and centrifuged (1620g;10 minutes; 4 degrees). Two layers were evident with an interface between them. The top aqueous layer was removed and discarded. A further wash of 0.04% $CaCl_2$:chloroform:methanol (1.76mls ; 47:3:48) was added to the remaining solution, centrifuged (1620g;10minutes;4 degrees) and the top layer discarded. The collected organic phase containing isolated lipids was dried under N_2 at 40°C in the dark. The extract was re-dissolved in 1.0 ml hexane and further purified by application to an aminopropyl solid phase extraction cartridge containing 1.0g of sorbent and preconditioned with hexane (6.0ml). Retinol, retinyl acetate and retinyl palmitate were eluted with chloroform:methanol (6.0ml; 2:1 v/v). The elutant was dried under N_2 at 40°C in the dark and stored at –20°C until analysis by HPLC.

3.3.5.2 Analysis.

Dried extracts were reconstituted in chloroform methanol (100µL; 2:1 v/v). Retinol and retinyl palmitate were separated and quantified by HPLC. The HPLC system on which the stool samples were analysed was different to that used for the analysis of vitamin A, due to time constraints of analysis. The HPLC system comprised a Hewlett-Packard 1050 series quaternary pump and absorption spectrophotometer set at 325 nm, a manual 20µL loop injector and a Supelcosil ODS reverse phase column fitted with a C18 guard column (details in appendix 3.1). Retinol and retinyl esters were separated using a binary solvent system. Both solvents were vacuum filtered and degassed. Solvent A was 100% methanol and solvent B was methanol:propan-2-ol (1:1 v/v). The column was equilibrated with the initial mobile phase by pumping at 2ml/min for 20 minutes and then at 1ml/min for 5 minutes before each sample injection. Following sample injection, solvent A was pumped at 1ml/min for 8.5 minutes after which the composition was changed linearly to 100% solvent B over 0.5 minutes and then maintained until 30 minutes. Retinol, retinyl acetate and retinyl palmitate peaks were detected at retention times of 5.6, 7.8 and 20.2 minutes respectively and the peak areas were quantified using the Beckman System Gold software. Figure 3.7 illustrates a typical HPLC chromatogram from an extracted stool sample.

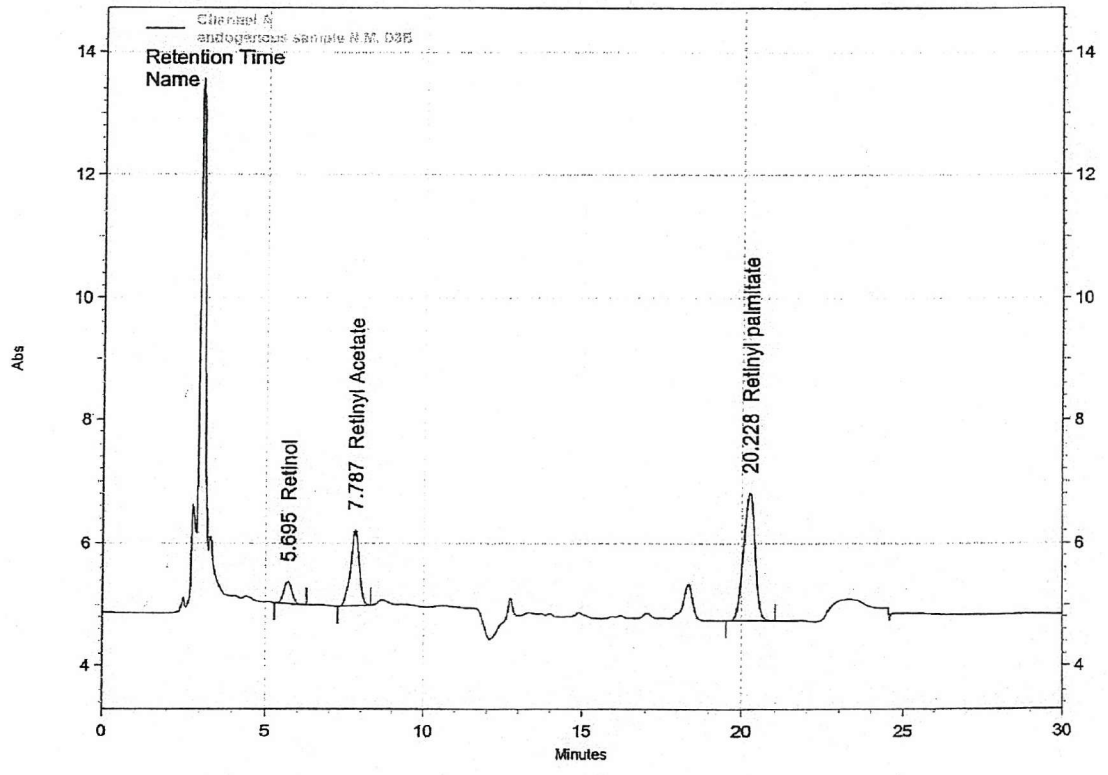
3.3.5.3 Calculation of results.

For each sample, the peak area ratios of retinol: retinyl acetate and retinyl palmitate:retinyl acetate were calculated. For each specimen, the retinol and retinyl palmitate concentration of the unspiked samples was calculated using the method of standard addition. The reasoning behind using this method is discussed in section 3.4.2. The mean unspiked sample peak area ratio (U) was quantified by comparison with the difference (S-U) between the ratios of the spiked (S) and unspiked samples. The difference calibrates the peak area ratio resulting from the retinol and retinyl palmitate spikes of 500ng/0.5 g homogenised stool as described above. Thus, Sample concentration = U x 500/(S-U) ng/0.5 g homogenised stool. This method corrects for differences in recovery between specimens and calculates the concentration of total retinol and total retinyl palmitate of 0.5 g homogenised stool. Daily excretion was

calculated as µmol/ day using the molecular weight and the recorded weight of the homogenised stool.

Figure 3.7

Typical HPLC chromatogram obtained from an extracted stool sample.
(Chromatogram illustrates retinol, retinyl acetate and retinyl palmitate peaks.)



HP 1050 Results

Name	Retention Time	Area	Height
Retinol	5.695	63940	3578
Retinyl Acetate	7.787	234232	12292
Retinyl palmitate	20.228	485120	20717
Totals		783292	36587

3.3.6 STOOL AND PLASMA: DEUTERIUM ENRICHED SAMPLES

3.3.6.1 Sample preparation.

Total plasma and stool vitamin A concentrations were used to assess the movement of vitamin A following the test meal. Using this method alone it is not possible to differentiate between endogenous and exogenous retinyl palmitate. Labelling retinyl palmitate in the test meal makes it possible to trace the label through the body in both stool and plasma. Figure 3.8 illustrates how orally administered label can be detected in stool and plasma. To analyse deuterated vitamin A by GCMS, samples of both plasma and stool must be first prepared as described in sections 3.3.4 & 3.3.5, by lipid extraction, and separation by HPLC.

Peaks of retinol and retinyl palmitate that contain d_4 label from plasma, were separated by HPLC and collected into clean glass tubes using a fraction collector. Fractions containing retinol and retinyl palmitate from stool were manually collected from 30 seconds before to 30 seconds after the retention time. Fractions of duplicate samples were pooled, dried under nitrogen at 40°C and stored at -20°C until preparation for analysis of isotopic composition. Native retinol peaks were reconstituted in hexane and transferred to amber vials with inserts. Samples containing retinyl palmitate were first saponified before transfer to amber vial with inserts. The pooled retinyl palmitate fraction was saponified by the addition of potassium hydroxide (1% KOH; 1ml) in ethanol. The solution was vortexed (30 seconds) and left in the fridge (30 minutes). Distilled water (1ml) followed by hexane (2ml) was added. The mixture was vortexed and centrifuged (1620g ;5 minutes ; 4 degrees). The top hexane layer was pipette into a fresh-labelled 10 ml glass tube. The aqueous layer was re-extracted with hexane (2ml). The hexane fraction was removed and combined with the initial extract and dried under nitrogen at 40°C. The dried retinol derived from the retinyl palmitate peak was transferred in hexane to amber gas chromatography vials with inserts and re-dried. Prior to analysis by GCMS, samples were derivatised with trimethylsilyl imidazole (TMSI) (100μL) and sealed under nitrogen. Analysis by GCMS was carried out within 24 hours by direct injection of the reaction mixture.

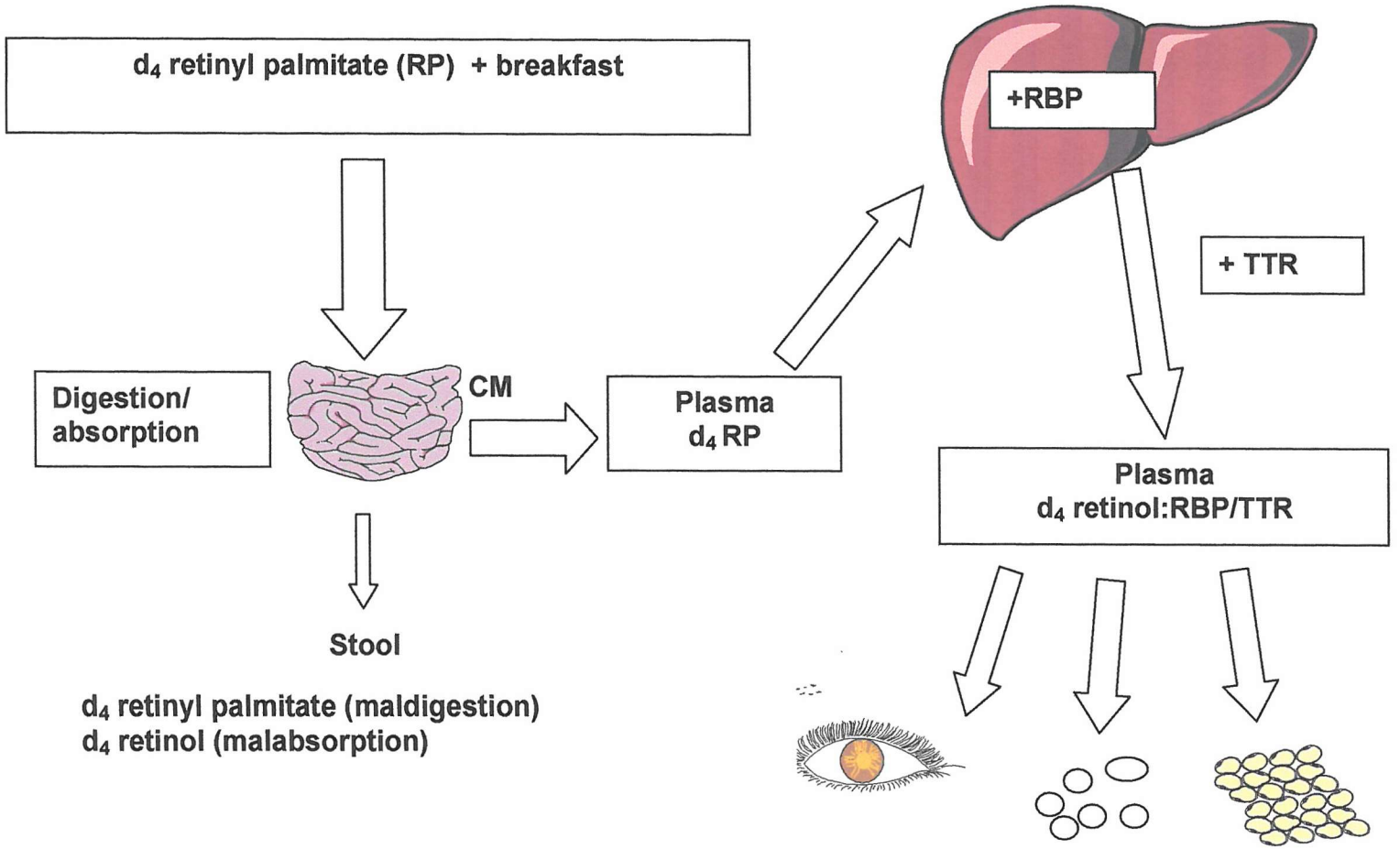


Figure 3.8: Schema illustrating stable isotope methodology to characterise the partitioning of exogenous retinyl palmitate.

3.3.6.2 Analysis

Derivatised samples of native retinol and retinol derived from the retinyl palmitate peak were analysed by GCMS, to obtain the ratio of unlabelled: labelled retinol (details of GCMS system in appendix 3.1) Retinol and [10,19,19,19] ^{2}H-retinol were monitored in selected ion mode using the fragments m/z 268 and m/z 272 respectively at the retention times of 9.85 and 9.79 minutes. The isotopic ratios of the samples were calculated by comparison of the fragment peak areas with a range of retinol and [10,19,19,19] ^{2}H-retinol standards (derived by saponification of mixtures of palmitate esters of known isotopic composition) analysed under identical conditions. The isotopic ratio was used to calculate retinol, [10,19,19,19] ^{2}H-retinol, retinyl palmitate and [10,19,19,19] ^{2}H-retinyl palmitate from the total retinol and retinyl palmitate concentrations measured by HPLC.

3.3.7 PLASMA LIPID ANALYSIS.

Total plasma TAG concentrations presented in chapter 7 were analysed by enzymatic assay, using Infinity Triglyceride Reagent, details presented in appendix 3.5.

For the results presented in chapter 8 lipid analysis was carried out on the GC-C-IRMS. Use of this instrument enables the measurement of both the concentration and the enrichment of individual fatty acids in the TAG fraction. A number of essential steps in the preparation of samples for analysis included, 1) lipid extraction, 2) isolation of the TAG fraction, and 3) formation of fatty acid methyl esters (FAME). The methodological stages are described in detail below. This method of lipid analysis is a routine method in use within the Institute of Human Nutrition Laboratory [Evans *et al* 2002].

3.3.7.1 Extraction.

Plasma (1ml) collected at each time point during the study (chapter 8) was pipette into clean 10ml glass tubes. Internal standard in chloroform methanol (2:1 v/v) was added to the plasma using a Hamilton Syringe and mixed (C17 TAG; Heneicosanoic acid; 100μg). The mass of standard used was chosen to reflect the approximate concentration of TAG within the plasma samples. Chloroform methanol with BHT (5.0ml; 2:1 v/v) was added to the plasma

sample, vortexed and shaken (15 minutes). Sodium chloride solution (NaCl; 1M;1.0ml) was added with mixing to the samples, which were centrifuged (720g; 10minutes; 4 degrees) to separate the solvent and aqueous phases. Three distinct phases were evident, the top aqueous phase, a protein rich interface plug, and a lower solvent layer. The aqueous phase was removed and discarded, and the solvent layer was removed into a clean tube by carefully passing the Pasteur pipette through the protein plug. Sodium chloride (NaCl; 1.0ml 0.9% w/v) was added to the remaining protein plug and the complete process was repeated by re-extracting the protein plug to ensure high recovery of the lipid from the extraction. Samples were dried under nitrogen, sealed and stored at -20°C.

3.3.7.2 Isolation of TAG fraction by Solid Phase Extraction.

The method of solid phase extraction (SPE) for separation of the lipid classes was developed within the laboratory of the Institute of Human Nutrition [Burdge *et al* 2000]. Bond-elute cartridges (aminopropylsilica; NH_2 100mg) were placed over clean 10ml glass tubes in a vacuum extraction tank. Chloroform:methanol (1.0ml; 2:1 v/v) was added to the lipid extracted from plasma and vortexed. Samples were applied to the cartridges and allowed to drip through under gravity. A further two washes of chloroform methanol (1.0ml; 2:1 v/v) were added to the cartridges and drawn through under vacuum. Tubes containing elutent (1) were removed from the apparatus, dried under nitrogen and stored at -20°C for further processing later. With fresh glass tubes in the apparatus, chloroform:methanol (1.0ml; 60:40) followed by methanol (1ml) was applied to the cartridge and drawn though under vacuum and the resulting pooled elutent was discarded. With fresh labelled glass tubes in place two washes of chloroform: methanol: acetic acid (1.0ml; 100:2:2) were added to the cartridge and washed through under vacuum (this eluted fraction contains NEFA). The bond-elute cartridges were discarded and replaced with new cartridges that were preconditioned with 4 washes of hexane (1.0ml). The stored dried elutent (1) was re-suspended in hexane (1.0ml) and vortexed. With fresh-labelled glass tubes in place the sample was added to the cartridges and allowed to drip through under gravity. The cartridges were washed twice with hexane (1.0ml) under vacuum and the elutent in the glass tubes discarded. Again with fresh

labelled tubes in place, two washes of hexane:chloroform:ethyl acetate (1.0ml; 100:5:5) were applied under vacuum. The resulting elutent (TAG fraction) was dried under nitrogen at 40°C.

3.3.7.3 Formation of fatty acid methyl esters.

In order to analyse the samples by GC-C-IRMS, the isolated TAG fractions need to be methylated. The reason for methylation is twofold. Firstly, as TAG is a large molecule with more than one fatty acid it has a high boiling point, removal of the fatty acids from the glycerol backbone reduces the boiling point of the molecule. Secondly, resolution and identification of molecules with multiple fatty acids would be difficult especially when trying to identify the isotopic enrichment in one of those fatty acids. TAG is hydrolysed to individual fatty acids and methylated to produce fatty acid methyl esters (FAME) that are sufficiently volatile to permit separation by gas chromatography.

To achieve methylation, toluene (1.0ml) and 2% sulphuric acid in methanol (2.0ml) was added to the dry TAG samples collected from SPE. Samples were sealed and placed in a heating block at 50°C overnight to allow complete methylation of the fatty acids. After incubation samples were removed and allowed to cool. Neutralising reagent (25g $KHCO_3$ + 34.55g K_2CO_3 in 500ml distilled water; 2.0ml) was added to the samples to prevent further reaction. Hexane (2.0ml) was added to each sample, vortexed, shaken (15 minutes) and centrifuged (720g; 14°C; 10 minutes) to separate the aqueous phase from the solvent phase. The upper solvent phase was completely removed into round bottom tubes, hexane was added (2.0ml) to the remaining aqueous phase and the whole process was repeated. The solvent layer in the round bottom tubes was gently dried down under nitrogen. These round-bottomed tubes were washed with dry hexane (200 μL), vortexed and the solvent transferred to GC vials containing inserts. The process was repeated three times and the solvent in the GC vials dried under nitrogen to complete dryness. Once dry, an isotopic enrichment standard (C23; tricosanoic acid methyl ester) was added to the sample in the same mass as the original standard (100μg). Samples were completely dried, sealed under nitrogen and stored at -20°C until analysed.

3.3.7.4 Analysis.

Total plasma concentrations of palmitic acid were used to determine the pattern of TAG appearance into the circulation following the test meal. Using this method alone it is not possible to differentiate between endogenous TAG (from adipose tissues or excreted from the liver (VLDL-TAG), and exogenous TAG from the meal (TAG rich CM). By labelling the lipid within the test meal it is possible to trace the ^{13}C label though the body. The FAMES generated (from the methods described) were analysed on the ORCHID GC- combustion isotope ratio mass spectrometer (GC-C-IRMS), which indicates the proportions of fatty acids present in the samples and the enrichment of each fatty acid

The concentration ratio of the isotopic standard to the internal standard within the samples was used to assess the recovery of lipid from the samples. FAME's were resolved on a fused silica capillary column using an HP6890 GC (for details refer to appendix 3.1). Prior to injection samples were re-suspended in dry hexane (100μL) and vortexed. Samples were loaded onto a carousel and a small aliquot (1μL) of each sample was automatically injected into the machine. The FAMEs were combusted to CO_2 by heating to 860°C in the presence of platinised copper oxide (PtCuO) using a Orchid IRMS interface and $^{13}CO_2$:$^{12}CO_2$ ratio was determined by a 20/20 stable isotope analyser. Data collection and isotope post-processing was performed by ORCHID software. Palmitic acid was resolved to baseline and identified by retention time relative to standards. Repeated analysis of the same specimen showed that the CV for measurement of the concentration of ^{13}C labelled fatty acid in individual plasma lipid classes was consistently less than 4%. This technique was able to resolve ^{13}C enrichment at 0.002% total fatty acid mass.

3.3.7.5 Calculation of results

Concentrations of ^{12}C palmitic acid and ^{13}C palmitic acid were calculated by integration of baseline corrected peak areas on chromatograms generated from the total ion current of the GC-C-IRMS.

For 1.0ml of plasma:

(A) Total Palmitic acid concentration (μmol/L) =

(Peak area of palmitic acid / peak area of internal standard (C17:0)) X (concentration of internal standard μmol/L)

(B) Fractional enrichment of palmitic acid =

Determined from extrapolation using an enrichment calibration curve

(C) ^{13}C Palmitic acid concentration =

Total palmitic acid concentration (A) x fractional enrichment of palmitic acid (B)

3.3.8 STOOL – LIPID ANALYSIS

In order to assess stool lipid losses, samples obtained from the three-day stool collections were analysed for total lipid content (chapter 7), by a method that is in routine use within the Institute of Human Nutrition Laboratory [Murphy *et al* 1995; Murphy *et al* 2001].

3.3.8.1 Total stool lipid.

All samples were analysed in duplicate. Stool homogenate was dried on a freeze dryer. Dried stool (50mg ± 1mg) was weighed into clean 10ml glass tubes and the sample weight recorded. Chloroform methanol (1.75ml; 2:1 v/v including 50mg/L of BHT), glacial acetic acid (500μL) and chloroform methanol (20ml) were added to the tube. Samples were vortexed between each addition of solvent. Samples were sealed and placed in the fridge overnight. The following day samples were centrifuged (1620g; 10 minutes; 4 degrees) to separate the particulate matter from the solvent. Using a glass Pasteur pipette the solvent (top layer) was removed into a clean pre-weighed 10ml glass tube. The process was repeated by re-extracting the particulate matter (dry stool) and combining the solvents. The combined solvents were washed with calcium chloride (0.04% $CaCl_2$; 1.76ml) and centrifuged (1620g; 10 minutes; 4 degrees). Two layers were evident with an interface between. The top aqueous layer was removed and discarded. A second wash of 0.04% $CaCl_2$:chloroform:methanol (1.76ml; 47:3:48) was added to the remaining solution, centrifuged (1620g; 10 minutes; 4 degrees) and top layer discarded again. Ethanol (200μL) was added to each tube and the sample dried under nitrogen. The dry weight of the tube containing lipid was measured and the mass of lipid calculated by difference.

Further calculation allowed the presentation of lipid mass as % dry weight, g/d of lipid from total daily dry weight.

3.3.9 ANALYSIS OF RBP AND TTR BY NEPHELOMETER (Appendix 3.6).

Nephelometry is defined as the measurement of light scattered by a particulate solution. RBP and TTR are proteins with short biological half-lives, 11 hours and 2 days respectively and serum concentrations reflect the synthesis capacity of the liver. The method is an immunochemical reaction, where the proteins in the sample of serum form immune complexes with specific antibodies. These complexes scatter a beam of light passed through the sample. The intensity of the scattered light is proportional to the concentration of the relevant protein in the sample. The results are evaluated by comparison with a standard of known concentration.

Samples of serum were bar-coded, gently mixed by inversion and loaded onto the analyser along with reagents (Antiserum Human Prealbumin; Antiserum Human RBP). Quality control specimens were loaded onto the analyser and pre-programmed to run at the start of the assay and at intervals throughout the run. Serum samples were automatically diluted 1:5 with N diluent. Results were calculated automatically. Defined reference intervals for serum samples from healthy adults are 0.2-0.4g/L (3.92-7.84μmol/L) prealbumin (TTR) and 0.03-0.06g/L (1.43-2.86μmol/L) RBP.

Reference curves were constructed by multi-point calibration. Serial dilutions of the N protein standard SL were automatically prepared using N diluent. For accuracy and precision the protein controls low, medium and high were used at the beginning of each run, after every 15 samples and at the end of the run. The confidence interval for the controls is assigned the value ± 15%. The coefficients of variation for the assays of RBP and TTR in serum with N-antiserum on the Behring nephelometer were, 1.9% and 3.1% for TTR and RBP intra assay, and 1.4% and 2.2% for TTR and RBP inter-assay, respectively

3.3.10 STATISTICAL ANALYSIS.

The data from the studies was prepared and analysed using a computerised data package (SPSS for Windows, SPSS inc., Chicago, USA). Results were reported as median and ranges. Differences were considered to be significant at the 95% confidence level; $P< 0.05$, above which values were considered to be non-significant (NS). Patients with CF are a heterogeneous population; strictly speaking it is unlikely that they represent a single population. It would inappropriate to attempt to normalise the data from a study population of this size, as although mathematically desirable it may well obscure functional differences of physiological interest [Waterlow, 1992]. For these reason non-parametric statistics were used. Mann Whitney U statistical analysis was used to assess differences between the two groups, and Spearman Rank was used to assess associations between two variables. The graphical data shown within the results were produced by a graphical package (GraphPad Prism 2.01, GraphPad Software Inc., USA).

3.4 VALIDATION OF METHODS.

3.4.1 PLASMA VITAMIN A

The internal standard (retinyl acetate) was used to correct for losses of retinol and retinyl palmitate that may occur throughout the extraction procedure. The internal standard was calibrated by spectrophotometry before the start of each analysis by measuring absorbance at 325nm, and the actual concentration calculated using a molar absorption value of 51090 absorbance units / cm [Merck Index, 1983].

Factors that may affect the reproducibility of the results are the skills of the operator to pipette the internal standard, accuracy of the pipette and the reproducibility of the method and HPLC procedure. Addition of internal standard was by positive displacement pipette, reproducibility of the pipette as determined by the manufacturer was 0.3% (CV). The range of volumes obtained for measuring 50μL was 49.5-50.5μL. Repeat analysis was carried out as the method described in section 3.3.4, on a pooled plasma samples. The mean, SD, and coefficient of variation for repeat analysis is presented in Table 3.5. Variation of the HPLC instrument was calculated by repeat injection of a single sample. Mean plasma retinol concentrations were 1.17 ± 0.03 μmol/L, and mean plasma retinyl palmitate concentrations were 0.16±0.01μmol/L. Thus most of the variation is in the extraction procedure. Recovery of an added spike of retinol and retinyl palmitate to plasma was found to be approximately 100% when calculated by use of the internal standard.

The limits of detection were determined to be 0.06μmol/L - 4.4μmol/L for retinol and 0.01μmol/L - 3μmol/L for retinyl palmitate. The ranges for retinol concentrations (R=0.9978) and retinyl palmitate concentrations (R=0.9992) were linear.

A commercial quality control specimen (QC HI & LO, Chromsystems,Munich, Germany) was used to assess both accuracy and precision of the analytical procedure and was analysed with each batch of samples. The specimen was a lyophilised control, based on human serum that was handled in exactly the

same manner as the subject specimen. The batch was accepted if the QC specimens fell within the defined accepted ranges. Further quality assurance was implemented by membership to the UK External Quality Assurance Scheme (EQA scheme, Department of Chemical Pathology, St Helier Hospital, Carshalton, Surrey). Samples were analysed every eight weeks and all results during the studies from the laboratory at the Institute of Human Nutrition were designated as acceptable by the scheme.

Table 3.5:

Results of the repeat analysis of retinol and retinyl palmitate concentrations from a pooled sample of plasma.

	Mean Concentration	SD	CV
Retinol	2.47μmol/L	0.134	5.4%
Retinyl Palmitate	0.9μmol/L	0.05	5.29%

3.4.2 STOOL VITAMIN A

Preliminary studies show that the recovery of retinol and retinyl palmitate added to stool was reproducible within a sample but that between samples variability was much greater. Recovery of retinol and retinyl palmitate was measured by addition of 500ng of retinol to 0.5g of stool homogenate before extraction. For retinol, with a mean recovery of 62.7%, the between sample s.d. and within sample s.d. were 13.4% and 4.3 % respectively. For retinyl palmitate, with a mean recovery of 72.6%, the between sample s.d. and within sample s.d. were 14.6% and 6.5 % respectively. Consequently extraction was standardised by the method of standard addition to correct for the between sample component of variance. Analytical precision was calculated from the difference between duplicate faecal extractions analysed and standardised in this manner. Table 3.6 shows the sample coefficient of variation at different faecal excretion rates.

Table 3.6 :

Table illustrating the sample coefficient of variation at different faecal excretion rates for the excretion of both retinol and retinyl palmitate (RP).

Range of faecal excretions	Mean retinol excretion	Sample CV	Number of samples results based on (n=)
Retinol excretion 0.02-0.75μmol/d	0.3μmol/d	6.7%	11
Retinol excretion 0.75-1.5μmol/d	1.1μmol/d	9.0%	8
RP excretion 0.01-0.75μmol/d	0.16μmol/d	10.8%	11
RP excretion 0.75-1.6μmol/d	1.04μmol/d	11.5%	11

RESULTS CHAPTERS

Results obtained from studies undertaken are reported within the following chapters. Section A of the results chapter includes chapter 4-6 and addresses issues relating to retinol concentrations in adult patients with Cystic Fibrosis compared to healthy adults. The section investigates the prevalence of low retinol concentrations in CF patients who are undergoing current CF care regimens. The section begins to address firstly factors that may contribute to low retinol, including supply of vitamin A and the presence of infection and secondly the clinical relevance of a low retinol. Despite current care regimens and excluding patients who exhibit the factors that effect plasma retinol (illustrated in section A) some CF patients still have low retinol concentrations in their plasma. Results presented in section B (chapters 7 & 8) focus on the movement of vitamin A within the body and addresses in particular, questions relating to both the availability of vitamin A across the GI tract and the mobilisation of retinol from the liver. Results investigate the appearance of vitamin A in stool and retinyl palmitate in plasma following an oral bolus dose. The use of stable isotopic tracers also enables the appearance of mobilised retinol from the liver to be investigated. A general discussion of results reported within this thesis is presented at the end of the results chapters (chapter 9).

SECTION A RESULTS

CHAPTER 4

CIRCULATING RETINOL CONCENTRATIONS IN A GROUP OF PATIENTS WITH CF AND HEALTHY ADULTS.

4.1 INTRODUCTION.

Anderson first described the existence of a relationship between vitamin A and the disease CF in 1939. Clinical evidence of xerophthalmia was evident in 10 of her 49 cases with pancreatic fibrosis. One subject was administered carotene by mouth and directly to the eyes, which allowed the slow healing of ulcers. At post-mortem it was revealed that vitamin A therapy in this subject had been fairly effective in restoring epithelium when compared to a sibling that had received no therapy. Since this time there have been other reports of vitamin A deficiency [O'Donnell 1987], xerophthalmia [Brooks *et al* 1990], abnormal dark adaptation [Raynor *et al* 1989], and poor nocturnal vision [Huet *et al* 1997] despite patients receiving both vitamin A supplements and enzyme therapy. These findings have been attributed to vitamin A being malabsorbed along with dietary lipid. If true this perceived deficiency could have important implications within the body as vitamin A is an essential nutrient required for many functions other than vision, which include cell differentiation, barrier function, maintenance of epithelia and immune function. Along with reports of functional manifestations a series of studies in CF (mainly children) report low plasma retinol concentrations [Bennet *et al* 1967; Jacob *et al* 1978; Palin *et al* 1979; Smith *et al* 1972; Fulton *et al* 1982]. The current literature with relation to plasma concentrations of retinol is presented in table 4.1; the table illustrates 15 studies from 1967-2002. The lowest retinol concentrations were reported in the earlier studies when vitamin A was measured by the method of Neeld & Pearson [1979] (discussed in chapter 3), patients used crude preparations of pancreatic enzymes, did not attend routine clinical assessment, and nutritional advice was to consume diets low in fat and therefore low in vitamin A. When considering all studies plasma retinol concentrations were significantly related

to the year of study (Spearman R=0.707; P=0.007). In that plasma retinol concentrations were reported to be lower in the 1960's compared to the 1990's.

In current practice, all patients with CF who attend clinics within the U.K receive specialist CF care. Nutritional care, aggressive enzyme therapy, routine supplementation and annual reviews are all central to the management of these patients. It is recommended that all patients with CF who are pancreatic insufficient require supplementation of vitamin A [Ramsey *et al* 1992; Congdon *et al* 1981; Littllewood & Wolfe 2000; Anderson *et al* 1939; Peters & Rolles 1993, CF trust, 2002], and vitamin A (as plasma retinol) should be reviewed annually to monitor the risk of vitamin A deficiency and adjust supplementation accordingly [Ramsey *et al* 1992, CF trust 2002]. Currently both pancreatic insufficient patients and pancreatic sufficient patients with low concentrations of plasma retinol are routinely supplemented with prophylactic vitamin A in an attempt to increase plasma retinol concentrations, and overcome the widely held belief that the availability of vitamin A is compromised [Durie *et al* 1989; Eid *et al* 1990]. The true requirement for vitamin A has not been established within this group of patients and there are considerable differences in the doses recommended and prescribed in clinical practice [Peters *et al* 1993]. Routine supplementation is often in excess of 2mg/d, nearly four times the Reference Nutrient Intake (RNI) for healthy children and adults [Department of Health 1991].

The prevalence of low plasma retinol concentrations in adults with CF and factors that may contribute to this has not been investigated in adult patients who receive modern CF management with routine supplementation of vitamin A and pancreatic enzymes. It is important to estimate the prevalence of CF patients with low retinol concentrations in order to determine the proportion of the population at risk and inform clinical supplementation practices. It will provide valuable information to enable a balance to be gained between adequate supplementation of at risk individuals against the potential toxic risks of vitamin A over-supplementation.

4.2 AIM

The aim of the present study was two fold. As a first step we sought to characterise the prevalence of low retinol concentrations, compared to a reference population, in a cohort of adult patients with CF where nutritional care, enzyme therapy and routine supplementation of vitamin A were central to management. Secondly some of the factors that may impact upon a patient's plasma retinol were investigated.

Questions to be addressed within this chapter include:

1) How prevalent are low retinol concentrations in a group of adult patients with CF at Southampton?
2) What factors contribute to a low plasma retinol concentration?
3) Does a low plasma retinol concentration matter in terms of a patient's clinical course?
4) Are low concentrations of plasma retinol associated with a poor dietary supply of vitamin A?

4.3 HYPOTHESIS

Despite current practices patients with CF will have an increased prevalence of low concentrations of retinol when compared to healthy adults.

Table 4.1: Current literature on plasma retinol concentrations in patients with CF. (supp =supplement)

REFERENCE	PERT	VITAMIN A SUPP	DIET	SUBJECTS	PLASMA RETINOL μmol/L
Bennett 1967	Yes	No info	No info	45 CF children	0.44μmol/L no range
Smith 1972	yes	Daily supplement	No info	43 CF mean 12.3y	0.82μmol/L
Underwood 1972	yes	Daily multivitamin	High fat	48 CF 6m-22y	0.94μmol/L (0.24-2.69)
Jacob 1978	16 subjects	Daily multivitamin	No info	17 CF 6-17y	1.07μmol/L (0.48-1.67) calc from S.D.
Palin 1979	No info	No info	No info	36 CF; 15y ±4.9y	0.81μmol/L (0.21-1.41) calc from S.D.
Congdon 1981	32 subjects	27 subjects	Low fat	36 CF 10m-16y	1.98μmol/L (0.5-4.5) from graph
Fulton 1982	No info	No info	No info	34 CF 4-34y	0.73μmol/L no range
Raynor 1989	41 subjects	2 daily multivitamins	No info	43 CF 8-44y median 16y	34 normal dark adaptation - 1.1μmol/L (0.5-2.3) 9 abnormal dark adaptation - 0.53μmol/L (0.2-1.4)
James 1992	8 subjects	Yes	No info	9 CF median 22y	1.61μmol/L (1.14-2.39)
Winklhofer-Roob 1995	yes	12 subjects on supps	No info	35 CF 11.5±7.5y	1.05μmol/L (0.33-1.77) calc from S.D.
Duggan 1996	34 subjects	23 on multivitamins	No info	35 CF (CRP 9.8mg/L)	1.70μmol/L
Huet 1997	Yes	Yes	No info	10 CF mean 15.2y	1.47μmol/L (0.87-2.80)
Leonard 1998	Yes	Yes ADEK	No info	58 CF 19 children (7-15y) 35 adults (17-36y)	7-10y; 1.40μmol/L (0.7-2.0) 11-15y; 1.85μmol/L (1.2-2.2) 17-36y; 2.00μmol/L (0.7-3.0)
Ansari 1999	Yes	Yes	No info	28 CF Median 20y (12-33y)	1.68μmol/L (1.08-2.80)

4.4 METHODS.

This section will describe the methods used to assess both prevalence of low circulating retinol concentrations in a group of CF patients at Southampton and factors that may contribute to differences in plasma retinol.

4.4.1 CYSTIC FIBROSIS PATIENTS.

At the CF centre, Southampton University Hospitals Trust, a team of clinicians, specialist nurses, physiotherapists and dieticians annually review all adult patients. All investigations and data recorded at these reviews are documented in the patient's notes. Variables, that are recorded for all patients, include a dietetic review, clinical review, blood investigations, and lung function. In order to audit the patients' annual reviews, firstly it was necessary to identify those patients that regularly attend the clinic at Southampton, and locate patient notes. For all patients included in the audit complete notes were read and information of interest was recorded onto a prepared data collection sheet. Once data collection was complete all data was input into a SPSS database for analysis.

The complete audit of clinical notes was carried out at the last annual clinical review of 63 CF adult patients attending the CF centre at Southampton University Hospitals trust (SUHT) between January 1999 and July 2001. The only inclusion criteria to the study were that patients had to have attended an annual review between 1999-2001. The population studied was therefore representative of the whole population at SUHT. Specific aspects of the clinical assessment were recorded including, height, weight, body mass index (BMI), use of pancreatic enzymes, vitamin supplements, adherence to regimens, FEV1 (as a marker of lung function), number of admissions to hospital over the prior year and biochemical analyses of serum immunoglobulins, CRP and plasma retinol. As this analysis was retrospective, plasma retinol concentrations were measured by reverse phase HPLC within the routine Chemical Pathology Laboratory at Southampton General Hospital. The formation of reference range related to this analysis was from an ad hoc analysis of a small number of unspecified individuals using methodologies that predate existing routine methodology. It would not be appropriate to use these ranges to classify

subjects analysed by the current routine method. For these reasons a group of healthy adults were recruited to assess plasma retinol concentrations in a reference population. Due to practical limitations of the use of routine equipment within Chemical Pathology, and the need for further analyses of the reference blood, plasma retinol concentrations of the healthy adults were determined by reverse phase HPLC methodology within the Institute of Human Nutrition Laboratory. Both methods use common quality control sera (Chromsystems) and were routinely assessed within the external quality assurance scheme. A subset of 10 subjects had plasma retinol concentrations measured within both laboratories to assess the variability in results obtained from the two methods (table 4.2). Results obtained on the same specimen by the two different methods were not different from each other ($P>0.05$; Wilcoxon Signed Rank test). The mean difference observed between the two methods was 0.1µmol/L. Samples analysed by either method could therefore be compared.

4.4.2 HEALTHY ADULTS

Fasted healthy adults were recruited to the Clinical Nutrition and Metabolism Unit, Institute of Human Nutrition. Consent was obtained and blood drawn for measurement of plasma retinol. Plasma retinol concentrations were measured by HPLC within the Institute of Human Nutrition, methods described in chapter 3. Healthy adult recruits had measures taken of age, height, weight, CRP and BMI.

4.4.3 STATISTICAL METHODS.

The data presented within this chapter are reported as median and ranges. Differences were considered to be significant at $P < 0.05$. Non-parametric statistics were used as data was not normally distributed. Mann Whitney U statistical analysis was used to assess differences between the two groups, and Spearman Rank was used to assess the association between two variables.

Table 4.2:

Plasma retinol concentrations of 10 subjects obtained from the same specimen run by methods within the Nutrition and Chemical Pathology laboratories.

	PLASMA RETINOL μmol/L		
Subject	**Institute of Human Nutrition**	**Chemical Pathology, SUHT**	**Difference μmol/L**
1	3.47	3.30	0.17
2	1.65	1.50	0.15
3	1.92	1.90	0.02
4	0.81	1.00	0.19
5	2.08	1.90	0.18
6	1.15	1.25	0.10
7	1.85	1.90	0.05
8	3.26	3.30	0.04
9	0.74	0.90	0.16
10	0.95	1.00	0.05
MEAN	**1.79**	**1.80**	**0.1**

4.5 RESULTS.

Results are presented in two main sections. The first section investigates the prevalence of low plasma retinol concentrations in the group of CF patients compared to healthy adults. The second part begins to investigate, from the audit data, factors that may be influencing a patient's plasma retinol concentration and if a low plasma retinol impacts upon the patients clinical course.

4.5.1 SUBJECTS

Forty-seven fasted healthy adults participated in the study (31M; 16F; median age 28 years range 20-63 years) (table 4.3). All healthy adults had measures of weight, height, CRP and plasma retinol. Inclusion into the study criteria included

CRP <6mg/L, weight stable, and no evidence of infection or disease. All healthy adults had CRP concentrations <2mg/L and the group had a median BMI of 23.64kg/m^2 (20.66-31.31kg/m^2).

Of the 63 CF adult patients assessed in the audit, 8 were new patients who had not had an annual review, 7 patients had not completed an annual review since 1999, and 4 patients did not have plasma retinol measured (figure 4.1). The final study population of retrospective plasma retinol concentrations comprised of 44 CF adults, 23 men and 21 women, median age 21 years (range 17 – 45years). The group characteristics can be seen in table 4.4. There appears to be wide variability in patients studied, this is probably related to the heterogeneity of the disease. BMI was measured as a proxy for overall nutritional status; patients had a median BMI of 20.1 kg/m^2 (range 16.2 – 26.3 kg/m^2) with 46% of patients having a BMI of less than 20 kg/m^2. The median recorded estimated energy intake from food diaries was 2691 kcal/d (1104 – 4985 kcal/d) and the median lipase units were 4975 units/kg/d (0 – 20618 units/kg/d).

Table 4.3:

Subject characteristics of the 47 healthy adults.

	Median	Range
Age (years)	28	20-63
Height (metres)	1.73	1.58-1.92
Weight (kg)	70.00	58.40-99.20
BMI (kg/m2)	24.2	20.66-31.31

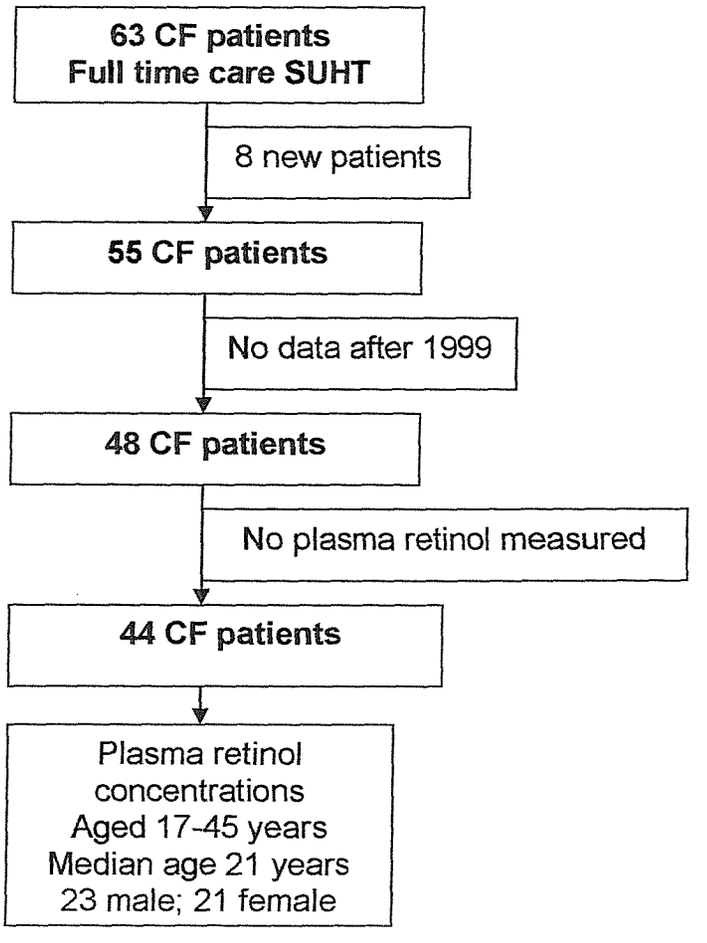
Table 4.4 :

Subject characteristics of the 44 adults with CF.

	Median	Range
Age (years)	21	17 - 45
Height (metres)	1.67	1.52 - 1.92
Weight (kg)	57.25	41.20 - 96.50
BMI (kg/m2)	20.15	16.20 - 26.20
Energy Intake (kcals)	2691	1104 - 4985
Lipase units (kg/d)	4975	0 - 20618
Vitamin A (mg retinol per day)	1.20	0.00 - 3.60
CRP (mg/L)	5.0	0.5 - 90.1
FEV1 (%)	63.50	27.00 - 88.10

Figure 4.1 :

Inclusion of patients with Cystic Fibrosis into the study flow diagram.



4.5.2 PLASMA RETINOL.

Plasma retinol concentrations of the 47 healthy adults and 44 CF patients are shown in figure 4.2. A table summarising the prevalence of low retinol concentrations in both CF patients and healthy adults is shown in table 4.5.

Median plasma retinol concentrations of the group of healthy adults were 1.80μmol/L ranging from 1.07 – 3.58 μmol/L. The majority of healthy adults (37 subjects; 79%) fell within the 95% CI of plasma retinol for adults observed from a national surveillance cohort (1.4 - 3.2 μmol/L; DoH / MAFF 1990). Nine healthy adults (19%) had plasma retinol concentrations lower than 1.4 μmol/L. There were no significant differences in plasma retinol concentrations between the genders (figure 4.3) (median (range): males 1.84μmol/L (1.07-2.97); females 1.71μmol/L (1.17-3.58); P=0.745), nor was there any obvious association with age (data not shown) (Spearman R value = 0.125; P=0.403).

The group of CF patients showed a greater variability in plasma retinol concentrations (figure 4.2). Median plasma retinol of the group was 1.45 μmol/L ranging from 0.4 - 4.8 μmol/L. When the CF group was compared to the 95% CI of plasma retinol for adults (1.4 - 3.2 μmol/L; DoH / MAFF 1990), 20 patients (46%) had plasma retinol concentrations lower than 1.4 μmol/L. Eight patients (18%) had plasma retinol concentrations lower than the minimum concentration (1.07μmol/L) found in the group of healthy adults. The plasma retinol concentrations of the patients with CF (median 1.45μmol/L) were significantly lower (Mann Whitney P=0.035) than the healthy adults (median 1.8μmol/L). Similar to the group of healthy adults there were no significant difference in plasma retinol between genders (figure 4.3), (median (range): males 1.4μmol/L (0.4-3.2); females 1.5μmol/L (0.4—4.8); P=0.596) or age (Spearman R=-0.086; P=0.58).

Figure 4.2

Individual and median plasma retinol concentrations (μmol/L) for the two subjects groups, 47 healthy adults (HA) and 44 patients with CF.

DoH/MAFF reference ranges for plasma retinol are also shown.

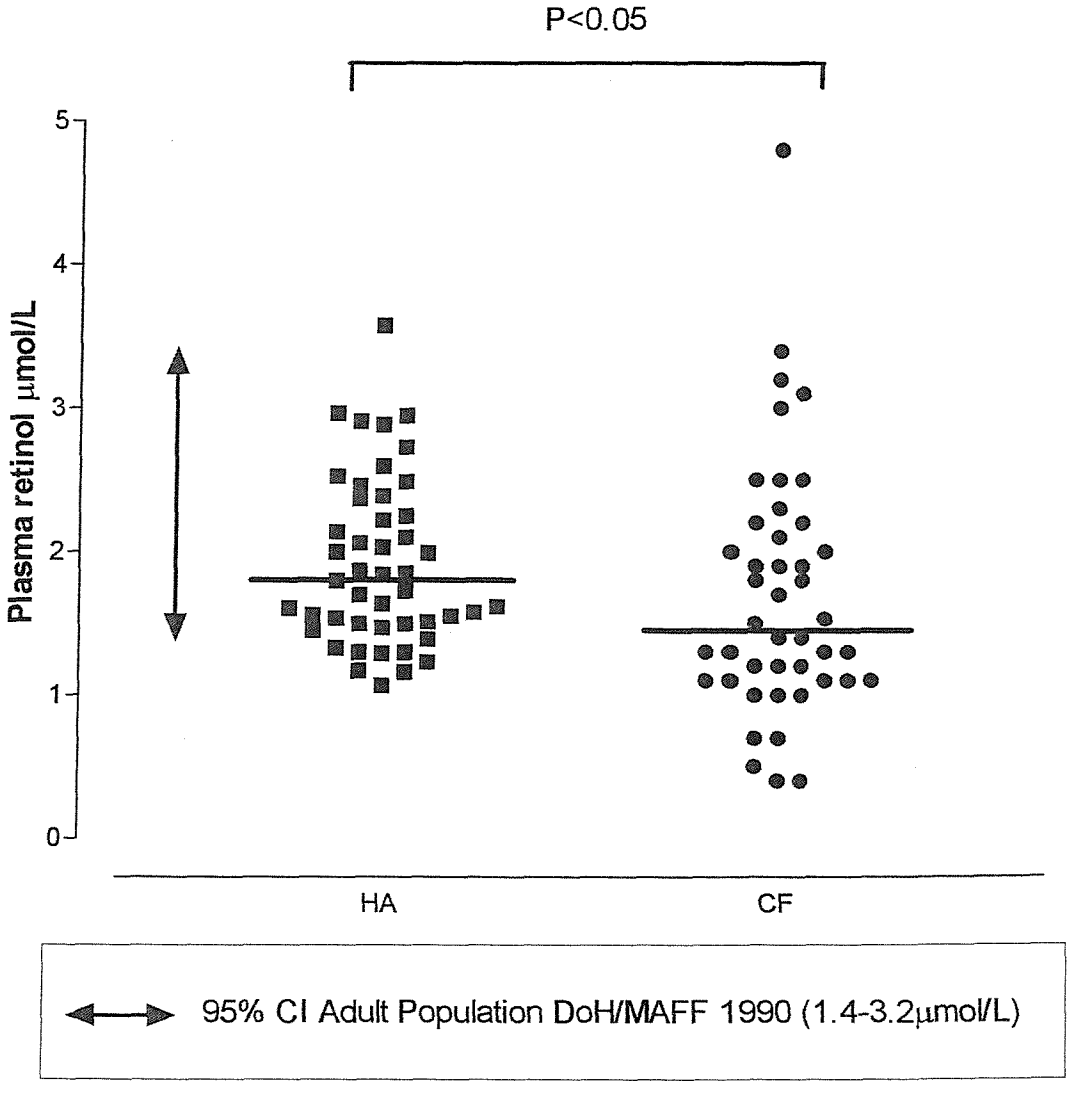


Table 4.5:

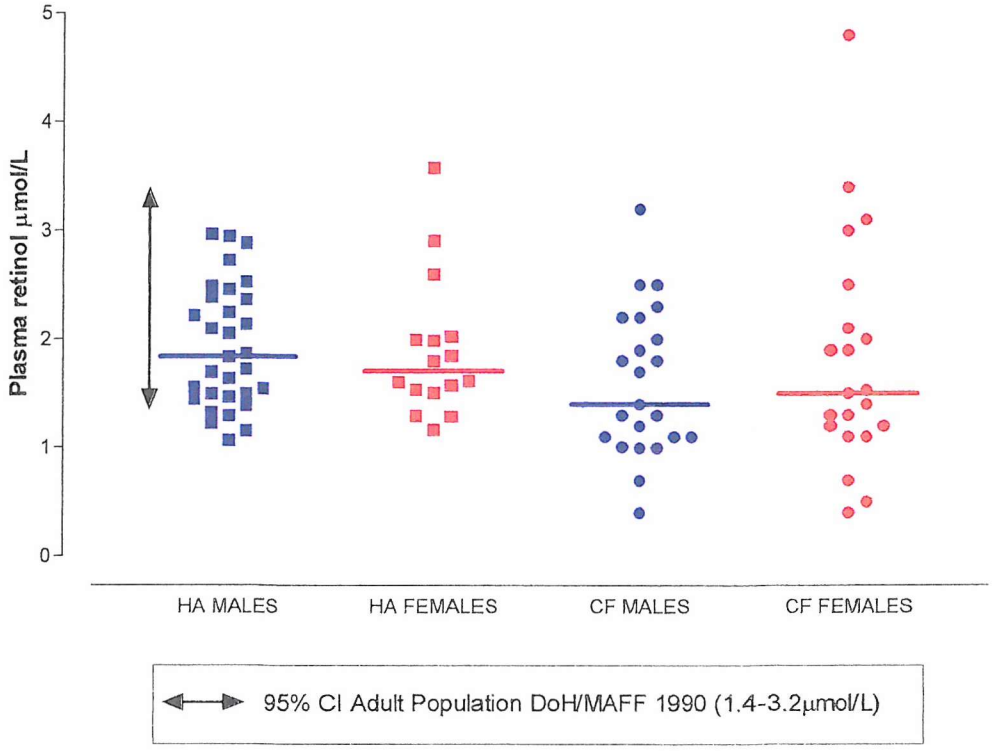
Table showing the prevalence of low retinol concentrations in both the patients with CF and healthy adults (HA).

	PREVALENCE			
	HA		CF	
	No. Patients	%	No. Patients	%
Below 95% CI for DoH (<1.4μmol/L)	9/47	19%	20/44	46%
Below min. value observed in HA (<1.07μmol/L)	N/A	N.A	8/44	18%

Figure 4.3

Individual and median plasma retinol concentrations (μmol/L) of both males and females in the two subjects groups, 47 healthy adults (HA) and 44 patients with CF.

Graph illustrates DoH/MAFF reference ranges for plasma retinol; there are no gender specific reference ranges.



4.5.3 FACTORS THAT INFLUENCE PLASMA RETINOL CONCENTRATIONS IN PATIENTS WITH CF.

The following section focuses on the audit of patients with CF and investigates variables measured within the audit, which may impact upon the variation seen in plasma retinol concentrations within this population. Particular interest will be placed on the impact of nutritional status, supply of vitamin A through the use of supplements and the effect of infection.

4.5.3.1 NUTRITIONAL STATUS

BMI can be used as a proxy marker to assess the nutritional status of an individual, in that those patients with the lowest BMI have the poorest nutritional status and are sicker. Plasma retinol concentrations were significantly correlated with BMI in patients with CF, in that those patients with the lowest body mass index appear to have lower plasma retinol concentrations (Spearman R=0.365; P<0.05) (figure 4.4). Those patients who fall within the box on the graph have plasma retinol concentrations within the DoH retinol reference range and BMI's between 20-25kg/m^2. The majority of patients fall outside of the box and 18 patients (41%) had BMI <20kg/m^2, of these, 11 (61%), had plasma retinol concentrations <1.4μmol/L (table 4.6.). In contrast there is no correlation between plasma retinol and BMI in the group of healthy adults (P=0.928) (figure 4.5). The majority of healthy adults fall within the DoH reference range for retinol, no patients have BMI <20kg/m^2 and 38% of subjects had BMI >25kg/m^2. In the healthy adults approximately 20% of subjects had plasma retinol concentrations <1.4μmol/L in both groups (BMI 20-25kg/m^{2}and BMI >25kg/m^2).

Other measures of nutrition and patients well being are those of energy intake estimated from food diaries and the amount of pancreatic enzymes required for adequate digestion and absorption of lipid. For example those patients requiring more enzymes and ingesting less energy tend to be sicker. Plasma retinol concentrations were not related to a patients estimated energy intake (P=0.151), or the amount of pancreatic enzymes taken measured either as an absolute amount enzyme dosage units per day (P=0.937), or corrected for body

weight, lipase units per kg per day (P=0.729). Energy intake was estimated from five-day diet diaries kept by the patients. There is always the factor of validity of estimates during self-reporting, however patients with CF are unlikely to under-report as there is no benefit, patients may however over report to please the investigator. For these reasons patients who report low intakes may be eating poorly and there is a weak indication of a relationship between plasma retinol concentrations and energy intake at low reported intakes (figure 4.6).

4.5.3.2 SUPPLY OF VITAMIN A

The simplistic explanation of a low plasma retinol is that the primary limitation is that of poor dietary supply either with or without poor compliance to supplement regimens. It has been reported [Nelson *et al* 1989; Russell-Briefel *et al* 1985] that there are a number of difficulties in adequately measuring vitamin A intake from the diet. All pancreatic insufficient patients with CF are routinely prescribed supplements of vitamin A greatly in excess of any habitual dietary supply and this would overcome any dietary deficiency that may be present. For these reasons in order to assess supply of vitamin A in patients with CF, supplement regimens were assessed. In the population investigated 40 of the 44 patients were routinely prescribed supplements as vitamins A and D and / or multivitamins (figure 4.7). The median dose of retinol per day in the form of a supplement was 1.2mg (range 1 - 3.6 mg/d of retinol). Supplement dose was in addition to their habitual dietary intake. The patients actual vitamin A intake is not known however based upon data from the National Diet and Nutrition Survey (NDNS, 1990) this is likely to be around 1mg/day (average intake; men 1.23mg, women 1.1mg retinol). Four patients were not prescribed supplements, as they were judged to be pancreatic sufficient and perceived of being well. There was no difference in the supplemented dose of retinol between patients in the lowest plasma retinol tertile (median dose 1.2mg range 1-3.4mg) to patients in middle (median dose 1.2mg range 0-3.6mg) and highest plasma retinol tertile (median dose 1.2mg range 0-2.4mg) (table 4.7). Whole group analysis revealed that supplement dose had no effect on the variation in plasma retinol concentrations (Spearman R = -0.108; P=0.497) (figure 4.8).

Adherence was measured by consensus clinical score and 64% of patients were assessed as being adherent to their supplement regimen. Statistical analysis revealed that adherence to supplement regimens had no effect on differences in plasma retinol concentrations (P=0.68) (figure 4.9). In those patients with the lowest plasma retinol (n=16 with 1 missing data point) 11 were assessed as being adherent, 3 partially adherent and only 1 patient as being non-adherent. This non-adherent patient had a plasma retinol of 1.1 μmol/L. Adherence is similar to other published data where 82% of CF patients were assessed as being always and usually compliant to vitamin supplementation [Abbott *et al* 1994].

4.5.3.3 IMMUNE RESPONSE AND DISEASE PROCESS

Table 4.7 shows data relating to relationships between plasma retinol concentrations, with markers of the immune response and measures of disease severity. Results are presented by tertiles of plasma retinol obtained from the population of 44 patients with CF. Tertile analysis of data between the highest tertile of plasma vitamin A (1.91 - 4.8 μmol/L) and lowest tertile (0.4 – 1.2 μmol/L) revealed significant differences between the tertiles with BMI (P=0.02), IgM (P=0.03), and number of admissions over 1 year (P=0.03) (table 4.3). There also appeared to be trends towards poorer lung function (FEV1%), higher ESR, IgE and CRP concentrations in patients with lower vitamin A when compared to those with the highest vitamin A. There were no differences between the groups in relation to supplement dose, plasma selenium and zinc. Further analysis revealed that patients with a greater number of admissions to hospital (4+ over 1 year) had significantly lower (P=0.02) plasma retinol concentrations (median 1.25 μmol/L) than patients with less admissions (median plasma retinol 1.9 μmol/L). There was no significant difference of this kind noted with any other audit variable.

Of the patients in the lowest tertile (n=16) the majority had measures of immune function recorded. Trends show that patients with the lowest retinol had elevated markers of the immune response (IgG, IgA, IgE, ESR, CRP, WCC) when compared to patients with the highest retinol. Of the patients in the lowest retinol tertile, 9/14 (64%) had elevated IgE and IgG concentrations, and 8/14

(57%) had elevated IgA and CRP concentrations, 8/9 (89%) had elevated WCC and 4/10 had elevated ESR concentrations when compared to SUHT normal reference ranges. Patients with an elevated CRP (median 19.9mg/L (7.1-90.1mg/L)) had a median plasma vitamin A concentration lower (1.30μmol/L (0.4-3.4μmol/L) than that seen in patients with a CRP less than 6mg/L (1.62μmol/L (0.4-4.8μmol/L). The association between plasma concentrations of retinol and CRP is significant ($P<0.05$) when CRP concentrations are raised (>10mg/L) (figure 4.10).

The only variable relating to the immune system to show a significant correlation with plasma retinol was serum IgG such that those patients with a high IgG had a low plasma retinol (Spearman $R = -0.334$; $P = 0.041$) (Figure 4.11). Multiple linear regression revealed that IgG was the only variable of significance to influence plasma retinol and 16% of the variance in plasma retinol can be attributed to differences in serum IgG. When all measured markers of immune function were forced into the analysis, infection accounted for 27% of the variance in plasma retinol concentrations. Quadrant analysis of the graph (figure 4.11) allowed stratification of patients into four distinct subclasses, those with 1) "normal" retinol and IgG, 2) low retinol and normal IgG, 3) normal retinol and low IgG, and 4) low retinol and IgG. The hypothesis to be tested was that those patients with the lowest retinol and highest IgG (group A) would have poorer clinical outcomes when compared to patients with retinol and IgG concentrations within the normal reference ranges (group B). Analysis revealed (table 4.6) that patients in group A had significantly higher IgA, and ESR concentrations ($P<0.05$), and significantly lower BMI, and FEV1% ($P<0.05$). Results also revealed trends within group A towards increased IgE, CRP and WCC, increased supplement dose, and number of admissions over 1 year.

Figure 4.4: Relationship between plasma retinol concentrations (μmol/L) and BMI (kg/m^2) in 44 patients with CF.

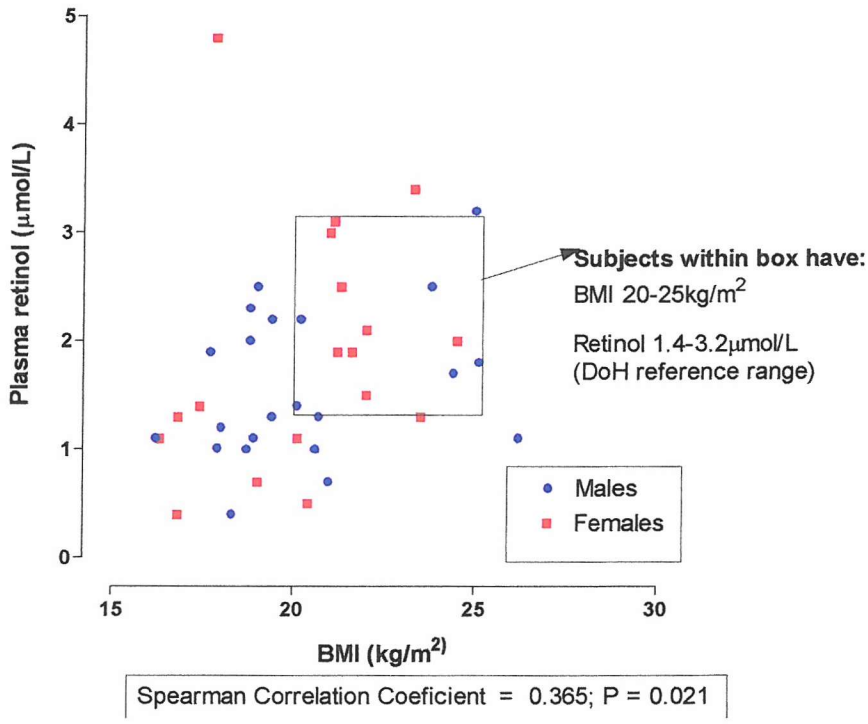


Figure 4.5: Plasma retinol concentrations (μmol/L) and BMI (kg/m^2) in 47 healthy adults.

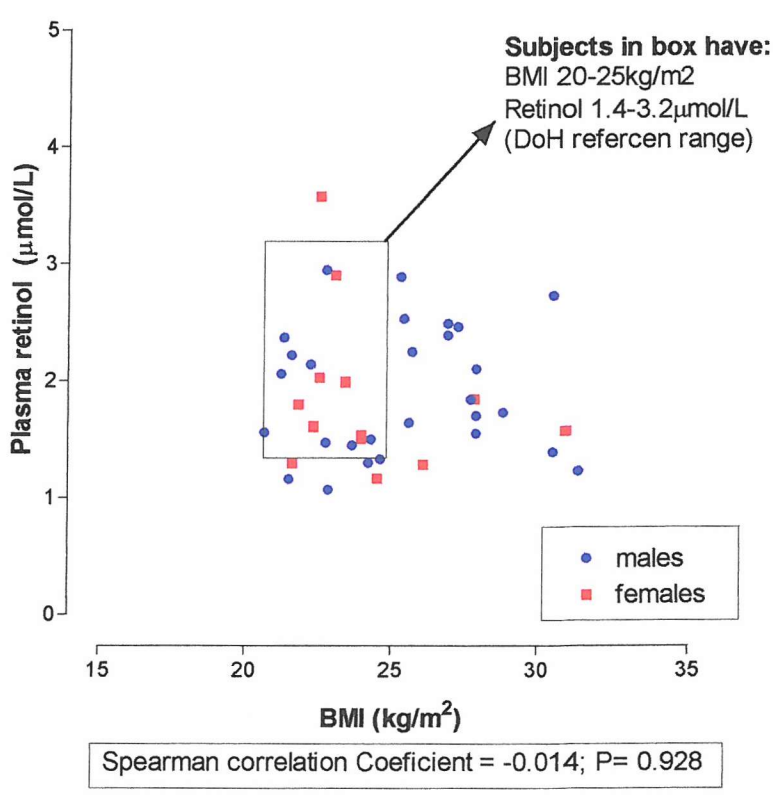


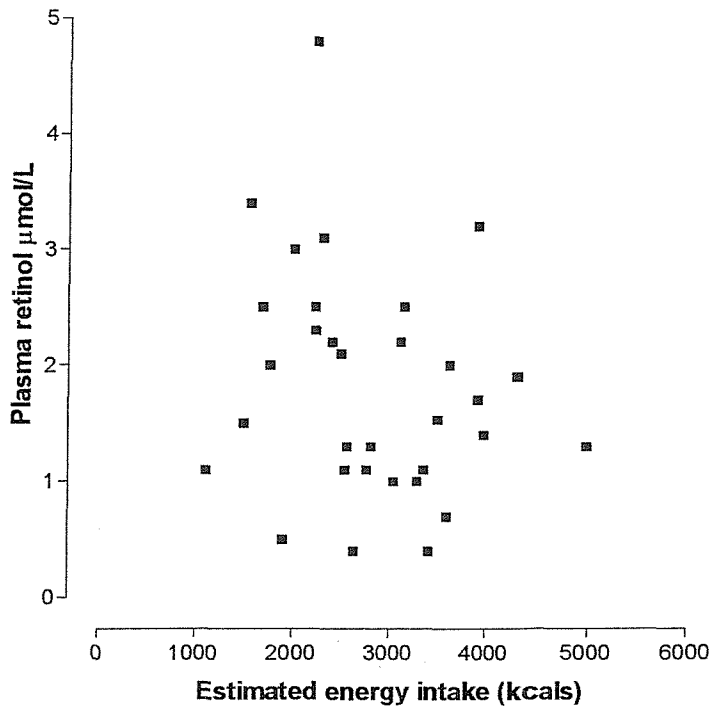
Table 4.6:

Table showing results for healthy adults and patients with CF stratified for BMI and plasma retinol.

	HA n=43; 4 missing		CF n=38; 4 missing	
	Retinol median (range)	**Retinol <1.4µmol/L**	**Retinol median (range)**	**Retinol <1.4µmol/L**
BMI <20kg/m²	n=0	0/0	n=18 1.25 (0.4-4.8)	11/18; 61%
BMI 20-25kg/m²	n=25 1.61 (1.07-3.58)	6/25; 24%	n=20 1.9 (0.5-3.4)	6/20; 30%
BMI >25kg/m²	n=18 1.84 (1.23-2.89)	3/18; 17%	n=2 1.45 (1.1-1.8)	1/2; 50%

Figure 4.6:

Plasma retinol concentrations (µmol/L) and estimated energy intake (kg/m²) in patients with CF.



P>0.05

Figure 4.7:

A visual representation of the types of supplements taken by the group of 44 patients with CF within the clinic at Southampton.

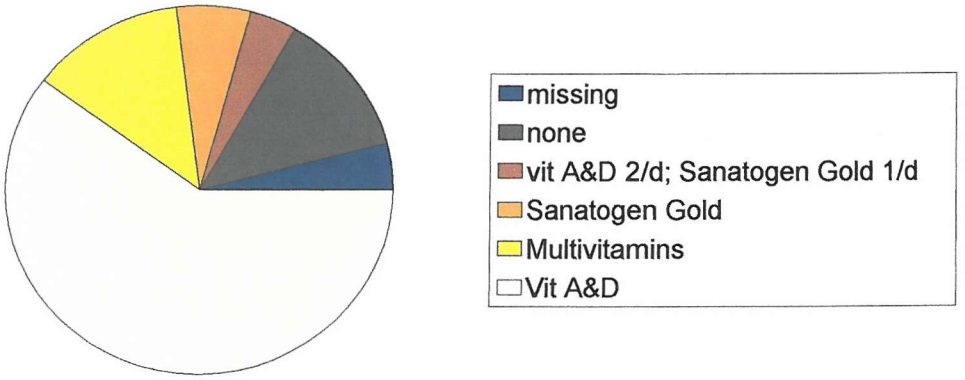


Figure 4.8:

Individual values for plasma retinol concentrations (μmol/L) and supplement dose of retinol (mg of retinol per day) in 44 patients with CF.

Graph illustrates DoH/MAFF retinol reference for healthy adults.

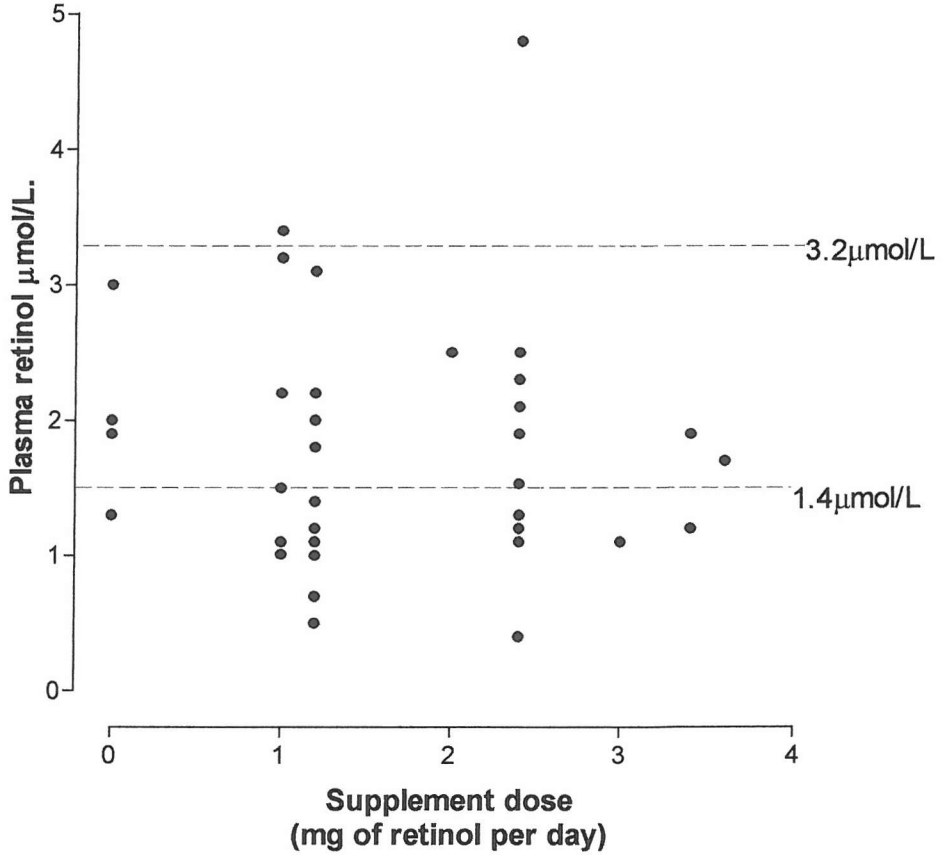


Figure 4.9:

Individual values for plasma retinol concentrations (μmol/L) and adherence to supplement regimens.

Graph illustrates DoH/MAFF retinol reference for healthy adults.

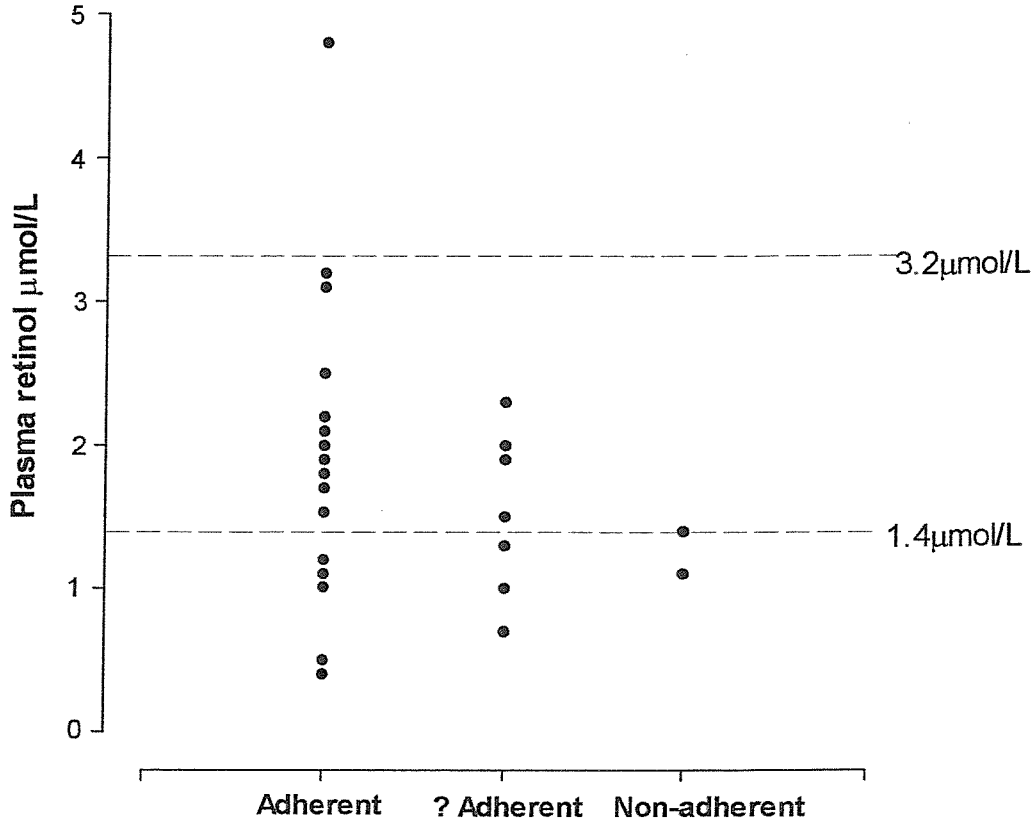
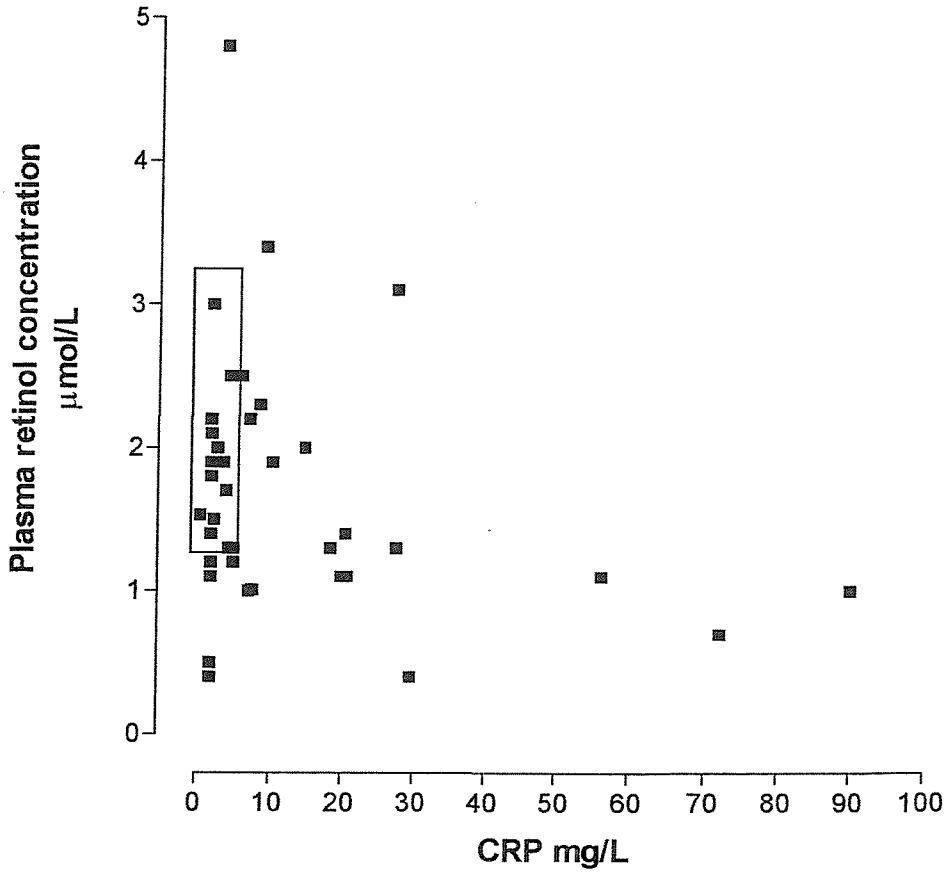


Figure 4.10:

Plasma retinol (μmol/L) and CRP concentrations (mg/L) in the group of 44 patients with CF.

Values that fall within the box, fall within the reference ranges for both plasma retinol and CRP. Table below illustrates that the relationship is only significant at raised CRP concentrations.

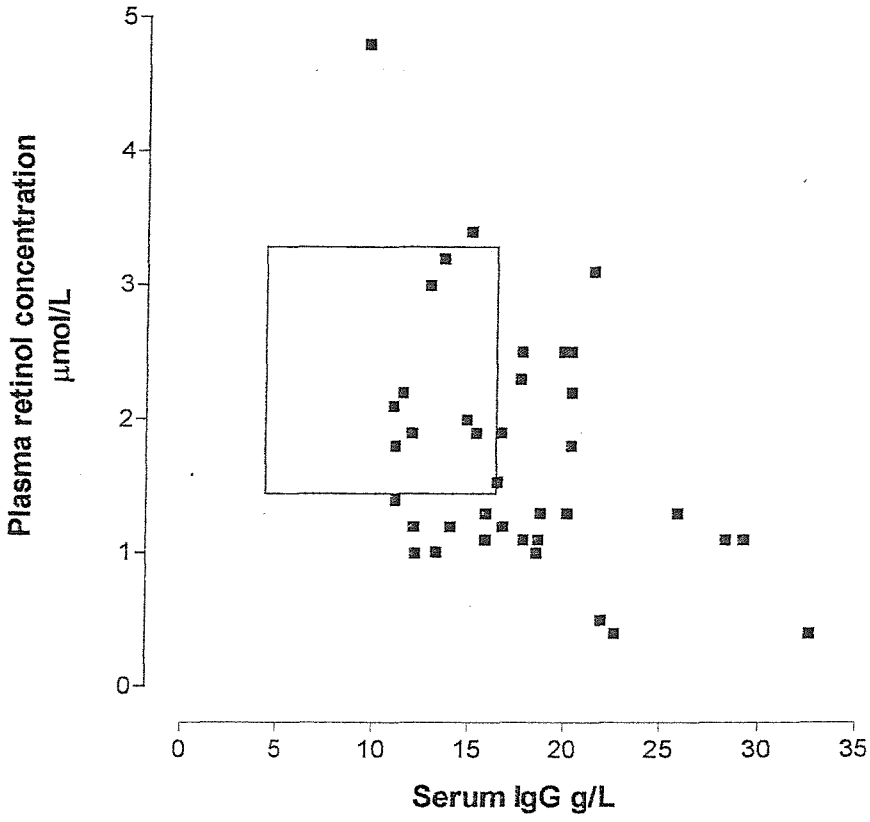


Data included in analysis	Spearman Correlation Coefficient	P value
All CRP concentrations	-0.199	P = 0.224
CRP >6mg/L only	-0.445	P = 0.073
CRP >10mg/L only	-0.681	P=0.015*

Figure 4.11

Association between plasma retinol concentrations (μmol/L) and IgG concentrations (mg/L) in 44 patients with CF measured at annual routine assessment.

Values that fall within the box fall within the reference ranges for both plasma retinol and serum IgG.



Spearman R = -0.334; P=0.041

Table 4.7

Table illustrating median results for variables measured within the audit.

Subjects were placed into groups corresponding with their plasma retinol concentrations. Tertiles were determined from the results obtained within the audit of 44 adults with CF.

	Lowest retinol tertile 0.4-1.2μmol/L	**Medium retinol tertile 1.21-1.9μmol/L**	**Highest retinol tertile 1.91-4.8μmol/L**	**Lowest compared to highest**
	Median	**Median**	**Median**	
Lipase Units (kg/d)	3561	4975	7252	⇓
Energy Intake (kcals)	2895	3709	2264	⇔
BMI (kg/m^2)	18.8	20.95	21.05	⇓ P<0.05
Supp dose (mg retinol)	1.20	1.20	1.20	⇔
IgG (g/L)	18.2	16.4	15.0	⇑
IgA (g/L)	4.5	2.8	2.6	⇑
IgE (IU/ml)	39.5	25.0	1.0	⇑
Zinc (μmol/L)	13	13.2	13.5	⇔
ESR (mm/h)	18	16.0	7.50	⇑
CRP (mg/L)	7.4	4.0	5.1	⇑
WCC (10^9/L)	11.50	10.40	8.50	⇑
FEV1%	57.20	63.80	69.9	⇓
Selenium (μmol/L)	0.78	0.82	0.82	⇔
Vitamin E	18.50	17.85	25.5	⇓
Number of admiss 1 year	5	3	0	⇑ P<0.05

Table 4.8

Table illustrating median results for variables measured within the audit.

Subjects were placed into two groups according to their plasma IgG and retinol concentrations.

Group A – low retinol and high IgG

Group B – normal retinol and IgG.

	Normal retinol & IgG Group B		**Low retinol & high IgG Group A**		**Median Group A compared to Group B**
	Median	**Range**	**Median**	**Range**	
Lipase Units (kg/d)	690	0 - 14019	6706	0 - 13158	⇑
BMI (kg/m^2)	21.8	17.7 - 25.0	18.7	16.2 - 26.2	⇓ P<0.05
Supp dose (mg retinol)	1.0	0.0 - 2.4	1.80	0.00 – 3.00	⇑
IgA (g/L)	2.1	1.2 - 5.1	4.7	0.1 - 8.6	⇑ P<0.05
IgE (IU/ml)	1.0	1.0 - 1933	56	1.0 - 968	⇑
Zinc (µmol/L)	15.5	8.4 – 17.0	13	9.7 - 20	⇔
ESR (mm/h)	5.5	1.0 – 18.0	20	2 - 55	⇑ P<0.05
CRP (mg/L)	3.3	1.9 - 14.8	5	2 - 90.1	⇑
WCC (10^9/L)	9.50	6.70 - 14.2	11.30	3.40 – 97.00	⇑
FEV1%	75.7	56.0 - 82.9	60	41 - 79.6	⇓ P<0.05
Selenium (µmol/L)	0.83	0.75 - 1.42	0.80	0.68 - 1.45	⇔
Vitamin E	26.1	12.3 - 30.3	17.70	5.10 - 35.20	⇓
Number of admiss (1 year)	0	0 - 9	5.5	0 - 11	⇑

4.6 DISCUSSION

The study reported in this chapter examined the prevalence of low plasma retinol concentrations in a group of CF patients compared to healthy adults. It began to address questions relating to factors, in particular those of supply and infection, which may be acting to depress plasma retinol. Finally it investigated the clinical impact of a patient exhibiting a low plasma retinol. The hypothesis to be tested was that despite current practices patients with CF will have an increased prevalence of low concentrations of retinol when compared to healthy adults.

4.6.1 Previous literature

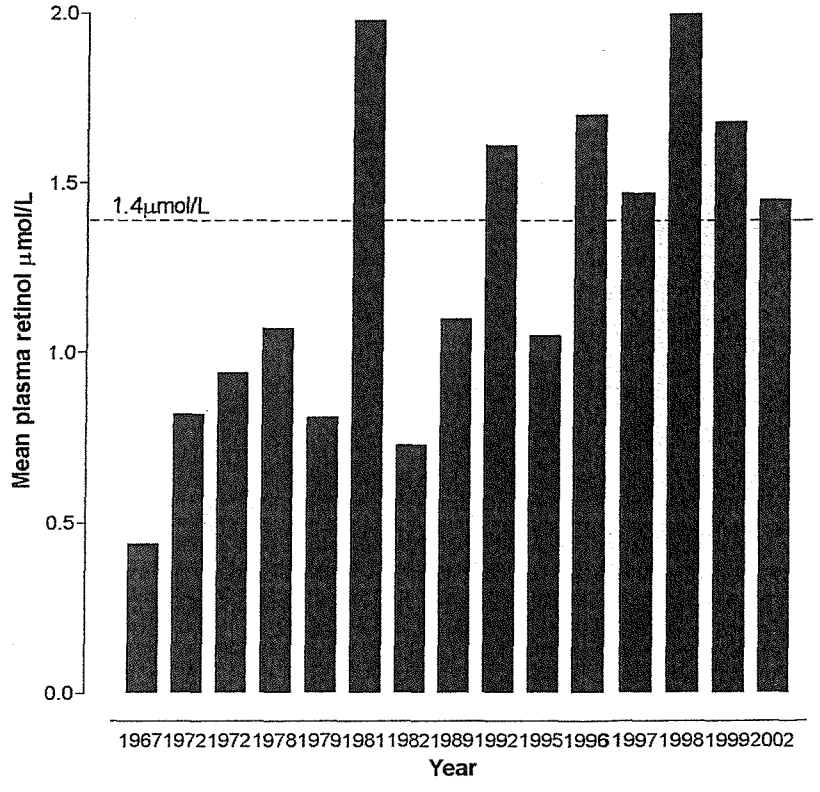
Figure 4.12 (and table 4.1) illustrates the current literature on plasma retinol concentrations in patients with CF. Most studies present the mean plasma retinol concentration with a range or S.D. The majority of reports are from studies in children, only five studies have recruited young adults and only one of these has investigated adults plasma retinol concentrations separate to that of children [Leonard *et al* 1998]. The literature reports limited information on the study populations making it hard to determine if the subjects studied provide data that reflects of the total CF population. There is also little data on the subject's clinical regimens such as pancreatic enzymes and vitamin A supplement use. The data may be restricted by the primary aims of the investigations. Only five of the studies main aim was to assess vitamin A status (as plasma retinol) in CF patients compared to controls [Bennett *et al* 1967, Smith *et al* 1972, Underwood 1972, Congdon *et al* 1981, James *et al* 1992]. All the other studies present plasma retinol concentrations in CF although the primary aims of the studies all differ and include; the assessment zinc status [Jacob et al 1978], effect of zinc supplementation [Palin *et al* 1979], effect of β-carotene supplementation [Winklhofer-Roob *et al* 1995], effect of exacerbations [Duggan *et al* 1996], vitamin A deficiency functional consequences [Fulton *et al* 1982; Raynor *et al* 1989; Huet *et al* 1997; Ansari *et al* 1999] and the study of the use of a high potency multivitamin [Leonard *et al* 1998].

The studies reported compare group means of retinol in CF patients and controls to determine if the populations are significantly different from each

other. However given the heterogeneity of the disease CF, a difference in mean values may depend on the mix of patients within the population studied. For example if more patients who are well and comply to supplement and enzyme regimens are studied no difference in mean results may be evident although there may still variation in the range of values observed. Of the 15 studies the mean plasma retinol concentrations reported, ranges from 0.44-2.00μmol/L, over half of the studies report mean plasma retinol concentrations less than 1.4μmol/L (95%CI for adult population; DoH/MAFF 1990). Reported mean retinol concentrations appear to improve during the 1990's when retinol was measured by HPLC and annual assessment and supplementation of patients was routine. Mean values appear to be higher over the last decade, and retinol concentrations are significantly related to the year of study (Spearman R=0.707; P=0.007), however the range of concentrations reported appear not to differ over the years. The prevalence of low concentrations of retinol cannot be determined from these studies and in particular these studies do not investigate plasma retinol concentrations in adult CF patients.

Figure 4.12

Graph illustrating mean plasma retinol concentrations reported in the literature and the dates in which they were published.



4.6.2 Plasma retinol.

Data presented in this chapter found that despite current CF management practices median plasma retinol concentrations observed in a group of healthy adults (1.8μmol/L) was significantly higher than that observed in the group of CF adults (1.45μmol/L). The population of CF patients exhibited a wide range retinol concentrations from those that would be classified in the deficient range (0.4μmol/L) to high concentrations (4.8 μmol/L), these ranges are similar to those reported by Congdon [1981] (0.5-4.5μmol/L). The median and lower values observed in this group of CF adults was comparable to those reported in the literature over the last decade (figure 4.1).

The prevalence of low retinol concentrations has not been previously stated in a group of CF adults. Our results (table 4.5) found the majority of the healthy adults (79%) fell within the 95% CI (DoH;MAFF 1990), only nine subjects (19%) had plasma retinol below the lower CI. By comparison the prevalence of low retinol concentrations in the group of CF patients was around 50%. These low concentrations are evident despite current CF care, pancreatic enzyme replacement therapy, diets high in fat and routine prescription of supplements. The implications of this prevalence will be discussed later.

4.6.3 Factors that are associated with plasma retinol concentrations in patients with CF.

In terms of nutrition the most simplistic explanation of a low plasma retinol is that of poor dietary supply. The diet can potentially be limiting either through poor supply of vitamin A as retinyl palmitate, poor availability of the vitamin across the GI tract due to maldigestion/malabsorption or poor compliance to supplement practices. Analysis of data collected from this retrospective audit revealed that plasma retinol concentrations were not associated with a patients estimated energy intake, dosage of pancreatic enzymes, dosage of supplemented retinol or adherence to supplement regimens. Therefore the high prevalence of low plasma retinol concentrations in this group of CF patients cannot be simply attributed to a poor dietary supply of the vitamin. Other investigators have also found low plasma vitamin A concentrations in patients with CF who have an adequate dietary intake and oral supplementation

regimens [Palin *et al* 1979; Smith *et al* 1972; Lancellotti *et al* 1996]. It should be acknowledged that those patients who do eat poorly and are not compliant to supplement regimens are at risk, but even when these patients are excluded from the analysis there is still a high prevalence of low retinol concentrations within this group of patients. There is a need to specifically investigate those CF adult patients who exhibit low retinol concentrations, which cannot simply be explained by diet supply, compliance or infection.

Results revealed that low plasma retinol concentrations were associated with a worse clinical course in that low retinol concentrations correlated with patients having a lower BMI (poorer nutritional status and sicker), greater admissions to hospital, poorer lung function (FEV1%), and elevated markers of immune function. Other investigators have also reported associations between plasma vitamin A concentrations and markers of clinical status. One study reported that plasma retinol concentrations measured near the time of death were lower than samples obtained earlier in the clinical course of the disease [Underwood & Denning 1972], and another found that low plasma retinol concentrations were associated with a low Shwachmann Score (poor clinical state of the patient) [Congdon *et al* 1981]. Two preliminary reports found that plasma retinol concentrations were associated with a low Shwachmann Score [Raynor *et al* 1989], and poor lung function [Carr *et al* 1996].

Data from the audit revealed that an activated immune system is associated with low concentrations of retinol. The effect of infection on plasma retinol concentrations and the pathogenesis of disease is of particular interest in CF. Over recent years, the important role that vitamin A has in maintaining both cellular and humoral immunity has become increasingly evident. Vitamin A appears to have immune stimulating effects and insufficient vitamin A has been associated with impaired immune competence as shown by decreased lymphocyte proliferation in response to mitogens and increased bacterial binding to respiratory epithelia [Gerster 1997]. This role of vitamin A in the immune system and the potential effect on clinical course requires further investigation.

From both our data and data from the literature we are unable to differentiate if these differences in plasma retinol concentrations are cause or effect. They could be interpreted in two ways, firstly low circulating retinol worsens the clinical course by impairing the immune response, or secondly that the activation the immune response in exacerbations of CF depress plasma retinol and worsen the clinical course. It may be that sicker patients have an activated immune system, lower BMI, poor energy intake, poor availability of the vitamin across the GI tract, poor hepatic function and potentially greater demands of the vitamin.

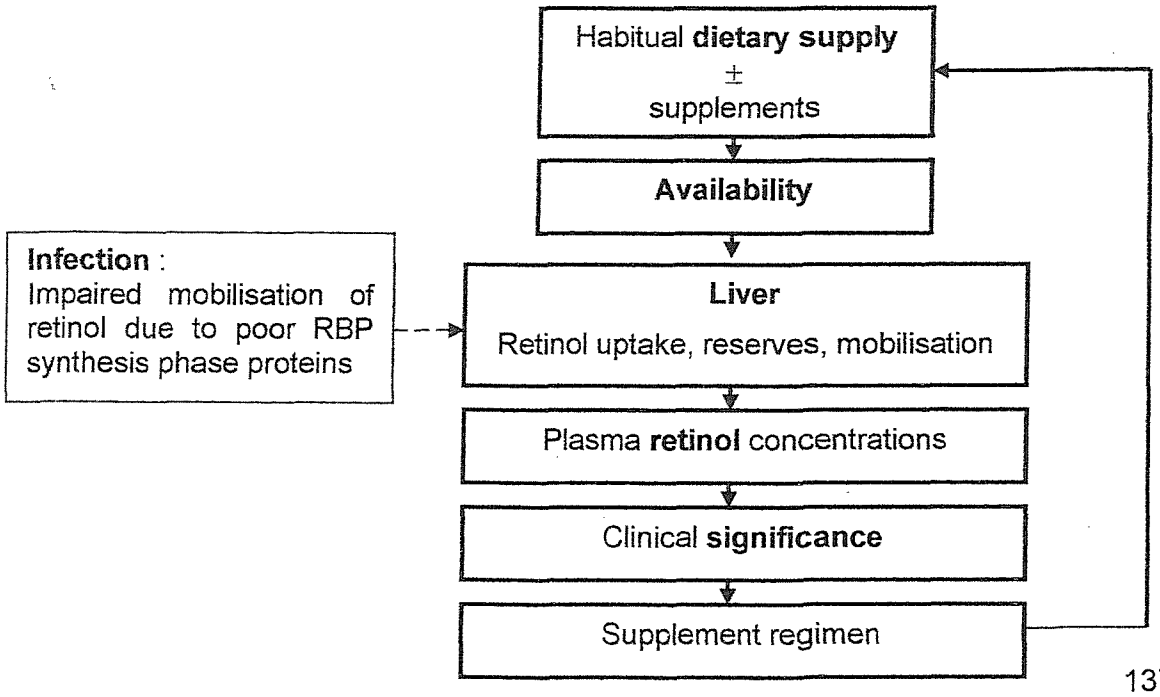
4.6.4 CONCLUSION

In summary this is the first time the prevalence of low concentrations have been reported in adult patients at a CF centre in the UK. Median plasma retinol concentrations in the adult group were similar to those reported previously in children during the last decade. The prevalence of low retinol concentrations was ~50% and these low concentrations are associated with both the clinical course of the patient and inflammatory response. Despite this prevalence routine practice is to supplement all pancreatic insufficient CF patients and not only those patients at risk. A number of patients within this study who are dosed on 3.6mg/d of retinol alongside their habitual intake of approximately 1mg/d, (~4mg/day) are taking over six times the recommended daily allowance which does not appear to be having the intended rise in plasma retinol concentrations. If effectively absorbed, these doses may exceed safe levels and potentially be harmful not beneficial to the patient. Data from healthy individuals reported that prolonged and continuous supplementation of doses in the therapeutic range can result in liver damage [Geubel *et al* 1991]. This may have particular important implications to children with CF, who are routinely supplemented between 1.2-3mg of retinol in excess of their habitual intake (~0.5mg/d). The Department of Heath recommends that regular daily intakes in children should not exceed 0.9mg/d in infants, 1.8mg/d between 1-3years, 3mg between 4-6years and 4.5mg between 6-12years. Recommended doses in childhood may therefore exceed government guidelines and could cause harm. High doses in childhood may be the foundation of the effects seen in adulthood, particularly if vitamin A stores are able to accumulate. The information presented within this

chapter has important implications in informing supplementation regimens in clinical practice to provide a balance between those patients who are at risk and the potential toxic effect of the vitamin.

The whole body processing of vitamin A and factors that impact upon plasma retinol concentrations have not been systematically investigated (figure 4.13). The three main factors that may influence the processing of vitamin A within the body, and may act to effect circulating plasma retinol are 1) supply of the vitamin, 2) availability of the vitamin across the GI tract and 3) mobilisation of retinol from the liver (figure 4.13). As data presented in this chapter does not support the view that supply of vitamin is the primary determinant of low circulating retinol concentrations other factors require investigation. These include the well-held belief that vitamin A is malabsorbed and maldigested along with dietary lipid, causing the vitamin to be less available to the body [Anderson *et al* 1939; Huet *et al* 1997; Congdon *et al* 1981] and the potential that retinol mobilisation from the liver is disturbed. Both the availability of the vitamin across the GI tract and the mobilisation of retinol from the liver in CF patients and healthy adults were studied and results are presented within this thesis (chapter 7 & 8).

Figure 4.13:
Schematic representation of the factors potentially impacting upon plasma retinol concentrations.



4.6.5 SUMMARY.

- Patients with CF have an increased prevalence of low retinol concentrations when compared to healthy adults, despite modern advances in care.
- Plasma retinol concentrations are low in half of the group of CF patients and were significantly lower than that seen in healthy adults.
- Plasma retinol concentrations are not correlated with; the dose of supplemented vitamin A, adherence to supplement regimens, nutrient intake, and enzyme dosage.
- 16% of the variance in plasma retinol concentrations can be accounted for by differences in serum IgG concentrations.
- 27% of the variance in the population of CF patients studied can be accounted for by raised inflammatory markers.
- Patients with low retinol concentrations have poorer clinical outcomes.
- Retinol concentrations alone should not be used to assess vitamin A requirement.
- Supplementation of vitamin A may only be appropriate when dietary supply and / or availability is the limiting factor.
- Other factors that may be impacting on retinol concentrations need to be systemically investigated in order to better understand the clinical relevance of a low plasma retinol and to inform further clinical practice.

Further studies are required to better understand the contribution made by other factors that may contribute to an alteration in vitamin A status including GI function, and disturbances in retinol mobilisation to establish better guidelines to promote the safe and effective management of vitamin A status in CF.

CHAPTER 5

CIRCULATING RETINOL AND RBP CONCENTRATIONS IN PATIENTS WITH CF AND HEALTHY ADULTS.

5.1 INTRODUCTION.

Transport of retinol from stores in the liver is accomplished by means of the specific transport protein RBP. RBP has one high affinity-binding site for retinol and consequently the molar ratio of retinol to RBP in the circulation is approximately 1:1 [Gamble *et al* 2001]. Once in the circulation retinol-RBP forms a complex with TTR thereby reducing glomerular filtration. Normal concentrations of RBP within the circulation range between 30-60mg/L (1.43-2.86μmol/L) [Dati *et al* 1996].

Serum RBP concentrations are not routinely measured in patients with CF. Only a handful of studies in the literature have measured RBP concentrations in patients with CF, these studies have mainly been conducted in children (table 5.1). The range of concentrations reported within the literature is 0.43-2.24μmol/L, median value 1.33μmol/L. Six of nine the reports found mean RBP concentrations below the normal range (1.43-2.86μmol/L), despite supplementation of vitamin A adequate to maintain hepatic stores. It is hard to draw comparisons between these studies and make an informed decision on the prevalence of low RBP concentrations in populations of CF patients, due to the characteristic differences in each CF population studied. There does not appear to be a simple trend in RBP concentrations over time as was noted in chapter 4 for plasma retinol concentrations (figure 5.1).

It has been suggested that low concentrations of retinol in CF can be attributed to an impaired mobilisation of retinol from the liver due to a shortage of the protein RBP. It has been hypothesised that depressed concentrations of retinol and RBP occur in patients with an activated immune system, associated with an acute phase shift in hepatic protein synthesis [Duggan *et al* 1996]. However the actual mechanism associated with lowered concentrations during inflammation

and infection has not been systematically investigated. The prevalence of low RBP concentrations, the relationship between serum RBP and plasma retinol concentrations and the effect of infection have not been adequately investigated in patients with CF.

5.2 AIMS

The aim of the present study was three fold. As a first step we sought to determine the prevalence of low RBP concentrations in patients with CF compared to healthy adults. The second aim was to determine if the ratio of retinol:RBP in the circulation differed in patients with CF compared to healthy adults, and thirdly if low concentrations of RBP are correlated with the inflammatory state of the patient.

5.3 HYPOTHESIS

Hypothesis 1: Compared to healthy adults patients with CF will have an increased prevalence of low concentrations of RBP. As serum RBP concentrations circulate in a 1:1 molar complex with retinol, low circulating retinol concentrations will be associated with low RBP concentrations.

Hypothesis 2: The molar ratio of retinol:RBP will be unaltered in CF when compared to healthy adults.

Hypothesis 3: CF patients with low circulating RBP and retinol concentrations are those patients with an elevated CRP (infective state).

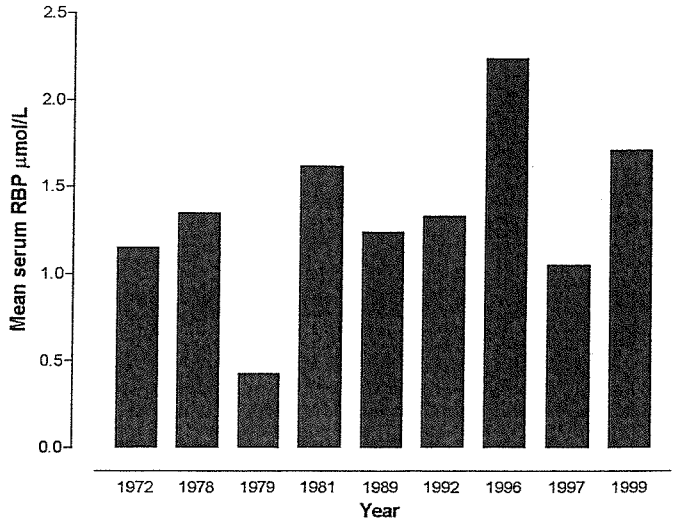
Table 5.1:

Current literature on serum RBP concentrations in patients with CF.

REFERENCE	PERT	VIT A SUPP	DIET	SUBJECTS	Serum RBP μmol/L	PLASMA RETINOL μmol/L
Smith 1972	yes	Daily supplement	No info	43 CF mean 12.3y	1.15μmol/L	0.82μmol/L
Jacob 1978	16 subjects	Daily multivitamin	No info	12 CF 6-17y	1.35μmol/L (0.51-2.19) calc for S.D.	1.07μmol/L; (0.48-1.67) calc from S.D.
Navarro 1979	No info	No info	No info	14 CF 5m-14y	0.43μmol/L no range stated	No retinol
Congdon 1981	32 subjects	27 subjects	Low fat	36 CF 10m-16y	1.62μmol/L (0.89-4.10)	1.98μmol/L (0.5-4.5) from graph
Raynor 1989	41 subjects	2 daily multivitamins	No info	43 CF 8-44y median 16y	34 normal dark adaptation 1.24μmol/L (0.21-2.19) 9 abnormal dark adaptation 0.36μmol/L (0.05-1.48)	34 normal dark adaptation 1.1μmol/L (0.5-2.3) 9 abnormal dark adaptation - 0.53μmol/L (0.2-1.4)
James 1992	8 subjects	Yes	No info	9 CF median 22y	1.33μmol/L (1.05-1.76)	1.61μmol/L (1.14-2.39)
Duggan 1996	34 subjects	23 on multivitamins	No info	35 CF (CRP 9.8mg/L)	2.24μmol/L	1.70μmol/L
Huet 1997	Yes	Yes	No info	10 CF mean 15.2y	1.05μmol/L (0.66-1.62)	1.47μmol/L (0.87-2.80)
Ansari 1999	Yes	Yes	No info	28 CF Median 20y (12-33y)	1.71μmol/L (0.61-3.86)	1.68μmol/L (1.08-2.80)
Thesis results	40 subjects	40 subjects	High Fat	44 CF median 21y (17-45y)	1.29 (0.52-3.14)	1.21μmol/L (0.47-3.47

Figure 5.1: Graph illustrating mean serum RBP concentrations reported in the literature and the dates in which they were published.

(Concentrations of RBP determined by radial immuno-diffusion (RID)).



5.4 METHODS

All methods used within this chapter are described within the methodology section, chapter 3. Plasma retinol concentrations were measured by HPLC (Institute of Human Nutrition) and serum RBP concentrations were determined by Nephelometry.

5.4.1 SUBJECTS

A cohort of 46 patients with CF and 30 healthy adults were used for the concurrent analysis of both plasma retinol and serum RBP concentrations. Healthy adults were recruited to the Clinical Nutrition and Metabolism Unit, Institute of Human Nutrition, and fasting blood samples obtained. Fasted blood specimens drawn as part of other studies presented within this thesis were used for the analysis of plasma retinol and RBP concentrations in the patients with CF. All subjects had measures of age, height, weight, BMI and CRP.

5.4.2 STATISTICAL METHODS.

Data presented within this chapter are reported as median and ranges. Differences were considered to be significant at $P< 0.05$. Non-parametric statistics were used, as data was not normally distributed, Mann Whitey U statistical analysis was used to assess differences between the two groups and Spearman Rank was used to assess the association between two variables.

5.5 RESULTS

5.5.1 SUBJECTS

Subject characteristics are presented in table 5.2. Forty-six adult patients with CF (26F; 20M), median age 23 years (17-37y), and 30 healthy adults (9F; 21M), median age 23 years (20-57y) were included in the analysis. CF patients (median 19.93kg/m^2) had significantly lower BMI when compared to healthy adults (median 24.22kg/m^2). BMI can be used as a proxy marker of nutritional status, 19 (50%), (8 missing data points) of the patients with CF had BMI measures less than 20kg/m^2, at risk, 13 patients (34%) had values that fell within the range (20-25kg/m^2) and only 6 patients (16%) had values greater than 25kg/m^2. In comparison none of the healthy adults had values less than 20kg/m^2, 60% of subjects fell between 20-25kg/m^2, and 40% had BMI values greater than 25kg/m^2. Concentrations of plasma retinol were significantly correlated with BMI in patients with CF (Spearman R = 0.514; P=0.001), in that those patients with the lowest retinol also had the lowest BMI (graph not shown). This association was not evident in the group of healthy adults (Spearman R = -0.024, P=0.901), these results are similar to those reported in chapter 4.

Table 5.2:

Characteristics of the 46 adult patients with CF and the 30 healthy adults.
(Values presented as median and ranges.)

	HA (n=30)		CF (n=46)	
	Median	**Range**	**Median**	**Range**
AGE (years)	23	20-57	22	17-37
HEIGHT (m)	1.73	1.58-1.99	1.66	1.37-1.92
WEIGHT (kg)	72.45	58.40-107.50	55.45 *	41.50-96.50
BMI (kg/m2)	24.22	20.66-31.03	19.93 *	16.01-27.70
Plasma retinol (μmol/L)	1.63	1.07-2.91	1.21 *	0.47-3.47
Serum RBP (μmol/L)	1.76	1.05-2.67	1.29 *	0.52-3.14
Ratio retinol:RBP	0.99	0.69-1.47	1.07	0.45-1.78
CRP (mg/L)	1.00	1-7.02	6.94 *	1-139

Mann Whitney U – Significantly different from HA P<0.05

5.5.2 RETINOL AND RBP CONCENTRATIONS

Plasma retinol and RBP concentrations of the two groups are presented in table 5.2 and figure 5.2. The median plasma retinol concentration observed in the 30 healthy adults was 1.63μmol/L (1.07-2.91μmol/L), which was significantly higher (P=0.001) than the median value observed in the 46 CF patients (median (range) 1.21μmol/L (0.47-3.47μmol/L)). This data is similar to that presented in chapter 4. The median RBP concentration observed in the 30 healthy adults was 1.76μmol/L (1.05-2.67μmol/L). This was significantly higher (P=0.005) when compared to the median concentration observed in the 46 CF patients (median;(range) 1.29μmol/L; (0.52-3.14μmol/L)). Concentrations of retinol and RBP were not associated with either the gender or age of the subjects (P>0.05) (data not shown).

Table 5.3 shows the prevalence of low circulating retinol and RBP concentrations in the cohort of healthy adults and patients with CF. CF patients have a higher prevalence of low retinol and RBP concentrations when compared to the group of healthy adults. Twenty-seven of the 46 (59%) patients with CF had plasma retinol concentrations that fell below 1.4μmol/L (95%CI for adults), compared to only 7 of the 30 (23%) healthy adults. Twenty-three of the 43 patients with CF (53%) had low serum RBP concentrations compared to 5 of the 29 (17%) healthy adults.

When analysing the previous literature on the molar ratio retinol:RBP (figure 5.3), it would appear that results fall near the line of unity reflecting a 1:1 molar complex. The median ratio observed from previous data was 1.01 ranging from 0.68-1.40, however this small number of studies does not provide an evidence base to enable us to assume that all patients with CF have circulating retinol and RBP in a 1:1 molar ratio. In the cohort of CF adults investigated at Southampton plasma retinol concentrations were positively associated with serum RBP concentrations (Spearman R = 0.838; P=0.000) in both healthy adults and patients with CF (figure 5.4). The ratio of retinol:RBP was not different between the two groups (P>0.05).The median ratio of retinol: RBP was 1.01 and 0.93 for healthy adults and CF patients respectively. Despite this patients with CF had a wider range of values (0.56-2.21), when compared to the group of healthy adults (0.68-1.44).

Of particular interest, three patients deviate from the line of unity. Two CF patients have more retinol than RBP in their circulation (ratios 2.21 and 1.40) and one CF patient appears to have more RBP than retinol in their circulation (ratio 0.56). As the data set is small, there is no conclusive evidence to determine if the ratio of RBP:retinol in these patients is associated with an inflammatory response. However it would appear that the two patients with low retinol:RBP ratios (high retinol concentration with respect to RBP), have low CRP concentrations (<4mg/L) compared to the patient with a high ratio of retinol:RBP (CRP = 18.8mg/L).

5.5.3 CRP

The group of CF patients had significantly higher (P=0.000) CRP concentrations when compared to the group of healthy adults (median (range): CF 6.94mg/L (1.00-139.00mg/L); HA 1.00mg/L (1.00-7.02mg/L)). CRP concentrations were significantly correlated with both plasma retinol concentrations (Spearman R= -0.437; P=0.004) and RBP concentrations (Spearman R = -0.428; P=0.007) concentrations, in that those patients with an elevated CRP had lower retinol and RBP concentrations (figure 5.5). There was no difference in the ratios of retinol:RBP between patients with an elevated CRP (>6mg/L) and those patients with a low CRP (<6mg/L), (P=0.488).

Figure 5.2:

Individual and median plasma retinol and serum RBP concentrations (μmol/L) in the two subject groups, healthy adults (HA) and patients with CF.

(Graph illustrates reference ranges for plasma retinol and serum RBP.)

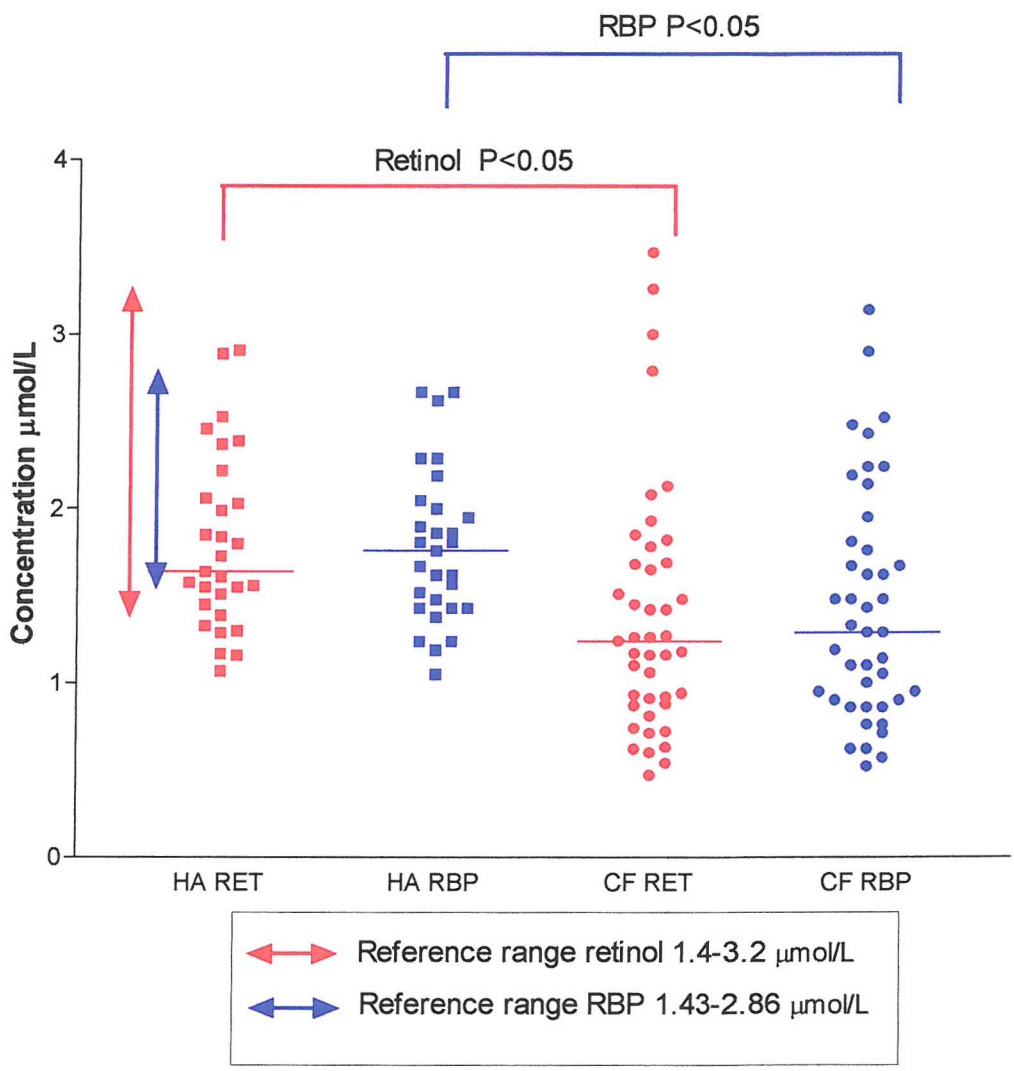


Table 5.3:

Table illustrating the prevalence of low retinol and RBP concentrations in patients with CF and healthy adults (HA).

(N.B. RBP concentrations; HA 1 missing value. CF 3 missing values)

	PREVALENCE			
	HA		CF	
	No. patients	%	No. patients	%
Plasma retinol concentrations below 95% CI for DoH (<1.4μmol/L)	7/30	23%	27/46	59%
Serum RBP concentrations below the reference range (<1.42μmol/L)	5/29	17%	23/43	53%
Plasma retinol concentrations that fall within 95% CI for DoH (1.4-3.2μmol/L)	23/30	77%	17/46	37%
Serum RBP concentrations that fall within ref range (1.42-2.86μmol/L)	24/29	83%	18/43	42%

Figure 5.3:

Relationship between plasma retinol and serum RBP concentrations (μmol/L) in the studies reported from the current literature.

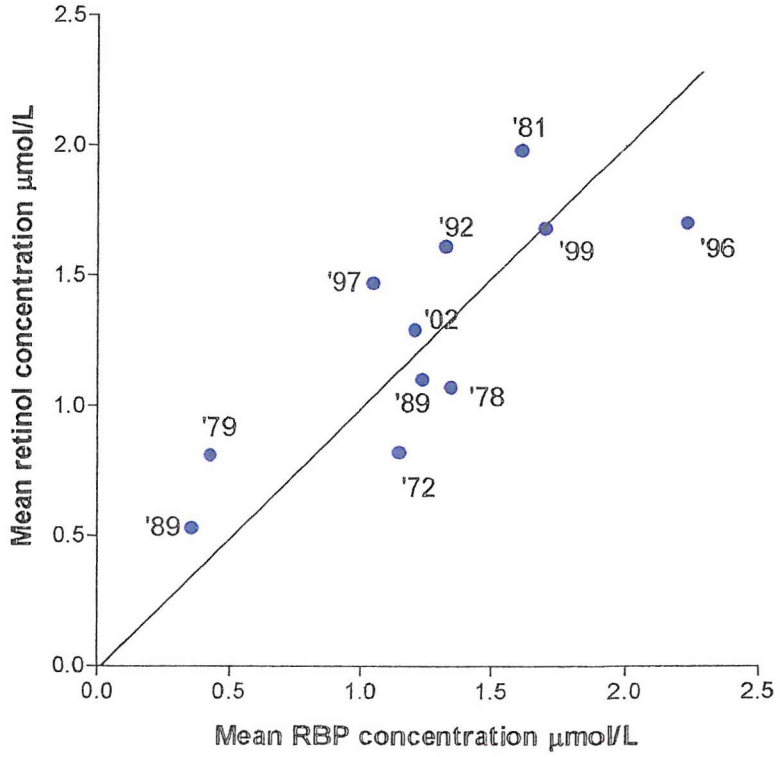


Figure 5.4

Relationship between plasma retinol and serum RBP concentrations (µmol/L) in patients with CF and healthy adults (HA).

(Results on the line have a 1:1 molar ratio).

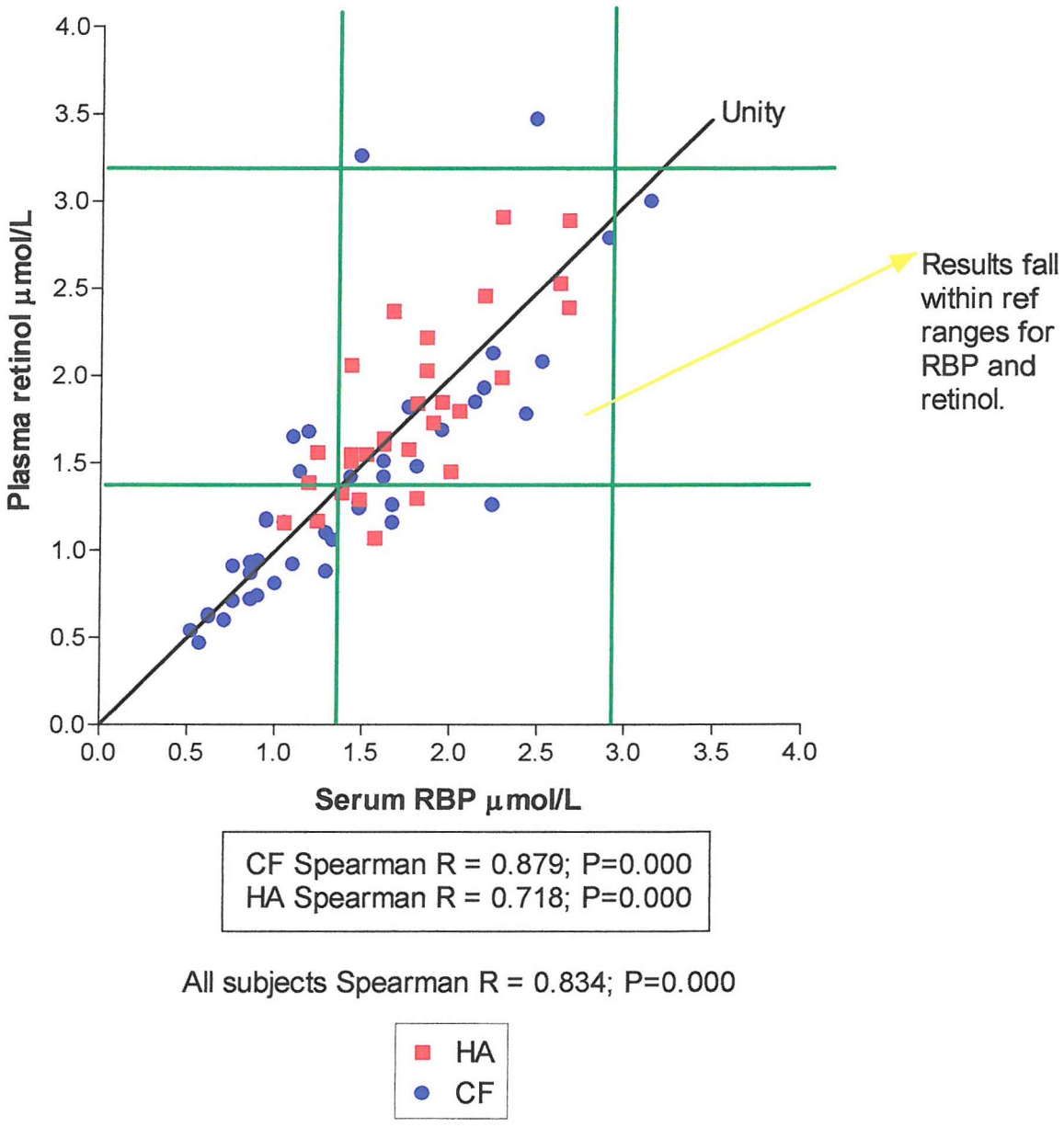


Table 5.4

Table illustrating whole group (CF and HA) differences in the ratio of retinol:RBP when subjects are stratified by plasma retinol concentrations.

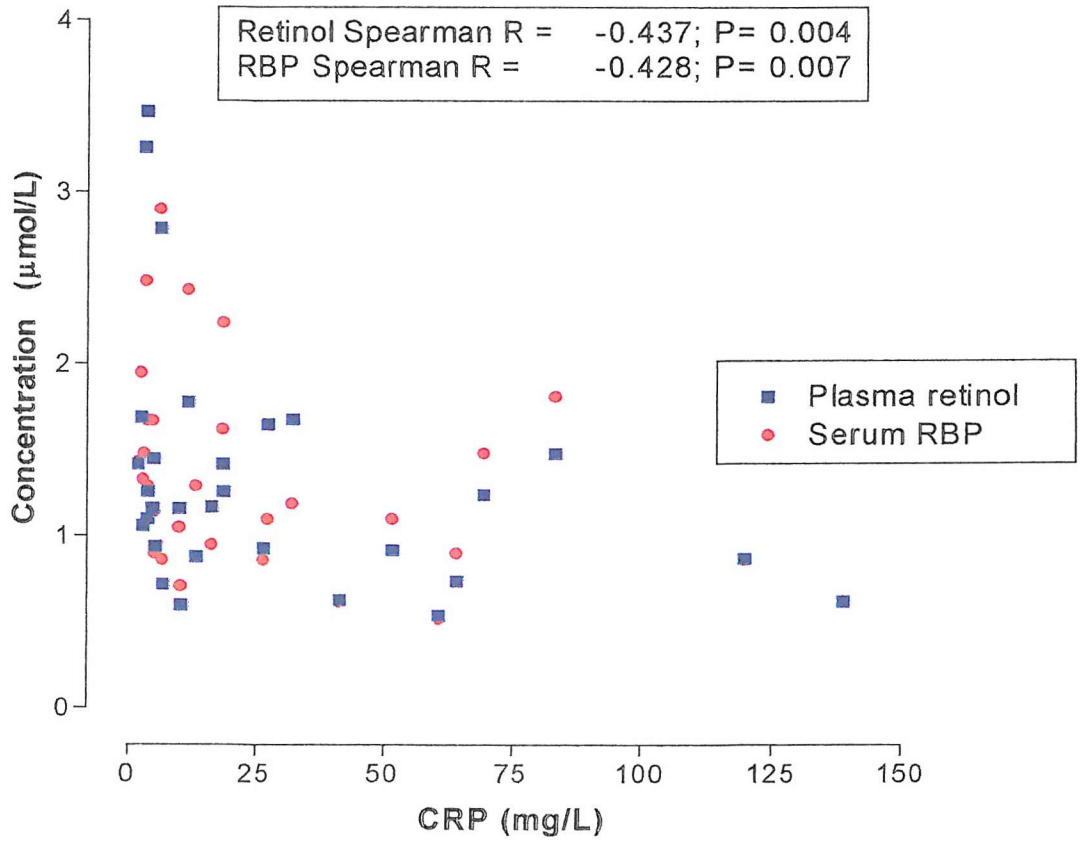
(Ratios of retinol:RBP were not different between the two groups; HA and CF)

	n	Retinol (μmol/L		RBP (μmol/L)		Ratio	
Retinol Classification		Median	Range	Median	Range	Median	Range
<1.4 μmol/L	34	1.07	0.47-1.39	1.05	0.52-2.24	0.87	0.56-1.24
1.4-3.2μmol/L	40	1.81	1.42-3.00	1.86	1.1-3.14	0.98	0.73-1.51
>3.2μmol/L	2	3.36**	3.26-3.47	1.98	1.48-2.48	1.80**	1.40-2.21

Mann Whitney U = HI retinol (>3.2μmol/L) significantly different from LO retinol (<1.4μmol/L).* P<0.05; ** P<0.01

Figure 5.5

Graph illustrating the association between retinol and RBP concentrations (μmol/L) with CRP concentrations (mg/L) in the group of patients with CF.



5.6 DISCUSSION

The study reported in this chapter has investigated; 1) the prevalence of low RBP concentrations in a group of CF patients and healthy adults, 2) the molar relationship between circulating retinol and RBP concentrations, and 3) the association between infection and concentrations of retinol and RBP in paitients with CF. The hypotheses to be tested were, 1) compared to healthy adults patients with CF will have an increased prevalence of low RBP concentrations, 2) the molar ratio of retinol:RBP will be unaltered in CF when compared to healthy adults, and 3) low circulating RBP and retinol concentrations are associated with an elevated CRP (infective state).

Serum RBP concentrations were associated with retinol concentrations in both the group of healthy adults and patients with CF. Median RBP concentrations in the cohort of CF patients were significantly lower than those observed in healthy adults. The population of CF patients studied exhibited a wide range of RBP values (0.52-3.14μmol/L) from those that would be classified below the normal range (<1.43μmol/L) to high concentrations (>2.86μmol/L), these were similar to those previously reported within the literature (table 5.1). The prevalence of low RBP concentrations has not been previously investigated in a group of CF adults. Results indicate that the majority of healthy adults fall within the normal reference range for serum RBP (83%), and only 17% had RBP concentrations below the reference range. However the prevalence of low RBP concentrations (<1.42μmol/L) in the group of CF patients was 53%, similar to the prevalence of low retinol concentrations (<1.4μmol/L). This data supports hypothesis one, in that patients with CF have an increased prevalence of low RBP concentrations compared to healthy adults.

Compared to healthy adults the molar association between retinol and RBP was unaltered in CF, which supports hypothesis two. However there were 3 CF patients of particular interest, all exhibiting RBP concentrations within the reference range. Two patients had more retinol, (>3.2μmol/L) than RBP in their circulation. This could be interpreted as "spill over" of "free" retinol from the liver. It may reflect that the livers capacity to store vitamin A has been reached and vitamin A that is unable to be stored is released into the circulation not bound to RBP. It could be bound and transported by another mechanism, potentially

VLDL. The other patient had low retinol in their circulation (<1.4μmol/L) with respect to RBP; this may reflect "free" RBP. This situation could arise from two potential mechanisms (figure 5.6). The first is if the liver has low stores of vitamin A, but RBP is still synthesised and released from the liver. In this instance low retinol with respect to RBP in the circulation may reflect a lack of stored vitamin A and could potentially be detrimental to the host. Alternatively, a low retinol concentration in the face of an adequate RBP concentration may reflect an up-regulation of retinol uptake by cells and tissues. For example during infection there may be an increased demand for retinol. In this instance retinol maybe taken up by cells and tissues at a rate that exceeds the capacity of the liver to release retinol. In this case low concentrations of retinol in the circulation may not reflect low liver stores of vitamin A, but a process that is beneficial to the host. Due to the small data set it is not possible to determine the mechanisms that affect retinol and RBP concentrations, nor if there is an increased value of measuring both retinol and RBP concentrations in CF patients. However it may be important to measure both concentrations in the blood if the ratio in some way reflects liver stores (figure 5.7).

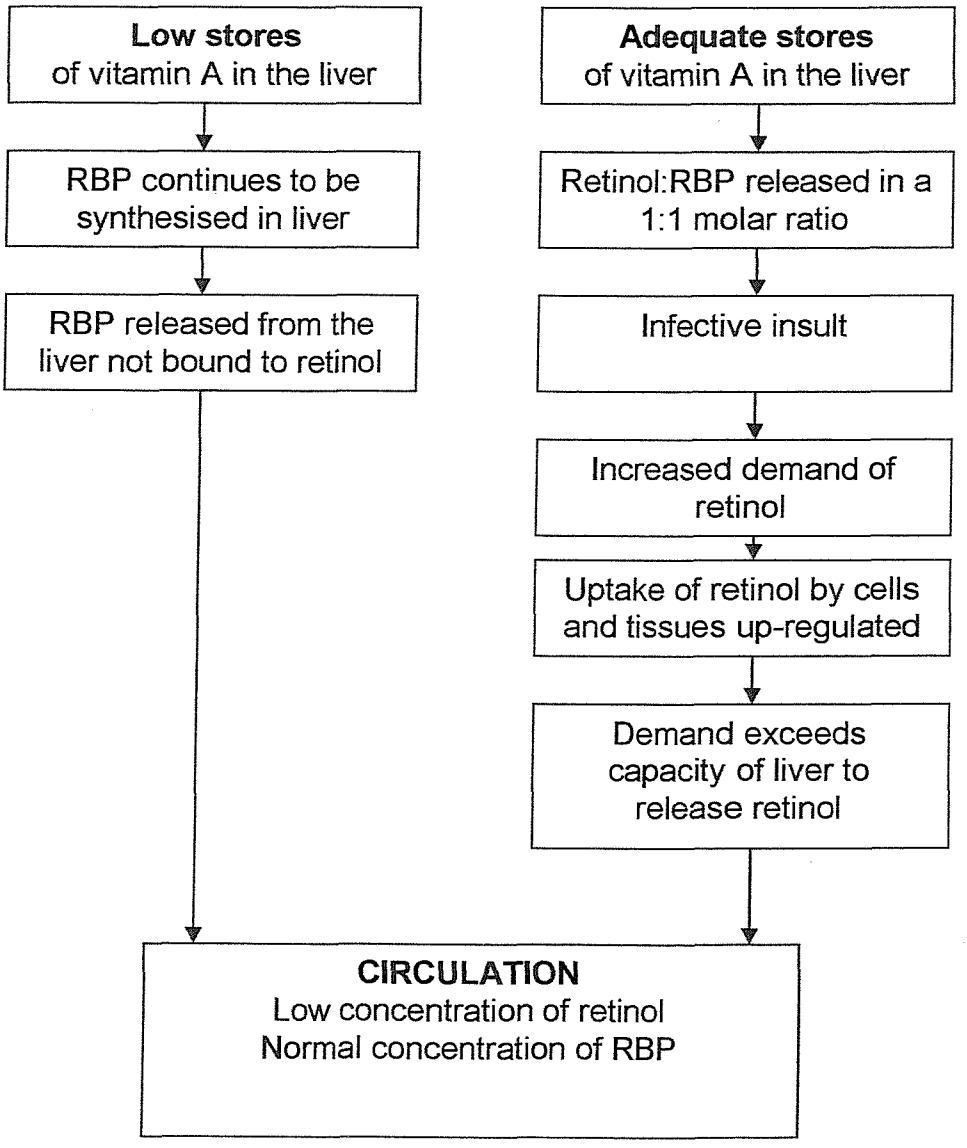
Results, presented in chapter 4, found that an activated immune system was associated with low retinol concentrations. As concentrations of RBP and retinol are associated in an 1:1 molar ratio an activated immune system may also be a factor associated with the prevalence of low RBP concentrations. The group of patients with CF had significantly higher CRP concentrations when compared to the healthy adults and these elevated concentrations were associated with both low retinol and RBP concentrations, which supports hypothesis three. It is not known whether this is a cause or consequence of the infective insult. It could be hypothesised to be a consequence, as low concentrations of RBP are a consequence of a constraint in the synthesis of RBP caused by an acute phase shift in the priorities of the liver produced by the infective insult (figure 5.8).

As supply of vitamin A is not a limiting factor associated with low retinol and RBP concentrations there is a potential risk of the liver storing high amounts of vitamin A during infection. Stores of vitamin A may accumulate in the liver during infection, and if mobilisation of retinol from the liver is regulated these stores may stay increased, and over time with each infective insult be further

enlarged. Underwood and colleagues [1972], reported stores of vitamin A in the liver to be around three times greater than that found in controls. This information could therefore have important implications relating to the current clinical management of these adult patients with CF.

Figure 5.6:

Diagram illustrating a conceptual framework to interpret the molar association of retinol:RBP in patients with CF.



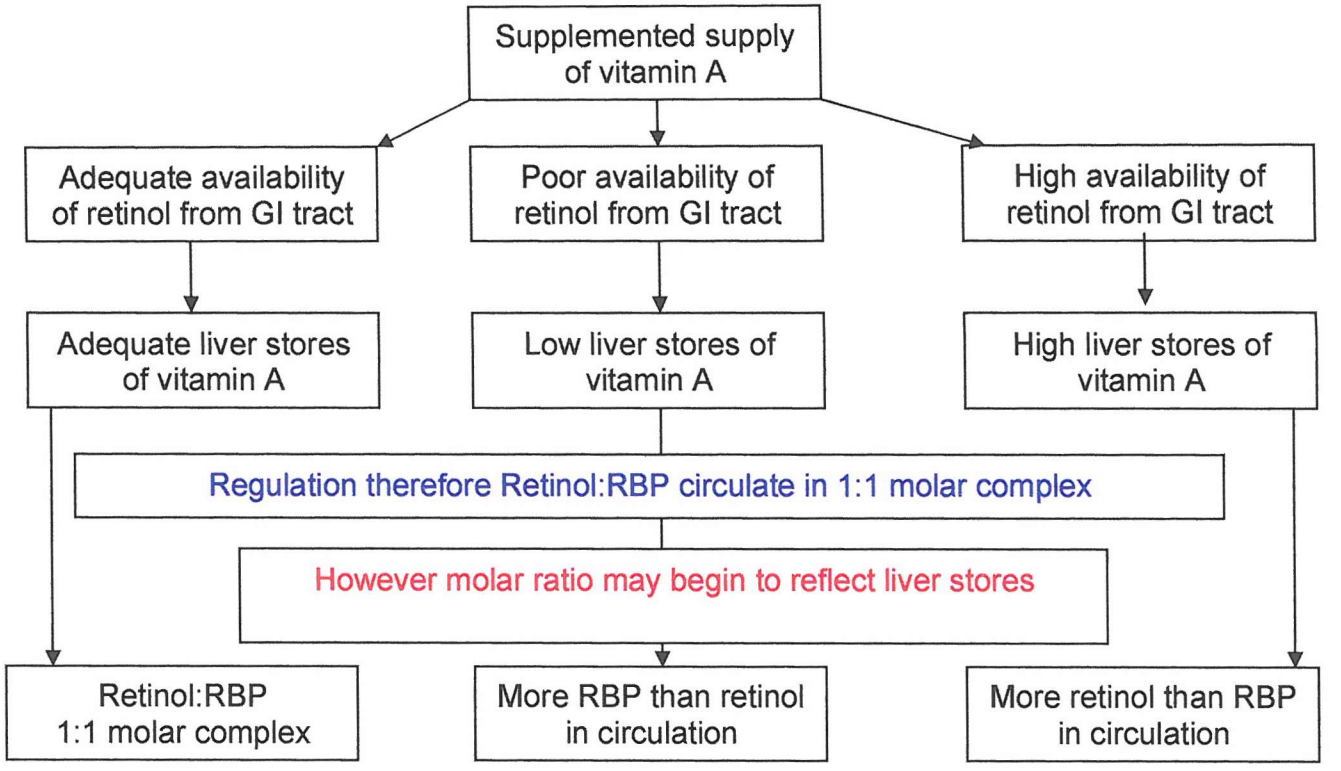
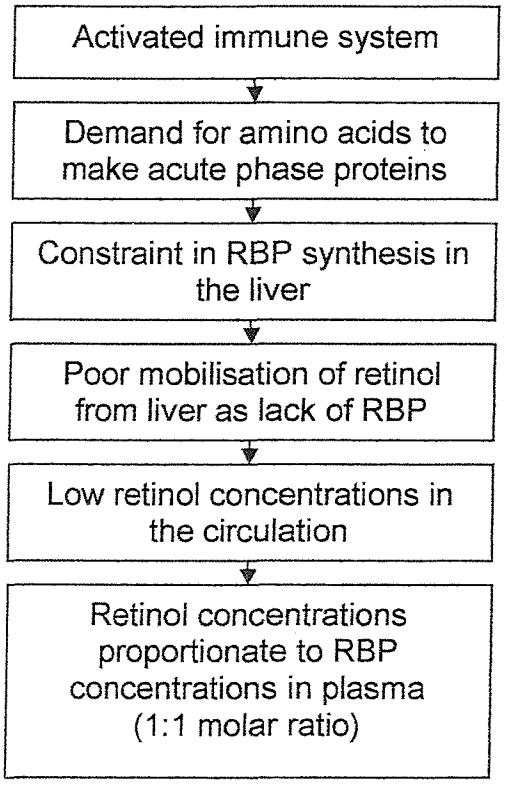


Figure 5.7: Diagram illustrating how the ratio of retinol:RBP in the circulation could reflect liver stores.

Figure 5.8:

Flow diagram illustrating how low retinol and RBP concentrations could be a consequence of the acute phase response.



5.7 SUMMARY

- In this cohort of patients with CF 53% have low concentrations of serum RBP and 59% have low concentrations of plasma retinol.
- Concentrations of retinol and RBP in the circulation are positively associated with each other, and the majority of CF patients exhibit a molar ratio around 1:1 which does not alter during inflammation.
- Retinol and RBP concentrations were correlated with CRP in that those patients with an elevated CRP had lower retinol and RBP concentrations.
- Low retinol and RBP concentrations during infection probably reflect poor mobilisation of retinol-RBP from the liver due to a constraint in the synthesis of RBP during the acute phase response.
- Low retinol and RBP concentrations are not necessarily associated with low stores of vitamin A in the liver, this therefore has important implications relating to supplement regimens of vitamin A in patients with CF.

CHAPTER 6

CIRCULATING RETINOL AND RBP CONCENTRATIONS IN EXACERBATIONS OF CYSTIC FIBROSIS.

6.1 INTRODUCTION.

Patients with CF have a high prevalence of low circulating retinol and RBP concentrations despite current therapy (chapter 4 & 5). Data presented in these chapters has reported evidence that low concentrations of retinol and RBP in patients with CF was associated with an activated immune system and patients with the lowest plasma retinol concentrations had greater admissions to hospital. The important role vitamin A plays within the immune system has been recognised for a number of years, one important observation noted in measles was that plasma vitamin A concentrations were low during the acute phase response [Frieden *et al* 1992]. One study in young patients with CF has reported low retinol and RBP concentrations during infective exacerbations [Duggan *et al* 1996], but the mechanisms, timeframes, and the potential nature of the recovery of plasma retinol and RBP concentrations are unclear.

CF is characterised by recurrent pulmonary infections, which require treatment with antibiotics and physiotherapy. In CF, an activated immune system may depress plasma retinol through infection-induced changes in the nutrient transport proteins due to increased demands for acute phase proteins during the immune response. Mechanistically a reduced plasma retinol may simply be the consequence of impaired synthesis of RBP through the competition for amino acids and not a true dietary deficiency [Rosales *et al* 1996]. A conceptual framework for the mechanism of depressed retinol and RBP concentrations in CF during exacerbations and the potential recovery of concentrations to those that fall within the reference range on treatment with antibiotics is presented in figure 6.1.

As supplementation of vitamin A in CF is based on circulating concentrations of retinol, it may be important when detecting a low plasma retinol to differentiate between infection induced hyporetinolaemia or a true deficiency in order to provide appropriate management.

6.2 AIM

The aim of the present study was to characterise the changes in concentrations of retinol, RBP and CRP over the treatment of an infective exacerbation in patients with CF. This will enable us to determine if patients retinol and RBP concentrations fall within the "normal" reference range on recovery from an exacerbation.

6.3 HYPOTHESIS

Acute pulmonary exacerbations of CF will depress plasma retinol and RBP concentrations; these concentrations will rise again on treatment of the exacerbation.

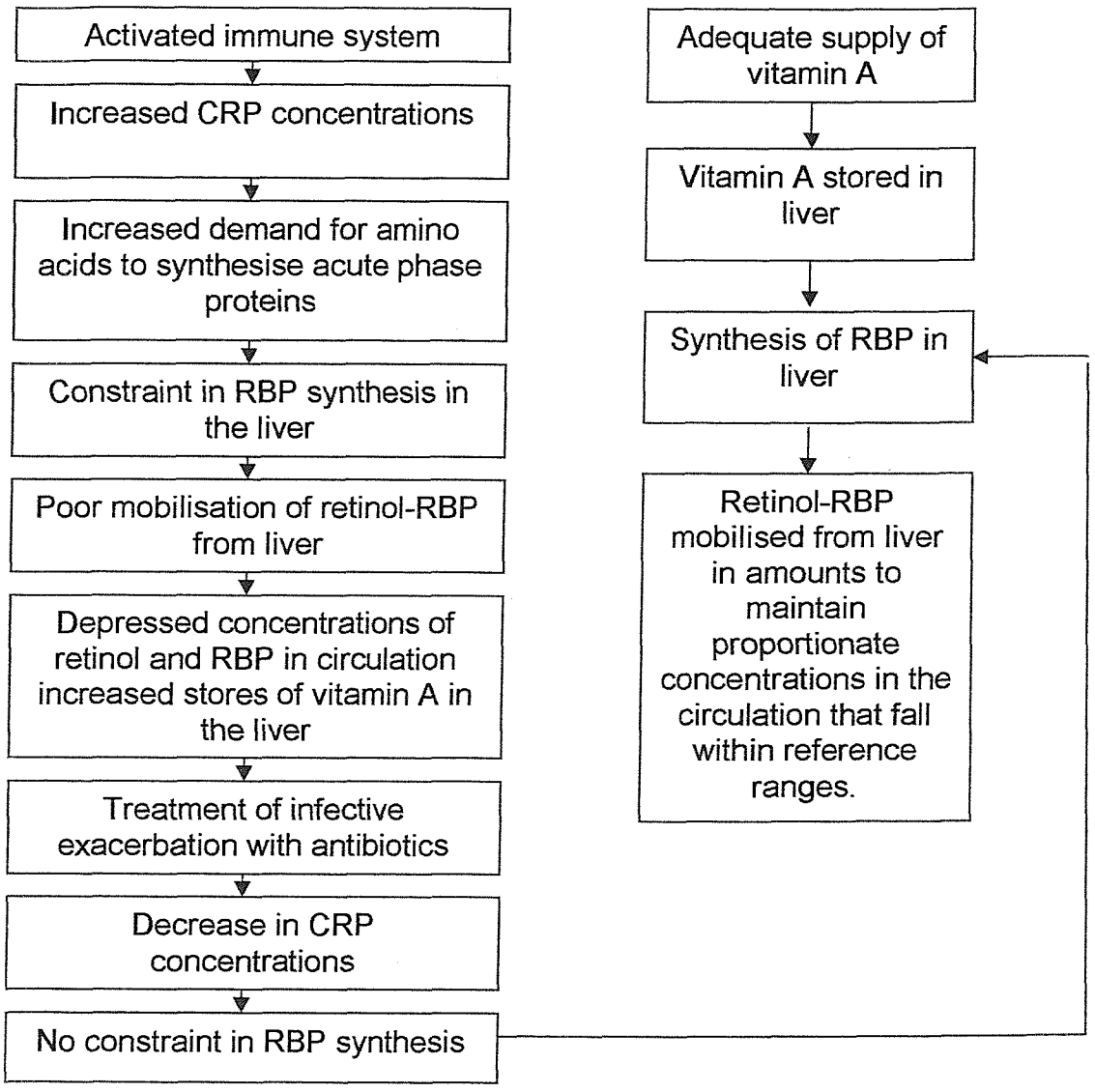
6.4 METHODS

During the prospective study patients with CF were recruited on admission to hospital with an infective exacerbation. A pulmonary exacerbation was defined as a clinical worsening of a patient's lung disease, resulting in increased shortness of breath, decreased exercise tolerance and / or forced expiratory volume (FEV). These alterations were judged by clinicians as requiring hospital admission, and treatment with antibiotics.

Six patients with CF were recruited during the period of the study. All subjects had 20ml of blood drawn by venesection on admission to hospital for the analysis of CRP, plasma retinol and serum RBP concentrations. Subjects were prescribed 14 days of antibiotic treatment on admission. Blood specimens were taken at days 7 and 14 of treatment for measurement of plasma retinol, CRP and serum RBP. A retrospective audit of the recruited patients was undertaken to assess the patient's previous retinol concentrations.

Figure 6.1:

Conceptual framework illustrating the effect in acute exacerbations of CF on circulating concentrations of retinol and RBP.



6.4.1 STATISTICS

The data presented within this chapter is reported as median and ranges. Differences were considered to be significant at $P<0.05$. Results were not normally distributed therefore non-parametric analysis was used. Differences between two related samples were analysed by Wicoxon signed ranks test.

6.5 RESULTS

6.5.1 SUBJECTS

Over the period of the study 6 subjects (5F; 1M), were admitted to Southampton General Hospital with infective exacerbations, median age 22 years (18-27 years), median BMI 18.95 kg/m^2 (16.80-25.10 kg/m^2).

6.5.2 PLASMA RETINOL

Plasma retinol concentrations rose significantly over the period of the study. Median concentrations on admission were 0.9μmol/L (0.6-1.42μmol/L) and rose to 1.34μmol/L (0.72-2.13μmol/L) and 1.74μmol/L (1.1-1.93μmol/L) at days 7 and 14 respectively. Retinol concentrations at day 14 were significantly higher than those at day zero (P=0.028). Retinol concentrations were nearly twice as high (mean 1.73 times greater (1.25-2.82)) on day 14 following treatment compared to those at day zero, onset of exacerbation.

It should be noted that 5 of the 6 patients (83%) had plasma retinol concentrations less than 1.4μmol/L (95%CI DoH/MAFF healthy adults) on admission with an infective exacerbation, 4 of the 6 patients had plasma retinol concentrations that were less than 1μmol/L. By comparison only 1 of the patients had plasma retinol <1.4μmol/L by day 14 of antibiotic treatment, this patient had not responded well to the antibiotics. Plasma retinol concentrations rose, in all subjects, over the period of study (figure 6.2). Five of the six patients had previously had plasma retinol concentrations measured as part of routine annual assessment (table 6.1). Of these only three did not have elevated CRP concentrations (>6mg/L) at the time of measure, these patients retinol concentrations were not different (P =0.655) with those measured at 14 day post antibiotic treatment.

6.5.3 PLASMA RBP and TTR.

Over the study period median serum RBP concentrations rose from 1.19μmol/L, (0.71-2.23μmol/L) at admission to 1.57μmol/L (0.86-2.23μmol/L) at day 7, and 1.95μmol/L (1.29-2.43μmolL) by day 14 of antibiotic treatment. Individual subjects data points are presented in figure 6.3. RBP concentrations were on average 1.35 times higher by day 14 compared to those at day zero. Four of the

6 subjects had serum RBP concentrations below the normal reference range at day zero compared to only 1 patient by day 14 of treatment. This patient was the same patient who also exhibited a low plasma retinol at day 14.

The relationship between plasma retinol and serum RBP concentrations is presented in figure 6.4. There was a significant correlation between plasma retinol and RBP concentrations over the whole study period (Spearman $R=0.919$; $P<0.001$). The ratio of retinol to RBP was approximately 1:1 during the whole study. The median ratio at day 0 was 1.19 (1.07-1.78) and did not alter by day 7 and 14 respectively, 1.19 (1.01-1.32), and 1.15 (1.07-1.36). It should be noted that over the period of the study, in all cases patients have more circulating RBP than retinol.

In the circulation retinol-RBP is complexed with TTR (prealbumin) to prevent filtration by the kidney. Median concentrations of TTR were 3.09µmol/L, 4.45µmol/L and 5.70µmol/L at days 0, 7 and 14 respectively. The normal reference range for TTR in the circulation is 3.64-7.27µmol/L. The ratio of TTR:RBP was 2.59, 2.83 and 2.91 at days 0, 7 and 14 respectively.

6.5.4 CRP.

Rises in concentrations of retinol and RBP occurred concurrently as median CRP concentrations fell from 18.6mg/L to 4.8 mg/L over the study period. All subjects had CRP concentrations greater than 10mg/L at the onset of exacerbation and by day 14, 5 of the subjects had CRP concentrations less than 6mg/L (figure 6.5).

6.5.5 CHANGES OVER TIME.

Figure 6.6 shows the changes in concentrations of retinol, RBP and CRP over the period of the study. Median retinol and RBP concentrations significantly rose during treatment of the exacerbation with antibiotics, concurrently as CRP concentrations fell. Table 6.2 illustrates the prevalence of low circulating retinol and RBP concentrations over the period of the study. At onset of exacerbation 83% (5/6) of the patients have plasma retinol concentrations below 1.4µmol/L

(DoH;MAFF 1990), and 67% (4/6) patients have serum RBP concentrations below the reference range (1.46μmol/L), all patients have an elevated CRP (>6mg/L). By day 14 of antibiotic treatment 83% of patients (5/6) have low CRP concentrations (<6mg/L), and only 1 patient has low retinol and RBP concentration.

Figure 6.2:

Subject's individual changes in plasma retinol concentrations over the 14-day treatment of infective exacerbation, in 6 patients with CF.

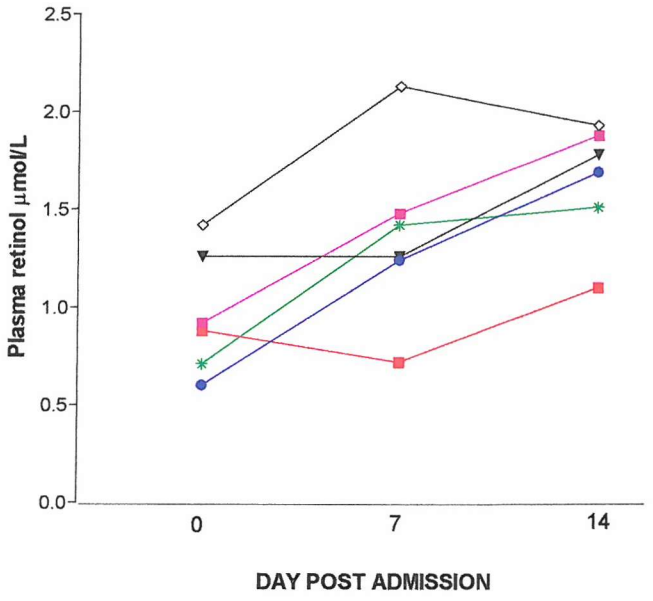


Table 6.1:

Data of individual subjects plasma retinol and CRP concentrations on days zero and 14 of the study, alongside retinol and CRP concentrations for the subjects at the previous annual assessment.

Subject	Retinol Day 0 study (μmol/L)	CRP Day 0 study (mg/L)	Retinol Day 14 study (μmol/L)	CRP Day 14 study (mg/L)	Retinol. Annual Assessment (μmol/L)	CRP. Annual Assessment (mg/L)
1	0.92	51.90	1.88	4.80	1.40	29.20
2	0.60	10.50	1.69	2.69	0.70	120.00
3	1.26	18.80	1.78	11.80	1.80	1.20
4	0.88	13.40	1.10	4.01	1.30	5.00
5	0.71	50.90	1.51	<2	-	-
6	1.42	18.60	1.93	<2	1.70	7.12

Figure 6.3:

Subject's individual changes in serum RBP concentrations over the 14-day treatment of infective exacerbation in 6 patients with CF.

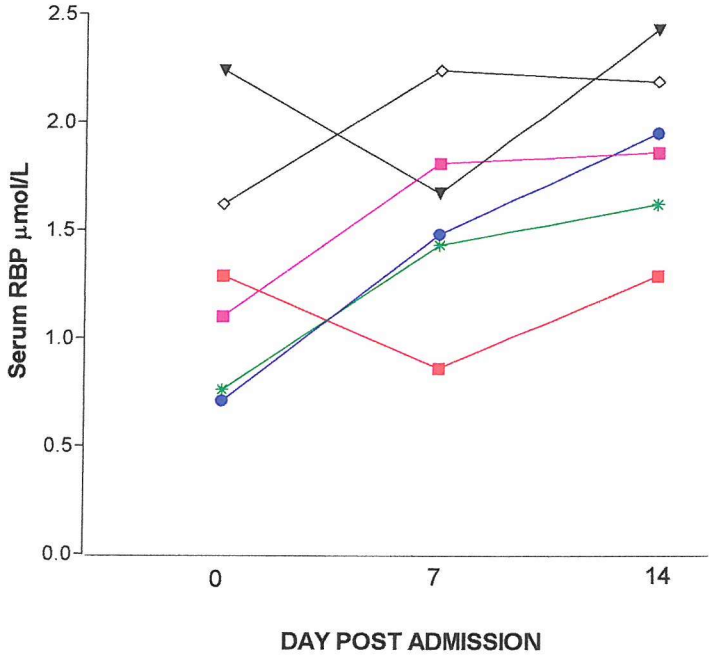


Figure 6.4:

Relationship between plasma retinol and serum RBP concentrations in 6 patients with CF over the period of treatment of an infective exacerbation.
(Each individual subject is represented by a different symbol).

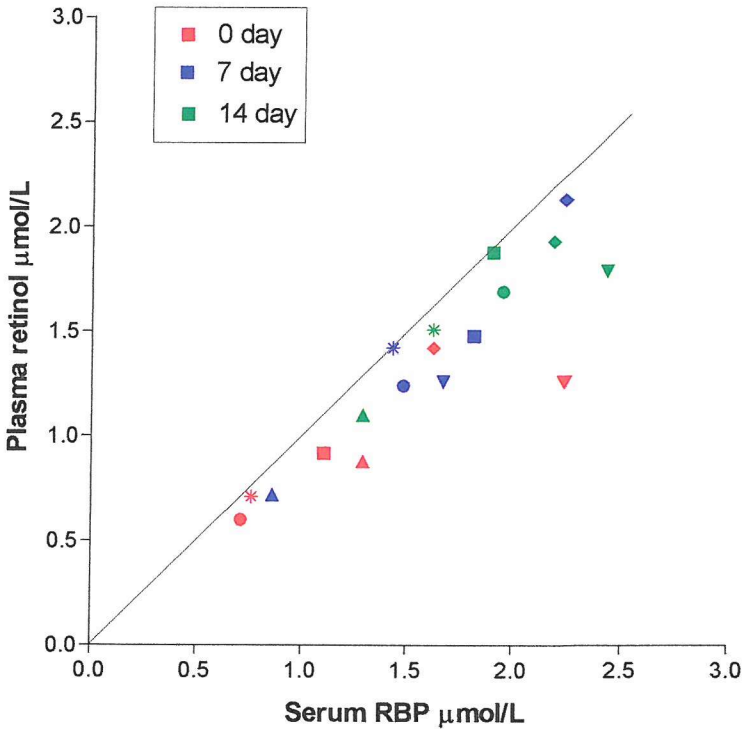


Figure 6.5:

Subject's individual changes in CRP concentrations over the 14-day treatment of infective exacerbation in 6 patients with CF.

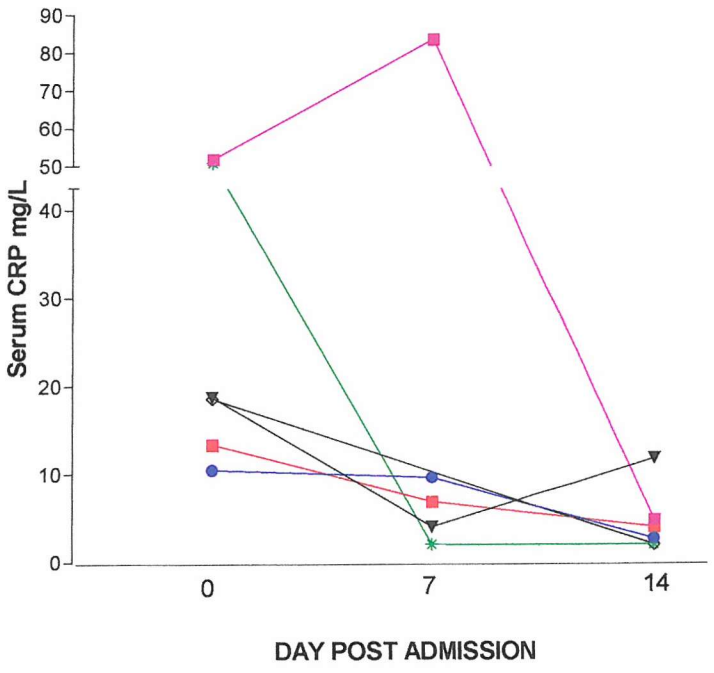


Figure 6.6:

Median changes in plasma retinol, RBP and CRP concentrations over 14 days of treatment of an infective exacerbation in 6 patients with CF.

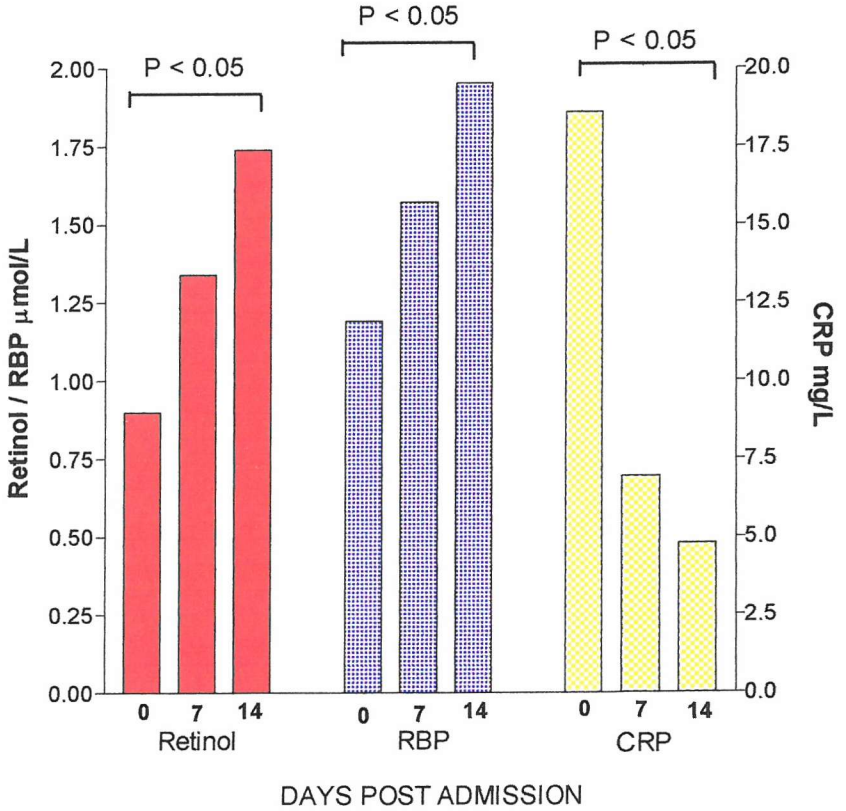


Table 6.2 :

Data indicating the prevalence of low concentrations of retinol and RBP over the period of the study in 6 patients with CF.

	Days of antibiotic treatment					
	Day 0		Day 7		Day 14	
	Number	%	Number	%	Number	%
Low Retinol <1.4μmol/L (DoH, 1990)	5/6	83%	4/6	67%	1/6	17%
Low RBP <1.46μmol/L (Dati, 1996)	4/6	67%	2/6	33%	1/6	17%
Low CRP <6mg/L	0/6	0%	3/6	50%	5/6	83%

6.6 DISCUSSION.

The study reported in this chapter has investigated the effect of the acute phase response on concentrations of retinol and RBP in patients with CF. The hypothesis to be tested was that acute pulmonary exacerbations of CF will depress plasma retinol and RBP concentrations, which rise again on treatment of the exacerbation.

Data presented in this chapter found that during antibiotic treatment of an infective exacerbation, plasma retinol and RBP concentrations significantly increased concomitant with a reduction in CRP concentrations. Retinol concentrations at onset of the infection fell into the deficient range but during treatment of the exacerbation rose to within the normal range. The most probable mechanism for the reduction in plasma retinol and RBP concentrations is an acute phase shift in hepatic protein synthesis of RBP to proteins required for the acute phase response (figure 6.1). A constraint to synthesise RBP, causes poor mobilisation of retinol-RBP from the liver, and therefore depressed concentrations of retinol and RBP in the circulation. On treatment of the infection with antibiotics the constraint in the synthesis of RBP is removed and therefore retinol can be mobilised from the liver to enable circulating retinol and

RBP concentrations to return to concentrations that are not considered to be low.

However it should be considered that low retinol concentrations during infection could also be attributed to a number of other possible factors. These include increased losses of vitamin A in the urine [Alvarez *et al* 1995; Stephensen *et al* 1994], increased uptake of retinol to tissues [Willumsen *et al* 1997], or loss of RBP into extra cellular fluid due to increased vascular permeability during infection [Thurnham *et al* 1991]. The data presented in this chapter provides some preliminary findings that relate to these factors. Two previous studies from the literature [Stephensen *et al* 1994; Alvarez *et al* 1995], found that patients with pneumonia and sepsis have high urinary losses of retinol compared to healthy control subjects. In our data set urinary losses of vitamin A were not directly measured during the infective exacerbation, however TTR was in excess of RBP throughout the study period. Increased uptake of retinol to cells and tissues during the infective exacerbation may also contribute to low concentrations of retinol observed in the circulation. The data presented in this chapter found in the circulation that RBP was in excess of retinol. This may occur if there are low stores of vitamin A from the liver, but more importantly during infection this phenomenon may occur if the uptake of retinol to cells and tissues in up-regulated. During an infective stress there maybe increased demands by the cells and tissues, so uptake of retinol is up-regulated. In this case low retinol concentrations may not be detrimental to the host but a beneficial adaptation during infection.

6.7 SUMMARY AND CONCLUSIONS.

- Plasma retinol concentrations are depressed into ranges that would be classified as deficient in patients with CF during acute pulmonary exacerbations.
- Serum RBP concentrations are depressed in patients with CF during acute pulmonary exacerbations.
- Concentrations of retinol, and RBP rise into ranges that would be classified as "normal" following the treatment of exacerbation.
- The depression of retinol and RBP concentrations maybe associated with an acute phase shift in hepatic protein synthesis during the acute phase response.
- Increased metabolic demands may also be contributing factors in the reduced concentrations seen at the onset of an exacerbation.
- In clinical practice plasma retinol concentrations should be monitored together with a marker of infective status to enable the differentiation between infection-induced hyporetinolaemia or a problem associated with status before any adjustment to vitamin A supplementation is made.

SECTION B RESULTS

CHAPTER 7

FAECAL LOSSES AND POSTPRANDIAL EXCURSIONS OF VITAMIN A AND LIPID IN HEALTHY ADULTS AND PATIENTS WITH CYSTIC FIBROSIS.

7.1 INTRODUCTION

Data presented in section A of the results chapters found that there was ~ 50% prevalence of low retinol concentrations in patients with CF and patients with low retinol concentrations have a poorer clinical course. Data indicated that supply of the vitamin and adherence to supplement regimens were not the primary limitations resulting in low retinol concentrations, although the presence of an immune response appeared to be associated with low concentrations. It should be acknowledged that patients who do eat poorly and are not compliant to supplement regimens are at risk of low retinol concentrations from poor dietary supply. However when these patients are excluded from analysis there is still a high prevalence of low retinol concentrations. There is a need to specifically investigate factors associated with low retinol concentrations in patients with CF that cannot be simply explained by dietary supply, compliance or infection.

There are three main factors within the processing of vitamin A that may influence plasma retinol concentrations. These include, dietary supply, availability of the vitamin across the GI tract and mobilisation of retinol from the liver (figure 7.1). Availability of the vitamin across the GI tract will be systematically investigated within this chapter; the conceptual framework in which availability may influence plasma retinol concentrations is illustrated in figure 7.2.

A major area in the patho-physiology of the disease CF is pancreatic insufficiency. Patients secrete inadequate amounts of enzymes and bicarbonate, which in turn have a direct effect on the GI tract. All pancreatic

insufficient patients receive PERT but many patients exhibit, to some extent, lipid maldigestion and malabsorption, despite enzyme therapy [Murphy *et al* 1991]. Limited data within the literature appears to suggest that availability of vitamin A is lower in patients with diseases associated with impaired lipid handling when compared to controls [Barnes *et al* 1950; Johnson *et al* 1992b; Rasmusen *et al* 1986, Katsampes *et al* 1944, Chesney *et al* 1934, Ahmed *et al* 1990]. It is with this information and knowledge of the chemical similarities of lipid and the lipid soluble vitamin, which forms the basis of the well-held belief that availability of dietary vitamin A is compromised, in patients with CF. Low circulating plasma retinol concentrations seen in these patients are often attributed to this perceived decrease in apparent availability, despite current routine use of oral PERT. For these reasons patients with CF are routinely prescribed supplements of retinyl palmitate often in excess of 2mg/day.

Figure 7.1

Factors that may influence the processing of vitamin A.

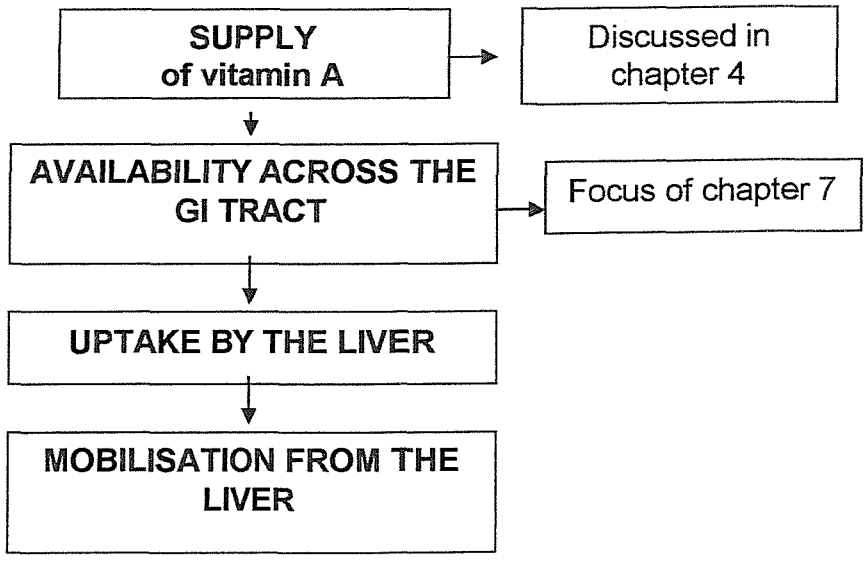
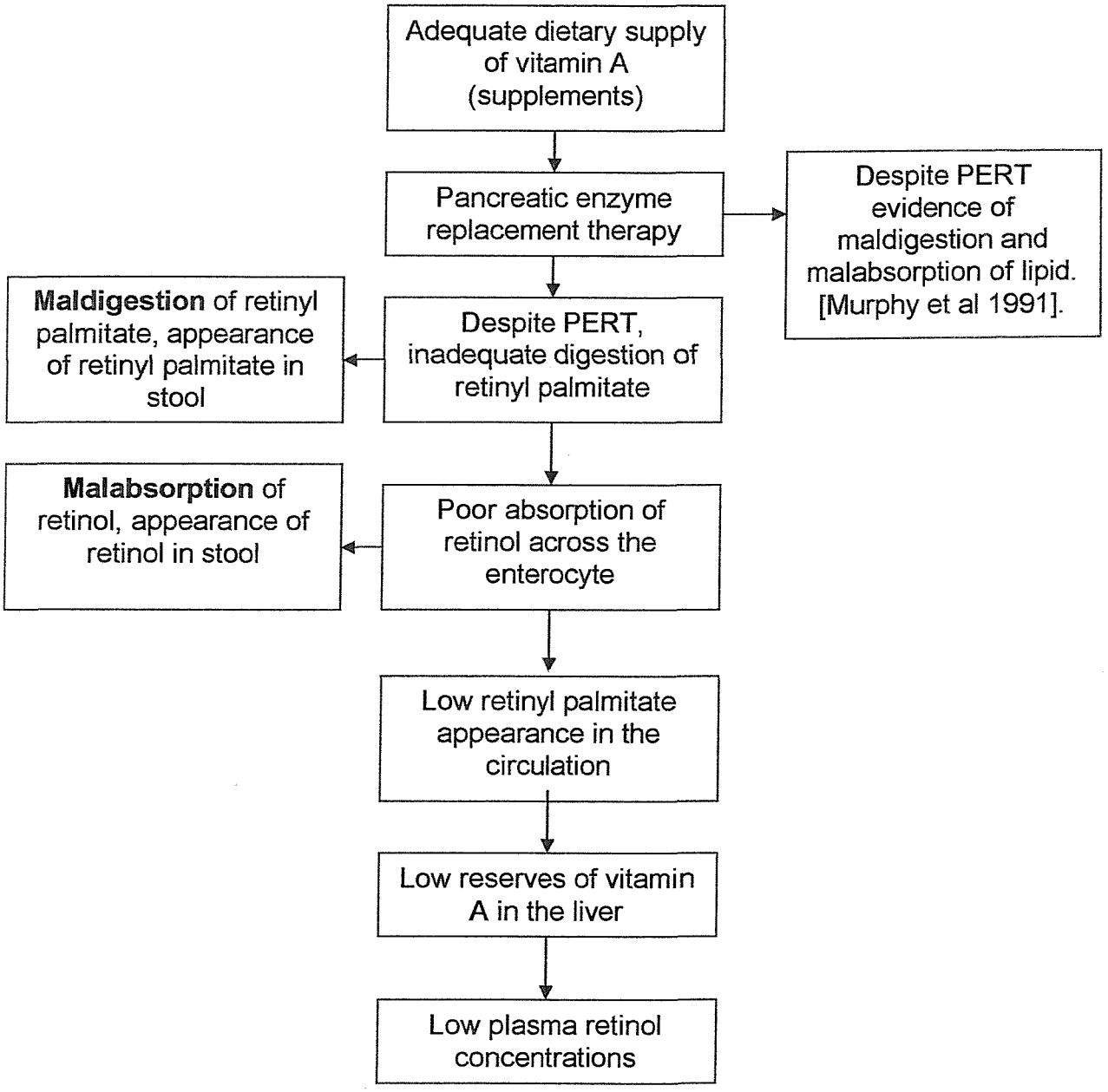


Figure 7.2

Conceptual framework illustrating how low retinol concentrations may potentially arise in patients with CF who are routinely supplemented vitamin A and PERT.



7.2 AIMS AND QUESTIONS

The aims of the present study were two fold. The primary aim was to determine availability of retinyl palmitate across the GI tract by characterising faecal losses of vitamin A in association with the postprandial changes in plasma retinyl palmitate following a test meal containing isotopically labelled retinyl palmitate. The second aim was to determine the extent to which the handling of vitamin A is associated with that of lipid.

Questions to be addressed include:

1) Do patients with CF excrete higher amounts of vitamin A in stool compared to healthy adults?
2) Do patients with CF excrete higher amounts of lipid in stool compared to healthy adults?
3) Is there a relationship between faecal lipid and vitamin A excretion?
4) What is the apparent absorption of vitamin A across the GI tract?
5) Are losses of vitamin A in stool related to the process of maldigestion or malabsorption?
6) Do patients with CF have lower postprandial excursions of retinyl palmitate and TAG?
7) Do losses of vitamin A in stool correlate with the appearance of retinyl palmitate in the circulation?
8) Do losses of lipid in stool correlate with the appearance of TAG in the circulation?

7.3 HYPOTHESIS

Patients with CF have disturbed GI handling of dietary lipid which is associated with impaired GI handling of vitamin A. Availability of vitamin A across the GI tract contributes to decreased retinol concentrations.

7.4 METHODS

Six healthy adults, median age 22.5 years (22-23 years; 6 M), and 6 patients with CF median age 21 years (17-39 years; 5M;1F) participated in the study. Informed consent was obtained from each of the subjects after the nature of the study had been explained. The healthy subjects were either employees or students at the University of Southampton. All subjects were in good health prior to and during the course of the study. The CF subjects were recruited from the CF clinic at Southampton General Hospital. Inclusion criteria of CF patients included those who were compliant to supplement regimens, exhibited no dietary constraints and were well at the time of study. None of the subjects were taking vitamin supplements for the period of the study. The study had the approval of the Southampton Hospitals and South West Hampshire Health Authority Ethical Committee.

A schema illustrating the methods of emulsion administration, sample collection times and movement of tracer around the body is presented in figure 7.3. All methods used within this chapter are described in detail in chapter 3. Following an overnight fast, baseline venous blood samples were collected, for measurements of CRP, plasma retinol, retinyl palmitate, serum RBP and zinc concentrations. All subjects received an oral bolus dose of [10,19,19,19] ^{2}H-retinyl palmitate (300μg/kg body weight) in an emulsion along with a test breakfast (time zero). Post dose bloods were drawn at 2, 5, 10 and 24 hours, 7, 14 and 28 days. All subjects only drank water between blood samples. After the 5-hour sample subjects received a test lunch (low in vitamin A). All stools were collected for 3 days. Subjects continued on their habitual diet for samples collected after the study day. Vitamin A as retinol and retinyl palmitate was determined by HPLC. [$^{2}H_4$] retinol was determined by GCMS following fractionation by HPLC and derivitisation. Total faecal lipid was measured by a modified method of Folch *et al* 1957. Total plasma TAG concentrations were analysed by an enzymatic method. Serum RBP concentrations were measured by Nephelometry. Plasma CRP was measured by chemical pathology (SUHT), and plasma zinc was measured by the Faculty of Medicine, University of Southampton.

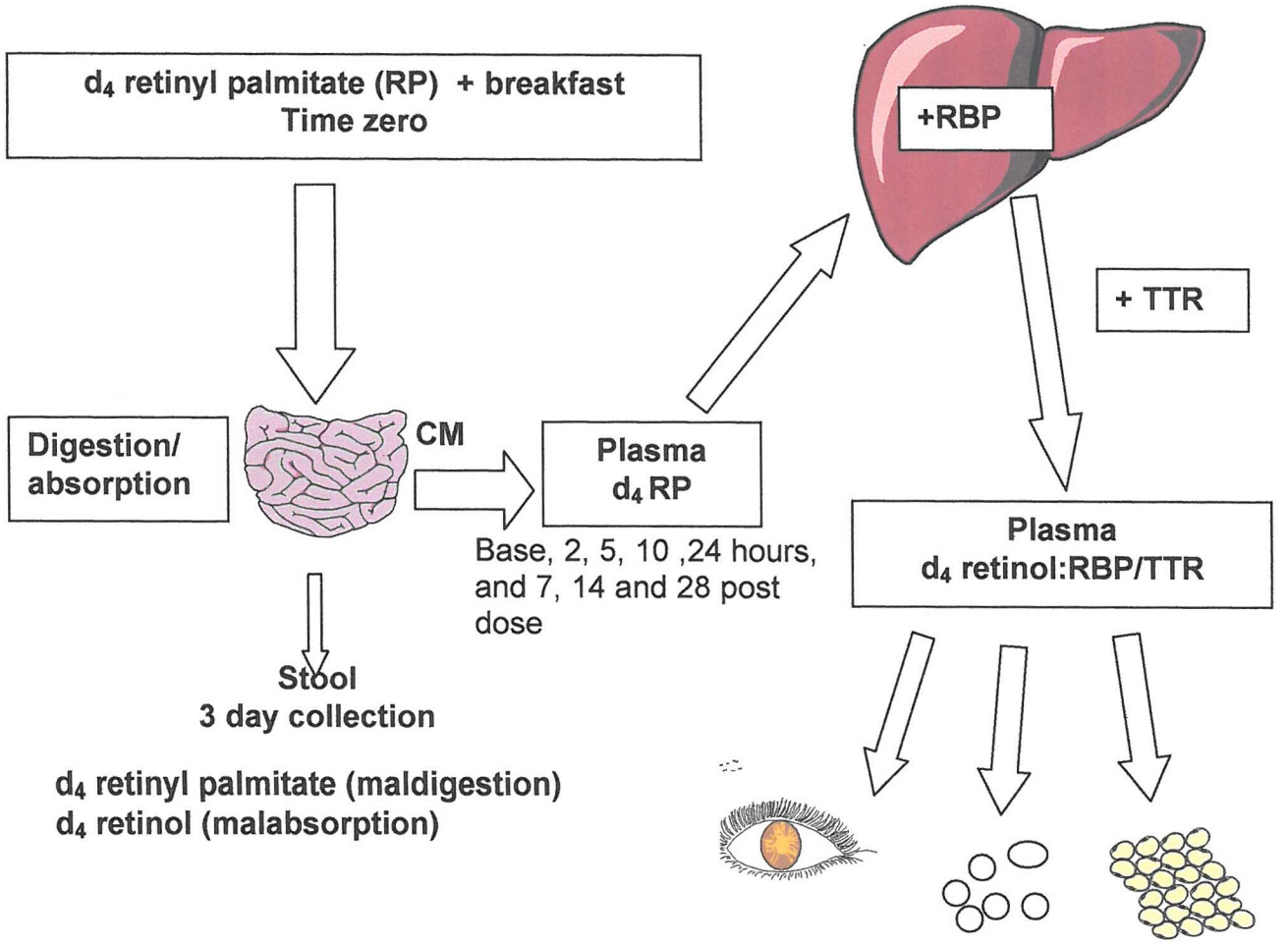


Figure 7.3:

Schema illustrating stable isotope methodology to characterise the partitioning of exogenous retinyl palmitate.

7.5 RESULTS

7.5.1 SUBJECT CHARACTERISTICS

The study was carried out in a group of 12 adults of similar ages, comprising 6 healthy adults (median age 22.5 years) and 6 patients with Cystic Fibrosis (median age 21 years). Subject characteristics are shown in table 7.1. Patients with CF were recruited to the study when well however despite this, patients with CF still had elevated concentrations of CRP indicative of an acute phase response. There were significant differences between the two groups for the variables of CRP and BMI, and the dose of deuterated retinyl palmitate administered.

7.5.2 PLASMA RETINOL and RBP CONCENTRATIONS.

Patients with CF had trends, although not significant, towards lower concentrations of retinol and RBP (median (range); Retinol 1.14μmol/L (0.60-1.82μmol/L); RBP 1.12μmol/L (0.86-1.76μmol/L)) when compared to healthy adults (median (range); Retinol 1.50μmol/L (1.07-2.22μmol/L); RBP 1.55μmol/L (1.05-2.00μmol/L)) (figures 7.4 and 7.5). Baseline plasma retinol concentrations were significantly related to baseline serum RBP concentrations ($P<0.05$) data not shown. The molar ratio of retinol:RBP was not different between the two groups (HA median 1.06 range 0.68-1.26; CF median 1.07 range 1.02-1.41). When compared to the healthy adults, CF patients had lower retinol concentrations at each time point, although significant differences between the two groups were only obvious at 14 and 28 days post dose (table 7.2). Individual subjects plasma retinol concentrations did not significantly alter over the period of the study ($P>0.05$).

Of the healthy adults, 4 (66.6%) had plasma retinol and RBP concentrations that fell within the reference ranges (1.4-3.2μmol/L retinol; 1.43-2.86μmol/L RBP). In comparison only 2 (33.3%) patients with CF had plasma retinol concentrations within the reference range and only 1 (25%) patient (2 missing data points) had serum RBP concentrations within the reference range. The two CF patients with the lowest plasma baseline retinol concentrations (0.60μmol/L and 0.87μmol/L) had the greatest CRP concentrations (62mg/L and 120mg/L respectively).

Table 7.1:

Characteristics of the study population, 6 healthy adults and 6 patients with CF.

(Data presented as median and ranges).

	Healthy adults (HA) n=6	Cystic Fibrosis Adults n=6
Age (years)	22.50 (22 - 23)	21 (17 - 39)
BMI (kg/m2)	21.95 (20.61 - 23.77)	19.00* (16.01 - 22.09)
Weight (kg)	65.75 (64.6 - 80.90)	51.65** (41.50 - 64.60)
Height (m)	1.74 (1.70 - 1.85)	1.65* (1.55 - 1.71)
CRP (mg/L) Ref range 0-6mg/L	1.00 (1.00 - 7.02)	21.30* (1.00 - 120.00)
Dose Retinyl Palmitate (mg)	19.75 (19.40 - 24.30)	15.50** (12.40 - 19.38)
Dose Retinyl Palmitate (μmol)	37.64 (36.97 - 46.30)	29.54** (23.63 - 36.93)
Zinc (μmol/L) Ref range 11-24μmol/L	12.55 (10.90 - 15.80)	11.85 (9.70 - 17.30)
RBP (μmol/L) Ref range 30-60mg/L	1.55 (1.05-2.00)	1.12 (0.86-1.76)
Baseline retinol (μmol/L) Ref range 1.4-3.2μmol/L	1.50 (1.07 - 2.22)	1.14 (0.60 - 1.82)

Mann Whitney U Significantly different from healthy individuals

* $P < 0.05$

** $P < 0.01$

Table 7.2:

Plasma retinol concentrations (μmol/L) of the 6 healthy adults and 6 patients with CF over the period of the study.

(Presented as median and ranges).

	Plasma retinol concentration μmol/L	
Time point	**CF adult**	**Healthy adult**
Base	1.14 (0.60-1.82)	1.50 (1.07-2.22)
2 hour	1.09 (0.54-1.80)	1.41 (1.10-2.43)
5 hour	1.19 (0.53-1.94)	1.50 (1.28-2.32)
10 hour	1.13 (0.61-1.99)	1.53 (1.27-2.32)
24 hour	1.10 (0.55-1.79)	1.37 (1.17-2.09)
7 day	1.49 (0.83-2.38)	1.65 (1.40-2.37)
14 day	1.00 * (0.67-1.51)	1.61 (1.41-1.93)
28 day	0.98 * (0.75-1.50)	1.54 (1.32-1.87)

Mann Whitney U Significantly different from healthy individuals
* $P < 0.05$

Figure 7.4

Baseline plasma retinol concentrations (μmol/L) in the 6 patients with CF and 6 healthy adults (HA). (Line depicts median value).

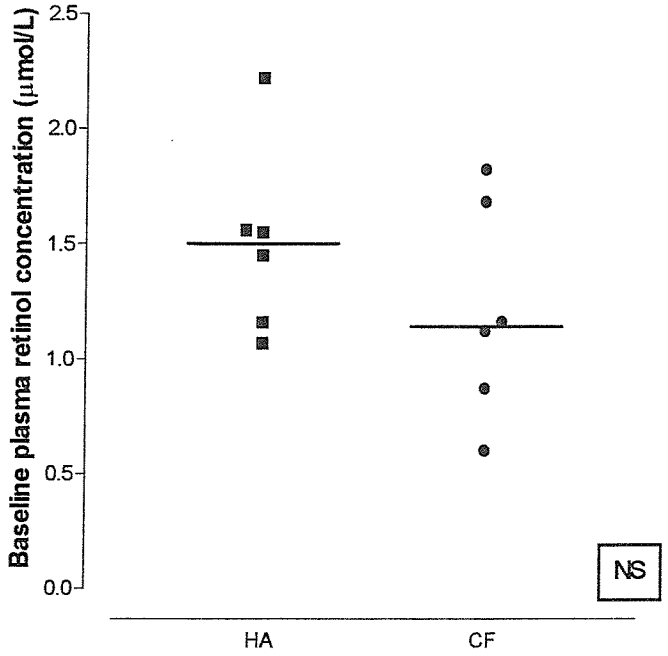
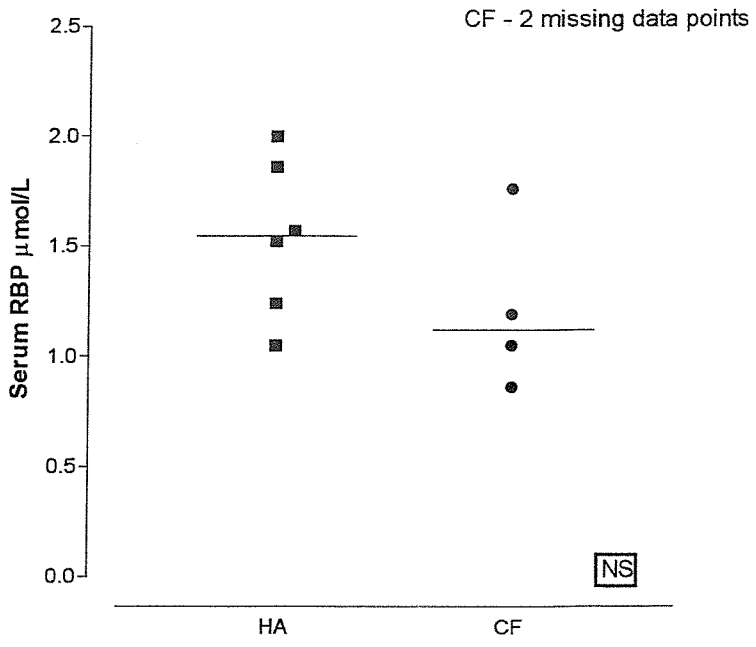


Figure 7.5

Baseline serum RBP concentrations (μmol/L) in the 6 patients with CF and 6 healthy adults (HA). (Line depicts median value)



7.5.3 INTAKE OF SUPPLEMENTS.

None of the CF patients took prescribed supplements on the day of the study. However 5 of the CF study subjects were routinely prescribed supplements in excess of their habitual diet. One female subject until recently was not prescribed supplements as she was assessed as being pancreatic sufficient. However, approximately 6 months prior to the study she began supplements. Doses of supplement ranged from 1.2 - 3.4mg of retinol per day (median 1.2mg/d). All subjects were assessed as being adherent to supplement regimens. Four subjects took vitamin A and D capsules, 1 subject took Sanatogen Gold, and 1 subject took vitamin A and D and Sanatogen gold.

7.5.4 FAECAL LOSSES vitamin A and lipid.

CF patients had daily stool weights of 75 – 381.1g/d (median 222.02g/d) these were not significantly different to healthy individuals (daily stool weight 71.47-209.8g/d; median 128.30g/d). The individual's data relating to stool losses of both vitamin A (as retinol and retinyl palmitate) and lipid are presented in table 7.3.

There was marked variability (2.5 - 27.2 g/d) in faecal lipid losses in patients with CF compared to that seen in healthy adults (5 – 7.4 g/d) (figure 7.6). Adults with CF had greater, although not significant, median lipid losses when compared to healthy adults (11.9 g/d CF; 6.2 g/d HA). Faecal vitamin A losses were positively correlated with faecal lipid losses in the patients with CF (Spearman R = 0.886 P=0.019) (figure 7.7).

Median losses of vitamin A excreted in stool as a percentage of labelled vitamin A ingested were significantly different between the two groups (Mann Whitney P=0.031). Results ranged from 0 – 5.18% (median 0%) for healthy adults and 0 - 29.5% (median 6.51%) for patients with CF. The median apparent absorption of vitamin A for the dose administered in both the CF group and the healthy adults was estimated to be 93.49 % and 100 % respectively (figure 7.8).

Adults with CF loose significantly greater total retinol and retinyl palmitate (RP) (1.05μmol/3d retinol; 0.78μmol/3d RP) in faeces compared to healthy

individuals (0μmol/ 3d for both retinol and RP) ($P<0.05$) (figure 7.9). In patients with CF (excluding the pancreatectomised patient) the d_4-RPA:d_4-retinol ratio in faeces ranged from 0.57-1.49 indicating both maldigestion and malabsorption of the vitamin. The ratio of the pancreatectomised patient was 5.06 reflecting mainly maldigestion. There were no differences between losses of retinol and retinyl palmitate in the stool of patients with CF ($P>0.05$), reflecting both maldigestion and malabsorption of vitamin A in patients with CF.

Table 7.3:

Faecal losses of vitamin A and lipid in stool.

Total unlabeled and labelled retinol and retinyl palmitate (RP), total vitamin A and total labelled vitamin A, total lipid.
(Px = Pancreatectomised patient).

	Total unlabelled retinol (µmol/3d)	Total unlabelled RP (µmol/3d)	Total d_4-retinol (µmol/3d)	Total d_4-RP (µmol/3d)	Total Vitamin A (µmol/3d)	Total d_4-vitamin A (µmol/3d)	Total Lipid (g/d)
HA 1	0.07	0.05	0.04	0.00	0.16	0.04	5.6
HA 2	0.00	0.00	0.00	0.00	0.00	0.00	7.4
HA 3	0.00	0.00	0.00	0.00	0.00	0.00	6.8
HA 4	0.00	0.00	0.00	0.00	0.00	0.00	5.0
HA 5	0.02	0.00	0.00	0.00	0.02	0.00	6.9
HA 6	0.03	0.01	0.01	0.00	0.05	0.01	5.2
CF 1	2.76	4.30	1.82	2.71	11.59	4.53	27.2
CF 2	1.06	0.39	1.16	0.66	3.27	1.82	4.0
CF 3	0.45	0.33	0.94	0.83	2.55	1.77	5.5
CF 4	0.00	0.00	0.00	0.00	0.00	0.00	2.5
CF 5 (Px)	0.22	0.44	1.42	7.18	9.26	8.60	18.3
CF 6	2.07	1.37	0.81	0.74	4.99	1.55	24.0

Figure 7.6:

Total faecal lipid (g/d) in the stool of patients with CF and healthy adults (HA). (Px = Pancreatectomised patient)

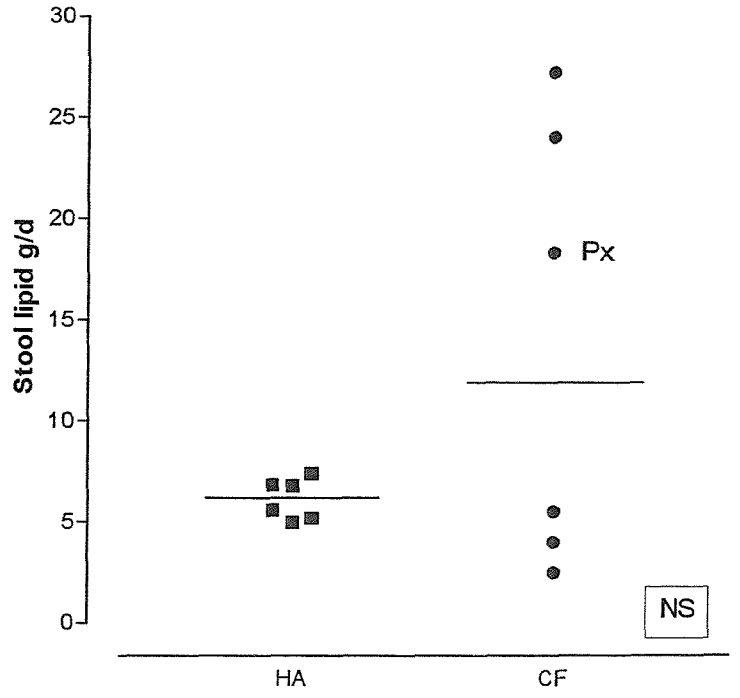
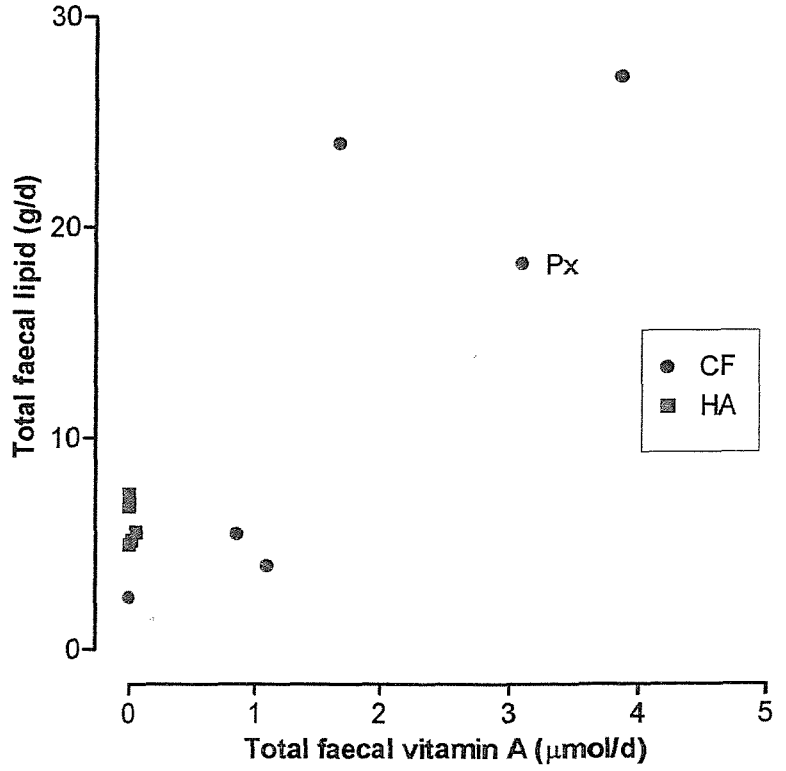


Figure 7.7:

Relationship between total faecal vitamin A and total faecal lipid losses in patients with CF and healthy adults (HA). (Px = Pancreatectomised patient)



Sperman R = 0.886; P=0.019

Figure 7.8:

Loss of vitamin A in stool as a percentage of administered dose, in both patients with CF and healthy adults (HA). (Px = Pancreatectomised)

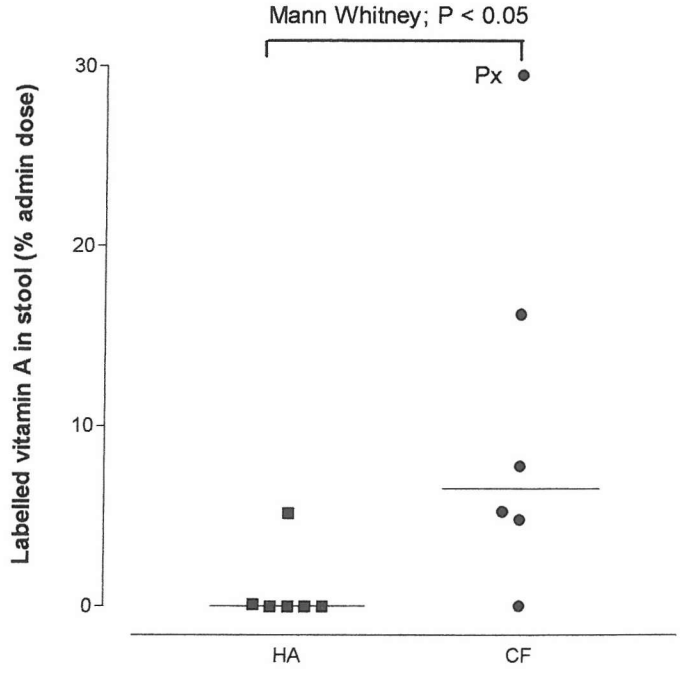
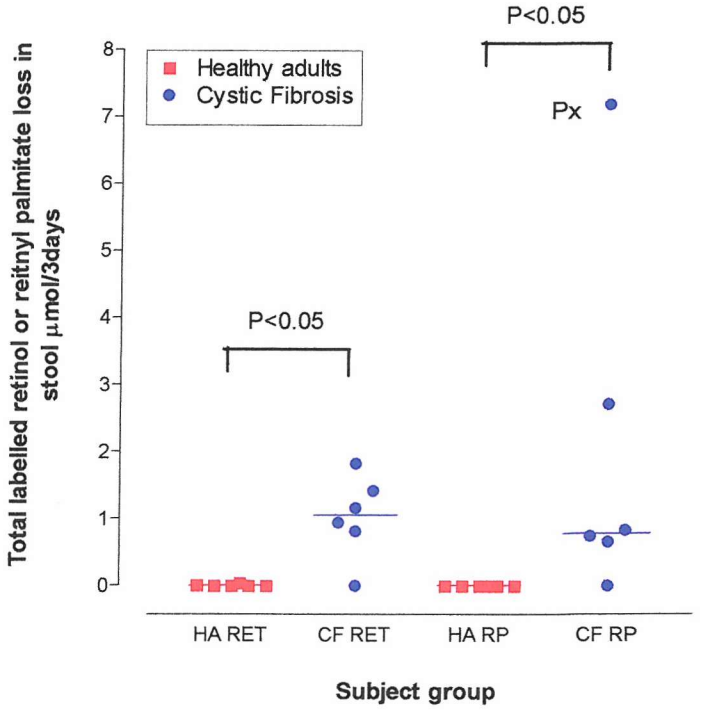


Figure 7.9:

Total labelled vitamin A losses in stool as retinol (RET) and retinyl palmitate (RP) in patients with CF and healthy adults (HA).



7.5.5 PLASMA EXCURSIONS OF VITAMIN A AND LIPID.

7.5.5.1 PLASMA RETINYL PALMITATE.

The patterns of appearance of retinyl palmitate in the circulation following the oral dose in both healthy adults and patients with CF are shown in figures 7.10 & 7.11 respectively. There was individual variability both within and between the groups. The healthy adults appear to follow a similar pattern, a peak rise at 2 hours followed by a decline to 24 hours, and then a plateau. Two patients with CF appear to have similar post-prandial profiles in the circulation to the healthy adults although four of the group of patients with CF have different profiles (maximum concentration reached at 10 hours) when compared to the healthy adults.

Postprandial excursions of plasma retinyl palmitate were not different between the two groups. There were no significant differences between the groups in concentrations achieved at each time point (Table 7.4). Median maximum retinyl palmitate concentration observed for the patients with CF was 0.4159μmol/L (0.163-0.708μmol/L) and for the group of healthy adults was 0.4550 μmol/L (0.230-0.990μmol/L) (figure 7.12). Median area under the retinyl palmitate time course curve at 10 hours for the healthy adults and the CF group was 2.255 (μmol/L)x10h (1.283-4.615 (μmol/L)x10h) and 1.543 (μmol/L)x10h (0.415-2.981 (μmol/L)x10h) respectively (figure 7.13). The time at which plasma retinyl palmitate appears in the circulation after an oral bolus dose significantly differs between the two groups ($P<0.05$) (figure 7.14). Median time taken to reach maximum concentration of retinyl palmitate (over the 10 hour study period) was 2 hours for healthy adults and 10 hours for patients with CF.

There was an altered pattern of tracer appearance within the circulating plasma retinyl palmitate pool between the two groups (figure 7.15). In healthy adults the greatest enrichment in the retinyl palmitate pool appeared at 2 hours (median 91.21%), where nearly all of the retinyl palmitate was labelled (comparable to that seen in the meal) enrichment remains elevated for 10-24 hours. In contrast in patients with CF less than half of the retinyl palmitate at 2 hours was label (median 39.63%) with the peak enrichment at 10h (median 81.32%).

Enrichments in the retinyl palmitate pool were statistically different between the two groups at 2 hours. In both groups all the tracer had appeared in the circulation by 24 hours post dose, there was no detectable label in the retinyl palmitate pool over the subsequent time points (7 – 28 days). Table 7.5 shows the changes in enrichment within the plasma retinyl palmitate pool over the study period.

7.5.5.2 PLASMA TAG.

Median baseline TAG concentrations for healthy adults and CF adults were 0.83mmol/L (0.62 - 1.00mmol/L) and 0.93mmol/L (0.78 - 1.31mmol/L) respectively. There were no significant differences in the area under the TAG concentration time course (Figure 7.16) and maximum concentration achieved in plasma over the 10-hour study period (figure 7.17). The time taken to reach maximum concentration in the plasma followed the same pattern in the CF patients as previously seen for retinyl palmitate (data not shown), in that 4 of the patients reached the maximum concentration achieved over the study period at 10 hours and 2 patients at 2 hours.

7.5.6 RELEASE OF RETINOL FROM THE LIVER.

By using stable isotope tracers we are able to assess the release of retinol from liver stores. If d_4-retinol appears in the circulation over the study period released from the liver, it has been derived for the d_4-retinyl palmitate in the test meal. Enrichment within the retinol pool is evident in both groups, by 5 hours post dose. Healthy adults have greater enrichments in the retinol pool than patients with CF. There was an altered pattern of tracer appearance within the circulating plasma retinol pool between the two groups (figure 7.18; table 7.6), In healthy adults enrichment at 5 hours was 19.29%, the greatest enrichment in the retinol pool appeared at 10 hours (median 29.42%), where nearly 30% of the retinol was labelled (from the test meal 10 hours previous). Enriched retinol was evident in the healthy adults at 7, 14 and 28 days post dose. In contrast in patients with CF only 1.97% of retinol at 5 hours was enriched and less than half of the retinol was enriched at 10 hours when compared to the healthy adults (median 13.35%). There was no detected enrichment in the retinol pool after 24 hours post dose in the patients with CF. Enrichments in the retinol pool

were statistically different between the two groups at 5, 10 and 24 hour, and 7 days post dose.

7.5.7 RELATIONSHIP BETWEEN LIPID AND VITAMIN A PROCESSING.

If the movement of lipid were associated with the movement of vitamin A then it would follow that there would be associations between; 1) losses of vitamin A and lipid in stool and 2) appearance of vitamin A and lipid in the circulation. In that patients with the greatest losses of lipid in stool would have the lowest postprandial excursions of lipid in plasma, and patients with the greatest losses of vitamin A in stool would have the lowest postprandial excursions of vitamin A in plasma

Data indicates that stool lipid losses are associated with stool vitamin A losses in patients with CF (Spearman R=0.886; P=0.019). However, there is no simple association between maximum concentration of TAG and retinyl palmitate achieved in the plasma of patients with CF (Spearman R= -0.314; P=0.544). The areas under the curve for retinyl palmitate and TAG in the circulation are also not associated in patients with CF (Spearman R = 0.371; P=0.468). There is however an association between the times in which both TAG and retinyl palmitate enter the circulation from the meal (Spearman R = 1.00; P=0.00). There was no simple relationship between the loss of vitamin A in stool and postprandial excursions of vitamin A in plasma.

If availability of vitamin A was a limiting factor associated with low retinol concentrations in patients with CF then it would follow that, patients with the lowest retinol concentrations would have the greatest losses of vitamin A in stool and patients with the lowest plasma retinol concentrations would have the lowest postprandial excursions of vitamin A in plasma. However data reveals that there are no associations between plasma retinol concentrations and the appearance of vitamin A in stool and plasma.

Figure 7.10:

Total retinyl palmitate concentrations (µmol/L) over time in 6 healthy adults.

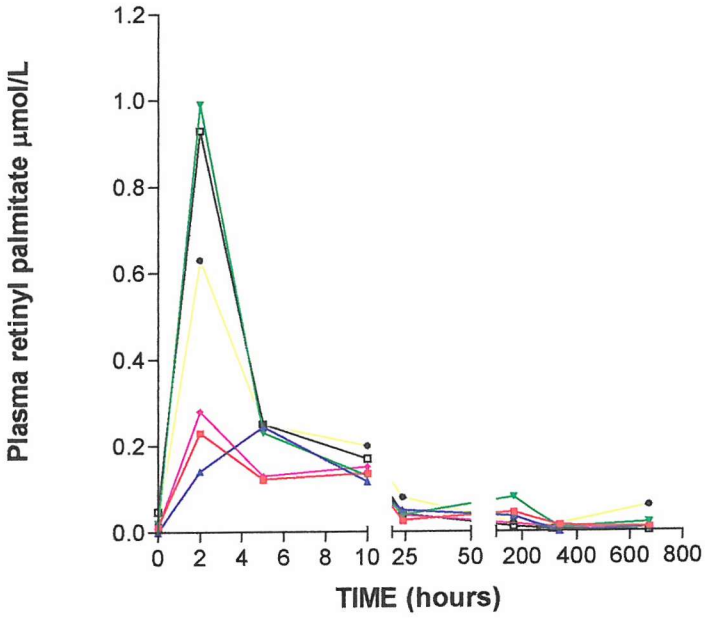


Figure 7.11:

Total retinyl palmitate concentrations (µmol/L) over time in 6 patients with CF.

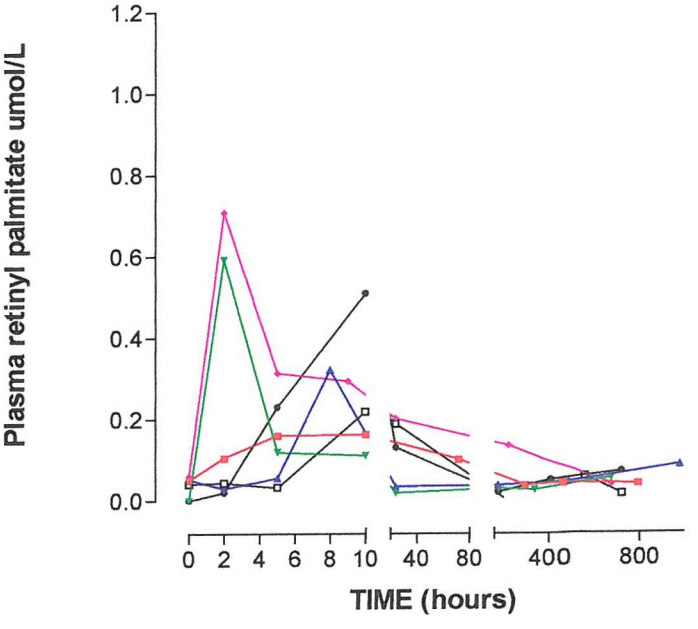


Table 7.4 :

Summary of the plasma retinyl palmitate concentrations for both groups (patients with CF and healthy adults (HA)) over the first 10 hours post dose. No significant differences between the groups at any of the time points.

	PLASMA RETINOL PALMITATE CONCENTRATIONS (μmol/L) (Median and range)			
	Base	**2 hour**	**5 hour**	**10 hour**
CF	0.04 (0.00-0.06)	0.07 (0.02-0.71)	0.14 (0.03-0.31)	0.26 (0.11-0.51)
HA	0.02 (0.00-0.05)	0.46 (0.14-0.99)	0.24 (0.12-0.25)	0.14 (0.12-0.20)

Figure 7.12:

Maximal concentration of retinyl palmitate in the plasma of patients with CF and healthy adults (HA). (Lines represent median value).

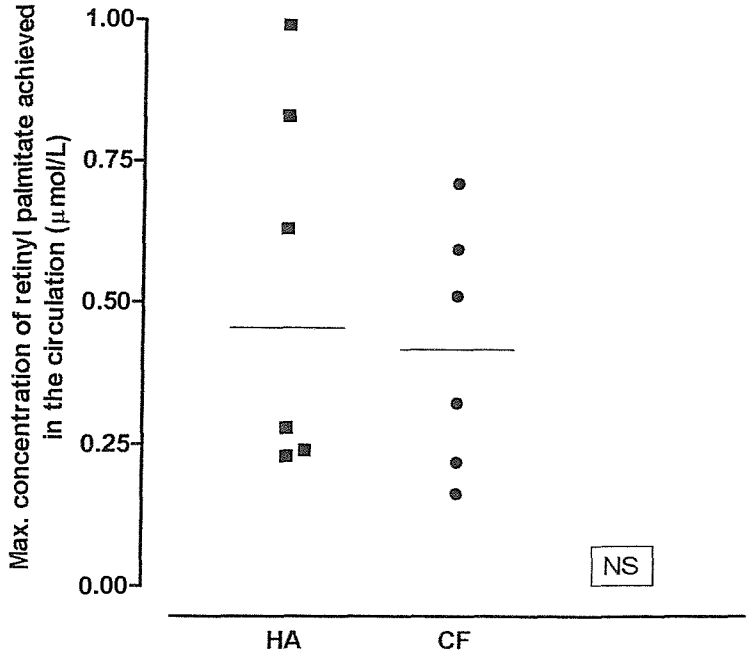


Figure 7.13:

Area under the concentration time cure for d_4-retinyl palmitate in the plasma of patients with CF and healthy adults (HA).

(Lines represent median value).

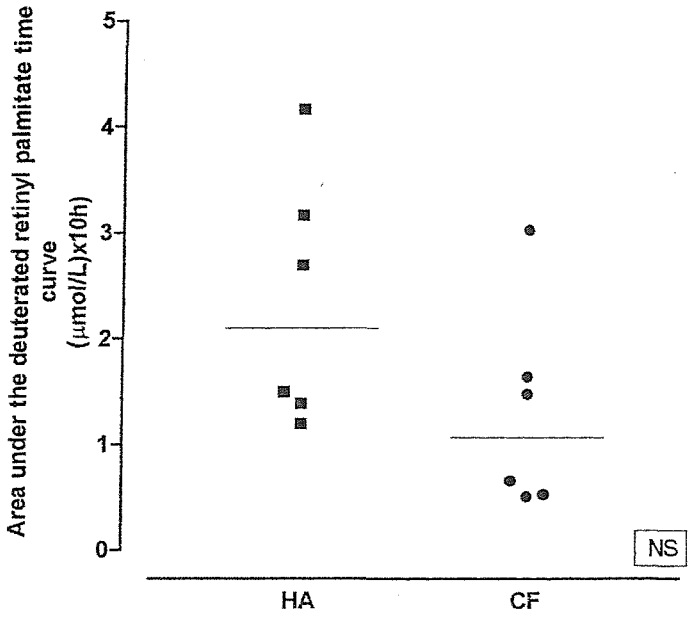


Figure 7.14:

Time to reach maximal concentration of retinyl palmitate in the plasma of patients with CF and healthy adults (HA). (Lines represent median values).

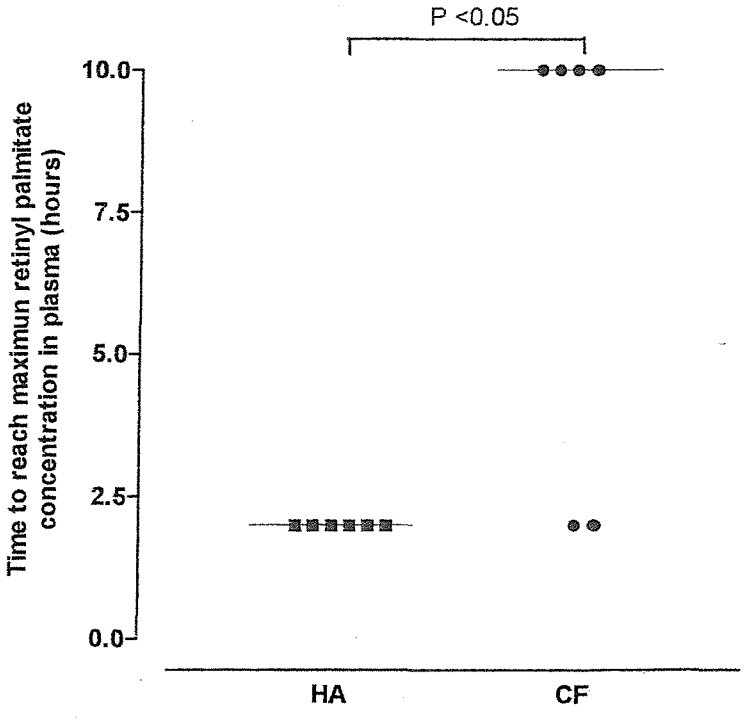


Figure 7.15:

Enrichment in the retinyl palmitate pool at each time point for both healthy adults (HA) and patients with CF.

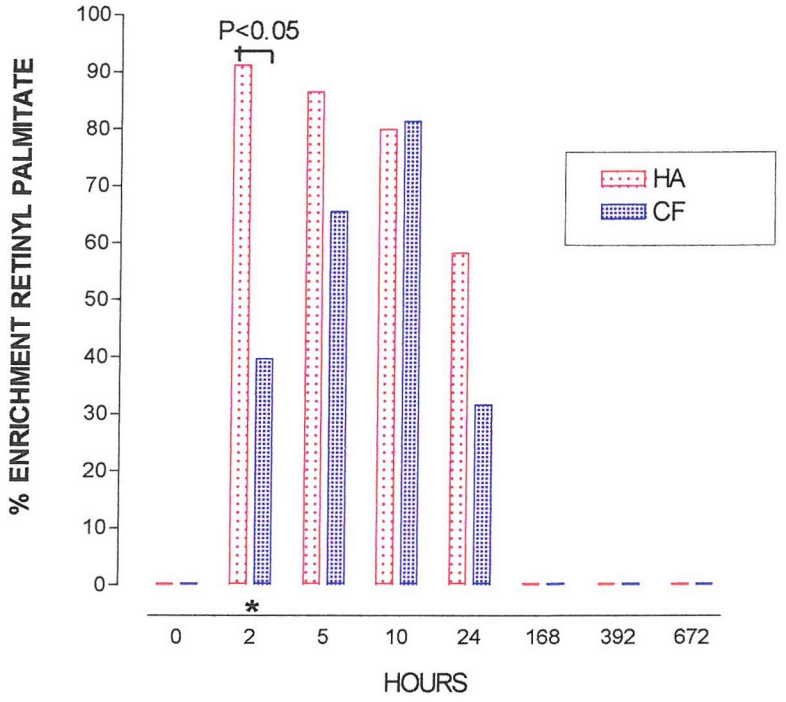


Table 7.5:

Summary of the enrichment in the retinyl palmitate pool for both groups; (healthy adults (HA) and patients with CF), over the first 7 days post dose.

Data presented as median and ranges. No enrichment detected after 7 hours.

	% ENRICHMENT OF RETINYL PALMITATE	
	CF	HA
BASE	0.00%	0.00%
2 HOURS	39.63% * 0.00 - 91.79%	91.21% 86.38 - 100%
5 HOURS	65.44% 46.69 - 83.91%	86.47% 79.34 - 100%
10 HOURS	81.32% 67.53 - 89.44%	79.96% 71.99 - 83.95%
24 HOURS	31.61% 0.00 - 62.81%	58.21% 42.47 - 60.26%
7 days	0% 0.00 - 0.00%	0% 0.00 - 14.45%

Mann Whitney U Significantly different from healthy individuals; *P < 0.05

Figure 7.16:

Area under the concentration time curve for TAG in the plasma of patients with CF and healthy adults (HA). (Lines represent median value).

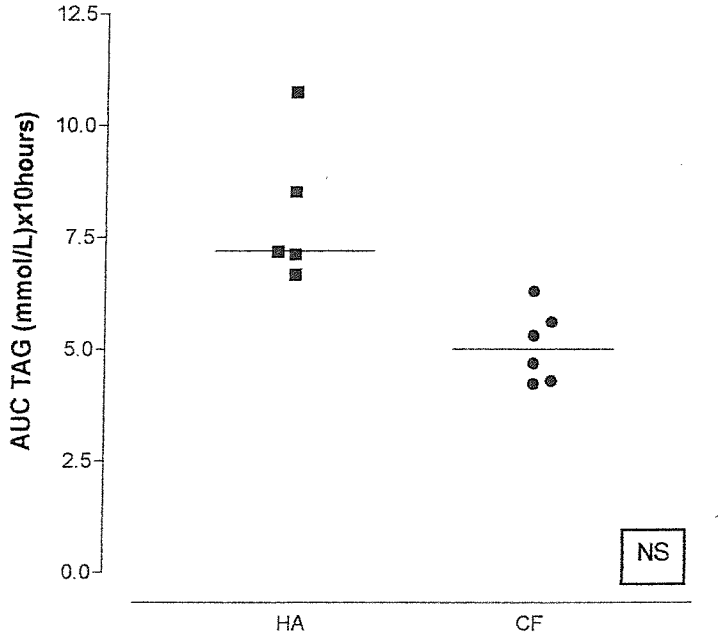


Figure 7.17:

Maximal concentration of TAG in the plasma of patients with CF and healthy adults (HA). (Lines represent median value).

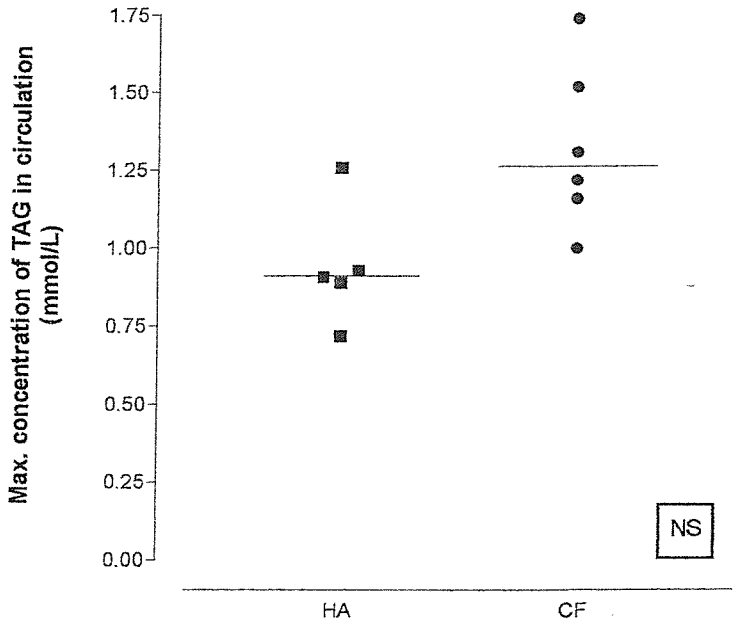


Figure 7.18:

Enrichment in the retinol pool at each time point for both healthy adults (HA) and patients with CF.

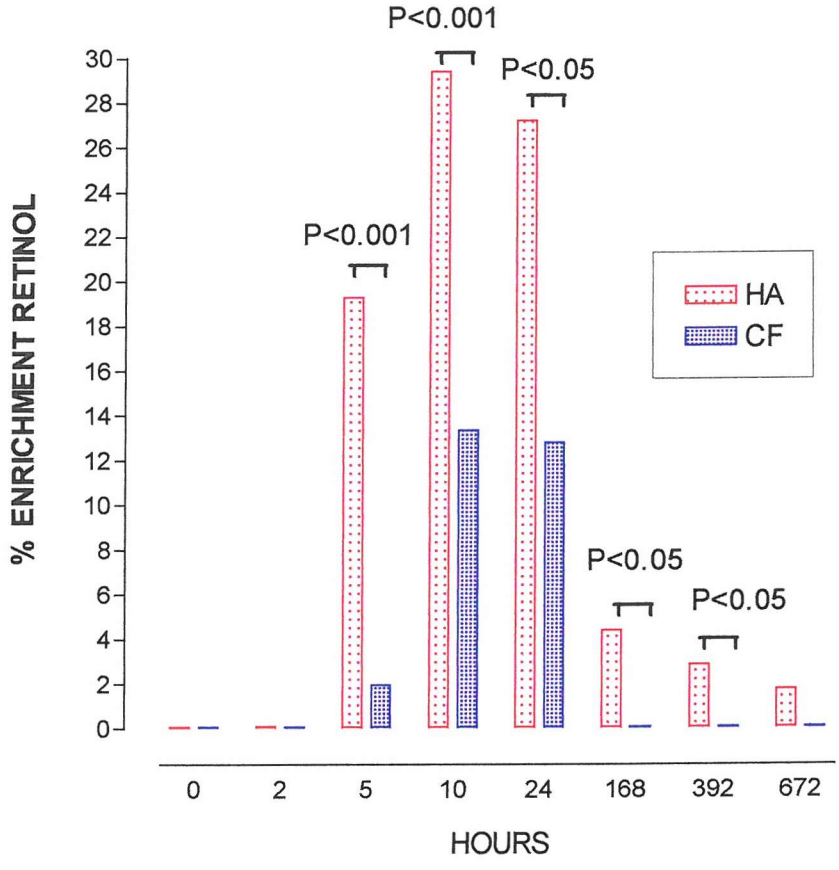


Table 7.6:

Summary of the enrichment in the retinol pool for both groups, (patients with CF and healthy adults (HA), over the 28 day study period.

Data presented as median and ranges.

	% ENRICHMENT OF RETINOL	
	CF	HA
BASE	0.00%	0.00%
2 HOURS	0.00% 0.00 – 0.00%	0.10% 0.00 – 4.30%
5 HOURS	1.97% * 0.00 - 26.53%	19.29% 11.56 – 26.68%
10 HOURS	13.35% * 5.80 – 34.50%	29.42% 24.81 – 39.80%
24 HOURS	12.79% ** 0.00 - 15.02%	27.21% 23.39 – 34.00%
7 DAYS	0.00% * 0.00 – 3.07%	4.40% 1.63 – 8.46%
14 DAYS	0.00% * 0.00 – 0.00%	2.87% 0.00 - 5.49%
28 DAYS	0.00% * 0.00 – 0.00%	1.76% 0.00 - 5.49%

Mann Whitney U Significantly different from healthy individuals
* $P < 0.05$
** $P < 0.01$

7.5.8 DISCUSSION.

The present study investigated availability of retinyl palmitate across the GI tract by characterising faecal losses in association with postprandial changes in plasma retinyl palmitate, following a test meal containing isotope. The hypothesis to be tested was that patients with CF, despite routine PERT, have disturbed GI handling of dietary lipid which is associated with impaired GI handling of vitamin A. In this case poor availability of vitamin A across the GI tract will contribute to decreased retinol concentrations in patients with CF.

Data presented in this chapter reiterates that patients with CF on supplements and PERT have low concentrations of plasma retinol compared to healthy adults. However in this study the difference was not significant between the two groups, which is probably related to the low study subject number. It is interesting to acknowledge that patients with CF were recruited to the study whilst "well", however four of the six subjects had elevated concentrations of CRP, indicative of an acute phase response.

When addressing total stool losses of vitamin A, data indicates that despite routine prescription of PERT and supplements patients with CF have significantly increased, but variable total faecal vitamin A losses. Losses were comparable to those reported previously from Southampton [Ahmed *et al* 1990]. Figure 7.19 illustrates total faecal losses from the previous literature alongside results presented in this chapter. Results from these two data sets were obtained from different patients groups. Ahmed and colleagues investigated total stool losses in children with CF, not routinely supplemented with vitamin A, and data reported in this chapter is from adult CF patients who are prophylactically supplemented. Both data sets report similar results however data from the current study reports a higher median value along with a greater range of losses, with only one CF subject having similar vitamin A losses to the healthy adults. This may reflect a more complicated disease process in adults compared to children with CF. It is interesting to note that the patient with the lowest stool vitamin A loss in the current data set had the greatest CRP concentration (120mg/L), and one of the lowest plasma retinol concentrations (0.87μmol/L). This patient was only classified as pancreatic insufficient and

commenced on PERT and vitamin A supplements 6 months prior to the study. For the purpose of this discussion this patient will be termed patient one, and will be considered throughout this section.

The current literature is conflicting as to the extent of the relationship between losses of vitamin A and lipid in stool. One study reports faecal losses of vitamin A unrelated to the degree of fat in the stool [Ahmed *et al* 1990], however the work of Halford [1990], found a relationship between the loss of lipid and vitamin A. Data presented in this chapter found that faecal lipid losses were associated with total vitamin A losses, in that those patients with the greatest lipid loss on habitual PERT also exhibited the greatest loss of vitamin A. This data therefore supports the hypothesis that patients with CF have disturbed GI handling of dietary lipid, which is associated with impaired GI handling of vitamin A.

The data discussed up until this point was obtained from traditional gross balance techniques, which reflect total losses of vitamin A and in themselves have practical limitations and constraints. These include analytical limitations in the ability to; i) differentiate between retinol and retinyl esters in faeces, and ii) differentiate between exogenous and endogenous vitamin A. Methodological issues addressed as part of this thesis has allowed advances in both the extraction and isolation techniques. The new method has made it possible to improve both precision and fractionation of retinol metabolites. To overcome gross balance limitations, isotopic tracer techniques were employed in this study. The inclusion of isotopic tracers make it possible to; a) differentiate between dietary residue that has not been digested or absorbed from endogenous secretions, and b) follow the partitioning of absorbed retinyl palmitate in the circulation.

Data obtained from the isotope tracer studies re-confirms the gross balance data, in that patients with CF have increased but variable losses of labelled vitamin A in stool compared to healthy adults (figure 7.20). The variability of these results is probably related to the heterogeneity of the disease CF and the patients studied. In every subject, losses of labelled vitamin A were less than those of total vitamin A. This could be attributed to losses of total vitamin A

reflecting both the unlabelled and labelled vitamin A from the meal along with other endogenous losses, whereas labelled vitamin A losses only reflect the loss of the administered label. One of the benefits of isotopic tracers is the ability to differentiate between maldigested and malabsorbed material (figure 7.21). Losses of labelled retinyl palmitate in stool will reflect dietary material that has been maldigested, and losses of labelled retinol will reflect dietary material that has been digested but has been malabsorbed. Losses of both labelled retinol and retinyl palmitate were greater in the stool of CF patients compared to healthy adults. Analysis of results suggests that both maldigestion and malabsorption of ingested retinyl palmitate occurs in patients with CF (figure 7.9 results). However the pancreatectomised patient exhibits mainly maldigestion of retinyl palmitate. This probably reflects a lack of both bile acids and enzymes that are required for adequate digestion and which are normally produced by the pancreas.

When losses of vitamin A are considered as a proportion of intake the greatest loss was seen in the pancreatectomised patient which was equivalent to ~30% of the intake. When accounting for this loss ~70% of the intake is still available to the body. In the other 5 patients losses were <20% administered dose, allowing 80% of the dose to be available to the body. Previous unpublished data from a gross balance study in CF children found that despite elevated faecal retinol losses, higher intakes resulting from supplementation give rise to on average a net availability over 3 times greater than that seen in controls [Halford, 1993b]. Figure 7.22 illustrates the unpublished data from Halford, [1993b], alongside the data reported in this chapter. Halford estimated daily vitamin A intake in children on supplements from a 5-day food diary. For comparison of data it has been assumed that habitual intake of vitamin A is ~1mg/d, over and above that obtained from the documented use supplements (median 1.2mg/d). The data illustrates that patients with CF have 2-3 times greater availability of vitamin A when compared to control subjects. When considering these observations in light of the greatest intake of vitamin A from supplements recorded in the audit (3.6mg/d; chapter 4), availability of vitamin A could be over 4 times greater than that of healthy adults. This suggests that although losses of vitamin A in CF are greater than healthy adults, increased

intake from supplements is more than adequate to overcome modest losses. Availability across the GI tract is not the primary factor associated with low retinol concentrations. This data has important implications for the clinical management of patients with CF, as intake from prophylactically prescribed supplements greatly exceeds faecal losses such that net intake is higher than that usually consumed by healthy adults. These observations highlight the potential risk of toxicity associated with the excess prescription of vitamin A.

Availability can also be reflected in the postprandial excursions of retinyl palmitate following absorption. Although overall excursions appear lower in patients with CF, the area under the curve and maximum concentrations achieved are not significantly different between the two groups (figures 7.11 &7.12 results). These observations imply that whilst retinyl palmitate handling within the GI tract may be altered, net availability is not limited by maldigestion or malabsorption in this group of CF patients on habitual PERT. However the time in which retinyl palmitate enters the circulation appears to differ between the subject groups. Delayed appearance of retinyl palmitate in four of the six patients with CF may reflect altered GI processing of vitamin A. Interestingly subject one, had similar postprandial excursions and time course for appearance of retinyl palmitate to the healthy adults. Use of the label allows us to better assess appearance in the circulation of retinyl palmitate from the diet. Similar to total retinyl palmitate results, the tracer also appears in the circulation later in the patients with CF. At 2 hours post dose the healthy adults exhibit enrichment in the circulation similar to the amount ingested in the meal (~90%), however median enrichment in the CF patients was much lower (~40%). The greatest enrichment observed for the CF patients (10 hours post dose) was ~10% less than the greatest enrichment seen in the healthy adults (90% at 2 hours). Figure 7.23 illustrates that peak enrichment is similar to that observed for the healthy adults and there is no difference between the two groups. Delayed appearance of tracer in the patients with CF may reflect an alteration in the GI processing of vitamin A or delayed transit time. The use of PERT ingested before the meal in CF may in some way influence the GI processing, in that the enteric-coated capsules may require longer to digest the ingested material within the GI tract. This could be associated with the delayed

appearance of retinyl palmitate in the circulation. It should however be noted that although as a group appearance of tracer is delayed and enrichment is around 10% less than that observed in healthy adults, there is variability in the results obtained (table 7.5 results). This variability can be accounted for by the pancreatectomised patient, and patient one (table 7.7). For example patient one had no loss of vitamin A in stool and has similar levels of enrichment and a similar time course in the circulation as the healthy adults. In contrast the pancreatectomised patient lost ~30% of the administered dose in stool, and achieved a corresponding ~70% enrichment of retinyl palmitate in plasma 10 hours post dose. The remaining four CF patients had a peak enrichment of ~80% at 10 hours compared to a peak enrichment of ~90% at 2 hours in the healthy adults.

Table 7.7:

Table analysing the subject data on enrichment in the retinyl palmitate pool post dose. (Px = Pancreatectomised patient)

	Retinyl palmitate enrichment post dose %			
	2 hours	**5 hours**	**10 hours**	**24 hours**
CF Subject 1	91.79%	83.91%	81.32%	0%
CF Px Patient	0%	59.72%	67.53%	0%
CF (4 patients median results)	39.63%	58.9%	81.64%	37.35%
HA (6 subjects median results)	91.21%	86.47%	79.96%	58.21%

Finally data presented within this chapter investigated enrichment in the plasma retinol pool. Enrichment was detected in the retinol pool 5 hours post dose in both groups. This could be interpreted as the liver releasing retinol on a last in first out basis, in that some of the retinyl palmitate ingested within the breakfast is mobilised from the liver by 5 hours post ingestion. Patients with CF have significantly less enriched retinol released from the liver over the period of the study. At 5 hours post dose healthy adults have 20 time greater enrichment of retinol and at 10 and 24 hours post dose they have 2 times greater enrichment

of retinol. Low enrichment in the retinol pool may reflect a large liver store of vitamin A, in that CF patients have a greater store of vitamin A and therefore the label is diluted in a large pool. For instance if the label enters a large unlabelled vitamin A storage pool in the liver, the label will be more diluted (less percentage enrichment) than if it enters a small unlabelled storage liver pool (greater percentage enrichment). These increased stores may be a consequence of both increased availability from the use of supplements, which exceed the demands of the vitamin, and / or it may reflect a constraint to effectively mobilise retinol from the liver. A constraint in mobilisation may be a consequence of a shift in priorities in the liver during the acute phase response or it could reflect impaired metabolic machinery in the liver of patients with CF.

Whilst addressing the variability in the results of enrichment in the retinol pool, it is again interesting to consider enrichment of both subject one and the pancreatectomised patient (table 7.8). Firstly enrichment in the retinol pool of the pancreatectomised patient at each time point is lower than the healthy adults. However subject one at each time point has a similar enrichment in the retinol pool as the healthy adults. As patient one only began routine prescription of vitamin A 6 months prior to the study, this data could be interpreted as patient one having similar liver stores to the healthy adults. At the time of study subject one had a CRP concentration of 120mg/L and a plasma retinol concentration of 0.87μmol/L indicative of an acute phase response. Previous hypotheses have attributed low retinol concentrations during infection to a constraint in mobilisation. However data from this one subject suggests that retinol, from the previously ingested diet is being released from the liver during the infective episode, so the low plasma retinol concentration may reflect increased demands for retinol by cells and tissues and / or increased losses during infection.

Table 7.8:

Table analysing the subject data on enrichment in the retinol pool post dose. (Px = Pancreatectomised patient)

	5 hours	10 hours	24 hours	CRP mg/L	Retinol μmol/L
CF Subject 1	26.53%	34.50%	25.32%	120	0.87
CF Px Patient	3.93%	13.70%	2.09%	7.4	1.12
CF (4 patients median results)	0%	12.6%	13.2%	8.2	1.42
HA (6 subjects median results)	19.29%	29.43%	27.21%	1.0	1.50

7.5.9 SUMMARY

In summary, patients with CF have disturbed GI handling of dietary lipid, which is associated with impaired GI handling of vitamin A. Although faecal vitamin A losses are greater in patients with CF, availability of vitamin A across the enterocyte is not limiting. The additional increase in vitamin A intake necessary to compensate for these modest faecal losses is considerably less than that routinely prescribed in clinical practice, and raises concerns over the possibility of excessively high intakes. Further attention needs to be directed towards examining the subsequent metabolism of retinol and in particular, the extent to which the liver is able to mobilise retinol to the peripheral tissues. These results therefore do not support the view that retinyl palmitate availability is limited by impaired GI function in CF subjects and is unlikely to be the primary cause of the low plasma retinol concentrations often seen in this group of patients.

Following these results a modified conceptual framework is presented in figure 7.24. The framework hypotheses that stores of vitamin A in the liver of CF patients are greater than healthy adults due to the increased intake from supplements. Low retinol concentrations could therefore be a consequence of a number of factors which include, increased demands for retinol by cells and

tissues, increased losses of vitamin A, impaired metabolic machinery within the liver which effects mobilisation, or the effect of the acute phase response and chronic inflammation on effective mobilisation from the liver.

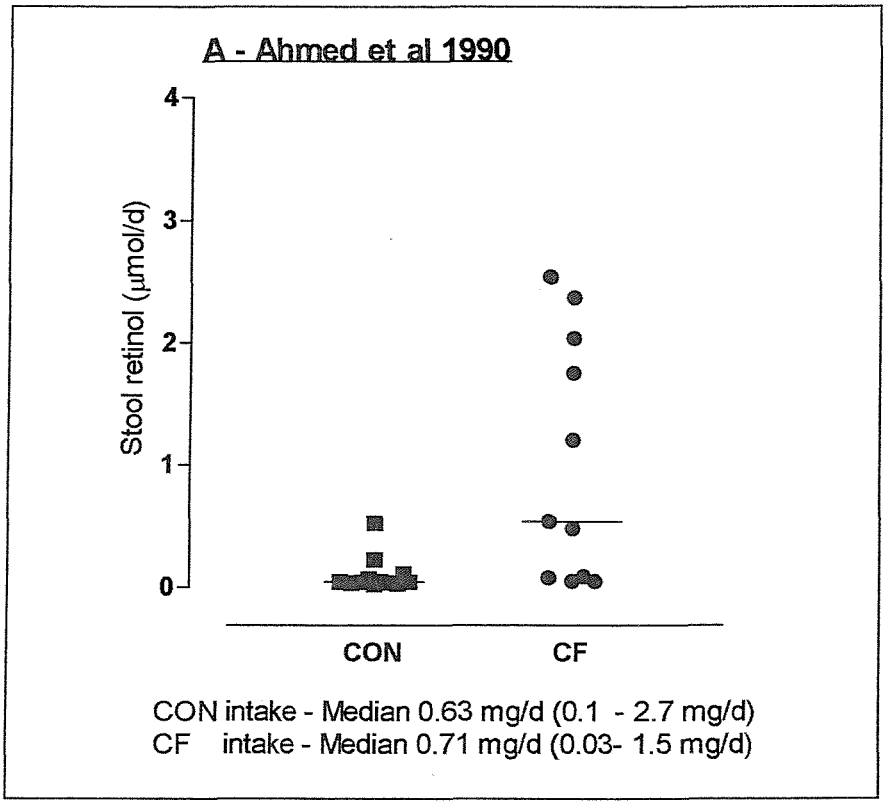
- Patients with CF have increased losses of vitamin A in stool (approximately 10% of intake).
- Losses of vitamin A are associated with losses of lipid.
- Patients with CF suffer from both maldigestion and malabsorption of vitamin A.
- Due to the increased intake of vitamin A by the use of supplements patients with CF have an increased availability of vitamin A from the GI tract.
- Retinyl palmitate appears in the circulation later in patients with CF compared to HA.
- Enrichment in the retinol pool is lower in CF patients compared to HA, this may reflect a greater hepatic store in patients with CF.
- Increased availability of vitamin A across the GI tract along with the potential of an increased hepatic stores raises concerns over current CF management.

Figure 7.19

Total stool losses of vitamn A (μmol/L), previous and current data.

A : Ahmed et al 1993. Total stool retinol losses. (20 CF children)

B: Data presented in thesis. Total vitamin A losses. (6 CF adults)



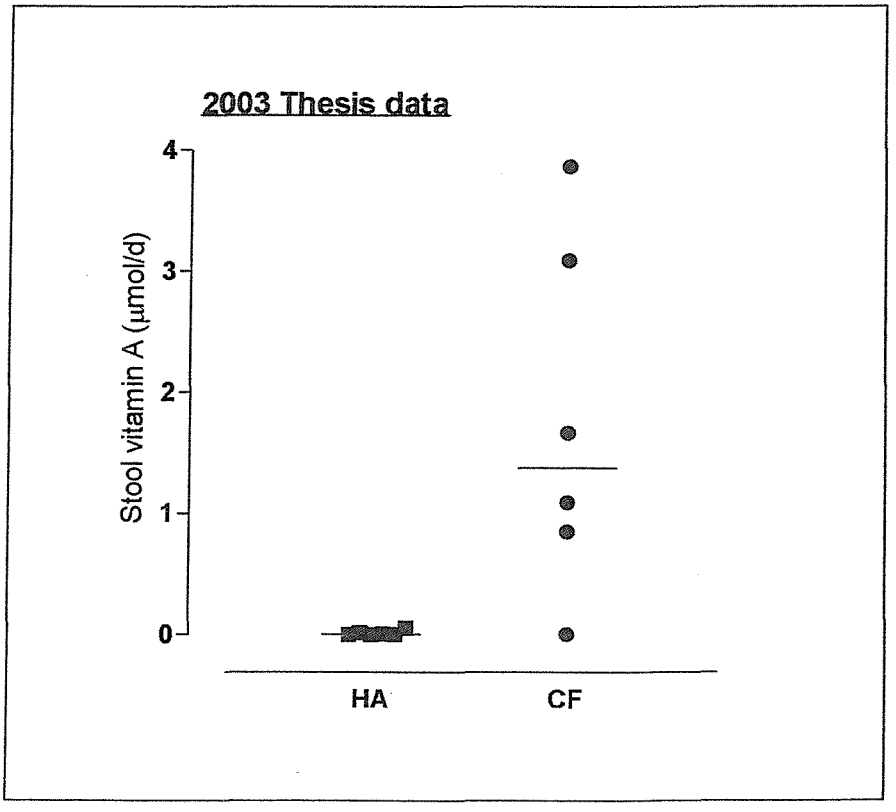


Figure 7.20:

Total labelled vitamin A losses per day (μmol/L) in both patients with CF and healthy adults (HA). (Px = Pancreatectomised patient)

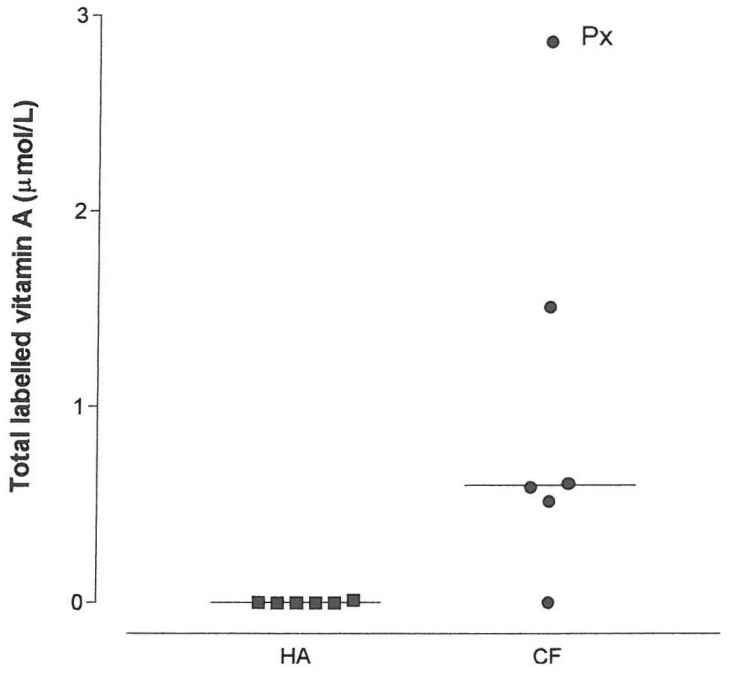


Figure 7.21:

Isotopic tracer methodology to differentiate between maldigestion and malabsorption of vitamin A.

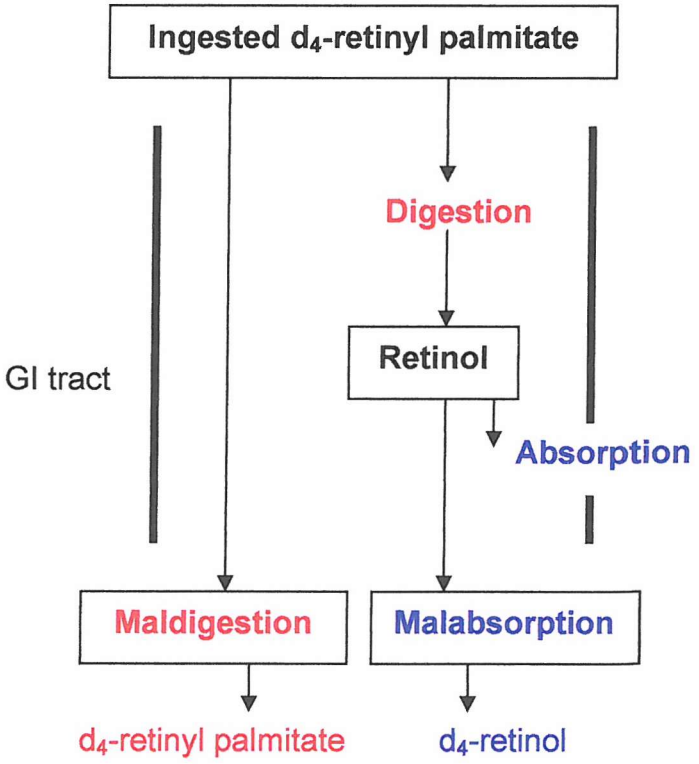
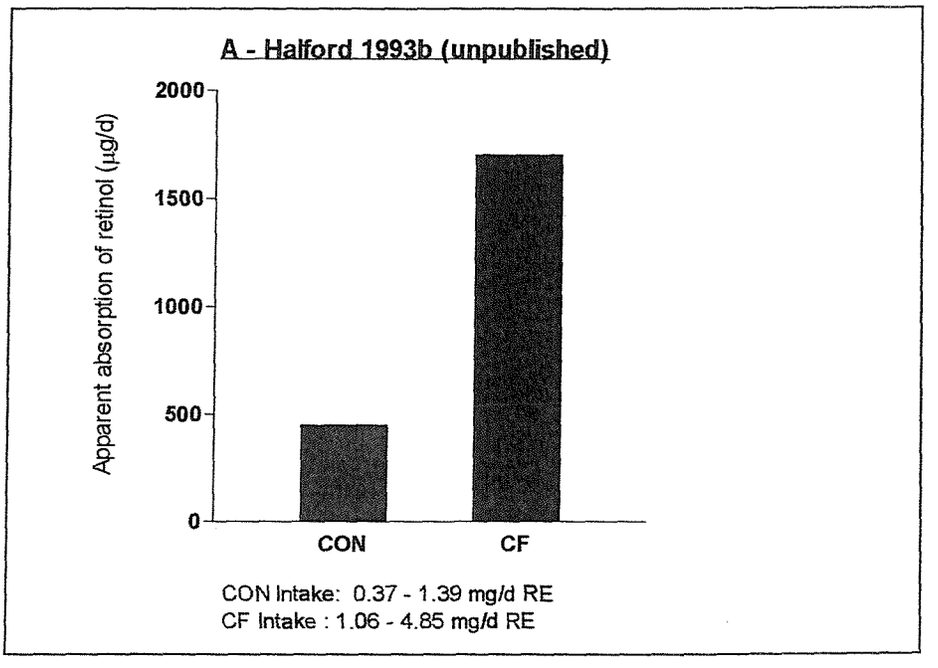


Figure 7.22

Availability of ingested vitamin A from the GI tract.

A: Halford et al 1993b Controls (CON) and CF.

B: Data from thesis Healthy adults (HA) and CF adults.



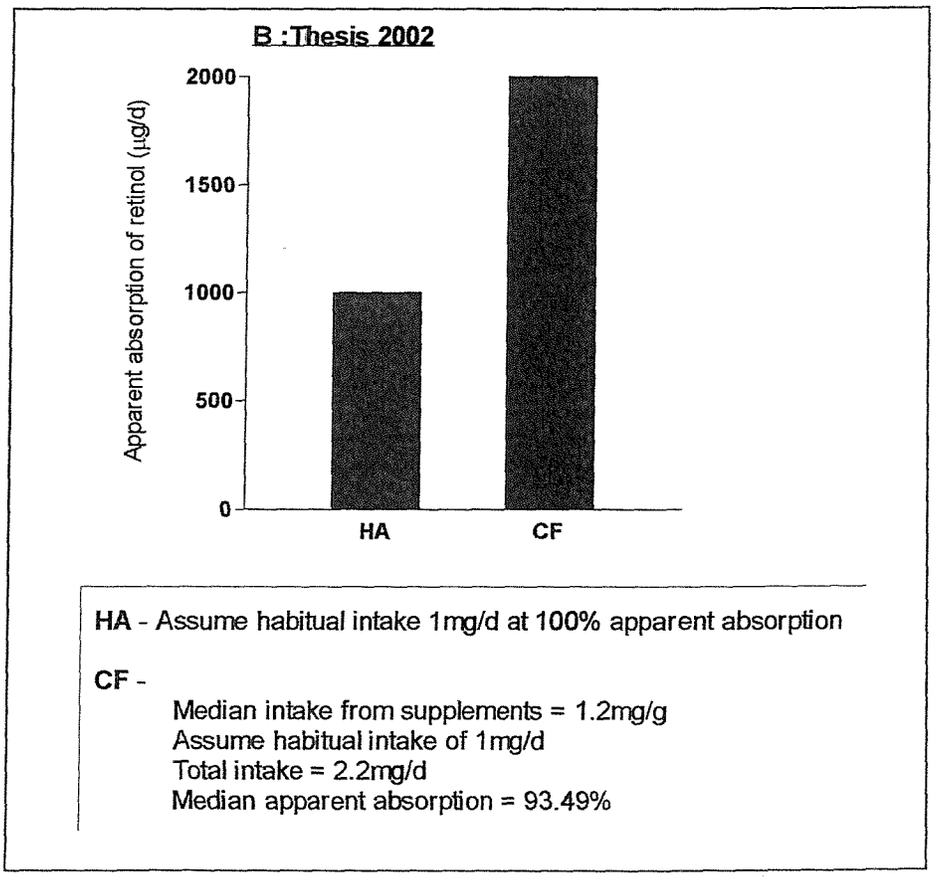


Figure 7.23

Graph illustrating peak enrichment in the retinyl palmitate pool for both healthy adults and paitients with CF.

(Lines represent median values).

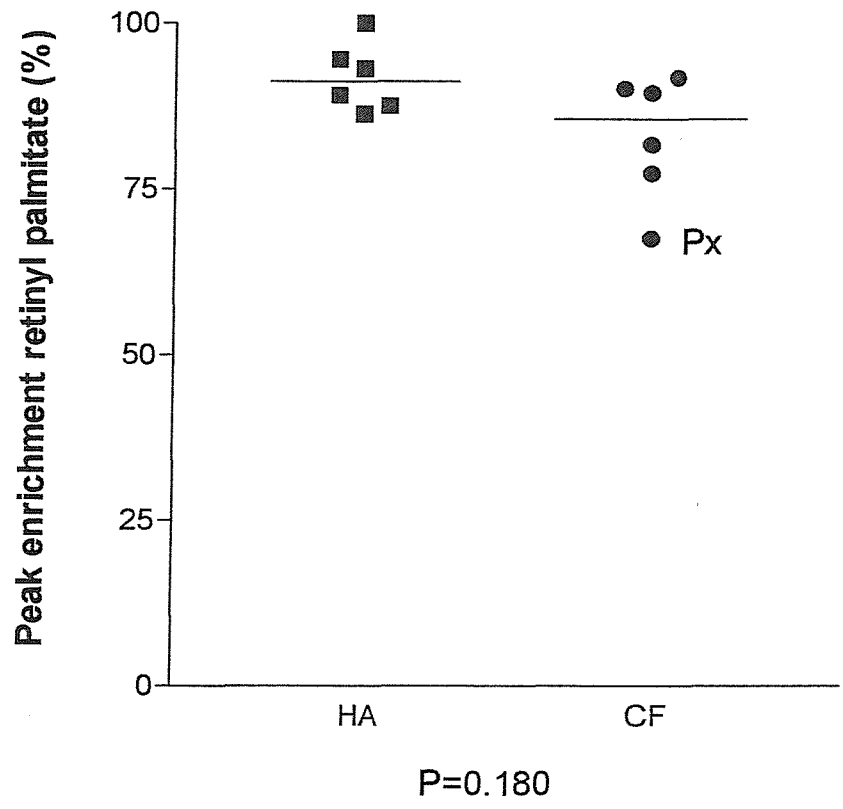
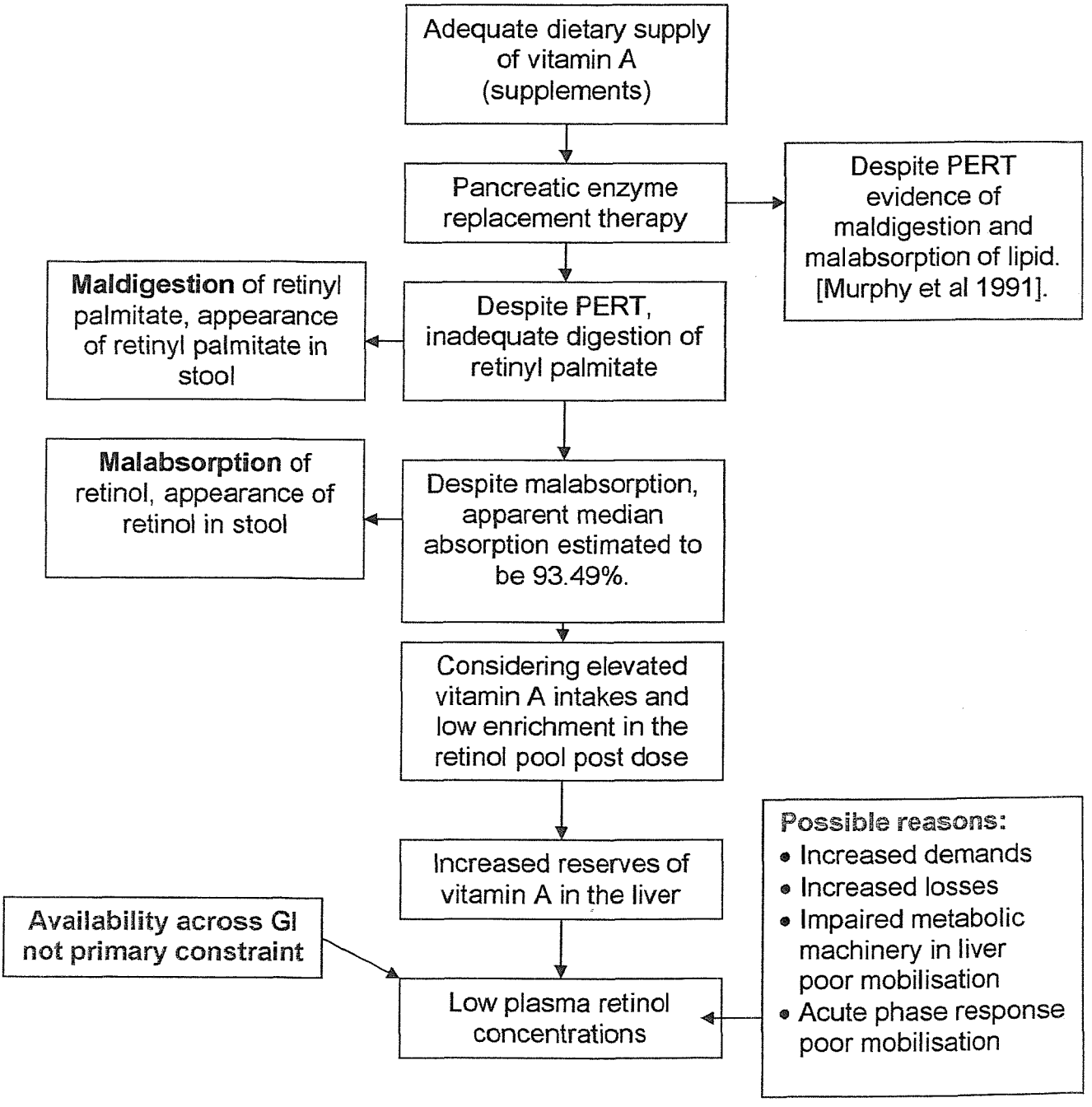


Figure 7.24

Modified conceptual framework illustrating how low retinol concentrations may potentially arise in patients with CF who are routinely supplemented with both vitamin A and PERT.



CHAPTER 8

POSTPRANDIAL EXCURSIONS OF VITAMIN A AND LIPID IN HEALTHY ADULTS AND PATIENTS WITH CYSTIC FIBROSIS.

8.1 INTRODUCTION

From the data presented in chapter 7 it would appear that availability of vitamin A across the GI tract is not the primary limitation associated with low circulating concentrations of retinol. Results indicated that although losses of vitamin A in stool were increased in patients with CF, there were no differences in the maximum concentration of retinyl palmitate achieved in the circulation or area under the retinyl palmitate concentration time curve however, it would seem that retinyl palmitate appears later in the circulation when compared to controls. Data revealed that stool lipid losses were associated with stool vitamin A losses, but postprandial excursions of vitamin A were not related to that of lipid. Measures of total plasma lipid in chapter 7 were determined by enzymatic assay. Total lipid in this instance would reflect both lipid that was absorbed from the meal (within the CM), and lipid in VLDL released from the liver. The use of an isotopically labelled lipid within the meal, increased sampling time points, and increased subjects numbers, will enable the handling of exogenous lipid and vitamin A to be better investigated. Using both labelled lipid and vitamin A will allow the relationship between the postprandial excursions of vitamin A and lipid in patients with CF to be assessed. There is no direct evidence of the relationship between the postprandial handling of vitamin A and lipid in patients with CF. The study presented within this chapter investigates the postprandial excursions of both labelled vitamin A and lipid, in a greater number of CF patients with increased sampling time points.

8.2 AIMS

The aims of the present study were firstly to investigate postprandial excursions of labelled retinyl palmitate and labelled lipid in healthy adults and patients with CF. The second aim was to investigate if differences in retinol concentrations can be attributed to differences in postprandial responses between the two groups.

Aims to be addressed include to:

1) Determine postprandial excursions of isotopically labelled ^{13}C Tripalmitin (TAG) and d_4-retinyl palmitate (vitamin A) in the circulation of healthy adults and patients with CF, following a test meal.
2) Determine the extent to which handling of vitamin A is associated with handling of lipid.
3) Determine if results obtained in chapter 7 are repeatable in a larger number of subjects.
4) Determine if availability of exogenous retinyl palmitate (measured as appearance in the circulation) is related to an individual's plasma retinol.
5) Indirectly investigating liver stores of vitamin A, by determining enrichment in the retinol pool and change in plasma retinol concentrations following an oral bolus dose of vitamin A.

8.3 HYPOTHESES

Based on current literature, patients with CF will have lower postprandial excursions of both lipid and vitamin A compared to healthy adults and there will be an association between the postprandial TAG and retinyl palmitate responses. Postprandial excursions of vitamin A in patients with CF will be associated with the patient's plasma retinol concentration.

8.4 METHODS

Six healthy adults, (3F;3M), median age 25 years (23-28 years) participated in the study. The healthy adults were either employees or students at the University of Southampton. Twelve patients with CF were recruited to the study from the CF clinic at Southampton General Hospital. These patients comprised of 7 males and 5 females, median age 24 years (18-29 years). Inclusion criteria of CF patients included those that were compliant to supplement regimens, exhibited no dietary constraints and had low plasma retinol concentrations at the time of the study. Informed consent was obtained from each of the subjects after the nature of the study had been explained. All subjects were in good health prior to and during the course of the study. None of the subjects took vitamin supplements on the day of the study. The study had the approval of the

Southampton Hospitals and South West Hampshire Health Authority Ethical Committee.

On the study day, following an overnight fast, subjects attended the Wellcome trust Clinical research facility at Southampton General Hospital. All methods used within this chapter are described in chapter 3 of this thesis. Initial baseline venous blood samples were collected, for measurements of CRP, plasma retinol, retinyl palmitate, lipid and RBP concentrations. All subjects received two separate emulsions containing isotope along with a test breakfast (time zero). The two emulsions consisted of; 1) an oral bolus dose of [10,19,19,19] ^{2}H-retinyl palmitate (300μg/kg body weight), and 2) an oral bolus dose of [1,1,1-^{13}C] tripalmitin (10mg/kg body weight). Post dose bloods were drawn for analysis of retinol, retinyl palmitate and lipid at 1, 2, 3, 5, 6, and 8 hours post dose. All subjects only drank water between blood samples. After the 5-hour sample subjects received a test lunch (low in vitamin A). Vitamin A as retinol and retinyl palmitate was determined by HPLC. [$^{2}H_4$] retinol was determined by GCMS following fractionation by HPLC and derivitisation. Plasma lipid was measured by GC-C-IRMS and serum RBP concentrations were determined by nephelometry. Plasma CRP concentrations were measured by the routine Chemical Pathology Laboratory (SUHT).

8.5 RESULTS

8.5.1 SUBJECT CHARACTERISTICS

The study was carried out in a group of 18 adults of similar ages, 6 healthy adults (median age 25 years; range 23-28y) and 12 patients with CF (median age 24 years; range 18-29y). Subject characteristics are shown in table 8.1. Subjects were recruited whilst "well". All healthy adults had CRP concentrations less than 6mg/L however 5 of the 10 patients with CF (2 missing data points) had CRP concentrations >6mg/L. Patients with CF had significantly lower BMI (median 20.80kg/m^2) and higher CRP concentrations (10.98mg/L), when compared to healthy adults (BMI 25.00kg/m^2, CRP 1.00mg/L).

8.5.2 PLASMA RETINOL AND RBP CONCENTRATIONS

Patients with CF had significantly lower median baseline plasma retinol (figure 8.1) (0.94μmol/L; P=0.003) and RBP concentrations (figure 8.2) (0.9μmol/L; P=0.001) when compared to healthy adults (HA) (retinol 1.52μmol/L; RBP 1.45μmol/L). Only 1 of the 6 healthy adults (16.7%) had plasma retinol and RBP concentrations that fell below the reference ranges, compared to 11 of the 12 CF patients (92%) (Table 8.2). Plasma retinol concentrations were significantly lower at each time point in the patients with CF when compared to healthy adults (table 8.3), but an individual's retinol concentration did not significantly alter over the 8-hour study period (P>0.05).

Plasma retinol and RBP concentrations were significantly associated (figure 8.3) (Spearman R = 0.913; P=0.000) and ratios of retinol: RBP did not differ between the two groups (P=0.385) (median (range); CF 1.03 (0.80-1.27); HA 1.09 (0.87-1.42)). Interestingly, the majority of subjects (5 HA, 9 CF) had ratios greater than 1, reflecting more retinol than RBP in their circulation. Four patients with CF had ratios less than 1 (0.87, 0.86, 0.82, 0.80), reflecting more RBP than retinol in the circulation; these patients did not have elevated CRP concentrations.

Whole group plasma retinol concentrations were correlated with CRP concentrations (Spearman R= -0.664; P=0.005), in that those patients with the lowest plasma retinol had the highest CRP (figure 8.4). All the healthy adults and 5 of the patients with CF (2 missing data points) had CRP concentrations less than 6mg/L indicating no presence of infection. However 5 patients with CF had elevated concentrations of CRP, these patients had significantly lower plasma retinol (P=0.037) and serum RBP concentrations (P=0.005).

8.5.2.1. RELATIVE DOSE RESPONSE TEST.

The relative dose response test has been proposed to be an indirect indicator of liver stores. The test is based on the findings that vitamin A administration to a person with depleted stores results in an immediate release of retinol-RBP into the circulation, with a maximum increase in plasma retinol after 5 hours. When liver stores are adequate no increase in retinol is observed, and an increase in

excess of 20% of baseline is considered a positive result that indicates that hepatic vitamin A reserves are not adequate. Results indicate (figure 8.5; table 8.4) that at 5 hours post administration of vitamin A; none of the patients with CF have an increase in plasma retinol concentrations greater than 20% of baseline. In comparison two of the healthy adults had a percentage increase over baseline concentrations greater than 20%. Median percentage increase in plasma retinol concentrations over baseline was 6% (-12% - 19%) for patients with CF and 6% (-8% – 24%) for the healthy adults. There was no significant difference between the two groups (P=0.713). Figure 8.6 illustrates the collective results (CF n=18; HA n=12), from the relative dose response from patients studied in both this chapter and chapter 7.

Table 8.1:

Characteristics of the study populations, 6 healthy adults and 12 patients with CF. Data presented as median and ranges.

	Healthy adults n=6	**Cystic Fibrosis Adults n=12**
Age (years)	25 (23 – 28)	24 (18 – 29)
BMI (kg/m2)	25.00 (21.30 – 31.00)	20.80** (17.06 – 27.66)
Weight (kg)	70.30 (59.80 – 97.80)	54.34 (45.90 - 72.10)
Height (m)	1.73 (1.58 - 1.80)	1.67 (1.37 - 1.82)
CRP (mg/L) Ref range 0-6mg/L	All < 2mg/L	11* (1 – 139)
Dose Retinyl Palmitate (mg)	21.10 (17.94 - 29.34)	16.08** (6.10 - 21.63)
Dose Lipid (Tripalmitin) (mg)	755 (610 - 978)	558** (458 – 722)
RBP (µmol/L) Ref range 1.43-2.86µmol/L	1.45 (1.19 - 1.86)	0.88* (0.52 - 1.48)
Baseline retinol (µmol/L) Ref range 1.4-3.2µmol/L	1.53 (1.29 - 2.37)	0.94** (0.47 - 1.45)

Mann Whitney U Significantly different from HA; *P < 0.05;** P < 0.01

Table 8.2

Table illustrating the prevalence of low retinol and RBP concentrations in the group of patients with CF (n=12) and the group of healthy adults (HA; n=6).

	PREVALENCE			
	HA		CF	
	No. Patients	%	No. Patients	%
Plasma retinol below 95% CI for DoH (<1.4μmol/L)	1/6	17%	11/12	92%
Serum RBP concentrations below the reference range (<1.42μmol/L)	1/6	17%	11/12	92%
Plasma retinol concentrations that fall within the 95% CI for DoH (1.4-3.2μmol/L)	5/6	83%	1/12	8%
Serum RBP concentrations that fall within the reference range (1.42-2.86μmol/L)	5/6	83%	1/12	8%
CRP concentrations <6mg/L	6/6	100%	5/10	50%
CRP concentrations >6mg/L	0/6	0%	5/10	50%

Table 8.3:

Plasma retinol concentrations of the 6 healthy adults and 12 patients with CF over the period of the study (8 hours).

Data presented as median and ranges.

	Plasma retinol concentration μmol/L	
Time point	**CF adult**	**Healthy adult**
Base	0.94 ** (0.47-1.45)	1.53 (1.29-2.37)
1 hour	1.00 * (0.5-1.42)	1.72 (1.29-2.23)
2 hour	0.89 ** (0.46-1.43)	1.79 (1.34-2.27)
3 hour	0.96 ** (0.47-1.39)	1.73 (1.38-2.39)
5 hour	1.07 * (0.55-1.42)	1.81 (1.38-2.47)
6 hour	1.08 (0.57-1.46)	2.03 (1.94-2.11)
8 hour	0.96 ** (0.52-1.51)	1.90 (1.60-2.13)
24 hour	1.13 * (0.67-1.37)	1.87 (1.28-2.15)

Significantly difference from healthy adults (Mann Whitney)

* P=<0.05

* P=<0.01

Table 8.4:

Table illustrating plasma retinol concentrations at baseline and 5 hours post dose – Relative Dose Response.

	Baseline retinol concentration µmol/L	5 hour retinol concentration µmol/L	Difference in concentrations	% Difference in plasma retinol above baseline
HA	2.03	2.13	0.10	4.93
HA	1.29	1.39	0.10	7.75
HA	1.51	1.38	-0.13	-8.61
HA	1.39	1.70	0.31	22.30 +ve result
HA	2.37	2.47	0.10	4.22
HA	1.55	1.92	0.37	23.86 +ve result
CF	1.45	1.42	-0.03	-2.07
CF	0.93	1.11	0.18	19.35
CF	0.91	0.95	0.04	4.40
CF	1.18	1.28	0.10	8.47
CF	0.62	No sample	-	-
CF	0.94	0.91	-0.03	-3.19
CF	1.27	1.34	0.07	5.51
CF	1.17	1.02	-0.15	-12.82
CF	0.47	0.55	0.08	17.02
CF	0.54	No sample	-	-
CF	0.63	0.69	0.06	9.52
CF	1.06	1.13	0.07	6.60

Figure 8.1:

Plasma retinol concentrations (μmol/L) in the group of 6 healthy adults (HA) and 12 patients with CF. (Lines represent median value).

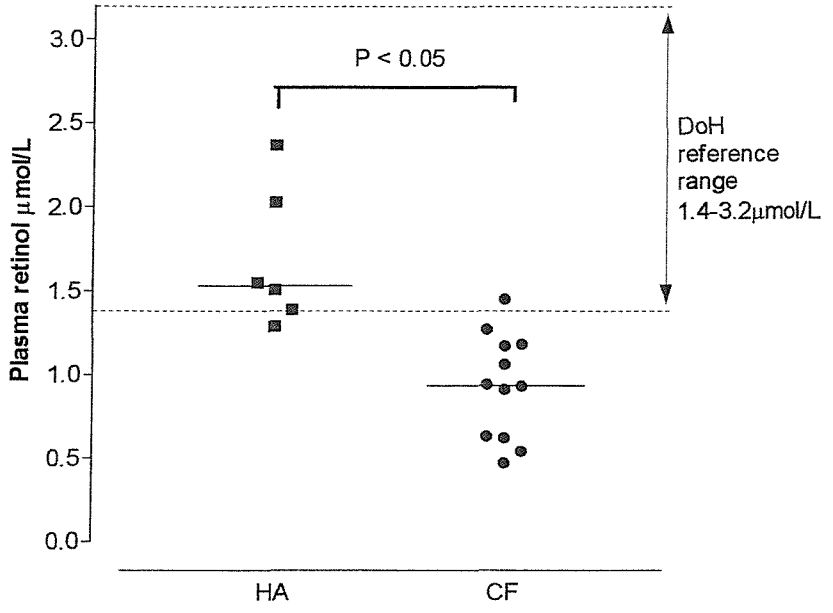


Figure 8.2:

Serum RBP concentrations (μmol/L) in the group of 6 healthy adults (HA) and 12 patients with CF. (Lines represent median value).

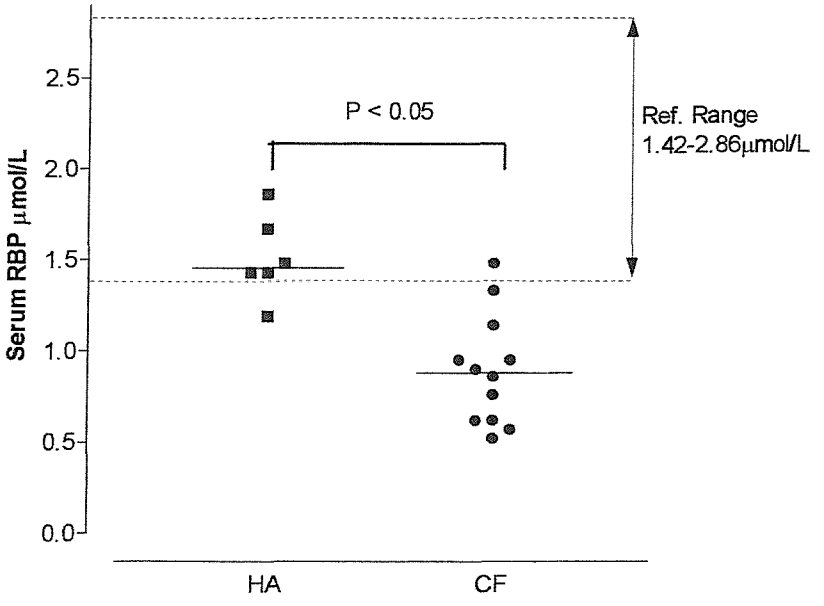


Figure 8.3:

Relationship between plasma retinol and serum RBP concentrations in the group of 6 healthy adults (HA) and 12 patients with CF.

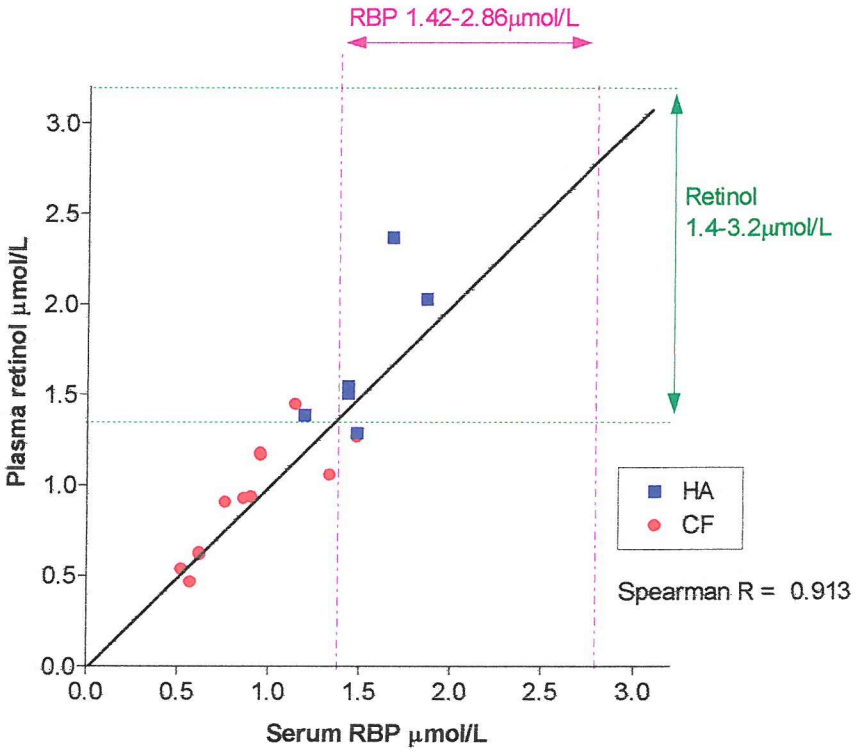
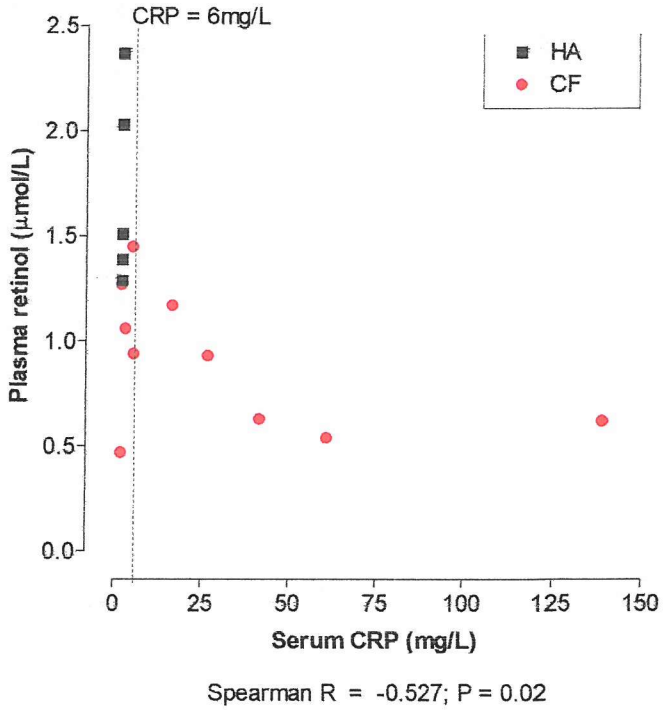


Figure 8.4:

Plasma retinol, relationship with CRP concentrations in the group of 6 healthy adults (HA) and 12 patients with CF.



Spearman R = -0.527; P = 0.02

Figure 8.5:

Relative dose response results for 12 patients with CF and 6 healthy adults. Graph illustrates percentage increase in plasma retinol concentrations from baseline following and oral bolus dose.

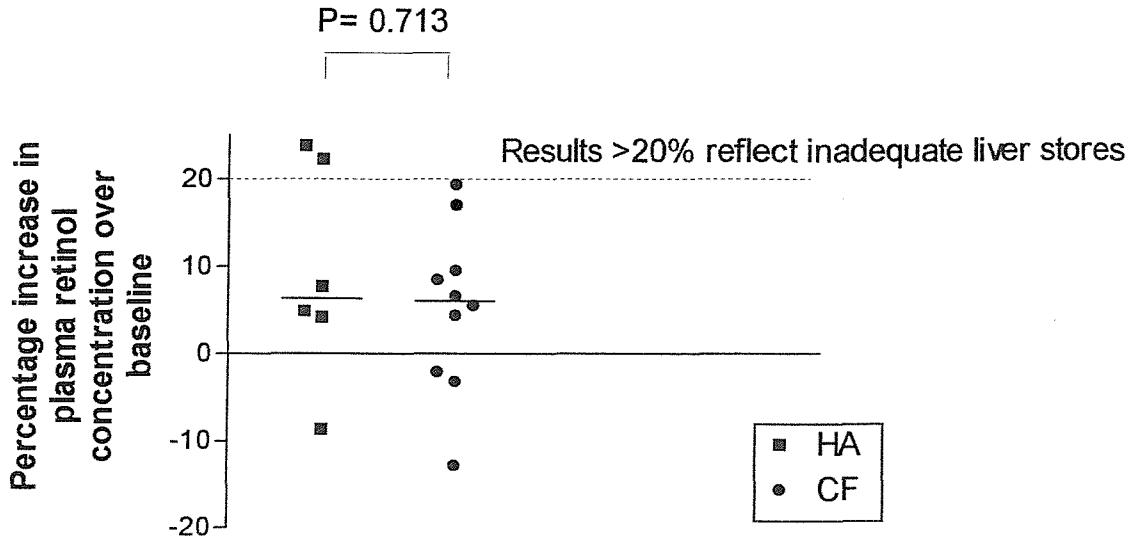
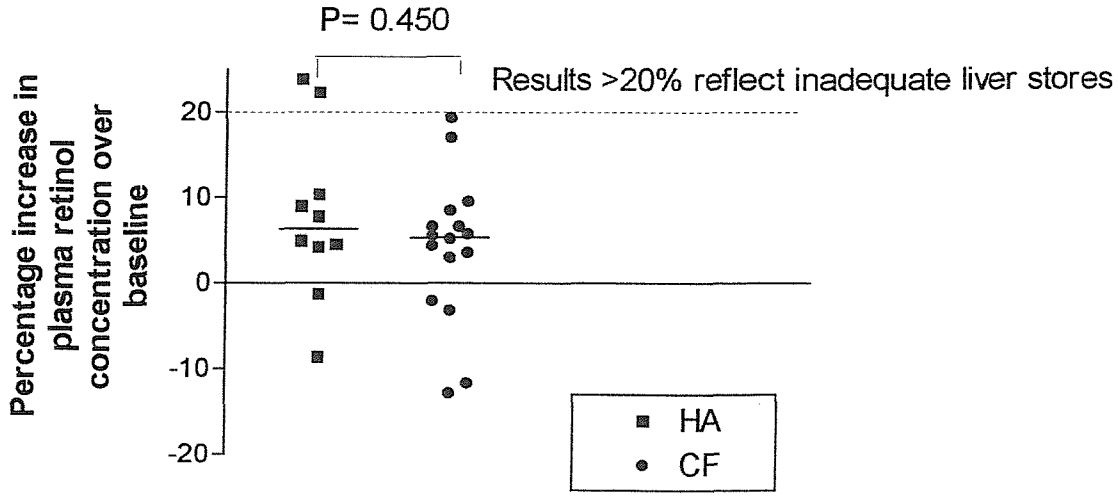


Figure 8.6:

Relative dose response results for 18 patients with CF and 12 healthy adults (chap 7 & chap 8).



8.5.3 POSTPRANDIAL EXCURSIONS IN PLASMA

8.5.3.1 LABELLED RETINYL PALMITATE

Plasma d_4-retinyl palmitate concentrations were variable both within and between subject groups (figure 8.7 and 8.8). Median plasma d_4-retinyl palmitate concentrations at each time point were greater in healthy adults when compared to patients with CF. Significant differences between the two groups were present at 1,2,and 6 hours post dose (table 8.5). Median maximum concentrations of d_4-retinyl palmitate achieved over the 8-hour study period were 0.530μmol/L (0.197-0.86μmol/L) and 0.263μmol/L (0.01-0.82μmol/L) for healthy adults and patients with CF respectively (figure 8.9). Despite variability both within and between subject groups, patients with CF had significantly lower ($P=0.032$) maximum concentrations of d_4-retinyl palmitate compared to healthy adults. Area under the plasma d_4-retinyl palmitate concentration time curve was not different between the two groups (HA; median 1.56(μmol/L)x8h (0.52-3.31(μmol/L)x8h); CF; median 0.73(μmol/L)x8h (0.01-3.2(μmol/L)x8h)) (figure 8.10). This is similar to the data presented in chapter 7.

The time at which plasma retinyl palmitate appears in the circulation after an oral bolus dose significantly differs between the two groups ($P=0.007$), similar to the data presented in chapter 7. The median time taken to reach maximum concentration of retinyl palmitate (over the 8 hour study period) was 2.5 hours (1-3 hours) for healthy adults and 4 hours (3-8 hours) for patients with CF (figure 8.11). There was an altered pattern of tracer appearance within the circulating plasma retinyl palmitate pool between the two groups (figure 8.12; table 8.6). In healthy adults there was detectable enrichment within the retinyl palmitate pool by 1-hour post dose (74.71%), which was significantly higher than that observed in the patients with CF (0%). For the healthy adults the greatest enrichment appeared at 6 hours (median 92.39%), where nearly all of the retinyl palmitate was labelled (comparable to that seen in the meal). Enrichment was elevated between 1 and 8 hours post dose. In contrast enrichment was not detected till 2 hours in patients with CF (30.47%), and was less than half the value observed at 2 hours in the healthy adults (85.82%). The greatest enrichment in the retinyl palmitate pool for patients with CF appeared at 8 hours (median 81.82%).

Figure 8.7:

Individual variability in plasma d_4-retinyl palmitate concentrations over the 8-hour study period in 6 healthy adults.

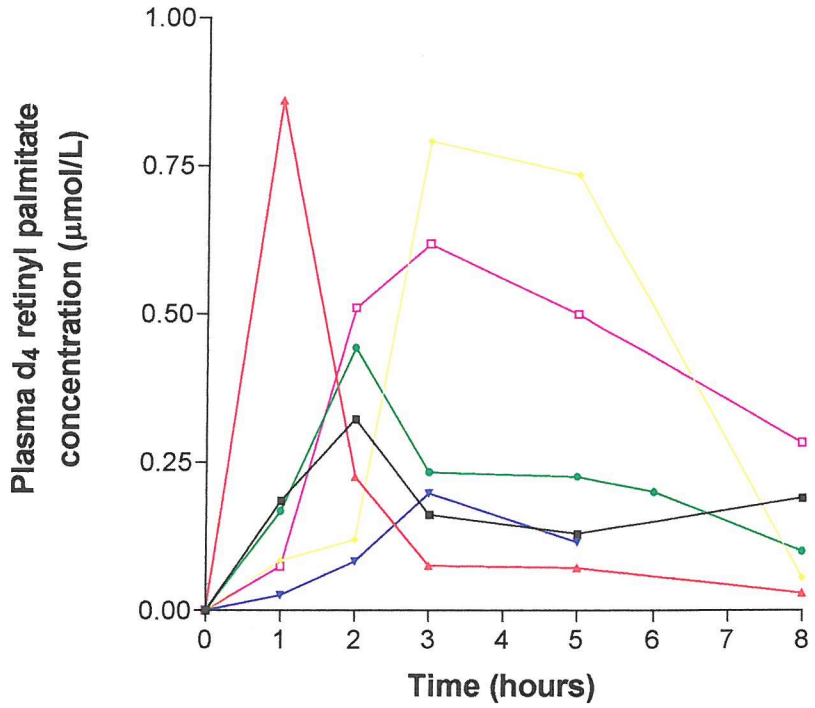


Figure 8.8:

Individual variability in plasma d_4-retinyl palmitate concentrations over the 8-hour study period in 12 patients with CF.

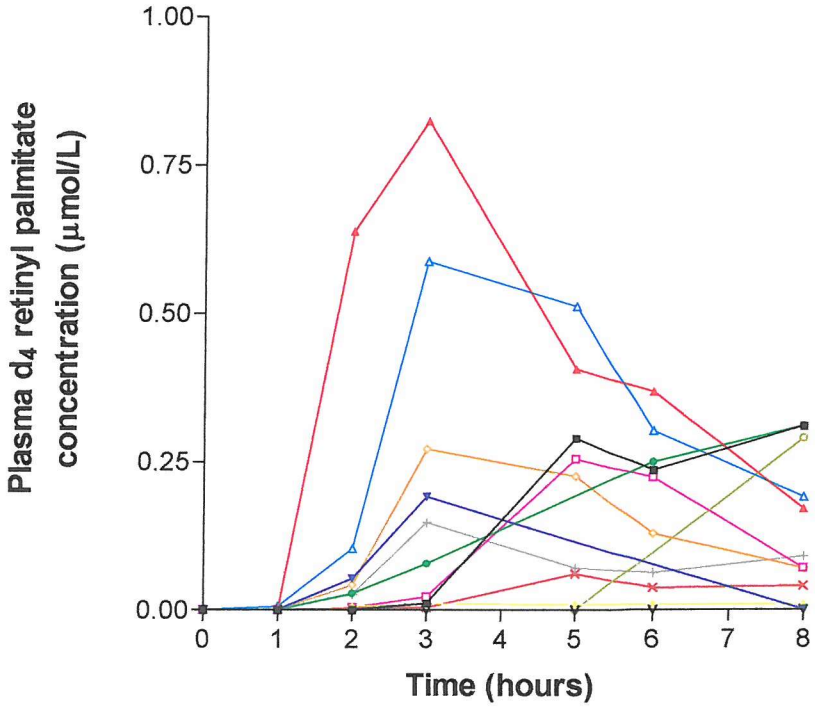


Table 8.5:

Plasma d_4-retinyl palmitate (nmol/L) of the 6 healthy adults and 12 patients with CF over the period of the study (8 hours).

Data presented as median and ranges.

	Plasma d_4-retinyl palmitate concentration nmol/L	
Time point	**CF adult**	**Healthy adult**
Base	0.0 (0.0-0.0)	0.0 (0.0-0.0)
1 hour	0.0** (0.0-6.0)	126 (26.3-860)
2 hour	16.1** (0.0-637.8)	273.9 (83.1-509.8)
3 hour	78.6 (0.0-823.9)	214.8 (75.2-791.9)
5 hour	148.4 (0.0-511.4)	178.2 (72.4-735.2)
6 hour	176.2 (0.0-367.5)	199.7 (199.7-199.7)
8 hour	80.0 (0.0-310.0)	100 (30-280)

Significantly difference from healthy adults (Mann Whitney)

* P=<0.05

* P=<0.01

Figure 8.9:

Maximal concentration (µmol/L) of d_4-retinyl palmitate in plasma over the 8-hour study period for both healthy adults and patients with CF.

(Lines represent median values).

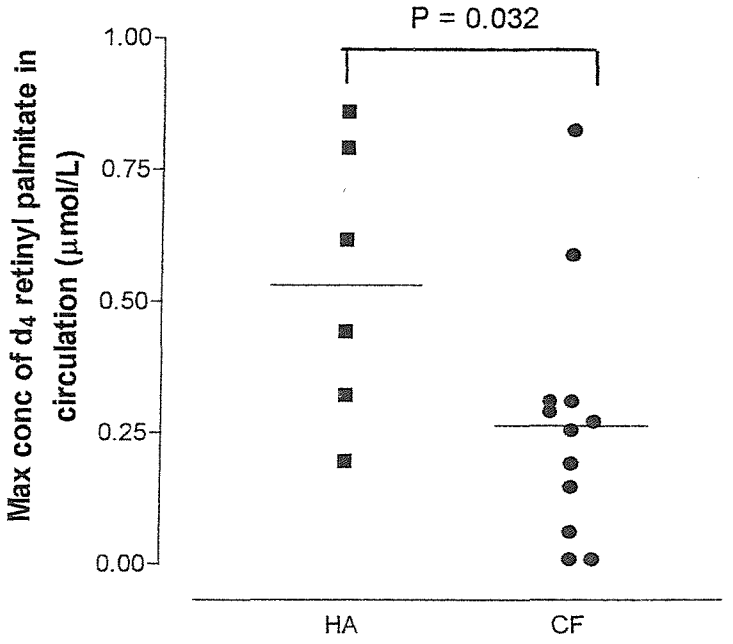


Figure 8.10:

Area under the d_4-retinyl palmitate concentration time curve (8 hours) for healthy adults (n=6) and patients with CF (n=12).

(Lines represent median values).

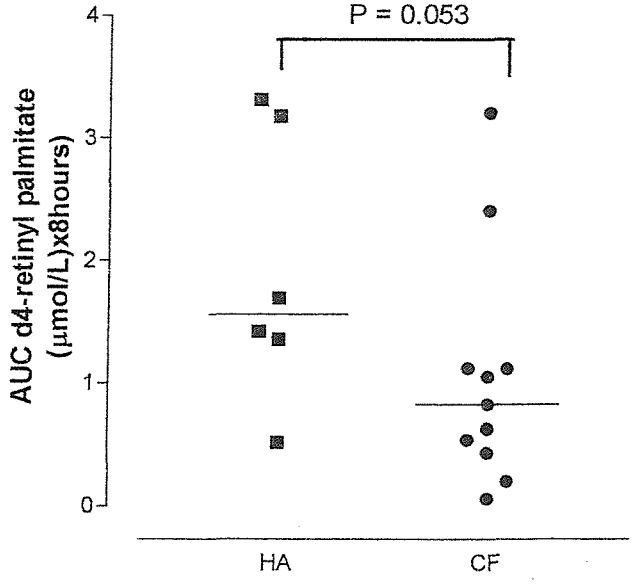


Figure 8.11:

Time taken to reach maximal concentration of d_4-retinyl palmitate over the 8-hour study period for both healthy adults (HA) and patients with CF patients. (Lines represent median values).

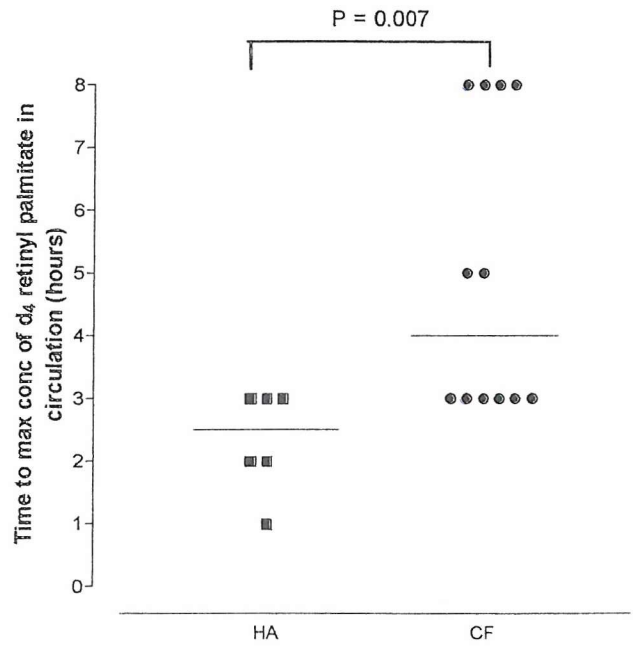


Figure 8.12:

Enrichment in the retinyl palmitate pool at each time point for 6 healthy adults (HA) and 12 patients with CF.

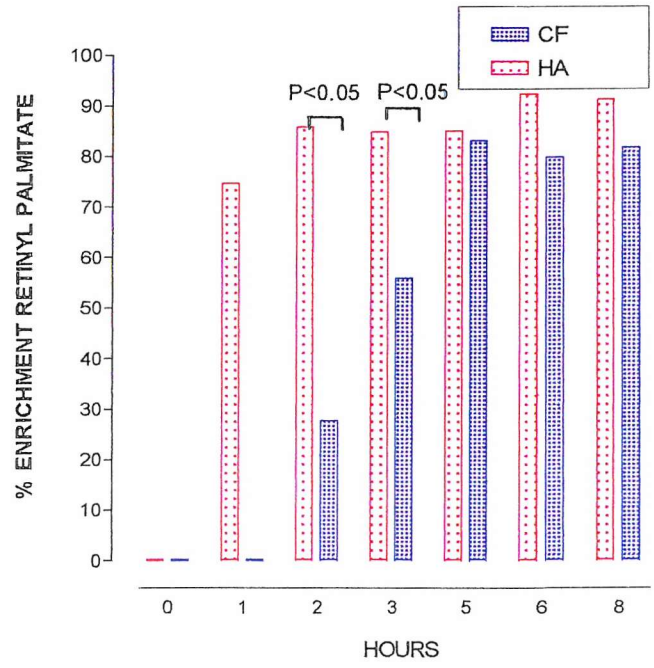


Table 8.6:

Summary of the enrichment in the retinyl palmitate pool for both groups, healthy adults (HA) and patients with CF, over the 8-hour study period.

Data presented as median and ranges.

	% ENRICHMENT OF RETINYL PALMITATE	
	CF	**HA**
BASE	0.00%	0.00%
1 HOUR	0.00% * 0.00 – 16.01%	74.41% 44.04 – 93.43%
2 HOURS	30.47% * 0.00 – 95.70%	85.82% 75.81 – 93.11%
3 HOURS	68.56% 0.00 – 95.57%	84.88% 72.02 – 93.59%
5 HOURS **(test lunch at 5 hours)**	72.43% 0.00- 94.19%	83.82% 69.51 – 93.54%
6 HOURS	80.63% 0.00 – 91.00%	92.39% 90.78 - 94.00%
8 HOURS	81.82% 19.10 - 92.40%	82.40% 30.00 - 92.15%

* Mann Whitney – Statistically difference from HA $P<0.05$

8.5.3.2 LABELLED LIPID.

Concentrations of plasma palmitic acid were determined as a proxy for plasma TAG. Median baseline palmitic acid concentrations for healthy adults and CF adults were 0.38mmol/L (0.29 – 0.67mmol/L) and 0.50mmol/L (0.26 – 2.74mmol/L) respectively. These values were not different between the two groups (P=0.349) and did not significantly differ between the two groups over the 8-hour period (table 8.7). It should be noted that 1 patient with CF had a particularly elevated palmitic acid concentration, fasting palmitic acid 2.74mmol/L. Median baseline concentrations of ^{13}C palmitic acid did not differ between the two groups; however median values were different at 1, 2 and 5 hours post dose (table 8.7). At 1-hour ^{13}C palmitic acid concentrations were nearly 10 times greater in healthy adults compared to patients with CF (median (range); HA 1.123µmol/L (0.242-1.803µmol/L); CF 0.116µmol/L (0.00-0.859µmol/L)).

Postprandial excursions of plasma palmitic acid (measured as area under the concentration time curve and maximal concentration achieved) were not different between the two groups (data not shown). However postprandial excursions of ^{13}C palmitic acid were significantly different between the two groups. Patients with CF had significantly lower areas under the ^{13}C palmitic acid concentration time curve at 8 hours (median 18.74(µmol/L)x8h (1.47-145.4(µmol/L)x8h)) when compared to healthy adults (median 53.71(µmol/L)x8h (16.56-82.40(µmol/L)x8h)) (figure 8.13). CF patients also had over three times lower maximum concentrations of ^{13}C palmitic acid when compared with the group of healthy adults (median (range); CF 4.11µmol/L (0.28-43.32µmol/L); HA 12.42µmol/L (3.32-16.35µmol/L)) (figure 8.14). There was no significant difference (P=0.844) in the time taken to reach the maximum concentration of ^{13}C palmitic acid in the plasma between the two groups (figure 8.15).

8.5.3.3 RELATIONSHIPS BETWEEN LIPID AND VITAMIN A.

There appears to be a relationship between the postprandial ^{13}C-palmitic acid responses and the postprandial d_4-retinyl palmitate responses in the circulation following an oral bolus dose of both ^{13}C-tripalmitin and d_4-retinyl palmitate. There are significant correlations between the ^{13}C and d_4 data for the maximum

concentrations achieved in the plasma over the 8-hour study period (figure 8.16; P=0.019), and area under the concentration time curves (figure 8.17; P=0.001). There was no relationship of this kind between the unlabelled retinyl palmitate and palmitic acid postprandial results. There was no association between the time to reach maximum concentrations of labelled lipid and labelled retinyl palmitate in the circulation (P=0.147) (figure 8.18).

8.5.3.4 RELATIONSHIPS WITH PLASMA RETINOL

If the appearance of d_4-retinyl palmitate in the plasma is associated with the prevalence of low retinol concentrations then it would follow that patients with the lowest retinol concentrations would have the lowest postprandial excursions of plasma retinyl palmitate. Analysis of data revealed that there were no correlations between plasma retinol concentrations and maximum concentration of d_4-retinyl palmitate in the plasma (P=0.141), or area under the d_4-retinyl palmitate concentration time curve (P=0.108).

Table 8.7:

Plasma palmitic acid and ^{13}C palmitic acid concentrations of the 6 healthy adults and 12 patients with CF over the period of the study (8 hours).

Data presented as median and ranges.

	CF (n=12)		HA (n=12)	
Time	**Palmitic Acid (µmol/L)**	**^{13}C palmitic acid (µmol/L)**	**Palmitic Acid (µmol/L)**	**^{13}C palmitic acid (µmol/L)**
Base	496.4 260.9-2736.6	0.12 0.00-0.30	377.8 291.7-668.8	0.11 0.02-0.27
1 hour	529.2 287.9-3324.2	0.12 ** 0.00-0.856	502.5 420.7-842.8	1.12 0.24-1.80
2 hour	512.4 322.9-3936.5	0.69 ** 0.22-18.05	554.2 372.0-826.7	5.98 1.44-10.33
3 hour	533.7 432.5-4245.6	2.26 0.11-43.32	654.6 297.5-831.0	6.25 1.06-13.29
5 hour	644.2 310.7-3228.1	3.16 * 0.19-21.42	620.4 392.5-712.3	5.65 3.32-13.88
6 hour	611.8 487.5-2136.2	2.45 * 0.33-13.57	978.4 801.3-1155.4	16.17 15.99-16.35
8 hour	643.1 402.2-2393.9	3.09 0.17-1.19	629.0 346.0-1332.8	6.04 2.59-13.73

Significantly difference from healthy adults (Mann Whitey)

* $P<0.05$

** $P<0.01$

Figure 8.13:

Area under the ^{13}C palmitic acid (in TAG fraction) concentration time curve (8 hours) for healthy adults (HA) (n=6) and patients with CF (n=12). (Lines represent median values).

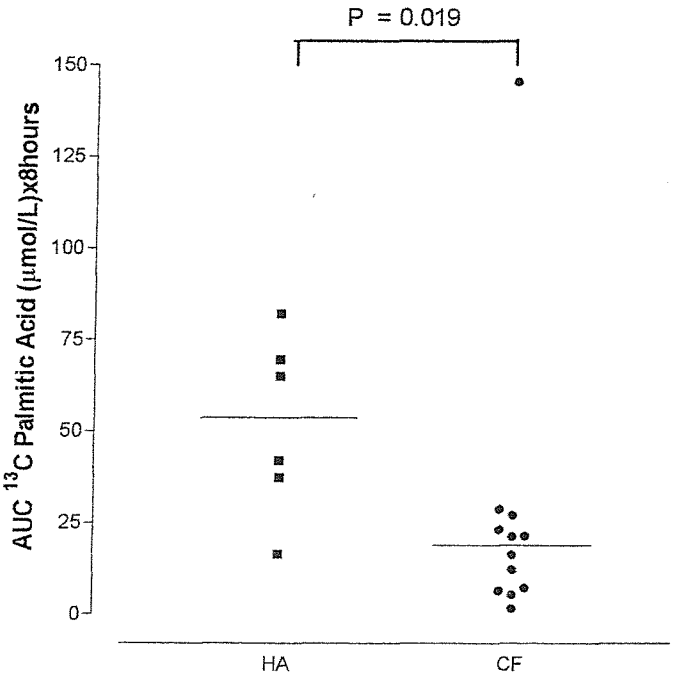


Figure 8.14:

Maximal concentration (µmol/L) of ^{13}C palmitic acid (in the TAG fraction) in plasma over the 8-hour study period for both healthy adults (HA) (n=6) and patients with CF (n=12). (Lines represent median values).

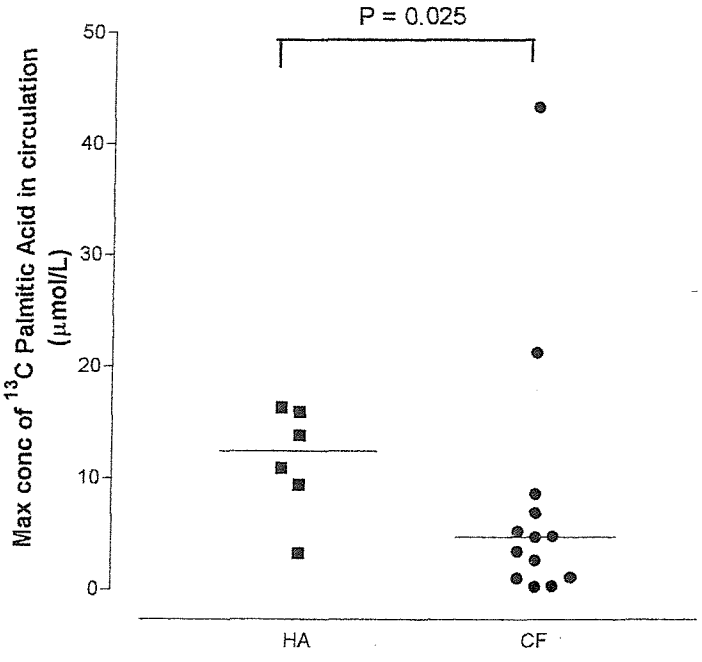


Figure 8.15:

Time taken to reach maximal concentration of ^{13}C palmitic acid (in the TAG fraction) over the 8-hour study period for both healthy adults (HA) (n=6) and patients with CF (n=12). (Lines represent median values).

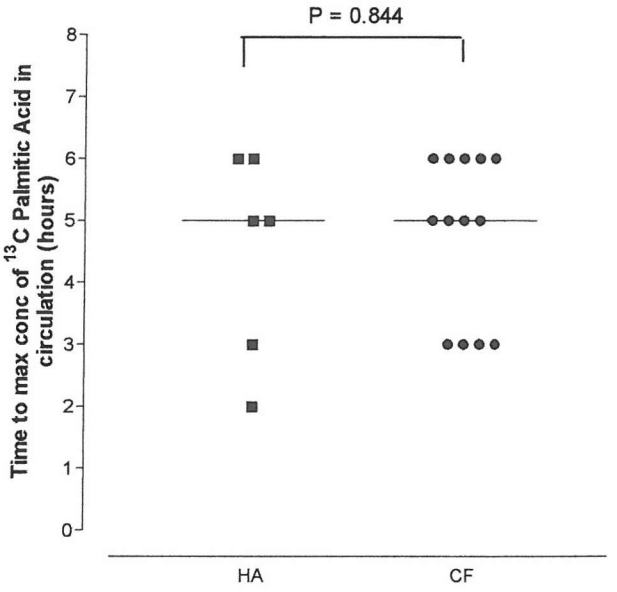


Figure 8.16:

Relationship between the labelled lipid and retinyl palmitate maximum concentrations achieved in the plasma over the 8-hour study period, in both healthy adults (HA) and patients with CF.

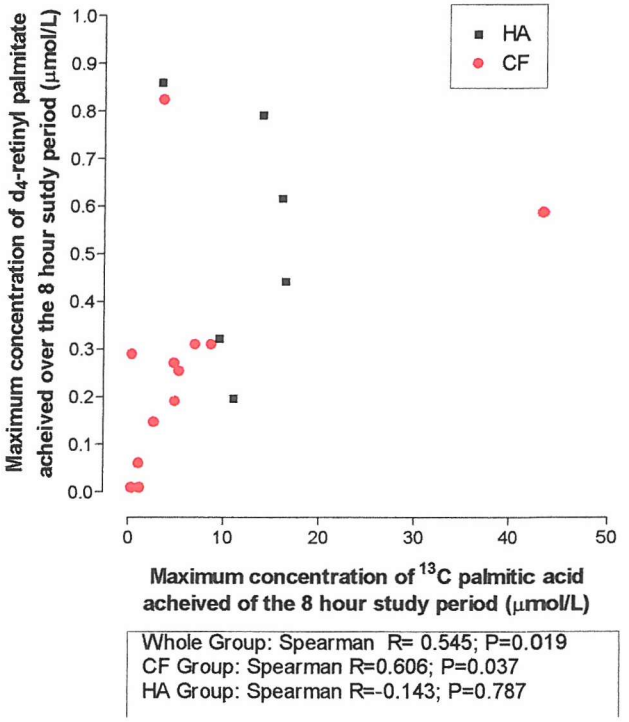


Figure 8.17:

Relationship between the labelled lipid and retinyl palmitate area under the concentrations time curve over the 8-hour study period, in both healthy adults (HA) and patients with CF.

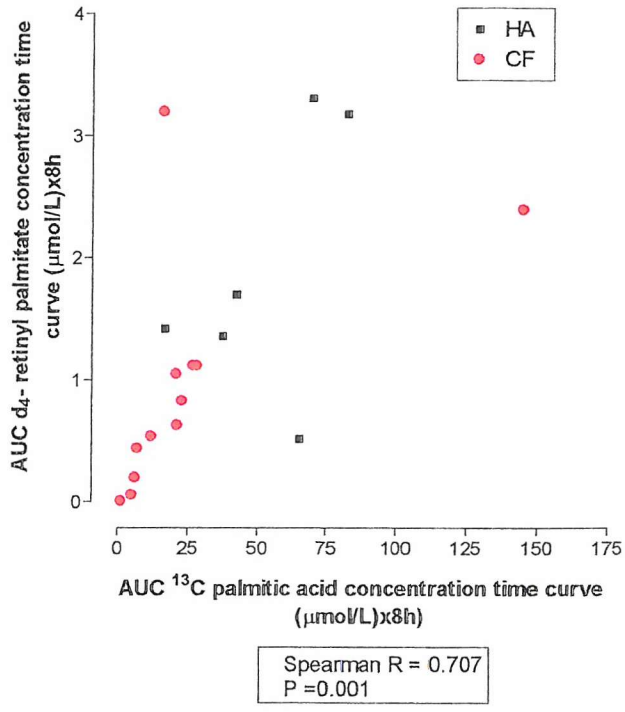
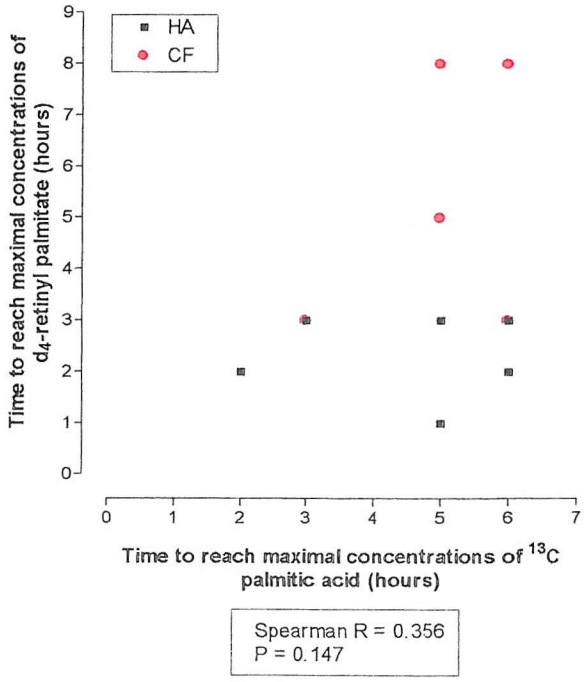


Figure 8.18:

Graph illustrating the labelled lipid and retinyl palmitate time to maximum concentrations achieved in the plasma over the 8-hour study period, in both healthy adults (HA) and patients with CF.



8.5.4 MOBILISATION OF RETINOL FROM THE LIVER.

By dosing d_4-retinyl palmitate to the subjects we were able to assess the release of retinol from liver stores. In theory if d_4-retinol appears in the circulation it has been mobilised from the liver as retinol that was derived from the d_4-retinyl palmitate in the test meal. The changes in enrichment within the retinol pool for the group of healthy adults and patients with CF are presented in table 8.8 and figure 8.19.

Low levels of enrichment within the retinol pool were evident by 3 hours in the group of healthy adults and by 5 hours in the patients with CF. Healthy adults appear to have greater enrichment in the retinol pool than patients with CF, in that patients with CF have significantly (P=0.001) lower area under their d_4-retinol curves when compared to healthy adults. The greatest enrichment within the retinol pool appeared at 8 hours in both subjects groups; however healthy adults (23.89%) had on average 2.5 times more enrichment than the patients with CF (9.95%). Enrichments in the retinol pool were statistically different between the two groups at 3 and 5 hours post dose. This data is similar to that presented in chapter 7 in that patients with CF appear to have less enriched retinol released from the liver over the 8-hour period. This may reflect a large liver store of vitamin A, in that patients with CF have a greater store of vitamin A and therefore the label is diluted in a larger pool.

Table 8.8

Summary of the percentage enrichment in the retinol pool for both groups, healthy adults (HA) and patients with CF, over the 8-hour study period.

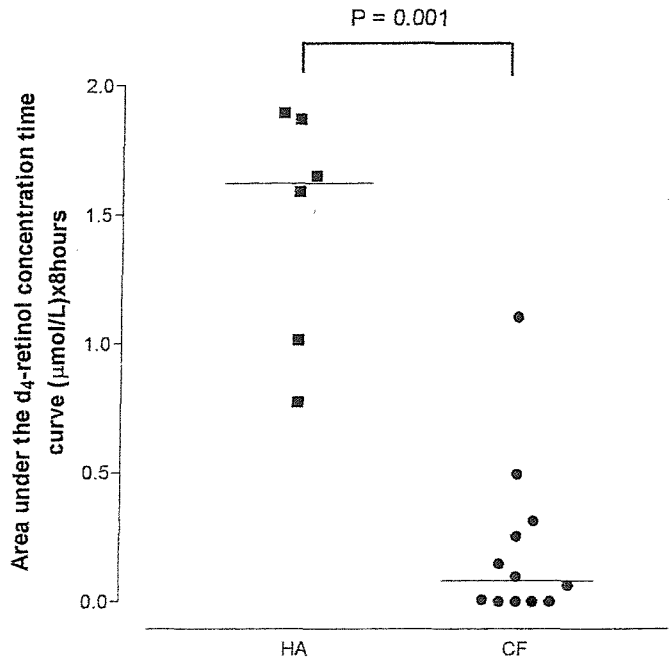
Data presented as median and ranges.

	% ENRICHMENT OF RETINOL	
	CF	**HA**
BASE	0.00% 0.00 – 0.00%	0.00% 0.00 – 0.00%
1 HOUR	0.00% 0.00 – 0.00%	0.00% 0.00 – 0.00%
2 HOURS	0.00% 0.00 – 0.00%	0.00% 0.00 – 4.19%
3 HOURS	0.00% * 0.00 – 6.13%	3.21% 0.00 – 20.88%
5 HOURS	0.135% * 0.00- 33.58%	15.06% 5.87 – 22.91%
6 HOURS	2.38% 0.00 –41.03%	12.34% 8.01 – 16.66%
8 HOURS	9.95% 0.00 – 30.02%	23.89% 14.32 – 32.26%

Mann Whitney – Statistically difference from HA $P<0.05$

Figure 8.19:

Area under the d_4-retinol concentration time curve (8 hours), for healthy (HA) (n=6) and patients with CF (n=12). (Lines represent median values).



8.5.5 DISCUSSION.

The present study investigated the postprandial excursions of both labelled retinyl palmitate and labelled lipid following a test meal. The hypothesis to be tested was that patients with CF will have lower postprandial excursions of both lipid and vitamin A compared to healthy adults, and there will be an association between the postprandial TAG and retinyl palmitate response. Postprandial excursions of vitamin A in patients with CF will be associated with the patient's plasma retinol concentration. This chapter differs from chapter 7, in that there are increased patient numbers, increased blood sampling times over the initial postprandial period and patients have low plasma retinol concentrations. In order to better assess the relationship between the processing of vitamin A and lipid the study presented in this chapter employed the use of both labelled retinyl palmitate and labelled lipid, unlike that in chapter 7.

Whilst assessing circulating concentrations of plasma retinol, results reaffirm that despite PERT and vitamin A supplements patients with CF have significantly lower plasma retinol concentrations than healthy adults. In the

study all but 1 patient with CF had plasma retinol concentrations below 1.4μmol/L [DoH, MAFF 1990]. Whole group concentrations of retinol were associated with CRP concentrations in that subjects with a low plasma retinol had increased CRP concentrations. Interestingly despite low concentrations of retinol only five patients with CF had raised concentrations of CRP indicative of infection-induced hyporetinolemia. However it should be noted that an absence of an elevated CRP does not necessarily reflect the absence of the acute phase response. The ratio of retinol:RBP did not differ between the two subject groups. The majority of subjects (13/19) had more retinol than RBP in the circulation, possibly reflecting "free" retinol as a consequence of spill over of the liver. However 3 patients with CF, had more RBP than retinol in their circulation which may reflect either increased demands for retinol or increased loss of retinol.

Postprandial excursions of labelled retinyl palmitate.

Recommendations for clinical practice in CF are based on the assumption that low concentrations of plasma retinol are a consequence of poor availability of vitamin A across the GI tract, associated with maldigestion and malabsorption of lipid. Results presented in chapter 7 found that despite increased losses of vitamin A in the stool of CF patients, availability of vitamin A across the GI tract is not limiting. These results however were based on studies in 6 patients with CF. In order to re-confirm these results the study presented in this chapter has specifically investigated postprandial excursions of labelled retinyl palmitate in 12 patients with CF with low concentrations of plasma retinol. Analysis of the results revealed similar observations to those reported in chapter 7.

Increased subject number and sampling time points have produced a greater within and between subject variability in the postprandial excursions of labelled retinyl palmitate. Despite this the area under the concentration time curve was not different between the two groups, similar to results in chapter 7. However three patients with CF had areas lower than the lowest area observed for the healthy adults which may reflect decreased availability in these subjects. The maximum concentration of retinyl palmitate achieved just reached significance between the two groups, in that as a group the patients with CF had lower

maximal concentrations of retinyl palmitate in their circulation compared to the healthy adults. On closer inspection the majority (8 patients) of patients with CF had maximum concentrations in the same range as the healthy adults, only 4 patients with CF had lower maximum concentrations of labelled retinyl palmitate compared to healthy adults. These results will be discussed in more depth later in this section.

Results also suggest that exogenous retinyl palmitate enters the circulation significantly earlier in healthy adults compared to patients with CF. These results are similar to those in chapter 7, however in this chapter there is a greater cross over of results. All healthy adults had a mean time to maximum concentration less than 3 hours and 6 patients with CF had similar times to the healthy adults (3 hours). However 6 patients with CF had greater times to reach maximum peak, 2 patients at 4 hours and 4 patients at 8 hours. It should however be recognised that as the study was only 8 hours long some CF patients could have greater time to reach maximum peak.

Whilst considering the enrichment of retinyl palmitate within the circulation it would appear that results correspond with those previously reported. Peak enrichment occurs later in patients with CF compared to the healthy adults (8h v 6h), and median peak enrichment is approximately 10% less in CF patients compared to that observed in healthy adults (81.82% v 92.39%). Due to the earlier sampling time points labelled retinyl palmitate was observed as early as 1 hour post dose in healthy adults and at 2 hours post dose in patients with CF. Although as a group enrichment of retinyl palmitate in patients with CF was similar to the healthy adults there was variability in the results. Figure 8.20 illustrates the variability in peak enrichment of retinyl palmitate. The majority of patients (8 patients) have peak enrichments similar to the healthy adults, representative of the label administered. However 4 patients with CF (A-D) had peak retinyl palmitate enrichments lower than the rest of the group. These 4 patients did not have the lowest concentrations of plasma retinol. In these cases results may suggest decreased availability of retinyl palmitate associated with increased stool losses. Decreased availability may be related with disturbed GI processing of vitamin A, which could be associated with incorrect prescription,

or incorrect usage of PERT, along with the severity of pancreatic insufficiently. Three of these four patients had previously attended the clinic at Southampton for routine annual assessment (table 8.9). Data presented in table 8.9 illustrates the potential availability of retinol to the body taking into consideration supplement dose, habitual intake and losses in stool. In theory only subject A will have less available retinol than healthy adults (~1mg/d).

To summarise as a group it would appear that availability of exogenous vitamin A is not a limiting factor associated with low concentrations of retinol in patients with CF, however some patients may exhibit poor availability of the vitamin. Patients with CF appear to have delayed appearance of retinyl palmitate post absorption, which may reflect altered GI processing.

Figure 8.20:
Graph illustrating the peak enrichment achieved in the retinyl palmitate pool over the study period for both healthy adults and patients with CF.

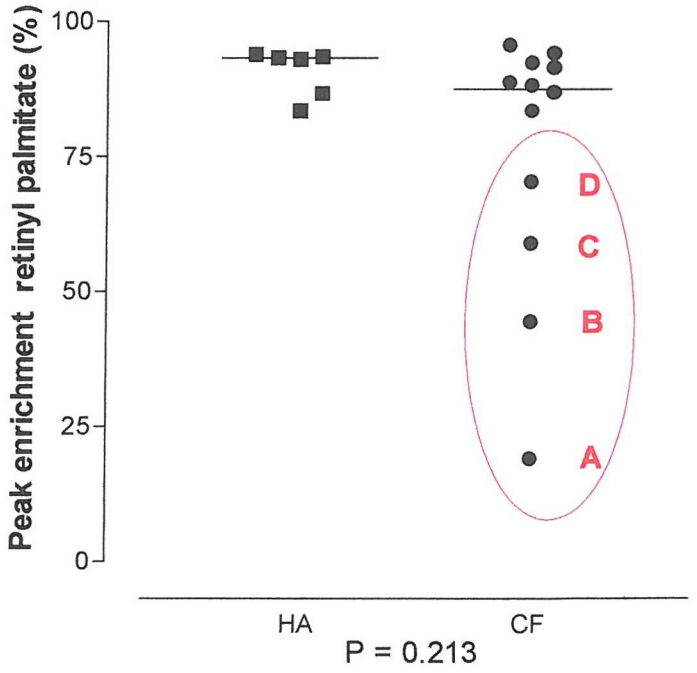


Table 8.9:

Table illustrating previous MOT data on subjects A-D.

Supplement dose and assumptions of availability.

Subject	Vitamin A supplement	Dose	PERT	Assumptions of availability.
A	Vitamin A & D (adherent)	2.4mg/d in xs of diet	Creon 25000	Peak enrichment 19.1% Assume loss of ~80% 0.6mg/d retinol available
B	Vitamin A & D (adherent)	2.4mg/d in xs of diet	Creon 10000	Peak enrichment 44.48% Assume loss of 55.52% 1.5mg/d retinol available
C	Vitamin A & D (adherent)	2.4mg/d in xs of diet	Pancrex (takes during meal)	Peak enrichment 58.98% Assume loss of 41.02% 2.0mg/d retinol available
D	New patient to clinic. No MOT Therefore assumptions.	Assume modest dose 1mg/d		Peak enrichment 65.64% Assume loss of 34.36% 1.29mg/d retinol available

Postprandial excursions of labelled lipid.

Postprandial excursions of total lipid do not differ between the two subject groups, similar to the data presented in chapter 7. However differences were evident on analysis of the labelled lipid data. The most probable explanation for no observed differences in the unlabelled data, is that total unlabelled palmitic acid, as the contribution of palmitic acid from the CM was counterbalanced by the contribution of palmitic acid from VLDL. For this reason in order to trace the CM TAG (the lipid within the meal) the ^{13}C palmitic acid results were most appropriate. Analysis of labelled lipid results revealed that patients with CF had lower postprandial excursions of lipid compared to healthy adults (as measured by AUC and maximal concentrations). However results again highlight variability both within and between subject groups. On closer inspection of the results the

same four patients who had low postprandial excursions of labelled retinyl palmitate (patients A-D) also have postprandial excursions of labelled lipid lower than the lowest point in the range of healthy adults. This highlights the associated between the GI processing of vitamin A and lipid.

Association between the processing of lipid and vitamin A.

Examining the labelled vitamin A and lipid data allowed the association between the processing of these two molecules to be assessed. Previous results (chapter 7) revealed an association between the losses of vitamin A and lipid in stool, however no association of this kind was seen in the postprandial excursions. Data reported in this chapter revealed a relationship between the postprandial handling of labelled lipid and labelled vitamin A. However the appearance of retinyl palmitate in the circulation was not associated with concentrations of retinol in the plasma. One explanation as to why this may be is that the downstream effects of processing and mobilisation of retinol from the liver, dominate over any GI effects that may be present. Previous data in the literature reported delayed appearance of retinyl esters in the circulation compared to lipid, when retinyl palmitate was presented in an oil soluble preparation [Krasinski *et al* 1990; Cohn *et al* 1993]. However this is not a consistent finding and the form in which retinyl palmitate is administered has been associated with this discrepancy. In the study presented in this chapter labelled retinyl palmitate was presented in the form of an emulsion and results indicate that there was no association between the times of appearance in the circulation of these two molecules.

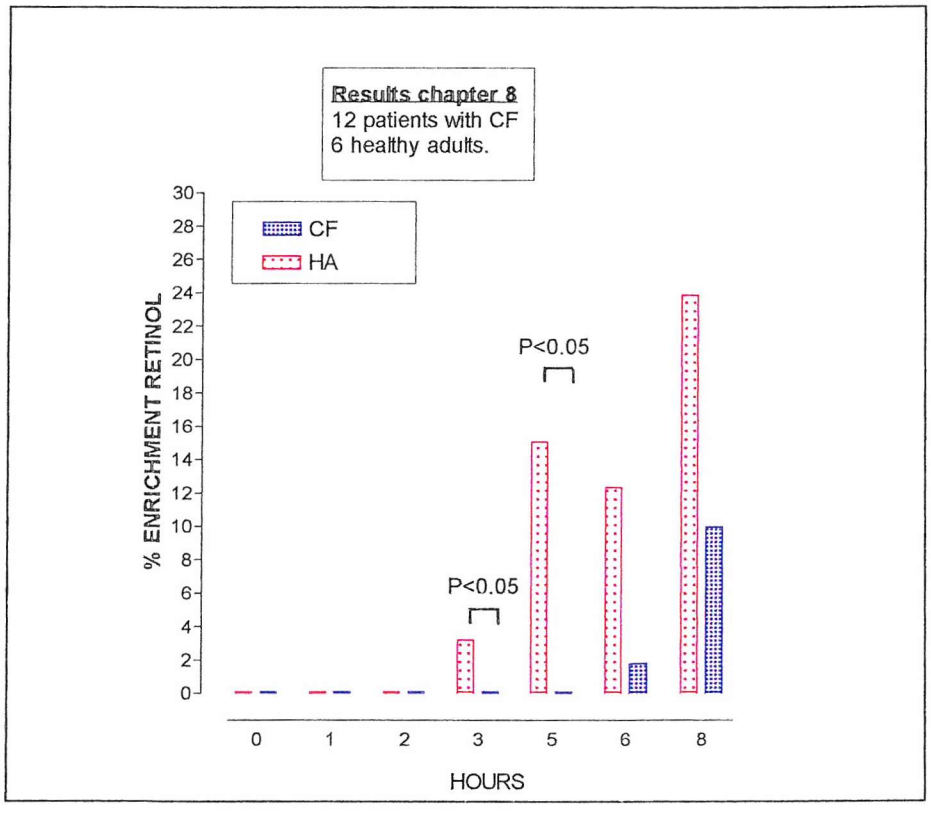
Mobilisation of retinol from the liver.

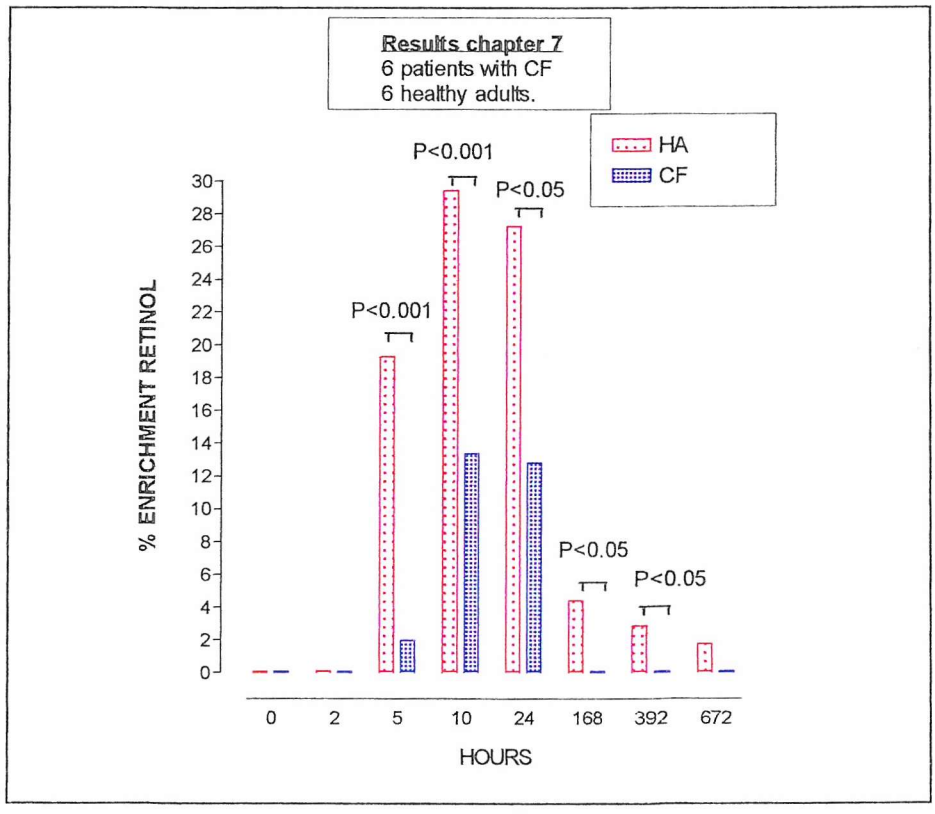
As d_4-retinyl palmitate was administered to the subjects, the appearance of d_4-retinol in the circulation can be measured to reflect retinol released from the liver that was derived from the meal. The study reported in this chapter sampled different time points to those reported in chapter 7. Despite this results were similar between the two studies (figure 8.21). On average the greatest enrichment observed in the healthy adults at 8 hours was 23.89%, compared to 29.42% at 10 hours in chapter 7. In comparison enrichment was less in the patients with CF. At 8 hours enrichment observed for the patients with CF was

9.95%, compared to 13.35% at 10 hours in chapter 7. Enrichment was detected as early as 3 hours post dose in the healthy adults compared to 5 hours in the patients with CF. This could be interpreted as the liver releasing retinol in a last in first out basis, for example retinol obtained from the dose of retinyl palmitate 3 hours previously is circulating in the plasma. It should be noted that patients A-D (low enrichment of retinyl palmitate) have correspondingly low enrichments of retinol. Low enrichment in the retinol pool in these cases may reflect poor availability of retinyl palmitate with or without poor mobilisation of retinol from the liver. However the majority of patients exhibit low enrichment in the retinol pool despite enrichment in the retinyl palmitate pool similar to the group of healthy adults. In this instance low enrichment in the retinol pool may reflect increased hepatic stores which could be associated with poor mobilisation of retinol from the liver. Enrichment in the retinol pool was not associated with plasma concentrations of retinol (P=0.642), or concentrations of CRP (P=0.383). To summarise on average enrichment within the circulating retinol pool appears to be generally low in patients with CF, whereas higher levels of enrichment are consistently evident in the healthy adults. Such a finding would arise if the labelled vitamin A was diluted within a large hepatic pool of unlabelled vitamin A, or if mobilisation of retinol was in some way constrained. This is further emphasised by the results obtained from the relative dose response, which suggest that liver stores of vitamin A in patients with CF are adequate.

Figure 8.21

Graphs illustrating enrichment in the retinol pool for the studies presented in chapters 7 and 8.





8.5.6 SUMMARY.

In summary, current recommendations in CF are based on the assumption that low concentrations of plasma retinol are a consequence of poor availability, associated with the maldigestion and malabsorption of dietary lipid. Considering this the hypothesis to be tested was that patients with CF patients will have lower postprandial excursions of both lipid and vitamin A compared to healthy adults which will be associated with the patient's plasma retinol concentration. Results presented in this chapter do not support this hypothesis. In general patients with CF do not have lower postprandial excursions of vitamin A compared to healthy adults, and postprandial excursions of exogenous retinyl palmitate are not associated with a patient's plasma concentration of retinol. Postprandial excursions of labelled retinyl palmitate are associated with postprandial excursions of labelled lipid. Further attention needs to be directed towards examining the relationship between mobilisation of retinol from the liver and plasma retinol concentrations in patients with CF.

- Patients with CF have similar postprandial excursions (as determined by area under the concentration time curve) of labelled retinyl palmitate when compared to healthy adults.
- Patients with CF have a delayed appearance of exogenous retinyl palmitate compared to healthy adults.
- There is a relationship between the postprandial excursions of exogenous vitamin A and lipid.
- The appearance of retinyl palmitate in the circulation is not related to plasma retinol concentrations.
- Patients with CF have lower levels of enrichment in their retinol pool when compared to healthy adults; this may reflect a large store of vitamin A in the liver.

CHAPTER 9

GENERAL DISCUSSION

9.1 INTRODUCTION

Vitamin A plays a pivotal role in mucus production, non-specific barrier function of the respiratory and GI tract and specific immune function. Supplements of retinyl palmitate, in excess of the RNI, are routinely prescribed to patients with CF, in response to low circulating plasma retinol concentrations. This practice assumes that either intake and/or availability of dietary retinyl palmitate, associated with impaired lipid handling, are compromised. However the clinical significance of low retinol concentrations and what constitutes the most appropriate approach to management, particularly with regard to the use of oral supplements, remains unclear. Present understanding of the role that vitamin A plays in the disease CF, is constrained by the lack of knowledge of how vitamin A is handled in the body. The GI processing of exogenous retinyl palmitate has not been systematically investigated nor has the possibility that low circulating retinol concentrations may reflect a constraint on the ability to mobilise retinol stored within the liver. This chapter will begin to consider some of these issues in the light of the research presented within this thesis.

The chapter will firstly summarise results in the context of the three original hypotheses, followed by a section reviewing how the work presented within this thesis contributes to our understanding. The next part considers implications for clinical practice, including toxicity, infection, mobilisation of retinol from the liver and whether current clinical approaches are justified. Finally the wider issues of the research are considered along with ideas for future studies and further research.

9.2 TESTING THE HYPOTHESES.

This thesis has characterised the metabolism of vitamin A, and examined a number of issues relating to the processing of vitamin A. Such information is needed to establish a conceptual framework to develop safe and effective approaches to clinical management.

Firstly, there was a need to establish and extensively validate analytical procedures that underpinned studies presented within this thesis (chapter 3), as these have never been previously applied to faecal material. A method that permits simultaneous measurement of retinol and retinyl palmitate in plasma and stool by HPLC, along with the proportion of isotopic enrichment within each fraction was developed. These methods enabled concurrent measures of both total concentrations and enrichment in plasma and stool so complete balance studies across the GI tract and handling within the circulation could be conducted. Following method development the principle aims were twofold. The first aim was to determine the prevalence of low retinol concentrations in CF patients and investigate if reductions in the functional pool affect clinical outcomes. The second aim was to apply tracer techniques to systematically determine the extent to which vitamin A handling is altered in CF along with factors that may be associated with low retinol concentrations. Three specific research hypotheses were tested.

Hypothesis One:

Patients with CF have lower plasma retinol concentrations compared to healthy adults that affect or are affected by their clinical outcomes.

Hypothesis Two:

Patients with CF have lower concentrations of retinol in their circulation due poor dietary supply, and / or impaired GI handling and availability of the vitamin.

Hypothesis Three:

Patients with CF have lower concentrations of retinol in their circulation that are unrelated to supply, but are a consequence of poor mobilisation of retinol from the liver.

9.2.1 HYPOTHESIS I:

An in-depth clinical audit of the cohort of adult patients with CF was conducted to determine the prevalence of low circulating retinol concentrations (results presented in chapters 4&5). Nearly half the adult patients attending annual assessment at the CF clinic, Southampton General Hospital, had circulating retinol concentrations beyond the lower limits observed in healthy adults. This is an important observation in that the clinic at Southampton is a specialist centre and has an aggressive approach to nutritional care. Patients have been prophylactically prescribed vitamin A supplements and when low circulating retinol concentrations have been observed, compliance and prescription has been re-assessed. As a population the patients with CF had significantly lower plasma retinol concentrations than the healthy adults. However within the population individual results varied from very low plasma retinol values (0.4μmol/L) to high plasma retinol values (4.8μmol/L). It would appear that individuals with the lowest plasma retinol concentrations have greater admissions to hospital, and poorest lung function. This data strengthens preliminary abstracts from the literature [Carr *et al* 1996; Raynor *et al* 1992], in that concentrations of plasma retinol in some way are associated with the clinical course of the patient. This data supports hypothesis I of the thesis.

Furthermore, in this set of patients, an initial indication of an interaction between plasma retinol concentrations, clinical course and immunological status were discernible from the data set. There was a significant correlation between plasma retinol concentrations and serum IgG such that those patients with a high IgG had low plasma retinol concentrations. Quadrant analysis of this data revealed that patients with low retinol concentrations and high IgG had trends towards poor clinical outcomes (e.g. lower Body Mass Index, plasma zinc and FEV1% with higher ESR, CRP and WCC) and more hospital admissions than that observed in patients with retinol concentrations and IgG within the normal range. It is not clear whether low circulating retinol concentrations play a causal role in the clinical course or whether the deteriorating clinical condition results in a concomitant fall in circulating retinol which in turn impacts upon progression of the disease. It is however clear that in CF, retinol concentrations fall in the face of an infective exacerbation, and increase again following treatment (chapter 6).

From the data it can not be determined if low retinol concentrations per se have a direct effect on patients clinical course, or if inflammation and infection act to effect concentrations of plasma retinol and therefore affect clinical outcomes.

9.2.2 HYPOTHESIS II:

To address hypothesis II, both the findings from the clinical audit and that obtained from gross balance techniques were used. Results from the audit support the view that low plasma retinol concentrations can not simply be attributed to inadequate dietary supply and / or poor adherence with vitamin A supplementation. There was no association between plasma retinol concentrations energy intake, intake of PERT, vitamin A supplement dose and adherence to supplement regimens. The median dose of vitamin A from supplements was 1.2mg of retinol per day, over and above habitual dietary intake, which was not different to that of healthy adults. It should be recognised that although supply of vitamin A does not appear to impact upon plasma retinol concentrations in this cohort of patients, those patients with poor controlled malabsorption, poor vitamin A intake and poor adherence to supplements may be at risk.

In order to determine availability across the GI tract an oral bolus dose of labelled retinyl palmitate was administered to both patients with CF on habitual PERT, and healthy adults. Three approaches were used to assess availability which included, i) gross stool losses, ii) isotopically labelled stool losses, and iii) the appearance of retinyl palmitate in the circulation. If availability were constrained, increased labelled vitamin A loss in faeces, and diminished postprandial excursions of labelled retinyl palmitate in the plasma following a meal, would be seen. Initially availability of vitamin A was investigated in 6 healthy adults and 6 patients with CF (on their habitual PERT regimen) (chapter 7). This study showed that whilst CF patients excreted relatively more vitamin A than that observed in healthy adults, the magnitude of such losses were relatively small (usually equivalent to <15% of intake) and could be easily compensated by higher intakes associated with supplementation (2-4 times greater than that consumed by healthy adults). Further analysis revealed a relationship between the handling of dietary lipid and vitamin A, in that those

patients, with the greatest lipid losses, on their habitual PERT, also exhibited the greatest losses of vitamin A. Whilst there was a trend towards delayed appearance of labelled retinyl palmitate in to the circulation, net excursions were similar. These observations imply that whilst retinyl palmitate handling within the GI tract may be altered, net availability does not appear to be limited by maldigestion or malabsorption in this group of CF patients. Following this study the postprandial excursions of retinyl palmitate and lipid following an oral bolus dose of labelled material, was examined in a greater number of subjects (n=12 CF) (chapter 8). The study followed the partitioning of labelled retinyl palmitate simultaneously with that of labelled fatty acid, in patients with low to low/normal concentrations of circulating retinol in the fasting state. Analysis revealed that, in support with the stool data, net excursions of labelled retinyl palmitate in the circulation of patients with CF was similar to healthy adults. Enrichment in the plasma retinyl palmitate pool was not different between the two groups, however, on average patients with CF had ~10% lower enrichment, consistent with modest losses of intake. Patients with CF appear to have delayed appearance of retinyl palmitate in the circulation compared to healthy adults. Delayed appearance may reflect altered GI processing possibly contributed to by the use of PERT. There was a relationship between postprandial responses of exogenous lipid and vitamin A, but there was no association between net excursions of labelled retinyl palmitate and circulating retinol concentrations in the fasting state.

These observations should be considered alongside previous literature on stool vitamin A losses in CF. Previous studies found increased stool losses of vitamin A with respect to healthy controls, investigators concluded that patients with CF require supplementation with vitamin A [Ahmed *et al* 1990; Halford *et al* 1993]. Results presented in this thesis similarly found elevated stool losses of vitamin A in patients with CF however the overall interpretation of the results differs. A comparison of the current literature with that presented in this thesis is presented in table 9.1.

It is hard to make direct comparisons between these studies due to differences in methods and study design. Firstly, both Ahmed and colleagues [1990], and

Halford and co-workers [1993], assessed losses of total vitamin A (as retinol) from habitual vitamin A intake by traditional gross balance techniques. This technique has a number of drawbacks; in particular it assumes all losses reflect dietary residue. In this thesis stable isotope tracer methods have been used for the first time to specifically investigate losses of dietary derived vitamin A. Secondly, Ahmed and colleagues investigated losses in children and young adults not receiving supplements, whereas Halford and co-workers assessed losses in children receiving supplements and the data presented in this thesis investigated losses in adults receiving supplements.

Taken together these studies report significantly increased losses of vitamin A in patients with CF compared to healthy individuals. Both Halford and co-workers and findings presented in this thesis found an association between vitamin A and lipid losses, however reported results from Ahmed and colleagues found no association. Results from this thesis are similar to those of Halford and co-workers in children. When the pancreatectomised patient is excluded from the present data set the range of losses as a percentage of intake would be 0-16%, a similar range to that of Halford (range 0-17%). When considering availability of retinol to the body, the median intake of vitamin A reported by Halford [1993], at ~98% apparent absorption would allow 1.98mg of retinol to be available [unpublished data]. Similarly assuming a median intake from supplements of 1.2mg/d in addition to habitual diet (~1mg/d), at ~93% apparent absorption (data from thesis), 2.05mg of retinol would be available to the body.

To summarise, taken together this evidence does not support the view that poor dietary supply, increased losses of vitamin A in stool, and poor availability across the GI tract, contribute to low retinol concentrations in the population of adults patients with CF a Southampton. Modest losses of vitamin A seen in this group of patients can easily be overcome by the intake of supplements. This data does not support Hypothesis II, in that low concentrations of plasma retinol are not related to availability of the vitamin across the GI tract.

Table 9.1 : Comparison of studies reporting losses of vitamin A in stool.

	Ahmed [1990]	Halford thesis unpublished work [1993b].	Cawood Thesis [2003]
Subjects	12 controls 8-21y; 11 CF 8-24y	10 controls 3-16y; 10 CF 3-16y	6 controls 22-23y; 6 CF 17-39y
Intake of Retinol	CF - 0.71 mg/d (0.03- 1.5 mg/d) Control – 0.63mg/d (0.112-2.7mg/g) Total intake from 7 day weighed food intake (only 3 CF on multivitamins)	CF 2mg/d (1.06 - 4.85 mg/d) Control 0.72mg/d (0.28-1.39mg/d) (75% of vitamin A intake from supplements in CF)	CF - Median 1.2mg/d (0-3.4mg/d) from supplements. Dose of retinyl palmitate on day of study.
Method of assessment	Traditional gross balance techniques, habitual intake of vitamin A.	Traditional gross balance techniques, habitual intake of vitamin A.	Bolus dose of ~15mg labelled retinyl palmitate. Gross balance.
Loss of Vit A in stool	CF - 154μg/d* (14-726μg/d) Control – 12.5μg/d (7-65μg/d)	CF – 27.6μg/d* (0-660μg/d) Control – 0μg/d (0-1.56μg/d)	CF - 394μg/d* (0-1105μg/d) Control – 0.95μg/d (0-15.25μg/d)
Loss of Vit A as % intake	CF – 40%* (0.8-9.8%) Control – 1.8% (8.7-95%)	CF – 1.3%* (0.2-17%) Control – 0% (0-0.063%)	CF – 6.51%* (0-29.5%) Control – 0% (0-5.18%
Apparent availability of intake	CF – 60% Control – 98.2%	CF – 98.7% Control – 100%	CF – 93.49% Control – 100%
Lipid loss	CF – 9.6g/d* (1.4-17g/d) Control – 2.2g/d (1.1-5.6g/d)	CF - 16g/d* (1-79g/d) Control – 3.5g/d (0-6g/d)	CF – 11.9g/d (2.5-27.2g/d) Control – 6.2g/d (5-7.4g/d)*
Relationship with lipid	No relationship between losses	Association between lipid and retinol losses	Association between lipid and vit A losses

* Significantly different from controls

9.2.3 HYPOTHESIS III:

Finally the functional capacity of the liver to mobilise stored retinol was determined by measuring the extent of dilution of labelled vitamin A in the retinol bound to RBP pool (chapter 7 & 8). These studies excluded patients who were assessed to be poor compliers and / or poor absorbers. Enrichment of retinol within the circulating retinol pool appears to be generally low and in some cases below the limits of detection in CF whereas much higher levels of enrichment were consistently evident in healthy adults. Such a finding would arise if labelled vitamin A were diluted within a large hepatic pool of unlabelled vitamin A. These findings are consistent with an accumulation of vitamin A in the liver of patients with CF. Further evidence to support adequate to high stores of vitamin A in the liver of patients with CF was demonstrated by the use of the RDR. Following administration of the bolus dose of vitamin A to patients with CF, concentrations of plasma retinol post dose were not different from baseline, reflecting adequate stores.

Increased vitamin A stores could arise from increased net availability of retinyl palmitate from the use of supplements, which exceed the demands for vitamin A by cells and tissues. Increased stores could also be a consequence of a constraint to mobilise retinol from the liver. A constraint to mobilise retinol may either be a result of a shift in hepatic priorities during the acute phase response, or impaired liver metabolic machinery, which in some way affects the livers ability to adequately process and release vitamin A. This data supports hypothesis III in that low concentrations of retinol are a consequence of poor mobilisation of retinol from the liver. These observations fit with previous data reported by Underwood and colleagues [1971]; they found increased concentrations vitamin A in the livers of CF patients at autopsy. However this is the first time in which liver stores of vitamin A have been assessed indirectly in human subjects with CF concurrently with the assessment of availability across the GI tract and plasma concentrations of retinol.

Taken together, interpretation of these results suggest that:

- At the clinic in Southampton patients with CF exhibit of low circulating retinol concentrations when compared to the healthy adult population.
- Low concentrations of retinol are observed in the face of adequate to high intake of vitamin A (5 times RNI) and good adherence to supplement regimens.
- Low circulating retinol concentrations can not be simply attributed to dietary inadequacy or poor compliance with vitamin A supplementation regimens.
- Low concentrations of retinol are associated with raised inflammatory markers in patients with CF.
- Stool losses of vitamin A are elevated in patients with CF.
- Availability of the vitamin across the GI tract may be altered, particularly in those patients with continuing maldigestion and/or malabsorption on their habitual PERT.
- However as intakes of vitamin A are so high in patients with CF these modest losses of vitamin A can easily be overcome with supplements.
- Patients with CF have increased liver stores of vitamin A compared to healthy adults. This may arise from a constraint in the process of mobilising retinol in association with RBP from the liver to the peripheral tissues.

To summarise, when all the evidence is reviewed together it would appear that supply and availability of vitamin A are not limited in patients with CF at Southampton. The CF centre at Southampton General Hospital follows CF guidelines for patient management; it is typical of a well-managed specialist centre. Currently, a high proportion of patients with CF, in the UK, are managed within specialist centres. As a generalised statement it may therefore follow that dietary supply and availability is less likely to be the limiting factors associated with low concentrations of plasma retinol for all patients managed at specialist centres. It should however equally be considered that low circulating concentrations of retinol may reflect poor supply, poor availability, and poor compliance in patients cared for outside of these centres.

It should be acknowledged, at this point, the potential limitations and problems associated with the interpretation of the results presented within this thesis. Detailed metabolic investigations, by their nature can not be conducted on large numbers of subjects due to the complexity of the studies, the expense of the isotopic label and issues with recruitment. There are a number of implications related to studying relatively small numbers of patients. These can include; i) the fact that those studied may not truly reflect the population as a whole, particularly when the population is so heterogeneous in nature, and ii) in statistical terms the power of such studies is generally low often producing a large variance in results. There is a risk of misinterpreting the data, for example accepting the hypothesis when it is false or rejecting the hypothesis when it is true. It should however be accepted that results presented within this thesis fit with previous literature and if availability were the most important factor effecting concentrations of plasma retinol, differences would be discernible even in a small amount of subjects. Overall, to summarise, although individual figures and results may be challenged, when all the available evidence is considered collectively the findings appear to be consistent.

9.3 HOW DOES THIS WORK CONTRIBUTE TO OUR PRESENT UNDERSTANDING?

Whist reviewing the current evidence base, with the benefit of hindsight, it would appear that there is a mismatch between current recommendations in CF and scientific evidence. Figure 9.1 highlights the current literature with respect to vitamin A processing in CF. There is evidence of decreased availability of vitamin A across the GI tract and low retinol concentrations. However it would appear from autopsy specimens that patients with CF may have increased stores of vitamin A in the liver.

Figure 9.1: Vitamin A processing in CF current evidence base.

SUPPLEMENTATION OF VITAMIN A All PI patients with CF, and patients with low retinol concentrations are routinely supplemented with vitamin A.
AVAILABILITY OF VITAMIN A **Ahmed 1990** – Increased losses of retinol in stool compared to controls **Halford 1993** - Increased losses of retinol in stool compared to controls **James 1992** - Decreased excursions of retinyl palmitate compared to controls.
LIVER STORES OF VITAMIN A. **Underwood 1971, 1972** - Increased concentrations (2.5 times) of vitamin A in CF livers at autopsy compared to controls.
MOBILISATION OF VITAMIN A FROM LIVER - Reported low concentrations of retinol, along with increased liver stores from autopsy form the basis of the hypothesis that poor mobilisation of retinol from the liver in CF. - **Duggan 1996** - Proposal that depressed retinol concentrations during the acute phase response are a consequence of a shift in protein synthesis and lack of RBP for mobilisation
RETINOL AND RBP CONCENTRATIONS - A number of investigators, but not all, report low concentrations of retinol and RBP - **Duggan 1996** – Plasma retinol concentrations fall during infective exacerbations of CF. - No evidence if demands for retinol are increased in CF - No evidence of prevalence or clinical consequences.
FUNCTIONAL CONSEQUENCES - Reports of patients with CF developing visual disturbances Raynor *et al* 1989, Leguire *et al* 1992, Huet *et al* 1997, Tsinopoulos *et al* 2000. - **Ansari et al 1999** Dark adaptation was normal in all patients with CF. - No evidence of other functional consequences in CF for example effects on barrier function, and the ability to mount an appropriate immune response.

This thesis makes several original contributions to our understanding. Firstly, in the U.K. it is recommended that all patients with CF be supplemented with vitamin A, therefore dietary supply in patients with CF is high. The RNI for vitamin A in adults is 0.7mg/d and 0.65mg/d of retinol for men and women respectively, and intakes should not exceed 9mg/d (men) and 7.5mg/d (women) [Department of Health 1991]. In the UK population median intakes of vitamin A are 1mg/d and 0.85mg/d of retinol for men and women respectively [Gregory, 1990]. In the cohort at Southampton the median dose of vitamin A supplement was 1.2mg/d of retinol (1-3.6mg/d retinol), over and above that habitually consumed (~1mg/d). Therefore some patients with CF consume in excess of 4mg/d of retinol. This may be particularly important in children. It is recommended that children under the age of 1 year be supplemented with 1.2mg/d and over 1 year 1.2-3mg/d of retinol over and above habitual intake [CF Trust, 2002]. However intakes should not exceed 0.9mg in infants; 1.8mg from 1-3 years of age; 3mg from 4-6 years; 4.5mg from 6-12years old or 6mg in adolescents [Department of Health, 1991].

Secondly, this is the first occasion in which exogenous faecal losses of vitamin A as both retinol and retinyl palmitate has been assessed. Results suggest that whilst GI dysfunction may impair vitamin A availability, the additional increase in intake required to overcome these modest losses is considerably less than that achieved with routine supplementation. For instance, the greatest loss of vitamin A in stool was 30% of the intake, which was seen in a pancreatectomised patient, the majority of patients lost <15% of their intake. This data can be considered theoretically in terms of the availability of retinol to the body (table 9.2).

Scenario one is a healthy subject ingesting habitually 1mg/d of retinol at 100% apparent absorption, allowing 1mg/d to be available to the body. In order for CF patients to have less retinol available than healthy individuals they would need to consume the same habitual diet, with no supplementation, and excrete 30% of intake, (equivalent to the pancreatectomised patient) (scenario 2). If we consider CF patients to consume the lowest recommended dose of supplement (1.2mg/d) on top of their habitual diet, and loose 30% of the intake in stool, they would still have greater retinol available to the body than the control (scenario

3), and availability would be doubled if losses accounted for 10% of the intake (scenario 4). If patients with CF were supplemented 3.6mg of retinol (greatest supplement dose reported in chapter 4), the amount of retinol available to the body would be three and four times greater (assuming 30% and 10% losses respectively) than that seen in healthy adults (scenario 5&6). Theoretically in order for CF patients to have the same availability of retinol as healthy adults, losses would have to be in the region of 50% when patients are on low dose supplementation (scenario 7), and nearly 80% on high dose supplementation (50% greater losses than that seen in the pancreatectomised patient) (scenario 8).

Table 9.2 :

Theoretical calculations illustrating relationships between intake of vitamin A, loss in stool and availability of vitamin A to the body.

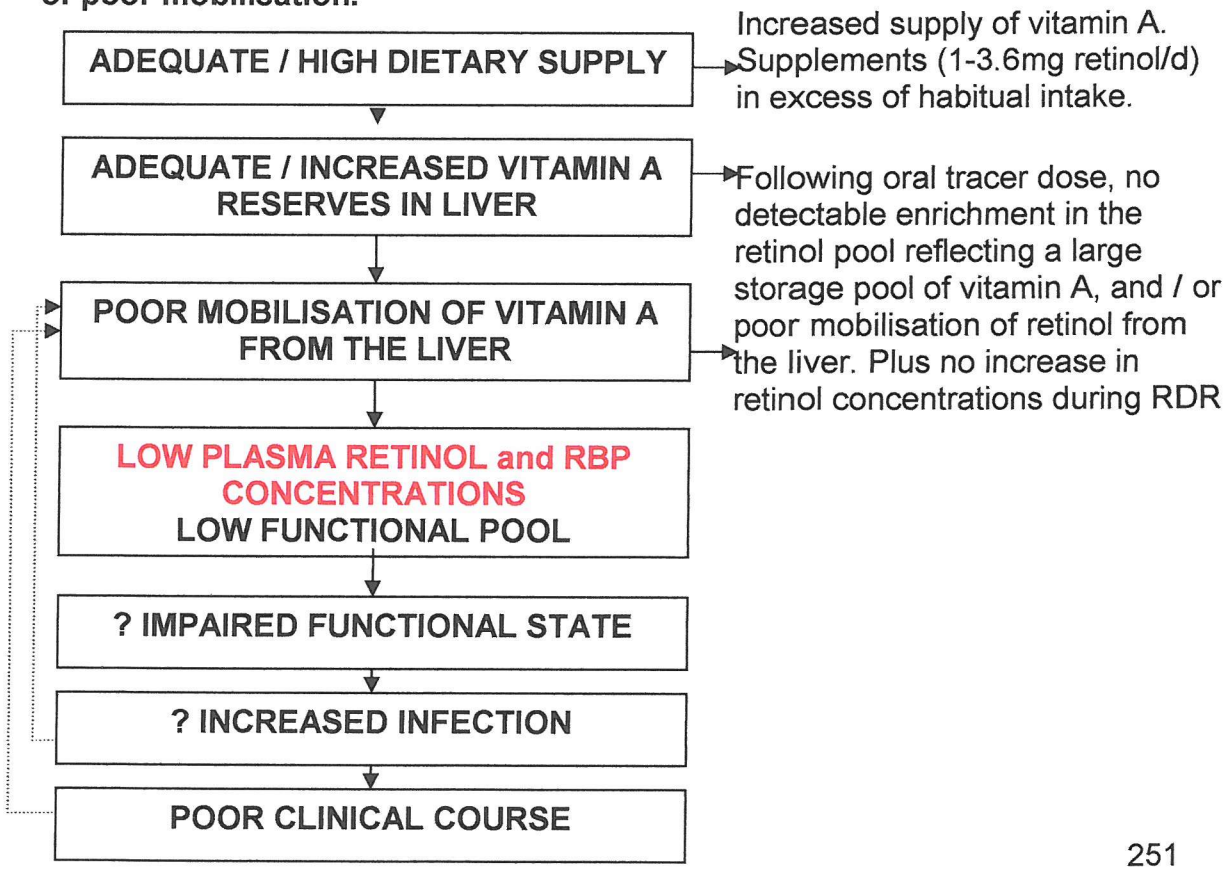
Scenario	Habitual intake mg/d	Supplement Intake mg/d	% Loss of intake	% Availability	Available to body mg/d retinol
1	1mg/d	0.0mg/d	0%	100%	1.00mg/d
2	1mg/d	0.0mg/d	30%	70%	0.70mg/d
3	1mg/d	1.2mg/d	30%	70%	1.54mg/d
4	1mg/d	1.2mg/d	10%	90%	1.98mg/d
5	1mg/d	3.6mg/d	30%	70%	3.22mg/d
6	1mg/d	3.6mg/d	10%	90%	4.14mg/d
7	1mg/d	1.2mg/d	55%	45%	1.00mg/d
8	1mg/d	3.6mg/d	78%	22%	1.00mg/d

Thirdly, it would appear that low plasma retinol concentrations relate to the clinical course of the patient although whether this reflects cause or effect is unclear. Finally, previously, elevated hepatic vitamin A concentrations were observed in post-mortem tissue at autopsy. In the absence of biopsied liver samples the ability to indirectly assess liver stores of vitamin A is valuable, and this is the first time that hepatic stores of vitamin A have been assessed in patients with CF. The inability to detect labelled retinol following tracer administration in patients with CF may reflect a large store of vitamin A within

the liver and/or a constraint in mobilisation compared to healthy adults. If a constraint in mobilisation of retinol from the liver is the primary cause of low circulating concentrations of retinol, continued supplementation of vitamin A may be inappropriate. For instance, if we assume an intake of 1mg/d at 100% absorption for healthy individuals; 1mg/d would be available to the body. In contrast if we assume a modest intake of 2mg/d at 70% absorption in CF, 1.4mg/d would be available to the body. On the assumption that both groups use retinol at the same rate it would follow that patients with CF would have an extra 0.4mg/d, 146mg/y, and 1.5g of retinol over ten years, which may be potentially harmful in conjunction with poor mobilisation from the liver. Chronic exposure of vitamin A may have detrimental effects on both the function of the liver, and the function of cells that require vitamin A. In conclusion, this research highlights the need to review current practice in order to prevent potential harm from excessively high intakes. With these findings in mind an adapted casual chain illustrates how low concentrations of retinol occur in the face of an adequate to high dietary intake (figure 9.2).

Figure 9.2:

Low retinol concentrations unrelated to effective supply, a consequence of poor mobilisation.



9.4 IMPLICATIONS FOR CLINICAL PRACTICE.

Current CF guidelines in the UK have been developed to inform and standardise practice [CF Trust, 2002]. They recommend; a) prophylactic oral vitamin A supplementation (1.2-3.0mg of retinol per day) to all CF patients with pancreatic insufficiency, b) that plasma concentrations be reviewed annually and supplement dose adjusted according to plasma concentrations, and c) supplementation commenced in pancreatic sufficient patients with low retinol concentrations [CF Trust Nutrition Working Group, Consensus Report, 2002]. The observations made within this thesis have important implications for clinical practice. For instance, when mobilisation is the primary constraint, continuing to persist with high oral intakes of vitamin A may result in potentially toxic accumulation of retinol. Increased liver stores may however not necessarily increase the supply of retinol to peripheral tissues. There is a pressing need to review guidelines associated with vitamin A supplementation in CF. The recent Expert Panel on Micronutrients commissioned by the Department of Health restated the consensus position reached by a previous COMA panel [DoH 1991, report 41], which highlighted the dangers of even modestly elevated intakes of vitamin A over prolonged periods in children and adults. These issues have not been adequately addressed in CF.

There is a need to reflect whether such an approach is justified and a number of lines of evidence will now be discussed. Firstly, initial reports of low plasma retinol concentrations were observed in patients when the fat content of the diet was restricted and PERT was relatively crude and inadequate. Current dietary practice recommends a more liberal approach to the fat content of the diet and PERT has developed to the point where the degree of maldigestion and malabsorption has been greatly reduced. Thus, even though losses maybe elevated, whether such high intakes are necessary to compensate for modest losses appears unlikely on theoretical grounds alone. This has been supported by the data presented in this thesis.

Secondly, low concentrations of plasma retinol continue to be reported even in patients who eat well, take high doses of vitamin A supplements and have established effective PERT to limit malabsorption [Lancellotti *et al* 1996; data reported in this thesis]. Current high dose supplementation may only be

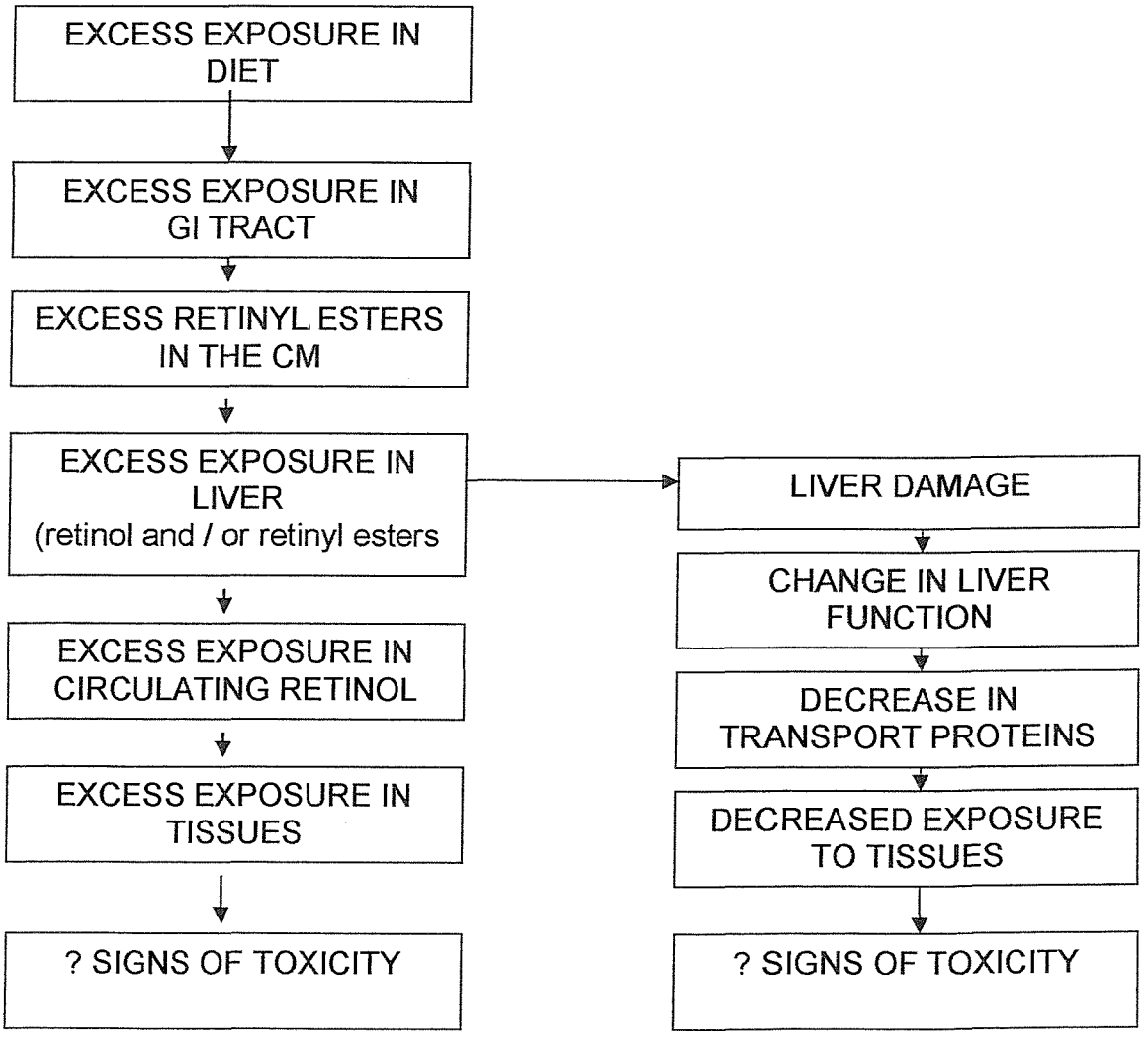
appropriate in patients whose dietary supply and / or availability in constrained. Measures of low plasma retinol concentrations are measures of a biochemical deficiency not a dietary deficiency; it may only be necessary to intervene in the face of a dietary deficiency. For this reason low plasma retinol concentrations may not always require correction by increasing the intake of vitamin A within the diet. All factors that contribute to low circulating concentrations of retinol require consideration before changing current therapy. A low plasma retinol concentration may simply require a reduction in demand for the vitamin by managing the infective process. For instance during infective exacerbations plasma retinol concentrations are depressed but rise again following antibiotic treatment of the exacerbation.

Thirdly, it is well accepted that large amounts of retinol cause harm, particularly in terms of damage to the liver and bone. Symptoms of chronic vitamin A intake include dry thickening skin, itching, cracking lips, brittle nails, bone and joint pain, and reduced bone mineral density [Expert Group On Vitamins And Minerals, 2002]. It is unclear whether these symptoms relate to high concentrations of vitamin A at the cellular level or low concentrations of vitamin A at the level of the cell (figure 9.3). For instance increased stores of vitamin A in the liver, could lead to increased circulating retinol concentrations and excess exposure of vitamin A at the level of tissues and cells which may cause the documented symptoms of high intake. However similarly increased liver stores could cause liver damage, alter liver function, cause a decrease in hepatic secretary proteins, which in turn would cause low plasma retinol concentrations and decreased exposure of vitamin A at the level of cells and tissues.

The term hypervitaminosis A is commonly used in the literature to describe toxicity of vitamin A. However, it is unclear what biological process this is meant to reflect. For instance does it reflect; i) high intake, ii) high concentrations of retinyl esters, iii) high stores of vitamin A, iv) high circulating concentrations of retinol, or v) high amounts of retinol at the functional cell? Or could all of these contribute. Expert committees in Europe and USA have repeatedly stressed that harm arises from chronic ingestion of retinol or retinyl esters, not necessarily in large amounts but sufficient over a period of time to build up stocks that exceed the liver's ability to destroy or store them. Hepatotoxicity

may therefore reflect a high intake of vitamin A that exceeds the storage capacity of the liver. Children are believed to be at a greater risk than adults to the toxic effects of retinol and at particular risk of prolonged exposure to even moderate amounts of vitamin A. The Department of Health recommend that regular intakes should not exceed 0.9mg/d in infants, 1.8mg/d between the ages of 1 to 3 years, 3 mg/d from 4 to 6 years, 4.5 mg/d from 6 to 12 years and 6 mg/d for adolescents whilst upper safe limits for adult men and women would be 9 mg/d and 7.5 mg/d respectively. Therefore, given that even very young patients with CF are recommended to consume 1-3 mg/d of retinol, over and above that habitually consumed, and will do so throughout their life, the potential harm associated with accumulating retinol within the body must be considered.

Figure 9.3. : What is vitamin a toxicity?



Fourthly, plasma retinol concentrations fall in the face of infection [Semba *et al* 2000; Filteau *et al* 1993; Christian *et al* 1998b; Filteau *et al* 1995; Stephensen *et al* 2000; Rosales *et al* 2000; Paracha *et al* 2000], and this phenomenon has been observed during exacerbations of CF [Duggan *et al* 1996; data presented in thesis]. It should be noted that these observations occur during acute exacerbations, and the effect of the ongoing more modest chronic inflammatory response in CF has not been considered. The fall in retinol concentrations during acute inflammation has traditionally been interpreted to be a consequence of a shift in hepatic protein synthesis. Synthesis of nutrient transport proteins such as RBP may be sacrificed in order to increase the synthesis of other proteins required to fight infection. Thus high intakes of vitamin A, in the face of compromised mobilisation, from the liver during infection may lead to a greater rate of accumulation of vitamin A within the liver. It should, however, be considered that low retinol concentrations may not only be an effect of a shift in hepatic priorities during infection. In theory low retinol concentrations during infection could be a consequence of a number of factors, which include:

1) Altered hepatic protein synthesis

During infection the liver may preferentially synthesise acute phase proteins at the expense of other proteins, therefore retinol concentrations fall even though liver stores maybe adequate.

2) Increased losses of vitamin A.

Data has reported increased losses of retinol in the urine of patients with pneumonia and sepsis [Stephensen *et al* 1994; Mitra *et al* 1998].

Retinol maybe either be preferentially cleared from the circulation to deprive pathogens of the micronutrients they need for proliferation, or a lack of TTR during infection may allow the retinol:RBP complex in the circulation to be excreted.

3) Increased loss of RBP.

RBP maybe lost into the extra cellular fluid due to increased vascular permeability [Thurnham *et al* 1991].

4) Increased uptake by cells and tissues.

During infection cells and tissues may require increased amounts of retinol.

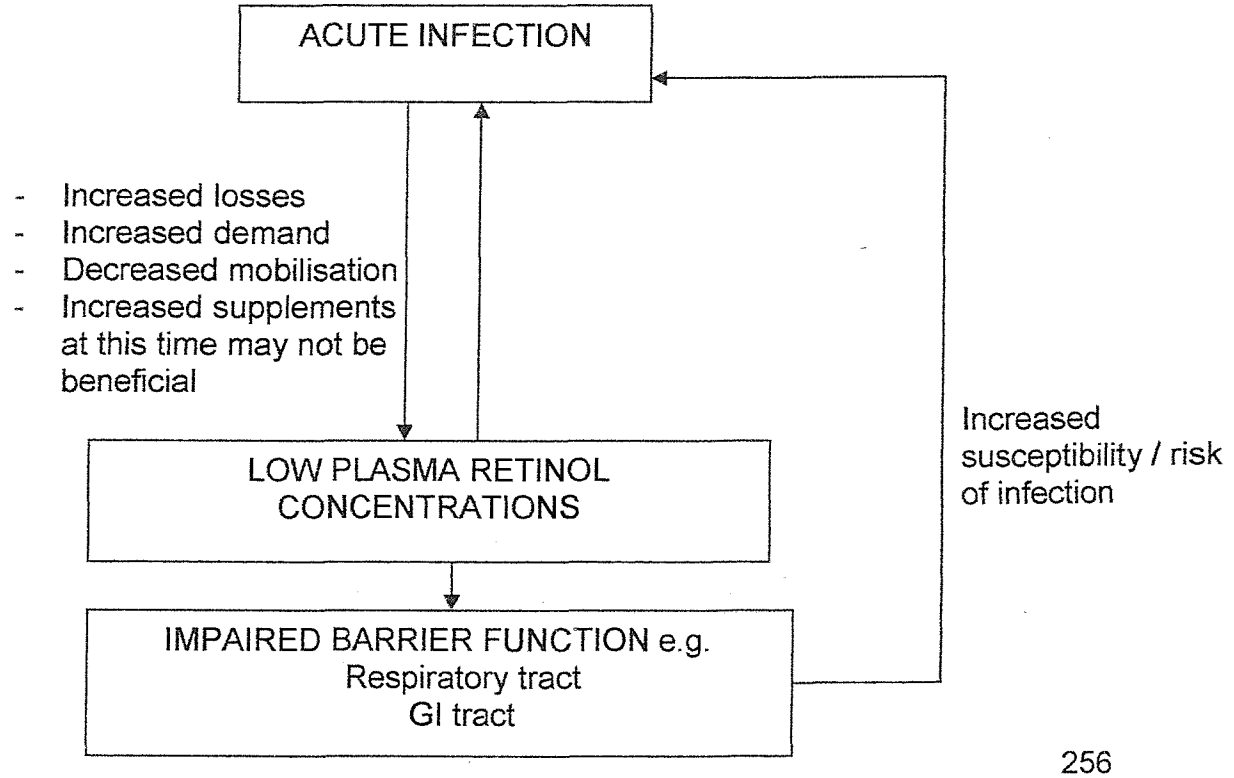
- Retinol maybe required to repair tissue damage caused by the infective episode [Thurnham *et al* 1991].
- Retinol may move to sites to participate in the host defence response to infection

An increased uptake of retinol to tissues resulting in low concentrations of retinol maybe a beneficial adaptation to the infection, and supplementation in the face of low retinol concentrations maybe detrimental during infection (discussed in more depth in following section).

Recurrent infections in CF may be a consequence of a vicious cycle (figure 9.4). For example acute infection for whichever reason depresses plasma concentrations of retinol in the circulation. As a consequence there may be an inadequate supply of vitamin A for cells and tissues. This can lead to impaired function of barriers, particularly in the GI and respiratory tract, which allows the immune system to be more susceptible to infection, and the cycle starts again. Each cycle could potentially further impair barrier function.

Figure 9.4:

The vicious cycle of recurrent infective exacerbations in CF.



CF guidelines state that supplement dose should be adjusted according to plasma concentrations [CF Trust Consensus Report, 2002]. However in the light of evidence relating to low concentrations of retinol during acute infection this may be inappropriate, and supplementation during infection may even be detrimental. If plasma retinol concentrations are low a number of avenues should be explored before any adjustment to supplement dose (figure 9.5). For instance, low plasma concentrations of retinol could be the consequence of, an acute infective episode, poor diet, poor compliance, or poor availability, all of which can be addressed before an increase in supplement dose is implemented. If the patient is a poor eater total food intake can be improved, if supply is not an issue but the patient has poor availability then the PERT regimen can be improved. A key aspect of the proposal is monitoring plasma retinol concentrations after any alteration. To summarise plasma retinol concentrations should be monitored with a marker of inflammatory status before any adjustment to supplement dose is made. Further investigations relating to the effect of the inflammatory response, the effect of raising retinol concentrations, and the potential of hepatotoxicity in patients with CF will further increase understanding.

There is a need to determine the capacity of the liver to mobilise retinol in patients with CF and it would be beneficial to determine the extent to which mobilisation occurs both with and without infection. This information would enable factors that are associated with low concentrations of retinol to be further investigated and may enable us to determine if adaptations are detrimental or beneficial to the host. Evidence from only one report highlights that liver stores of vitamin A are increased in CF in the face of low concentrations of retinol in the plasma [Underwood *et al* 1972]. Alongside this, evidence from the studies reported in this thesis highlight the potential of increased vitamin A stores in the liver. There is an urgent need to characterise liver vitamin A stores in patients with CF, particularly given that intakes of vitamin A have substantially increased over the last 30 years.

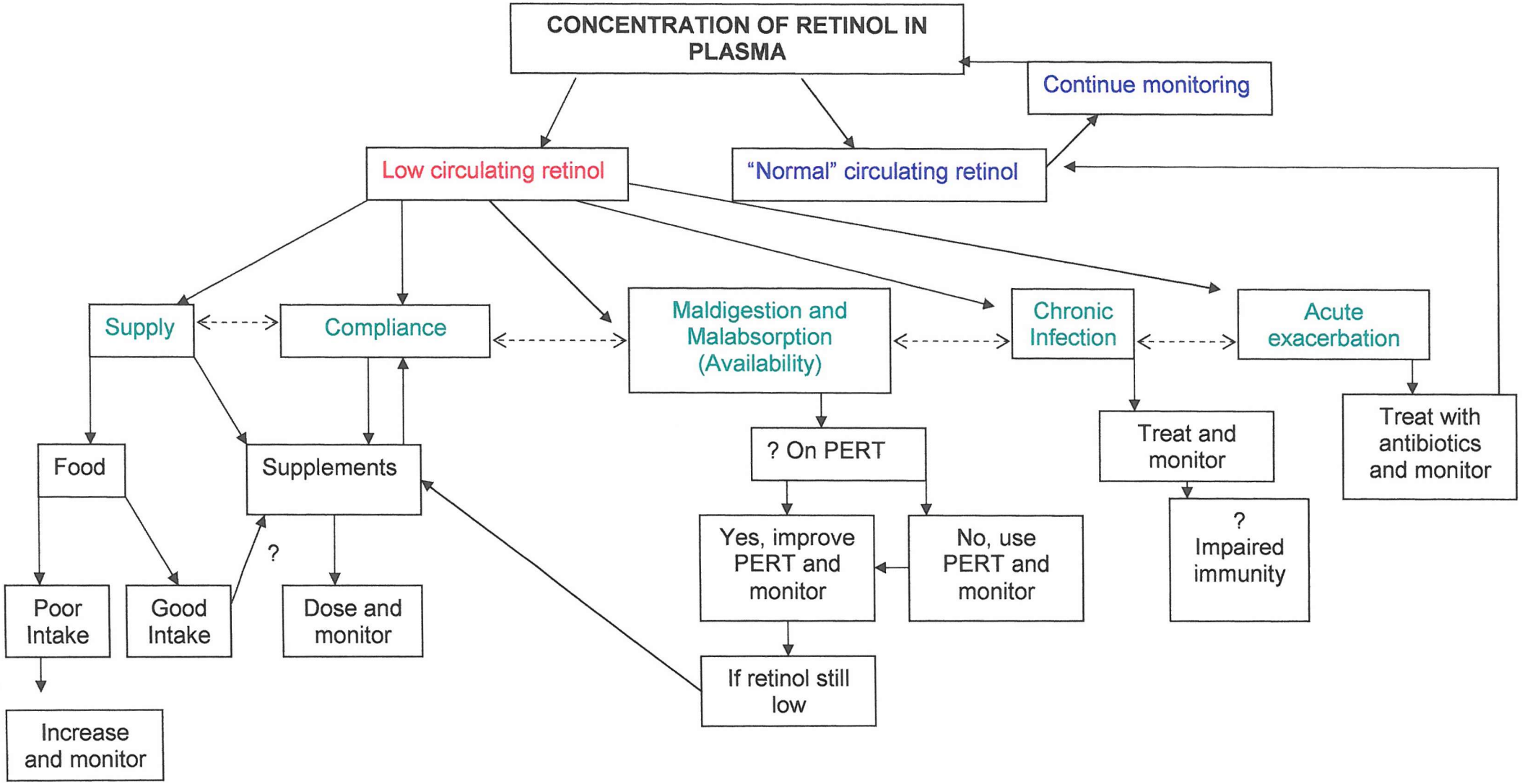


Figure 9.5: A conceptual framework for the management of vitamin A in patients with CF.

Finally, recent work has challenged the view that a reduction in plasma retinol is a consequence of the acute phase response, suggesting that the fall may be a necessary component of the body's response to infection. The T-helper type-1 (Th-1) response is macrophage dominated and would result in increased IFN-γ, IL-2 and TNF. The T-helper type-2 (Th-2) response is neutrophil dominated and would be associated with increased IL-4, IL-5 and IL-10. As retinol is known to drive the immune system towards a Th-2 dominated response, a fall in retinol may switch the system towards a Th-1 dominated response [Stephensen *et al* 2002]. The proposal that such a response may be more favourable to the host is supported by the observation that attempts to increase plasma retinol concentrations in the face of both respiratory infection and pneumonia by supplementation actually worsens, rather than improves, the clinical course [Stephensen *et al* 1998; Bresee *et al* 1996]. Thus high intakes of vitamin A in the face of chronic infection may even be detrimental and this phenomenon is illustrated in figure 9.6. Hypothesis one, relates to the prevailing belief in CF that decreased availability is associated with low plasma retinol concentrations. A lack of retinol is reflected in a Th-1 type response, which in itself may affect the clinical course of the patient. Hypotheses two and three could arise in the face of increased intake and increased liver reserves, as proposed in this thesis. Hypothesis two reflects an instance of increased plasma retinol, which will switch the system to drive a Th-2 type inflammatory response, which in itself may be detrimental to the host. The proposal from the literature is that a Th-1 type response may be most favourable to the host (hypothesis three). In this case, as previously discussed, additional supplements may not be beneficial. It is not known which response is the most appropriate, or which response would produce the worse clinical outcomes. The effect of retinol on the corresponding inflammatory response and the effect clinically to the patient requires further investigation in CF.

In summary, these observations support the view that there is an urgent need to reconsider the current approach to managing vitamin A metabolism in patients with CF, as current guidelines are not justified by evidence. Firstly we do not know what the true requirement is, nor do we know how much you should eat to satisfy that requirement. Given the heterogeneity of the condition, one dose may not fit all so a structured approach to the problem is required.

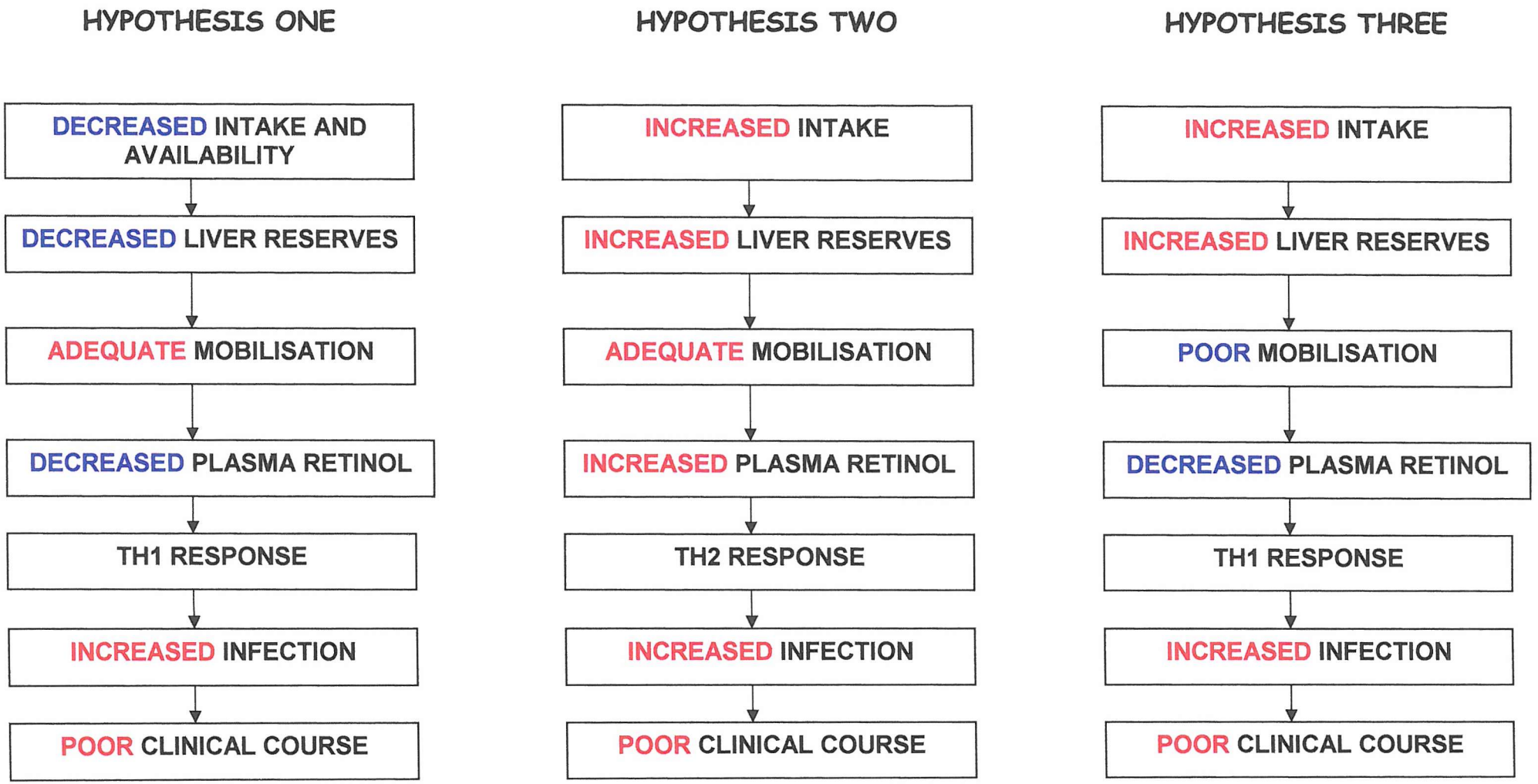


Figure 9.6: Hypotheses relating to the effect of retinol on the inflammatory response and clinical course.

One important issue is how to most appropriately assess status. An adequate vitamin A status has traditionally been accepted as reflecting an adequate intake to sustain an adequate liver store, which is able to maintain functional requirements of the cell. However plasma retinol concentrations, which are often used as a proxy measure of status, may not be an appropriate marker in patients with CF, due to a constant inflammatory burden. For instance during infection the functional pool of vitamin A may be low but the storage pool may be high, a biochemical deficiency in the face of a large storage pool.

In CF, a low plasma retinol has been seen as requiring correction with supplements, in the belief that availability is limiting. Rather than this supply-led concept, a more appropriate model may be that of a demand-led process, and status is at the level of the cell not the storage pool (figure 9.7). For example, poor vitamin A status of the cell could arise from a low circulating retinol, which is a consequence of low liver stores, low postprandial retinyl esters, and poor availability. However poor vitamin A status of the cell could also arise from a constraint to mobilise high liver stores from increased intake. Consideration of the effects of high intakes therefore should not be limited to the effect in the liver but should also reflect an increase of vitamin A in one or more of the vitamin A pools. For example too much retinol in the diet, leads to too much retinyl esters in the first transport pool and too much vitamin A in the storage / functional pool. An increased storage pool could cause direct liver damage, or indirect damage to non vitamin A related processes. If the system has no regulation or control this could allow too much retinol in the circulating pool and cells become over exposed to vitamin A and produce an inappropriate response. However it may not be too much vitamin A in one or more of the pools that is the issue but simply an inappropriate distribution of vitamin A between the pools. These issues may however become irrelevant if there is control in the system (figure 9.7). For instance, if there were a control mechanism affecting the uptake of retinol to target cells, the amount of vitamin A within the functional cell would be regulated, so even if retinol in the transport pool were in excess the cell would be regulated to its requirement. However if the plasma retinol pool is low, there may not be enough vitamin A entering the cell to meet its functional requirements. This may occur in CF, as these patients have high stores of vitamin A in the liver in the face of low concentrations of retinol in the plasma.

More functional markers, and an understanding of the demand for vitamin A is required to better assess the status of the cell in CF. Along with determining functional measures of vision, other functions require to be determined, for instance barrier function, and immunity.

It should be considered that vitamin A might not be the only nutrient having an effect on the processing of vitamin A, or the patients clinical course. Until now vitamin A has been considered in isolation as part of a direct pathway, in that low concentrations of retinol affect the clinical course of the patient (figure 9.2). However the overall micronutrient status of the individual may play a pivotal role. For example there may be interaction with other nutrients in the pathway and alterations to circulating concentrations of retinol may serve as a non-specific marker of other processes. Figure 9.8 illustrates two hypotheses with respect to how clinical outcomes of patients with CF may be altered. Hypothesis one illustrates that an inadequate availability of retinol will not meet the requirement of the functional cells and tissues and will affect the immune system and alter the clinical course of the patient. However this effect may not simply be due to vitamin A in isolation, but moreover inadequate availability of a number of micronutrients (hypothesis 2).

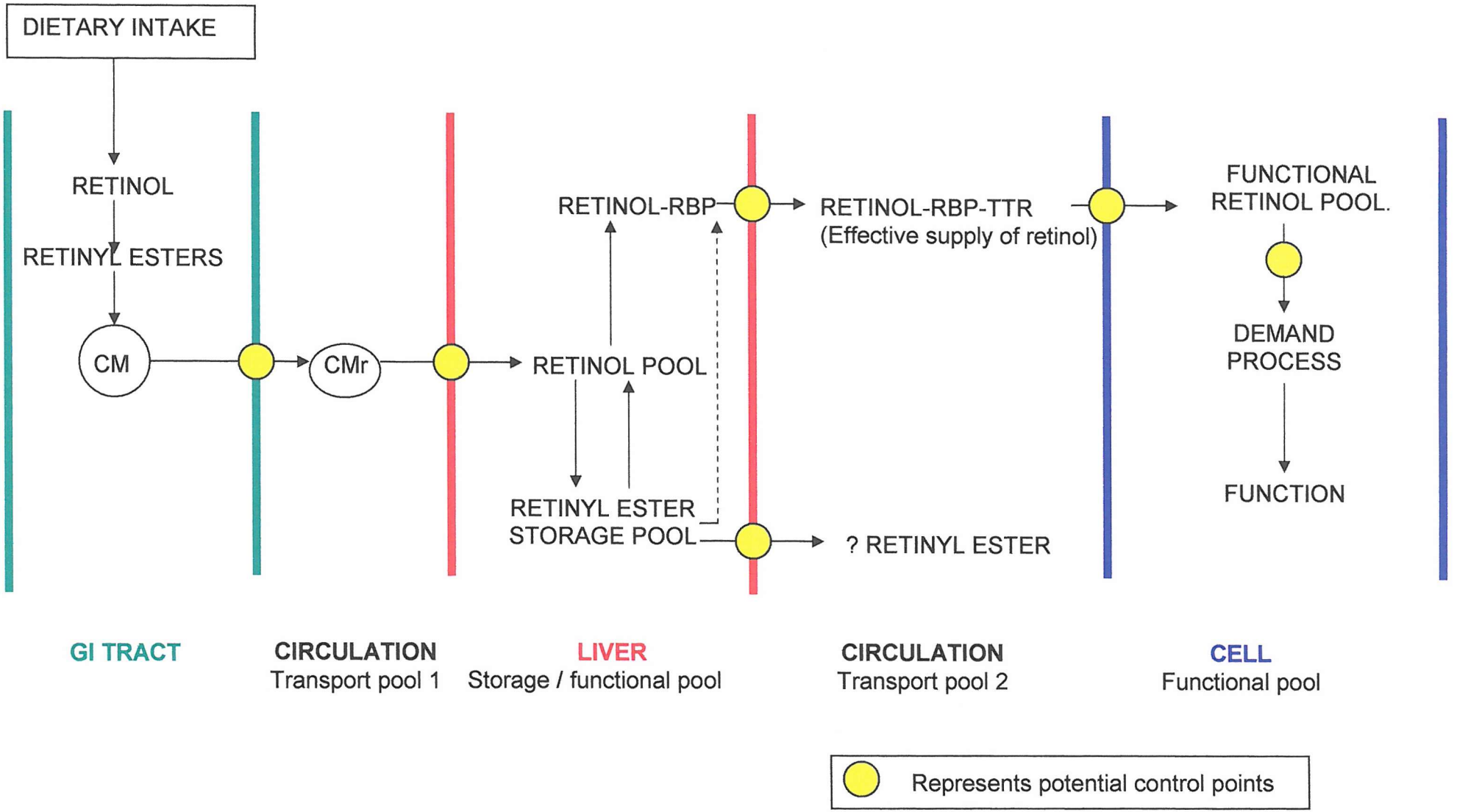


Figure 9.7: A demand led process, vitamin A status at the level of the cell.

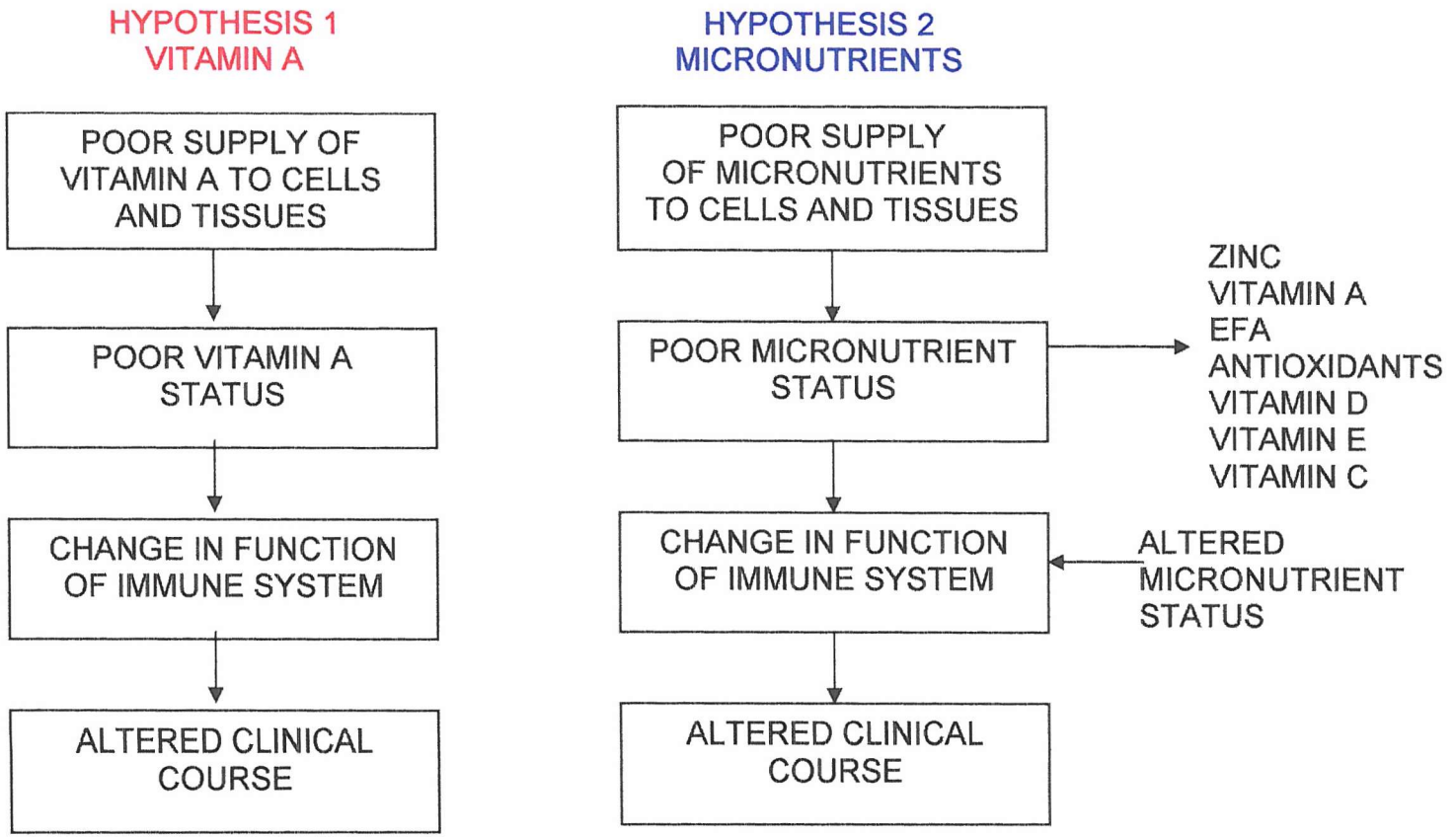


Figure 9.8: Effects related to vitamin A in isolation, or overall micronutrient status.

Throughout this discussion the processing of vitamin A has been considered to affect the disease process, it may however be the disease process, which affects the nutritional status of the patient (figure 9.9). The disease CF may in itself disrupt the metabolic handling of nutrients and this could affect the immune response. An activated immune system may alter nutritional intake, alter availability, alter mobilisation, alter excretion and alter the demand for nutrients. This disruption in the processing of nutrients, will affect the overall micronutrient status of the individual. Low circulating retinol concentrations reported in this thesis may simply mark an altered micronutrient status, and other micronutrients should also be assessed in the framework.

At this stage of the discussion it maybe important to highlight how research from this thesis could contribute to wider issues. The framework in which the processing of vitamin A in CF has been addressed could be applied to the processing of other nutrients in the disease CF. In particular other fat-soluble vitamins (vitamin E and D), and nutrients that are carried in the circulation bound to transport proteins of hepatic origin. The application of the model may also be beneficial when applied to other diseases. For example the framework could be equally applied to diseases like HIV, Crohns Disease, and Primary Biliary Cirrhosis.

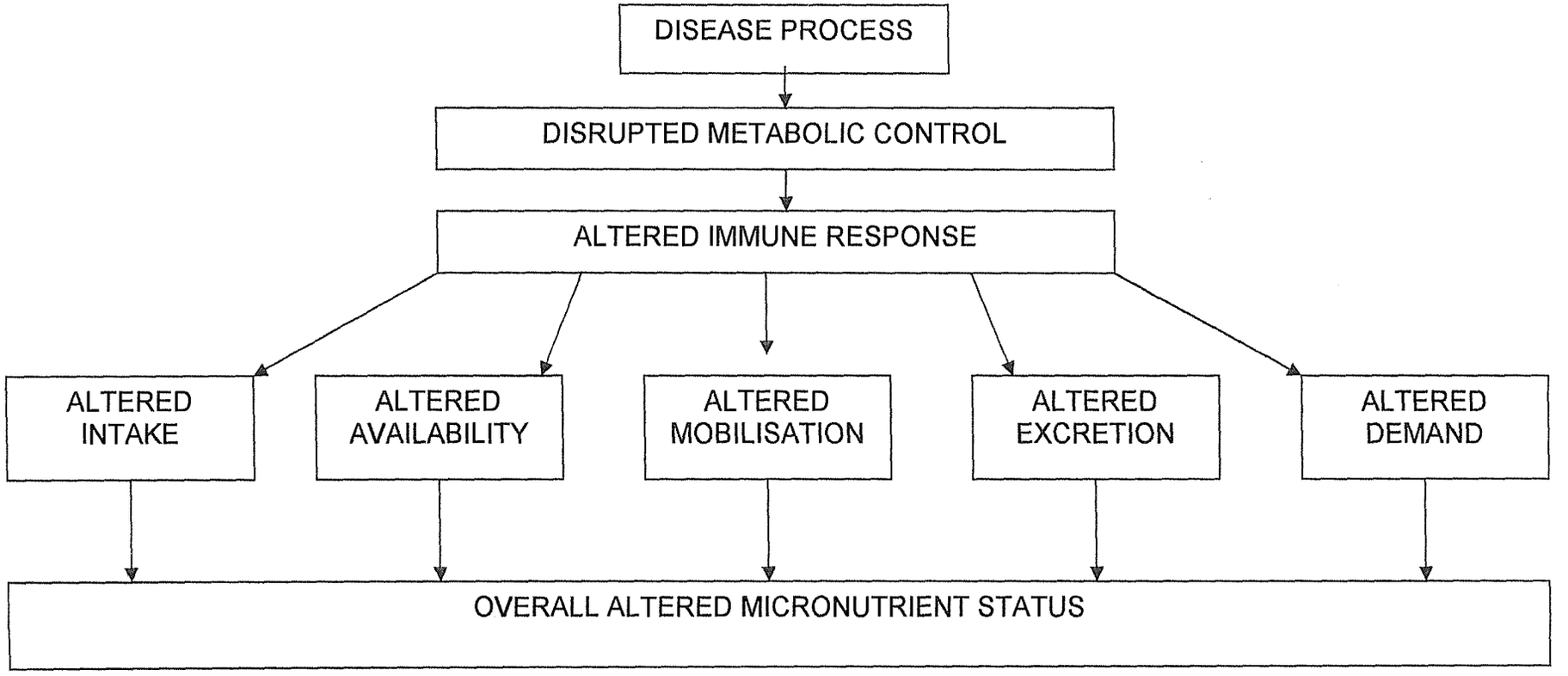


Figure 9.9: How the disease process may affect micronutrient status.

9.5 FURTHER STUDIES IN CF PATIENTS

A number of issues have been raised throughout this thesis relating to further studies in CF. Additional studies in CF may enhance the scientific evidence on which to base clinical practice. Before addressing issues relating to other nutrients in CF, and vitamin A in other diseases, further research is required into vitamin A metabolism in CF. Future areas of research which would help to aid the understanding of vitamin A metabolism in patients with CF include, liver storage and mobilisation of vitamin A, urinary excretion of vitamin A, along with functional and clinical consequences. These areas will be briefly discussed in the following section.

Liver storage and mobilisation.

Further work relating to the handling of vitamin A in the liver would provide a greater understanding in this area and future work has already been funded by the CF Trust. This work will assess if there is a constraint on RBP (and TTR) synthesis in patients with CF compared to healthy adults. The studies will determine the fractional synthesis of the protein following administration of a labelled amino acid. The ability to mobilise vitamin A from the liver will be reflected in the isotopic enrichment of the circulating pool of retinol and in the rate of hepatic protein synthesis. These studies will enable us to measure the fractional and absolute synthetic rate of RBP and TTR compared to other hepatic secretary proteins. This information will allow us to determine if effects are general or vitamin A specific.

Other future work associated with liver storage is related to the potential that patients with CF have increased liver stores, from possible high intakes, poor mobilisation or liver damage. It would be interesting to assess the vitamin A content in livers of adult patients who receive routine PERT and prophylactic supplementation of vitamin A, to determine if current practices are associated with elevated stores of vitamin A.

Urinary excretion.

The work presented within this thesis alludes to the potential of increased urinary losses of vitamin A in patients with CF, which may account in part for low concentrations of plasma retinol, however this has not been investigated.

Investigating excretory losses of vitamin A (as both retinol and retinyl glucuronides) would be important to determine if low concentrations of plasma retinol are associated with increased losses, which may require correction.

Functional and clinical consequences.

Evidence from this thesis provides an insight into the potential relationship between retinol concentrations and clinical outcomes, however there is no direct evidence to determine functionally if low concentrations of vitamin A matter. Functional consequences relating to low concentrations of retinol require assessment in CF in order to inform clinical practice. As well as assessing the potential visual consequences, such as dark adaptation, other functions of vitamin A should also be considered, for example affect to immune competence and epithelial barriers.

Collecting information as part of a well-designed study in a large number of patients with CF may provide information to address questions relating to functional consequences, immune competence and clinical outcomes. Best achieved within a multi-centred study, to include patients from specialist centres along with those who receive care outside of these centres. Associations could be explored in a subdivided population for instance patients with low concentrations, patients with normal concentrations and those with high concentrations in order to discern commonalties and differences.

Some of the potential factors to be assessed could include:

- Overall micronutrient status
- Adherence to practice
- Assessment of malabsorption
- Dietary intake
- Plasma concentrations of retinol and RBP, & other micronutrients
- Concentrations of markers of infection.
- The function of immune effector cells
- Number of admissions to hospital
- Number of exacerbations of CF
- Functional measures – visual, barrier function.
- Functional measures of other fat-soluble vitamins.

This type of study may enable us to address a number of questions associated with vitamin A and other micronutrients. With respect to vitamin A some of these questions could include:

- Within a given CF population what is the prevalence of low concentrations of plasma retinol and how are the patients distributed within the population? For example; poor supply, poor compliers, poor absorbers, and infection.
- Are low concentrations of retinol a cause or consequence of the immune response?
- Are low concentrations associated with the clinical course solely because low concentrations are a response of an activated immune system?
- Do low concentrations cause a change in function which effects clinical course, or does a change in function cause concentrations of retinol to fall and effect clinical course?
- Do low concentrations of retinol affect the response to infection or does infection effect nutrient metabolism?
- Do low concentrations of retinol have a direct effect on immune cells, or do they affect magnitude of response?
- Is the decrease in plasma concentrations of retinol during infection a desirable effect that does not require correction with supplements?
- Which type of T helper response dominates in CF?
- Is the T helper response affected by circulating concentrations of plasma retinol?

What to supplement?

It is important to address what is the most appropriate supplementation regimen for patients with CF. This information is of particular importance to individuals involved in the day-to-day clinical management of patients. As all patients with CF are prophylactically supplemented with vitamin A from diagnosis, addressing this particular issue will prove difficult. Whilst it may be considered unethical to supplement patients with placebo, as this is against the principle of the guidelines, undertaking a randomised dose controlled trial may be more appropriate. For example lowering the dose of vitamin A supplement received and examining the effects on concentrations of plasma retinol, immune competence, epithelial integrity, other functional markers and clinical outcomes.

9.6 OVERALL CONCLUSION:

In the CF clinic at Southampton, the majority of patients are routinely supplemented with vitamin A over 4 times the RNI. Despite this, nearly half the patients with CF have low concentrations of plasma retinol, which appear to be associated with the patient's clinical course. Evidence presented within this thesis indicates that patients with CF have greater losses of vitamin A in stool when compared to healthy adults. However, in contrast to the belief, dietary supply, and availability do not account for all the differences seen in plasma retinol concentrations. In fact, patients with CF may have a greater availability of vitamin A to meet their requirements when compared to healthy individuals. Preliminary findings suggest that the limiting factor in the handling of vitamin A, in patients with CF, occurs at the level of the liver. Results indicate that patients with CF have increased stores of vitamin A in the liver, which may be associated with a constraint to mobilise retinol. In this instance, high dose supplementation may be detrimental to the patient. One other important consideration is that an activated immune system plays an important role in determining plasma concentrations of retinol, which may further be acting to constrain mobilisation. With this in mind it may be inappropriate to supplement vitamin A to patients with infection-induced hyporetinolemia.

In light of the current evidence there is a pressing need to review present guidelines associated with vitamin A supplementation in CF. It would appear that correcting low plasma retinol concentrations with the use of supplements may not be the primary factor that requires consideration, but more importantly the potential high intakes of vitamin A.

Nutritional considerations need to move beyond simple dietary supplementation to understanding how the disease process affects the partitioning of nutrients to meet the metabolic demands of the individual.

APPENDIX 3.1 – LABORATORY EQUIPMENT LIST

ISOTOPES

[1,1,1-^{13}C] Tripalmitin **(TP)**	Masstrace Inc., Woburn, USA
[10,19,19,19] ^{2}H-retinyl palmitate **(d_4RP)**	Cambridge Isotope Laboratories, Andover, MA, USA

BLOOD COLLECTION

10ml K3E EDTA tubes	BD Vacutainer, Plymouth, UK
5ml SSP (serum separating tubes)	BD Vacutainer, Plymouth, UK
10ml lithium heparin vacutainer tubes	BD Vacutainer, Plymouth, UK

VITAMIN A STANDARDS AND QUALITY ASSURANCE

All-trans retinol	Sigma Aldrich Co. Ltd., Poole, Dorset. UK.
Retinyl acetate	Sigma Aldrich Co. Ltd., Poole, Dorset. UK.
Retinyl palmitate	Sigma Aldrich Co. Ltd., Poole, Dorset. UK.
EQA scheme, monthly serum	Department of Chemical Pathology, Carshalton, Surrey
Human standard reference material	Chromsystems, Munich, Germany

GENERAL SUPPLIES AND EQUIPMENT

HPLC grade water	Fisher Scientific, Loughborough,U.K
Methanol. HPLC grade	Fisher Scientific, Loughborough,U.K
Propan-2-ol. HPLC grade	Fisher Scientific, Loughborough,U.K
Chloroform. Residue analysis grade	Fisher Scientific, Loughborough,U.K
Hexane. Residue analysis grade	Fisher Scientific, Loughborough,U.K
1-(trimethylsilyl)imidazole (TMSI)	Sigma Aldrich Co. Ltd., Poole, Dorset. UK.
Foodbase.	Foodbase, Institute of Brain Chemistry and Nutrition, London.
Freeze-drier	Genevac, Ispwich
BondElut aminopropyl SPE cartridge	Varian, Walton-on-Thames, UK
Infinity triglyceride reagent	Sigma Aldrich Co. Ltd., Poole, Dorset. UK.

RBP and TTR

Nephelometer	Dade Behring Ltd, Milton Keynes, UK.
Antiserum Human Prealbumin	Dade Behring Ltd, Milton Keynes, UK.
Antiserum Human RBP	Dade Behring Ltd, Milton Keynes, UK.
N protein standard SL	Dade Behring Ltd, Milton Keynes, UK.
N diluent	Dade Behring Ltd, Milton Keynes, UK.
N/T protein controls low, medium and high	Dade Behring Ltd, Milton Keynes, UK.

HPLC equipment – PLASMA

System Gold 508 autosampler	Beckman Coulter UK Ltd, High Wickman, Bucks, UK.
System Gold 125 solvent delivery system	Beckman Coulter UK Ltd, High Wickman, Bucks, UK.
UV SP8480 XR absorbance detector	Spectra physics, UK
C18 ODS, APEX 2, 5 μm, 30cm x 4.6 mm	Jones Chromatography, Hengoed, U.K
2 cm guard cartridge packed with Perisorb 18	Anacham, Luton, U.K.
32 karet software.	Beckman Coulter UK Ltd, High Wickman, Bucks, UK.
Fraction collector SC100	Coulter UK Ltd, High Wickman, Bucks, UK.

HPLC equipment – STOOL

Hewlett-Packard 1050 series quaternary pump	Hewlett-Packard, Wokingham,Berks,UK.
Spectrophotometer detector	Hewlett-Packard, Wokingham,Berks,UK.
20μL loop injector	Rheodyne L.P., California, USA
Supelcosil ODS (5μ, 25cm x 4.6 mm)	Supelco, Sigma Aldrich Co. Ltd., Poole, Dorset. UK.
μBondapak C18 guard column	Waters, Elstree, Hertsford, U.K.

GCMS

HP 6890 Gas chromatograph (split/splitless injector)	Hewlett Packard, Wokingham,Berks,UK.
HP 5973 Mass detector with turbomolecular pump	Hewlett Packard, Wokingham,Berks,UK.
HP Chemstation software.	Hewlett Packard, Wokingham,Berks,UK.
Column, BPX5 (30m × 250μm i.d., 0.25μm film:	SGE Europe (UK))

Carrier gas, 2ml/min helium;Injection volume, 1μl; injector, splitless, 230°C; oven, 180°C for 2 min then 40°C/min to 280°C.

GC-C-IRMS

ORCHID GC-CIRMS	Europa Scientific Ltd, Crewe, U.K.
50mX0.25μmX0.32nn fused silca capillary column	BPX-70; SGE Eurpoe Ltd, Milton Keynes, U.K.
HP6890 GC	Hewlet Packard, Wokingham,Berks,UK.
Orchid IRMS interface	PDZ-Europa Scientific Ltd, Crewe, U.K.
20/20 stable isotope analyser	PDZ-Europa Scientific Ltd, Crewe, U.K.
ORCHID software	ORCHID GC-IRMS, Europa Scientific Ltd, Crewe, U.K

APPENDIX 3.2: METHODS IN USE FOR MEASURING EITHER RETINOL OR RETINOL AND RETINYL ESTERS

PAPER	WHAT IS MEASURED	EXTRACTION	HPLC SYSTEM	METHOD DETAILS.	INTERNAL STANDARD	NOTES
Bieri *et al* 1979	Retinol	Hexane or heptane	Reverse phase	Methanol: water 95:5.	R-acetate	
Bhat & LaCroix 1983	Retinol and retinyl esters.		Reverse phase	Methanol: water 98:2 1.5ml/min then 2ml.min		Does not baseline separate the retinyl esters
Bankson et al 1986	Retinol and Retinyl esters.	Hexane:dioxane 5/1000 by volume	Normal phase	Two solvent gradient: Hexane:dioxane 5/1000 by vol increase to 100%dioxane 2.5ml/min	Retinyl acetate	Esters elute in single peak.
Thurnham *et al* 1988	Retinol, carotenoids & tocopherol	Heptane.	Reverse phase	Methanol:acetonitrile:chloroform 47:47:6 1.5ml/min	Tocopherol acetate	
De Leenheer & Nelis 1990	Retinol	Acetonitrile and hexane	Reverse phase	Acetonitrile:dichloromethanol:methanol 70:15:15 1ml/min	Retinyl acetate	
Furr 1990	Retinyl esters		Reverse phase	acetonitrile : dichlorethane (80:20 v/v) 1.5ml/min 10min run.		
Krasinski *et al* 1990	Retinol and retinyl esters	Hexane.	Reverse phase.	hexane-dioxane gradient 2ml/min	Retinyl acetate	Bieri method retinol Bankson for retinyl esters
Eckhoff 1991	Retinol and retinyl esters	Isopropanol	Reverse phase	Ammonium acetate: methanol (1.5:8.5) Solvent A acetone:isoproanol (1.25:1000) Solvent B.	Retinyl nonanoate	R-PA and retinyl oleate co elute
Ruotolo *et al* 1992	Retinol and retinyl esters		Normal phase	Hexane:n-butyl chloride: acetonitrile:acetic acid (82:13:5 +0.01ml acetic acid) 2 ml/min	Retinyl acetate	

PAPER	WHAT IS MEASURED	EXTRACTION	HPLC SYSTEM	METHOD DETAILS.	INTERNAL STANDARD	NOTES
Zaman *et al* 1993	Retinol and carotenoids	Hexane	Reverse phase	Solvent A 100mmol/L ammonium acetate in methanol:acetonitrile (80:20) Solvent B 100mmol/L ammonium acetate in water.	Retinyl Acetate	
Sowell *et al* 1994	Retinol, tocopherol, retinyl esters, & carotenoids	Hexane	Reverse phase	Ethanol:acetonitrile equal volume Contained 0.1ml of diethylamine per L of solvent 0.9ml/min	Retinyl acetate for retinol and retinyl esters	Retinyl esters peaks not sharp.
Buss *et al* 1994	Retinol and retinyl palmitate	Hexane	Reverse phase	Gradient system. Solvent A = methanol:isopropanol:water 1:1:2 Solvent B = methanol:isopropanol 1:1 Detected at 320nm.	Retinyl acetate	
Reinersdorff D.V 1996	Retinol and retinyl esters	Ethanol and hexane	Reverse phase.	20% methylene chloride in acetonitrile 1.2ml/min for 6min then 2ml/min for the next 8mins.	Retinyl nonanoate	Retinol peak elutes very close to the void volume.
Talwar *et al* 1998	Retinol carotenoids tocopherol	Hexane	Reverse phase	Methanol:acetonitrile:tetrahydrofuran (70:20:5)	Retinyl acetate	

APPENDIX 3.3

TEST MEAL AND EMULSION PREPARATION

Vitamin A Emulsion

Casein	0.65g
Beet Sugar	0.25g
Glucose	0.5g
Chocolate Nesquick	0.6g
Sunflower oil	1ml
Water	6ml
Total d_4-Retinyl Palmitate	300μg/kg/body weight

1. Prior to preparation, allow label to come to room temperate

(The retinyl palmitate label becomes liquid at room temperature).

2. Connect up sonicator, using the cold-water tap.
3. Weigh casein into tin foil.
4. Weigh beet sugar into tin foil.
5. Weigh glucose into tin foil.
6. Weigh chocolate Nesquick into tin foil.
7. Pipette oil into a 10ml glass tube
8. Pipette d_4-retinyl palmitate in to a glass tube, which is covered in tin foil.
9. Weigh distilled water into a separate 10ml glass tube (1ml = 1gram) and warm.
10. Dissolve beet sugar and glucose in 6ml warm water.
11. Add casein to this sugar and glucose solution.
12. Sonicate until an emulsion is formed.
13. Add the sonicated solution of casein, glucose, sugar and water to the oil and label (in the 10ml tube)
14. Shake and sonicate.
15. Add chocolate Nesquick and sonicate.
16. After administering the emulsion rinse the 10ml tube with a further 10ml of water, to ensure all label has been administered.

    (Make sure the subject drinks all the emulsion and the water rinse).

Tripalmitin Emulsion

Casein	0.65g
Beet Sugar	0.25g
Glucose	0.5g
Chocolate Nesquick	0.6g
Sunflower oil	1ml
Water	6ml
^{13}C Tripalmitin (TP)	10mg/kg/body weight

1) Connect up sonicator, using the hot-water tap.
2) Heat a pan of water on the hob.
3) Weigh casein into tin foil.
4) Weigh beet sugar into tin foil.
5) Weigh glucose into tin foil.
6) Weigh chocolate Nesquick into tin foil.
7) Pipette oil into a 10ml glass tube
8) Weigh tripalmitin label in tin foil
9) Heat the sunflower oil with TP in the 10ml tube in a beaker in the pan of boiling water.
10) Weigh distilled water into a separate 10ml glass tube (1ml = 1gram)
11) Warm the 6ml of water and keep hot in a beaker of boiling water.
12) Dissolve beet sugar and glucose into this 6ml of water.
13) Add casein to this sugar and glucose solution.
14) Sonicate until an emulsion is formed.
15) Add the sonicated solution of casein, glucose, sugar and water to the oil and label (in the 10ml tube)
16) Shake and sonicate.
17) Add chocolate Nesquick and sonicate.
18) After administering the emulsion rinse the 10ml tube with a further 10ml of water, to ensure all label has been administered.
(Make sure the subject drinks all the emulsion and the water rinse).

APPENDIX 3.4

CALCULATIONS FOR CONCENTRATIONS OF VITAMIN A:

retinol & retinyl palmitate.

$$\frac{\text{Area of retinol or retinyl palmitate peak}}{\text{Area of retinyl acetate}} \times \text{Concentration of internal standard added (Retinyl acetate μmol/L)} = \textbf{Concentration of retinol or retinyl palmitate (μmol/L)}$$

Concentration of retinyl acetate added to sample :

- 10μg/ml concentration of retinyl acetate = 10ng/μL
- Take 50μL = 10 X 50 = 500ng in 500μL plasma
- 500ng in 500μL = 1000ng in ml = 1000μg in L
- 1000 / 328.5 = 3.04μmol/L of retinyl acetate added

Retinol or retinyl palmitate concentration:

$$\frac{\text{Area of retinol or retinyl palmitate peak}}{\text{Retinyl acetate peak area}} \times 3.04\mu\text{mol/L} = \textbf{Concentration of retinol or retinyl palmitate (μmol/L)}$$

WORKED THROUGH SAMPLE Chromatogram figure 3.6.

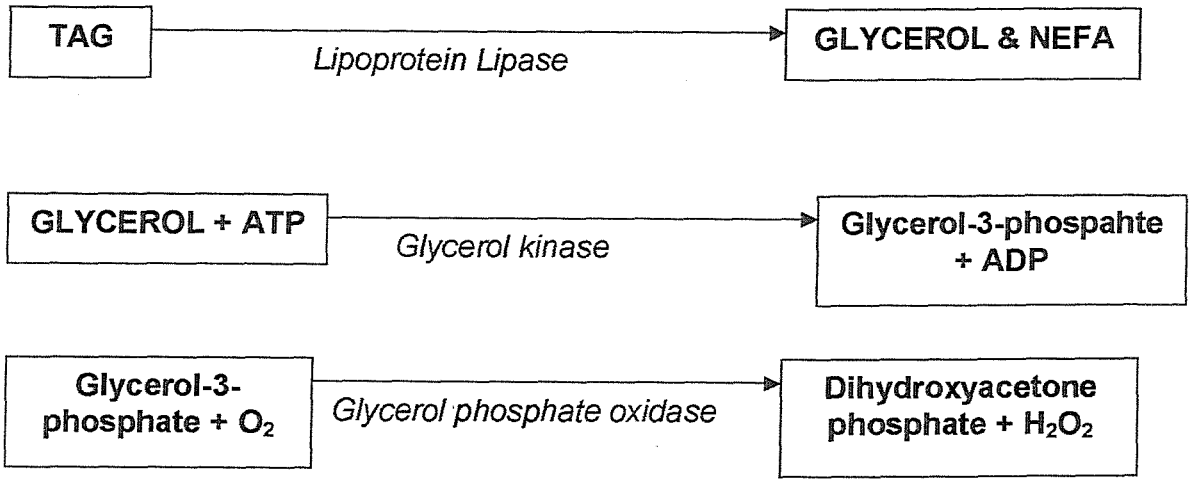
	PEAK AREA	Concentration retinol	Concentration Retinyl palmitate
RETINOL	101984	(101984/248088)X3.04= **1.25μmol/L**	
RETINYL ACETATE	248088		
RETINYL PALMITATE	121808		(121808/248088)X3.04= **1.49μmol/L**

APPENDIX 3.5

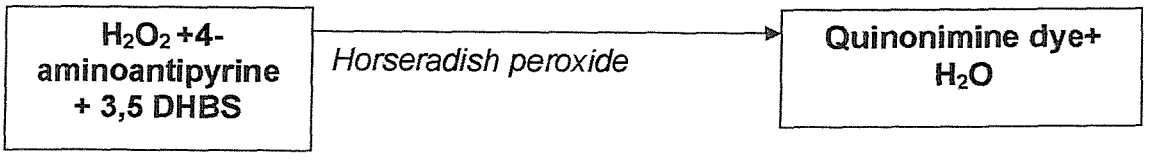
METHOD USED FOR THE ANALYSIS OF PLASMA TAG CONCENTRATIONS USING ENZYMATIC REAGENT KITS.

PRINCIPLE:

TAG is hydrolysed by lipase to glycerol and NEFA. Total glycerol is quantified by a Trinder type peroxidase reaction following generation of hydrogen peroxide by the sequential action of glycerol kinase and glycerol phosphate oxidase.



In a tinder type colour reaction catalysed by peroxidase H_2O_2 reacts with 4AAP and 3,5 DHBS to produce a red coloured dye. The absorbance of this dye is proportional to the concentration of TAG present in the sample.



REAGENT KIT: Triglyceride enzymatic determination of TAG using infinity TAG reagent. Reagent supplied ready for use.

STANDARD: 2.29µmol/L glycerol standard

MATERIALS REQUIRED:

- Clinical chemistry analyser capable of measuring 500 and 550nm.
- Control material
- Calibrator (glycerol standard)

SAMPLE PREPARATION : Sample volume 5µL, reagent volume 500µL, mix and incubate for 5 minutes. Measure absorbance of blank and set to zero, read absorbance of sample at 520nm.

CALCULATION:

$$\frac{\text{Absorbance of unknown}}{\text{Absorbance of calibrator}} \times \text{calibrator value}$$

e.g.

TAG mg/dl = 0.20 / 0.44 X 195 = 88.6mg/dl

TAG mmol/L = 0.20 / 0.44 X 2.2 = 1mmol/L

APPENDIX 3.6

ANALYSIS OF RBP AND TTR (prealbumin) FROM SERUM SAMPLES. Samples analysed automatically on Dade Behring, Nephelometer.

PRINCIPLE:
Immunochemical reaction, where the proteins in the sample of serum form immune complexes with specific antibodies. These complexes scatter a beam of light passed through the sample. The intensity of the scattered light is proportional to the concentration of the relevant protein in the sample. The results are evaluated by comparison with a standard of known concentration.

REAGENTS:
1 X 2ml vial of N antiserum to Human Prealbumin. Code no. OUIF
1 X 2 ml vial of N antiserum to Human Retinol Binding Protein. Code no. OUVO
N protein standard SL. Code no.OQIM
N/T protein controls. SL/L, M,H. Code no's OQIN, OQIO, OQIP
N diluent Code no. OUMT
N supplementary reagent. Code no. OUMU

1) Reagents and samples must be at room temperature
2) Reference curve constructed automatically by multi-point calibration. Serial dilutions of N protein standard SL automatically prepared using N diluent
3) Samples of serum were bar-coded, gently mixed by inversion, & loaded onto the analyser in the green position
4) Reagents (Antiserum Human Prealbumin; Antiserum Human RBP, Dade Behring) are loaded onto the analyser in the green position.
5) Quality controls (Dade Behring) are loaded onto the analyser in the grey position and pre-programmed to run at the start of the assay and subsequent intervals throughout the run.
6) Once fully loaded the nephelometer commences assaying.
7) Serum samples were automatically diluted 1:5 with N diluent.
8) Results were evaluated automatically by means of a logit-log function.

CHAPTER 11

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