THE ANTIOXIDANT VITAMIN STATUS OF INFLAMMATORY BOWEL DISEASE PATIENTS

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Glossary of Acronyms

5-ASA	5-aminosalicylate
6-MP	6-mercaptopurine
AA	Ascorbic Acid
Apo-RBP	Apo-retinol binding protein
ATBC	Alpha-tocopherol Beta-carotene
AZA	Azathioprine
BMI	Body Mass Index
CARET	Carotene and Retinol Efficacy trial
CD	Crohn's disease
CDAI	Crohn's Disease Activity Index
COV	Coefficient of Variation
CRP	C-reactive Protein
CTL	Cytotoxic T lymphocyte
DHAA	Dehydroascorbic acid
DNA	Deoxyribonucleic acid
ESR	Erythrocyte Sedimentation Rate
Fas-L	Fas-ligand
GCP	Good clinical practices
H_2O_2	Hydrogen Peroxide
HBI	Harvey Bradshaw Index
HOCL	Hypochlorous acid
Holo-RBP	Holo-retinol binding protein
HPN	Home Parenteral Nutrition
IκB	Inhibitory κB
IBD	Inflammatory Bowel Disease
IFNγ	Interferon γ
IL	Interleukin
MAC	Mid Upper Arm Circumference
NCHS	National Center for Health Statistics

NF-κB	Nuclear factor κB
NIK	Nuclear factor κB inducing enzyme
NO	Nitric Oxide
PGE_2	Prostoglandin E2
PGI_2	Prostocyclin I2
PUFA	Polyunsaturated Fatty Acids
QOL	Quality of Life
RBP	Retinol Binding Protein
RDA	Recommended dietary allowance
RE	Retinol Equivalents
RNA	Ribonucleic acid
ROMs	Reactive oxygen metabolites
SCFA	Short chain fatty acid
SD	Standard Deviation
SIL-6R	Soluble Interleukin-6R
SZP	Salazopyrin
TNF	Tumour necrosis factor
TNFR	Tumour necrosis factor receptor
TPN	Total Parenteral Nutrition
TRAF	Tumour necrosis factor receptor associated factor
TRADD	Tumour necrosis factor receptor associated death domaine
TSF	Tricep Skinfold Thickness
UC	Ulcerative colitis
VLDL	Very Low Density Lipoprotein

1. Abstract

Introduction

Free radical production may play an important pathogenic role in chronic inflammatory conditions, such as inflammatory bowel disease (IBD). Protective mechanisms against these agents, such as the antioxidant vitamins A, C and E may therefore be important in the control of the inflammatory process, and may have a therapeutic role in the disease.

Methods

100 consecutive IBD patients, attending the outpatient clinic at Groote Schuur Hospital were assessed regarding disease characteristics and activity, and nutritional and antioxidant vitamin status. This group comprised 56 patients with Crohn's disease (CD), and 44 with ulcerative colitis (UC). Results were evaluated in comparison to a group of 44 normal healthy controls. In order to evaluate the effect of antioxidant therapy on disease relapse rates, 23 CD patients were then randomised to receive either antioxidant vitamin supplement or placebo and monitored for a period of six months.

Results

Despite adequate intakes, serum concentrations of vitamins C, E and retinol were significantly lower than control values, whereas β-carotene levels were similar. The vitamin levels in CD and UC were similar. In IBD patients, age, smoking, nutritional status and medication did not appear to effect the serum concentrations of the vitamins. Active inflammation was associated with significant reduction of vitamin C and retinol levels and levels of α-tocopherol appeared to relate to the extent and duration of disease in UC patients. Retinol and β-carotene on the other hand, were low in both UC and CD patients with short duration of disease, and tended to increase with prolonged duration. In CD patients, recent relapses were associated with higher α-tocopherol levels while for UC, a similar phenomenon was shown for retinol. Antioxidant vitamin supplementation,

with 100% of the RDA for vitamins A and E, did not influence disease relapse rates or quality of life.

Conclusion

Serum levels of the antioxidant vitamins A, C and E are low in patients with IBD. The reasons for the depleted levels appear multifactorial with disease extent (α-tocopherol), and active inflammation (vitamin C and retinol) playing a significant role. We were unable to demonstrate a therapeutic role for vitamin A and E supplementation in the maintenance of remission in CD.

2. Literature Review

Part 1: Inflammatory Bowel Disease

2.1.1 Introduction

Inflammatory bowel disease (IBD) is a term used to describe two diseases, ulcerative colitis (UC) and Crohn's disease (CD). They are both chronic inflammatory conditions of uncertain aetiology affecting the gastrointestinal tract, with different clinical, endoscopic and histological characteristics. UC is mainly confined to the mucosa and submucosa, while in CD, the inflammation may extend through the intestinal wall from the mucosa to the serosa (transmural). UC is confined to the large bowel, whereas CD can occur in any area of the gastrointestinal tract, from the mouth to the anus.[1-3] Often a clear distinction between the two diseases may be difficult, and the term "indeterminate colitis" is then used.[1]

Although the diseases particularly affect the gut, the systemic nature of the conditions is evidenced by the extra-intestinal manifestiations including arthritis, skin conditions (erythema nodosum, pyoderma gangrenosum), eye problems (uveitis, iritis), and involvement of the liver (sclerosing cholangitis). Both diseases follow a relapsing and remitting course, and recurrence rates of approximately 70% per annum can be expected.[1]

2.1.2 Ulcerative colitis

The main symptom of UC is diarrhoea, which is often associated with blood in the stool. Increased frequency of bowel movements, with the feeling of incomplete evacuation (tenesmis), is common. Other symptoms include fever and abdominal pain.[1]

2.1.2.1 Complications of UC

Complications that occur in UC include haemorrhage, toxic megacolon and perforation. There is also increased incidence of carcinoma of the colon in patients with long standing disease.[1]

2.1.3 Crohn's disease

CD is often a more complex and difficult disease to manage than UC, mainly because of the diversity of its location (Table 1), and its associated complications.

Area affected	% of patients at presentation
Ileum and Caecum	40
Small intestine only	30
Colon only	25
Mouth/tongue/oesophagus/stomach/ duodenum	5

Table 1: Disease distribution in CD[1]

The main symptoms of CD are diarrhoea, abdominal pain and weight loss.[1,4] Patients with terminal ileal disease or who have had terminal ileal resections may have fat malabsorption and steatorrhoea[1,5,6] and may also develop vitamin B_{12} deficiency.[7,8]

2.1.3.1 Complications of Crohn's disease

Complications of CD include obstruction, fistulae, abscesses and peri anal disease.[1]

2.1.4 Epidemiology

The incidence and prevalence of CD and UC varies in different geographical regions.

The incidence of IBD is higher in Jews than non-Jews (Table 2). In the United States the incidence seems to be higher in the white population than the black population. However this seems to be changing, with more black people being diagnosed with IBD.[1,9]

The incidence of IBD may be higher in women, by as much as 30%.[1] The peak age of onset for both UC and CD is between the ages of 15 and 25 years. Another, smaller peak also seems to occur between the ages of 55 and 65 years.[1]

FACTOR	UC	CD
Incidence (per 100,000)	2-10	1-6
Prevalence (per 100,000)	35-100	10-100
Racial Incidence	High in whites	High in whites
Ethnic Incidence	High in Jews	High in Jews
Sex	Slight female preponderance	Slight female preponderance
Age at onset	15-25 ?55-65	15-25 ?55-65
Smoking	Fewer smokers than expected	More smokers than expected

Table 2: Epidemiology of IBD[1]

The prevalence of IBD continues to increase. However, latest data shows that the incidence of IBD worldwide is around 20/100000/year. The prevalence of CD, however, has been reported to be around 100/100000 in the developed world.[10] Even though this prevalence may seem small, the cost of the disease is high. In a study

undertaken in Sweden it was found that in 1994, the total cost of CD calculated from hospital admissions, ambulatory care, drugs, sickness leave and early retirement was US $43.1 million. Two percent of all patients were responsible for 10% of the total admissions during 1994. This finding was similar to findings in the USA and the UK. Only 6% of the total cost (US $2.6 million) was from drugs, and morbidity caused more than two-thirds (71%) of the total costs.[10] A recent study has reported that the average cost of care for CD is approximately $12,500 per patient.[11] Furthermore, 20% of the cases of CD account for 80% of the total cost of the disease, generally due to hospitalisation, with an average cost of $37,000 per hospitalised patient.[11] This highlights the need for efficient, cost effective patient care and treatment, in order to keep patients in remission and out of hospital.

2.1.5 Aetiology of IBD

The aetiology of IBD remains unknown, however a variety of factors have been reported to be associated with the disease.

2.1.5.1 Genetic Factors

There seems to be an ethnic and/or familial predisposition to IBD, and therefore, genetic traits may play a role in the pathogenesis of IBD. Family and twin studies have shown that between 10-20% of patients have another first-degree member of the family affected, and 41% of identical twins are affected.[12] Candidate gene studies have reported associations with genes for tumour necrosis factor (TNF) and other proinflammatory cytokines. Furthermore, genome wide linkage studies have identified potential susceptibility loci on a number of different chromosomes, with chromosomes 12 and 16[13,14] repeatedly identified. These two susceptibility loci have been termed the IBD1 and IBD2 "genes".[15]

2.1.5.2 Environmental factors

Smoking

Smoking has been shown to be beneficial in UC and detrimental in CD,[13,16] with current smokers being more prone to relapse,[16,17] and surgery.[18,19] The reason for this association is not clear, but may result from the adverse vascular effects of smoking (CD),[13] and possibly the effects of nicotine (UC).[20,21]

Diet

Although no specific foods have been linked conclusively to IBD,[13] dietary factors may have a primary effect in predisposed patients, or may act together with other environmental factors[22] in the development of IBD.

Increased carbohydrate intake in patients with IBD has been reported.[23] This however, may be due to patients avoiding fibrous foods[22-26] because of the risk of obstruction, and therefore consuming relatively greater amounts of refined carbohydrates and sucrose.[16,22,26-28]

However, fibre may be protective against IBD[22] due to the production of short chain fatty acids (SCFAs), during anaerobic fermentation in the intestine. These SCFAs, especially butyrate, provide a substantial energy source for the colonocyte,[29] and have a proliferative effect on ileocolonic epithelia.[30] Deficiency of SCFAs, and a decreased capacity to oxidise SCFAs have been demonstrated in UC,[31] and SCFA enemas have been reported to be beneficial in patients with distal colitis.[31]

Protein intake has been found to be increased in some studies,[26,32] while other studies have reported no significant findings.[22] Furthermore, UC patients may have an increased consumption of fat in the pre-illness period, the relevance of which is uncertain at present.[22]

Vitamins and minerals

A study by Reif et al, reported that increased consumption of vitamin A precursors, and calcium in the pre-illness diet was positively associated with increased risk for IBD,[22] while other vitamins such as various B vitamins showed no clear association.[24] Magnesium and vitamin C intake showed a negative association with the risk for IBD, while consumption of potassium showed a negative association only with the risk of CD.[22] Furthermore, vitamin A,[33-36] E[35] and C[37,38] serum concentrations have been reported to be decreased in IBD patients.

Total caloric intake

Total energy intake in IBD may be high or similar to controls, paticularly, but not exclusively, in the pre-morbid diet, and in those in remission.[23,26,32] Despite this, the prevalence of malnutrition in IBD is high, indicating that other factors, such as malabsorption, and the metabolic response to inflammation, stress and medications are important in determining the nutritional state of patients.[32]

2.1.5.3 Microbial agents

No pathogen has been proven to be a transmittable agent that causes IBD.[13] However, bacterial species including *Escherichia coli,* and *Staphylococcus albus* and *aureus* may be present in a large proportion of IBD cases.[39] Association with *M. paratuberculosis* has been reported, but a direct aetiological relationship has not been established.[13,16,40] This may be the result of secondary invasion of a previously damaged mucosa.[40]

An association between the pathogenesis of CD and measles virus exposure has also been reported.[13,41,42]

2.1.5.4 Immunology

There is good evidence that UC and CD are autoimmune disorders. Mucosal T cells from children with CD exhibit a hyperactive response to interleukin (IL-) 2. This hyper-reactivity was independent of clinical activity, disease location, disease duration and treatment, and was found even in areas free of inflammation.[43] In UC mucosal T cells are particularly susceptible to fas-mediated apoptosis, a physiologic process of cell death, suggesting that fas-mediated apoptosis may be important in UC pathogenesis.[13] Furthermore, mucosal mononuclear cells from UC produce autoantibodies against epithelial cell-derived isoforms of tropomyosin.[13] Long-lasting clinical remissions have also occurred in CD patients who have undergone allogenic bone marrrow transplantations.[13]

Furthermore, the gut immune response in CD seems to differ from that in UC, and in normals. The normal gut is in a constant state of immune activation in response to luminal antigen. This is however of the Th2 type, characterised by the production of cytokines IL-4, IL-5 and IL-10 resulting in a non-inflammatory, humoural immunity with the production of IgA antibodies.[44,45] The mucosal inflammatory response in CD is, on the other hand, associated with the production of the cytokines TNF, interferon gamma (IFNγ), and IL-2 resulting in an inflammatory cell mediated (Th1) immune response.[44,45] Interestingly, the cytokine profile in UC seems to be of the Th2 type.[44]

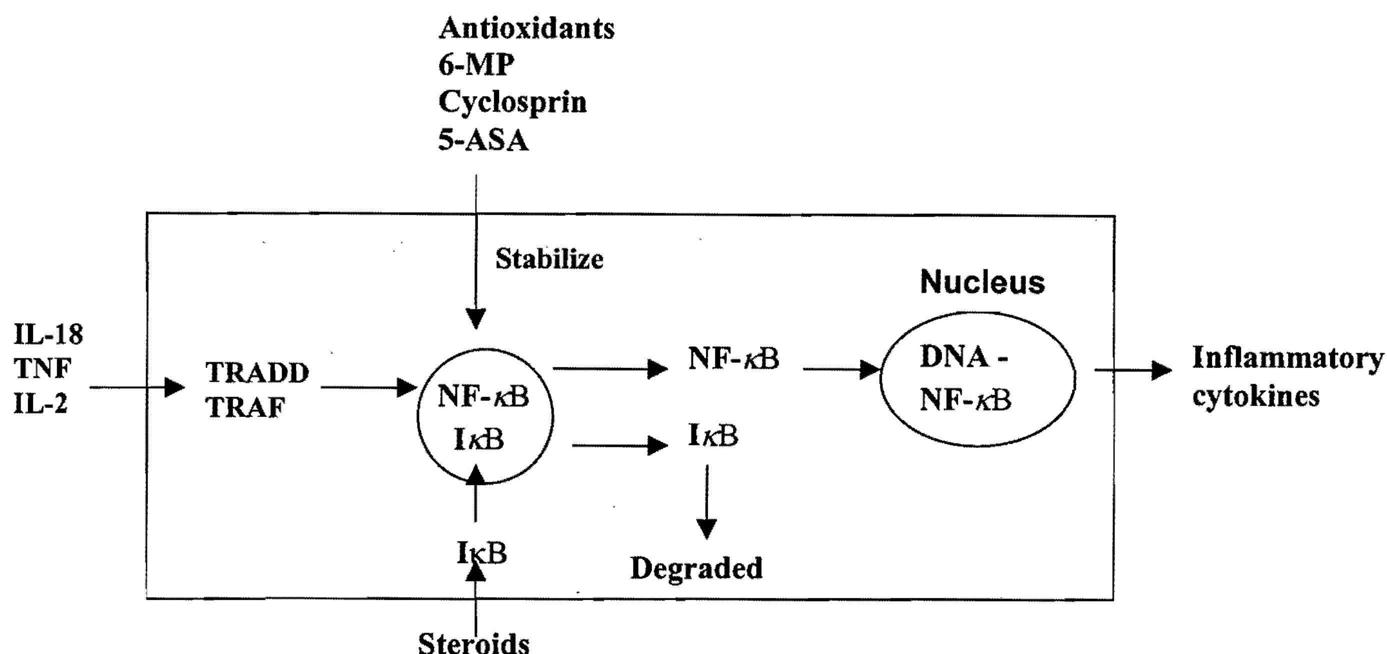
Recognition of the importance of the Th1 response in CD has lead to the use of specific monoclonal antibodies directed against TNF (Infliximab®), which have been shown to be efficacious in the treatment of steroid refractory CD, and in the treatment of fistulas.[13,29,46-48] IL-10 results in the down regulation of TNF and has been reported to be efficacious in active CD,[47,48] and drugs such as thalidomide, which also have anti-TNF affects, have been found to be useful in the management of CD.[47]

Transcription factors

Nuclear Factor κB (NF-κB) is a group of transcription factors which play a major role in the regulation of pro-inflammatory cytokine production. The group consists of several proteins including NF-κB_1, NF-κB_2, p65, c-Rel, and RelB, and is found in the cytoplasm of most cells as an inactive complex bound to the inhibitory protein, inhibitory κB (IκB). Activation of the cell with various stimuli, including TNF-α, IL-1 and IL-18, results in phosphorylation of IκB, and the disruption of inactive complex, and the release of NF-κB. NF-κB then translocates into the nucleus, binds to DNA regulatory sites and initiates transcription of various inflammatory cytokines[46,48,49,50] including IL-1, IL-6 and TNF-α,[46,47,50] as well as essential proteins for antigen presentation (Figure 1).

NF-κB expression has been shown to be increased in IBD, and CD has been associated with increased levels of Th1-type cytokines (eg. TNF-α, IL-2, and IFNγ), the promoters of which are regulated by the NF-κB pathway.[50] Control of NF-κB activity is therefore an attractive option for the management of IBD. Many of the drugs currently used in IBD have been shown to directly affect NF-κB activation.[50] Glucocorticosteroids repress NF-κB activity by inducing IκB production, and 5-aminosalisylates (5-ASA) compounds and immunosuppresive agents (azathioprine [AZA], 6-mercaptopurine [6-MP] and cyclosporin) inhibit NF-κB activation. Antioxidants have also been reported to suppress NF-κB activation, possibly by inhibiting the disruption of the inactive complex with IκB.[50]

Figure 1: Interaction between NF-κB, antioxidants and pro-inflammatory cytokines. NF-κB signal induction by TNF-α, IL-1, IL-18 via the TNF receptor associated factor (TRAF) and the TNF receptor associated death domaine (TRADD) resulting in phosphorylation of the NF-κB IκB complex and the release of cytosolic NF-κB. NF-κB translocates to the nucleus, binding to its target DNA sequences. Steroids directly induce the production of IκB, and antioxidants, immunosuppressants and 5-ASA inhibit the activation of NF-κB.



Apoptosis

Apoptosis (programmed cell death) is an important immune system mechanism that controls the overall size of cell clones which undergo expansion in response to antigenic stimuli.[51] It may also be involved in epithelial injury, and has been reported to play a role in the pathogenesis of IBD.[52] In fact mesalamine's beneficial effect in IBD has been reported to also relate to its involvement in the attenuation of peroxy-nitrite induced cell injury and apoptosis.[53]

Fas-ligand (Fas-L) is a transmembrane protein which belongs to the TNF receptor family. The Fas-L system induces programmed cell death and has been reported to aid in the colonic depletion of T lymphocytes to suppress autoimmune reactions.[51] Fas-L is expressed on the surface of cytotoxic T lymphocytes (CTLs), where it acts

as an effector for apoptosis. Fas-L transcripts are highly expressed in active UC, and Fas-L messenger ribonucleic acid (RNA) is strongly expressed in CD3 T lymphyocytes infiltrating the lamina propria of affected mucosa only. Furthermore, Fas-L may also have an indirect role in the progression of mucosal lesions in UC via the increased production and secretion of IL-8 from the colonic epithelium, which results in the promotion, migration and activation of lymphocytes and neutrophils.[51]

In CD, however, although cells for the lamina propria do express Fas on the cell surface, they seem to be less sensitive to Fas antigen cross-linking compared with healthy individuals, and decreased apoptosis compared to controls has been noted.[54] This may be due to increased concentration of antiapoptotic proteins such as Bcl-2. IL-2 induces Bcl-2 via a protein on the IL-2 molecule which serves as a signalling component of the receptor for IL-2, IL-4, IL-7 and IL-15. It is therefore up-regulated in cells on stimulation of various cytokines, and may be the mechanism used by cytokines to promote cellular survival. The increased levels of Bcl-2 found in the inflamed tissue of CD patients may be due to the increased exposure of these cells to various cytokines, and therefore, the increased levels of Bcl-2 are probably secondary to the inflammation that occurs in CD.[54] Furthermore, IL-6 may also play a role in the inflammation that occurs in CD, possibly due to resistance of mucosal T cells against apoptosis. IL-6 can bind to cells which don't have an IL-6 receptor when it forms a complex with soluble IL-6R (SIL-6R). In a study which blocked the IL-6 *trans* signalling, T-cell apoptosis was increased. Since IL-6 is increased in the lamina propria of CD patients compared to controls, and since the increased production of IL-6 in CD was associated with increased production of SIL-6R, the IL-6-SIL-6R complex may contribute to the perpetuation of chronic intestinal inflammation.[55]

Reactive metabolites

Reactive oxygen species and nitrogen metabolites are significantly raised in the circulation and in the inflamed mucosa[13,56,57] in both CD and UC compared to

controls.[13,57] Neutrophils,[41,42-50,56-60] eosinophils, monocytes,[50,56,58] macrophages,[56] and phagocytic leukocytes,[56,61] produce and release large amounts of reactive oxygen metabolites (ROMs) which are toxic[56] and can cause tissue injury and dysfunction. They may also further perpetuate inflammation.[62] Furthermore, neutrophil derived oxidants may also damage the epithelium and mucosal interstitium indirectly, by changing the protease/anti-protease balance in the intestine.[56,63] These factors are discussed further in part 3.

Inflamed colons produce increased amounts of reactive oxygen species when compared to non-inflamed colons.[56-58,62,63] Furthermore, there seems to be decreased endogenous intestinal protection against these oxygen-derived radicals.[64] The chronic gut inflammation that occurs in IBD may relate to an imbalance between pro-oxidant and antioxidant mechanisms, leading to the net accumulation of oxidatively modified proteins and lipids.[56]

2.1.6 Nutritional Status in IBD

The various causes of nutritional deficiency are illustrated in table 3.

Reduced food Intake	Anorexia Fear of eating from abdominal pain Restrictive diets without supplementation
Increased requirements and decreased synthesis	Active Inflammation Sepsis
Enteric Loss of nutrients	Exudation from intestinal mucosa Interrupted entero-hepatic circulation GI blood loss
Malabsorption	Loss of absorptive surface from disease, resection or bypass Stagnant loop syndrome from strictures, fistulae or surgically created blind loops Bile Salt deficiency after ileal resection Bacterial overgrowth Mucosal cell disease Lymphangectasia Rapid GI transit
Drug interference	Corticosteroids and calcium absorption/protein metabolism Sulfasalazine and folate absorption/haemolysis Cholestyramine and fat-soluble vitamin absorption

Table 3: Pathogenesis of malnutrition[2,28]

2.1.6.1 Malnutrition

The prevalence of malnutrition has been reported to be as high as 70-80% in some centers.[65,66] In CD without extensive small bowel involvement, weight loss is mainly due to a decrease in energy and protein intake,[65] however, fat malabsorption[30] and carbohydrate malabsorption[5] in the form of lactose intolerance, may also occur.

Decreased protein intake and increased intestinal losses of nitrogen,[67] may lead to major losses of body protein mass in IBD in a small number of patients. However,

low serum albumin concentrations are usually the result of active inflammation (negative acute phase response), increased intestinal losses, and maldistribution between intravascular and extravascular spaces.[67]

Bile acid metabolism may be altered in ileal CD, as well as in patients who have had small bowel resections, resulting in steatorrhoea.[30]

Diarrhoea may damage the intestinal villi resulting in decreased lactase activity in IBD patients. This often results in incomplete lactose intolerance, so that a small amount of lactose may be tolerated.[5]

2.1.6.2 *Vitamin abnormalities*

A number of serum vitamin level abnormalities have been reported in patients with IBD, with reduced levels of vitamin A,[34,35] vitamin E, pyridoxine, vitamin B_{12},[7,8] thiamin, riboflavin,[35] folate,[34,35] biotin,[34] β-carotene,[34] and vitamin C[34] being reported. It should be noted however, that a reduced serum level does not necessarily include vitamin deficiencies, and clinical signs of deficiency may only develop in severe longstanding disease.[35]

Vitamin C

Decreased serum vitamin C levels in patients with IBD have been reported.[7,37,38] Although the precise mechanism is unclear, dietary deficiency may play a role,[7,37] with reports indicating improved serum levels with increasing dietary intake.[7] Vitamin C serum levels seem to be lower in those with colonic disease.[38,68] Since vitamin C is a powerful antioxidant, and may be the first line of defence against oxidant damage,[69,70] the low levels may be the result of increased utilisation due to the relatively low stores of endogenous antioxidants in the colon, in comparison to the levels of ROMs.[38,71]

Vitamin A

Low serum vitamin A has been described in CD, but these low levels have not been clearly associated with any clinical signs of vitamin A deficiency.[34] However, in a study of 137 CD patients it was found that serum retinol concentrations were normal, despite 34% of the sample consuming less than 100% of the RDA.[33] Furthermore, low serum carotene levels were reported, and were not related to low dietary intakes, or to active CD, and were thought to rather be related to the presence of steatorrhoea.[33] Studies have also shown that vitamin A is absorbed normally in IBD patients,[72,73] and that the low serum carotene level does not indicate that the patients are at risk of developing vitamin A deficiency.[33] The low vitamin A levels may also be due to decreased retinol binding protein (RBP) concentrations as a result of increased protein catabolism or inadequate intakes.[33,73]

Vitamin E

Serum concentrations of vitamin E have been reported to be decreased in IBD.[34,35,74] However, when cholesterol was tested it was found that the vitamin E to cholesterol ratio was normal indicating that the low circulating vitamin E levels may be due to hypocholesterolaemia.[34,74]

Folate

Between 10 and 65% of patients with IBD are likely to have decreased serum and red blood cell folate (RBCF) levels,[7] which may lead to anaemia.[30] Salazopyrin (SZP) may also lead to a folate deficiency.[2,28]

Vitamin B_{12}

Low serum concentrations of vitamin B_{12} occur in 10-16% of CD patients[7] and may lead to anaemia.[30] This is usually associated with resection or disease of the terminal ileum,[7,8] and many patients require supplementation with vitamin B_{12} post ileal resection.

2.1.6.3 Mineral deficiencies

Various mineral deficiencies have been found to occur in IBD including calcium,[7] magnesium,[7,73] iron,[7,66] zinc,[73] copper,[75,76] and selenium.[76]

Calcium

Low serum calcium levels have been reported in approximately 20% of patients. Calcium depletion may occur as a result of vitamin D deficiency, inadequate intake especially for those on a low lactose or lactose-free diets, malabsorption, steatorrhoea and corticosteroid therapy.[7]

Magnesium

Fistulas, malabsorption, small bowel resection and any other condition that results in the abnormal loss of intestinal contents including diarrhoea or tube suction, as well as prolonged periods of inadequate oral intake, intravenous fluid supplementation without magnesium, corticosteroid therapy,[7] increased intestinal losses and malabsorption,[7,73] can result in depletion.

Iron

Twenty five to fifty percent of patients with CD, and up to two thirds of UC patients may have an iron deficiency,[66] usually as a consequence of chronic blood loss in the gut. This, together with folate and vitamin B_{12} deficiencies, is the major cause of anaemia in IBD.[7]

Zinc

Zinc status in IBD is controversial,[75,76] but may be of concern in patients with severe diarrhoea or enteric fistulae.[73] Zinc deficiency may occur as a result of

malabsorption, increased intestinal or urinary losses, increased requirements[7] or a decrease in albumin, the main zinc binding protein.[7,73]

Furthermore, in patients with active CD, plasma zinc may decrease due to intrahepatic sequestration of circulating metal.[7] Zinc also acts as a cofactor of superoxide dismutase, an antioxidant enzyme, which also seems to be reduced in IBD. Since oxidant stress may be increased in the inflamed mucosa, zinc requirements may increase in order to maintain adequate amounts of this enzyme.[75] Perhaps the high zinc levels found in some studies, are a result of the zinc being mobilised for this purpose.[76]

Zinc itself can act as an antioxidant and inhibits the direct oxidation of enzyme sulfhydryl groups.[60]

Copper

Serum copper levels have been reported to be elevated in IBD when compared to controls.[75,76] Excess total tissue copper, may catalyse free radical formation and increase the oxidative stress in the mucosa.[75,76]

Selenium

Serum concentrations of selenium may be reduced in IBD. Since selenium has known antioxidant properties, its lower level may contribute to inflammation, as well as to the increased prevalence of cancer in IBD.[76]

2.1.7 Methods of assessing disease activity in IBD

Various assessment methods have been developed to assess disease activity in IBD.

2.1.7.1 The Crohn's Disease Activity Index

The CDAI was designed as an objective assessment measure that could give a numerical value that would be proportional to severity of illness.[77]

The CDAI takes 8 variables into account: frequency of soft/liquid stools, abdominal pain, general well being, extraintestinal manifestations (arthritis, erythema nodosum, pyoderma gangrenosum, apthous stomatitis, iritis and uveitis, anal fissures and abscesses, and fevers), use of opiates or lomotil for diarrhoea, abdominal masses, haematocrit, and body weight.[77] The index requires a 7-day documentation by the patients of his or her symptoms.[78]

The CDAI, however, does have shortcomings. The predominance of subjective symptoms such as abdominal pain and general well being may introduce inaccuracy.[77-79] Furthermore, the medication that is often prescribed in CD may influence these subjective complaints. Also, patients with CD who have had a bowel resection, especially an ileocaecal resection, often have a higher defaecation rate and produce soft stools, even though no active disease is present. The CDAI also does not take into account other important objective parameters such as serum albumin level and ESR,[79] and the only laboratory parameter that is used in the index is that of haematocrit, which may be less discriminate than other acute phase reactants[80] and may not be an adequate measure of disease activity.[9,81] However, in a study in which three indices were compared – a simple clinical index, designed by the authors, specifically for the study (scored on a scale of 1-6 with 1 being well and 6 being fulminating attack. Clinical parameters used were diarrhoea, pain, systemic manifestations and a general feeling of well being), the Van Hees index and the CDAI , and it was found that the CDAI was the most sensitive indicator of an acute attack,[81] and is the index most often used in clinical trials.

2.1.7.2 The Van Hees Index

The Van Hees index was designed in response to a need for maximally objective, reproducible, quantitative manner of assessing disease activity in IBD, especially CD.[79] It contains nine variables; serum albumin, ESR, body weight related to length, abdominal mass, sex, temperature, stool consistency, bowel resection, and extraintestinal symptoms related to CD.[79]

The Van Hees index may be more objective than the CDAI as it uses parameters such as serum albumin and haemoglobin values. It is also less patient-dependent.[79] However, the index may vary according to the inter-laboratory variation in the determination of serum albumin[79] and complex and time-consuming calculations are necessary,[81,82] making it less ideal for routine use.[79]

2.1.7.3 The Harvey Bradshaw Index

The Harvey Bradshaw index (HBI) is a simplified form of the CDAI, which quantifies the patient's previous 24-hour symptoms.[78] It was first developed in 1980, as a means of assessing activity in either CD or UC.[78] It is based on 5 items; general well being, abdominal pain, the number of liquid stools per day, abdominal mass and complications.[82]

The HBI has easy calculations, and does not need to be planned in advance[82] but is heavily biased by the frequency of diarrhoea.[9,78] It is however, a useful index in the assessment of patients with UC.

The CDAI was selected as the tool for assessment of CD activity in this thesis as it has been shown to be widely tested and found to be the best indicator of active disease. The HBI will be used to assess clinical disease activity in UC.

2.1.7.4 Other indicators for assessing disease activity in IBD

Laboratory tests reflecting inflammatory activity should provide additional information in patient management. They should give an objective assessment of disease activity by identifying clinical deterioration not due to active inflammation, for example fibrotic intestinal strictures.[83]

Erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP) are often used as inflammatory markers and indicators of activity in IBD. They are however, not specific for IBD.[84] The CRP is an acute phase reactant which is sensitive to many types of inflammation[81,83] and has been reported to correlate significantly with clinical status in IBD.[80,83] Similarly, the ESR is commonly increased in active inflammatory disease and is often used as an indication of inflammation. It is however, subject to a large number of variables including changes in some serum proteins as well as changes to the erythrocyte. ESR has been reported to correlate significantly with clinical status in IBD patients.[80]

Since the ESR has been shown to correlate with clinical status in IBD patients, and since it is used routinely in our clinic, the ESR will be used as a direct indication of inflammation in this study.

2.1.7.5 Methods of assessing quality of life (QOL) in IBD.

The impact of inflammatory bowel disease on the lives of patients is often not reflected by inflammatory markers or extent of disease, as the emotional and social problems that occur are not accounted for.[85-87] The chronic nature and morbidity of IBD are major factors in the impairment of QOL in these patients.[88] However, the disease has such a relapsing-remitting course that the extent to which the QOL is affected may vary considerably, both inter-individually and intra-individually.[88]

A combination of generic and disease-specific scales may provide the best approach to assessing health related QOL in IBD.[85] Some of the more widely used questionnaires will be discussed below.

The Inflammatory Bowel Disease Questionnaire (IBDQ)

The IBDQ is a disease-specific scale, which can be administered by either the interviewer,[87,88] or it can be self-administered.[89] The scale has 32 items which are grouped into four sections; bowel symptoms, systemic symptoms, emotional factors and social factors.[85,86] Each item is scored on a scale of 1 to 7[85,86] and the total IBDQ score may range from 32 – 224. The higher the score, the better the health.[85] This QOL measure is well correlated with disease activity indices for both CD and UC.[87]

The Modified IBDQ and the Short-Form IBDQ

The modified version of the IBDQ is designed for patients with IBD, who are "clinically well". It uses 36 items, divided into 5 sections; bowel symptoms, systemic symptoms, functional impairment, social impairment and emotional function.[88]

The short-form IBDQ is a simplified version of the IBDQ. It only contains 10 questions and can be administered in 5 minutes. It has however, not been fully validated.[88]

The Medical Outcome Survey (MOS) Short Form-36 Health Survey (SF-36)

This QOL questionnaire has been validated in many different conditions and in the general population,[85,89-93] and has good test-retest properties.[90] In fact, in a study by Brazier et al, in which the reliability or reproducibility of this questionnaire was tested on 1980 people, the maximum mean difference in the dimension score was 0.8 meaning that a person who scored 70 on one test, may score 71 on re-test.[90] The SF-

36 is a shortened version of 149 health status questions. It consists of 36 items grouped into 10 sections; physical function, physical role limitation, mental role limitation, social function, mental health, energy and vitality, pain, health perception, and change in health. The dimension score is expressed as a value between 0 and 100, with higher scores showing better health.[85] The questionnaire takes only 5 –10 minutes to fill out,[91] can be self-administered,[94] is easily understandable[95] and therefore is highly acceptable to patients.[90] It also correlates well with the doctor's assessment of the severity of illness.[95] Therefore it is practical, and acceptable to the population, and is easy to use.[90] However, since the questionnaire is not disease specific, it should be used as part of a more detailed portfolio of measures to assess aspects of a patient's outcome.[92] But, the SF-36 is a good measure for detecting average group differences or changes over time.[93]

Since this questionnaire, has been widely tested, can be easily administered and understood, and is a good measure of detecting average group differences, the SF-36 QOL questionnaire will be used to assess QOL in this study.

2.1.8 Treatment of IBD

2.1.8.1 Drug therapy

Therapy of IBD is directed towards induction and maintenance of remission, and the avoidance of complications.

Corticosteroids

Steroids remain the mainstay of therapy in inducing remission in patients with active disease, and have been shown to be successful in approximately 70% of patients.[29,96-100] Despite this efficiency in inducing remission, steroids have not been found to be of value in maintaining remission.[29,96-100] Side effects from corticosteroids occur

often and include moon face, acne, hirsutism, striae, obesity, hypertension, diabetes, infection, osteoporosis, osteonecrosis, myopathy and cataracts.[29,101] Nutritionally, corticosteroids may inhibit calcium absorption, induce magnesuria and alter protein metabolism.[73]

Steroids with low systemic bioavailability, such as budesonide, are increasingly being used for therapy in IBD,[29,46,102] in order to decrease the risk of bone mineral abnormality and osteoporosis,[97] as well as other systemic side effects.[29,102]

Aminosalicylates

The main indication for the use of 5-aminosalicylate (5-ASA) compounds is for the maintenance of remission in patients with colonic disease. Sulphasalazine and olsalazine require colonic bacterial metabolism in order to release 5-ASA, and are therefore only effective in colonic disease. Formulations such as Asacol® and Pentasa® release 5-ASA in the small bowel and may also be of value in ileal CD.[29,96]

5-ASA may play a direct role in the scavenging of oxidants,[103-108] in particular hypochlorous acid (HOCL) that is produced by neutrophils in the inflamed colon.[103,106] However, 5-ASA may also act as a pro-oxidant, when the ratio of 5-ASA to iron is similar, thereby promoting oxidative degradation of deoxyribose. The tissue injury, mainly affecting the stomach, that may be seen with 5-ASA administration may be the result of this interaction.[108]

Immunomodulators

Azathioprine and 6-Mercaptopurine

6-MP and AZA are efficacious as steroid sparing agents in both UC and CD patients.[29,96,109,110] They are useful in the prevention of relapses in patients with Crohn's ileocolitis, and in patients with UC who have relapsed despite the use of 5-

ASA.[29,96,97,111] They have also been found to be useful in the management of patients with fistulising CD.[96,100,109,110]

AZA may also act as an oxidant scavenger. The compound undergoes hepatic cleavage releasing 6-MP which contains a thiol group. It is thought that oxidants such as HOCL, chloramines, nitric oxide (NO) and peroxide (H_2O_2) react with thiols. Furthermore, the thiol group on 6-MP may also protect against oxidant cellular damage by providing an alternative thiol target for inflammatory oxidants.[112]

Other Immunomodulators

Methotrexate and cyclosporin have also been reported to be useful in IBD,[29,97,102,113] however, their side effects, including hepatic and renal dysfunction, tend to limit their use.[96,114]

Antibiotics

Antibiotics have been shown to be useful in the management of CD. Ciprofloxacin has been shown to significantly reduce disease activity, and metronidazole has been reported to reduce relapses following surgery.[97,115] Metronidazole may also be useful in the treatment of fistulising CD.[116]

2.1.8.2 Dietary therapy

Patients should maintain a normal diet as far as possible. However, in certain instances specialised diets may be required. Patients with obstructive disease may benefit from low soluble fibre diets, whereas high insoluble fibre diets may benefit patients with colonic disease.[22,29] Fibre, is anaerobically fermented in the intestine to produce SCFAs; acetate, proprionate and butyrate. Butyrate is the preferred energy substrate of the colonocyte. It also has proliferative effects on ileocolonic epithelia together with the other SCFAs. In this way a high fibre diet may be beneficial in

colonic disease,[30] and soluble fibre may also be beneficial in obstructive disease as SCFAs are produced, but, the risk of obstruction is reduced.

Parenteral nutrition

Since the necessity for "bowel rest" in the management of active IBD was questioned,[73,117-120] and due to the importance of luminal nutrients in the maintenance and integrity of the bowel, early enteral feeding is now generally advocated. However, in patients with obstructive disease, or severe fistulising disease,[120] total parenteral nutrition (TPN) may be required as supportive nutritional therapy.[74]

Similarly, for patients with short bowel syndrome, home parenteral nutrition (HPN) may be essential.[74, 118,120,121]

Enteral nutrition

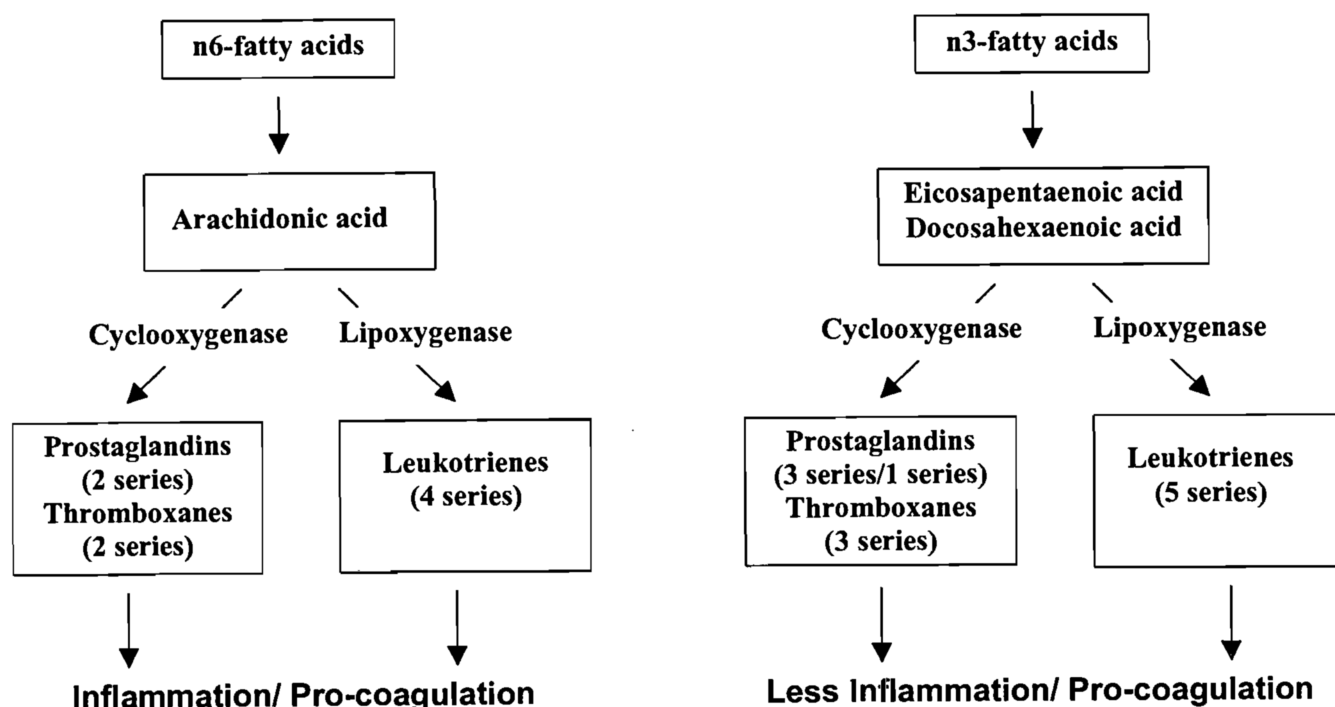
Randomised trials have shown that both elemental diets, and polymeric diets are effective in achieving remission in CD patients,[73,121,122] and have few side effects.[123,124] Elemental diets reduce intestinal inflammation and protein loss, while restoring the intestinal mucosal barrier function[125,126] and in adolescents they may reverse growth failure and increase lean body mass.[126]

The manner in which dietary therapies have their effects is still not entirely clear. Improving nutritional status may assist the healing process and elemental diets, by avoiding antigenic stimuli in the gut, may influence the immune response to down regulate. Also, the provision of essential nutrients such as glutamine, which influences the metabolism and differentiation of epithelial tissue, might enhance the repair of the diseased intestinal mucosa.[119]

Fish oil

Fish oil contains high concentrations of eicosapentaenoic acid and docosahexaenoic acid (n3-fatty acids). These fatty acids compete with arachidonic acid (n6-fatty acids), resulting in the production of prostaglandins and leukotrienes which are less inflammatory.[127] Although fish oil has been shown to decrease leukotrine B4, a pro-inflammatory factor in IBD, the clinical response in active IBD has been disappointing.[29,127] Beluzzi et al, however, showed that an enteric-coated fish oil preparation was effective in reducing the rate of relapse in CD[128] (Figure 2[129]).

Figure 2: Interaction beween n6-fatty acids and n3-fatty acids.
Ingestion of n6-fatty acids results in the metabolism of arachidonic acid and the production of inflammatory prostaglandins (of the 2 series) and leukotrienes (of the 4 series) such as leukotriene B4. Ingestion of large amounts of n3-fatty acids, however, alters the pathway, so that eicosapentaenoic acid and docosahexaenoic acid compete with arachidonic acid for the pathway, and produce prostaglandins of the 1 or 3 series and leukotrienes of the 5 series, which are less pro-inflammatory.



Short–chain fatty acids

Deficiency of SCFAs has been implicated in UC and in addition, UC patient's colonocytes may have a reduced capacity to oxidise SCFAs. Furthermore, SCFA enemas have been reported to be beneficial in treating distal disease.[29]

2.1.8.3 Surgery

Surgery, with total colectomy, may be considered curative in UC, and is indicated in patients with refractory disease, or those considered to be at high risk of complications such as malignancy. In CD however, surgery is generally restricted to complications, such as obstructive disease and fistulisation.[5]

Ileal resections may result in fat malabsorption and steatorrhoea, and inadequate vitamin A, D, E, K and B_{12} absorption can occur.[130]

Part 2: Dietary and Nutritional Assessment

2.2.1 Introduction

There are various methods of assessing dietary intake, and each has its advantages and disadvantages. All the methods, however, rely on information given by the subjects themselves.[131] Important sources of errors in all dietary surveys include inaccurate recall of foods eaten and incorrect estimation of portion sizes.[132] Reported current dietary habits may also be more representative of previous dietary habits, which may have since changed due to gastrointestinal disorders. Subjects may also be tempted to report a diet which they consider "prudent". The limitations of dietary assessment by recall should therefore be appreciated.[131]

2.2.2 Methods of dietary assessment

Dietary assessment methods include dietary histories, dietary records, dietary recalls, and food frequencies. Each of these will be discussed briefly.

2.2.2.1 *Dietary history*

This method of dietary assessment involves the questioning of a subject about a typical day's eating pattern. Each meal is discussed in turn to find out which foods were used, and in what proportions. Other alternatives, which may have been used on other days of the week, and any irregularities in the eating pattern are established, so that a menu for 7 days to a month can be calculated. This type of interview usually lasts for 1-1.5 hours[131] and has been found to be an "adequately reliable" method of dietary assessment.[133]

2.2.2.2 *Dietary records*

The subject weighs and records before eating and again after eating. The total value of the food that was eaten is then recorded in a journal. The subject must therefore be taught the correct method of food weighing, and instructed not to change their habitual diet. Any composite recipes should also be written down, as well as type of food that is eaten, and the method of preparation in order for intake to be analysed correctly.[131] Therefore, this method is time-consuming and needs a literate subject with a large amount of patience.[131,133]

2.2.2.3 *Dietary recall*

Dietary recall involves an interview in which the actual food intake of the subject for the immediate 24-48 hours is recalled. Food quantities are assessed by the use of household measures; food models, and/or photographs.[131] However, it has been

reported that this method leads to under-reporting of normal intake, and therefore a single 24 hour recall cannot be used to accurately assess an individual's intake.[133]

2.2.2.4 *Food frequency*

The food frequency method estimates how frequently certain foods are eaten during a particular period of time. To estimate the nutrient intake, food frequency scores for individual items are multiplied by the nutrient content of the local standard portion or estimated portion size.[131] A semi-quantitative food frequency questionnaire can be used in which portion sizes are assessed. During this questionnaire the subjects are asked to report their current intakes of various foods according to the frequency of consumption, as well as the typical serving size that is consumed of the particular food. Food models, measuring cups, and spoons are used throughout the interview in order to assess usual intake more accurately.[134] This method may be of greatest use in epidemiological studies.[132] It is cheap, quick and simple to use.[132,134] The frequency form can easily be filled out by the subjects themselves,[132] however, to establish the quantities of the food eaten, a trained investigator is needed.

Since this method of assessment relies on the subject's ability to recall what they have eaten, and omissions may be made, the estimates of intake may be lower than those that could be obtained from a dietary record method.[134] Furthermore, the relative validity of the food frequency questionnaire estimating a limited number of dietary components is, in general, better than the relative validity of these type of questionnaires trying to estimate the whole diet.[131]

Accuracy of the Food Frequency questionnaire for the analysis of antioxidant vitamins

In a study by Russell-Briefel et al, in which three dietary methods (the 24 hour recall, the three-day food record and a food frequency questionnaire) were compared for their ability to evaluate vitamin A intake, it was found that the food frequency questionnaire method produced vitamin A results more similar to the 3-day record

(which is considered the most reliable method of the three), than the 24-hour recall.[135] Similarly, in a previous study in our clinic where we assessed the nutrient intake of our patients with both the 24 hour recall and the semiquantitative food frequency questionnaire, we found that the 24 hour recall seemed to underestimate intake in these patients.[9] Since the food frequency questionnaire covers the longest period of time and usual intake is collected, the intra-individual variation is decreased. And therefore, the food frequency questionnaire may be the preferable method for the estimation of vitamin A intake in individuals. Furthermore, the reliability of food frequency questionnaires has been reported to be high.[135]

Similarly, other investigators have found that a food frequency questionnaire was a valid instrument for assessing the intakes of carotenoids and α-tocopherol,[136-139] and this nutritional assessment measure has been used in a number of trials assessing intake of antioxidant vitamins including the Scottish heart health study,[140] the Alpha-tocopherol Beta-carotene (ATBC) Cancer Prevention study,[141] and a study by Persson et al, looking at the effect of diet in IBD.[142]

Therefore, since the food frequency questionnaire has been shown to be a valid instrument for assessing intake of antioxidant vitamins,[136-139] is cheap, quick and simple to use,[132,134] is the method that may be of greatest use in epidemiological studies,[133] and has been used in similar studies to ours,[140-142] a semi-quantitative food frequency questionnaire will be used in this study.

2.2.3 Nutritional assessment

2.2.3.1 Anthropometry

Anthropometric assessment of nutritional status has been shown to be a simple, reproducible method of detecting patients at risk from malnutrition,[66] either from over or undernutrition.

The nutritional status of an adult is easily indicated by relating their weight to their height. The body mass index (BMI) relates the weight to the square of the height, an estimate of the body surface area (BMI =weight/height^2).[5] By convention a BMI of greater than 30 is obese, 25-30 is overweight, 18.5-25 is normal, 16.0–18.4 is undernutrition and a BMI below 16 is severe malnutrition.[9]

Another simple method of identifying malnourished patients is the measurement of the mid arm circumference. Mid arm circumference (MAC) measurements correlate positively with body weight in patients with CD.[143] Tricep skinfold thicknesses (TSF) however, may be a useful assessment measure of fat stores, particularly in large scale population studies.[144] Apart from the lack of need for accurate weight and height scales, the MAC does not offer any real advantage over the BMI.

For the purposes of our study, the BMI, MAC, and TSF will be used as an indication of nutritional status.

2.2.3.2 Laboratory measurements

Serum albumin levels and total protein concentrations correlate poorly with lean body mass.[145] They also tend to decrease rapidly during inflammation (negative acute phase response), and may also be lost from the bowel as a result of inflammatory exudation (protein-losing enteropathy). Therefore, albumin is not a good indicator of

nutritional status,[146,147] however low levels may compromise the healing process, and therefore may reflect a poor prognostic sign.

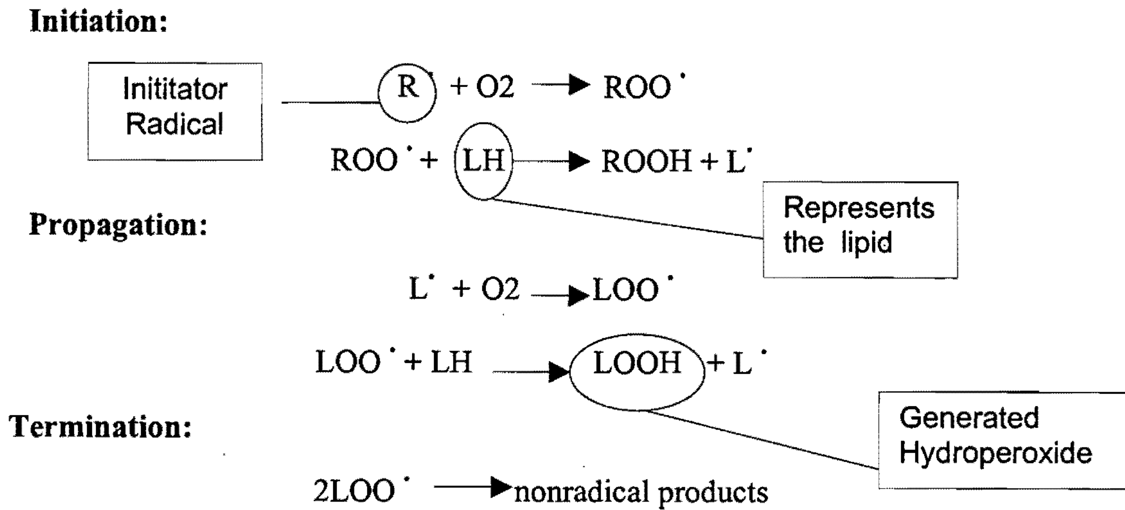
Part 3: Free Radicals and Antioxidants

2.3.1 Introduction

2.3.1.1 Free Radicals

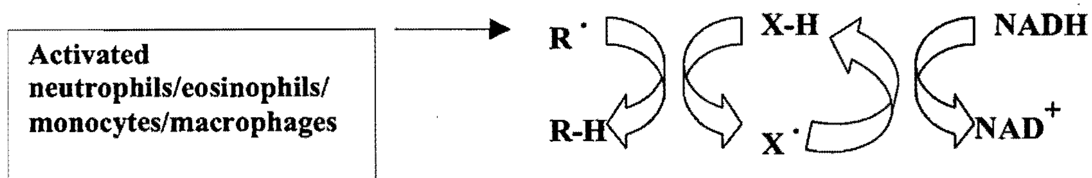
Free radicals are compounds with an unpaired electron or proton, which are unstable and react readily with other molecules, forming new free radicals.[3] They can be formed in biological systems in various ways, and are often released by activated neutrophils,[41,42-50,56-60] eosinophils, monocytes,[50,56,58] and macrophages.[56] Organic compounds may be cleaved and each of the new molecules keeps one electron from the original pair, or a receptor molecule may capture an electron. Furthermore, a chain reaction can occur during the formation of free radicals in polyunsaturated fatty acids (PUFA), in which the resulting fatty acid radicals can react spontaneously with oxygen to form a fatty acid peroxyl radical. This may then abstract hydrogen atoms to form hydroperoxide and new fatty acid radicals, thus propagating the process[148] (Figure 3[148]).

Figure 3: The free radical chain reaction that occurs during lipid peroxidation. An initiator radical forms new free radicals in the lipid membranes and hydroperoxide is generated. If two free radicals join together, they may share the extra electron or proton, thereby terminating the chain reaction



Oxygen containing free radicals (e.g. hydroxyl radical, superoxide anion radical, H_2O_2, singlet oxygen, HOCL, NO radical and peroxynitrite) are highly reactive, and are capable of damaging molecules such as DNA, protein, carbohydrates and lipids in the membranes of cells and on the nucleus. The destructive chain reaction that is started by free radicals can be broken by antioxidants, which are able to convert harmful free radicals into harmless derivatives[149] (Figure 4).

Figure 4: Free radical propagation and antioxidant interaction. Free radical R˙, produced from activated neutrophils, for example, tries to propagate the free radical but passes the extra electron/proton to the antioxidant X, which passes the free proton/electron to NADH, thereby ending the chain reaction.



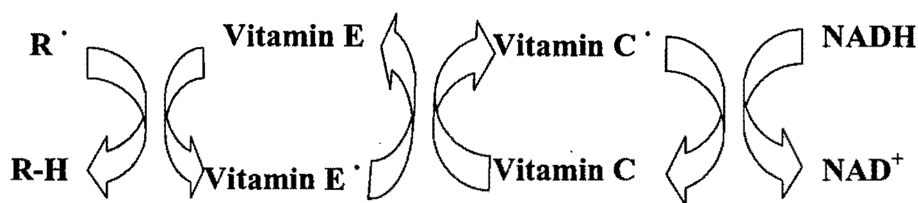
2.3.1.2 *Antioxidants*

A broad definition of an antioxidant is "any substance that, when present at low concentrations compared with those of an oxidisable substrate, significantly delays or

inhibits oxidation of the substrate".[150] Vitamin E, vitamin C, β-carotene and vitamin A all may act as antioxidants.[149]

Vitamin E (α-tocopherol), the major lipid-soluble antioxidant,[149,151] protects against lipid peroxidation. Vitamin C quenches free radicals as well as singlet oxygen and can also regenerate the reduced antioxidant form of vitamin E[69,149,151] (Figure 5[69]). β-carotene is a quencher of singlet oxygen, and vitamin A scavengers the lipoperoxyl radical,[152-154] and therefore, they both have antioxidant properties.[149]

Figure 5: Interaction between free radicals, and vitamins E and C. The free radical R˙ transfers its extra charge to vitamin E, thereby converting the vitamin to the tocopheroxyl radical. This then passes the charge to vitamin C, which itself becomes a free radical, while vitamin E is regenerated. The vitamin C then passes the charge to NADH thereby, terminating the free radical propagation.



The western world has been reported to have a relatively low intake of antioxidant vitamins or a borderline antioxidant vitamin status.[149] Other nutrients such as flavenoids and other food components (in vegetables, fruits, seeds, nuts, tea and wine), as well as zinc and selenium, may also contribute to the antioxidant and free radical scavenging capacity of the diet.[149]

The antioxidants act synergistically, and metabolic interrelations exist between antioxidant nutrients with beneficial protection and regeneration.[149]

Vitamin E and vitamin C synergistically inhibit the oxidation of liposomal membranes and LDL.[69,149] Ascorbic acid scavenges aqueous radicals and also acts as a synergist to regenerate α–tocopherol.[69,149,151] β-carotene and vitamin E may also have an additive synergistic effect,[149] as does retinol. In fact retinol may have greater

antioxidant potential when acting together with α–tocopherol, than when acting alone.[148] Furthermore, retinol may also regenerate α–tocopherol from the tocopheroxyl radical.[154]

The location of the antioxidant in the membranes and lipoproteins and its mobility in this environment are also important. β-carotene, which is less reactive than α-tocopherol and ascorbic acid, is found in the inside of the lipophilic domain in the membrane, making it easier for it to scavenge lipophilic radicals. α-tocopherol, on the other hand, is found at or near to the membrane surface, and the efficiency of this antioxidant decreases as it gets deeper in to the membrane.[149] Ascorbic acid, which acts in the aqueous phase, and α-tocopherol, in the lipophilic compartment, interact at the interface between the membrane of lipoprotein and water. They inhibit oxidation synergistically, but α-tocopherol is more potent and more effective at scavenging radicals.[149]

There is also a complementary effect of antioxidant nutrients in protecting different tissues or cellular compartments. Glutathione, ascorbic acid and α-tocopherol are all components of a regenerating redox cycle. α-tocopherol, is the major membrane-associated antioxidant. It scavenges lipid peroxy free radicals and interrupts the chain reaction of lipid peroxidation, becoming oxidised in the process. Vitamin C (ascorbic acid) is a scavenger of neutrophil oxidants and converts the tocopheroxyl radical back to the active α-tocopherol. In this way the antioxidant activity of vitamin E is replenished,[69,149,151] and further oxidation induced by this radical is prevented.[69] Glutathione, found in high concentrations in red blood cells, is capable of reducing oxidised vitamin C. Therefore these three antioxidants act together to protect tissues from oxidative damage. A change in any one of these antioxidants may, therefore, be balanced by changes in the other two, and in this way α-tocopherol may be spared.[149]

Recent evidence suggests that there may a link between increased oxidative stress, and a decreased antioxidant status in chronic conditions such as pancreatitis,[155,156] HIV,[157] coronary heart disease,[158] cancers,[158] and more recently IBD.

2.3.2.1 Antioxidants and Pancreatitis

Oxidative stress has been implicated in the pathogenesis of pancreatitis. Increased free radical activity is a common finding in pancreatitis.[155] Suboptimal availability of antioxidants in the face of increased demand, especially for those substances that protect cells against non-biological free radicals – may be the most important factor.[156] In fact an antioxidant supplement, has been shown by Uden et al, to significantly reduce the attack rate and background pain in chronic pancreatitis, as well as to normalise the suboptimal serum levels of selenium, β-carotene and vitamin E in these patients.[159] The reason for the low serum concentrations of these vitamins, and vitamin A remains controversial,[160] but may be due to inadequate intakes.[161]

2.3.2.2 Antioxidants and HIV

HIV infection results in the progressive development of opportunistic infections and malignancy due to immunological changes, which eventually results in AIDS. Oxidative stress induced by the production of ROMs may add to the progression of the disease. In HIV patients without active opportunistic infections, there may be increased oxidative stress and weakened antioxidant defences with decreased serum levels of vitamin C, α-tocopherol, β-carotene and selenium, and increased levels of lipid peroxides.[162] Furthermore, oxidative stress has also been implicated in HIV replication.[162,163]

2.3.2.3 Antioxidants and Coronary Heart Disease

Recent evidence indicates that it is the oxidised products of fats, cholesterol and linoleic acid, and not the fats themselves, that are deposited in the arterial walls

forming atherosclerotic lesions. The oxidisation may be consequent to cigarette smoking and industrial pollutants.[164] Antioxidants including vitamin A, β-carotene, vitamin C and vitamin E, are thought to prevent atherosclerosis, by preventing the oxidation of low density lipoprotein.[149,165] Vitamin C and retinol, however, may also exert a protective effect through the restoration of tocopherol to the reduced state.[154,166]

In the last two decades, studies have suggested an inverse relationship between the amount of fruit and vegetables that are eaten, and coronary heart disease.[149] More recently, studies have shown that there is a significantly decreased incidence of cardiovascular disease among those who have high plasma levels of β-carotene,[149] vitamin E,[149,167-169] and C.[166] This however, remains controversial.[149,169]

2.3.2.4 Antioxidants and Cancer

Dietary intake of ascorbic acid, tocopherols, vitamin A and β-carotene, has been linked to a decreased risk of various cancers[149,158,170-172] including colorectal cancer,[149,170] breast cancer,[149,171] pancreatic and gastric cancers,[149,172,173] lung cancer [149,170] and bladder cancers.[149] However, in the ATBC lung cancer study, in which 29133 male heavy smokers between the ages of 50-59 years were studied, it was found that the use of β-carotene supplements of 20mg/day was associated with a significant increase in the incidence of lung cancer.[149,174] Similarly, the Carotene and Retinol Efficacy Trial (CARET), which tested the chemopreventative effects of administering β-carotene and vitamin A together, found lung cancer was significantly more frequent in the group which received relatively high doses of β-carotene (30mg/day) and vitamin A (25 000IU retinol) for an average of 4 years, than in the placebo group.[149,169]

The mechanism by which antioxidants are involved in carcinogenesis is not known. Free radicals may damage the DNA which can lead to cancers, however, they may

also act independently as tumour promoters. All antioxidants might also, to a certain extent, modulate secondary messengers, such as by the inhibition of adenylate cylase.[157] Furthermore, antioxidants themselves may act as pro-oxidants under certain circumstances,[174] and indeed, β-carotene in high doses under highly oxidative conditions, such as in smokers and alcohol users, results in oxidative breakdown products which may interfere with retinoic acid binding to retinoid receptors.[175] Furthermore, these breakdown products may induce local enzymes in the lung such as the P450 enzymes, which increase the catabolism of retinoic acid.[176] This may explain the increased incidence of lung cancer in the above studies, but further research is needed in this regard.[177]

2.3.2 Antioxidants and Inflammatory Bowel Disease

Due to the potential role of free radicals and reactive oxygen metabolites in the pathogenesis of IBD, recent research has examined the role that antioxidants play in the disease.

Various studies have shown decreased serum antioxidant vitamin concentrations in IBD patients, however, results remain conflicting and controversial. Geerling et al, found decreased serum concentrations of β-carotene, vitamin C, vitamin E, selenium and zinc in 32 CD patients compared to controls.[74] Kuroki et al, showed that although their CD patients were depleted in vitamin A and E, they were not deficient in vitamin C. The authors postulated that vitamin C remains unchanged until the disease has severely progressed.[35] Another similar study of both UC and CD patients, reported that patients had lower serum levels of vitamin A, β-carotene, vitamin C and vitamin E. However, serum lipid levels were noted to be low, and when vitamin E was expressed as a vitamin E/lipid ratio, no difference to controls was evident.[178] A further study of 137 CD patients, also reported low levels of serum β-carotene levels, but normal serum retinol values.[33] Ascorbate levels were also reduced in these CD patients, and did not appear to be affected during therapy (prednisone or

sulfasalzine). A reduced dietary intake of vitamin C was considered the most likely explanation for the low levels.[37] In a study by Hoffenberg et al, in which 24 children with inflammatory bowel disease were studied, it was shown that the children with CD were particularly malnourished, compared to those with UC, and the control group. The children with CD had decreased ascorbic acid plasma levels, and increased glutathione peroxidase, glutathione, and α-tocopherol levels compared to the controls. The UC group had a trend toward lower ascorbic acid concentrations, and they also had decreased retinol concentrations compared to CD patients. The low serum vitamin C concentrations were thought to be consistent with colonic inflammatory disease. There were no significant differences in plasma selenium, β-carotene, γ-tocopherol or retinol binding protein (RBP) values between UC, CD and controls.[38]

There are reduced concentrations of superoxide dismutase and metallothionein in the intestinal mucosa of those with IBD. These enzymes contain zinc and copper and are known antioxidants. Therefore, their reduction in IBD, may decrease intestinal protection against oxidant damage and treatments aimed at increasing the mucosal concentrations of copper/zinc containing antioxidant proteins may be useful in the treatment of IBD.[64]

Although several studies have reported reduced antioxidant status in IBD, few have studied the role of supplementation in IBD. Wright et al,[179] was unable to demonstrate any effect on relapse rates in eighty six CD patients supplemented with 50 000U vitamin A twice a day.

The alterations in circulating concentrations of antioxidant vitamins and minerals, suggests an imbalance in antioxidant defences in IBD, primarily in those with CD.[38] The precise role of this in the promulgation of active disease is, as yet, unclear.

2.3.3 Vitamin C

2.3.3.1 Chemistry

Ascorbic acid (AA) is soluble in water, slightly soluble in alcohol, and insoluble in organic solvents. In aqueous solution, the compound is easily oxidised to the diketo form, dehydroascorbic acid (DHAA). The term vitamin C is used for all compounds that have qualitatively the same biological activity as ascorbic acid. Therefore, this term refers to either or both of the common biologically active forms, AA and DHAA.[70]

2.3.3.2 Dietary Intake and Requirements

More than 80% of the vitamin C in western diets comes from food or vegetable origin, mainly citrus fruits, green vegetables, peppers, tomatoes, berries and potatoes. A small portion comes from fortified products, meat, fish, poultry, eggs and dairy products. Essentially none comes from grains.[70] AA is easily destroyed by oxidation, especially in the presence of heat and alkalinity. It is also highly water soluble.[143] Therefore, the amount of available vitamin C in foods may be significantly reduced because of the destruction that occurs during cooking and loss in cooking water,[70,180,181] as well as the addition of sodium bicarbonate to preserve the colour of cooked vegetables.[182]

The dietary requirement for vitamin C remains controversial and ranges from 30mg per day (United Kingdom and WHO) to 75mg per day (West Germany).[70] However, an optimal vitamin C intake, of 200mg/day has been proposed, in order to decrease risk of various diseases such as coronary heart disease.[180,183] The recommended dietary allowance (RDA) however, recommends a daily intake of 60mg per day,[184] which provides plasma and leukocyte AA levels above the "at risk" or low levels of 0.4mg/dl. An intake of 60mg of AA per day is easily achievable in a normal western

diet.[70] However, it has been proposed that an increased intake should be recommended for smokers,[70,185,186] the elderly, and for the ill.[70]

2.3.3.3 Status Assessment

There are a number of methods for assessing vitamin C status, however, only the most practical methods will be discussed in this section.

Measurements of plasma and leukocyte AA levels are currently the most practical and reliable tests of assessing human vitamin C status. Plasma levels are responsive to recent dietary intake, whereas leukocyte levels reflect tissue contents. Plasma AA tests are preferred for large population studies because the test requires less blood, is easier to perform, and the results are relatively easy to interpret.[70] In healthy, fasting subjects the concentration of vitamin C in plasma can range from 25-80μmol/L (0.4 – 1.4mg/dl).[180,187,188] Serum/plasma measurements will therefore be used in this study.

Whole blood or erythrocyte AA levels are thought to be less sensitive indicators of AA deficiency, but are qualitatively the same as plasma.[70]

Urinary AA content is not useful for differentiating between subjects with normal or low AA status as the rate of excretion of urinary AA is not linear with AA intake due to efficient renal reabsorption at low AA intakes, and renal clearance at high intakes. However, measurement of urinary AA levels after a loading dose of 0.5 to 2g of AA over 4 days can be useful for assessing a tissue ascorbate deficit. Excretion of less than 60% of the dose indicates depletion of tissue AA.[70]

Women tend to have higher vitamin C concentrations than do men.[70,187,188] Smokers have been shown to have lower AA levels in plasma and leukocytes, and lower

dietary AA intake of smokers only partially explains the lower levels. Plasma levels of AA may also decrease with age.[187,189]

Interpretive Guidelines

The generally accepted interpretation of plasma levels is illustrated in table 4.

	Plasma umol/L (mg/dl)
Normal Range	23 – 84 (0.4 – 1.5)
Low	11.4 – 23 (0.2 – 0.4)
Deficient	<11.4 (<0.2)

μmol/L ÷ 56.8 = mg/dl

Table 4: Plasma levels of vitamin C for status assessment[70]

2.3.3.5 *Metabolism*

The intestinal absorption of AA and its entry into cells may be facilitated by conversion into DHAA, which penetrates membranes better than the reduced form at physiological pH, and is readily reduced back to AA in cells.[70,190] Seventy to 90% of the usual dietary intake of AA is absorbed. Ingestion of several spaced doses of less than 1gram throughout the day, are absorbed maximally, rather than the ingestion of a single megadose. This is probably due to a saturable absorption mechanism.[70]

Intracellularly, AA is almost entirely in the reduced form, and is localised to the cytosol which suggests a protective antioxidant function. In the plasma vitamin C exists in the free (nonprotein-bound) and reduced form.[70,191]

The amount of AA excreted depends on the amount of AA cleared by glomerular filtration which depends on the plasma AA level and the glomerular filtration rate.[70]

As this amount increases, the ability of the renal tubules to resorb AA reaches a maximum, at the urinary re-absoprtion threshold of about 85μmol/L, and the unresorbed excess AA is excreted in the urine,[185,192] which is the main route of AA elimination.[70] Negligible amounts of AA or its metabolites are excreted via faeces. The percentage of unmetabolised AA excreted in the urine relative to catabolic products increases with increasing dietary intake. A small amount of AA is converted to oxalic acid (5-10%), and is found even at very low dietary AA intakes. With increasing AA intakes, however, conversion to oxalate is limited.[70]

The average half-life of AA in the adult human is 16–20 days. The whole body turnover of vitamin C or catabolic rate depends on the AA body pool size. Intake of 8–10 mg per day of the vitamin is sufficient to provide enough AA to satisfy critical function and prevent deficiency.[70]

2.3.3.5 Functions

AA has numerous functions including collagen formation, carnitine biosynthesis, cross linking reactions and formation of connective tissue,[70,193,194] neurotransmitter synthesis, and the formation of norepinephrine.[180,195] It also has a role in the drug metabolising system that operates in liver microsomes and reticuloendothelial tissues,[70,196] as well as increasing the intestinal absorption of non-haeme iron.[70] The functions of AA which are directly related to this thesis, such as anticarcinogensis,[170,182] immunocompetence[70] and apoptosis,[197-199] are discussed below.

Anti-carcinogenesis

Vitamin C may also have anti-cancer properties[170,182] at high concentrations[182] by inhibiting nitrosamine formation, preventing activation of carcinogens, enhancing detoxification of carcinogens, improving the immune response, and inhibiting the promotion phase.[170]

Immunocompetence

A variety of immune-related functions have been associated with AA status, including decreased resistance to a variety of infectious agents during AA deficiency, effects on neutrophil activity, and lymphocyte blastogensis. Certain immune system modulators are affected by ingestion of AA, including levels of cyclic nucleotides in B and T cells, IL-1 and levels of histamine, prostaglandins, and prostacylin.[70]

Apoptosis

At high concentrations, vitamin C induces apoptotic cell death, possibly due to its pro-oxidant activity. At lower concentrations however, AA acts as an antioxidant, and therefore inhibits apoptosis.[197-199]

2.3.3.6 Deficiency

If dietary intake is inadequate, scurvy can result. Some historically reported symptoms of scurvy may be due to coexisting nutrient deficiencies such as of thiamin, "wet beriberi" (oedema), vitamin A (night blindness), vitamin D (rickets) and folic acid (megaloblastic anaemia).[70]

Clinical scurvy is rare, but usually occurs in those with exceptionally poor diets, such as alcoholics and drug abusers, or in those who have a near total lack of AA-containing foods. A greater proportion of low blood AA levels is found in the institutionalised elderly population, or those who are homebound or sick.

2.3.4 Vitamin A

2.3.4.1 Chemistry

Chemically, vitamin A is a subgroup of retinoids. The term vitamin A is used as a generic term for retinoids which have the same biological activity as all-trans retinol.[182,200] Precursor forms of vitamin A are those carotenoids that have the same biological activity of vitamin A after intestinal conversion of retinol. The most predominant carotenoid is β-carotene.[201]

Except for water, vitamin A and carotenoids are soluble in most organic solvents. Their increased sensitivity to light, oxygen, acid and high temperatures[202] demands that vitamin A (retinol) be stored in frozen serum in the dark at -20° C in sealed vials.[203] Carotenoids are less stable than retinol[200] and therefore require storage at -40°C.[203]

The primary unit of biological activity for vitamin A is 1 μg of all-trans retinol. Although retinyl esters are more common than retinol in food and are almost always present in synthetic preparations of vitamin A, the biological activity is still calculated as the amount of all-trans retinol present.[200]

About 50 carotenoids show pro-vitamin A activity. The most active pro-vitamin, is all-trans β-carotene, which is often found in food. Most other pro-vitamins are carotenoids, e.g. α- and γ–carotene which show biological activities of between 20-60%.[200]

In order to express both preformed vitamin A and β-carotene equivalents as a single value, retinol equivalents (RE) were created. 1μg RE is the equivalent of 1μg all-trans retinol, 6μg of all-trans β-carotene, or 12μg of other pro-vitamin A carotenoids in food.[200]

2.3.4.2 Dietary considerations

Food and other sources

Good dietary sources of preformed vitamin A (retinyl esters) are liver, other internal organs, whole eggs, dairy products and whole small fish. Good sources of pro-vitamin A carotenoids include carrots, dark green leafy vegetables, tomatoes, yellow maize, papayas, ripe mangoes,[200,204] spinach, oranges[200] and plums.[204] The richest sources of preformed vitamin A are the liver oils of marine fish and of marine mammals, whereas those of carotenoids are red palm oil and carrot oil.[200]

Requirements and recommended intakes

The RDA of vitamin A in μg RE is shown in table 5. 1μg RE = 3.5nmol, and is assumed to be nutritionally equivalent to 11nmol (6μg) of β-carotene and to 22nmol (12μg) of other pro-vitamin A carotenoids.[200]

RDA for Vitamin A		
Category	**Age (yrs)**	**Vitamin A (μg RE)**
Males	10-12	1000
	12-70+	1000
Females	10-12	800
	12-70	800
Pregnancy		+0
Lactation	0-0.5	+500
	>0.5	+400

Table 5: Recommended dietary allowances for vitamin A[184]

Nutrient Interrelations

Protein

Protein deficiency reduces the carotenoid cleavage and the synthesis of retinoid-binding proteins and receptors.[200]

Fat

Ten to twenty grams of fat per day is needed for efficient intestinal absorption of vitamin A and carotenoids. Vitamin A and fat must be eaten together.[200]

Iron

Plasma haemoglobin levels or iron status, are depressed in vitamin A deficiency and enhanced by vitamin A supplements. Vitamin A may act on the metabolism and storage of iron, or more probably, on the differentiation of red blood cells in the bone marrow.[200]

Zinc

In zinc deficiency, vitamin A in the plasma decreases, but that in the liver increases. Embryogenesis is impaired by deficiencies of both vitamin A and zinc. Zinc seems to play a general role in gene expression, due to its requirement for the binding of any nuclear transcription factor to deoxyribonucleic acid (DNA).[200]

Vitamin E

Vitamin E protects vitamin A from oxidation in the gut and presumably in the storage globules of the liver and other organs as well. Vitamin E reduces the rate of hydrolysis of retinyl esters in the liver.[200]

Other Nutrients

Vitamin A may also react in various ways with vitamin C, vitamin K, vitamin D, calcium, copper and iodine.[200]

2.3.4.3 Status Assessment

Large variations, from 0.15-0.45μmol/L, were found the plasma levels of retinol concentrations in a study by Dimitrov et al.[205] Plasma vitamin A concentrations lower than 0.35μmol/L (<10μg/dl) are indicative of vitamin A deficiency. Levels higher than 1.05umol/L (>30μg/dl) are associated with a satisfactory status.[200] Values above 21.5μg/dl are desirable for β-carotene.[206]

Serum levels of β-carotene are generally 10-15% higher in women,[207-209] while retinol levels have been found to be higher in men.[207,208]

2.3.4.5 Metabolism

Preformed vitamin A and carotenoids in foods are released in the stomach during proteolysis. They pass with lipids into the small intestine where they are emulsified by bile, to form micelles. Bile salts also stimulate pancreatic lipase, which in turn hydrolyses retinyl esters to retinol. Seventy to ninety percent of retinol in a bile-salt containing micelle is absorbed from the small intestine, while retinyl esters are poorly absorbed.[201] β-carotene is converted in the cytoplasm of the intestinal mucosal cells to retinyl esters while most pro-vitamin A carotenoids can be converted in the cytosol of the mucosal, hepatic and other tissue cells to retinol or retinoic acid. The bioavalibility of carotenoids is not known, as there is great variability in its absorption and conversion to retinol. β-carotene yields two molecules of retinol, which are mostly reduced and esterified to retinyl esters.[200] Conversion of β-carotene

to vitamin A is regulated so that excess vitamin A is not absorbed from carotenoid sources.[182]

After absoprtion into intestinal cells, newly formed chylomicrons, containing retinyl esters, are secreted into the plasma and converted to chylomicron remnants, which increases the relative concentration of retinyl esters.[200] The chylomicron remnants are then internalised in the liver. Retinyl esters are hydrolysed and combine with cellular RBP in the cytosol of the hepatocyte. Retinol may be stored as retinyl palmitate in the hepatocyte, or it may be esterified and stored in stellate cells. Under normal physiological conditions, stellate cells contain 80-90% of the stored vitamin A, hepatocytes 10-20% and other liver cells only a few percent. The stored vitamin A can be readily and completely mobilised.[200]

The release of retinol into the plasma from storage sites includes a number of proteins including apo-retinol binding protein (apo-RBP) which is formed in the hepatocyte. All–trans retinol combines with apo-RBP to form holo-retinol binding protein (Holo-RBP). This is then transported through the golgi apparatus, and is secreted in to the plasma where it combines with pre albumin.[200] From the plasma Holo-RBP interacts with cell-surface receptors for RBP on target tissue cells. Retinol is internalised, while apo-RBP is modified in conformation and released. This modified apo–RBP no longer binds retinol and is finally catabolised, mainly by the kidney.[200]

Although vitamin A is mainly stored in the liver, all tissues contain some vitamin A, with the main stores in the fat depots, lungs and kidneys.[182]

Between 5 to 20% of ingested vitamin A and a large percentage of carotenoids, are not absorbed from the intestinal tract and are excreted in the faeces. Ten to forty percent of absorbed vitamin A is oxidised or conjugated in the liver and then secreted

into the bile. Most of these biliary metabolites are excreted in the faeces, and some vitamin A is excreted in the urine. Of the dietary vitamin A taken in, on average 10% is not absorbed, 20% appears in the faeces through the bile, 17% is excreted in the urine, 3% is released as carbon dioxide, and 50% is stored, mainly in the liver.[200] A reserve supply gradually accumulates, which reaches its peak in adult life. This storage capacity allows for a temporarily reduced daily intake of vitamin A.[200]

Factors which affect absorption

Fat

A higher fat diet seems to increase absorption.[205]

Supplementation

Long term-supplementation with β-carotene may affect the serum concentrations of other carotenoids and retinol. The mechanism for this is not certain, but may be due to supplemental β-carotene modifying non β-carotene fractions by altering their absorption, distribution, storage, utilisation or clearance. Metabolic substitution by β-carotene for biological functions of other carotenoids, for example larger conversion of β-carotene to vitamin A, with less conversion of other carotenoids, e.g. α-carotene, may also change plasma levels. Alternatively, β-carotene might cause the release of other carotenoids from specific tissues into serum.[141,174]

Medications

Antihypertensive medication, in the form of diuretics mainly, may increase levels of retinol, but decrease levels of β-carotene, possibly due to an increase in RBP concentration as a result of interference with its catabolism in the kidney. These higher levels of RBP, together with an adequate intake of retinol, will result in higher levels of retinol in serum.[208] The lower levels of β–carotene, however, could be result of the haemoconcentration.[208]

Cholinergic or adrenergic agents, beta-blockers, acetylcholinesterase inhibitors, alpha-methyldopa, clonidine, hydralazine, replacement hormones and calcium channel blockers may also result in increased retinol concentrations.[137]

Certain drugs such as sulfonamides, aspirin and other vitamins, as well as nitrates, potassium chloride or antacids may decrease bioavailability of β-carotene.[137,205]

Food and Gastrointestinal involvement

Inactivation by the gastrointestinal mucosa or by constituents of food, such as sulfides and acids, may reduce absorption of β-carotene.[205]

Alcohol

Alcohol consumers may have lower β–carotene concentrations,[206] due to a reduced intake.[209] Retinol concentrations may however, increase.[141] The exact mechanism for this remains unclear. Alcohol may cause oxidative stress which leads to greater antioxidant use and increased conversion of carotenoids to retinol.[141] However, smokers tend to have a greater alcohol consumption than non-smokers,[211] therefore, the association between alcohol consumption and serum carotene levels may actually be a result of the strong correlation between smoking and drinking.[167,209,212]

Smoking

Cigarette smoking may decrease serum β-carotene concentrations,[141,167,205,212] however retinol concentrations seem to be unaffected.[207,213,214] The smoking-induced oxidative stress may increase β-carotene utilisation.[207,214,215] Furthermore smokers tend to have a lower intake of fruits and vegetables, and hence the antioxidant vitamins.[167, 214,215]

Oral contraceptives

The use of oral contraceptives seems to be associated with large decreases in β-carotene levels among 35-44 year old women.[206] Increased oxidative stress due to oral contraceptive use, which increases serum concentrations of copper, may result in the decreased β-carotene levels.[206]

Idiopathic

Certain individuals absorb β-carotene poorly, for no apparent reason, and would require greater supplementation doses to maintain the same tissue levels as others.[205]

2.3.4.6 Functions

Vitamin A is needed for the proper functioning of most organs of the body. Vitamin A is a component of the visual pigments, and therefore, is essential for the correct workings of the photoreceptors in the rods and cones of the retina.[182] It ensures proper bone development and maintenance, and may also influence the synthesis and secretion of various cytokines and growth factors.[200]

Carotenoids and retinol prevent the oxidation of low-density lipoproteins and therefore reduce the formation of atherosclerotic lesions.[149,152-154] They may also protect against the development of cortical cataract, and reduce the oxidative stress induced by smoking.[200] Vitamin A aids in the immune response[200] and may decrease the risk of certain cancers.[149,216] Retinol, may also effect apoptosis.[217,218] Since these functions may have an association with the topic of this thesis, they are discussed briefly below.

Immune Response

In vitamin A deficiency, both specific and non-specific protective mechanisms are impaired. These include the humoural response to various infections, cell-mediated

immunity, mucosal immunity, natural killer cell activity and phagocytosis. When vitamin A deficient subjects are supplemented with vitamin A immunity improves.[200]

Vitamin A deficiency also results in the depression of phagocytosis, especially the "oxygen burst" after the ingestion of a foreign body and the secretion of IgA. Furthermore, the reduction in the production of goblet cell mucins, both in the intestinal mucosa and in the conjunctiva of the eye may also be depressed.[200]

In vitamin A sufficient animals, carotenoids also enhance the immune response.[200]

Malignancy

Vitamin A intake has been associated with the reduced risk of various cancers.[149,216] The mechanism of this anti-cancer action is not entirely clear. Retinoids may enhance differentiation and maturation of stem cells.[200]

Despite the reported anticarcinogenic effects, carotenoid therapy has resulted in an increase in the risk of lung cancer in smokers.[150] Excess intake of retinol or its related compounds have been reported to be carcinogenic, especially when taken together with alcohol.[141]

Apoptosis

Retinol induces cell differentiation and apoptosis,[217] possibly by enhancing caspase-3 activity.[218]

2.3.4.7 Vitamin A status

Vitamin A status can be classified in to five categories:

1. deficient – characterised by clinical signs.
2. marginal - there are no clinical signs of deficiency, but individuals may show some impaired clinical responses, such as an impaired immune response.
3. adequate.
4. excessive - individuals also do not show clinical signs of hypervitaminosis, but these signs may be induced by larger vitamin A intakes, or by infection, such as viral hepatitis.
5. toxic -characterised by clinical signs.[200]

Eye conditions

Vitamin A deficiency is a serious nutritional problem in preschool children in southern and southeastern Asia, in parts of Africa and South America. The major signs of nutritional deficiency in preschool children are a history of night blindness, low serum vitamin A, and a sequence of abnormalities of increasing severity in the conjunctiva and cornea of the eye (xeropthalmia). Keratomalacia may also occur.[200]

Skin changes

Vitamin A is necessary for maintenance of the skin. Retinoids stimulate basal cell proliferation but inhibit the transcription of several epidermal keratins.[200] Therefore, vitamin A deficiency causes changes in skin structure and metabolism such as follicular hyperkeratosis and phrynoderma.[200]

Hair follicles are particularly sensitive to vitamin A, and become obstructed and enlarged during vitamin A deficiency.[200]

High risk clinical situations

Any fat malabsorption syndrome, such as cholestasis, cystic fibrosis, sprue, chronic diarrhoea, pancreatic insufficiency, and biliary cirrhosis decreases the digestion and absorption of vitamin A and carotenoids and may eventually lead to a state of vitamin A deficiency or depletion.[200] Similarly, those on TPN require regular monitoring as the plastic tubing may absorb the vitamin A in the infusion fluid.[200]

The liver is of primary importance in the storage and utilisation of vitamin A Therefore, long-term alcohol intake, even in relatively small amounts, may decrease vitamin A storage. In alcoholics, the symptoms of vitamin A deficiency that occur e.g. decreased dark adaptation, can be improved by supplemental vitamin A.[200]

Even though oestrogens and oestrogen containing contraceptives cause an increase in the steady-state concentrations of Holo-RBP in the plasma, the rate of mobilisation of vitamin A from liver reserves is only slightly affected. Therefore, in otherwise healthy women, contraceptive use does not significantly change their vitamin A requirement.[200]

2.3.5 Vitamin E

2.3.5.1 Chemistry

There are eight naturally occurring vitamin E compounds with characteristic biological activity. Four vitamers are part of the tocopherol family, and four are tocotrienols.[216,219] For the purposes of this thesis, only the tocopherols will be considered.

[d]-α-tocopherol, is the most active isomer, and is found naturally in food, in addition, β-, δ-, and γ-tocopherols are also found in food.[216] The vitamin E activity of

a vitamin E isomer depends not only on the compound's structure, but also on its relative absorption, uptake by target tissues, and turnover rate. [d]-α-tocopherol has the highest vitamin activity of all the tocopherols at 1.1IU/mg.[216]

2.3.5.2 Nutritional requirements

The tocopherol content of diets shows great variation depending on harvesting, processing, storage, and final food preparation.[216]

The minimum daily requirement of vitamin E is not known however, but for adult men, the level is above 2mg per day for dietary α-tocopherol.[216]

The RDA for vitamin E is shown in table 6.

RDA for vitamin E		
Category	**Age (yrs)**	**Vitamin E (mg)**
Infants	0-0.5 0.5-1	3 4
Children	1-3 4-6 7-10	6 7 7
Males	11-51+	10
Females	11-51+	8
Pregnant		10
Lactation	1st 6 months 2nd 6 months	12 11

Table 6: Recommended dietary allowances for vitamin E[184]

2.3.5.3 Status Assessment

Serum has generally been used to assess total tocopherol concentrations.[216]

Because of the marked influence of plasma lipids on tocopherol concentrations, various tocopherol/lipid ratios have been used to determine tocopherol concentrations more accurately.[216] Since tocopherol is transported by the low density B-lipoproteins which are rich in cholesterol, the tocopherol to cholesterol ratio is one of these methods of determining vitamin E status. Furthermore, in a study investigating the use of different lipids to express serum tocopherol levels, it was found that the tocopherol to cholesterol ratio gave the best results when comparing tocopherol with individual lipid ratios, with 86% sensitivity and 94% specificity, and was also the most convenient assessment method.[220] But, expressing tocopherol to apoliproteins A1 and B may be a more accurate measure of tocopherol concentrations.[221] However, since tocopherol to cholesterol ratios are the most convenient, and have high specificy and sensitivity, the tocopherol to cholesterol ratio will be used to assess vitamin E status in this study.

Blood levels of vitamin E often do not correlate with reported dietary intake,[211] but are affected by supplementation. This may be the result of inaccurate assessments of vitamin E content of foods due to variability following cooking, processing and storage.[167]

The lower limit of "normal" concentrations of vitamin E in plasma is +/- 11.5μmol/L.[150] Blood levels tend to increase appreciably within a few days with doses of 100mg or more, but this is accumulated in the tissues at a slower rate.[222]

2.3.5.4 Metabolism

The amount of tocopherol absorbed will vary depending on the amount of total lipids absorbed. However, the efficiency of absorption decreases as large amounts of tocopherol are consumed. An average absorption rate seems to be between 50-70%.[216]

Once absorbed, vitamin E isomers are transported with fat mainly by the lymphatic systems to the venous system. Tocopherols are distributed in plasma in association with lipoproteins; mainly low-density lipoproteins[222] but no specific carrier protein has been identified.[216,222] Most tissues take up α-tocopherol, including the liver, lung, heart, skeletal muscle and adipose tissue. Fat continuously takes up vitamin E and the vitamin is concentrated wherever there are large amounts of fatty acids. But, the tocopherol stored in the adipocytes is not readily available to other tissues. If the vitamin intake is high, the majority of the vitamin E is stored in the liver, however the total body pool also increases.[216]

In healthy individuals the concentration of tocopherol present in the tissues is related to the amount of tocopherol consumed, and the lipid content of the organ. In blood, the amount of lipid present also has a major influence, if not a determining role, on the level of circulating plasma tocopherols.

The liver secretes very low-density lipoprotein (VLDL), which is enriched in α–tocopherol. It is thought that a hepatic transfer protein is needed for this process. It is found only in hepatocytes, and transfers α–tocopherol between liposomes and microsomes. The ability to transfer tocopherol is needed for the incorporation of α–tocopherol into VLDL.[219]

Other tissues may also have binding proteins for tocopherol, which are necessary for regulation and delivery to specific intracellular sites. Metabolism of vitamin E

involves the hydrolysis and shortening of the molecule to ultimately form tocopheronolactone. The major route of excretion of tocopherol metabolites appears to be faecal elimination, possibly in association with bile secretion.[216]

Factors affecting absorption

Vitamin E is fairly stable to heat and acids and unstable to alkalis, ultraviolet light and oxygen. It is destroyed when in contact with rancid fats, lead, and iron. There is no loss by extraction in cooking; however, freezing and deep-fat frying destroys most of the tocopherol present. Esters of tocopherol, e.g. tocopherol acetate, are not appreciably destroyed.[182]

Vitamin E levels are not affected by cigarette smoking[167,208,210,214,215] or alcohol consumption.[167,208,210,211]

Medications

Anti-hypertensive medication (usually diuretics) may increase α-tocopherol levels,[208] due to the diuretics causing haemoconcentration. Furthermore, cholinergic or adrenergic agents, beta-blockers, acetylcholinesterase inhibitors, alpha-methyldopa, clinidine, hydralazine, replacement hormones and/or calcium channel blockers may also result in increased serum concentrations.[136]

Nitrates, potassium chloride and antacids may decrease vitamin E.[136]

Fat malabsorption

Fat malabsorption often results in decreased absorption of tocopherols.[216]

Interaction between vitamin E and vitamin C

As previously discussed, vitamin E and vitamin C act synergistically as AA acts in the aqueous phase, while α-tocopherol acts in the lipophilic compartment, and the actions of the two vitamins meet between the membrane of lipoprotein and water.[150] Vitamin C also acts to regenerate vitamin E.[69,151]

2.3.5.5 Functions

Vitamin E has many important functions including its ability to acts as a membrane stabiliser and inhibitor of 5-lipoxygenase activity.[223] It also prevents protein kinase C membrane translocation and activation, thereby playing a role in cell growth and differentiation,[219,223] as well as modulating gene expression[219,224] preserving erythrocytes and preventing haemolysis. [219] Furthermore, vitamin E acts as a sparing agent,[182,216] inhibits cellular proliferation,[219,226] has a role in arachidonic acid metabolism,[219] the NF-κB pathway,[219,224] and apoptosis.[229] Since these functions may directly relate to the topic of this thesis, they are discussed below.

Sparing agent

Vitamin E may protect protein sulfhydryl groups and act as a "sparing agent" during oxidative stress.[216] In the gut, vitamin E may enhance the activity of vitamin A by preventing its oxidation in the intestinal tract.[182]

Nuclear Factor κB

Vitamin E may exert an effect on the signal transduction pathway of NF-κB, which may depend on its antioxidant function. NF-κB may be activated by oxidative stressors[219,224] such as hydrogen peroxide and lipid hydroperoxides, and therefore, antioxidants may inhibit NF-κB activation[219] (Figure 1). In a study undertaken by Suzuki and Packer in 1993, vitamin E acetate almost completely blocked the

activation of NF-κB, while α-tocopherol did not. However, vitamin E acetate does become esterified to α-tocopherol.[224] This may imply that the free radical process that activates NF-κB may occur proximal to the membrane, possibly in the mitochondria[219] and therefore membrane oxidation may be an integral step in the signal-transduction pathway.[224] Furthermore, since the vitamin E content of cells does not decrease in response to TNF–α treatment, it is thought that the cell possesses a vitamin E recycling mechanism.[224]

Arachidonic metabolism

Arachidonic acid is acted on by the enzymes cyclooxygenase and lipoxygenase, producing eicosonoids. The products of the cyclooxygenase dependent pathway include prostaglandins, thromboxanes, and predominantly prostacyclins. Vitamin E modifies the production of prostacyclin (PGI_2). α-tocopherol, as well as β-, γ – and ծ-tocopherol potentiate the release of arachidonic acid and thereby stimulate $PG1_2$ synthesis. It has been shown that oxidative stress decreases the production of prostacyclin. This may be due to a decrease in the effective concentrations of vitamin E in cells.[219] Furthermore, because NF-κB sequences have been found in the upstream, regulatory portion of the gene for cyclooxygenase, the cellular redox state may be an important modulator of arachidonic acid metabolism.[219]

Vitamin E may alter prostaglandin synthesis, via the modulation of phospholipase A_2 activity,[219,225] which then inhibits prostaglandin E_2 (PGE_2) synthesis.[219]

Inhibition of cellular proliferation

All forms (α–, γ- and ծ-) of tocopherol, except β-tocopherol seem to have antiproliferative properties,[219,226] at relatively high concentrations (>20mg/L). But, an ester of α–tocopherol, α-tocopherol succinate, is an effective antiproliferative agent

at much lower (<1mg/L) concentrations.[219] However, this remains highly controversial.

The succinate ester, which is delivered more effectively in culture than α–tocopherol, is highly effective in tissue culture systems and is the most active type of vitamin E in these systems. In culture systems, α–tocopherol succinate may be taken up and the ester bond may then be hydrolysed, releasing α-tocopherol. Since this α–tocopherol is then transported intracellularly, it may be the α–tocopherol which is the effector molecule.[219] However, α-tocopherol succinate itself may have antiproliferative properties which may involve the modulation of gene expression.[219,223]

The exact mechanism of how α-tocopherol inhibits proliferation is not known. Vitamin E may directly inhibit the stimulation of phospholipase A2 by protein kinase C,[219,226,227] which would prevent the release of arachidonic acid. Also, functioning as an antioxidant, vitamin E could protect highly unsaturated n-3 and n-6 fatty acids, which have antiproliferative effects,[228] thereby preventing proliferation.[219]

Apoptosis

Vitamin E impairs apoptosis. The mechanism for this is unclear, but may be related to its interaction with cytochorome c or its interaction with free radicals produced by 7-ketocholesterol, a potent inducer of apoptosis.[229]

2.3.5.6 Deficiency

Human vitamin E deficiency can be defined as "a low plasma (or serum) tocopherol level (below at least 0.5mg/dl), accompanied by a low ratio of tocopherol to lipid and/or haemolysis of erythrocytes incubated in 2% hydrogen peroxide".[216] The occurrence of vitamin E deficiency due to dietary origin is rare in developed

countries. However premature infants, patients with gastrointestinal diseases leading to steatorrhoea and malabsorption, and individuals with abetalipoproteinaemia have been shown to be susceptible to this kind of deficiency.[216,222]

Human vitamin E deficiency is associated with *in vitro* haemolysis as well as decreased erythrocyte survival *in vivo* from diminished antioxidant activity. Therefore, vitamin E deficiency may also have haematological significance in those with intestinal malabsorption.[216]

Neurological disease

Neurological disease may occur as a result of vitamin E deficiency, especially in children. However, the characteristic neurological symptoms improve with vitamin E treatment and may be preventable in those with abetalipoproteinaemia.[216]

Destruction of cellular membranes

Destruction of cellular membranes may occur in patients with vitamin E deficiency, and lipopigment (ceroid) may be deposited in human tissues such as small intestine due to modified PUFA metabolism.[216]

2.3.6 Vitamins C, A and E as antioxidants

Vitamin C is the first line of defence against free radicals in the aqueous phase of the cell membrane in blood[69,70] In fact, it contributes up to 24% of the total antioxidant power of plasma.[180,230] It is the only scavenging antioxidant which can prevent initiation of lipid peroxidation.[180,231]

Vitamin E may be the major lipid-soluble, chain breaking antioxidant in human plasma,[151] and it helps to protect fatty acids in plasma membranes from peroxidation,[215,219,232] by preventing propagation of peroxidation of lipids.[233] Vitamin E may be recycled or regenerated thereafter by vitamin C[150] thereby inhibiting it's depletion.[38] α-tocopherol, is found at or near to the membrane surface, and the efficiency of this antioxidant decreases as it gets deeper in to the membrane.[150] AA and α-tocopherol interact at the interface between the membrane of lipoprotein and water.

Although the importance of carotenoids as antioxidants remains controversial,[234] a number of studies have indicated that both β-carotene and retinol do have antioxidant properties,[149,152-154,235,236] and in certain tissues retinol may in fact be the most potent antioxidant.[152] Studies investigating antioxidant vitamins have generally included both retinol and β-carotene,[140,237-241] and we therefore felt that they should be included in this thesis.

Beta-carotene is found in the inside of the lipophilic domain in the membrane, which makes it easier for it to scavenge lipophilic radicals. Chemically β-carotene is less reactive than α-tocopherol and AA,[150,234] but it may exert a cooperative effect with α-tocopherol by residing and scavenging radicals at different positions in the lipophilic compartment.[149]

All-trans retinol also acts as a chain-breaking antioxidant[152,153,236] and intervenes during lipid peroxidation by scavenging the lipoperoxyl radical.[152,153,235] Furthermore, it acts synergystically with α-tocopherol, and may interact with the tocopheroxyl radical, thereby regenerating α-tocopherol.[154] Retinol also seems to be a more effective antioxidant when it acts together with α-tocopherol than when it acts alone,[148] possibly due to the limitation of the auto-oxidation of retinol.[148]

2.4 Conclusion and motivation for this study

Recent research indicates that free radical production plays an important role in the pathogenesis of chronic inflammatory conditions such as UC and CD,[56,57,61] and therefore protective mechanisms against these agents may be important in the control of these diseases. Several studies have indicated low antioxidant vitamin status in patients with IBD, however reports are conflicting,[29,30,34,74] and the cause, and subsequent effects of these abnormalities remains uncertain.

3. Aims and Objectives

3.1 Aim

The aim of this study was to investigate serum antioxidant vitamin status in patients with IBD.

3.2 Objectives

The objectives of this study included:

1. To assess the nutritional and antioxidant vitamin status of patients attending the IBD clinic at Groote Schuur Hospital.
2. To investigate the relationship between dietary intake, nutritional status and antioxidant vitamin serum concentrations.
3. To relate nutritional status and antioxidant vitamin levels with disease type, extent, activity, relapse rates, and medications.
4. To assess the effect of antioxidant supplementation on serum concentrations, disease activity, and quality of life in CD.

4. Methodology

4.1 Study Design

One hundred consecutive patients attending the IBD Clinic at Groote Schuur Hospital were interviewed and studied. These patients were matched with 44 healthy controls recruited from the staff and students in the hospital. Twenty three patients with inactive CD, who had experienced a relapse in the previous year, were randomised to receive antioxidant or placebo, administered as a buccal spray and were monitored for a period of 6 months. The criteria of an active episode in the previous year was used in order to attempt to identify a group of patients who were most likely to relapse during the study period.

The study was divided into three parts:

1. Assessment of nutritional status.
2. Assessment of antioxidant status.
3. Double blind, placebo controlled trial assessing the effect of an antioxidant vitamin supplement on disease relapse rates.

Permission to perform the study was obtained by the Research and Ethics Committee, University of Cape Town and informed, written consent was obtained from all subjects. Good clinical practices (GCP) were maintained throughout the study.

4.1.1 Inclusion Criteria

1. Definite diagnosis of IBD based on histologic or radiologic findings.
2. Above the age of 18 years.

4.1.2 Exclusion Criteria

1. Pregnant females.
2. Clinically relevant organ failure or other diseases.
3. Inability to participate because of comprehension reasons.
4. Use of antioxidant vitamin supplements within 2 weeks before the start of the trial.
5. Extensive resectional surgery (>100cm) with short bowel.

Part 1: Assessment of nutritional status

4.2.1 Methodology

4.2.1.1 Baseline data

Baseline data was gathered from all subjects.

1. Case notes were reviewed in order to gain a thorough medical history of each patient.
2. Dietary information was obtained.
3. Smoking habits were determined.
4. Anthropometric measurements were taken.
5. Disease activity was assessed.
6. A blood specimen (20mls) was taken from each patient.

Controls

Results were evaluated in comparison to a group of normal healthy volunteers who were matched for age and sex. The controls were recruited from the staff and students in the hospital, and were not on any form of vitamin supplementation or medications.

4.2.1.2 Review of case notes

Each patient's folder was reviewed in order to obtain the following information:

Biographical data

1. Name.
2. Gender.
3. Date of birth.

Medical history

1. Type of IBD.
2. Date of diagnosis.
3. The extent of disease.
4. The number and duration of clinical relapses in the last year.
5. The number of operations and extent and site of surgery.
6. The type and dosage of present medication.
7. Any form of vitamin or nutrient supplementation being taken.

Disease activity

The CDAI was used to assess disease activity in CD, while the HBI was used in UC patients. Patients with a CDAI value of greater than 200, or a HBI value of greater than 10 were considered to have active disease (Appendix 4 and 5).

4.2.1.3 Anthropometric data

Anthropometric measurements were taken by the principal investigator throughout the study.

Each subject was weighed in light street clothing using a Healthometer beam balance scale to the nearest 100g, and height was measured in stocking feet using a Healthometer measuring stick. The scale was checked for accuracy using a weight, and was corrected accordingly. Three measurements were taken and an average measurement was used. Body mass index (BMI = weight/height^2) was then calculated from this data. When analysing this data, BMI was used to group patients into weight categories. A BMI of less than or equal to 16kg/m^2, was considered to be severely malnourished. A BMI of between 16kg/m^2 and 19kg/m^2 was considered to be moderately malnourished. A BMI of between 19kg/m^2 and 25kg/m^2 was in the normal range. A BMI between 25 and 30 kg/m^2 was considered overweight, while a BMI of greater than 30kg/m^2 was termed obese.

The patient's TSFs were measured to the nearest 1.0mm, using a set of Harpendon skinfold calipers placed midway between the olecranon and acromium of the right arm, with the arm relaxed and bent at the patient's side. MACs were also measured at the same point, with a tape measure to the nearest 1mm, as a measure of muscle mass. Three measurements were taken, and average measurement was used. For analysis these measurements were divided into percentiles using national center for health statistics (NCHS) tables.[9]

4.2.1.5 Dietary Information

A semi-quantitative food frequency questionnaire, originally designed by Gladys Block and colleagues,[133] was used in order to assess usual daily intake. The questionnaire used was based on questionnaires used in previous studies,[135-138] but was changed slightly to include South African terminology and available and commonly eaten foods, as found in a previous study[9] (Appendix 3). The subjects were asked specific questions about the foods that were eaten, and were asked how many times a week or month they were eaten.

Portion sizes were calculated using household food measures. Each subject was asked how much of each food was eaten at a time, and they were then shown various household utensils of different sizes. Furthermore, patients were also asked about the preparation method of the food, and whether the entire portion had been eaten. These were then recorded. In order to evaluate the portion sizes for analysis, the actual foods were weighed using the utensils, and a Soehnley kitchen scale. In order to analyse the intake on the Food Finder program, the analysis was reduced to a daily intake.

Part 2: Antioxidant Vitamin Status

4.3.1 Blood analysis

Twenty millilitres of blood was collected for antioxidant vitamin analysis. These tubes were covered in foil, to protect them from light, and were centrifuged within 10 minutes of being drawn. Serum was extracted, stored in foil covered test tubes, and coded. All vitamin determinations were subsequently performed, using recognised, validated techniques, by the principal investigator in a blinded fashion. Training and supervision was provided by Dr. J Ogden, a PhD research scientist in the Gastroenterology Clinic, and Mrs. M. Callanan (National Diploma Medical Technology), a medical technologist with 4 years of experience working on HPLC.

4.3.1.1 Vitamin C analysis

Serum for vitamin C analysis was kept at 4° C until it was analysed. All the blood was analysed within 3 hours after being drawn. The vitamin C assay was performed colourometrically, according to the method described by Tietz et al[242] (Appendix 7). Standards for the vitamin C analysis were freshly made up according to the method,[242] for each day of analysis. Quality assurance data are shown in table 7. We were unable to calculate the between batch coefficient of variation for vitamin C

analysis, as fresh blood was required for the analysis method,[242] and therefore the same blood could not be compared between batches.

			Coeffecient of variatiation (%)
Measurement	Material	Method	Within Batch
AA	Serum	Colourometric[242]	1.34

Table 7: Quality Assurance Data for AA Analysis

4.3.1.2 Analysis for vitamin A and E

Blood serum vitamin levels for vitamins A, E and β-carotene were analysed using HPLC[243] (Appendix 8), and were kept in foil covered containers, in the dark at -65° C until analysed. Furthermore, the samples were also analysed in the dark in order to minimise vitamin loss during analysis. The standards for the HPLC analysis were made up according to the method used[243] and were also stored in foil covered containers, in the dark at -65°C until used. Each standard was defrosted a maximum of 4 times before being discarded and were run at the beginning of a days' analysis. Quality assurance data are shown in table 8, and the precision of the analysis has been reported to be "acceptable for this type of analysis".[243]

			Coeffecient of variatiation (%)	
Measurement	Material	Method	Within Batch	Between Batch
Retinol	Serum	HPLC[243]	5.17 (3.8)#	10.03 (8.2)#
β-carotene	Serum	HPLC[243]	8.46 (7.0)#	14.73 (13.9)#
α-tocopherol	Serum	HPLC[243]	5.48 (4.9)#	14.63 (13.2)#

- coeffecient of variation (COV) as a percentage are shown: COV from our data (COV from method[243])

Table 8: Quality Assurance Data for Retinol, β-carotene, and α-tocopherol Analysis

Part 3: Antioxidant supplementation trial

4.4.1 Study population

Previous studies have indicated that approximately 70% of patients with CD, who have had a previous active episode within the previous year, would experience a relapse within 6 months.[111] In the same study, use of AZA as a maintenance agent reduced the relapse rate to 35%. With these figures in mind, and an α value of 0.05, at a power of 90%, a sample size of 20 was calculated to be sufficient to demonstrate a significant difference. Therefore, 23 patients with CD who had experienced a relapse within the previous year were randomised into a supplementation trial.

Vitamin supplementation was provided by Vitamist antioxidant vitamin sprays®. The contents of the spray is shown in table 9.

Vitamin	Amount	% RDA
Vit A	1000 RE	100% RDA
β-Carotene	25mg	10% RDA
Niacin	3mg	20% RDA
Vit C	6mg	10% RDA
Vit E	10mg α-tocopherol equivalents	100% RDA

Table 9: Contents of the antioxidant spray

These vitamin sprays are sprayed into the mouth, and may be absorbed through the buccal membrane,[244-247] or through the stomach when swallowed. Buccal spray formulations have been reported to have an absorption rate of 96%,[248] which is greater than oral fomulations.[248] Furthermore, ease of application, and patient interest in this form of delivery mechanism, as well as claims of the manufacturers regarding the efficacy of the product in the management of chronic inflammatory conditions

persuaded us to submit the product to clinical trial. The antioxidant sprays and placebo sprays were provided by the company (Karemore: Vitamist) in identical containers, and were coded for subsequent analysis following the study.

Although the dosages used were modest in comparison to some supplementation trials,[178,249,250] the dosages used for vitamins A and E were 100% of the RDA, and the β-carotene dosage was equivalent to other supplementation trials.[140,249,251,252] We had noted that the subjects were taking in adequate amounts of vitamins in their diets (greater than 100% RDA). Since, over supplementation of certain vitamins, such as carotenoids may be associated with adverse effects including an increased risk of certain cancers,[173,253] we elected to use the dosages recommended by the manufacturer of the formulation for the management of chronic inflammatory disease. In view of the formulation of the product, the study was concerned with the effect of supplementation of vitamins A and E. The role of supplementing vitamin C was not fully assessed in this study.

In order to assess vitamin status, results were evaluated in comparison to 23 of the controls who were matched directly to patients on the supplementation trial for age, sex and BMI, and were also studied further.

4.4.1.1 Inclusion Criteria

1. Patients who had previously qualified for the antioxidant trial.
2. Known CD as identified by pathological and/or radiological findings.
3. A history of at least one active episode in the previous year.
4. Clinically inactive disease reflected by a CDAI score of less than 150, at the time of entry into the study.

4.4.1.2 Exclusion Criteria

1. A CDAI score of greater than 150.
2. Those on immunosuppressive therapy.
3. Those on corticosteroid therapy.
4. Those with abscesses or fistulae.

4.4.2 Methods

1. Patients were randomised to receive either active supplement or placebo for a period of 6 months.
2. The sprays were administered by using 2 sprays, 4 times per day which delivered 100% of the RDA for both vitamin A and E, and 10% of the RDA for vitamin C and β-carotene in the active group.
3. Patients were instructed to be nil per mouth from 10 o' clock the previous night for each visit.
4. Patients were assessed monthly for a period of 6 months.

At the 1st visit or baseline visit for the supplementation trial

1. Blood was taken, for vitamin analysis, cholesterol levels, full blood count and ESR.
2. Each patient completed a semi-quantitative food frequency questionnaire.
3. Instructions on the use of the supplement were given.
4. Patients were encouraged not to change their eating habits during the course of the trial.
5. Patients were also encouraged not to take any other antioxidant vitamin supplements during the trial.
6. A clinical disease activity index (CDAI) was completed for each patient. Another form was given to each patient to take home, to be filled out every day for a week

before the following visit to ensure accuracy of the data. Instructions were given about how to fill in the form.

7. A quality of life questionnaire using the SF-36 QOL survey was also completed.
8. Weight and height were measured, and recorded as above, and BMI was calculated.

Subsequent visits

1. Compliance was checked and noted.
2. Any adverse events, or change in medications were noted. Any adverse event that was reported was then also assessed by a doctor who determined severity of symptoms. The patient then continued on the trial or was withdrawn.
3. The semi-quantitative food frequency questionnaire was completed at each visit in order to ensure that patients had not altered their diets in any significant way from month to month.
4. A fasting blood specimen was taken.
5. Disease activity was assessed, and a new form was supplied for the next visit.
6. QOL questionnaire was completed.
7. Weight was measured and recorded, and BMI was calculated.

4.4.2.1 Withdrawal Criteria

Patients were withdrawn from the trial on the following criteria:

1. A CDAI score greater than 200.
2. The development of disease complications.
3. Users of outlawed medications or vitamin supplements.
4. Patients who were lost to follow up.

4.4.3 Blood analysis

The blood analysis for this part of the trial was the same as above. Analysis of retinol, β-carotene and α-tocopherol was by HPLC,[243] and that of vitamin C was by colourometric method.[242] Furthermore, a sample of blood was also sent to the Chemical Pathology Laboratory at Groote Schuur hospital for cholesterol analysis, which was then used to determine the α-tocopherol to cholesterol ratio, in order to determine the vitamin E status of the patients, more accurately.

4.4.4 Data Analysis

All data from the questionnaires and from blood results was coded and captured into Microsoft Excel 7.0 computer programme.

The semi-quantitative food frequency questionnaires were reduced to one day's intake, and were analysed using the Food Finder programme (version 4) which correlated the data into macro and micronutrients. American recommended dietary allowances (RDA) as well as the amounts of micronutrients in milligrams were calculated.

QOL analysis was based on the SF-36 QOL questionnaire (Appendix 9), and permission was granted to use this QOL form by Prof. John E. Ware. Because the QOL has not been fully validated in South African patients, the QOL assessment was based on analysis of change from baseline (Statistics department, U.C.T). Patients who defaulted from the intervention trial, were considered to be treatment failures in the analysis of data.

Data was analysed using the Statistica for Windows computer package. Pearsson's correlations, students T-tests (for parametric data), Mann-Whitney U-test (for non-parametric data), Chi-squared tests, and ANOVA were performed as was applicable. Results were considered to be significant when the p value was found to be less than 0.05.

All results will be shown as means ± standard deviations (SD) for parametric data, and median (Lower Quartile-Upper Quartile) for non-parametric data.

5. Results

5.1 Subject data

One hundred consecutive patients (65 females and 35 males) were studied. The mean age of the group was 42.52±13.08 years. The patients were matched to a group of 44 controls (Table 10).

	CD (n=56)	UC (n=44)	Controls (n=44)
age (yrs)	43.74±11.62	41.55±14.00	40.10±12.10
male/female	19/37	16/28	18/26

Table 10: Subject data

5.2 Anthropometry

The mean weight of the patients was 65.50±15.25 kg, with a mean BMI of 24.94±5.47 kg/m^2 (normal range 19-25 kg/m^2). Mean TSF was 2.63±0.61 cm (50th-75th percentile 2.5-3 cm), and the MAC was 27.79±5.29 cm (50th-75th percentile 25.8–28.3 cm) (Table 11).

There were no significant differences in mean anthropometric measurements between controls and subjects, and between CD and UC.

	CD (n=56)	UC (n=44)	Controls (n=44)
BMI (kg/m^2)	24.53±5.64	25.46±5.28	25.03±3.22
TSF (cm)	2.68± 0.73	2.59±0.52	2.43±0.61
TSF (percentile)	50-85	50-85	50-85
MAC (cm)	27.79±4.72	26.65±2.71	26.87±3.50
MAC (percentile)	50-75	50-75	50-75

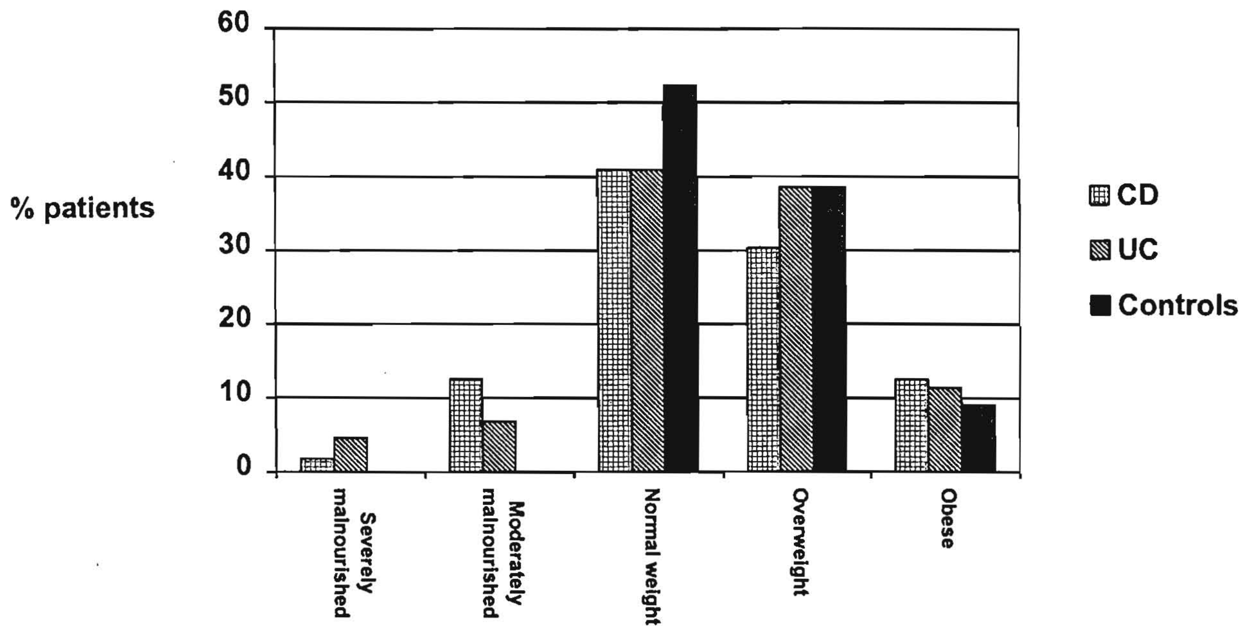
Table 11: Anthropometry

5.2.1 BMI

Forty six percent of the patients interviewed had BMI values greater than 25 (Figure 6). Thirty four percent of patients were overweight (30.35% CD; 38.64% UC), and 12% were obese (12.5% CD; 11.36% UC). Forty one percent of the patients were of normal weight (41.07% CD; 28.64% UC), and 13% were underweight with 10% being moderately malnourished (12.51% CD; 6.81% UC), and 3% severely malnourished (1.79% CD; 4.54% UC). None of the controls were underweight, 52.27% were of normal weight and 47.72% had BMI values of greater then 25 (38.63 % overweight; 9.09% obese) (Figure 6).

BMI was found to be correlated with age for both CD (r=0.32; p=0.02) and UC (r= 0.37; p=0.02).

Figure 6: Nutritional status distribution.
BMI of greater than 30 is obese, 25-30 is overweight, 19-24.9 is normal, 16.0–18.9 is undernutrition (moderately malnourished) and a BMI below 16 is severe malnutrition.[13]



Mean albumin concentrations were within the normal ranges for all groups and were similar between all the groups (Table 12).

	Total	CD (n=56)	UC (n=44)	Normal
Albumin	44.01±5.08	43.02±5.71	45.63±3.35	35-50g/l

Table 12: Albumin concentrations

Only 3 patients were found to have subnormal albumin concentrations. All these patients had CD, were smokers and had ESR values above 20, indicating active inflammation.

5.2.2 Nutrient Intakes

	CD (n=56)	UC (n=44)	Controls (n=44)
Energy (kcal/day)	2407±850.6	2790±375.4	1565±747.1
% protein	10.89±2.27*	11.28±2.28*	13.45±2.59
% fat	37.20±8.03*	35.02±7.02*	33.65±3.91
% carbohydrate	50.12±9.12*	52.38±7.43*	46.37±5.43
% alcohol	0.234±0.64*	0.255±0.78*	6.91±4.91

* $p<0.01$ vs controls

Table 13: Intakes of macronutrients

Although the total energy intake of subjects was higher than controls, the difference did not reach significance ($p=0.12$). Protein intake was significantly lower ($p=0.001$) (Table 13) and carbohydrate intake was significantly higher ($p=0.016$) than controls (Table 13). Furthermore, alcohol intake was significantly higher in controls ($p<0.01$) (Table 13).

Nutrient intake did not correlate with BMI.

5.3 Smoking

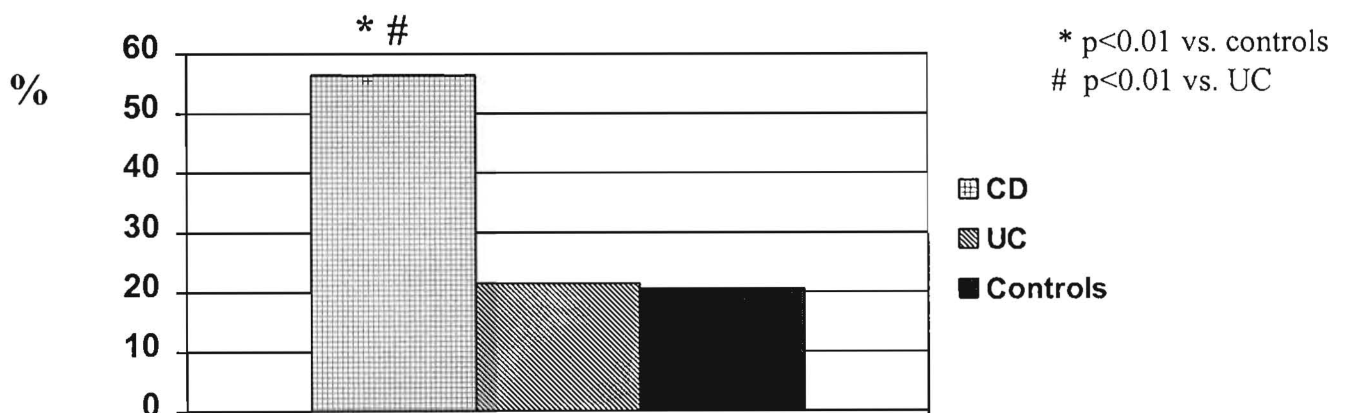
Forty percent of patients with IBD compared to 20.5% of the controls smoked ($p<0.01$) (Table 14; Figure 7).

	CD (n=55)	UC (n=44)	Controls (n=44)
non-smokers n (%)	24 (43.63)	35 (79.54)	35 (79.54)
smokers n (%)	31 (56.36)	9 (21.43)	9 (20.45)

Table 14: Distribution of smokers

Significantly greater numbers of smokers were found in CD compared to UC (χ^2=10.6; p<0.01) and controls (χ^2=11.64; p<0.01). The number of smokers in UC and controls were similar (Figure 7).

Figure 7: Smoking prevalence. Percentage of patients in each group is shown



There were no associations between the number of cigarettes smoked and BMI (r=0.074; p=0.64).

5.4 Drugs

At the time of interview the medications that patients were taking included steroids, salazopyrin and 5-ASA compounds, and immunosuppresives (Table 15).

	CD (n=56)	UC (n=44)
Steroids n (%)	8 (15.38%)	6 (13.95%)
SZP/ASA n (%)	14 (25%)	34 (79.07%)
AZA/6-MP n (%)	6 (10.71%)	3 (6.98%)

Table 15: Number of patients on each medication at time of interview

Other medications included iron preparations (CD 8.92%; UC 16.28%), antidiarrhoeals eg immodium and codeine phosphate (CD 8.92%, UC 11.63%) and in smaller amounts paracetomol, flagyl, and antidepressants. At the time of interview, no patients were taking any antioxidant vitamin supplements.

There were no significant differences between the nutritional status of those patients on medication and those patients not on medication both within and between the 2 groups.

5.5 Duration of disease

The median duration of disease for the IBD patients was 7.32 years (Quartile range 5.0-15.0 yrs) (Table 16).

	CD (n=56)	UC (n=44)
Duration of disease (yrs)	8.63 (2.0-13.0)	6.00 (2.5-18.0)

Table 16: Median duration of disease (Quartile Range)

The duration of disease for both groups was similar (Table 16). There was no association between duration of disease and BMI (CD r=0.065; p=0.66, UC r=0.37; p=0.24).

5.6 Site of disease

In patients with CD, disease location was assessed as ileal, colonic, and ileocolonic. UC patients were divided into total colitis, left sided disease and proctitis (Table 17).

CD Site	Number (%)	UC Site	Number (%)
Ileal	17 (30.35%)	Total Colitis	19 (45.23%)
Colitis	25 (44.64%)	Left sided disease	10 (23.81%)
Ileocolitis	14 (25%)	Proctitis	13 (30.95%)

Table 17: Location of disease for CD and UC

There was no significant association between site and extent of disease and nutritional status.

5.7 Disease activity

The mean ESR of the IBD patients was 24.99±22.41 mm/hr. This was similar between the two groups, as shown in table 18. The mean CDAI of the CD patients was 103.25±75.24, and the mean HBI for the UC patients was 7.25±13.39 (Table 18).

Disease Activity marker	CD	UC	Normal Range
ESR (mm/hr)	26.84 ±23.38	21.85±20.73	0-10
CDAI	103.25±75.24	———	0-150
Harvey Bradshaw	———	7.25±13.39	0-10

Table 18: Disease activity markers

5.7.1 Disease activity in CD

Forty two patients with CD were in remission (CDAI<150) at the time of interview, and 3 had evidence of active disease (CDAI >200) (Table 19).

	CDAI <150	150< CDAI <200	CDAI <200
n (%)	42 (75.00)	11 (25.00)	3 (6.82)

Table 19: Disease activity as measured by the CDAI

Neither the CDAI or the ESR were significantly correlated to BMI (CDAI r=0.22; p=0.16, ESR r=-0.0002; p=0.99).

5.7.2 Disease activity in UC

Using the HBI, 33 of the UC patients (75.00%) were in remission at time of interview. The HBI was not significantly correlated with BMI (r=0.17; p=0.54).

5.8 Disease Relapse Rates

Seventy-six percent of the sample had experienced one or more relapses in the previous year (Table 20).

	No of people with relapses	% of group
CD	47	83.9
UC	29	65.9

Table 20: Relapse rates

There was no association between relapse rates and BMI (CD r=0.089; p=0.54, UC r=0.15; p=0.68), the number of cigarettes smoked (CD r=-0.03; p=0.83, UC r=0.096; p=0.54), site of disease (CD p=0.31, UC p=0.57), and duration of disease (CD r=-0.18; p=0.22, UC r=0.11; p=0.48). Furthermore, none of the weight categories were more prone to relapse than others. In CD however, relapse rates were significantly positively correlated with fat intake as a percentage of energy (r=0.49; p=0.002) and were negatively associated with carbohydrate intake as a percentage of energy (r=-0.45; p=0.006) (Figures 8 and 9).

Figure 8: Correlation between relapse rates per year and fat intake as a percentage of energy in CD

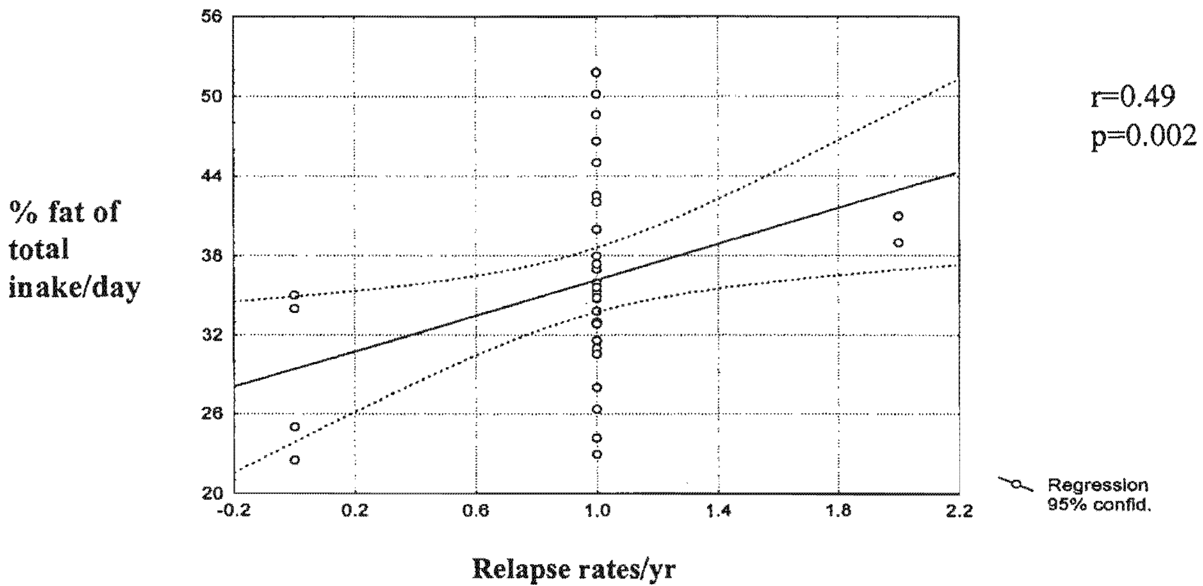
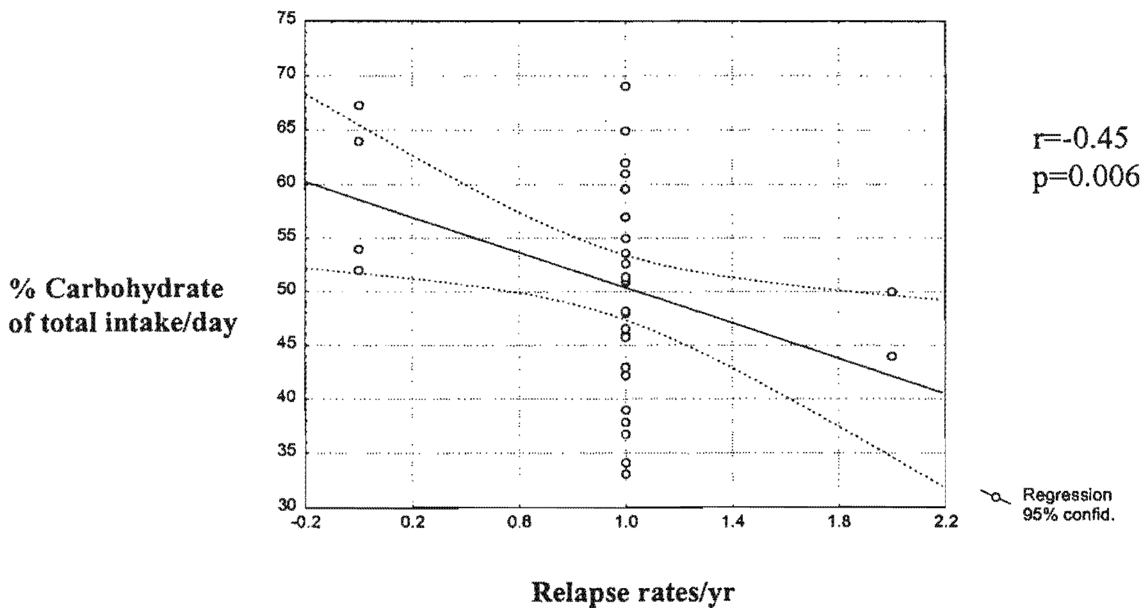


Figure 9: Correlation between relapse rates over the previous year and carbohydrate intake as a percentage of energy in CD



5.9 Surgery

Eight (14.29%) CD patients had undergone surgery to the small bowel, 3 (5.36%) patients had undergone surgery to the large bowel, and a further 8 (14.29%) patients had both their small bowel and large bowel operated on. Furthermore, 3 (5.36%) patients had experienced complications which had required surgical intervention. In all these patients, surgery was resectional, but a maximum of 50cm of small bowel had been removed, and none of the patients had clinical evidence of short bowel syndrome.

For UC, only 1 (2.27%) patient interviewed had undergone a colectomy.

There was no association with BMI and surgical procedures.

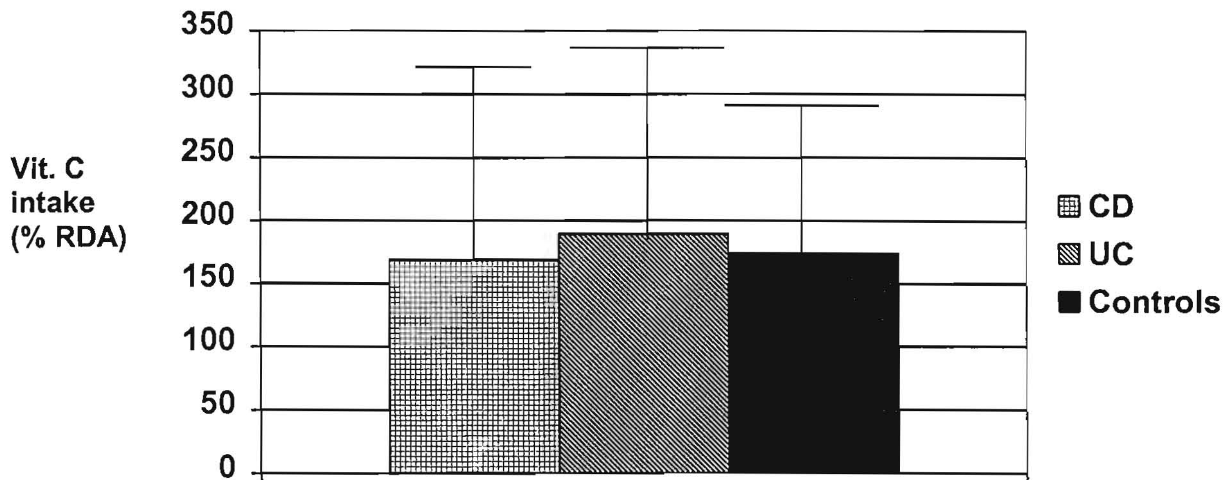
5.10 Vitamin Status

5.10.1 Vitamin C

5.10.1.1 Intake

The mean vitamin C intakes are illustrated in figure 10. All groups had mean intakes greater than 150% of the RDA with the intake in CD 168.9±133.7% RDA, UC 189.1±132.3% RDA, and controls 173.9±94.3% RDA (Figure 10). There were no significant differences between the mean intakes of the 3 groups.

Figure 10: Vitamin C intake (% RDA).
Means of intake are shown with errror bars representing SDs



	Total	CD	UC	Controls	RDA[184]
Vit C (mg)	120.6±76.23	123.6±167.9	116.4±77.5	116.2±63.0	60.0
Vit C (%RDA)	163.5±126.2	168.9±133.7	189.1±132.3	173.9±94.3	100.0

Significantly more patients took in less than 100% of the RDA for vitamin C compared to controls (CD χ^2=11.09; p=0.01, UC χ^2=7.2; p=0.01). The percentage of patients taking in less than 100% RDA were similar in the UC and CD groups (Table 21).

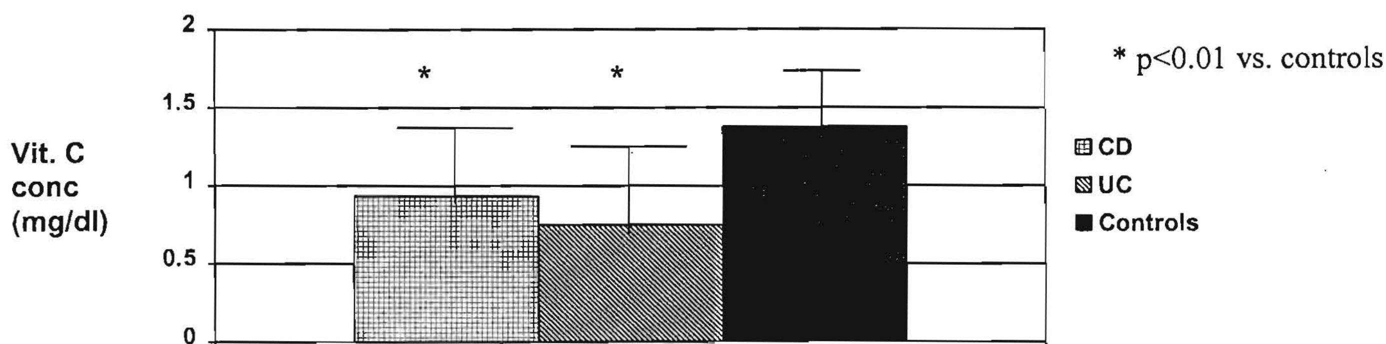
	Total	CD	UC	Controls
>100% RDA (%)	65	35 (62.5)	30 (68.2)	41 (93.2)
100%<RDA<67% (%)	13	7 (12.5)	6 (13.63)	2 (4.55)
67%<RDA<50% (%)	15	10 (17.86)	5 (11.36)	1 (2.27)
<50% RDA (%)	7	4 (7.14)	3 (6.82)	0

Table 21: Number of subjects according to %RDA for Vit C (RDA=60mg[184])

5.10.1.2 Serum concentrations

Both CD and UC patients had significantly lower mean serum vitamin C concentrations compared to controls (UC 0.75±0.54 mg/dl vs. 1.37±0.54 mg/dl; $p<0.01$, CD 0.93±0.48 mg/dl vs. 1.37±0.54 mg/dl; $p<0.01$). There was no significant difference between CD and UC (Figure 11).

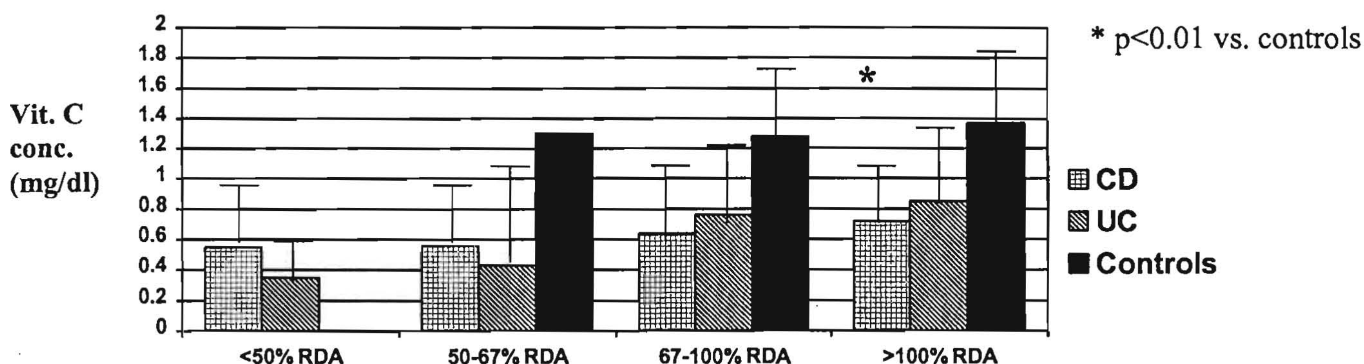
Figure 11: Serum concentrations of Vitamin C (mg/dl).
Means of serum concentrations are shown with errror bars representing SDs



	Total	CD	UC	Controls
Vit C conc (mg/dl)	0.81±0.53	0.93±0.48	0.75±0.54	1.37±0.54

There was no correlation between intake and serum concentrations (r=-0.08; p=0.52) and there was no significant difference in serum concentrations of those patients whose vitamin C intake was less than 100% RDA, and those in whom intake was higher (0.81±0.59 vs. 0.79±0.43; p=0.90). Although there was a trend to decreased vitamin C levels with decreasing intake, the differences did not achieve significance (Figure 12).

Figure 12: Serum vitamin C serum concentrations for the different vitamin C intakes according to the RDA.[184]
Means of serum concentration (mg/dl) are shown with errror bars representing SDs. If there was only one person in a group, SDs are not shown

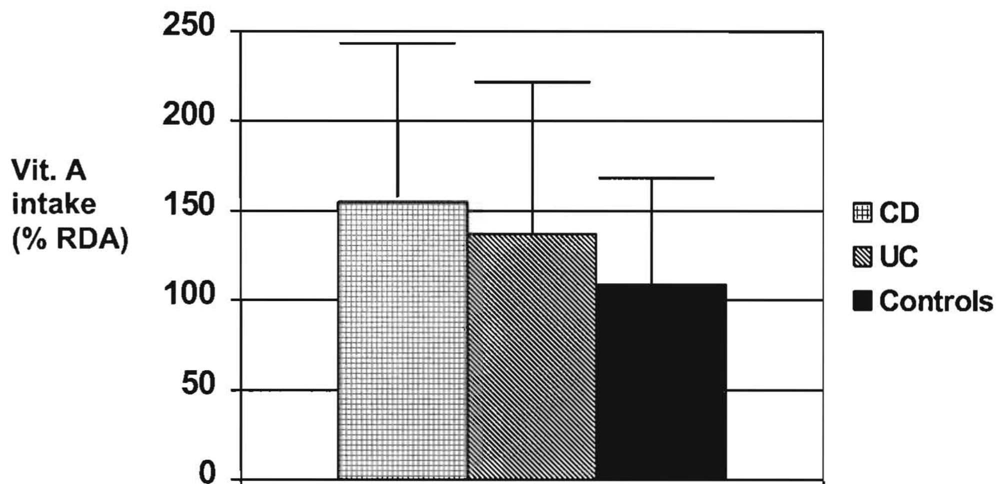


5.10.2 Vitamin A

5.10.2.1 Intake

There were no significant differences between mean vitamin A intakes of the various groups (Figure 13). The mean intakes of all groups was greater than 100% of the RDA with the intake of CD 154.9±89.86% RDA, UC 137.1±69.09% RDA and controls 108.8±49.21% RDA.

Figure 13: Vitamin A intake (% RDA).
Means of intake are shown with errror bars representing SDs



	Total	CD	UC	Controls	RDA[184]
Vit A (RE)	1255±763.0	1305±818.5	1194±693.1	902.1±444.1	1000
Vit A (%RDA)	146.9±81.3	154.9±89.9	137.1±69.0	108.8±49.2	100.0

Significantly more CD patients had intakes below the RDA for vitamin A compared to controls (χ^2=3.95; p=0.046) (Table 22). The number of UC patients who consumed less than the RDA was similar to controls (χ^2=2.25; p=0.13) and to CD (χ^2=0.04; p=0.84) (Table 22).

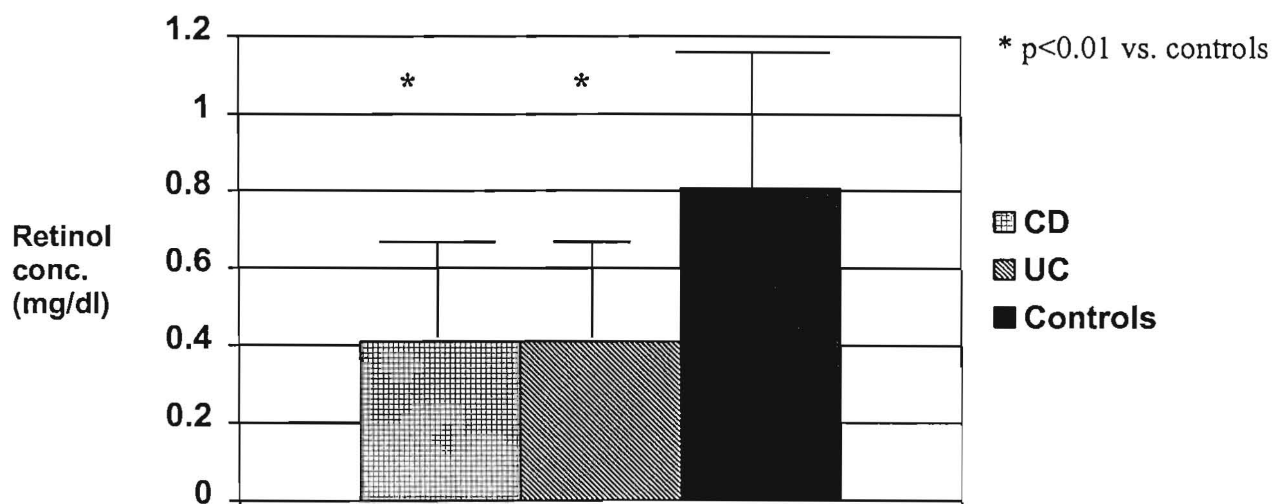
	Total	CD	UC	Controls
>100% RDA (%)	66	36 (64.28)	30 (68.18)	37 (84.09)
100%<RDA<67% (%)	27	17 (30.36)	10 (22.73)	3 (6.82)
67%<RDA<50% (%)	3	2 (3.57)	1 (2.27)	2 (4.55)
<50% RDA (%)	4	1 (1.79)	3 (6.82)	2 (4.55)

Table 22: Number of subjects according to %RDA for vit A intake (RDA=1000RE[184])

5.10.2.2 Serum concentrations of retinol

The mean serum retinol concentrations of the CD patients and the UC patients was significantly lower than controls (CD 0.41±0.22 mg/dl vs. 0.81±0.32 mg/dl; $p<0.01$, UC 0.41±0.23 mg/dl vs. 0.81±0.32 mg/dl; $p<0.01$). There was no difference between serum concentrations of CD and UC (Figure 14).

Figure 14: Serum concentrations of retinol (mg/dl).
Means of serum concentration are shown with errror bars representing SDs

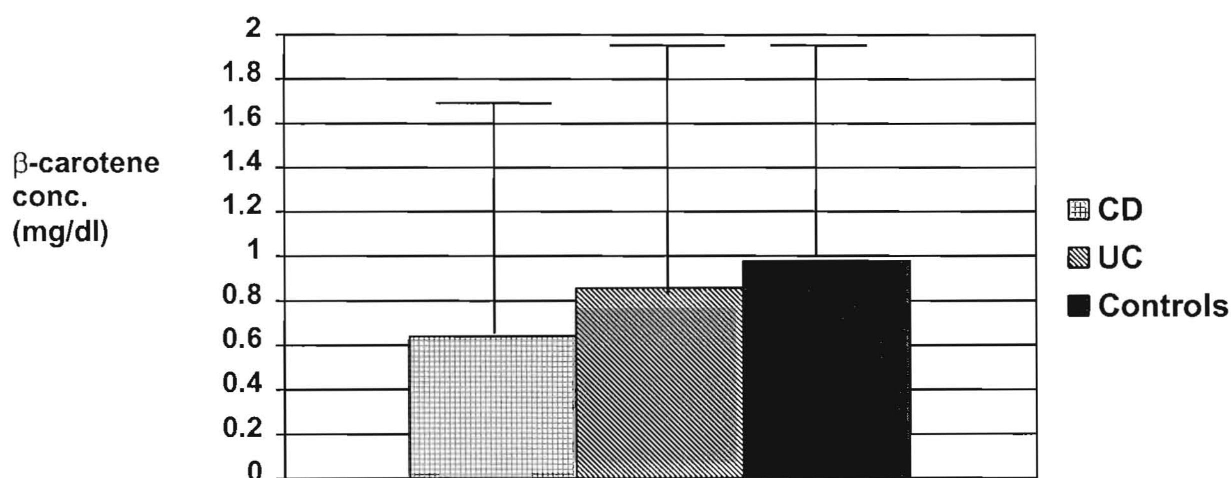


	Total	CD	UC	Controls
Vit A (mg/dl)	0.41±0.22	0.41± 0.22	0.41±0.23	0.81± 0.32

5.10.2.3 Serum concentrations of β-carotene

The mean serum β-carotene concentrations are shown in figure 15. There were no significant differences between the 3 groups (CD 0.64±1.01 mg/dl, UC 0.86±1.13 mg/dl, controls 0.98±1.02 mg/dl), although there was a trend toward lower serum levels for CD compared to controls (p=0.20).

Figure 15: Serum concentrations of β-carotene (mg/dl).
Means of serum concentration are shown with errror bars representing SDs



	Total	CD	UC	Controls
β-carotene (mg/dl)	0.72±1.10	0.64±1.01	0.86±1.13	0.98±1.02

There was no correlation between intakes and serum concentrations (r=-0.008; p=0.95) and there was no significant difference in retinol and β-carotene levels of those patients who consumed less than the RDA of vitamin A, and those who consumed more than the RDA (retinol 0.39±0.22 mg/dl vs. 0.42±0.23 mg/dl; p=0.54, β-carotene 0.49±0.86 mg/dl vs. 0.89±1.23 mg/dl; p=0.16). Similarly, there was no significant differences between the mean serum levels of the 4 different groups of intake according to RDA (Figure 16 and Figure 17). There was also no correlation between fat intake and serum retinol and/or β-carotene concentrations of subjects (r=0.11; p=0.83) and controls (r=-0.20; p=0.67).

Figure 16: Serum retinol concentrations for the different vitamin A intakes according to the RDA.[184]
Means of serum concentration (mg/dl) are shown with errror bars representing SDs. If only one person was in the group, no SDs are shown

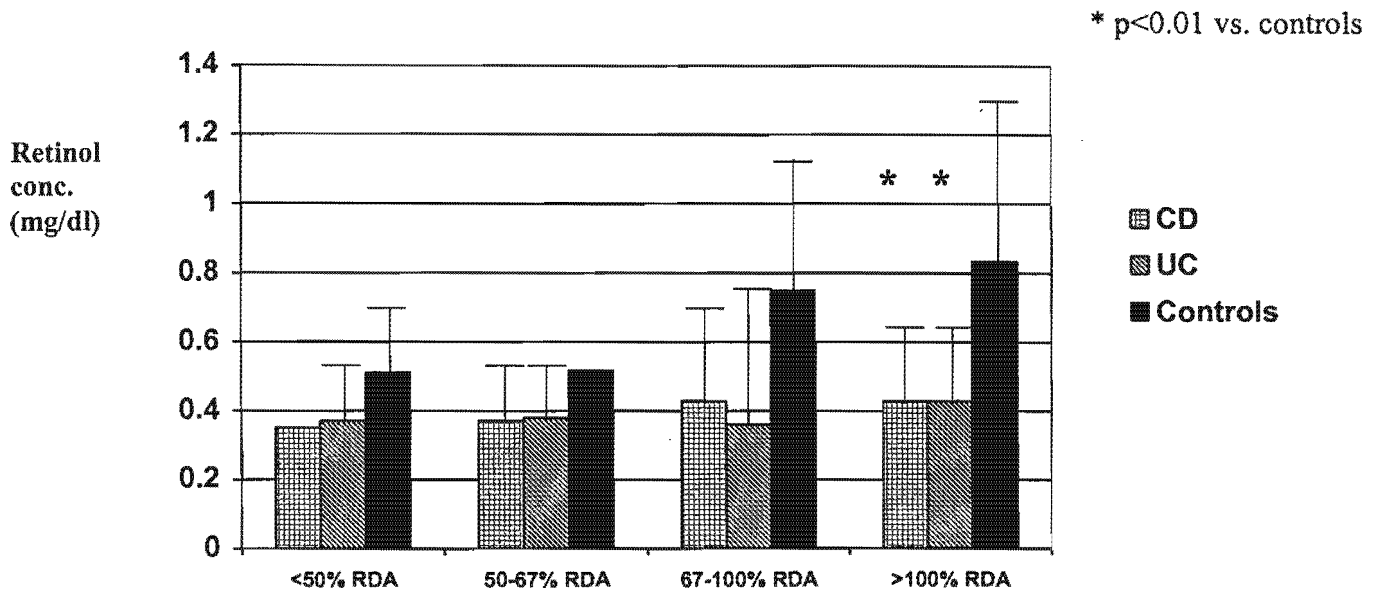
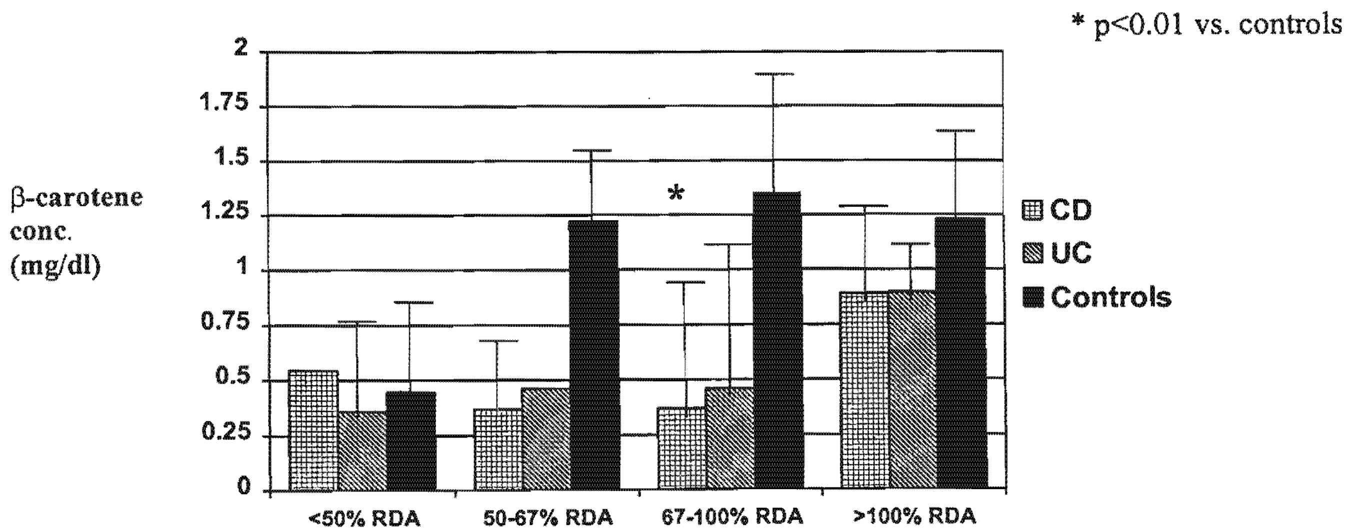


Figure 17: Serum β- carotene concentrations for the different vitamin A intakes according to the RDA.[184]
Means of serum concentration (mg/dl) are shown with errror bars representing SDs. If only one person was in the group, no SDs are shown

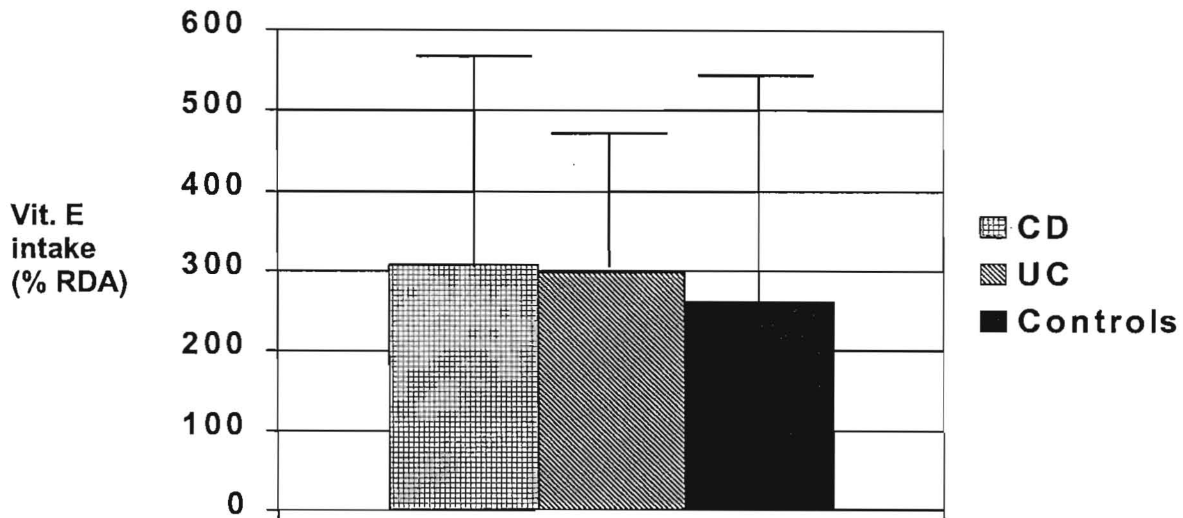


5.10.3 Vitamin E

5.10.3.1 Intake

Vitamin E intakes are shown in figure 18. Again, all groups had a mean intake of greater than 100% of the RDA. Mean intakes of UC, CD and controls were not significantly different (CD 308.19±257.35% RDA, UC 298.23±169.97% RDA, controls 260.60±297.50% RDA).

Figure 18: Vitamin E intake (% RDA).
Means of intake are shown with errror bars representing SDs



	Total	CD	UC	Controls	RDA[184]
Vit E (mg)	24.87±14.38	25.52±13.80	27.31±21.61	25.49±29.23	10
Vit E (%RDA)	303.7±221.5	308.2±257.3	298.2±169.9	260.6±297.5	100.0

The number of people in each group who took in less than the RDA for vitamin E were similar (Table 23).

	Total	CD	UC	Controls
>100% RDA (%)	94	54 (96.43)	40 (90.91)	43 (97.73)
100%<RDA<67% (%)	3	0	3 (6.82)	1 (2.27)
67%<RDA<50% (%)	0	0	0	0
<50% RDA (%)	3	2 (3.57)	1 (2.27)	0

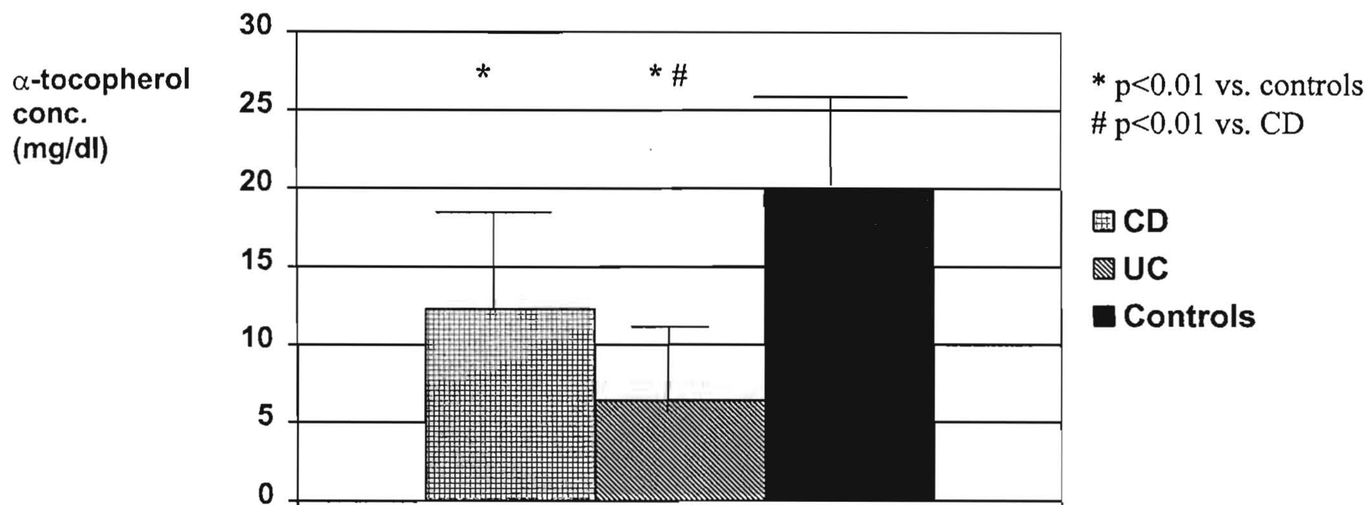
Table 23: number of people grouped according to % RDA taken in for Vitamin E (RDA=10mg[184])

5.10.3.2 Serum concentrations

The mean α-tocopherol serum concentrations of the UC and CD groups were significantly lower than that of controls (UC 6.41±5.54 mg/dl vs. 20.03±5.88 mg/dl; $p<0.01$, CD 12.25±7.31 mg/dl vs. 20.03±5.88 mg/dl; $p<0.01$). Furthermore the UC concentrations were significantly lower than CD ($p<0.01$). These concentrations for both patients and controls are shown in figure 19.

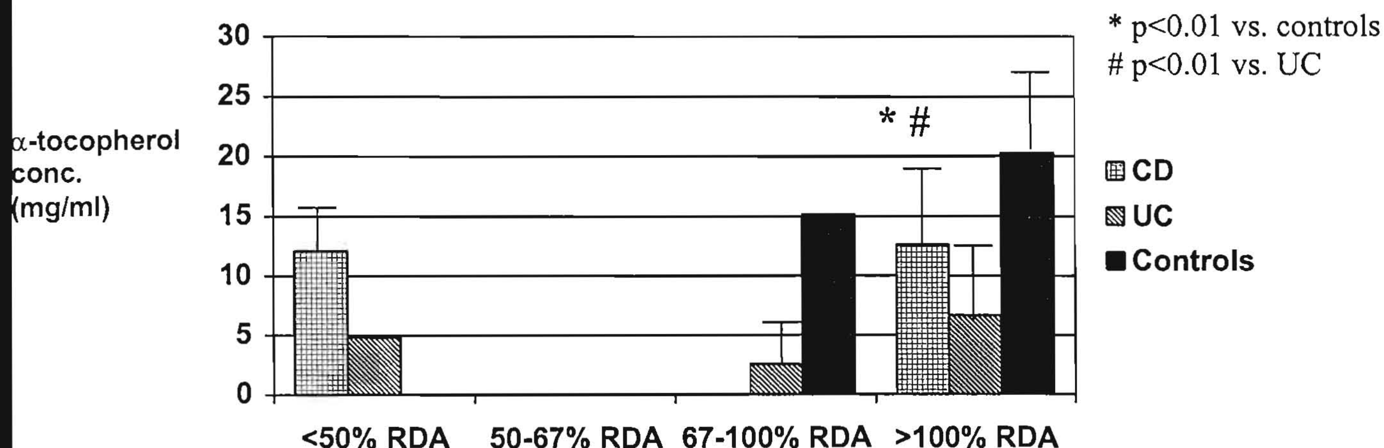
There was no correlation between serum concentrations and intakes of subjects ($r=0.23$; $p=0.074$) and controls ($r=0.72$; $p=0.10$). Similarly, those who took in less than the RDA did not have significantly different serum levels to those who consumed more than the RDA (8.20±6.54 mg/dl vs. 10.65±7.33 mg/dl; $p=0.43$). (Figure 20). There was also no significant correlation between fat intake and serum vitamin E concentrations of subjects ($r=0.046$; $p=0.19$) and controls ($r=-0.04$; $p=0.93$).

Figure 19: Serum concentrations of Vitamin E (mg/dl).
Means of serum concentration are shown with errror bars representing SDs



	Total	CD	UC	Controls
Vit E (mg/dl)	10.18 ±7.26	12.25±7.31	6.41±5.54	20.03±5.88

Figure 20: Serum α-tocopherol concentrations for the different vitamin E intakes according to the RDA.[184]
Means of serum concentration (mg/dl) are shown with errror bars representing SDs. If there was only one person in a group SDs are not shown

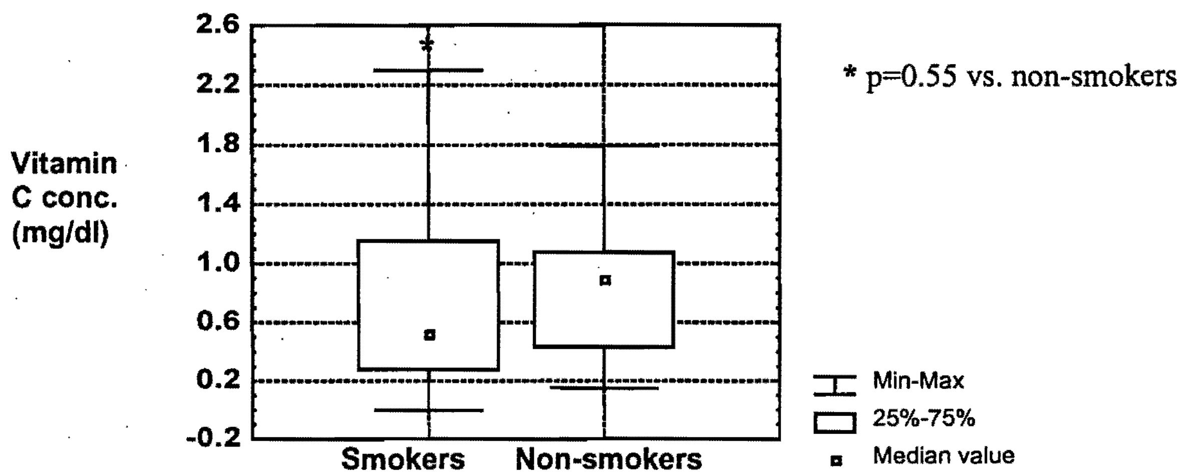


5.11 Associations with smoking and alcohol

Although in the group of normal controls, smokers tended to have lower mean serum concentrations of vitamin C than non–smokers (1.09±0.52 mg/dl vs. 1.46±0.53 mg/dl; p=0.056) (Figure 21), there was no significant association with smoking in the IBD patients (0.76±0.63 mg/dl vs. 0.83±0.45 mg/dl; p=0.55) (Figure 22).

Figure 21: Serum vitamin C concentrations of smokers and non-smokers (Controls)

Figure 22:Serum vitamin C concentrations of smokers and non-smokers (IBD patients)



Mean serum retinol and β-carotene concentrations were not significantly different between smokers and non-smokers in both controls (retinol 0.76±0.36 mg/dl vs. 0.82±0.31 mg/dl; p=0.65, β-carotene 0.95±0.63 mg/dl vs. 0.87±1.10 mg/dl; p=0.71) and IBD patients (retinol 0.41±0.22 mg/dl vs. 0.42±0.23 mg/dl; p=0.87, β-carotene 0.68±1.06 mg/dl vs. 0.75±1.15mg/dl; p=0.79). Similarly, there was no significant difference in mean vitamin E serum concentrations for smokers and non-smokers for

both patients (11.71±7.59 mg/dl vs. 9.08±6.90 mg/dl; p=0.15) and controls (20.80±6.85 mg/dl vs. 19.84±5.73 mg/dl; p=0.69).

There were no significant relationships between alcohol intake and serum vitamin concentrations (p>0.05).

5.12 Association with age

There were no significant correlations between any of serum vitamin concentrations and age (vitamin C r=0.04; p=0.75, retinol r=0.04; p=0.78, β-carotene r=0.06; p=0.63, α-tocopherol r=0.014; p=0.91).

5.13 Association with BMI

There was no correlation between BMI and serum concentrations of vitamin C (r=-0.09; p=0.48), retinol (r=-0.0043; p=0.97) and β-carotene (r=-0.010; p=0.94). Furthermore, there were no significant differences between the serum vitamin concentrations of the different weight categories. There was a trend, however, for correlation between serum vitamin E concentrations and BMI (r=0.24; p=0.057).

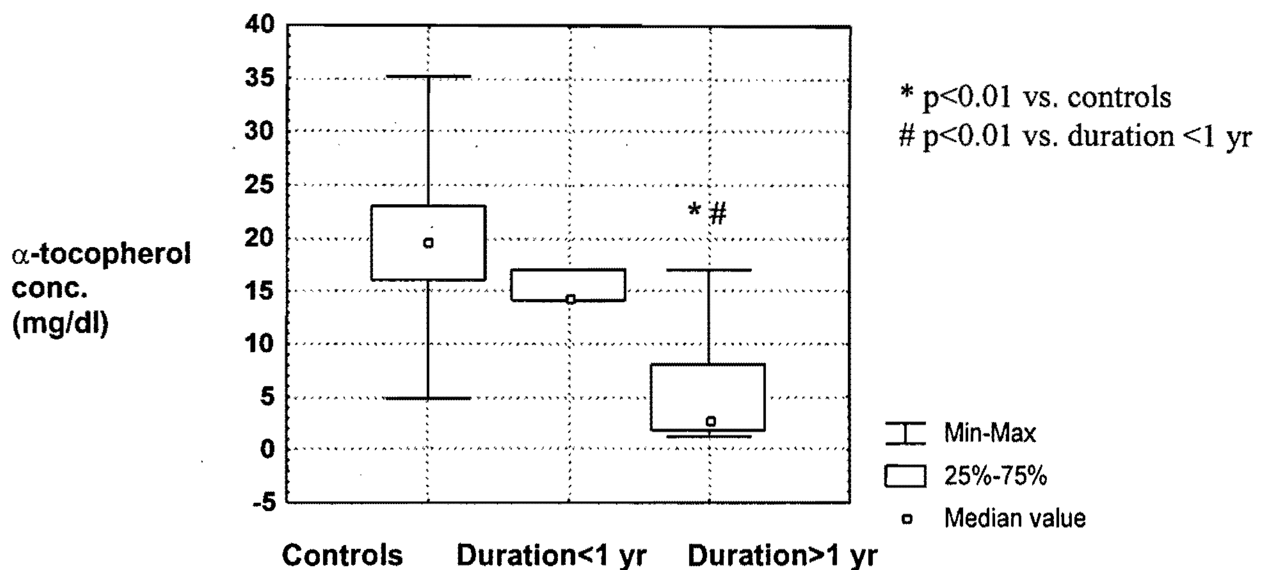
5.14 Association with duration of disease

There was no direct relationship between vitamin C levels and duration of disease in either CD (r=0.19; p=0.16) or UC (r=0.18; p=0.24).

However, UC patients who had recently been diagnosed (duration of disease of one year or less), had significantly higher levels of α-tocopherol than those who had disease for greater than one year (15.17±1.67 mg/dl vs. 4.53±3.9 mg/dl; p=0.002)

with levels not significantly different to controls (15.67± 1.67 mg/dl vs. 19.81±6.45 mg/dl; p=0.23) (Figure 23).

Figure 23: ***α-tocopherol serum concentrations of those with a duration of disease of less than or equal to 1 year, and those with a duration of disease of greater than 1 year***



On the other hand, mean serum retinol and β-carotene levels were significantly higher in patients with a long history of disease (greater than 10 years) than in those with a shorter duration (retinol 0.34±0.15 mg/dl [0.22-0.45 mg/dl] vs. 0.42±0.25 mg/dl [0.25-0.71 mg/dl]; p=0.018, β-carotene 0.32±0.71 mg/dl [0.03-4.35 mg/dl] vs. 0.51±1.53 mg/dl [0.14-0.65 mg/dl]; p=0.044) (Figures 24 and 25).

Figure 24: Retinol serum concentrations for those patients with duration of disease greater than 10 years and a duration of disease less than 10 years

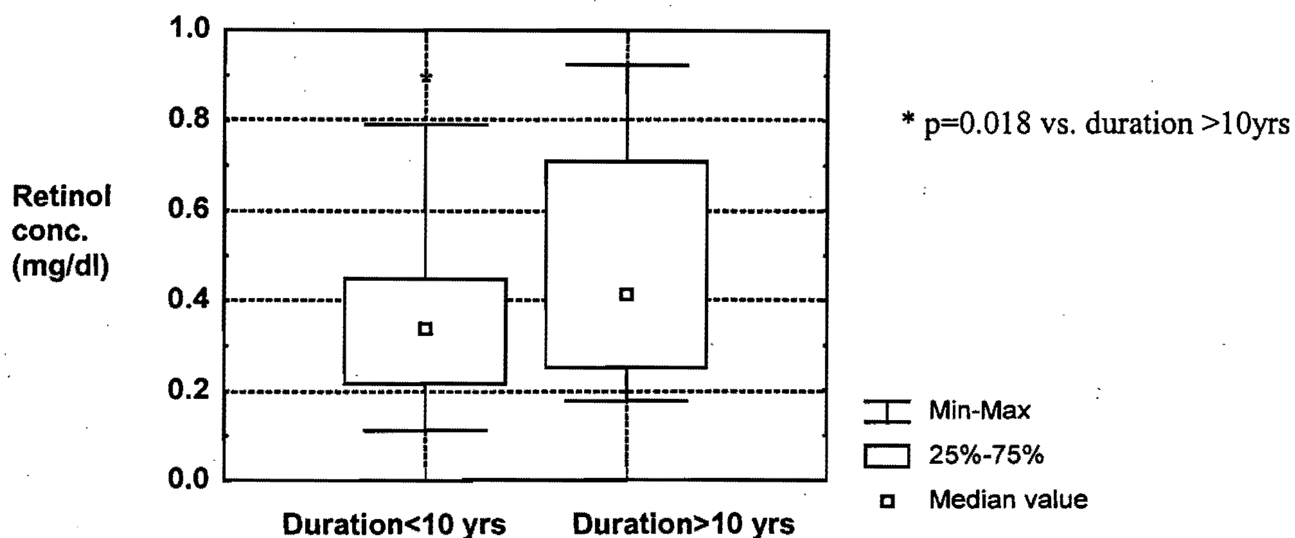


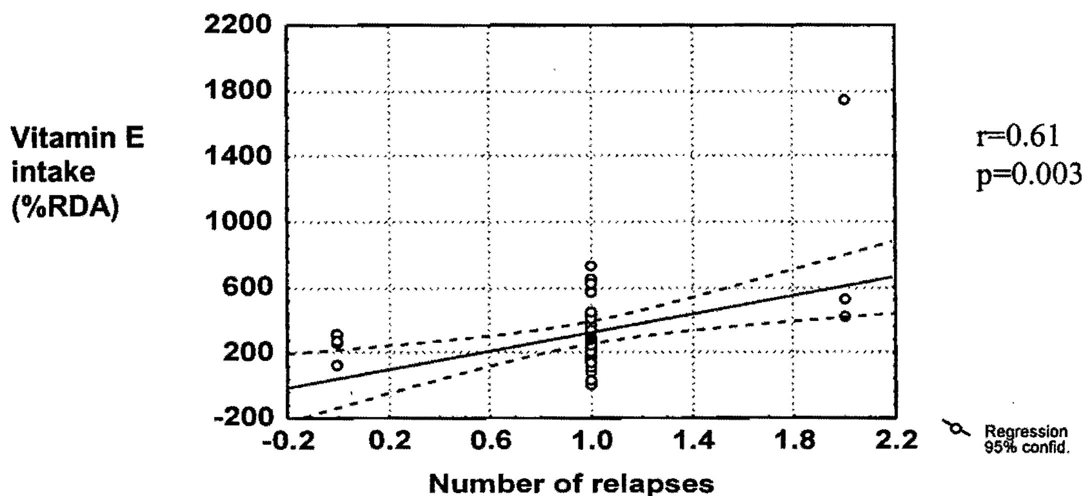
Figure 25: β-carotene serum concentrations of those with duration of disease greater than 10 years and those with duration of disease less than 10 years

5.15 Association with relapse rates

There were no significant associations between vitamin C and β-carotene and relapse rates in the previous 2 years (vitamin C r=-0.29; p=0.82, β-carotene r=-0.038; p=0.77). Retinol levels correlated significantly with relapse rates in UC patients (r=0.52; p=0.01), but not in CD (r=0.0029; p=0.99).

Although α-tocopherol seum levels did not correlate with relapse rates, intake of vitamin E did correlate with relapse rates for CD patients (r=0.61; p=0.003) (Figure 26), but not for UC (r=0.19; p=0.40).

Figure 26: Vitamin E intake (%RDA) for CD patients and relapse rates



5.16 Association with inflammation and disease activity

There was a trend towards negative correlation between serum vitamin C concentrations and ESR (r=-0.27; p=0.068).

Expressed catagorically, serum vitamin C concentrations were significantly lower in those with ESR of above 20mm/hr, than in those with ESR of below 20mm/hr (0.63±0.39 mg/dl vs. 1.097±0.61 mg/dl; p=0.004). This association appears to be mainly associated with CD patients (CD 0.56±0.21 mg/dl vs. 1.10±0.52 mg/dl; p<0.02, UC 0.76±0.30 mg/dl vs. 1.01±0.52 mg/dl; p=0.39) (Figure 27).

There was no direct correlation between serum retinol, β-carotene and α-tocopherol levels and ESR. However, even though serum retinol concentrations were not significantly different between those with an ESR of greater than 20mm/hr and those with an ESR of less than 20mm/hr (CD 0.43±0.22mg/dl vs. 0.43±0.22mg/dl; p=0.95, UC 0.38±0.11 mg/dl vs. 0.32±0.20 mg/dl, p=0.45), we did note that serum concentrations of retinol were significantly lower in those with an ESR of greater than 10mm/hr than in those less than 10mm/hr (0.42±0.23mg/dl vs. 0.38±0.28 mg/dl; p=0.009) (Figure 28).

There was no direct association between vitamin C, retinol and α-tocopherol levels and CDAI or HBI. However, mean β-carotene levels were significantly higher in patients with active CD (CDAI greater than 200), compared to those who were inactive (0.49±0.27 mg/dl vs. 0.39±0.20 mg/dl; p=0.008).

Figure 27: Vitamin C concentrations of CD patients with an ESR of less than 20 mm/hr, and those with an ESR of greater than 20 mm/hr

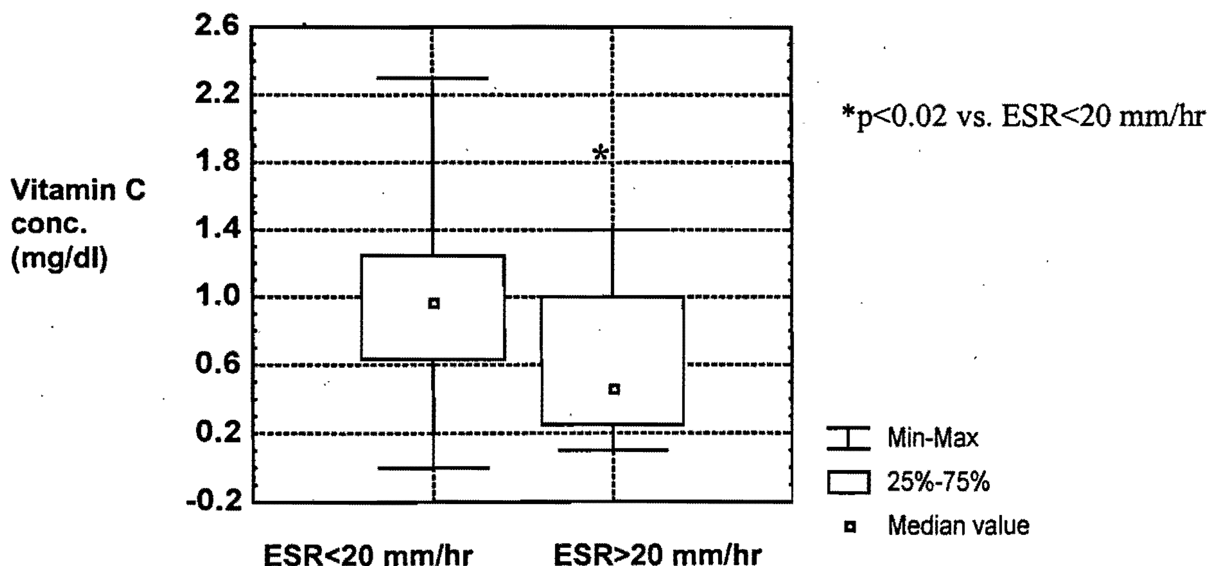
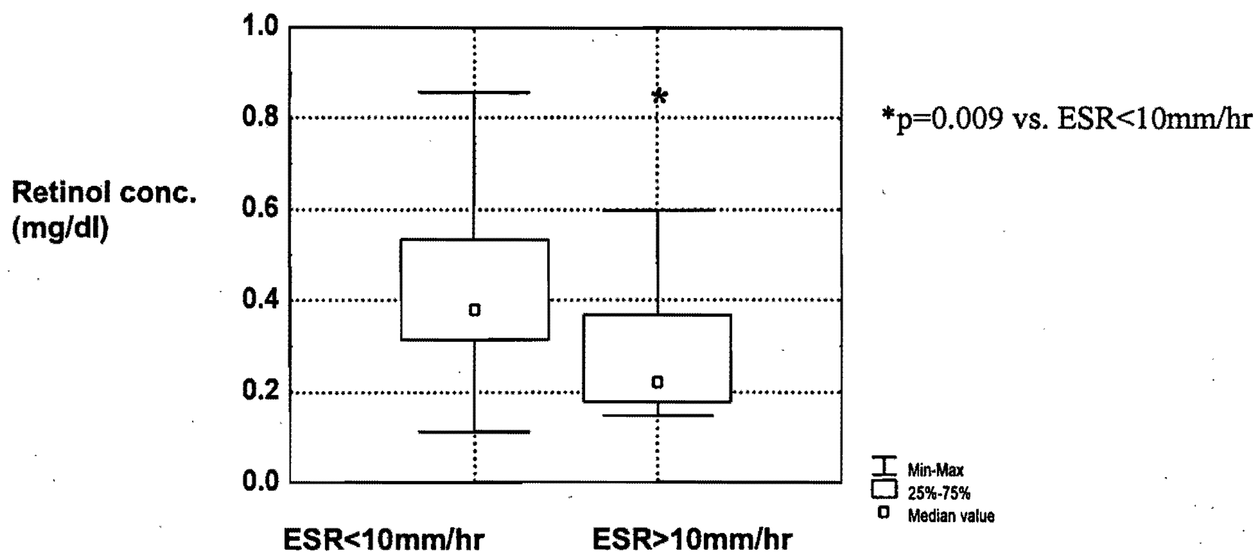


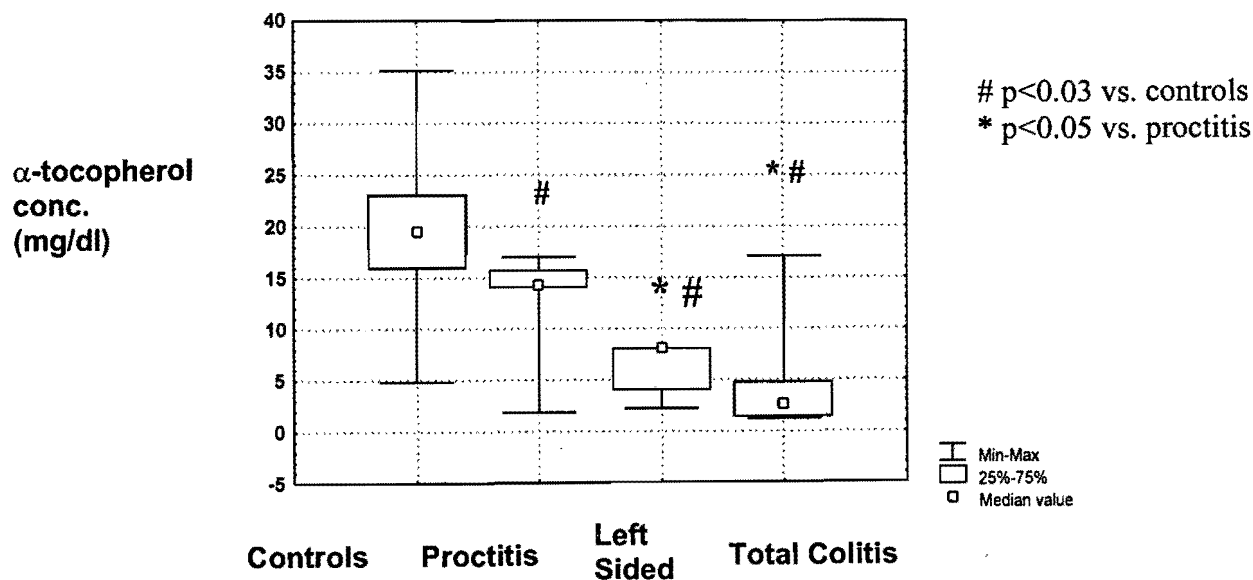
Figure 28: Retinol concentrations of UC patients with ESR less than 10 mm/hr and ESR greater than 10 mm/hr



5.17 Association with site of disease

There was no relationship between location of disease and vitamin C, retinol, β-carotene and α-tocopherol levels in patients with CD. However, α-tocopherol levels in UC patients with limited disease (proctitis), although significantly lower than controls (12.62±6.14 mg/dl vs. 19.81±6.45 mg/dl; p=0.03) were higher than patients with left sided disease (6.42±2.60 mg/dl vs. 12.62±6.14 mg/dl; p=0.05) and total colitis (4.11±4.86 mg/dl vs. 12.62±6.14 mg/dl; p=0.01) (Figure 29). Since these findings may be a reflection of inflammation, we examined the ESR concentrations in UC patients with different sites of disease, and no association was found (proctitis 11.67±10.96 mm/hr vs. left sided disease 30.57±25.62 mm/hr; p=0.26, left sided disease 30.57±25.62 mm/hr vs. total colitis 16.31±10.27 mm/hr; p=0.09, proctitis 11.67±10.96 mm/hr vs. total colitis 16.31±10.27 mm/hr; p=0.50).

Figure 29: α-tocopherol serum concentration for sites of disease in UC



5.18 Associations with drugs

Use of steroids, salazopyrine (SZP) and AZA did not appear to affect serum levels of antioxidant vitamins (Table 24).

Serum concentration	Steroids		SZP		AZA	
	+	-	+	-	+	-
Vit C (mg/dl)	0.89±0.68	0.82±0.53	0.94±0.53	0.70±0.54	0.99±0.65	0.81±0.53
Retinol (mg/dl)	0.45±0.20	0.44±0.25	0.45±0.24	0.42±0.25	0.36±0.25	0.45±0.24
β-carotene (mg/dl)	7.16±5.95	8.17±6.26	8.09±5.45	8.42±7.21	5.31±6.57	8.70±6.14
α-tocopherol (mg/dl)	0.40±0.49	0.86±1.39	1.20±1.67	1.36±1.34	1.14±2.16	0.76±1.14

Table 24: Serum concentrations of those on medications (+) and those not on medication (-).

5.19 Effect of antioxidant vitamin supplementation in patients with Crohn's Disease

5.19.1 Demographic data

There were no significant differences in sex, age, BMI and smoking habits between the normal healthy controls and the patients with CD randomised to receive active spray or placebo (Table 25). Duration of disease, disease extent and surgery details were also similar in those patients who received active antioxidant spray and those who received placebo, and the two groups of patients had similar CDAI, ESR and QOL scores at time of entry into the study (Table 25).

Of the 23 patients who entered the study, 5 (4 placebo, 1 active) patients defaulted follow up and 1 patient in the active group refused to continue due to nausea. This was recorded as an adverse event, possibly due to the treatment being tested. Seven

patients (30%), 3 in the placebo group (25%), and 4 in the active group (36%) were withdrawn due to the development of active disease (CDAI >200), and 1 further patient in the active group was withdrawn due to the development of an abscess (Figure 30).

	Controls	Placebo	Active
N	23	12	11
N males	8	6	2
N females	15	6	9
BMI (kg/m^2)	25.57±3.45	25.44±8.31	25.33±3.96
Smokers n (%)	5 (22)	7 (58)	7 (64)
Age (yrs)	40.91±13.25	39.67±14.26	48.09±16.91
Duration (yrs)		6.54±5.22	9.8±8.34
N surgery (small bowel) (%)		2 (17)	2 (18)
N surgery (large bowel) (%)		1 (8)	1 (9)
N surgery (large and small bowel) (%)		2 (17)	2 (18)
ESR (mm/hr)		28.1±24.52	30.13±20.18
CDAI		97.17±56.84	112.40±55.53
QOL		69.02±12.72	58.05±8.64

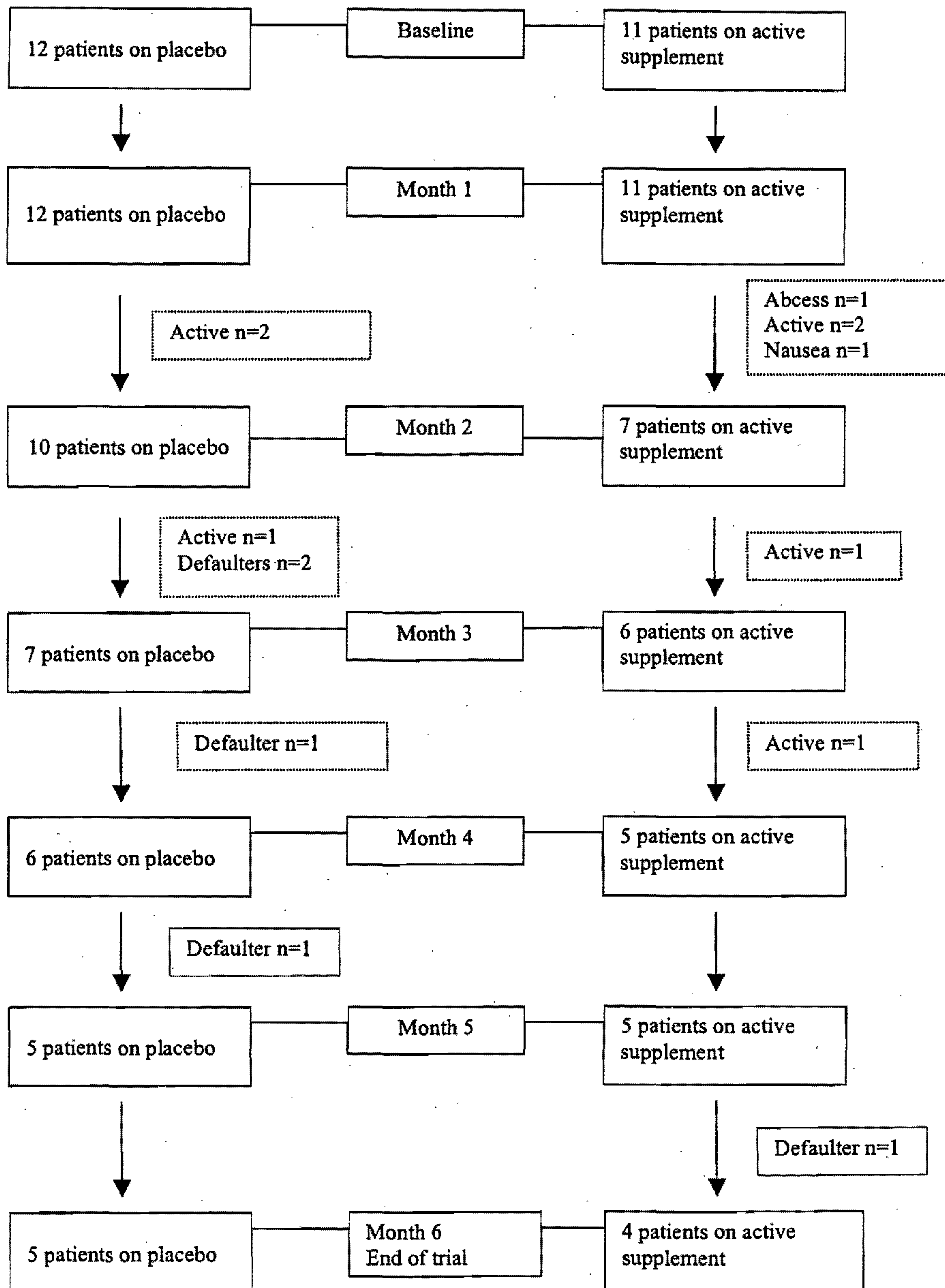
Table 25: Demographic Data

5.19.2 Vitamin status

5.19.2.1 Intake

There were no differences between the intakes of controls and subjects. Both subjects and controls took in more than 100% of the RDA for all the vitamins (Table 26), and there were no significant differences between the macro-and micronutrients in the 6 administrations of the food frequency questionnaire.

Figure 30: Organogram of patients who were withdrawn from the supplementation study



Mean Values	Controls (n=23)	Placebo (n=12)	Active (n=11)
Vit C (mg)	173.5±105.6	107.1±118.4	115.2± 93.67
Vit C %RDA	289.1±176.0	178.5±197.3	191.9±156.1
Vit A(mg)	863.6±409.4	974.3±625.4	1184±736.8
Vit A%RDA	100.9±49.54	135.6±89.54	142.6±92.03
Vit E(mg)	27.24±31.88	27.318±16.67	25.23±14.74
Vit E % RDA	301.5±411.2	300.17±166.56	299.5±172.6

Table 26:Mean vitamin intakes in milligrams and % RDA for controls and patients at entry into the study

5.19.2.2 Serum concentrations

At entry into the study, the serum levels of vitamin C, retinol, β-carotene and α-tocopherol of the group of CD patients who received the supplement were similar to those of the patients who received placebo. The serum levels of vitamin C, retinol and α-tocopherol in the CD patients was significantly lower than controls (vitamin C 0.78±0.51 mg/dl vs. 1.26±0.46 mg/dl; $p<0.01$, retinol 0.41±0.23 mg/dl vs. 0.85±0.32 mg/dl; $p<0.01$, α-tocopherol 16.32±5.51 mg/dl vs. 21.53±4.74 mg/dl; $p<0.01$). Furthermore, there was a trend toward lower β-carotene serum concentrations in the CD patients (0.54±0.39 mg/dl vs. 0.87±0.75mg/dl; $p=0.077$) (Table 27).

Serum conc. (mg/dl)	Controls (n=23)	Placebo (n=12)	Active (n=11)
Vit C	1.26±0.46	0.85±0.61*	0.67±0.32*
Retinol	0.85±0.32	0.42±0.18*	0.39±0.28*
α-tocopherol	21.53±4.74	16.24±4.66*	16.40±6.56*
β-carotene	0.89±0.75	0.52±0.44	0.60±1.73

* p<0.01 vs controls

Table 27: Serum vitamin concentrations at entry into the study

Serum cholesterol concentrations were significantly lower in the patients compared to controls (4.08±0.98 mmol/l vs. 5.31±1.09 mmol/l; p=0.006) (Table 28). The ratio of α-tocopherol to cholesterol, and β-carotene to cholesterol were not significantly different between the groups (p=0.60, p=0.4) (Table 28).

	Controls (n=23)	Placebo (n=11)	Active (n=12)
Total cholesterol (mmol/l)	5.31±1.09	3.93±0.63*	4.00±1.38*
α-tocopherol/cholesterol ratio	1.053±0.21	1.07±0.41	1.06±0.308
β-carotene/cholesterol ratio	0.046±0.043	0.034±0.025	0.038±0.023

* p<0.01 vs controls

Table 28: α-tocopherol, β-carotene and cholesterol concentrations

Cholesterol concentrations were not associated with BMI (r=0.17; p=0.68), and duration of disease (r=-0.02; p=0.91). Similarly, cholesterol concentrations did not correlate with ESR (r=-0.24; p=0.38) There was, however, a positive correlation between cholesterol concentrations and previous relapse rates (r=0.45; p=0.04). Furthermore, those who had relapsed more than once in the previous year, had significantly lower α-tocopherol to cholesterol ratios than those who had relapsed once or who had not relapsed at all (0.91±0.19 vs. 1.02±0.32; p=0.01). However, the

patients who relapsed during the study, did not have significantly different serum cholesterol levels to the patients who did not relapse (3.86±0.87 mmol/l vs. 4.30±1.04 mmol/l; p=0.34).

There were no significant correlations between vitamin intake and serum vitamin concentrations for all groups. There were also no significant correlations between serum vitamin concentrations and ESR or CDAI.

There were no differences between the serum concentrations of vitamin C, retinol, α-tocopherol and β-carotene of the 2 groups of CD patients who remained in remission over the 6 month period (Figures 31-34). Retinol concentrations increased over 3 months and were higher at the 3 month visit than at entry into the study, in both the actively supplemented (0.74±0.54 mg/dl vs. 0.39±0.28 mg/dl; p<0.01) and placebo groups (0.70±0.21 mg/dl vs. 0.43±0.17 mg/dl; p<0.01) (Figure 32). There was a significant increase from baseline levels in vitamin E serum concentration at 6 months in the actively supplemented group (22.5±3.96 mg/dl vs. 16.4±6.56 mg/dl; p=0.03), whereas the placebo group remained at baseline levels (16.47±11.04 mg/dl vs. 16.24±4.66 mg/dl; p=0.88) (Figure 34).

Figure 31: Vitamin C concentration over 6 months.
Means of serum concentrations are shown with errror bars representing SDs

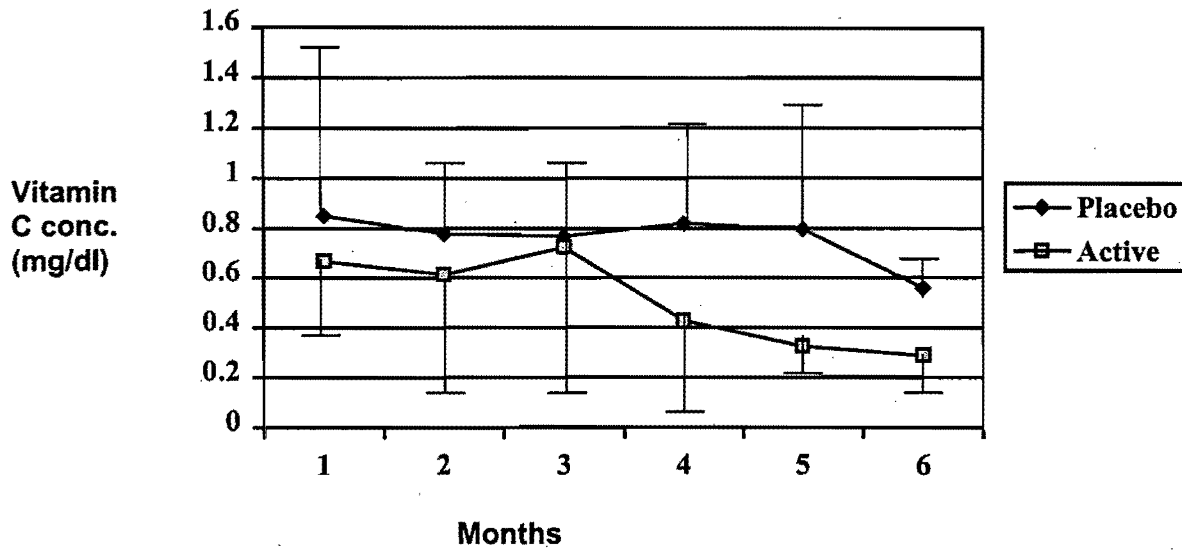


Figure 32: Retinol serum concentrations over 6 months.
Means of serum concentrations are shown with errror bars representing SDs

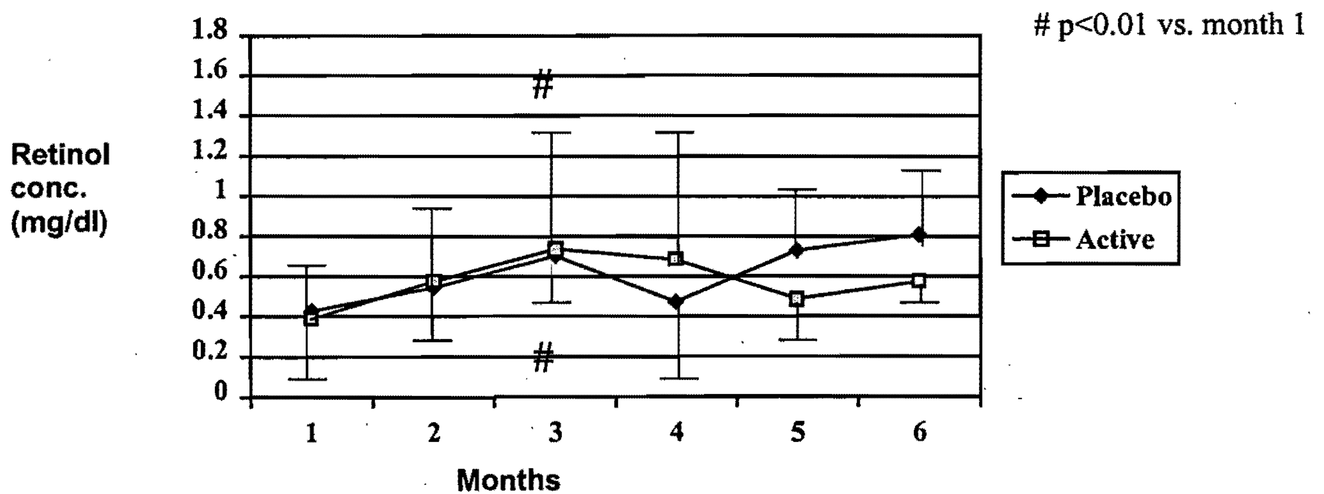


Figure 33: β-carotene serum concentrations over 6 months.
Means of serum concentrations are shown with errror bars representing SDs

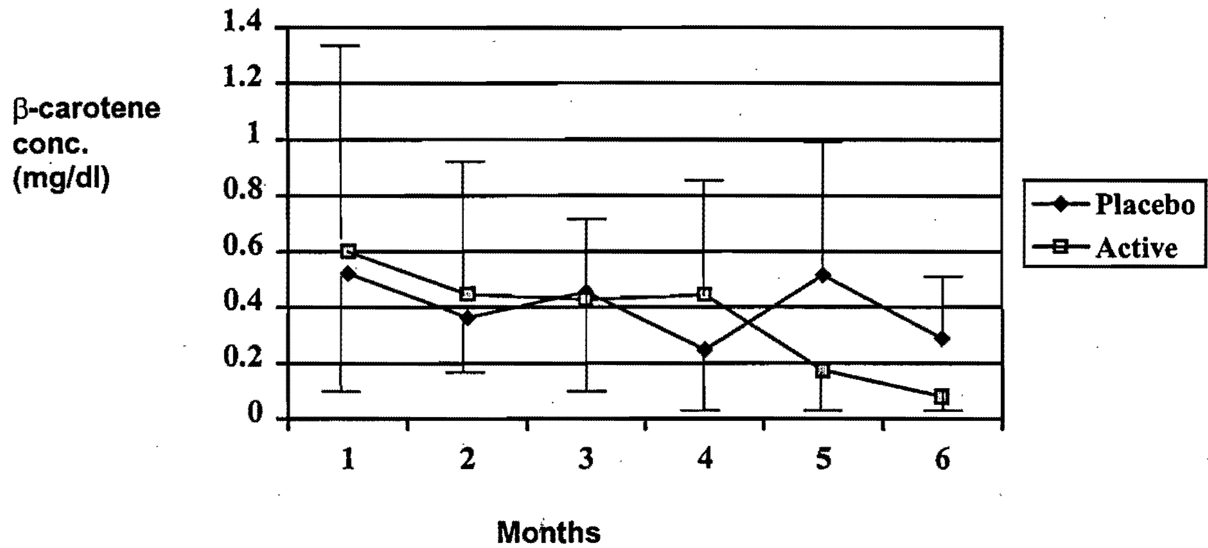
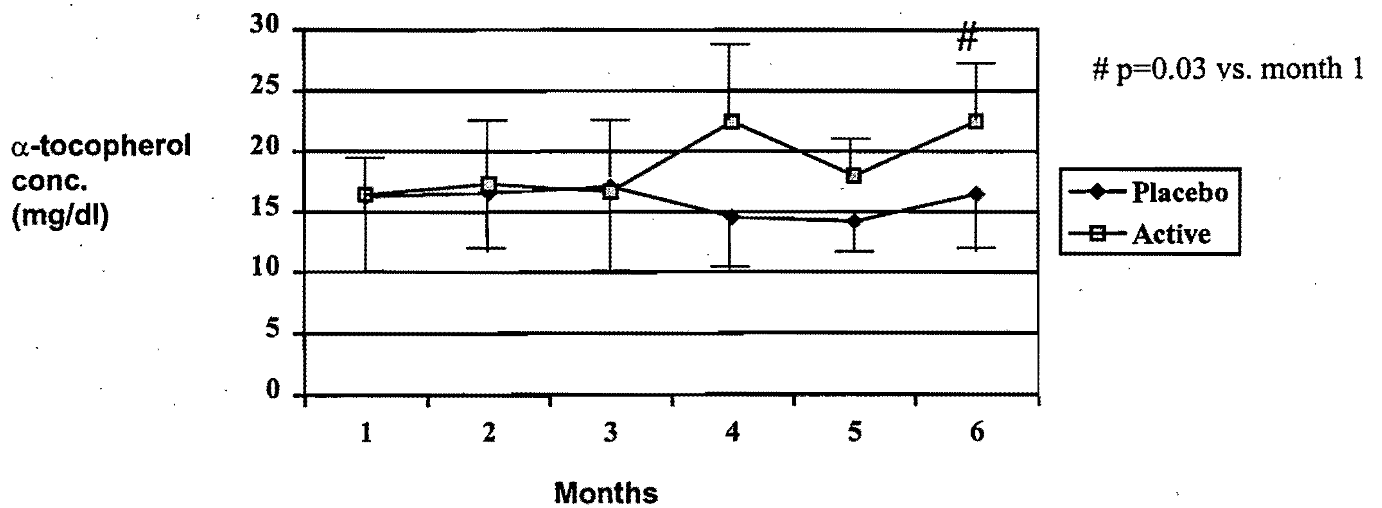


Figure 34: α-tocopherol serum concentrations over 6 months.
Means of serum concentrations are shown with errror bars representing SDs



5.19.3 Disease relapse

Relapse rates in the group of patients who received active spray were similar to the group who received placebo (Figure 35). At the end of the study, 5 patients in the placebo group (42%) and 4 patients in the active group (36%) were still in remission ($p>0.05$). Likewise there was no significant difference in mean CDAI scores or ESR values of the two groups over the 6 month period (Figures 36 and 37).

Figure 35: Cumulative proportion of patients in remission

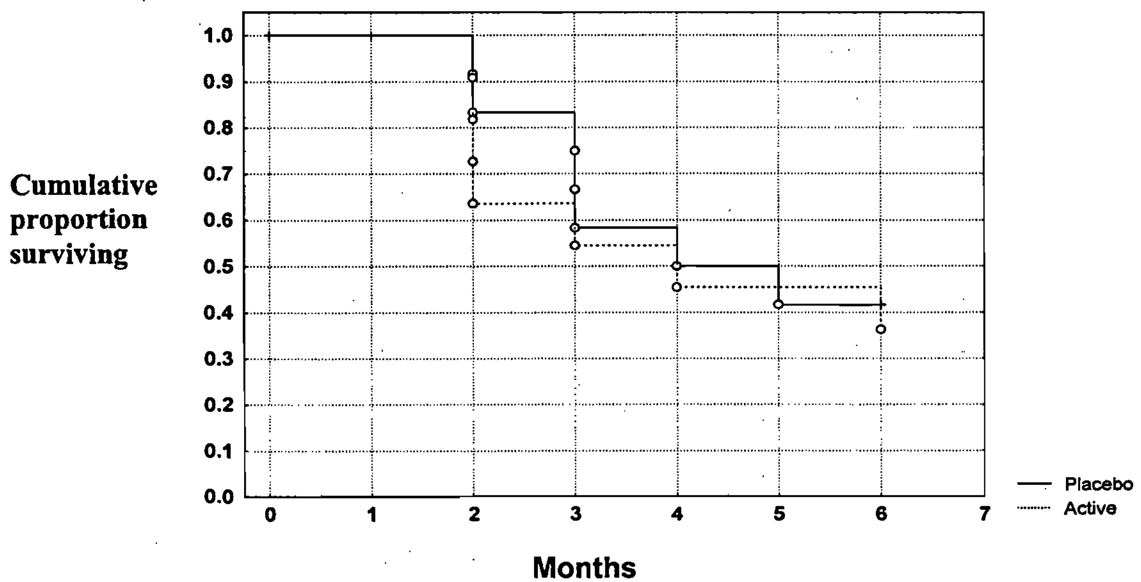


Figure 36: Mean CDAI over 6 months.
Means are shown with errror bars representing SDs

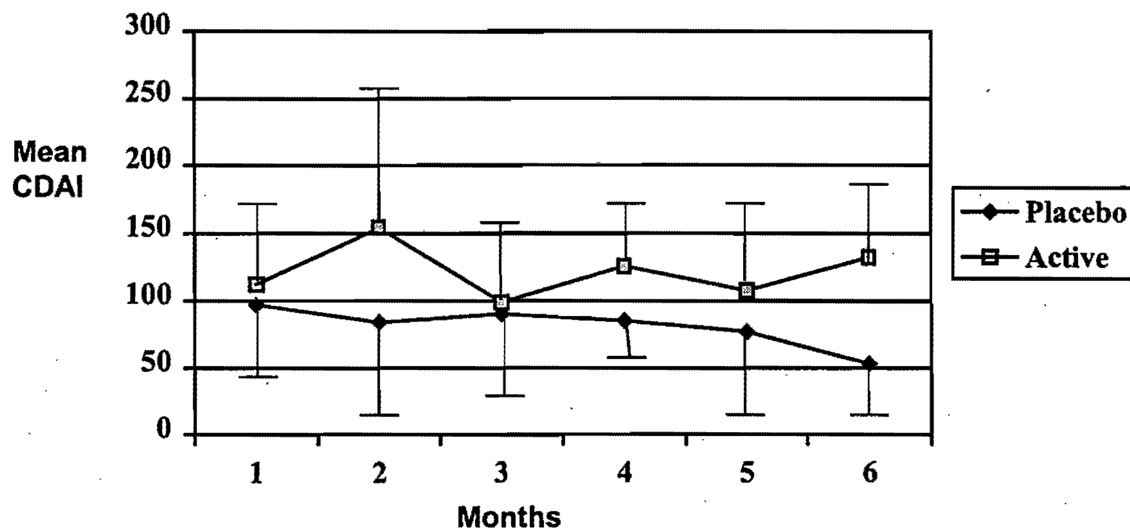
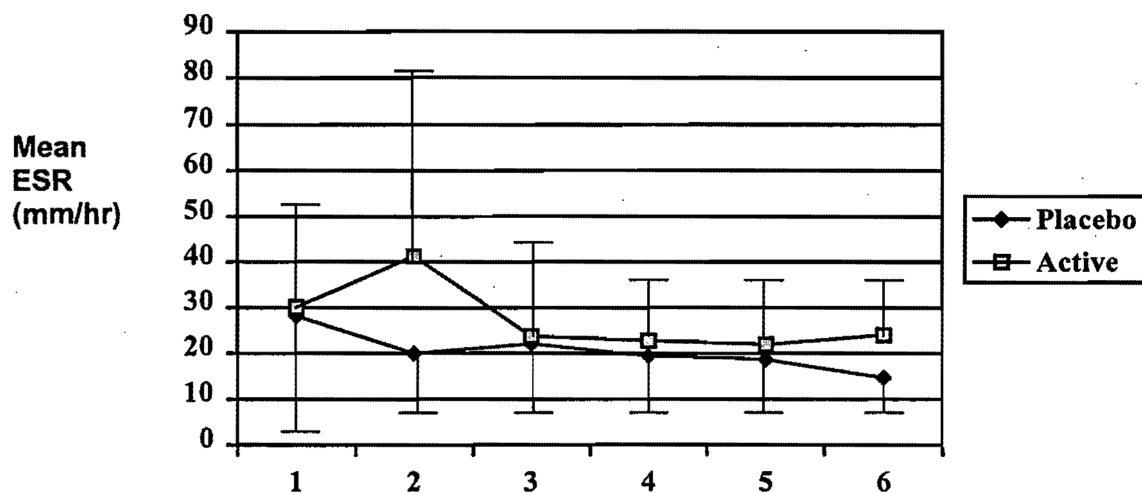


Figure 37: Mean ESR (mm/hr) over 6 months.
Means are shown with errror bars representing SDs



Quality of life scores remained static in both groups over the course of the study (Figure 38), and correlated with the CDAI (r=-0.69; p=0.008) (Figure 39).

Figure 38: Mean QOL score over 6 months.
Means of are shown with errror bars representing SDs

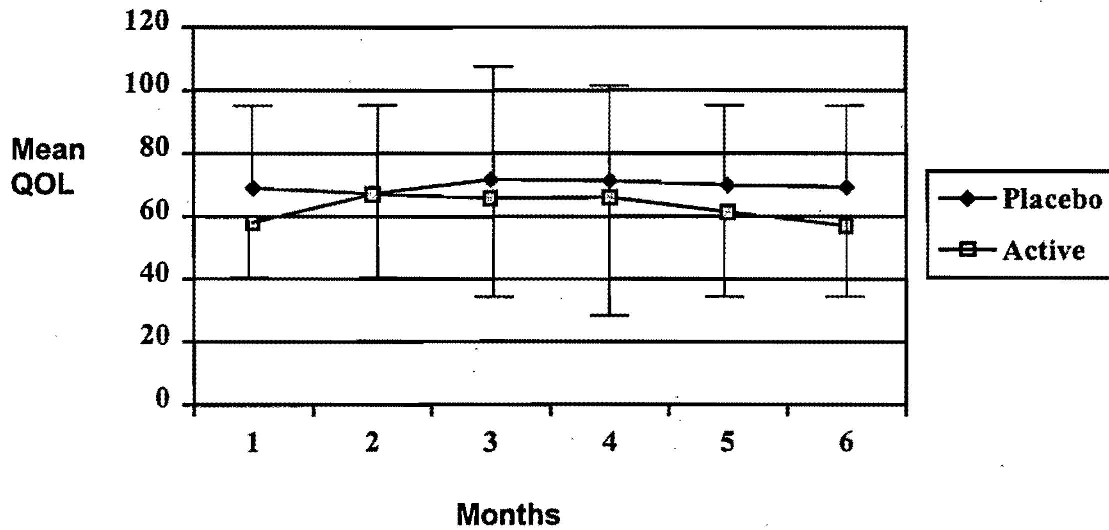
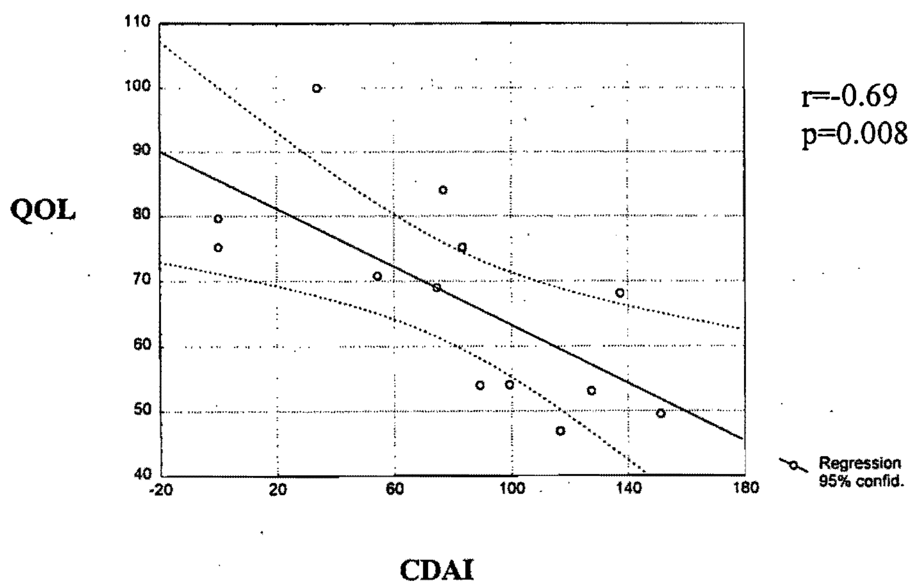


Figure 39: Correlation between CDAI and QOL form



At time of entry into the study, the serum vitamin levels of those patients who subsequently relapsed were similar to those who remained in remission (vitamin C 0.89±0.69 mg/dl vs. 0.64±0.42 mg/dl; $p=0.77$, retinol 0.46±0.32 mg/dl vs. 0.33±0.17 mg/dl; $p=0.14$, β-carotene 0.38±0.25 mg/dl vs. 0.38±0.36 mg/dl; $p=0.36$, vitamin E 15.05±5.87 mg/dl vs. 18.73±5.18 mg/dl; $p=0.13$). Furthermore, at the time of relapse and withdrawal from the study, the serum vitamin concentrations of those who were withdrawn, were not significantly different to their serum vitamin concentrations at entry (vitamin C 0.90±0.56 mg/dl vs. 0.89±0.69 mg/dl; $p=0.95$, retinol 0.69±0.45 mg/dl vs. 0.46±0.32 mg/dl; $p=0.17$, β-carotene 0.38±0.25 mg/dl vs. 0.63±0.28 mg/dl; $p=0.09$, vitamin E 18.03±5.46 mg/dl vs. 15.05±5.87 mg/dl; $p=0.49$).

6. Discussion

6.1 Nutritional Status and Role of Diet

The prevalence of malnutrition in IBD as has been reported to be as high as 60-70% in some centers.[1,254] Yet, despite this, using the BMI as an index of nutritional status, we found that in our clinic 47% of our patients were either obese or overweight, and only 13% were underweight. This may be the result of a greater awareness by clinics such as ours, of the nutritional problems associated with IBD, with positive steps being taken to avoid nutritional deficiencies, and to maintain remission in patients. Although 44% of patients were overweight, the prevalence of obesity was 12% which was similar to our control patients (9%). This prevalence however, may still be lower than general populations in which obesity may affect as many as 33% of individuals.[255]

At the time of interview, 68% of CD patients, and 89% of UC patients were assessed as being in clinical remission. It should be noted, however, that obesity affects the calculation of the CDAI, and may lead to some underestimation of disease activity in CD.[9] Eighty four percent of patients with CD and 66% of patients with UC had experienced a relapse in the past 2 years.

We were unable to show a significant direct relationship between present dietary intake and nutritional status in patients with IBD. Furthermore, nutritional status did not appear to relate to duration of disease, or to disease location and extent. Likewise, there was no apparent association between nutritional status and relapse rates assessed retrospectively. These results indicate that nutritional status in IBD is multifactorial, and also illustrates the possible shortcomings of dietary recall as a means of assessing nutrient intake, with both under-reporting and over-reporting occuring.[255] Furthermore, although the food frequency questionnaire is a valid instrument for assessing intake of antioxidant vitamins[136-138] and may be of greatest use in epidemiological studies,[56] a single dietary assessment method may not be representative of dietary intake if the study population includes several ethnic

groups with different dietary habits.[256] This study, however, was undertaken in a defined patient population, and even though two ethnic groups were included in this study, patients were of similar social and economical backgrounds, and tended to consume similar foods.[9]

Comparing the intakes of the macronutrients of the patients to that of controls it was found that patients consumed significantly less protein as a percentage of the energy consumed, than controls. In a previous study, 14% of patients complained of intolerance to red meat, and 40% said that they avoided eating nuts.[9] Similarly, patients often avoid fatty and/or fried foods such as sausages, pies, and fried fish possibly as a result of these foods increasing diarrhoea in patients with ileal disease.[9] Therefore, the patients may be consuming less protein containing foods than controls. Furthermore, patients also appeared to have higher caloric intake than controls. This has been reported in other studies[23,26,32] and may be due to patients increasing their intake while they are well.[26]

Patients also consumed significantly more carbohydrates than controls, a phenomenon which has been reported in other studies.[23,26,74] This may be a consequence of the decreased protein intake, with carbohydrate taken in place of protein.[23] The relatively low protein intake, and high carbohydrate intake, is unlikely to represent an intentional prudent diet, considering the relatively high fat intake (36%) of the patients.

The number of relapses in the last year was significantly associated with fat intake as a percentage of energy, and was significantly negatively associated with carbohydrate intake as a percentage of energy. Although the incidence of relapse rates was assessed retrospectively, the dietary consumption of the patients was considered reasonably constant, and therefore representative of the diet prior to relapse. Although we did not specifically assess the n6- and n3-fatty acid component of the diet, a normal diet contains a predominance of n6-fatty acids,[257] and it is possible that an increased intake of n6-fatty acids may result in an increased production of inflammatory mediators, such as

leukotriene B4, via the arachidonic pathway.[13,257,258] Leukotriene B4 has been reported to be increased in IBD,[257] and therefore, a diet rich in n6-fatty acids may be detrimental to IBD patients. This was further supported by Beluzzi's study where administration of n3-fatty acids in the form of fish oil capsules significantly reduced the rate of relapse in CD.[128] The n3-fatty acids (eicosapentaenoic acid) compete with arachidonic acid,[260] resulting in the production of eicosonoids which are less inflammatory.[128] The reduced carbohydrate intake in patients with recurrent disease may be a consequence of patients avoiding fibrous, carbohydrate rich foods due to obstructive symptoms.[22] Since fibre is fermented in the gut to produce SCFAs which provide an energy source for the colonocyte,[29] and have proliferative, yet anti-inflammatory effects on ileocolonic epithelia,[30] a diet low in these fatty acids may predispose IBD patients to relapse.

As in previous studies,[261,262] we noted a high prevalence of smoking in patients with CD (50%), which was significantly lower in patients with UC (21%) and controls (20%), ($p<0.01$). Despite the reported adverse effects of smoking in CD, and benefit in UC,[17,22] we were unable to demonstrate any significant association between smoking and relapse rates in our patients.

6.2 Vitamin Status

Mean dietary intakes of vitamin C, vitamin A and vitamin E of patients with IBD and the controls were all above 100% of the RDA, with no significant differences between the three groups. However, significantly more people with IBD compared to controls consumed less than the RDA for vitamin C, with 35% consuming less than the RDA. Likewise, significantly more patients with CD consumed less than the RDA for vitamin A. Despite the overall adequate intake, serum levels of vitamin C, retinol and α-tocopherol, but not β-carotene, were significantly lower in both CD and UC compared to controls. There was no direct relationship between serum levels and vitamin intake in both controls and IBD patients. This has been previously reported for vitamins A and E.[167,211] These two

vitamins are fat soluble, and therefore large body stores exist which can be mobilised when needed.[200,216] Decreased intake needs to occur for an extended period of time before serum concentrations are affected.[33,252] The lack of association between dietary intake and serum levels of vitamin E may relate to inaccurate food table estimates of vitamin E and variability of the vitamin E content of foods after cooking, storage and processing.[167] Vitamin E levels may also be affected by regeneration of α-tocopherol by vitamin C,[69] and possibly retinol.[154] One may have expected a better association between vitamin C levels and dietary intake in the control group.[37] However, serum levels may be more acutely affected by recent dietary intake, which may not have been reflected in the dietary assessment used. These factors would, however, have affected both controls and patients and are therefore unlikely to explain the poor correlation between intake and the low serum levels in the IBD patients. The low serum levels in the IBD patients are therefore unlikely to relate to dietary intake.

The effect of changes in serum cholesterol levels on serum levels of α-tocopherol and β-carotene in IBD patients was considered, as previous studies have reported lower cholesterol levels in IBD patients compared to controls.[74,178] In order to investigate this, serum cholesterol levels were assessed in the 23 patients with CD who participated in the supplementation study. We found cholesterol concentrations to be significantly lower than the control values. The reason for this reduced level is unclear as it does not appear to relate to dietary intake, or inflammation, and may reflect an alteration in lipid metabolism.[263,264] When expressed as the vitamin E/cholesterol ratio, levels of vitamin E were in fact no different to control values, suggesting that the apparent low levels of α-tocopherol merely reflect hypocholesterolaemia in these patients.[74,178] Although it has been suggested that the α-tocopherol/cholesterol ratio may be a more accurate index of vitamin E status,[205,216] this is usually in patients with hyperlipidaemia, and its value in hypolipidaemia has not been established.[265]

Serum retinol levels are affected by serum protein levels, particularly albumin and RBP.[178] However, mean albumin levels were normal in our patients, with only three patients, all

with evidence of active inflammation at the time, having below normal values. Therefore the low levels could be not be explained by low albumin levels.

Age and nutritional status did not significantly affect serum levels. There was however, a positive trend between vitamin E levels and BMI. The possibility that the association between vitamin E and BMI was a consequence of an association with cholesterol was considered. However, no direct association between cholesterol and BMI was evident, and this is unlikely to fully explain the trend. It has been reported that overweight and obese people may have decreased serum antioxidant vitamin concentrations[266] due to the vitamins being sequestered in increased body fat stores.[267] However, the antioxidant serum vitamin concentrations in our obese and overweight controls and patients were not significantly different to those found in our normal weight controls, or patients. Therefore, the decreased serum concentrations found in our IBD patients did not appear to be due to the large prevalence of overweight in this population.

Serum vitamin C concentrations of controls tended to be lower in those who smoked compared to those who did not smoke. This phenomenon has been previously documented,[70,182,183,213,268] but was not apparent in our IBD patients. The reason for this discrepancy is uncertain. Smoking is known to be a significant cause of free radical production, and therefore antioxidant depletion, and has been cited as a reason for decreased antioxidant vitamin levels.[74] It is possible that other factors affecting the serum levels of the vitamins in IBD mask the effects of smoking. Also, despite reports in the literature of smokers having lower β-carotene concentrations than non-smokers,[141,167,206,213] smoking did not seem to affect β-carotene concentrations in our sample. Retinol and α-tocopherol levels were also not affected by smoking.[167,206,209,210,213,214]

Other possible causes of the low vitamin C, retinol and α-tocopherol levels in our patients include increased consumption due to active inflammation. Although, like other

studies[33,35,74] we were unable to demonstrate any direct relationship between the serum levels of vitamin C, vitamin A and vitamin E and indices of disease activity such as CDAI and the HBI, these assess other parameters such as abdominal pain, diarrhoea, and general well being, which may not directly relate to inflammation. The blood ESR is a more direct indication of inflammation and it is interesting that patients with a raised ESR had significantly lower levels of vitamin C and retinol. This suggests that serum levels of vitamin C and retinol are affected by active inflammation. The effect of inflammation on vitamin C serum concentrations has been well documented,[269-272] but the mechanism behind this is not clear.[272] It is thought that in active inflammation serum ferritin falls (negative acute phase response). This results in the amount of free iron increasing due to inadequate binding capacity in the plasma. Under these circumstances vitamin C reacts with the iron, and may form free radicals. In order to prevent free radical formation, and further oxidant mediated damage,[271,272] neutrophils may increase their uptake of vitamin C from serum.[270] This increased uptake may also help improve the antioxidant function of the neutrophil.[270] Furthermore, the vitamin C may be transported to the tissue site where it is needed, thereby decreasing the circulating serum concentrations.[270] Vitamin C is also utilised to regenerate vitamin E.[69] The reduction in retinol levels with acute inflammation may be consequent to a reduction in RBP, also as part of the acute phase response,[270,271] as well as retinol utilisation to regenerate vitamin E.[155] The apparent lack of effect of active inflammation on vitamin E levels may be explained by the synergism between vitamin C and vitamin E,[69,149,152] and retinol and vitamin E,[155] where in active inflammation, vitamin C and retinol are utilised to regenerate vitamin E, thereby normalising vitamin E levels.[38,155]

The acute phase response has been associated with a number of other metabolic abnormalities. These include decreased albumin and cholesterol concentrations,[269] which can affect serum vitamin levels, particularly of vitamin A and E, which are protein or lipid bound. Although active inflammation does appear to result in decreased levels of vitamin C and retinol, it should be noted that serum levels were also low in patients with no evidence of active inflammation indicating that other factors are also responsible.

Site of disease, such as colonic, ileo-colonic and ileal involvement did not appear to influence the serum levels of the vitamin in patients with CD. Similar results were shown in a study by Kuroki et al, in 1993.[35] However, extent of disease did appear to affect serum levels of α-tocopherol in patients with UC. Serum levels of α-tocopherol in patients with disease restricted to the rectum although still significantly lower than controls, were significantly higher than those with disease in the left colon or with total colitis. The colon is a dynamic organ which is particularly prone to oxidative stress. Most of the antioxidant enzymes in the colon are located in the lamina propria. This leaves the mucosa with a relative deficiency,[110] and therefore the colon is at risk of oxidant mediated damage, and depletion of antioxidant vitamins. Vitamin C, retinol and β-carotene are antioxidants which are used preferentially in order to spare vitamin E, the most potent antioxidant.[151] Therefore, since vitamin C, retinol and β-carotene concentrations are low, and the colon is inflamed, vitamin E is being utilised to neutralise free radicals. Furthermore, the greater the extent of disease, the greater the free radical production and the more vitamin E will be used. These factors may explain the association between vitamin E levels and the extent of colitis. The role of serum cholesterol levels in UC was not investigated in this study, and further study is required in this regard.

Alpha-tocopherol serum concentrations in UC patients with recent onset of disease (less than one year), were significantly higher than those with a longer duration of disease. On the other hand, serum retinol and β-carotene levels appeared to be higher in both CD and UC with disease of longer duration (>10years). The reason for these associations is not clear. It has been shown, however, that vitamin A decreases with active inflammation[36] and it is possible that patients with a recent onset of illness may have experienced more active disease, than that in whom the condition is longstanding and stable. As regards the serum levels of α-tocopherol, in investigating the reason for the apparent decrease in levels with activity of disease in UC, it would be important to assess the effects of disease duration on serum cholesterol levels.

The relationship between the antioxidant vitamins and relapse rates was also considered. Patients with UC who had relapsed in the previous 2 years tended to have higher levels of retinol, and those with CD tended to have higher levels of α-tocopherol. There was no apparent association between relapse rates and vitamin C and β-carotene. However, in this aspect of the study, the rate of relapse was assessed retrospectively, and the present serum levels of vitamins may not reflect the levels prior to relapse. In fact the higher levels of retinol and α-tocopherol may reflect an adaptive response following the episode of acute inflammation.[36] This was shown in a study by Ramakrishna et al, when vitamin A decreased with active disease, but increased again, when the disease was treated. Furthermore, when levels had returned to normal, they were in fact non-significantly higher than controls.[36]

6.2 Effect of supplementation

In order to prospectively study a group of patients with IBD and to investigate the role of antioxidant vitamin supplementation, a double-blind, placebo controlled study was performed in a group of patients with CD. The study was designed to investigate the effect of supplementation of antioxidant vitamins, given as a buccal spray, on subsequent relapse rates. The supplemental spray contained 100% RDA for vitamin A, and E, and 10% for vitamin C and β-carotene. We elected to use the apparent rather modest dose, as when the supplementation was added to the normal diet, patients were in fact consuming greater than 200% of the RDA for vitamin A and 400% for vitamin E, and the dose used complied with the manufacturers recommendations. Furthermore, studies have indicated that carotenoids in large doses may be associated with adverse effects including an increased risk of certain cancers.[174,253] We also chose to use a multivitamin supplement as antioxidant vitamins act synergystically,[149,155] and since we had shown that our IBD patients have lower serum antioxidant vitamins for all the vitamins tested, it seemed that a combination of these vitamins was more likely to have a beneficial effect than any one vitamin alone. Furthermore, in supplementation trials on pancreatic, HIV and cancer

patients, multivitamin supplements have been used with beneficial effect,[249,250] while single vitamin supplementation trials in IBD have shown no beneficial effects.[179,273]

Antioxidant supplementation has been reported to be beneficial in several diseases, including pancreatitis, cardiovascular disease, cancer, and HIV. A number of different supplementation dosages have been used, and the ideal supplementation dose, as well as which vitamins need to be supplemented, remains controversial.[247,274] In a 20 week double-blind dummy cross over trial, Uden et al, 1990, reported that a supplement containing 600μg organic selenuim, 9000IU β-carotene, 270IU vitamin E, 2g methionine, and 0.54g vitamin C aided in the therapy of recurrent (non-gallstone) pancreatitis and/or pancreatic pain.[249] In cardiovascular disease, Enstrom et al, showed an inverse relationship between vitamin C supplementation of at least 50mg/day and risk of mortality,[275] and Vita et al reported that vitamin C doses at higher than the RDA are beneficial to subjects with deficient intakes or to patients with significant coronary artery disease.[276] In the CARET study, however, in which 18 314 smokers, former smokers, and workers exposed to asbestos were randomised to receive 30mg β-carotene per day and 25 000IU of retinol in the form of retinyl palmitate or placebo, no effect on cardiovascular disease was found.[175] Similarly in the ATBC lung cancer prevention study where 29 133 male subjects were randomised to receive a supplement of β-carotene (20mg/day) or α-tocopherol (50mg/day) alone, or both, or placebo, there was no reduction in incidence in lung cancer after 5-8 years of dietary supplementation.[174] In fact, there seemed to be an increase in the incidence of lung cancer in those on the β-carotene supplement.[174] In HIV, supplementation studies using vitamin A at a daily dose of 5000IU retinyl palmitate and 30mg β-carotene, showed no effect on reducing overall mother-to-child transmission of HIV.[277] Similarly in a study using a β-carotene supplement of 180mg/day for 4 weeks in patients with normal baseline serum levels of β-carotene and vitamin A, there was no effect on CD4 lymphocyte count or plasma HIV RNA copy number.[278] Furthermore, a supplement containing 10 500U vitamin A, 300mg vitamin C, 300mg vitamin E, 150ug selenium, and 200mg zinc, taken twice daily with 800mg albendezole, has been shown to have no effect on diarrhoea or morbidity or mortality in HIV.[279] However, in a trial of 49 HIV positive patients randomised to receive

supplements of both DL-α-tocopherol acetate (88IU daily) and vitamin C (1000mg daily) or placebo for 3 months, oxidative stress decreased significantly in the supplemented group as compared to controls, and there was a trend to reduction in viral load.[250]

Our supplementation provided similar β-carotene (25mg) but less vitamin E (10mg), vitamin C (6mg), and retinol (1000RE) than other supplementation trials.[174,177,250,275,276,279,280] Supplementation resulted in a significant increase in serum vitamin E levels, to a level not different to control values. There was also a significant increase in retinol levels, but this increase was noted in both the actively supplemented group as well as the placebo group, and is therefore unlikley to be solely the result of supplementation. There was no statistical change in vitamin C and β-carotene levels over the course of the study.

In view of the low dose of vitamin C in the supplement, and the normal serum levels of β-carotene in the patients, it is perhaps not surprising that the serum concentrations of these vitamins were unaffected during the course of the study. However, a number of factors may have affected the absorption and utilisation of vitamins in our patients. The mean BMI of our trial subjects was in the overweight range, there were a large amount of smokers in the group, and serum concentrations of the vitamins were significantly lower than controls at baseline. Nierenberg et al, reported that nonsmokers, lean people and those with higher baseline serum concentrations have larger increases in plasma concentrations with supplementation.[251] Therefore the response of our subjects to the supplement may have been retarded. Furthermore, the levels of vitamins in the supplement may not have been adequate for a population who may be malabsorbing the vitamins from their diets,[251] and larger doses, as in other supplementation studies,[177,249,250] should be considered for further investigation. There is however, large inter- and intra-individual variability in response to vitamin supplementation, and in a study of HIV patients using a supplement containing 10 500U vitamin A, 300mg vitamin C, 300mg vitamin E, and 150ug selenium and 200mg zinc, the serum levels of those in the

supplemented group, also did not rise significantly when compared to placebo.[279] In a study by Wright et al, 1985 in which 50 000U of vitamin A per day was given to 86 CD patients in remission, a significant difference in serum concentrations between the placebo group and the supplemented group was only noted after 5 months.[179] Furthermore, serum level measurements of these vitamins may not be an adequate measure of the effect of supplementation. Albanes et al, showed that adipose tissue concentrations of retinoic acid increased, in patients whose plasma or serum retinol levels did not change in response to a β-carotene supplement.[141] Furthermore, in one study in which high doses of β-carotene were fed to preruminent calves, vitamin A was found to have accumulated in the liver.[281] Leukocyte vitamin C concentrations, which reflect tissue levels, may also have been a more accurate measurement of vitamin C.[70] Moreover, in view of the method of delivery, it was difficult to assess compliance in our subjects, as we could not do a pill count. The default rate for the trial was high and several of our patients were lost to follow up.

Our study investigated the serum vitamin levels of CD patients during supplementation. We did not directly examine the absorption of the supplement, the metabolism of these vitamins in this group of patients, the consumption of these vitamins in the inflammatory process, or their excretion. Future studies looking at these aspects of antioxidant vitamin metabolism, in this population, are required.

In our study, results indicated similar relapse rates in the patients who received active antioxidant spray compared to placebo with 4 patients in the active group relapsing compared to 3 patients in the placebo goup. On an intent to treat analysis 4/11 (36%) of those receiving antioxidant were still in remission after 6 months compared to 5/12 (42%) of those receiving placebo. At time of entry into the study, the serum concentrations of the vitamins of those patients who subsequently relapsed were similar to those who remained in remission, and therefore gave no indication of risk of relapse. There was also no significant difference in CDAI scores between the 2 groups over the 6 month period. QOL scores were also similar.

It is possible that the doses delivered in our spray supplement may have been insufficient to have an effect on relapse rates. However, a previous study by Wright et al, using a vitamin A supplement of 55 000 IU BD (550% RDA) was also unable to show any effect on relapse rates.[179] As regards supplementation with α-tocopherol, when reflected as the α-tocopherol/cholesterol ratio, the levels in our CD patients were similar to controls, and the patients may in fact not have been deficient initially. Furthermore, we have also noted that increased dietary intake of vitamin E was in fact associated with an increased relapse rates in CD. It is therefore possible that increasing the vitamin E intake may actually be detrimental in CD. The mechanism behind this is unclear, but may be related to the role of vitamin E in apoptosis. In CD, apoptosis has been reported to be decreased compared to controls,[54] and may be an important pathogenic mechanism in the disease.[55] Vitamin E has also been shown to impair apoptosis,[229] and it is possible that an increased intake of vitamin E may actually perpetuate the inflammation in the disease.

The primary objective of our study was to investigate the effect of an antioxidant vitamin supplement on relapse rates in CD. Patients who relapsed were therefore discontinued from the study. The development of active disease during the study, determined by the CDAI, or the development of complications such as abscesses, did not appear to significantly affect the serum levels of the vitamins, and the levels at the time of relapse were similar to levels at the time of entry. The effect of subsequent therapy of active CD on vitamin levels was not assessed.

These results indicate that supplementation of vitamin A and E does not effect disease relapse rates in CD, and suggests that the low serum levels noted in patients may be a consequence of the chronic inflammatory process, and not necessarily the cause.

Considering the relatively small numbers of patients recruited into our study, one should, perhaps, be cautious in interpreting the results, and the trial should rather be considered a pilot study. However, considering our results, and the proportion of patients who

remained in remission over the duration of the study, we calculated that in order to demonstrate a significant difference between the two groups, a sample size of 712 subjects would have been required to demonstrate an advantage, in favour of placebo! We are therefore satisfied that there is no evidence that the supplementation at the doses used, and with the delivery method utilised, offered any advantage to CD patients. To our knowledge, the role of supplementing vitamin C to patients with IBD has not been reported, and further research on the role of vitamin C in IBD is indicated.

7. Conclusion

Both Crohn's disease and ulcerative colitis are associated with decreased serum levels of vitamin C, retinol and α-tocopherol, but not β-carotene, despite adequate dietary intakes. The cause of these reductions is likely to be multifactorial, with active inflammation directly affecting the levels of vitamin C and retinol, and α-tocopherol levels affected by disease extent in UC, as well as serum cholesterol concentrations. Nutritional status and smoking did not appear to significantly affect the vitamin levels in IBD patients. Supplementation of vitamin A and E did not appear to influence disease relapse rates in CD, suggesting that low levels of vitamins may be a consequence and not necessarily the cause of the inflammatory state.

7.1 Recommendations for future studies

1. This study assessed the role of antioxidant supplementation in preventing relapse in Crohn's disease. The effect in active disease was not assessed and should be evaluated in further studies. Furthermore, the effect of higher doses of antioxidant vitamins should also be considered.

2. The effect of antioxidant supplementation in UC should also be assessed.

3. The role of vitamin C appears to be central to the anti-inflammatory process, both with its specific antioxidant properties, as well as in regeneration of α-tocopherol. The role of vitamin C in treating active disease, as well as in preventing relapses should be assessed.

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APPENDIX 1: BASELINE CASE REPORT FORM

CRF for baseline subjects

Name: **Folder no:** **Gender: m/f**

Age:

Diagnosis: **Smoking:**
Diagnosis date:
Duration:

Relapses in last 2 years:

Surgery in last 2 years:

Medications:

Anthropometry

Weight: **Height:** **BMI:**

TSF: 1 2 3 average

MAC 1 2 3 average

Food Frequency:
Cals:
%prot:
%fat:
% CHO
% alcohol

APPENDIX 2: INFORMED CONSENT FORM

The term "Inflammatory Bowel Disease" (IBD) encompasses two diseases, Crohn's Disease and Ulcerative Coltits. The exact cause of these diseases remains unknown.

However, recent research suggests that reactive molecules known as free radicals, which are produced in the tissues, may play a role in many inflammatory conditions including IBD. Certain vitamins, such as Vit A, E and C have antioxidant properties, and may help to decrease the level of free radicals. This may be beneficial in reducing the inflammation in IBD.

Therefore, the aim of this study is to:

1. assess the antioxidant status of patients and to relate this to nutritional status and disease activity
2. assess the effect of supplementation of antioxidant vitamins, especially Vit A, C, E and B-carotene, on serum levels, disease activity and quality of life.

This study will require subjects to undergo monthly interviews and blood tests to ascertain vitamin intake, as well as taking a vitamin supplement.

I ...., hereby give my informed consent to be a subject in the aforementioned trial.

Signature..............................

Date...................................

APPENDIX 3: DIETARY QUESTIONNAIRE

Food Frequency

FOOD FREQUENCY QUESTIONNAIRE

	TYPE	TIMES PER WEEK	1-2 TIMES PER MONTH	AMOUNT	PREPARATION
MILK					
FULL CREAM					
LOW FAT/2%					
SKIM					
ICE-CREAM					
SORBET					
FROZEN DESSERT					
YOGHURT					
LOW FAT					
FAT FREE					
YELLOW CHEESE					
COTTAGE CHEESE					
EGGS					
FRIED					
SCRAMBLED					
BOILED					
POACHED					
RED MEAT EG MUTTON					
MINCE MEAT					
CHICKEN					
WITH SKIN					
WITHOUT SKIN					
LIVER					
PORK					
FISH					
TUNA					
SHELLFISH					
POLONY					
VIENNAS					
BOEREWORS					
VEG					
CARROTS					
TOMATOES					
SWEET POTATOES					
POTATO					
GEM SQUASH					
SPINACH (COOKED)					
MIXED VEG					

LETTUCE					
BROCOLLI					
GREEN BEANS					
PEAS					
CABBAGE					
CORN/ MIELIES					
FRUIT					
MELON					
ORANGES					
ORANGE JUICE					
NAARTIES					
PEACHES					
WATER-MELON					
APPLES					
BANANAS					
PRUNES					
RAISINS					
GRAPEFRUIT TOMATO SAUCE					
OIL VINEGAR DRESSING					
MAYO					
CHUTNEY					
PIZZA					
POTATO CHIPS					
NUTS					
PEANUT BUTTER					
PIE (READY MADE)					
SLAP CHIPS					
CEREAL					
RICE					
WHITE					
BROWN					
PASTA					
BREAD/ ROLLS					
WHITE					
BROWN					
WW					
RYE					
CEREAL					
CHOCOLATE					
SWEETS					
PUDDINGS					
JAM					

SUGAR					
TEA					
COFFEE					
ROOIBOS					
ALCOHOL					
COLDRINKS					
GAS					
DIET					
FRESH FRUIT					
MIX A DRINK					
OTHER					
BUTTER					
OIL					
CREAM					
CREAMER					
MARG					

APPENDIX 4: CLINICAL ASSESSMENT INDEX

Crohn's Disease Activity Index (CDAI)

Name:

Sex:

folder number:

Liquid/soft stools: (days one it seven in consecutive blocks)

total 1:

Abdominal pain rating: (0=none; 1=mild; 2=moderate; 3=severe)

total 2:

General well-being: (0=generally well, 1=slightly under par, 2=poor, 3=very poor, 4=terrible)

total 3:

Complications

- ☐ Arthritis/Arthralgia.
- ☐ Iritis/Uveitis
- ☐ Erythema Nodosum/Pyoderma Gangrenosum/Aphthous Stomatitis
- ☐ Anal fissure, fistula or abscess
- ☐ Other fistula
- ☐ Fever over 37.8 degrees celsius during the past week

total 4: 0

Use of diphenoxylate,loperamide, or other opiates for diarrhoea

yes

no

total 5:

Abdominal mass: 0=none, 2=questionable, 5=definite

total 6:

Haematocrit: %

total 7:

Body weight: kilograms

Standard weight: kilograms

total 8:

CROHN'S DISEASE ACTIVITY INDEX (CDAI) is:

Todays date: Thursday, October 26, 2000

Data entered by:

Notes:

APPENDIX 5: CLINICAL ASSESSMENT INDEX

Harvey Bradshaw Index (HBI)

COLITIS ASSESSMENT

Diarrhoe (no. of daily stools)

0 - 2	0
3 - 4	1
5 - 6	2
7 - 9	3
10	4

Nocturnal Diarrhoea

No	0
Yes	1

Visible blood in stools (% of movements)

0%	0
<50%	1
>50%	2
100%	3

Faecal Incompetence

No	0
Yes	1

Abdominal pain/cramping

None	0
Mild	1
Moderate	2
Severe	3

General well being

Perfect	0
Very good	1
Good	2
Average	3
Poor	4
Terrible	5

Abdominal Tenderness

None	0
Mild and localised	1
Mild to moderate and diffuse	2
Severe or rebound	3

Need for antidiarrhoels

No	0
Yes	1

APPENDIX 6: QUALITY OF LIFE ASSESSMENT

The SF- 36 quality of life form

INSTRUCTIONS: This questionnaire asks for your views about your health, how you feel and how well you are able to do your usual activities.

Answer every question by marking the answer as indicated. If you are unsure about how to answer a question, please give the best answer you can.

1. In general, would you say your health is:

(circle one)

Excellent 1

Very good 2

Good 3

Fair 4

Poor 5

2. Compared to one week ago, how would you rate your health in general now?

(circle one)

Much better now than one week ago 1

Somewhat better now than one week ago 2

About the same as one week ago 3

Somewhat worse now than one week ago 4

Much worse now than one week ago 5

3. The following questions are about activities you might do during a typical day. Does your health now limit you in these activities? If so, how much?

(circle one number on each line)

ACTIVITIES	Yes, Limited A Lot	Yes, Limited A Little	No, Not Limited At All
a. **Vigorous activities**, such as running, lifting heavy objects, participating in strenuous sports	1	2	3
b. **Moderate activities**, such as moving a table, pushing a vacuum cleaner, bowling, or playing golf	1	2	3
c. Lifting or carrying groceries	1	2	3
d. Climbing **several** flights of stairs	1	2	3
e. Climbing **one** flight of stairs	1	2	3
f. Bending, kneeling or stooping	1	2	3
g. Walking **more than one kilometre**	1	2	3
h. Walking **half a kilometre**	1	2	3
i. Walking **100 metres**	1	2	3
j. Bathing or dressing yourself	1	2	3

4. During the past week, have you had any of the following problems with your work or other regular daily activities as a result of your physical health?

(circle one number on each line)

	YES	NO
a. Cut down on the **amount of time** you spent on work or other activities	1	2
b. **Accomplished less** than you would like	1	2
c. Were limited in the **kind** of work or other activities	1	2
d. Had **difficulty** performing the work or other activities (for example, it took extra effort)	1	2

5. During the past week, have you had any of the following problems with your work or other regular daily activities as a result of any emotional problems (such as feeling depressed or anxious)?

(circle one number on each line)

	YES	NO
a. Cut down on the **amount of time** you spent on work or other activities	1	2
b. **Accomplished less** than you would like	1	2
c. Didn't do work or other activities as **carefully** as usual	1	2

6. During the past week, to what extent has your physical health or emotional problems interfered with your normal social activities with family, friends, neighbours, or groups?

(circle one)

Not at all 1

Slightly 2

Moderately 3

Quite a bit 4

Extremely 5

7. How much bodily pain have you had during the past week?

(circle one)

No bodily pain 1

Very mild 2

Mild 3

Moderate 4

Severe 5

Very severe 6

8. During the past week, how much did pain interfere with your normal work (including both work outside the home and housework)?

(circle one)

Not at all 1

A little bit 2

Moderately 3

Quite a bit 4

Extremely 5

9. These questions are about how you feel and how things have been with you during the past week. For each question, please give the one answer that comes closest to the way you have been feeling. How much of the time during the past week -

(circle one number on each line)

	All of the Time	Most of the Time	A Good Bit of the Time	Some of the Time	A Little of the Time	None of the Time
a. Did you feel full of life?	1	2	3	4	5	6
b. Have you been a very nervous person?	1	2	3	4	5	6
c. Have you felt so down in the dumps that nothing could cheer you up?	1	2	3	4	5	6
d. Have you felt calm and peaceful?	1	2	3	4	5	6
e. Did you have a lot of energy?	1	2	3	4	5	6
f. Have you felt down?	1	2	3	4	5	6
g. Did you feel worn out?	1	2	3	4	5	6
h. Have you been a happy person?	1	2	3	4	5	6
i. Did you feel tired?	1	2	3	4	5	6

10. During the past week, how much of the time has your physical health or emotional problems interfered with your social activities (like visiting with friends, relatives, etc.)?

(circle one)

All of the time 1

Most of the time 2

Some of the time 3

A little of the time 4

None of the time 5

11. How TRUE or FALSE is each of the following statements for you?

(circle one number on each line)

	Definitely True	Mostly True	Don't Know	Mostly False	Definitely False
a. I seem to get sick a little easier than other people	1	2	3	4	5
b. I am as healthy as anybody I know	1	2	3	4	5
c. I expect my health to get worse	1	2	3	4	5
d. My health is excellent	1	2	3	4	5

APPENDIX 7: SERUM VITAMIN ANALYSIS

Colourometric vitamin C analysis[242]

Vitamin C analysis[242]

Blood was analysed within 3 hours of being drawn, as stated in the methodology section (4.3.1.1).

Reagents

All reagents were supplied by Merck NT Laboratory Supplies

1. Vitamin C
2. Metaphosphoric acid
3. Thiourea
4. Cupric sulphate (anhydrous)
5. 2,4 Dinitrophenylhydrazine
6. Sulphuric acid

Preparation of reagents for blood analysis

Sulfuric acid solutions of 12mol/L and 4.5mol/L were prepared in advance by adding 650ml and 250ml of sulfuric acid to cold double-distilled water. The concentrated sulfuric acid was stored at 4^0C.

A copper sulfate solution (0.6g/dl) was also made up by dissolving 0.6g of anhydrous copper sulfate solution in double-distilled water, and diluted to a final volume of 100mls.

Each month, a thiourea solution (5.0g/dl) was made by dissolving 5g of thiourea in double distilled water and diluted to 100ml. This was also stored at 4^0C, and discarded after one month.

Once a week, 2,4-dinitropheylhydrazine reagent, 2.0g/dl was made. 5g of 2,4-dinitrophenylhydrazine was dissolved in sulfuric acid (4.5mol/L), and diluted to a final volume of 250ml with the 4.5mol/l sulfuric acid. This was stored at 4^0C overnight, and filtered the next day. The filtered solution was also stored at 4^0C, and was discarded after a week.

Similarly a solution of dinitrophenylhydrozine-thiourea-copper sulfate (DTCS) reagent was prepared once a week by the addition of 5ml of the thiourea solution, 5ml of the copper sulfate solution and 100ml of the 2,4-dinitrophenylhydrazine solution. This was stored in a glass container in the fridge at 4^0C for no longer than a week, when it was discarded.

Apparatus

Beckman 25 spectrophotmeter and recorder

Analysis

Once the serum was centrifuged and stored away from light and air in foil covered sealed test-tubes, in the fridge at 4^0C, a solution of metaphosphoric acid (6.0g/dL) was prepared by dissolving 15.0g of metaphosphoric acid in double-distilled water, and bringing it to a final volume of 250ml. This was prepared directly before use.

Standards

The ascorbic acid stock standard was then prepared by dissolving 25mg of ascorbic acid in the metaphosphoric acid and bringing it to a final volume of 50ml. An intermediate standard was then prepared by pipetting 5mls of this solution into a volumetric flask, and diluting it to 50ml to make a 5.0mg/dl solution.

Analysis of vitamin C

Once this task had been accomplished 0.5 ml of plasma was pipetted into 2.0ml of the metaphosphoric acid in a 13 X 10mm test tube. This was mixed well on the vortex mixer and then centrifuged for 10 minutes on 2500g. Each sample was prepared in duplicate.

While the samples were spinning working standards were prepared by pipetting the following amounts of intermediate standards into test tubes (see table below).

Working Standard concentration (mg/dl)	Amount intermediate Standard (ml)	Amount metaphosphoric acid (ml)
0.10	0.05	2.45
0.4	0.2	2.3
0.8	0.4	2.1
1.2	0.6	1.9
2.00	1.0	1.5
3.00	1.5	2
4.00	2.0	1.5

Once prepared, these standards were then vortex mixed to ensure homogeneity

Once prepared, 1.2 mls of each working standard was pipetted into teflon lined test tubes, in duplicate. Furthermore, 1.2ml of metaphosphoric acid was added to two of these test tubes as blanks. Once centrifuged, 1.2ml of the clear supernatant of the samples was also pipetted into teflon-lined test tubes.

0.4mls of the DTCS reagent was added to each tube. Caps were placed on the test tubes, and they were all vortex mixed, and then placed in a water bath at 37^0C for 3 hours.

The test tubes were removed form the water bath after 3 hours, and placed in a ice-bath for 10 minutes. 2mls of cold 12mol/L sulfuric acid was then added while vortex mixing. The temperature of the samples did not exceed room temperature at any time.

The absorbence of samples was read on a spectrophotometer at 520nm. Each working standard concentration was plotted on a graph against the absorbence, creating a standard curve. Sample absorbence readings were then used to plot the samples on the standard graph. The concentrations found from the graph were then multiplied by 5, in order to correct for the dilution of the serum by metaphosphoric acid, and the true concentrations of the samples were recorded in mg/dl.

APPENDIX 8: SERUM VITAMIN ANALYSIS

HPLC method for vitamin A and E analysis[243]

Method for analysis of α-tocopherol, Retinol and β-carotene by HPLC[243]

Vitamin Standards were purchased from Anatech and Sigma Aldrich PTY LTD respectively. All Standards were kept at –65°C until the stock standards were made up. All the standards were made up in a darkened room, and were stored in foil covered containers at -65°C until used. All chemicals used in the HPLC analysis were of HPLC grade, and were purchased from Anatech and Merck Chemicals.

Apparatus

1. SP8800 ternary HPLC pump
2. SP4400 Crom Jet integrator
3. Supelcosil LC-18 column

Standards

Stock Standards:

The stock standards were of the concentrations below:

α-carotene 10μg/ml

β-carotene 1mg/ml

Retinyl Palmitate 40mg/ml

Retinol 1mg/ml

Retinyl Acetate 1mg/ml

α-tocopherol 500μg/ml

γ-tocopherol 500μg/ml

α-tocopherol acetate 10mg/ml

Therefore we used:

α-carotene:

To make up 10μg/ml:

Used 5mg standard per 500ml chloroform

β-**carotene**:

To make up 1mg/ml

Used 25mg standardd per 25ml chloroform

Retinyl Palmitate:

To make up 40mg/ml

Used 100mg per 2.5 ml chloroform

Retinol:

To make up 1mg/ml

Used 100mg per 100ml ethanol

Retinyl Acetate:

To make up 1mg/ml

Used 100mg per 100ml ethanol

α-**tocopherol**

To make up 500ug/ml

Used 100mg per 200ml ethanol

γ-**tocopherol**

To make up 500ug/ml

Used 25mg per 50ml ethanol.

α-**tocopherol acetate**

To make up 10mg/ml

Used 100mg per 10ml ethanol

<u>Secondary Stock Standards:</u>

The secondary stock standards were of the concentrations below:

β-carotene 20μg/ml

Retinol 100μg/ml

Retinyl acetate 100μg/ml

Retinyl palmitate 400μg/ml

Therefore we used:

β-carotene:

To make up 20μg/ml

Took 200ul β-carotene standard. Add 9.8ml ethanol to make up to 10ml.

Retinol:

To make 100μg/ml

Took 1ml of retinol standard. Add 9ml ethanol to make up to 10ml

Retinyl acetate

To make 100μg/ml

Took 1ml of retinyl acetate standard. Add 9ml ethanol to make up to 10ml.

Retinyl palmitate

To make 400μg/ml

Took 100ul of retinyl palmitate STD. Add 9.9ml ethanol to make up to 10ml.

<u>Working Combined internal Standard 1:</u>

This standard consisted of:

0.5μg/ml Retinyl acetate

1μg/ml. Retinyl palmitate

25μg/ml α–tocopherol acetate

Therefore add:

50µl Retinyl acetate from secondary stock

25µl α-tocopherol - acetate from stock

25µl of Retinyl palmitate from secondary stock

9.9ml Ethanol

Working combined internal standard 2

This standard consisted of:

2µg/ml Retinyl acetate.

4µg/ml Retinyl palmitate

100µg/ml α-tocopherol acetate

Therefore for 10 mls used:

200µl Retinyl acetate secondary standard

100µl Retinyl palmitate secondary standard

100µl α-tocopherol primary standard

9.6ml ethanol.

Working combined standard

The working combined standard contained

0.2µg/ml α-carotene

50µg/ml α-tocopherol

50µg/ml γ-tocopherol

0.4µg/ml β-carotene

2µg/ml Retinol

So took:

200µl of α-carotene from the stock standard

1ml α-tocopherol from the stock standard

1ml γ-tocopherol from the stock standard

200µl β-carotene from the secondary stock

200µl Retinol from the secondary stock

7.4 ml ethanol

Buffer/Mobile Phase

per litre:

acetonitrile: 780ml

Chloroform: 160ml

2 propanolol 35ml

water: 25ml

Samples

1. Place 1ml of serum in 16x100 culture tube with teflon sealed screw cap.
2. Slowly add 1ml of internal standard 1 to each tube while vortex mixing.
3. Add 3mls of petroleum ether.
4. Cap
5. Vortex mix for 2 minutes.
6. Centrifuge at 500g for 5 minutes
7. Transfer as much of upper layer as possible into foil covered 10x100mm test tube.
8. Evaporate to dryness at 37° under stream of N2.
9. Reconstitute in 200ul of mobile phase/buffer

Analysis

1. Set detector at 460nm to measure carotenoids, and at 292nm to measure retinoids and tocopherols.
2. Recorder chart speeds set at 0.5cm/minute.
3. Eluting time 20min
4. At the beginning of each day's analysis, run the standards on each wavelength
5. Analyse +/- 40ul of reconstituted extract
6. Run each sample in duplicate

The standards were defrosted a maximum of 4 times, and then discarded in order to prevent degradation. All samples were analysed in duplicate. We had only one detector; therefore each sample was run four times – in duplicate on each wavelength.

Calculations

$$\text{Concentration of sample} = \frac{\text{area of sample X concentration of standard}}{\text{area of standard}}$$

APPENDIX 9: METHOD FOR QUALITY OF LIFE ANALYSIS

Quality of life assessment method

Permission was granted to use the SF-36 quality of life form by Prof. John E. Ware. With the help of the Statistics department of UCT; we devised our own analysis method for the form, which approximates the analysis method described in the journals, and was used to determine any differences in quality of life over the 6 month period. This analysis method was used only as a method for determining improvement or deterioration in QOL of our patients over the 6 month supplementation period, and not as an indication of QOL in general, or any deficiency in the 10 sections of health status that are measured in the form.

Since the dimension score for the form could have been between 0-100 with a higher score showing better health, we transformed all the scores so that health was associated with the highest score. This was achieved by the calculations below. Each question was calculated separately, and then a total score was calculated from these. Please refer to the appendix for the SF-36 quality of life questionnaire.

If a particular questions' score was lower for better health, these scores were then subtracted form a larger no, in order to make the highest score a higher numerical value. For example for the first question in the questionnaire, which asks how one would describe one's health, Excellent scored 1, while poor scored 5. Therefore, it makes the healthiest answer a higher numerical value than an unhealthy answer; we subtracted the score for 6. In this way the answer excellent, would give a score of 5, while the answer poor, would give a score of 1. If a question's score was already in this order, just the score was used. The following table shows which questions were altered in this way:

Question no:	Total Score equal to:
1	6-score
2	6-score
3a-j	Score
4a-d	Score
5a-c	Score

6	Score
7	7-score
8	6-score
9a	7-score
9b	Score
9c	Score
9d	7-score
9e	7-score
9f	Score
9g	Score
9h	7-score
9I	Score
10	Score
11a	Score
11b	6-score
11c	Score
11d	6-score

Therefore, with this method the minimum score that could be obtained was 36, while the maximum was 148. Since the SF-36 has a dimension score of 0-100, we transformed our data with the formula.

Total score= (total-36)/113 x100 so that our final score fell within this range.

APPENDIX 10: CASE REPORT FORM FOR SUPPLEMENTATION TRIAL

CRF for subjects on vitamin supplement

Name:

No:

Visit:	1	2	3	4	5	6
Date:						
Weight:						
Height:						
CDAI:						
Q.O.L.						
Fasting:						
Bloods:						
Adverse Events:						
Concomtant Meds:						

	units	% RDA
Vitamin A		
Vitamin C		
Vitamin E		

CDAI/Harvey Bradshaw:

Blood Results:

Haematocrit (%)
Albumin (g/dl)
ESR (mm/hr)
Vitamin C (mg/dl)
Retinol (mg/dl)
B-carotene (mg/dl)
α-Tocopherol (mg/dl)

Visit 1: Baseline Data

Date of Diagnosis:

Extent of Disease:

Smoking habits:

Surgery:

Concomitant meds: