

Epidemiological & Clinical Studies
of Vitamin A
in Black South African
pre-school children

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in Black South African
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by

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SUMMARY

The ocular complications of vitamin A deficiency have been known for many years, however, recent studies have suggested that marginal vitamin A status enlarges the risk of common childhood infections and increases mortality. It is therefore important to assess the vitamin A status, and some of its consequences, in children who are most likely to be at risk for vitamin A deficiency as this has important implications for promoting the health of children and for formulating appropriate primary health care policies. In South Africa very little data is available on vitamin A nutrition of communities; therefore one of the objectives of this research programme was to document the vitamin A status of African children who, because of historical inequities, are most likely to be at risk for deficiency. Sound, epidemiologically based surveys of vitamin A intake and body levels were conducted in a typical established township (using dietary intake as the measuring tool) and in a typical peri-urban informal settlement (using serum retinol and conjunctival impression cytology as the measuring tools). These studies revealed that the majority (97%) of children living in the established township surveyed had an adequate intake of vitamin A, whereas 44% of the children in the informal settlement had low serum retinol levels ($< 20 \mu\text{g}/\text{dL}$), and 18% had insufficient vitamin A, as assessed by 2 abnormal disc specimens, using the conjunctival impression cytology test.

In order to investigate the interrelationships between vitamin A, other micronutrients and some risk factors, an analysis was undertaken of anthropometry, parasite infestation and blood concentrations of vitamin E, calcium, magnesium, phosphorous, albumin, haemaglobin, serum iron and ferritin and percent transferrin saturation. Significant positive correlations were found between serum retinol and all the biochemical indicators of iron metabolism studied except for serum ferritin. Ninety one percent of the children sampled were infested with parasites. These results highlight the fact that in this population close interconnections exist among nutrients and suggest that attempts at correcting vitamin A deficiency in such communities should be based on comprehensive intervention programmes rather than on single nutrient replacement.

The impact of infections on blood levels of vitamin A was investigated in African children with severe measles. In addition, substances related to vitamin A metabolism such as other micronutrients (zinc, vitamin E) and proteins (retinol binding protein, prealbumin, albumin) were measured in serum. In addition the changes induced in these substances by vitamin A supplementation (offered in a randomised, double blind, placebo controlled trial) were studied. Serum retinol as well as the other nutrients measured were significantly reduced early in the exanthem in measles patients as compared to healthy controls. Vitamin A and prealbumin levels on day 8 (of the intervention trial) were significantly increased in

the supplemented group compared to the placebo group. Vitamin A levels in serum correlated with those of retinol binding protein (RBP), prealbumin and zinc. These findings strengthen the hypothesis that hyporetinaemia during measles is the consequence of impaired mobilisation of retinol stores from the liver.

The effect of reversing the temporary lowering of serum retinol concentrations during acute measles infections by supplementation with vitamin A was investigated in a hospital based, randomized, double-blind, placebo controlled trial. The two groups were comparable in known covariates of measles severity : weight/age centiles; overcrowding; rash; total blood lymphocytes; serum levels of zinc, albumin, pre-albumin, RBP, vitamins A and E. 90% of the patients had hyporetinaemia. Integrated Morbidity Scores (IMS) derived from diarrhoea, herpes and respiratory tract infection (radiologically confirmed) were assigned on day 8, at 6 weeks and 6 months - these were reduced by 82%, 61% and 85% respectively in the supplemented group. This was mainly due to reduced respiratory tract infection. There was one death in the placebo group. At 6 weeks there was significant weight gain in the supplemented group. Despite the selected sample, attention to multiple covariates enhances the validity of the data obtained and supports the current WHO recommendations for vitamin A supplementation during measles.

There are several mechanisms by which vitamin A is thought to have its effect of reducing morbidity, one of which is by improving immune responsiveness. This particular mechanism has not been adequately studied in children; most of the studies having been conducted in animals. The effect of vitamin A supplementation on selected factors of immunity in African children with complicated measles was therefore investigated during the randomized double-blind, placebo controlled, intervention trial described above. Placebo and treated groups had similar baseline characteristics. In the treated group there was a significant increase in total number of lymphocytes (day 42, $p = .05$) and measles IgG antibody concentrations (day 8, $p = .02$), both of which have consistently been shown to correlate more closely with outcome in measles than other immunological, clinical and radiological factors. Interleukin-2 (IL-2) and plasma complement (C_3) values were unaffected by vitamin A supplementation. These findings reinforce results from animal studies which show that the pathways of vitamin A activity in decreasing morbidity and mortality are partly founded on selective immunopotentialiation.

In conclusion epidemiological and biochemical methods which were used to assess the vitamin A status of African children in South Africa revealed that overt vitamin A deficiency is not a public health issue to the extent it is in the poor rice eating nations of the world. Marginal vitamin A deficiency is however prevalent in informal settlements. Interventions to reverse this marginal vitamin A deficiency should be incorporated in comprehensive programmes to ensure food security. Infections such as measles which increase utilisation and inhibit mobilisation from body stores are damaging to vitamin A homeostasis in the individual. The morbidity associated with measles can be reversed by high dose vitamin A supplementation during the acute phase of the infection. Improving immune responsiveness is one of the likely paths of vitamin A activity in decreasing morbidity from measles.

In this research programme the statistical planning and analyses, and recommendations arising from these analyses, have been done with the support of the Institute for Biostatistics of the Medical Research Council.

PREFACE

The majority of the work reported in this thesis was performed in the Department of Paediatrics and Child Health, University of Natal, Durban, under the supervision of Professor H.M. Coovadia. The High Pressure Liquid Chromatography work was performed in the Analytical Unit, Department of Physiology, University of Natal, Durban, under the supervision of Mr Roger Salisbury.

These studies represent original work by the author and have not been submitted in any other form to another University. Where use was made of the work of others it has been duly acknowledged in the text.

The studies reported in this thesis have been published in scientific journals. Research workers who were closely associated in these studies are co-authors in these publications.

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8. Coutsooudis A, Jinabhai CC, Coovadia HM, Mametja LD. Determining appropriate interventions for nutritional problems of South African children living in urbanised informal settlements. In press: S Afr Med J, 1993.

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LIST OF CONTENTS

	Page
SUMMARY	i
DECLARATION	vi
PREFACE	vii
PUBLICATIONS	viii
ACKNOWLEDGEMENTS	x
LIST OF CONTENTS	xii
LIST OF TABLES	xviii
LIST OF FIGURES	xx
LIST OF APPENDICES	xxii
CHAPTER 1. VITAMIN A BACKGROUND INFORMATION	
1.1 DISCOVERY OF VITAMIN A	1
1.2 CHEMISTRY AND TERMINOLOGY	2
1.3 OVERVIEW OF VITAMIN A METABOLISM	4
1.4 PHYSIOLOGICAL ROLE OF VITAMIN A	4
1.5 FACTORS AFFECTING VITAMIN A STATUS	5
1.5.1 Nutrient interactions	5
1.5.2 Environmental factors	6
1.5.3 Age	7
1.5.4 Infections	7
1.6 MEASURING VITAMIN A NUTRITIONAL STATUS	8
1.7 THE EPIDEMIOLOGY OF VITAMIN A DEFICIENCY	9
1.7.1 The global magnitude of the problem	9
1.7.2 South African data	11
1.8 REFERENCES	14
CHAPTER 2. LABORATORY METHODS	
2.1 HIGH PRESSURE LIQUID CHROMATOGRAPHY (HPLC) FOR VITAMIN A AND E DETERMINATIONS	17
2.1.1 Principle of method	17
2.1.2 Reagents	20
2.1.3 Apparatus	20

2.1.4	Preparation of standards	20
2.1.4.1	Vitamin A (retinol)	20
2.1.4.2	Vitamin E (tocopherol)	21
2.1.4.3	Tocol	21
2.1.5	Standard curves and calculations	22
2.1.6	Extraction procedure	23
2.1.7	Chromatographic conditions	24
2.1.8	Analytical recovery of analytes	26
2.1.9	Analytical precision	27
2.1.10	Sensitivity	28
2.1.11	Problems encountered in setting up the method	28
2.2	SINGLE RADIAL IMMUNODIFFUSION	31
2.2.1	Principle of method	31
2.2.2	Materials and methods	32
2.2.3	Preparation of plates	32
2.2.4	Calculation of results	33
2.2.5	Reproducibility of results	33
2.2.5.1	Intra-batch reproducibility	33
2.2.5.2	Inter-batch reproducibility	33
2.3	ENZYME-LINKED IMMUNO-SORBENT ASSAY (ELISA) FOR IMMUNOGLOBULIN G (IgG) ANTIBODIES TO MEASLES VIRUS	34
2.3.1	Principle of method	34
2.3.2	Materials and apparatus	34
2.3.3	Preparation of plates	35
2.3.4	Standards for ELISA	37
2.3.5	Calculation of results	37
2.3.6	Conversion of OD readings into international unitage	39
2.4	RADIOIMMUNOASSAY FOR INTERLEUKIN-2 (IL-2)	40
2.4.1	Principle of method	40
2.4.2	Materials and apparatus	40
2.4.3	Assay procedure	41
2.4.4	Calculation of results	44
2.4.5	Reproducibility of results	45
2.4.5.1	Intra-assay reproducibility	45
2.4.5.2	Inter-assay reproducibility	45

2.5	CONJUNCTIVAL IMPRESSION CYTOLOGY (CIC) FOR DETECTING MARGINAL VITAMIN A DEFICIENCY	47
2.5.1	Principle of method	47
2.5.2	Materials and apparatus	47
2.5.3	Sampling method	49
2.5.4	Staining procedure	49
2.5.5	Interpretation of specimens	50
2.5.6	Reproducibility of results	50
2.6	REFERENCES	52
CHAPTER 3. VITAMIN A STATUS OF AFRICAN PRE-SCHOOL CHILDREN IN A TYPICAL ESTABLISHED TOWNSHIP		
3.1	INTRODUCTION	55
3.1.1	Choice of dietary questionnaire	56
3.1.1.1	Seven-day record of actual intake	56
3.1.1.2	24 hour recall	56
3.1.1.3	7 day food frequency recall	57
3.2	METHODS	58
3.2.1	The study population	58
3.2.2	The dietary questionnaire	58
3.2.3	Validating the questionnaire	59
3.2.4	Statistical analysis	60
3.3	RESULTS	61
3.4	DISCUSSION	67
3.5	SUMMARY	69
3.6	REFERENCES	70
CHAPTER 4. VITAMIN A STATUS OF AFRICAN PRE-SCHOOL CHILDREN IN A TYPICAL PERI-URBAN INFORMAL SETTLEMENT		
4.1	INTRODUCTION	74
4.2	SUBJECTS	75
4.3	METHODS	77
4.3.1	Serum retinol determinations	77
4.3.2	Assessment of vitamin A status by CIC	77
4.3.3	Ethical approval	79
4.3.4	Statistical analysis	80

	xv	
4.4	RESULTS	81
4.4.1	CIC exclusions	82
4.4.2	Vitamin A status according to CIC	82
4.4.3	Reproducibility of the CIC test	84
4.4.4	Serum retinol exclusions	84
4.4.5	Serum retinol status	84
4.4.6	Correlations between serum retinol and CIC	86
4.4.7	Summary of vitamin A status	86
4.5	DISCUSSION	88
4.6	SUMMARY	91
4.7	REFERENCES	92
CHAPTER 5. EFFECT OF VITAMIN A SUPPLEMENTATION ON MORBIDITY		
IN AFRICAN CHILDREN WITH MEASLES		
5.1	INTRODUCTION	96
5.2	METHODS	97
5.2.1	Patients	97
5.2.2	Exclusion criteria	98
5.2.3	Hospital management of patients	98
5.2.4	Study design	98
5.2.5	Sample size calculation	99
5.2.6	Determinants of measles severity which were considered	99
5.2.7	Morbidity assessment	100
5.2.8	Biochemical assessment procedures	103
5.2.9	Ethical approval	103
5.2.10	Statistical methods	104
5.3	RESULTS	104
5.3.1	Baseline comparability	104
5.3.2	Hospital, acute morbidity outcome	104
5.3.3	Six week morbidity outcome	109
5.3.4	Six month morbidity outcome	109
5.4	DISCUSSION	114
5.5	SUMMARY	117
5.6	REFERENCES	118

CHAPTER 6. EFFECT OF VITAMIN A SUPPLEMENTATION ON		
IMMUNOLOGICAL PARAMETERS IN MEASLES PATIENTS		
6.1	INTRODUCTION	124
6.2	METHODS	125
6.2.1	Subjects	125
6.2.2	Biochemical and immunological investigations	126
6.2.3	Controls	127
6.2.4	Statistical methods	127
6.3	RESULTS	128
6.3.1	Baseline comparibility	128
6.3.2	Morbidity outcome	128
6.3.3	Immune factors	129
6.4	DISCUSSION	138
6.5	SUMMARY	142
6.6	REFERENCES	143
CHAPTER 7. INTERRELATIONSHIPS BETWEEN VITAMIN A AND OTHER		
MICRONUTRIENTS IN MEASLES PATIENTS		
7.1	INTRODUCTION	145
7.2	METHODS	146
7.2.1	Patients	146
7.2.2	Controls	146
7.2.3	Serological investigations	146
7.2.4	Ethical approval	147
7.2.5	Statistical methods	147
7.3	RESULTS	149
7.3.1	Measles patients compared to controls	149
7.3.2	Comparison between vitamin A and placebo treated measles patients	151
7.3.3	Sequential changes in micronutrients in measles patients	151
7.3.4	Correlations between micronutrients in measles patients and controls	154
7.4	DISCUSSION	154
7.5	SUMMARY	158
7.6	REFERENCES	159

**CHAPTER 8. INTERRELATIONSHIPS BETWEEN VITAMIN A AND OTHER
FACTORS IN CHILDREN LIVING IN AN INFORMAL
SETTLEMENT**

8.1	INTRODUCTION	161
8.2	METHODS	161
8.2.1	Subjects	161
8.2.2	Anthropometry	161
8.2.3	Investigations	162
8.2.4	Statistical methods	163
8.3	RESULTS	164
8.4	DISCUSSION	169
8.5	SUMMARY	172
8.6	REFERENCES	173

LIST OF TABLES

	Page
Table I. Vitamin A conversion table	3
Table II. Countries with vitamin A deficiency	10
Table III. Vitamin A status of South African children: hospital studies	12
Table IV. Vitamin A status of South African children: community studies	13
Table V. Recovery of retinol and tocopherol	26
Table VI. Short and long term precision for HPLC determinations of retinol and tocopherol	27
Table VII. Radioimmunoassay protocol	43
Table VIII. Inter-assay reproducibility	46
Table IX. Daily intake of vitamin A according to age	63
Table X. Relative contribution of some foods to the total intake of vitamin A in 2 sample populations of pre-school children	66
Table XI. Demographic profile of subjects	81
Table XII. Mean serum retinol values according to CIC status	86
Table XIII. Baseline characteristics of children with measles, by treatment group	105
Table XIV. Baseline haematological and serum micronutrient concentrations in children with measles, by treatment group	106
Table XV. Morbidity outcome in children hospitalised with measles, according to treatment group	107
Table XVI. Morbidity outcome, according to treatment group, in children six weeks after being hospitalised for measles	110
Table XVII. Morbidity outcome, according to treatment group, in children six months after being hospitalised for measles	111

Table XVIII. Immune factors studied in healthy controls and in placebo and vitamin A treated groups of children with measles	130
Table XIX. Serum micronutrient levels in healthy controls and in measles patients (on admission)	149
Table XX. Serum levels in children on days 1, 8 and 42 after being hospitalised for measles (according to treatment group)	152
Table XXI. Demographic profile of subjects	164
Table XXII. Definitions of some nutrients in blood and serum of children living in an informal settlement	167
Table XXIII. Coefficient of correlation (r) between biochemical and anthropometric factors	168

LIST OF FIGURES

	Page	
Figure 1.	Structure of all- <u>trans</u> retinol	2
Figure 2.	HPLC chromatogram of a serum extract	25
Figure 3.	Deterioration of chromatogram after a few weeks of using hexane/ethanol mobile phase	30
Figure 4.	Chromatogram after a few weeks of using hexane/isopropanol mobile phase	30
Figure 5.	Example of a computer print out with OD readings	38
Figure 6.	Frequency histogram of daily intake of vitamin A of children in Umlazi	62
Figure 7.	Mean daily intake of vitamin A according to age	64
Figure 8.	Percentage of children at risk for vitamin A deficiency according to age	65
Figure 9.	CIC status of 185 children from Besters Farm	83
Figure 10.	Serum retinol status of 169 children from Besters Farm	85
Figure 11.	Mean serum retinol values according to CIC status	87
Figure 12.	Duration of common measles complication according to treatment group	108
Figure 13.	Integrated morbidity scores according to treatment group, during and up to 6 months after measles	112
Figure 14.	Weight gain according to treatment group, at 6 weeks and 6 months after measles	113
Figure 15.	Mean total lymphocyte numbers in children on days 1 and 42 after being hospitalised for measles, according to treatment group	131
Figure 16.	Changes in total lymphocyte numbers, in children on day 42 after being hospitalised for measles, according to treatment group	132

Figure 17.	Mean measles IgG antibody levels in children on days 1, 8 and 42 after being hospitalised for measles, according to treatment group	133
Figure 18.	Changes in IgG antibody levels, in children on days 8 and 42 after being hospitalised for measles, according to treatment group	134
Figure 19.	Mean C ₃ concentrations, in children on days 1, 8 and 42 after being hospitalised for measles, according to treatment group	136
Figure 20.	Mean IL-2 concentrations, in children on days 1, 8 and 42 after being hospitalised for measles, according to treatment group	137
Figure 21.	Mean serum micronutrient concentrations in healthy controls and in measles patients on hospital admission (day 1 of the study)	150
Figure 22.	Mean serum micronutrient concentration in children 8 days after being hospitalised for measles (according to treatment group)	153
Figure 23.	Percentage of children < 3rd NCHS weight for age; height for age; and weight for height centiles	165

LIST OF APPENDICES

	Page
Appendix i. Map showing location of Umlazi, Malukazi and Besters Farm	72
Appendix ii. Vitamin A intake questionnaire	73
Appendix iii. Photographs taken in Besters Farm	94
Appendix iv. Photograph showing conjunctival sampling in Besters Farm	95
Appendix v. Newspaper cutting documenting political violence which adversely affected the follow-up in this study	122
Appendix vi. Newspaper cutting with photographs documenting political violence which adversely affected the follow-up in this study	123

CHAPTER 1

Vitamin A Background Information

CHAPTER 1

VITAMIN A BACKGROUND INFORMATION

1.1 DISCOVERY OF VITAMIN A

Symptoms which are now known to be the result of vitamin A deficiency have been described and treated for centuries; the Ebers Papyrus written in about 1600 BC, probably referred to night blindness when liver was recommended for the eyes, while the Chinese in 1500 BC were prescribing liver and honey as a cure for night blindness (Barker 1983). In 1913 McCollum and Davis of the University of Wisconsin, and Osborne and Mendel of Yale University independently discovered that rats consuming purified diets, with lard as the only source of fat, failed to grow and developed soreness of the eyes. When butterfat or ether extract of egg yolk was added to the diet, growth resumed and the eye condition was corrected. The term "fat-soluble A" was the term used by McCollum and Simmonds (1917) to describe the organic complex present in the ether extract which was necessary for normal growth. Wolbach and Howe in 1928 showed that lack of fat-soluble A vitamin led to the replacement of various epithelia by stratified keratinised epithelium. The name "anti-infective" vitamin was first given to vitamin A by Green and Mellanby in 1928. In 1931 Dr Paul Karrer and his colleagues reported the chemical structure of retinol (Frickel 1984).

1.2 CHEMISTRY AND TERMINOLOGY

Historically vitamin A is a collective term denoting all compounds with vitamin A activity and include retinol (found naturally only in foods of animal origin) and various provitamin A carotenoids (found in both plant and animal products). Beta-carotene is the most biologically active of the provitamin A carotenoids, and the most widely distributed in plant products; alpha-carotene and gamma-carotene have only about half the biological activity of beta-carotene (Gibson, 1990).

Retinol is a primary alcohol and a polyene, and in the pure state exists as pale yellow crystals which are soluble in fats and most organic solvents. It is known to exist in several isomeric forms but it is only the all-*trans* form which occurs to any extent in nature. The structure is shown in Figure 1.

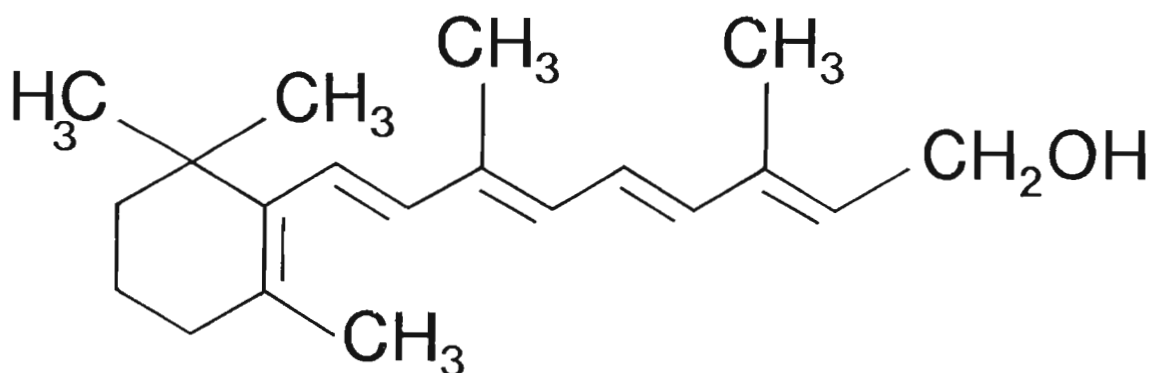


Figure 1. Structure of all-*trans* retinol

The bioavailability of carotenoids in many foods is not as great as that of retinol or of pure carotenoid supplements (NRC, 1989). Therefore, the vitamin A activity in foods is currently expressed as retinol equivalents (RE), which adjusts for assumed biological effectiveness of the different forms of vitamin A (see Table 1).

Table 1. Vitamin A conversion table

Unit	Equivalent Unit
1 RE	1 μg of all-trans retinol 6 μg of all-trans beta-carotene 12 μg of other provitamin A carotenoids 3.33 IU vitamin A activity from retinol 10 IU vitamin A activity from beta-carotene

1.3 OVERVIEW OF VITAMIN A METABOLISM

Retinol is a low molecular weight compound (molecular weight, 286) that can partition into membranes and can, if present in excessive amounts, disrupt normal membrane structure and function (Roels et al. 1969). Thus to be transported through an aqueous environment, and to limit its level in membranes, retinol is normally bound to proteins; extracellularly, retinol is transported in the plasma by retinol binding protein (RBP) and intracellularly, retinol is bound to cellular RBP. Preformed vitamin A present in food as retinyl esters is hydrolyzed by pancreatic and intestinal enzymes to form free retinol. After absorption into the mucosal cell, retinol is reesterified to form retinyl esters and incorporated into the chylomicrons for transport to the liver, the main storage organ for retinol. Carotenes are split in the intestinal mucosa to form retinaldehyde which is then reduced to retinol (Blomhoff et al. 1991). The presence of dietary fat enhances the absorption of retinol and beta-carotene (de Luca et al. 1981).

1.4 PHYSIOLOGICAL ROLE OF VITAMIN A

Vitamin A is essential for vision, growth, reproduction, cellular differentiation and proliferation, normal function of the mucosal epithelium, and the integrity of the immune system (Barker 1983, Flodin 1988, NRC 1989). Accumulating evidence also suggests that carotenoids have important antitumour effects in the body, which may be related to their biological role as antioxidants, as suggested by Goodman in 1984.

Early signs of vitamin A deficiency in humans include growth failure, loss of appetite, and impaired immune response with lowered resistance to infection (Gibson 1990). Aspects of the interaction of vitamin A deficiency and infection are investigated in chapters 6,7, and 8. Night blindness develops when liver reserves of vitamin A are nearly exhausted. Later, ocular lesions include conjunctival xerosis (abnormal dryness

of the conjunctiva), corneal xerosis (abnormal dryness of the cornea), Bitot's spots (triangular, shiny, gray spots on the conjunctiva), and keratomalacia (irreversible corneal lesions associated with partial or total blindness). Collectively, these ocular lesions are referred to as xerophthalmia (WHO, 1976).

1.5 FACTORS AFFECTING VITAMIN A STATUS

1.5.1 Nutrient interactions

The absorption of retinol and carotenoids is dependent on the presence of fat in the diet to stimulate secretion of the required bile acids. Several studies have suggested that fat intake in children and lactating women in developing countries is low (McClaren, 1986; Brown and Solomons, 1991) which could be a factor involved in the low vitamin A status of children and women in some developing countries.

Dietary protein is necessary for the production of enzymes required for the absorption of retinol and carotenoids, the transformation of carotenoids into retinol, and the transportation of retinol in the blood (Olson 1988). The small amounts of protein in the diet in most developing countries seem adequate when enough vitamin A, either as retinol or as beta-carotene, is available (FAO, 1988). However, in protein-energy malnutrition, the activity of enzymes required for vitamin A absorption, the conversion of carotene and other provitamin A carotenoids to retinol in the intestine, and the synthesis and release of plasma RBP are all depressed (Schwarz and Olson, 1981; FAO, 1988). Thus, the addition of protein alone, to the diet of protein-deficient, but vitamin A-sufficient children, can markedly increase plasma retinol levels (Olson 1988).

Retinol is dependent on RBP for its transport in the plasma and RBP in turn is dependent on Zn for its synthesis; the interactions of vitamin A status and zinc are discussed in more detail in chapter 7.

There also appears to be some interaction between vitamin A and E; vitamin E enhances the absorption of vitamin A (McLaren, 1986) and the storage of vitamin A in the liver (Olson, 1988).

Interactions between vitamin A and iron are discussed in chapter 8.

Dietary fibre appears to reduce the bioavailability of beta-carotene. Rao and Rao (1970) compared consumption of vegetables and fruits to beta-carotene supplements in four healthy subjects in a metabolic unit. Chromatographic measurements indicated that nearly all of the purified beta-carotene was absorbed, but less-efficient absorption occurred from the vegetable sources. Results from other similar studies (Brown et al. 1989, Micozzi et al. 1992) also confirm the above findings and suggest that in populations which obtain most of their vitamin A in the form of carotenoids from fruit and vegetables, dietary fibre is likely to affect their vitamin A status adversely if carotenoid intake is low.

1.5.2 Environmental factors

Season affects availability of vitamin A-rich fruits and vegetables, and thus the vitamin A status in those populations which depend on these foods for most of their vitamin A. In poorly-nourished populations, the seasonal variation in dietary intake and serum levels of vitamin A, as well as the occurrence of clinical signs of vitamin A deficiency have been well documented (McClaren, 1986).

1.5.3 Age

Low vitamin A status and xerophthalmia is more prevalent in young pre-school age children than in older children and adults (Barker 1983). The age distribution of poor vitamin A status and xerophthalmia is closely associated with that of weaning. After six months of life the child requires supplementary feeding with foods rich in vitamin A or carotenes. For a variety of reasons, chiefly economic, unavailability, and cultural taboos, these may not be consumed in adequate amounts. For example in Indonesia, xerophthalmic children were found to consume less fruit, dark green leafy vegetables and eggs than non-xerophthalmic children (Sommer, 1982; Tarwotjo et al. 1982). The weaning period is also a time when children, in developing countries especially, are at great risk for infections and therefore (as will be discussed in the next section) are also at risk for poor vitamin A status.

Gestational age also has an affect on the vitamin A status of the infant (Brandt et al. 1978; Hustead et al. 1984, Peeples et al. 1991).

1.5.4 Infections

Both acute and chronic infections accelerate the catabolism and excretion of vitamin A (Flodin, 1988; West et al. 1992). In addition to increasing metabolic demand, respiratory infections, measles, and other febrile illnesses interrupt normal feeding patterns and thereby reduce vitamin A intake (DeMaeyer, 1986). Further details are provided in chapter 7. Furthermore, severe infections may possibly interfere with proper utilization of vitamin A by target tissues, and give rise to a localized tissue deprivation of the vitamin, even when total body stores of vitamin A are adequate (de Luca et al. 1981). In addition if the immune system is depressed, as it appears to be in

vitamin A deficiency, then even children with marginal vitamin A status may have increased susceptibility to infections, which may further impair vitamin A status and this in turn may increase the severity of the infection, creating a vicious cycle (West et al. 1989). Two of the studies discussed in this thesis have shown that vitamin A supplementation can reverse some of the morbidity and immune suppression normally associated with measles (see Chapters 5 and 6).

1.6 MEASURING VITAMIN A NUTRITIONAL STATUS

The vitamin A nutritional status can be assessed using a clinical, dietary or biochemical approach. A combination of these is considered most accurate (IVACG, 1989). Habitual dietary inadequacy is a useful early indicator of possible vitamin A depletion; dietary assessment as a technique for assessing vitamin A status is discussed in detail in chapter 3. Clinical assessment includes documentation of the presence or absence of functional signs of vitamin A deficiency such as night blindness, Bitot's spots, and xerophthalmia. Unlike dietary and clinical assessment, which are subjective, biochemical assessment has the advantage of being objective. Biochemical assessment involves measuring the vitamin A concentration in the blood or liver. Because more than 90 percent of the vitamin A in the body is stored in the form of retinyl esters in the liver, a measure of liver stores of vitamin A is the best index of vitamin A nutriture. However liver biopsies are impractical in population studies, and thus other measures are used. The use of serum retinol levels to assess the vitamin A status of a community is discussed in detail in chapter 4. A new method of assessing vitamin A status known as conjunctival impression cytology has been recently tested in several studies; details of this test are described in chapter 2, section 5. This method was also used to assess the vitamin A status of a community in South Africa as discussed in chapter 4.

1.7 THE EPIDEMIOLOGY OF VITAMIN A DEFICIENCY

1.7.1 The global magnitude of the problem

Vitamin A deficiency is a major public health problem in developing countries (see Table 2) being second only in incidence and prevalence to protein-energy malnutrition. In addition it outnumbers all other causes of blindness in children (Thylefors, 1985). It is estimated that seven percent of all children under the age of five years (43 million) are vitamin A deficient (VITAL, 1991), and countless other children have marginal vitamin A status, a condition associated with decreased resistance to infectious diseases and increased mortality. The World Health Organization estimates that worldwide one child dies needlessly from vitamin A deficiency every minute! (Sommer, 1992; Humphrey et al. 1992). For these reasons, the elimination of vitamin A deficiency by the year 2000 was targeted in the "Declaration on Children" endorsed by political leaders around the world at the World Summit for Children held in New York in September in 1990.

Table II. Countries with vitamin A deficiency*

WHO Region	Countries with an established problem	Countries with a high probability of a problem (more evidence needed)
Africa	Benin Burkina Faso Chad Ethiopia Ghana Malawi Mali Mauritania Niger Nigeria Sudan Tanzania Zambia	Angola Burundi Kenya Mozambique Rwanda Uganda
Americas	Brazil Haiti	Bolivia El Salvador Guatemala Honduras
South-East Asia	Bangladesh India Indonesia Nepal Sri Lanka	Bhutan Myanmar
Eastern Mediterranean		Afghanistan Oman Pakistan Sudan
Western Pacific	Phillipines Vietnam	Kampuchea Lao Peoples Rep.

* Sources: adapted from FAO/WHO, 1988; EPI, 1989

1.7.2 South African Data

The data on the vitamin A status of children in South Africa is insufficient and fragmentary, presumably because the magnitude of the problem of general protein-energy malnutrition (PEM) has diverted nutritionists away from single nutrient deficiencies. The available data from hospital and community based studies documenting the vitamin A status of children in South Africa is summarised in Tables III and IV respectively.

Because of scarcity of data on the vitamin A status of the children in South Africa the vitamin A status of children living in a typical urban formal settlement and a typical peri-urban informal settlement were assessed (see Chapter 3 and 4 respectively).

Table III. Vitamin A status of South African children: hospital studies

Author	Year	Place of study	Age	Findings
Scragg and Rubidge	1960	King Edward VIII Hospital, Durban	< 9 yrs	0.9% of children with PEM had xerophthalmia
Kuming and Politzer	1967	Baragwanath Hospital, Johannesburg	< 8 yrs	9.4% of children with PEM had xerophthalmia
Konno et al.	1968	Cape Town hospitals	< 12 yrs	0.3 - 0.6% of children with PEM had xerophthalmia and low vitamin A levels were found in association with PEM and gastro-enteritis
Argent et al.	1989	Elim (rural), Baragwanath and Johannesburg hospitals	full-term neonates	rural neonates had significantly lower vitamin A levels than neonates from the 2 urban hospitals; 18% of the rural neonates had levels < 10 $\mu\text{g}/\text{dL}$

Table IV. Vitamin A status of South African children: community studies

Author	Year	Place of study	Age	Findings
Du Plessis et al.	1966	Pretoria, school based study	7-11 yrs	low serum vitamin A levels in 0.2% of white; 15.9% of African; 19.4% of Indian; and 10.1% of coloured children
Hill et al	1989	Transkei	< 15 yrs	0.58% of children had xerophthalmia and serum vitamin A levels were reduced in siblings of those with xerophthalmia
Peberdy	1991	Malukazi - informal settlement near Durban	3 - 6 yrs	mean vitamin A intake was considerably lower than the RDA for the vitamin
Steyn et al.	1991	Richtersveld	7 - 14 yrs	median nutrient intakes of vitamin A fell about 50% below the RDA for the vitamin although the range of intakes was very wide.

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CHAPTER 2

Laboratory Methods

CHAPTER 2

LABORATORY METHODS

2.1 HIGH PRESSURE LIQUID CHROMATOGRAPHY (HPLC) FOR VITAMIN A AND E DETERMINATIONS

2.1.1 Principle of method

In 1926 Carr and Price described a method for determining vitamin A in serum which was based on the formation of a blue colour following the reaction of antimony trichloride in chloroform, with vitamin A. This method was used widely in clinical laboratories although it had 4 main drawbacks viz: large quantities of serum were required (± 3 mL); the antimony trichloride reagent develops turbidity in the presence of small amounts of moisture; the blue colour upon which the measurement of the vitamin A concentration is based, lasts only for a short time and critical timing of the corresponding absorbance reading is therefore essential for accuracy; fourthly the reagents are corrosive and poisonous and could pose a danger to laboratory personnel. On the micro-scale the turbidity problem becomes unmanageable which led Bessey et al. (1946) to devise a microspectrophotometric method that has been widely employed. More recently Neeld and Pearson (1963) and Bayfield (1971) described colorimetric methods based on the Carr-Price method but using different reagents viz. trifluoroacetic acid and trichloroacetic acid respectively. These reagents were an improvement on the antimony trichloride reagent as they did not exhibit the same turbidity. All these above methods however which not only require large sample volumes also exhibit interferences from endogenous compounds (e.g. beta-carotene and phytofluene) thus reducing their sensitivity. In 1976 De Ruyter and De Leenheer published the first specific assay for determining vitamin A in serum using

HPLC - a method which eliminated the problems experienced with the earlier methods.

The HPLC method makes use of a pump to deliver a mobile phase solvent at a uniform rate, at pressures that are typically 500 to 5000 psi. Samples can therefore be separated more quickly than by gravity liquid chromatography. The sample to be separated passes over a solid stationary phase e.g. silica gel. The mobile phase and solute (components in the sample) are in competition for active adsorption sites on the stationary phase particles. Thus, more strongly adsorbed components are retained longer than weakly adsorbed components. Because more polar compounds adsorb on a polar surface to a greater degree than do less polar compounds, retention in the column is related to sample polarity. If the stationary phase is polar (eg silanol groups of silica gel) and the mobile phase is non-polar (eg hexane) then polar components are retained more strongly and elute last. This is known as normal phase HPLC. Where the opposite occurs i.e. the mobile phase is polar resulting in polar components favouring the mobile phase and eluting faster this is known as reverse phase HPLC. Once the sample components have been separated they pass through a detector; usually ultraviolet (UV) absorption or fluorescence detection. The amount of absorbance or fluorescence relative to known standards is then used to quantify the unknowns.

The main analyte of interest in the studies following is vitamin A. However since it has been suggested that vitamin E has a sparing action on the expenditure of vitamin A (Horwitt 1976) it was decided that setting up an analysis for the simultaneous determination of vitamin E and A could prove to give additional useful information.

The majority of HPLC methods for simultaneous measurement of vitamin A and E use reverse phase HPLC, for example, De Leenheer et al. 1979, Driskell et al. 1982 and Catignani and Bieri 1983. However the method used in the studies following in this thesis, was normal phase HPLC.

The choice of normal phase was based on two criteria:-

1. To obtain the most sensitive assay - in normal phase (non-polar) eluent, both tocopherol and retinol show an increase in fluorescence intensity over that in a reversed phase eluent.
2. To maintain compatibility between the extraction procedure and the HPLC solvent. The reverse phase method requires evaporation of the extracting solvent and redissolving of the residue in the HPLC solvent to be used. This step may cause loss of vitamin A and it makes large-scale analysis more time consuming.

The normal phase method used was similar to that described by Rhys Williams, 1985 with the major difference being that the method described below employed an internal standard. The reason for choosing to use an internal standard was based on the fact that an internal standard compensates for any losses caused by spillage and for pipetting variability. The particular standard used was dl-tocol which was chosen on the basis of its structural analogy to d- α -tocopherol, and because of its retention characteristics (it elutes between the 2 vitamins being assayed). Tocol however is not readily commercially available. Fluorescence was chosen as the method of choice for detecting the analytes because of the possibility that by using specific excitation and emission wavelengths for the analytes being studied there is less chance of interference for any other analytes as documented for example by Shephard et al. 1985. In addition many of the previous methods of simultaneous determination of the vitamins using UV absorption, only monitored absorption at

a single wavelength. As the absorption (and fluorescence emission) of the vitamins differ considerably, monitoring at one wavelength is a compromise. In our method we used a fluorescence detector which automatically changes the excitation and emission wavelengths with respect to retention time so optimising the selectivity and sensitivity of the analysis.

The predominant form (>95%) of vitamin A in the serum is all-*trans*-retinol (De Leenheer et al. 1979) and d- α -tocopherol represents about 90% of the vitamin E activity in the total pool in the serum (Bieri and Prival 1965). Hence vitamin A was determined as serum all-*trans*-retinol (retinol) and vitamin E as d- α -tocopherol (tocopherol).

2.1.2 Reagents

All-*trans*-retinol and d- α -tocopherol, the standards, were obtained from Sigma, St. Louis, MO, USA.

Dl-Tocol (Tocol), the internal standard, was donated by Mr Kirk Gemell, Roche Products, South Africa.

Ethanol, isopropanol and n-hexane (hexane), all HPLC grade, were obtained from BDH Chemicals, Poole, U.K.

2.1.3 Apparatus

HPLC was performed using a Hewlett-Packard instrument, HP 1090 (Hewlett-Packard, Waldbronn, FRG), which was attached to a programmable fluorescence detector (HP 1046A). The instruments were linked up with a Hewlett Packard integrator (HP 3392A) which was programmed to produce a computer print out with retention times and peak heights. The column used was a 15cm x 1mm normal phase microbore column (Sperisorb S3W, Phase Sep, Queensferry, Wales, UK). An ultralow dead volume precolumn filter was attached upstream of the column.

2.1.4 Preparation of standards

2.1.4.1 Vitamin A (retinol)

A retinol stock solution of 200 $\mu\text{g}/\text{dL}$ of hexane was prepared and aliquots were kept frozen at -70°C . The stock solution was used to prepare working standards. For the standard curve the following concentrations were prepared in a series of doubling dilutions: 100; 50; 25; 12.5; 6.25 and 3.1 $\mu\text{g}/\text{dL}$.

2.1.4.2 Vitamin E (tocopherol)

A tocopherol stock solution of 25 mg/dL was prepared in hexane and aliquots were stored at -70°C until they were needed for making up the working standards. The working stock was 2.0 mg/dL . For the standard curve the following dilutions were prepared: 1.0 mg/dL and 0.5 mg/dL .

2.1.4.3 Tocol

A tocol stock solution of 10 mg/dl was prepared in ethanol and aliquots were stored at -70°C . The stock solution was diluted 1 in 20 to give a working standard of 0.5 mg/dL which was freshly prepared each day.

The concentration of the standard solutions was verified by using the Beer Lambert law (Wootton and Freeman, 1982) viz. $A = KRC$

where: A = absorbance
 K = absorptivity in a 1% solution
 R = light path of solution in cm.
 C = concentration of substance in g/dL

Where the cuvette used gives a light path of 1 cm the concentration of the solution (C) is then determined by the formula $C = A/K$. The absorbance of the solution was measured in a UV/Visible spectrophotometer (LKB Biochrom Ultrospec II, Model 4050). The respective absorptivities

of the standards are: retinol in hexane 1780 at 326 nm; tocopherol in hexane 75.8 at 292nm; tocol in ethanol 100.3 at 292nm (Wheeler and Kaplan 1956-7).

2.1.5 Standard curves and calculations

For both retinol and tocopherol a linear relationship between peak height and concentration was observed over the working range (3.1 - 100 $\mu\text{g}/\text{dL}$) for retinol and tocopherol (0.5 - 2.0 mg/dL). The regression coefficients of the equations were .9999 for retinol and .9997 for tocopherol. For each 5 consecutive days of analysis a retinol standard curve was set up plotting peak height ratios (ratio of retinol peak height to tocol peak height) against concentration. Three concentrations were used for the curve viz. 5; 20 and 40 $\mu\text{g}/\text{dL}$. For each concentration 6 extractions and injections were performed and the mean peak height ratio was plotted. The relationship between the peak height ratio and the concentration was defined by the equation $y = mx$ where the peak height ratio is defined by y , the concentration by x and the slope of the line by m .

Similarly for tocopherol a standard curve was set up plotting peak height ratios (ratio of tocopherol peak height to tocol peak height) against concentration. Two concentrations were used for the regression line viz. .5 and 1 mg/dL and similarly an equation was determined for the regression line and the concentration was determined by dividing the peak height ratio by the slope of the regression line ($x = y/m$).

2.1.6 Extraction procedure

1. The following were added to 10 x 75mm disposable borosilicate glass tubes (Kimble, code GKM 7350002):-
 - 100 μ L serum sample
 - 100 μ L ethanol containing the internal standard
2. The contents were vortex mixed for 1 minute to allow for precipitation of proteins (ethanol deproteinizes the serum).
3. The following were then added:-
 - 100 μ L distilled and millipored water
 - 100 μ L hexane
4. The samples were then vortex-mixed for 1 minute and then centrifuged at 4500 g for 3 minutes.
5. The hexane layer being lighter and immiscible with ethanol formed a layer on the top and retinol, tocol and tocopherol being lipids dissolved in this hexane layer. This hexane layer was then carefully transferred into a 0.1 mL crimp seal vial (code 3-3208, Supelco, Inc.) which was then placed into the HPLC instrument for injection onto the column.
6. Each sample was prepared/cleaned up in duplicate. A coefficient of variation of > 5% between the duplicates resulted in the analysis being repeated.
7. All procedures were conducted away from direct sunlight and no artificial lighting was on in the laboratory because vitamin A is sensitive to photodestruction.
8. The standards were prepared in the same way except that instead of 100 μ L of serum an equal volume of standard solution was used.
9. All solvents and water were filtered through a millipore cartridge to ensure no contaminants would block the column.

2.1.7 Chromatographic conditions

Before starting the day's runs the mobile phase was desparged with helium to purge out any oxygen which would interfere with the chromatography. The fluorescence detector was initially set to monitor for vitamin E and tocol at excitation wavelength of 295 nm and emission wavelength of 330nm and the photomultiplier tube (PMT) gain set at 10. At 4.2 minutes the detector was programmed to change the excitation wavelength to 325nm and the emission wavelength to 480nm with a PMT gain of 16. Serum samples containing mean values of both vitamins at the maximum excitation and emission wavelengths would give tocopherol peaks about 20 times more intense than that of retinol. Rhys Williams (1985) coped with this problem by changing the emission wavelength for monitoring tocopherol to 390 instead of its maximum of 330 nm. Our method chose to rather reduce the intensity of the tocopherol peak by monitoring at a lower PMT gain than that used for monitoring retinol i.e. 10 instead of 16.

The following conditions were used:

Mobile phase = Hexane 99.1%, Isopropanol .9%
Flow rate = .1 ml/min
Oven temperature = 45°C
Injection volume = 10 μ L
Maximum pressure = 200 psi
Chart speed = 0.5 cm/min

An elution profile of a normal serum sample is shown in Figure 2.

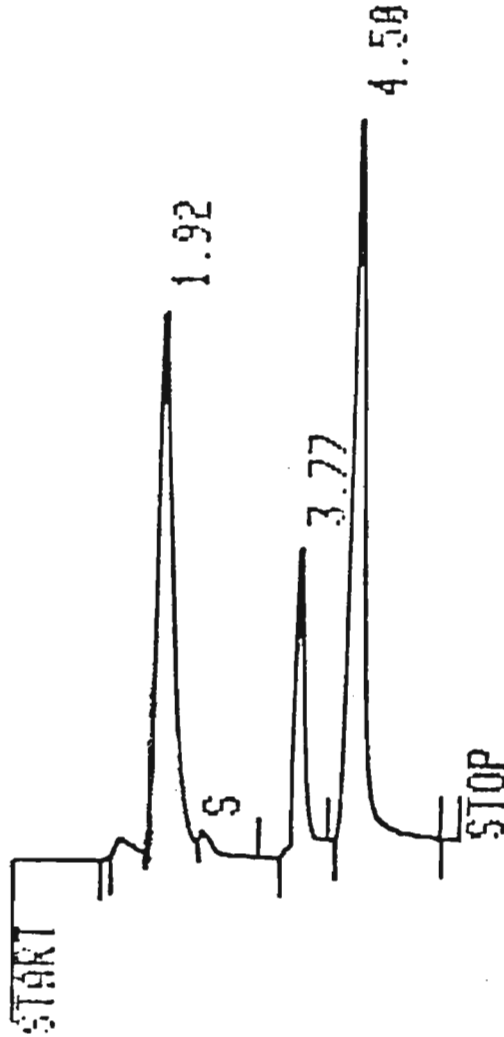


Figure 2. HPLC chromatogram of a serum extract
peak 1, retention time 1.92 min, is tocopherol
peak 2, retention time 3.77 min, is tocol the internal
standard
peak 3, retention time 4.50 min, is retinol

2.1.8 Analytical recovery of analytes

The efficiency of recovery of retinol and tocopherol after standard treatment for their extraction in preparation for chromatography was performed by determining the peak height of a pure standard in hexane without any treatment. This was repeated 6 times and the mean peak height was determined. The standard was then treated by the standard method for extraction before being injected onto the column and the peak height was determined. This was repeated 6 times and the mean peak height was determined. The percentage recovery of retinol ranged from 98.8% to 101% depending on the concentration of the solution, and for tocopherol (1.0mg/dL) the percentage recovery was 100.1% (table I). Therefore the 2 analytes of interest were essentially completely extracted from the aqueous into the organic matrix which is injected onto the column for separation and quantification of the analytes.

Table V. Recovery of retinol and tocopherol

Analyte and concentration	Mean peak height of pure standard	Mean peak height of extracted standard	‡ Recovery
Retinol 5 μ g/dL	303058 μ V	299308 μ V	98.8%
Retinol 25 μ g/dL	1431516 μ V	1428829 μ V	99.8%
Retinol 50 μ g/dL	3138775 μ V	3171069 μ V	101%
Tocopherol 1.0mg/dL	3202174 μ V	3204363 μ V	100.1%

2.1.9 Analytic precision

In Table VI are shown the results of a precision study in which a serum pool was assayed 10 times during one day and ten times on different days over a period of 1 month. The short term (intra-day) coefficient of variation (CV) was 4.4% for retinol and 4.9% for tocopherol. The long term (inter-day) CV was 4.7% for retinol and 4.8% for tocopherol.

Table VI. Short and long term precision for HPLC determinations of retinol (in $\mu\text{g}/\text{dL}$) and tocopherol (in mg/dL)

	Retinol short-term	Tocopherol short-term	Retinol long-term	Tocopherol long-term
1	25.6	.66	27.9	.67
2	26.5	.59	25.0	.56
3	23.8	.61	24.1	.61
4	23.8	.60	24.1	.62
5	23.8	.57	24.7	.58
6	23.8	.58	25.0	.60
7	24.1	.61	26.7	.62
8	25.0	.60	25.3	.60
9	25.3	.56	25.3	.67
10	26.7	.57	27.0	.64
Mean	24.8	.61	25.5	.62
S.D.	1.09	.03	1.21	.03
CV %	4.4	4.9	4.7	4.8

2.1.10 Sensitivity

The lowest quantifiable level using a 100 μL sample and a 10 μL injection volume was 2 $\mu\text{g}/\text{dL}$ for retinol and 0.1 mg/dL for tocopherol using the basis of a signal/noise ratio of 4:1. The sensitivity of the method was therefore such that the serum sample could easily be reduced to 10 μL which would therefore make it suitable for using capillary blood samples which would be advantageous in large scale community surveys.

2.1.11 Problems encountered in setting up the method

In the first attempts made to set up the method the mobile phase suggested by Rhys Williams (1985) was used, viz. 99% hexane and 1% ethanol. Ethanol is very hygroscopic and any water in the mobile phase would deactivate the column therefore extra care was taken to keep the ethanol moisture-free. However even though these precautions were taken it was observed that after a few weeks there were changes in retention times of tocol and retinol so that the analyte peaks were not being completely resolved. It appeared that the column was being deactivated. It was therefore decided to change the mobile phase and after several experiments it was found that the best mobile phase was a mixture of 99.1% hexane and .9% isopropanol. Isopropanol is less volatile than ethanol so it was assumed that there would be less changes in the mobile phase (and therefore in retention times) with the hexane/isopropanol mix than with the hexane/ethanol mix. An example of deterioration in chromatogram resolution after a few weeks of using the hexane/ethanol mixture is shown in Figure 3. In Figure 4 an example is shown of a chromatogram after a few weeks of using the hexane/isopropanol mixture. There was no deterioration in resolution and both the tocol and retinol peaks were completely resolved to base line. Because Rhys Williams was not using an internal standard the changes in retention time and therefore deterioration in resolution

between tocol and retinol would not have caused a problem as there is sufficient time between the elution of tocopherol and retinol for the monitoring wavelengths to be altered and the peaks to therefore be completely resolved.

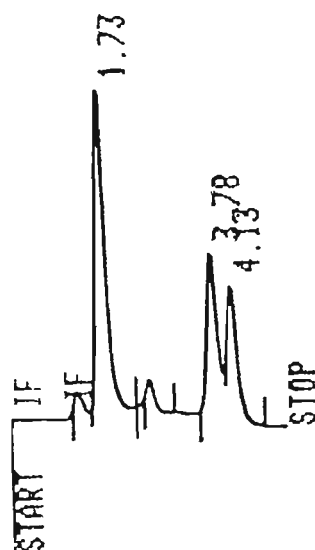


Figure 3. Deterioration of chromatogram after a few weeks of using hexane/ethanol mobile phase, tocol and retinol retention times have changed so that the peaks are no longer completely resolved

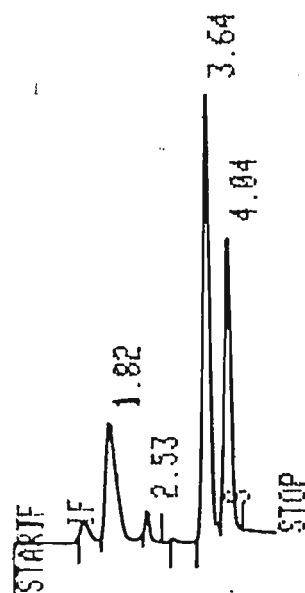


Figure 4. Chromatogram after a few weeks of using hexane/isopropanol mobile phase; tocol and retinol peaks are completely resolved.

2.2 SINGLE RADIAL IMMUNODIFFUSION

2.2.1 Principle of method

In the single radial immunodiffusion technique, a protein antigen in solution (in this case retinol binding protein, RBP) is applied to a cylindrical well, and it then diffuses radially into a thin film of agar gel in which a specific precipitating antiserum is present in uniform concentration (Mancini et al. 1965). The antigen applied is quantified most accurately by determining the amount of specific antibody (in this case anti-RBP) with which it combines at equivalent proportions, indirectly measured by the volume of agar in which the antiserum is evenly distributed. This volume is determined by the volume of the precipitin cylinder when diffusion ceases, i.e. when all the applied antigen is combined with antibody at equivalence.

As the thickness of the gel is kept constant, this corresponds to the area of the precipitin ring and it is seen that this area therefore varies directly with the amount of antigen applied, and inversely with the concentration of antibody in the gel. It follows that when diffusion is complete, plotting the squares of the precipitin ring diameters (D^2) on the y axis against the different concentrations of antigen on the x axis will give a straight line. A standard curve may thus be set up from which the concentration of the unknown may be interpolated from the standard curve using the diameter squared of the unknown's precipitin ring.

2.2.2 Materials and apparatus

1. Commercially prepared radial immunodiffusion plates containing anti-RBP (LC-Partigen RBP, Behringwerke AG, Marburg, FRG; lot number: 057325B).
2. Protein standard plasma (human) supplied by Behringwerke AG, Marburg, FRG; lot number: 048622. The concentration of RBP in the standard plasma was 10.8 mg/dl.
3. Control plasma (human) supplied by Behringwerke AG, Marburg, FRG; lot number: 047016B.
4. Physiological saline for diluting standards.
5. Digital adjustable micropipette with glass capillaries (SMI Liquid Handling Products, Miami, USA).

2.2.3 Preparation of plates

1. For each batch of 6 plates the stock standard solution (10.8 mg/dl) was diluted with saline to give the following 3 dilutions; 1.35 mg/dl, 2.7 mg/dl and 7.2 mg/dl.
2. The plates were opened and allowed to stand for 5 minutes at room temperature before loading.
3. 20 μ l of each of the 3 standard dilutions was applied to plate 1 and 2 of each batch.
4. 20 μ l of control plasma was added to one well of each plate.
5. 20 μ l of each of the unknown samples was applied to the remaining wells. All samples were measured in duplicate on different plates to allow for intra-batch variability. Differences of > 10% between duplicates resulted in the analysis being repeated.
6. In order to allow for intra- and inter-batch variability, approximately equal numbers of samples from the 2 treatment groups and the control group were included in each batch of RBP assays for the

studies described in Chapters 5, 6 and 7.

7. After loading the plates the lids were firmly closed and left to stand at room temperature and allowed to diffuse for 72 hours.
8. At the end of the 72 hours, the diameters (D) of each precipitin ring were measured with a special measuring device (Behringwerke AG, Marburg, FRG). Measurement was made from the underside of the plate. The squares of the diameters (D^2) were then calculated.

2.2.4 Calculation of results

1. A standard calibration curve was prepared from the 3 standards; plotting D^2 as a function of RBP concentration on linear graph paper.
2. The mean diameter of the duplicate samples was determined and then the diameter was squared to give a D^2 value for each sample. The concentration of unknown samples was then calculated by taking intercepts from their D^2 values on the standard curve.

2.2.5 Reproducibility of results

2.2.5.1 Intra-batch reproducibility

The control sample was measured on six plates of one batch and had a mean \pm SD of 3.65 ± 0.32 with a coefficient of variation of 8.8%.

2.2.5.2 Inter-batch reproducibility

The control sample was measured on plate 1 of each of 6 batches and had a mean \pm SD of 3.61 ± 0.33 with a coefficient of variation of 9.1%.

2.3 ENZYME-LINKED IMMUNO-SORBENT ASSAY (ELISA) FOR IMMUNOGLOBULIN G (IgG) ANTIBODIES TO MEASLES VIRUS

2.3.1 Principle of method

In this method measles virus antigen is adsorbed onto the wells of a polystyrene microtitre plate. Patient's serum containing the measles IgG antibody to be tested is then added and after allowing time for binding of antigen and antibody, unbound antibody is washed away. A ligand is then added. The ligand in this case is a molecule which can detect the antibody and is covalently bound to an enzyme such as alkaline phosphatase. After allowing time for the ligand to bind to the antibody the free ligand is washed away. The bound ligand is visualised by the addition of chromogen - a colourless substrate which is acted on by the enzyme portion of the ligand to produce a coloured end-point. The amount of test antibody is measured by assessing the amount of coloured end-product by optical scanning of the plate.

2.3.2 Materials and Apparatus

1. Polystyrene microtitre plates, Enzygnost Measles, supplied by Behringwerke AG, Marburg, FRG. Plates consist of 6 strips in a special holder. Each strip has 2 x 8 reaction wells, one row in each case being coated with antigen and the other with control antigen. Antigen and control antigen (negative) are obtained from human cell cultures infected and not infected respectively with measles virus, and inactivated before coating. All plates used in the study outlined in Chapter 6 were supplied from the same batch (OSOK 02013, Lot 406446A).

2. Dilution buffer consisting of sterile phosphate buffer (pH 7.0) with the addition of 40 ml of Tween 20 and 0.2 g of sodium azide and bovine protein per litre.
3. Washing solution which consisted of sterile phosphate buffer (pH 7.0) with the addition of 200 ml of Tween 20 per litre.
4. Anti-human IgG Conjugate which is produced by the coupling of alkaline phosphatase to highly avid antibodies from the rabbit.
5. Substrate tablets which consisted of p-nitrophenyl phosphate. The substrate solution was prepared five minutes before use by adding 5 ml of substrate buffer to each substrate tablet (2 tablets were necessary for each plate).
6. Substrate buffer (pH 9.8) which consisted of 100ml of diethanolamine and 102mg of $MgCl_2 \cdot 6H_2O$ per litre of distilled water.
7. Stopping solution (2N sodium hydroxide solution).
8. Variable volume pipettes with disposable polypropylene tips.
9. Moisture chamber - a Tupperware container with moistened absorbent cloths at the base.
10. Incubator.
11. Behring ELISA automatic processor.

2.3.3 Preparation of plates

1. 20 μ l of patient's serum and each of 7 standards was diluted in 0,2 ml of dilution buffer (1:11 dilution) in a test tube.
2. 150 μ l dilution buffer was added to each well of the test plate.
3. 50 μ l of each of the 7 diluted standards was added to a test and control well according to the pre-planned protocol.

4. 50 μ l of each diluted patient serum being tested was added to a test and control well according to the protocol and the plate was then incubated for 60 minutes at 37°C in a moist chamber.
5. After incubation the plate was washed 6 times with washing solution which was automatically dispensed and then suctioned off in the Behring ELISA processor.
6. The plate was then tapped dry.
7. 50 μ L of IgG conjugate (coupled with alkaline phosphatase) was added to each well and the plate was incubated for 60 minutes at 37°C in a moist chamber.
8. After incubation the plate was washed 6 times with washing solution which was automatically dispensed and then suctioned off in the Behring ELISA processor.
9. 100 μ l of substrate solution was added to each well.
10. The plate was incubated for 30 minutes at 37°C in a moisture chamber.
11. The reaction was stopped by adding 50 μ l of stopping solution to each well in the same sequence as the substrate solution - both substrate solution and stopping solution was dispensed by the Behring ELISA processor.
12. The plate was tapped gently to mix the substrate solution and the stopping solution.
13. Optical densities (OD) of the solutions in each well were measured at a wavelength of 405 nm. The OD reading of the control well was subtracted from the OD reading of the test well for each sample.
14. Each plate was set up in duplicate so that standards and patients sera were in duplicate. If the coefficient of variation between the duplicate results were greater than 10% the sera were retested.

2.3.4 Standards for the ELISA

Both local and international standards (2 of which were donated by Dr. Roger Glass, Centers for Disease Control (CDC), Atlanta), of various degree of positivity ranging from negative to strongly positive were repeated ten times in order to obtain a mean OD reading for each standard (original standard readings). In each run of 4 plates these same local and international standards were always included in the same order in the first seven pairs of wells of plate 1 and 2. A correlation coefficient of $\geq 0,987$ between original standard readings and the day to day standard readings had to be obtained in order to accept that the ELISA was working optimally.

2.3.5 Calculation of results

The optical density data from the Behring ELISA processor was automatically captured and analysed in a special computer programme written by Mr P.J. Swanepoel, Department of Virology, University of the Orange Free State. The programme utilised the Lotus 1-2-3 software. The programme was set up to determine the correlation coefficient between the original standards and the day to day standards and then to correct all readings according to the standards. An example of a computer print out is shown in Figure 5.

DATE:	RUN NO:		STANDARDS		Y-RANGE VAL
	PlateNo1		PlateNo2		0.125 NEG.
CONTROL:	OD Val	Corr Val	PAT:OD Val	Corr Val	0.125 NEG
NEG.	0.094		42 0.217	0.290	0.223 HI8
HI8	0.113		43 0.270	0.351	0.370 HI128
HI128	0.352		44 0.183	0.251	0.693 GTS
GTS	0.566		45 1.073	1.277	0.852 HI16
HI16	0.629		46 0.629	0.765	1.093 INTHI
INTHI	0.962		47 1.183	1.404	1.745 POSCT
POSCT	1.465		48 1.391	1.644	
PATIENT: 1	0.035	0.080	49 -.015	0.022	Regression Output
2	0.897	1.074	50 0.940	1.124	Constant 0.040
3	0.798	0.960	51 0.891	1.068	Std Err Y est 0.063
4	0.189	0.258	52 0.235	0.311	R Squared 0.090
5	0.313	0.401	53 0.378	0.476	No.observ's 7.000
6	-.013	0.025	54 0.203	0.274	Deg. freedom 5.000
7	0.178	0.245	55 0.668	0.810	
8	0.488	0.603	56 0.508	0.626	X Coeff 1.154
9	0.494	0.610	57 0.640	0.778	Std Err 0.053
10	1.060	1.262	58 1.215	1.441	
11	0.829	0.996	59 1.266	1.500	
12	0.310	0.397	60 0.283	0.366	
13	0.566	0.693	61 0.760	0.916	
14	1.207	1.432	62 1.335	1.580	
15	0.194	0.263	63 0.276	0.358	
16	0.877	1.051	64 0.971	1.160	
17	0.175	0.242	65 0.187	0.255	
18	0.365	0.461	66 0.421	0.525	
19	1.138	1.352	67 1.173	1.393	
20	0.091	0.145	68 -.003	0.036	
21	0.435	0.542	69 0.531	0.652	
22	0.386	0.485	70 0.474	0.586	
23	0.981	1.171	71 1.152	1.369	
24	1.313	1.554	72 1.221	1.448	
25	0.538	0.660	73 0.556	0.681	
26	1.034	1.233	74 1.022	1.291	
27	0.402	0.503	75 0.359	0.454	
28	0.974	1.163	76 1.020	1.216	
29	0.059	0.108	77 0.076	0.127	
30	0.527	0.648	78 0.691	0.837	
31	1.048	1.249	79 0.996	1.189	
32	0.571	0.698	80 0.558	0.683	
33	1.082	1.288	81 0.855	1.026	
34	0.124	0.183	82 0.002	0.042	
35	-.182	-.170	83 0.138	0.199	
36	0.017	0.059	84 0.045	0.092	
37	0.024	0.067	85 -.018	0.019	
38	0.116	0.174	86 0.144	0.206	
39	0.670	0.813	87 0.670	0.813	
40	1.129	1.342	88 1.132	1.346	
41	0.016	0.058	89 -.034	0.000	

Figure 5. Example of a computer print out with OD readings

2.3.6 Conversion of OD Readings into International Unitage

World Health Organisation (WHO) international measles serum was obtained as a gift from WHO. This was diluted with dilution buffer in a series of doubling dilutions to produce a series of 11 dilutions ranging from 20,000 milli international units per ml (mIU/ml) to 19,5 mIU/ml. Each of these dilutions was set up in an ELISA test in the same way as the patients' sera according to the method described in section 3.3.3 and 3.3.4 and the OD readings were determined. A standard curve of OD against mIU/ml was then plotted. The relationship between the level of measles IgG antibody in mIU/ml and the OD readings was determined using polynomial regression. The relationship between $\sqrt{\text{mIU/ml}}$ and the OD readings was described by the equation:

$$\sqrt{\text{mIU/ml}} = 3.0031 + 12.794(\text{OD}) + 5.791(\text{OD})^2$$

The adjusted coefficient of determination was $R^2 = 0.97$, i.e., 97% of the variation in $\sqrt{\text{mIU/ml}}$ is accounted for by the variation in absorbance. The fit for the above equation was limited to the observed range of absorbance values (0 -1.5). The mIU/ml value for a single specimen was calculated by squaring the result obtained after substitution of the appropriate OD value into the above equation and the results were recorded to the nearest integer.

2.4 RADIOIMMUNOASSAY FOR INTERLEUKIN-2 (IL-2)

2.4.1 Principle of method

The assay is based on the competition between unlabelled IL-2 and a fixed quantity of ^{125}I -labelled IL-2(Met⁰,Ala¹²⁵)(human, recombinant) for a limited number of binding sites on an IL-2 specific antibody. With fixed amounts of antibody and radioactive ligand, the amount of radioactive ligand bound by the antibody will be inversely proportional to concentration of added non-radioactive ligand. The antibody bound IL-2 is then reacted with a second specially prepared antibody reagent, the Amerlex-M antibody reagent, which is a second antibody that is bound to magnetizable polymer particles. Separation of the antibody bound fraction is then effected by magnetic separation and decantation of the supernatant. Measurement of the radioactivity in the pellet enables the amount of labelled IL-2 in the bound fraction to be calculated. The concentration of unlabelled IL-2 in the sample is then determined by interpolation from a standard curve.

2.4.2. Materials and apparatus

1. Assay buffer - 0.025M phosphate buffer (pH 7.5).
2. Radioactive tracer - human, recombinant ^{125}I -labelled IL-2(Met⁰,Ala¹²⁵).
3. Standard recombinant human IL-2(Met⁰,Ala¹²⁵) in assay buffer at a concentration of 6.41 pmol/ml.
4. First antibody - rabbit anti-IL-2 IgG.
5. Amerlex-M second antibody reagent - donkey anti-rabbit serum coated on to magnetizable polymer particles, colour coded blue-green.

Note: the above reagents were all supplied by Amersham

(Aylesbury, Buckinghamshire, United Kingdom; code RPA 531). All reagents used in the study described in chapter 6 were supplied from the same batch (Batch 9B, Lot 85).

6. Variable volume pipettes with disposable polypropylene tips.
7. Vortex mixer.
8. Disposable polypropylene tubes (12 x 75mm)
9. Amerlex-M separator, code N.4001 comprising magnetic base and assay rack.
10. Gamma Scintillation Counter (Berthold, LB 2100).

2.4.3 Assay procedure

1. For each batch of 40 duplicate tests the standard recombinant human IL-2(Met⁰,Ala¹²⁵) was diluted in a series of doubling dilutions to give 8 standard concentrations ranging from 2.5 to 320 fmol/ml.
2. 200 μ l assay buffer was pipetted into the non-specific binding (NSB) tubes.
3. 100 μ l assay buffer was pipetted into the zero standard (Bo) tubes.
4. Starting with the most dilute, 100 μ l of each standard was pipetted into appropriately labelled tubes.
5. 100 μ l of each unknown sample (serum) was pipetted into the appropriately labelled tubes.
6. 100 μ l of antiserum was pipetted into all tubes except NSB and TC tubes.
7. All tubes were thoroughly vortex mixed and then covered with plastic film and incubated for 1 hour at room temperature.
8. 100 μ l of radioactive tracer was pipetted into all tubes. At this stage the TC tubes were stoppered and put aside for counting.

9. All tubes were thoroughly vortex mixed and then covered with plastic film and incubated for 3 hours at room temperature.
10. 250 μ l of Amerlex-M second antibody reagent was added to all the tubes except the TC.
11. The tubes were incubated for 10 minutes at room temperature.
12. The antibody bound fraction was separated using magnetic separation; the rack of tubes was placed in contact with the Amerlex-M separator base making sure that all the tubes were in contact with the base.
13. After 15 minutes the supernatant was discarded and the tubes were inverted onto a pad of absorbent tissue and allowed to drain for 5 minutes.
14. The rims of the tubes were then blotted to remove any adhering liquid and the radioactivity present in each tube was counted for 60 seconds in a gamma scintillation counter.

The above methodology is summarized in the radio-immunoassay protocol in Table VII.

Table VII. Radioimmunoassay protocol (all volumes are in microlitres)

	Total Counts (TC)	Non-specific binding (NSB)	Zero standard (Bo)	Standards	Samples
Buffer	-	200	100	-	-
Standard	-	-	-	100	-
Sample	-	-	-	-	100
Antiserum	-	-	100	100	100
Vortex mix, cover tubes and incubate at room temperature for 1 hour					
Tracer	100	100	100	100	100
Vortex mix, cover tubes and incubate at room temperature for 3 hours					
Amerlex-M second antibody	-	250	250	250	250
Vortex mix. Incubate for 10 minutes at room temperature. Separate using Amerlex-M separator for 15 minutes. Decant supernatants, drain for 5 minutes and count.					

2.4.4 Calculation of results

1. The average counts per minute (cpm) was calculated for each set of replicate tubes
2. The percent NSB/TC was calculated using the following equation:

$$\% \text{ NSB/TC} = \frac{\text{NSB cpm}}{\text{TC cpm}} \times 100$$

This was determined to check the counter background, if this was too high then it was subtracted from all counts.

3. The percent Bo/TC was calculated using the following equation:

$$\% \text{ Bo/TC} = \frac{(\text{Bo cpm} - \text{NSB cpm})}{\text{TC cpm}} \times 100$$

4. The percent bound/Bo for each standard and sample was calculated using the following equation:

$$\% \text{ B/Bo} = \frac{(\text{Standard or sample cpm} - \text{NSB cpm})}{(\text{Bo cpm} - \text{NSB cpm})} \times 100$$

5. Using computer software a linear regression model was fitted to the 8 standards which had been repeated seven times each in order to obtain a formula for which %B/Bo values could be converted to fmol values. The following model provided a good fit with a coefficient of correlation equal to 0.9645:

$$\log(\text{fmol}) = 5.5301 - 0.04789(\% \text{ B/Bo})$$

6. Using the above formula the %B/Bo values were converted into fmol's. The particular standard used in our analyses had been calibrated by Amersham against the IL-2 WHO First International Standard 86/504 and I International Unit (IU) was found to be equivalent to 5.3fmol IL-2 (Met₀, Ala₁₂₅). Therefore all results in fmol's were converted to international units according to the formula IU = fmol/5.3

2.4.5 Reproducibility of results

2.4.5.1 Intra-assay reproducibility

The intra-assay reproducibility was calculated by measuring one unknown sample ten times in the same assay. The result obtained was 48.6 ± 2.91 (mean \pm SD) with a coefficient of variation (CV) of 6%.

2.4.5.2 Inter-assay reproducibility

The inter-assay reproducibility was assessed by 7 repeat measurements of 6 samples in 7 successive assays. The coefficient of variation for the 6 samples ranged from 3.8% to 11.2%, with the greatest variation being found in the samples with the lower concentrations. The results are shown in Table VIII. In order to allow for the possible inter-assay variation approximately equal numbers of samples from the two treatment groups and the control group were included in each assay of IL-2 for the study described in Chapter 6.

Table VIII. Inter-assay reproducibility

Sample	Mean fmol/ml	± SD	CV
1	93.45	3.57	3.8%
2	89.52	4.99	5.6%
3	73.60	5.74	7.8%
4	52.69	3.72	7.1%
5	29.16	2.63	9.0%
6	15.98	1.79	11.2%

2.5 CONJUNCTIVAL IMPRESSION CYTOLOGY (CIC) FOR DETECTING MARGINAL VITAMIN A DEFICIENCY

2.5.1 Principle of method

It is well known that vitamin A is essential for normal cellular differentiation, particularly of mucous-secreting epithelium (Wolbach and Howe, 1925). In early vitamin A deficiency histopathologic changes have been described viz. loss of mucous-secreting goblet cells and metaplasia of conjunctival epithelial cells (Hatchell and Sommer, 1984; Wittpenn et al., 1986; Natadisastra et al., 1987,1988; Amedee-Manesme et al., 1988). Conjunctival impression cytology (CIC) is a method of obtaining surface cells from the bulbar conjunctiva and staining them in order to observe histologic changes. The principle of the staining procedure used is based on the fact that Periodic Acid-Schiff Reagent (PAS) stains mucin spots and goblet cells a bright pink. Sodium metabisulfite fixes the PAS stain. Harris haematoxylin counterstains the epithelial nuclei bluish-purple. Ethanol baths are used for drying the discs samples. The final stage of placing the discs in xylene renders the paper transparent to enable the conjunctival cells to be examined.

2.5.2 Materials and apparatus

1. Cellulose acetate filter paper, pore size $0.45\mu\text{m}$ (HAWP 304FO, Millipore Corp., Bedford, Mass.).
2. Stainless steel, filter paper disc applicator attached to a Nalgene hand-operated vacuum pump with pressure gauge; obtainable from the International Center for Epidemiologic and Preventive Ophthalmology, Johns Hopkins University, (ICEPO).
3. A 0.5 cm hole punch to cut filter paper into appropriately sized discs which fit exactly on the tip of the applicator probe.

4. Plastic container with lid to hold precut filter paper discs.
5. One small screw topped wide mouthed bottle for each child to be sampled. The bottles were filled with fixative which was constituted as follows:
 - 75 ml 95% ethanol (BDH Chemicals, Poole, UK)
 - 25 ml distilled water
 - 5 ml glacial acetic acid (BDH Chemicals, Poole, UK)
 - 5 ml 37% formaldehyde (BDH Chemicals, Poole, UK)
6. Sterile 70% isopropyl alcohol swabs (Preptic, Johnson & Johnson, South Africa) for sterilizing applicator tips.
7. Tissue staining tray (24-well tissue culture cluster, Costar, Mass.) The tray was modified by drilling 5 holes in the top and bottom of each well to allow the staining materials to drain out of the tray in between staining in different reagents. A perspex handle was fashioned to fit onto the tray to allow for easy handling during the staining procedure.
8. Staining materials:
 - Periodic acid (0.5% solution)
 - Schiff's Reagent (catalog number: 395-2-016)
 - Sodium metabisulfite (0.5% solution)
 - Harris Haematoxylin Solution (catalog number: HHS-1-16)

The above 4 reagents were supplied by Sigma Diagnostics (St. Louis, Missouri).

 - 95% ethanol
 - 100% ethanol
 - xylene

The above 3 reagents were supplied by BDH Chemicals, Poole, UK.
8. Glass staining baths.

2.5.3 Sampling method

1. The child was seated on the mother's lap. The mother wrapped her arms around the child so that the child felt secure.
2. The child's gaze was directed away from the site to be sampled and at the correct moment a sample was taken from the inferior temporal conjunctiva by gently but firmly pressing the applicator onto the conjunctiva for one second.
3. Both eyes were sampled and the disc specimens were placed in fixative until they were stained.

2.5.4 Staining Procedure

The staining protocol used has been described in detail in the ICEPO Training Manual (Wittpenn et al. 1988). Briefly the staining procedure involved transferring the discs into the wells of a tissue culture tray. The discs were treated with 0.5% periodic acid for 2 minutes after which they were stained with Schiff's reagent for 8 minutes. This Periodic Acid - Schiff's (PAS) stain, stains mucin spots and goblet cells a bright pink. At this stage the stain was fixed by immersing the discs in 0.5% sodium metabisulfite solution for 2 minutes. The well-tray was then dipped in Harris haematoxylin 3 times for 1 second each time in order to counterstain the epithelial nuclei, bluish-purple. In between all these stages the well-plate was rinsed with fresh tap water. The next stages involved a series of ethanol baths for drying out the discs and then the discs were placed into bottles of xylene to fix the specimen and also to turn the paper from opaque to transparent to allow the cells to be viewed easily.

2.5.5 Interpretation of specimens

The exact procedure used for interpreting the disc specimens has been outlined in detail in the ICEPO Training Manual (Wittpenn et al. 1988). Briefly the specimens were graded as "normal" if at least 5 goblet cells were present on a sheet of normal epithelium and "abnormal" if there were fewer than five goblet cells among abnormal epithelium or if keratinized cells were present. Because goblet cell density and conjunctival epithelial morphologic features can vary with location (Sommer et al. 1981), a child was considered to be vitamin A sufficient if either of the two disc specimens were graded as normal and was considered to have poor vitamin A status if either of the 2 disc specimens was abnormal. If one disc was abnormal, the status assigned was borderline abnormal.

2.5.6 Reproducibility of results

In order to assess intra-reader reproducibility of interpretations of CIC specimens, a 7% subsample of the discs which were interpreted in the study described in chapter 4 were randomly selected to be re-interpreted by the same reader. In order to minimize recall bias this second interpretation was undertaken 4 months after the original interpretation, using a blind procedure. The kappa statistic (k) was used to assess intra-observer variability in these interpretations. According to Fleiss (1981) this test is defined by the formula:-

$$k = \frac{2(ad - bc)}{p_1q_2 + p_2q_1}$$

where a,b,c,d are proportions of the grand total of a 2-way contingency table and;

$$p_1 = a + c, p_2 = a + b, q_1 = b + d, q_2 = c + d.$$

The 2-way contingency table is constructed in the following way:

	Normal (N)	Abnormal (Ab)	
Normal (N)	a	b	P_1
Abnormal (Ab)	c	d	Q_1
	P_2	Q_2	

For most purposes a kappa value greater than 0.75 may be taken to represent excellent agreement beyond chance, and values below 0.40 may be taken to represent poor agreement beyond chance.

The results of the reinterpretation of the specimens were as follows:

	Normal (N)	Abnormal (Ab)	
Normal (N)	19	0	19
Abnormal (Ab)	2	5	7
	21	5	

The kappa test showed a kappa statistic equal to 0.785 which is above the suggested threshold of 0.75 for excellent agreement between tests.

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CHAPTER 3

Vitamin A status of
African pre-school children
in a typical
established township

CHAPTER 3

VITAMIN A STATUS OF AFRICAN PRE-SCHOOL CHILDREN IN A TYPICAL ESTABLISHED TOWNSHIP

3.1 INTRODUCTION

Vitamin A deficiency has long been identified as a cause of xerophthalmia and blindness. In recent years however it has been suggested that vitamin A deficiency has an impact on mortality and morbidity and that this effect may be reversed by vitamin A supplementation (Milton et al. 1987, Sommer et al. 1983, 1984, 1986, Rahmathullah et al. 1990). Vitamin A deficiency is a common and major nutritional problem among pre-school children in communities of poor socio-economic status. Because of the original apartheid policies of South Africa the African communities generally constitute communities of poor socio-economic status with overcrowded homes and a high burden of infections. It is therefore a possibility that the children in these communities could be at risk for developing vitamin A deficiency. Data on the prevalence of vitamin A deficiency in South Africa are insufficient and fragmentary - vitamin A deficiency is closely linked with severe PEM and some rural populations have been shown to have reduced serum levels of vitamin A (see chapter 1, section 7.2).

At the time of preparing the protocol for this study no published epidemiological study had been conducted amongst pre-school children in South Africa to establish the prevalence of vitamin A deficiency. For this reason the aim of this study was to conduct an epidemiological survey in a typical established Black urban township to determine the vitamin A status of pre-school children and therefore to estimate the risk of vitamin A deficiency. This information is necessary to make policy decisions regarding the role of vitamin A in nutrition intervention projects.

Several methods exist for assessing vitamin A status, all of which have attendant advantages and drawbacks. One such method is assessing the dietary intake of vitamin A as a proxy indicator of vitamin A status. Where economic and personnel restraints exist, a dietary questionnaire could serve as a valuable tool. The International Vitamin A Consultative Group (IVACG) has suggested the use of a dietary questionnaire applied at the home level by auxiliary health personnel as a simplified method for evaluating the risk of vitamin A deficiency (IVACG Report, 1989).

3.1.1 Choice of dietary questionnaire

The 3 usual methods of assessing the intake of vitamin A are the following:

3.1.1.1 Seven-day record of actual intake

This is believed to give a reasonably accurate measurement of actual intake (Block 1982) and is carried out by weighing or measuring of portion sizes of foods eaten over a seven day period. It is impractical for large surveys because of the high degree of co-operation required from the respondents.

3.1.1.2 24 hour recall

Respondents are required to recall food intake over the previous 24 hours. The limitations of this method are that dietary intake is not constant from day to day making a single 24 hour recall, subject to being non-representative of the person's long-term consumption (Rasanen 1967, Emmons and Hayers 1973).

3.1.1.3 7 day food frequency recall

Respondents are asked to recall over a period of seven days the frequency with which certain foods are eaten.

They are also asked to estimate the amount eaten.

Both the 24 hour recall and the 7 day food frequency recall obviously have serious limitations because of the reliance they place on the respondent's memory. Recall may often be incomplete because most people do not devote full attention to the type or especially the amounts of food eaten (Dwyer et al. 1987). The ability therefore of these 2 methods to provide a reliable estimate of dietary intake for the individual is questionable (Rasanen 1967, Emmons and Hayers 1973). However on a population basis, it is accepted that both can provide a reasonable estimate of central tendency and of variance (Madden et al. 1976, Young et al. 1952, Gershovitz et al. 1978).

For this study however it was decided that a 7 day frequency recall for estimating vitamin A intake would be more reliable than a 24 hour recall because vitamin A intake is believed to be one of the most variable of all intakes (Beaton et al. 1979, Anderson et al. 1982). Portacarrero et al. (1991) compared the 24 hour recall method with the 7 day food frequency method to estimate the dietary intake of vitamin A. They found that the correlation between repeated interviews was far better for the food frequency method as compared to the 24 hour recall method (correlation coefficients were 0.49 and -0.07 respectively).

3.2 METHODS

3.2.1 The study population

203 children between the ages of 6 months and 6 years were randomly selected to participate in the study. This number was chosen in order to show up a 5% deficiency with a 3% margin of error. The children were from the Black African community of Umlazi township, Durban, South Africa (see number 1 on the map in Appendix i). The housing in Umlazi is of both formal and informal construction. Only the main streets are paved. Approximately 60% of the houses are served by electricity, and intradomiciliary sanitation and running water are not universal.

Six out of the 26 administrative sections in Umlazi were randomly selected for house visits. In each section every 36th house was visited. If there was no child who was eligible for the study in that particular house then the next closest house from the back door was sampled. If the person caring for the child in question had not been involved in the feeding of the child over the whole of the last week then she was not eligible for interviewing.

3.2.2 The dietary questionnaire

A simple quantitative questionnaire (Appendix ii), for our local situation, was designed to evaluate the dietary intake of vitamin A using the 7 day recall method. The questionnaire was designed to be administered at the home level by a trained nutritionist and the respondents would be the child minders. The nutritionist involved in this study was a resident of Umlazi and spoke Zulu, the language of the community, fluently and was therefore accepted by the people she was interviewing. The foods listed in the questionnaire were all the vitamin A containing foods eaten by the community which supplied

more than 30 retinol equivalents (RE) per 100g. The nutritionist used three dimensional food models, samples of real food, food packages and utensils to assist the respondents in assessing food quantities more accurately. The food intake recorded was converted to grams using the National Research Institute for Nutritional Diseases (NRIND) Food Quantities Manual (Gouws and Langenhoven, 1986). The vitamin A content of the foods was determined by using the NRIND Food Composition Manual (1986). The quantity eaten over 7 days was divided by 7 to provide an estimate of average daily intake. The questionnaire was administered during December 1990 and January 1991 which included the summer months and therefore it was a time of maximum vitamin A intake from fruit sources.

3.2.3 Validating the Questionnaire

The questionnaire was validated by conducting two pilot surveys. One pilot survey tested the questionnaire on 33 randomly chosen children from the study population. The other survey tested the questionnaire on 33 randomly chosen White pre-school children (age range 1-5 years) from a high socio-economic background. The mothers of these children had all had tertiary education and it was therefore decided to allow the mothers to administer the questionnaire themselves after they were given the necessary information to enable them to complete the questionnaire.

The object of these pilot surveys was to validate the questionnaire by determining whether the questionnaire would be able to differentiate between the amount and type of vitamin A foods eaten by two groups from different cultural and socio-economic backgrounds.

The relative validity of the questionnaire proved to be satisfactory when the results of these two pilot surveys were considered. The mean intake (\pm SD) of the White children was as expected much higher (1374.4 ± 500.4) than that of the Umlazi children (915.3 ± 572.7). In addition the relative contribution of dairy products to the total intake was high (double that of the Umlazi children) which is in keeping with data from other affluent communities (Table X). The validity of the questionnaire was further supported by the fact that it confirmed a correlation between age and daily intake of vitamin A (Table IX) which is consistent with the findings in other studies.

3.2.4 Statistical analysis

All the data from the questionnaires were recorded on computer using the Epi-info programme (Version 5, USD Inc, 2156-D West Park Court, Stone Mountain, GA 30087) the data were then exported and analysed using the Statistical Analysis System (SAS) - Release 6.03 edition, 1988 (SAS Institute Inc, Cary, NC, USA). Descriptive statistics consisting of means and standard deviations (SD) in the case of continuous variables, and frequencies and percentages in the case of categorical variables were calculated for the total sample as well as for the different age groupings. The Students T test was used to compare the age groups 0 - 3 and 4 - 5 years with relationship to dietary intake.

3.3 RESULTS

The daily vitamin A intake among African pre-school children in Umlazi covered a wide range viz. 147 - 3158 RE. The mean daily intake was 915.3 ± 572.7 RE (mean \pm SD) and the median intake was 743 RE. Frequencies of vitamin A intake are recorded in Figure 6. The mean intake of vitamin A according to age is recorded in Table IX and Figure 7. Dietary intake was influenced by age; children 4-5 years of age had a higher intake compared to children under 4 years (1062.0 ± 711.3 vs 839.8 ± 471.8 RE respectively, $p = .021$).

In the group of children under 4 years of age, 8 children took in less than the recommended dietary allowance (RDA) of 400 RE (Subcommittee on RDAs, 1989) and in the age group 4 to 5 years, 11 children took in less than the RDA of 500 RE (Subcommittee on RDAs, 1989). Therefore altogether 19 (9.4%) children were at risk for vitamin A deficiency. The question on when the child had last eaten liver revealed that 130 (64%) of the children had eaten liver in the week of the questionnaire or in the week preceding the questionnaire. Included in this number were thirteen of the 19 children mentioned who took in less than the RDA. Excluding these 13 children who had recently consumed a very rich source of vitamin A leaves 6 children (3%) who are at risk for vitamin A deficiency. This information is summarised in Figure 8.

The relative contribution that some of the foods made to the overall intake of vitamin A is summarised in Table X.

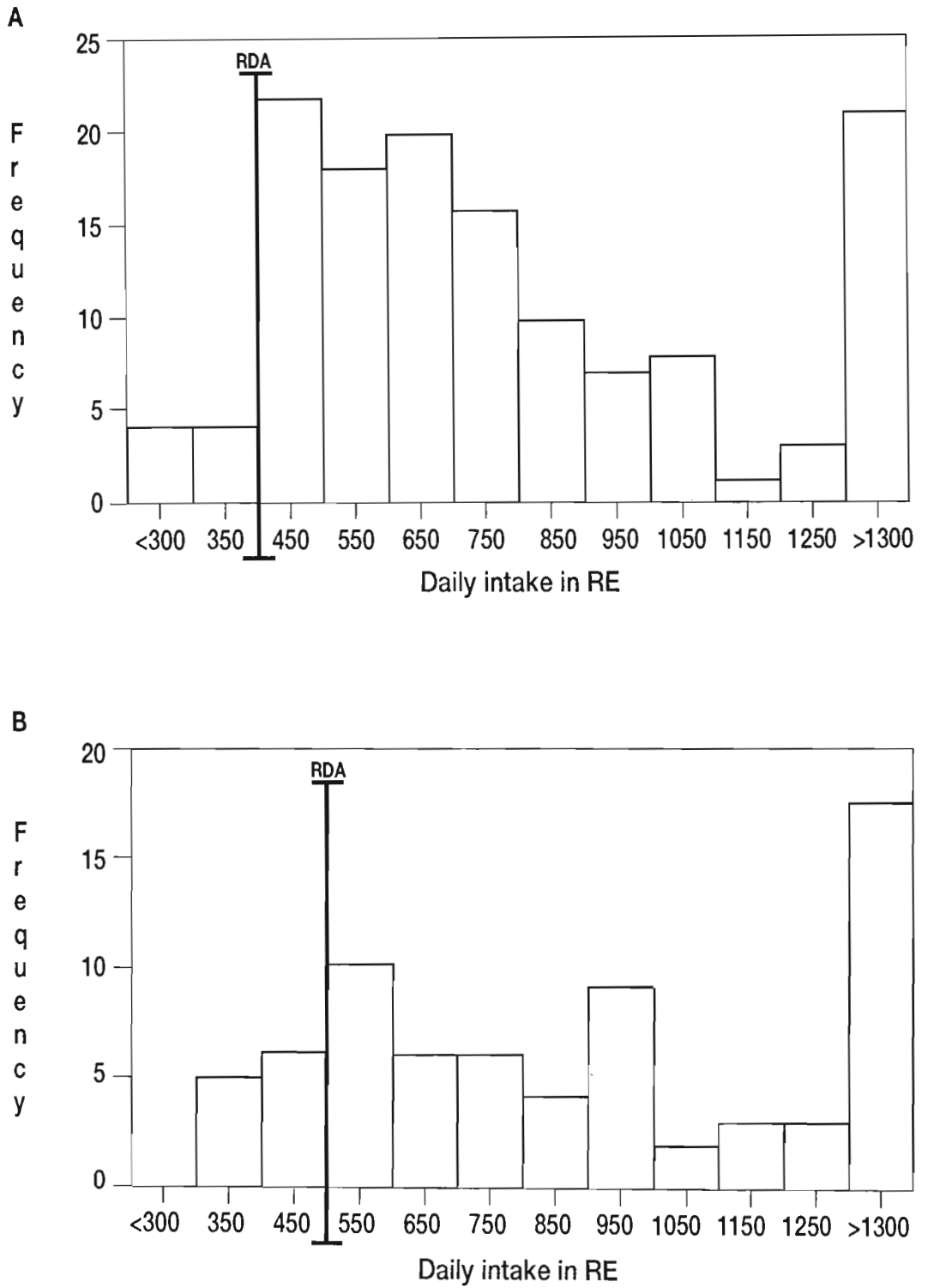


Figure 6. Frequency histogram of daily intake of vitamin A in retinol equivalents (RE) of children in Umlazi
A = 0-3 years (RDA = 400 RE) and B = 4-5 years (RDA = 500 RE)

Table IX: Daily Intake of Vitamin A according to age

Age (years)	n	Daily intake (RE)*
< 1	23	727.3 ± 448.5
1	36	768.1 ± 389.3
2	30	825.3 ± 508.9
3	45	964.4 ± 504.1
0 - 3 (RDA = 400 RE)	134	839.8 ± 471.8 ⁺
4	34	1079.8 ± 754.5
5	35	1044.7 ± 677.2
4 - 5 (RDA = 500 RE)	69	1062.0 ± 711.3 ⁺

* results presented as mean ± SD

+ difference in intake in 0-3 years and 4-5 years statistically significant, p = .021

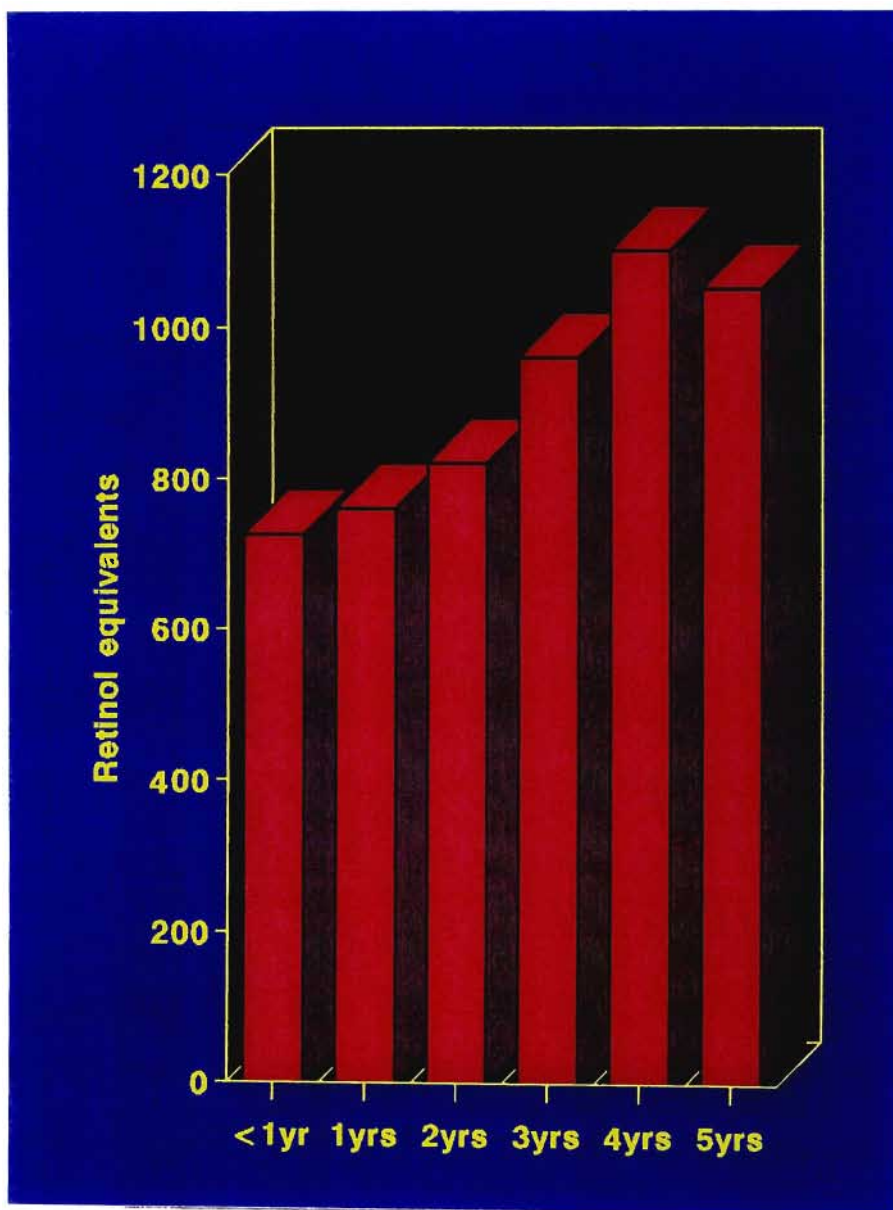


Figure 7. Mean daily intake of vitamin A according to age

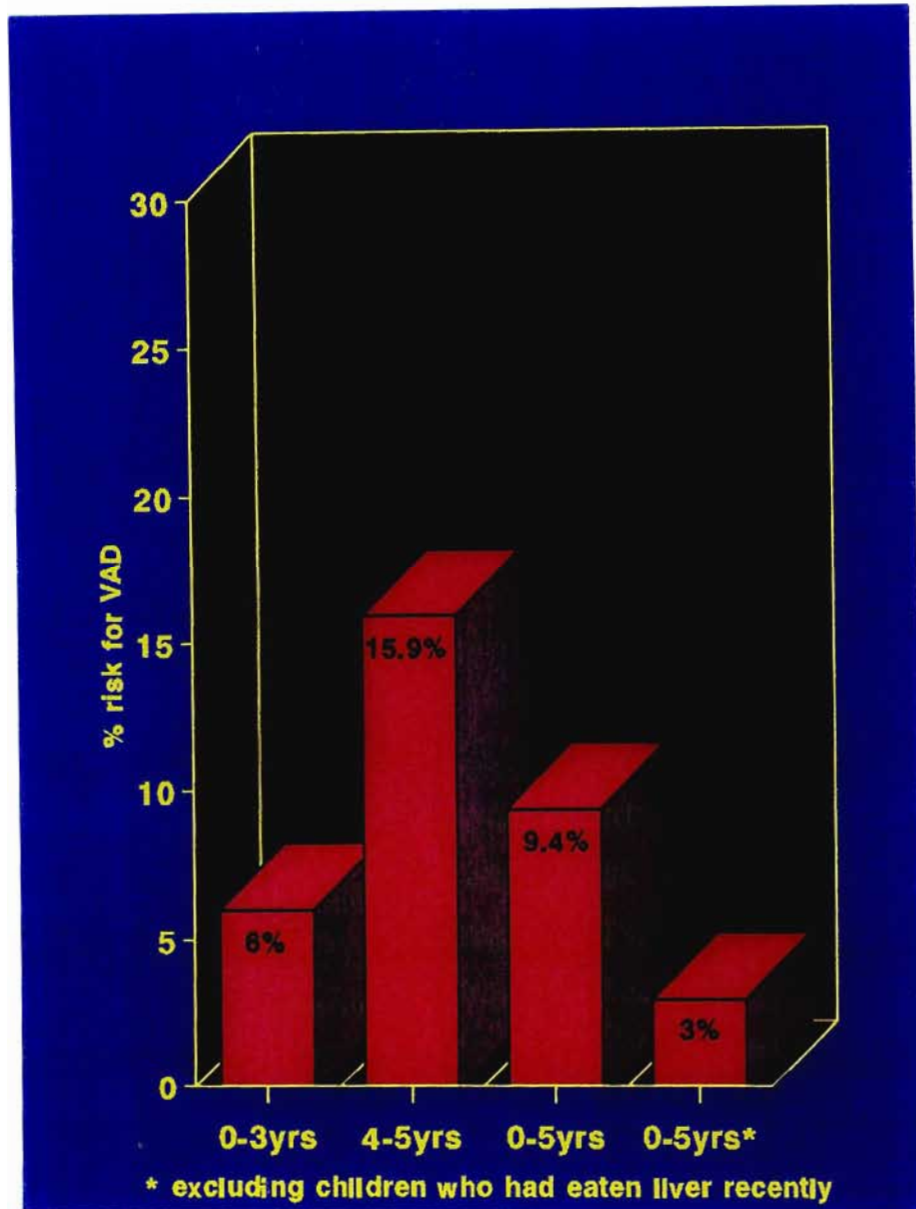


Figure 8. Percentage of children at risk for vitamin A deficiency (VAD) i.e. dietary intake below the RDA, according to age

Table X. Relative contribution of some foods to the total intake of vitamin A in 2 sample populations of pre-school children

	Black children n = 203	White children n = 33
Margarine	10%	5%
Imifino*	7%	0%
Spinach	6%	0%
Sweet potato	8%	0%
Carrots	8%	52%
Dairy products	11%	22%
Liver	26%	2%
Fruit	9%	6%

* locally consumed green leafy plant

3.4 DISCUSSION

Although assessment of dietary intake is subject to inaccuracies from several sources some of these inaccuracies could be minimised if locally-specific food composition tables are used. Such tables were at our disposal in this survey. One of the difficulties of using a 7 day recall type of questionnaire was illustrated by our finding that consumption of liver, a very rich source of pre-formed vitamin A, in the preceding weeks would have been missed if we had not used a few additional questions on usual dietary habits. This problem is particularly relevant when assessing intake of nutrients (such as vitamin A) which are stored in the body.

Other workers have shown that dietary questionnaires are reliable screening tools for identifying populations with poor vitamin A status (De Chavez et al., 1989). A dietary survey to assess vitamin A status was used in Cebu, Phillipines and it was clearly demonstrated that the questionnaire was able to pick up an expected inverse relationship between vitamin A intake and evidence of xerophthalmia (Solon et al., 1978).

The mean daily intake of vitamin A among the children in this study was considerably higher than that reported for other deprived communities (De Chavez et al., 1989, Solon et al., 1978). However when the data was analysed in respect of the number of children obtaining the RDA of vitamin A it was found that in the age group less than 4 years, 8 children took in less than the RDA of 400 RE and in the age group 4 years and older, 11 children took in less than the RDA of 500 RE. Therefore 19 of the 203 children (9%) could be at risk for developing vitamin A deficiency due to insufficient dietary intake.

This percentage is higher than expected because of the very low incidence of clinical signs of vitamin A deficiency detected at all levels of the health services. The situation may in fact not be as severe as it appears from the 7 day recall questionnaire which only measures very recent intake of the vitamin. Of the 19 children who had RDAs lower than the

recommended values, 13 had eaten liver in the week prior to the questionnaire being administered. As vitamin A is stored in the liver these 13 children are probably not at risk for developing vitamin A deficiency after all. A truer figure would thus be that 6 of the 203 children (3%) are in fact at risk for developing vitamin A deficiency.

The staple diet of the African community is maize meal which like rice is low in vitamin A. However the maize meal is traditionally prepared with carotene rich foods such as pumpkin, spinach and "imifino" (a local term referring to wild growing dark green leafy vegetables similar to spinach). Therefore the community is less at risk for developing vitamin A deficiency compared to other deprived communities.

The children often eat chicken liver a rich source of vitamin A. Margarine (which is fortified with vitamins A and D) contributed 10% of the total vitamin A intake. As a fat source it is also valuable for enhancing vitamin A absorption. The fact that 95% of the children consumed at least a teaspoon of margarine a day is probably an indication that nutrition education programmes have been effective. Mothers at the clinics have been advised to add a teaspoon of margarine to their children's maize meal porridge.

The results of this study imply that there is no serious vitamin A deficiency among urban African pre-school children in established townships and therefore a mass vitamin A capsule distribution campaign is not indicated as might be in other deprived communities. The results also confirm that a well designed dietary questionnaire (administered by a trained nutritionist who is well accepted by the community) is a suitable method for assessing the risk of vitamin A deficiency in a community.

3.5 SUMMARY

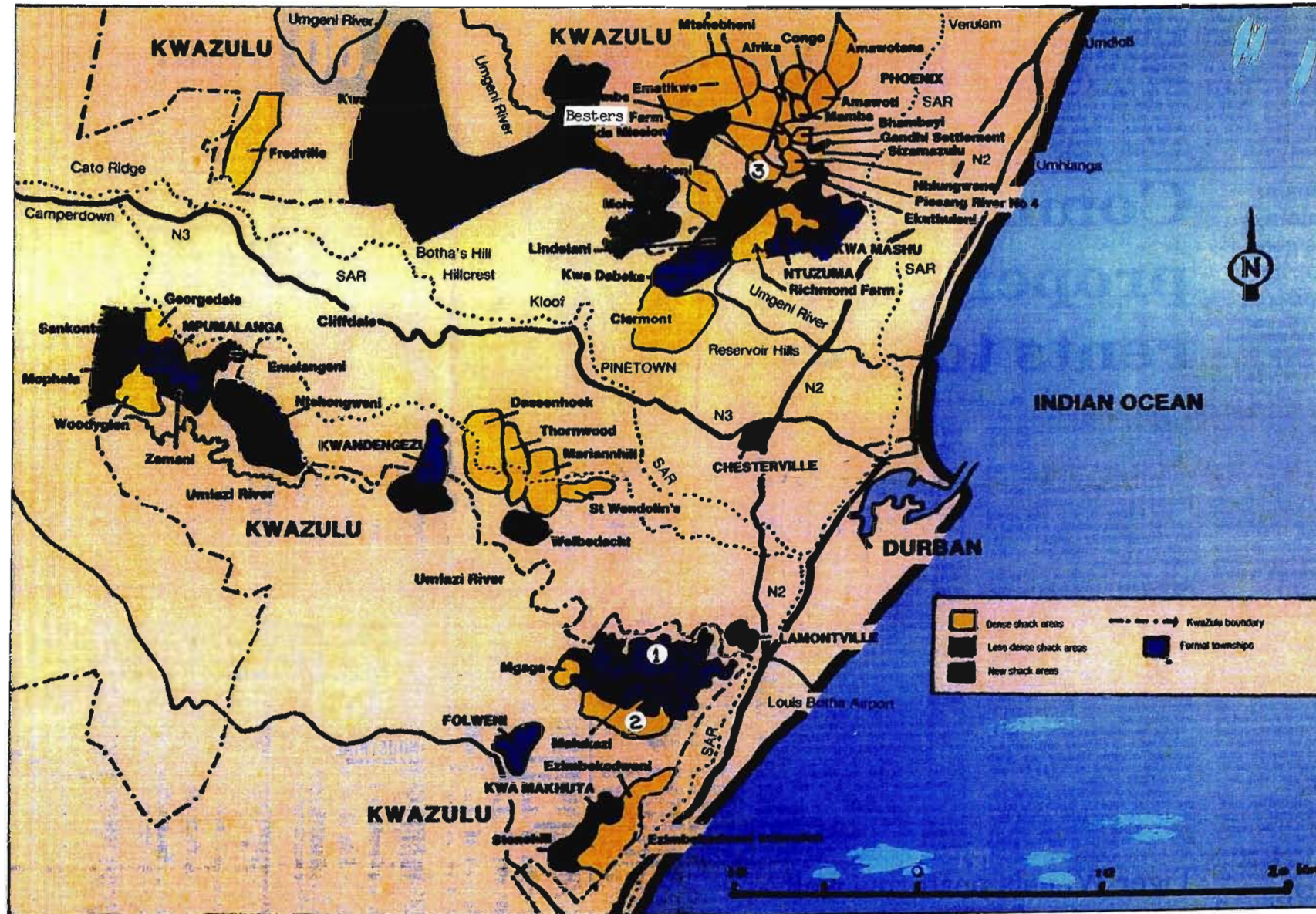
Vitamin A deficiency has been shown to have an impact on morbidity and mortality and therefore prevalence rates of vitamin A deficiency are important for public health policy. This study records the results of a 7-day recall, dietary questionnaire, which was validated and administered to 203 randomly selected urban African pre-school children in Umlazi, South Africa in order to assess their dietary intake of vitamin A. Common vitamin A containing foods eaten were leafy green vegetables, pumpkin, sweet potato, mangoes and chicken liver. Although the mean daily intake of vitamin A (915.3 ± 572.7 RE) was adequate, 19 children had intakes below the recommended dietary allowance (RDA). Thirteen of these children had eaten liver in the previous week, and as such were probably not at risk for developing vitamin A deficiency. It is therefore estimated that only 6 of the children (3%) were actually at risk for developing vitamin A deficiency. It is therefore suggested that African pre-school children living in established urban townships are not at risk for vitamin A deficiency and a mass vitamin A distribution campaign is not indicated as might be in other communities of poor socio-economic status.

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Appendix i. Map showing location of Umlazi (1), Malukazi (2), and Besters Farm (3)



Appendix ii. Vitamin A intake questionnaire

VITAMIN A INTAKE QUESTIONNAIRE				
Child's Name:				
Address:		Sex: M F	Age: yrs mth	
Responder's relationship to child:		1 Mother	2 Aunt	3 Grand
4 Sibling	5 Other Relative	6 Paid Childminder	7 Neighbour	
Who usually looks after child:		1 As above	2 Other	
Which vegetables do you grow:				
Which veges do you put in soups & stew:				
When did child last eat carrots:		liver:		

FOOD	Q/wk	VIT A	FOOD	Q/wk	VIT A
<u>Milk</u>			Imifino		
- breast			Spinach		
- whole cow's			Pumpkin		
- low fat milk			Squash		
- blend			Green beans		
- powdered milk			Peas		
Maas			Tomatoes		
Yogurt			Sweet potato		
<u>Cheese</u>			Carrots		
- gouda			Brocoli		
- cheddar			Apricots		
- cottage			Mangoes		
Butter			Guavas		
Ice cream			Pawpaws		
Chicken liver			Peach		
Lamb/beef liver			Naartjie		
Margarine			Guava		
Holsum			Pure fruit juice of above fruits		

CHAPTER 4

Vitamin A status of
African pre-school children
in a typical
peri-urban informal settlement

CHAPTER 4

VITAMIN A STATUS OF AFRICAN PRE-SCHOOL CHILDREN IN A TYPICAL PERI-URBAN INFORMAL SETTLEMENT

4.1 INTRODUCTION

In the previous chapter mention was made of the association between vitamin A deficiency and ocular complications and mortality (Cohen 1988, International Vitamin A Consultative Group 1981, World Health Organisation 1976). In addition it was noted that studies have suggested a close association between even subclinical vitamin A deficiency and increased childhood morbidity and mortality and that this effect may be reversed by vitamin A supplementation. For this reason the assessment of subclinical deficiency in populations at risk is becoming important.

Results were presented in the previous chapter of a study conducted amongst pre-school children living in a typical urban established township in Umlazi, Durban, which revealed that the prevalence of vitamin A deficiency as assessed by dietary intake is low (Coutsoudis et al. 1992). However, as the communities on the periphery of the major cities like Durban increase, and informal settlements mushroom - the potential exists for a large population with socio-economic conditions far more deprived than those existing in the established townships. These communities would therefore be at risk for developing vitamin A deficiency. A simple dietary survey in Malukazi, a typical urban shack community, on the periphery of Durban (see Appendix i, Chapter 3 for position) showed that the mean intake of vitamin A was below the RDA for the vitamin (Peberdy, 1991). Therefore, to test the hypothesis that pre-school children living in poor informal settlements have subclinical vitamin A deficiency, an epidemiological survey was conducted in a typical urban African shack community to establish the prevalence of vitamin A deficiency in order to

use this information in any future policy regarding vitamin A supplementation and/or other intervention measures.

Although a dietary intake survey is a very useful tool for the rapid assessment of the vitamin A status of a community, serum retinol distribution curves have traditionally been considered to be a more objective method of assessing the vitamin A status of a population (Olson 1984). Conjunctival impression cytology (CIC) has more recently been proposed as another method for detection of vitamin A deficiency. This test is based on the fact that loss of mucus-secreting goblet cells and metaplasia of conjunctival epithelial cells represent early histopathologic alterations due to vitamin A deficiency (Hatchell and Sommer 1984, Wittpenn et al. 1986, Natadisastra et al. 1987, Amedee-Manesme et al. 1988).

In this descriptive cross-sectional study CIC and serum retinol levels were used to assess vitamin A status. CIC as a method for assessing the vitamin A status of communities where overt vitamin A deficiency is not a public health problem has been used in some studies (Gadomski et al. 1989, Carlier et al. 1992) however it has not previously been used in South Africa, a country where overt vitamin A deficiency is also not prevalent. This study therefore provided the opportunity to field test the feasibility of CIC in our particular setting.

4.2 SUBJECTS

During November 1991, preschool children (3-6 years of age) living in Besters Farm, a typical urban shack settlement in the Natal/KwaZulu area of South Africa (see Appendix i, Chapter 3 for position), were assessed for vitamin A status. Being a typical urban shack settlement it manifests the typically poor socio-economic profile of other such settlements; the majority of houses (77%) are constructed with wattle and daub; there is no running water in the homes, with water being drawn from a communal kiosk in the majority of cases; there is no refuse removal service and refuse is usually dumped on the ground; the

mean monthly household income is R513.25 and mean monthly per capita income is R118.70. Photographs from the area are shown in Appendix iii.

Besters Farm has a population of approximately 16,000 with 3200 households in 11 units. A sample of 360 households was randomly selected using the stratified random sampling technique and allowing a 5% sampling error. This represented a sample of 182 children between the ages of 3 - 6 years. To allow for approximately 5% reluctance to be involved in the study, it was decided to recruit 192 subjects in order to be assured of a sample size of 182. If more than 1 child in a particular household was eligible for entry into the study only one child was randomly selected. Children outside the stipulated age range and those who were not normally resident in the target area were not eligible for the study. In addition any child who had a fever or who had been hospitalized for an infectious disease in the month prior to the study was excluded.

Community-based facilitators visited the randomly selected households and explained the importance of assessing children's vitamin A status and the procedure which would be carried out. Thereafter, written informed consent from the parent or guardian of the child was obtained.

All children were brought to the newly constructed clinic in the community on a pre-decided date. The age of the child was recorded in years and months; information being obtained in the first instance from the clinic card and then from the parent or guardian. Children between the age of 3 years, 0 months and 3 years, 11 months (inclusive) were classified as 3 years of age. Children between the age of 4 years, 0 months and 4 years, 11 months (inclusive) were classified as 4 years of age. Similar categories were used to classify children as 5 and 6 years of age.

4.3 METHODS

4.3.1 Serum retinol determinations

Blood samples were taken by ante-cubital venipuncture. The sera were kept frozen at -20°C until they were analyzed for vitamin A concentrations. All samples were analyzed in duplicate within 3 months of collection. In this particular study the HPLC apparatus in our institution was not available until 6 months after collection of specimens. Because this was a project in which the community was intimately involved and it was important to get the results to them as soon as possible the HPLC analysis of vitamin A needed to be done urgently. Therefore instead of doing the vitamin A analysis by the usual method described in chapter 3.1, serum samples were posted on dry ice to Cape Town, South Africa where the vitamin A analysis was performed in the Department of Human Nutrition, University of Stellenbosch, using a modified version of the method of Catignani et al. (1983) which allows for the simultaneous determination of vitamins A and E. Tocopherol acetate was used as an internal standard. Control sera were analyzed with each batch of samples and the interbatch coefficient of variation was $< 6\%$. Analytical recovery of added retinol was 97%.

4.3.2 Assessment of vitamin A status by CIC

Conjunctival samples were obtained by means of a special applicator which allows a disc of cellulose acetate filter paper (HAWP 304FO, Millipore Corp, Bedford, Mass) to be applied to the conjunctiva for one second before being removed by means of a special suction pump attached to the applicator probe (Keenum et al. 1990). This special applicator is relatively new and has not been extensively

used thus this study also afforded us the opportunity to test this new method in our population. After reassuring the child, the child was held on the mother's lap with her arms wrapped around the child's arms (see Appendix iv). The child was asked to look in the opposite direction to expose the conjunctiva. Samples were taken from the inferior temporal conjunctiva. Both eyes were sampled to reduce variability and samples were placed in fixative until they were stained with periodic acid-Schiff and hematoxylin (see section 3.5 of Chapter 2 on laboratory methods). The specimens were interpreted according to the guidelines suggested by the International Center for Epidemiologic and Preventitive Ophthalmology (ICEPO) viz. specimens were graded as "normal" if at least five goblet cells were present on a sheet of normal epithelium and "abnormal" if there were fewer than five goblet cells among abnormal epithelium or if keratinized cells were present (Wittpenn et al. 1988). CIC status was defined as normal if both disc specimens were "normal"; abnormal if both disc specimens were "abnormal" and borderline abnormal if one of the disc specimens was "abnormal". However because goblet cell density and conjunctival epithelial morphology can vary with location (Sommer et al. 1981) a child was considered to have poor vitamin A status only if both disc specimens were abnormal and borderline abnormal CIC status was classified as adequate vitamin A status. Interpretation of all conjunctival specimens was done by the author who had no information available as to the child's serum retinol status until after completion of the assessment. The author had been trained in the interpretation of CIC specimens by the CIC group at ICEPO. Any specimens which were difficult to interpret were posted to ICEPO for confirmatory interpretation.

In order to assess intra-reader reproducibility of interpretations of CIC specimens, a 7% subsample of discs (26 discs) were randomly selected to be re-interpreted by the same reader. In order to minimize recall bias this second interpretation was performed 4 months after the original interpretation using a blind procedure.

4.3.3 Ethical approval

The protocol and procedures for the study were approved by the Ethics Committee, University of Natal, Faculty of Medicine.

4.3.4 Statistical analysis

The differences in mean serum retinol values in the different CIC groups were tested by Analysis of Variance and Duncan's multiple range test for pairwise comparisons was used as a post hoc test. The kappa statistic (k) was used to assess intra-observer variability in the interpretations of CIC specimens. According to Fleiss (1981) this test is defined by the formula:-

$$k = \frac{2(ad - bc)}{p_1q_2 + p_2q_1}$$

where a,b,c,d are proportions of the grand total of a 2-way contingency table and $p_1 = a + b$; $p_2 = a + c$; $q_1 = c + d$; $q_2 = b + d$; as shown below:-

	Normal	Abnormal	
Normal	a	b	p_1
Abnormal	c	d	q_1
	p_2	q_2	

For most purposes a kappa value greater than 0.75 may be taken to represent excellent agreement beyond chance, and values below 0.40 may be taken to represent poor agreement beyond chance.

The software used for the computerized analysis of results was the Statistical Analysis System (SAS), release 6.03 edition, 1988 (SAS Institute Inc, Cary, NC).

4.4 RESULTS

Of the 192 children randomly selected for inclusion in the study, 2 children were excluded from the study (one was mentally retarded and the other was very ill with a high fever). None of the 190 children examined had clinical signs of vitamin A deficiency. Demographic data on the remaining 190 children are presented in Table XI.

Table XI. Demographic profile of subjects (n = 190)

AGE (years)	No.	%
3	47	25
4	61	32
5	40	21
6	42	22
SEX		
Male	91	48
Female	99	52

4.4.1 CIC exclusions

Three of the 190 children refused to co-operate for conjunctival sampling and in 2 children both specimens were unreadable. In the remaining 185 children, 6 had only 1 disc for interpretation; in 3 cases the child refused to cooperate for the second sample and in the other 3 cases the second disc was unreadable. In all these 6 cases the one disc was abnormal and status was defined as "borderline abnormal".

4.4.2 Vitamin A status according to CIC

The overall results of the CIC status of the 185 children from whom specimens were taken revealed (Figure 9) that 123 children (66%) had normal CIC status (i.e. 2 normal specimens); 30 children (16.2%) had borderline abnormal status (i.e. at least one abnormal specimen); and 32 children (17.3%) had abnormal CIC status (i.e. two abnormal specimens). The final decision on whether to classify a child as having poor vitamin A status according to ICEPO criteria required the child to have two abnormal disc specimens therefore the 6 children with only one specimen (which was abnormal) were excluded when calculating the percentage of children with poor vitamin A status according to CIC status, leaving 179 children to be included in this assessment. CIC assessment of vitamin A status revealed that 123 children (68.7%) had normal; 24 children (13.4%) had borderline abnormal; and 32 children (17.9%) had abnormal CIC status. According to ICEPO criteria children with at least one normal specimen have normal vitamin A status; therefore in this study 147 out of 179 children (82%) would be classified as having normal vitamin A status and 32 of 179 children (18%) would be classified as having poor vitamin A status.

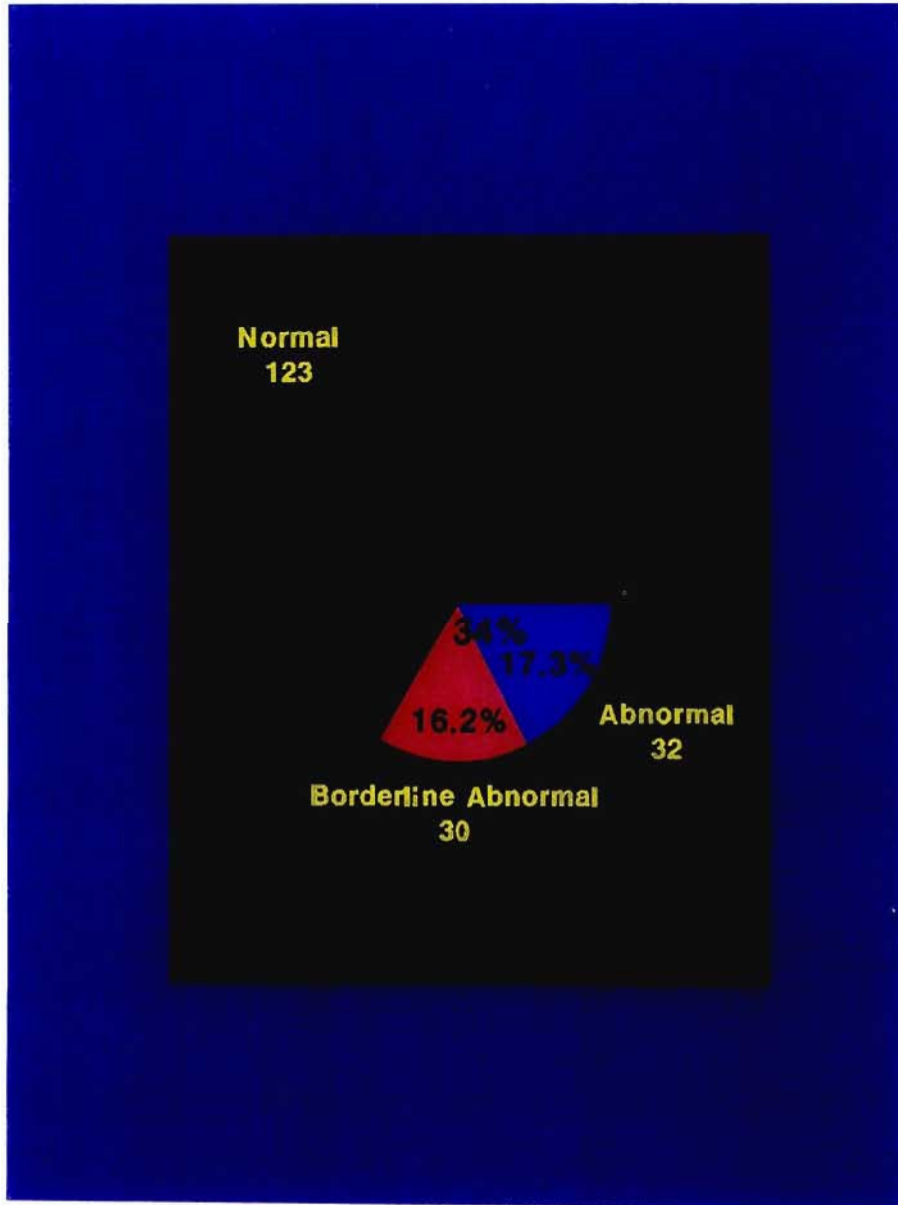


Figure 9. CIC status of 185 children from Besters Farm

4.4.3 Reproducibility of the CIC test

Results of the two sets of interpretations which were made 4 months apart are shown below.

	Normal	Abnormal	
Normal	19	0	19
Abnormal	2	5	7
	21	5	26

The kappa test to test the reproducibility of the test showed a kappa statistic equal to 0.785 which is above the suggested threshold of 0.75 for excellent agreement between tests.

4.4.4 Serum retinol exclusions

Five of the 190 children refused to co-operate for venipuncture and in 8 children a blood sample was not obtained in the first attempt and no repeat attempts were made. In 8 cases we had insufficient serum for serum retinol analysis. Sufficient serum was available for 169 analyses of serum retinol.

4.4.5 Serum retinol status

The mean serum retinol for the whole population was $20.8 \pm 7.4 \mu\text{g/dL}$ (mean \pm SD). Nine children (5%) had vitamin A deficiency as defined by serum retinol levels $< 10 \mu\text{g/dL}$ and 75 children (44%) had low levels ($< 20 \mu\text{g/dL}$); results are depicted in Figure 10.

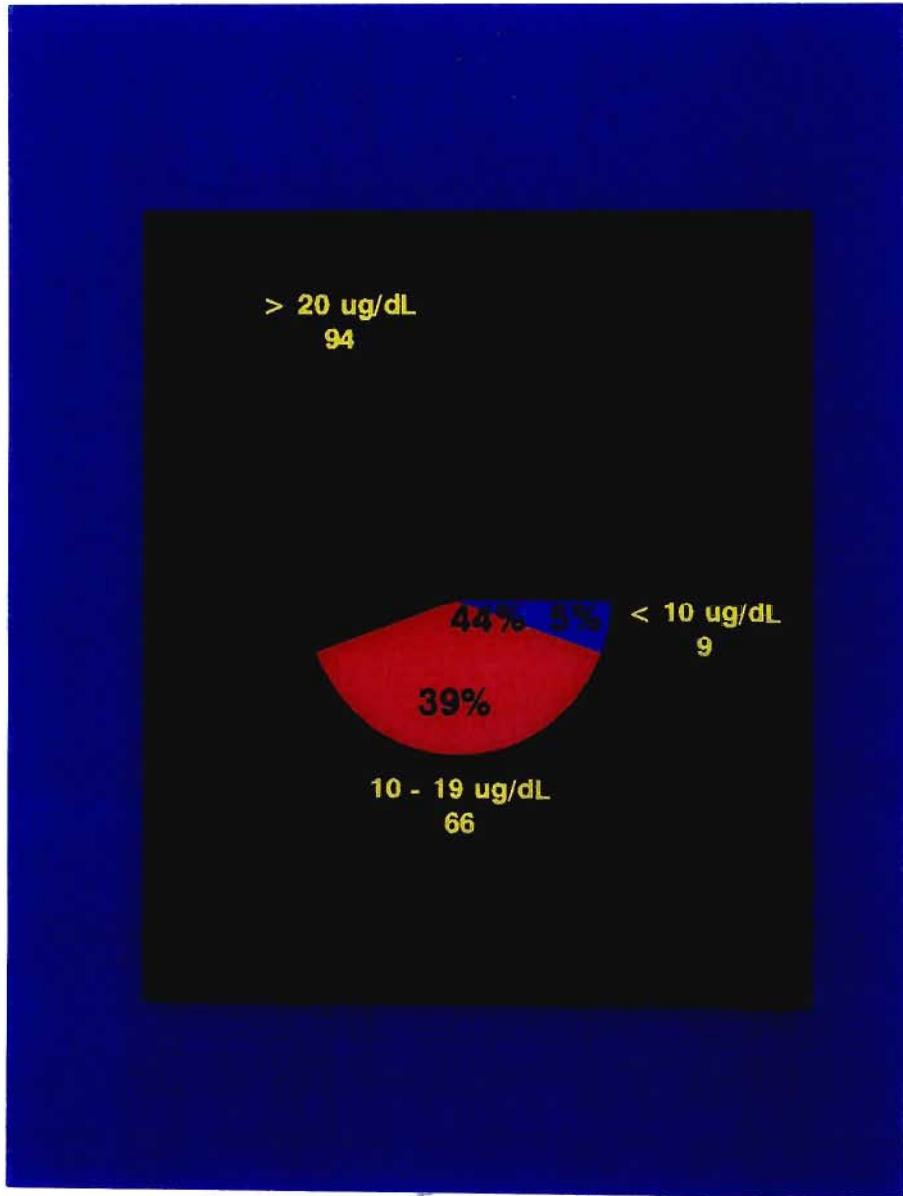


Figure 10. Serum retinol status of 169 children from Besters Farm

4.4.6 Correlations between serum retinol and CIC

Both serum retinol and CIC was available for 166 children. The mean serum retinol values for the different CIC groupings; normal, abnormal and borderline abnormal are given in Table XII and depicted in Figure 11. The mean serum retinol in the abnormal group was significantly different from that of the normal and borderline abnormal groups.

Table XII. Mean serum retinol values according to CIC status

No.	%	CIC status	Serum retinol*
123	66	Normal	21.9 ± 7.1 µg/dL
30	16	Borderline abnormal	20.2 ± 5.6 µg/dL
32	17	Abnormal	16.8 ± 8.9 µg/dL

* mean ± SD

4.4.7 Summary of vitamin A status

In summary 18% (32 of 179) of the population had poor vitamin A status as defined by 2 abnormal conjunctival specimens, and 44% (75 of 169 children) had poor vitamin A status as defined by serum retinol levels < 20 µg/dL.

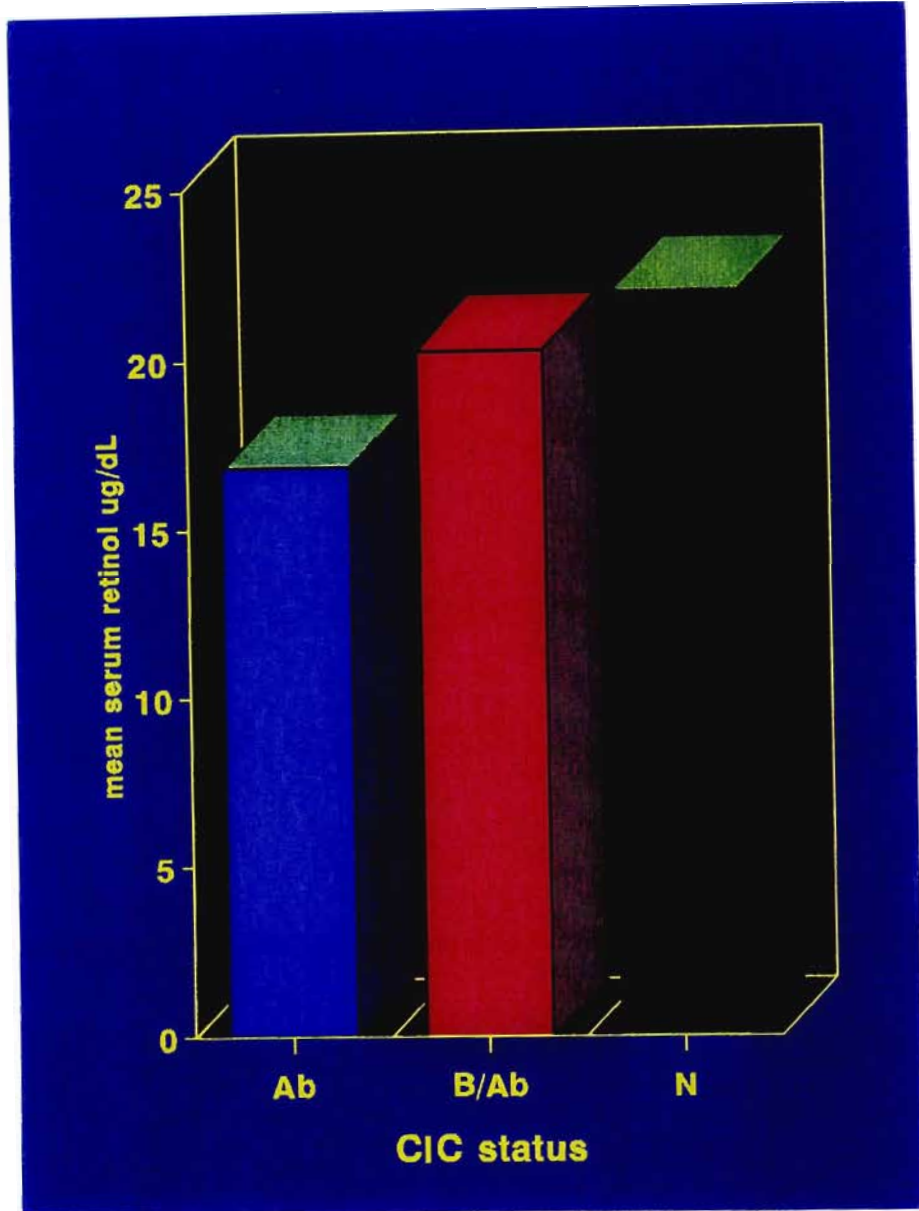


Figure 11. Mean serum retinol values according to CIC status

4.5 DISCUSSION

Poor vitamin A status (serum retinol $< 20 \mu\text{g/dL}$) was present in 44% of 3-6 year old children living in an urban South African informal settlement. This is well above the 15% which is considered to be the level at which vitamin A deficiency is regarded as a public health problem (IVACG 1977). This poor vitamin A status was also confirmed (although poorly) by the finding that 34% of the children (62 of 185) had at least one abnormal specimen and 18% of the children (32 of 179) had 2 abnormal CIC specimens.

This study which used CIC confirmed the feasibility of using CIC in pre-school children 3 - 6 years of age (Kjølhed et al. 1989). It was also shown, that in this particular age group no lid speculum, physical restraint in the form of a papoose board or local anaesthetic was necessary in order to obtain a conjunctival sample. The study also provided an opportunity to test the new applicator method for obtaining conjunctival samples (Keenum et al. 1990); it was found that targetting of the area to be sampled was easy and confirms the report by Keenum et al (1990) that higher quality specimens are obtained because of reduced variation in applied pressure and improved cell adhesion; it was found that only 7 discs out of 371 discs (1.9%) were unreadable. In addition the CIC test showed itself to have good reproducibility.

It was decided that a sensitivity and specificity analysis to determine the validity of the CIC test would not be very helpful when tested against the traditional cut off of $20 \mu\text{g/dL}$ of serum retinol, as serum retinol is generally recognized to be reliable only at the extremes of deficiency ($< 10 \mu\text{g/dL}$) and adequacy ($\geq 30 \mu\text{g/dL}$). In addition cytological and biochemical indicators vary differently with time; goblet cells reappear 3-8 weeks after vitamin A supplementation (Wittpenn et al. 1986) whereas serum retinol levels increase in 5 hours (Amedee-Manesme et al. 1986). Serum retinol is therefore an

unsatisfactory gold standard. The closest to a gold standard for vitamin A status would be liver retinol levels which are obviously not possible for population surveys. A very small study where CIC in children was compared with liver retinol levels showed that abnormal CIC was only found in children with liver retinol levels $< 20 \mu\text{g/g}$ (Amedee-Manesme 1988).

In summary this survey has shown that CIC is a practical and economically feasible test for use in population surveys in our setting. The feasibility of CIC in the field and the improved apparatus which is now available for obtaining conjunctival samples leads us to recommend the use of CIC in population surveys. It also has the advantage of being less traumatic for the child and economically viable in Third World communities compared to the standard method of blood sampling and HPLC analysis. From our findings we would emphasise the suggestions made by others (Kjølhed et al. 1989) that there should be a means for standardizing readers and/or a reference centre for the processing and interpreting of specimens. The ICEPO CIC Manual (Wittpenn et al. 1988) and the training provided by ICEPO should result in such standardisation. However as already mentioned, in the absence of an available standard for vitamin A status, commenting on the validity of the test is not possible. With the use of the ICEPO criteria the CIC survey indicated that only 18% of children had poor vitamin A status whereas the serum retinol survey that used a cut-off of $20 \mu\text{g/dL}$ found 44% with poor vitamin A status. Three explanations can be suggested for the above discrepancy:-

Firstly that the CIC test does not in fact identify the same group of children with marginal vitamin A deficiency as detected by biochemical means, in this population, where clinical vitamin A deficiency is not prevalent. Gądomski et al. (1989), in a similar population, in Guatemala made this suggestion; however Carlier et al. (1992), who studied a similar population, suggested that the CIC test and the biochemical evaluation were comparable. Both these studies however used different criteria for classifying poor and

adequate vitamin A status according to serum retinol. The second explanation is that further investigation and consensus is needed to ascribe poor vitamin A status according to CIC; if poor vitamin A status was classified by at least one of two abnormal CIC specimens then there would be closer agreement between the CIC results and serum retinol in assigning poor vitamin A status viz. 34% and 44% respectively.

The third possible explanation is that in our study population a cut off of 20 $\mu\text{g}/\text{dL}$ as an indicator of marginal vitamin A deficiency may be too stringent; this is borne out by the fact that if a precise threshold for vitamin A deficiency is not prescribed and the mean serum retinol concentrations are examined in the 3 different CIC groups it was found that these were in fact different, with the abnormal CIC status group having significantly lower mean serum retinol than either of the two other CIC groups. Further investigation is obviously needed to clarify this dilemma of the validity of the CIC test in our setting.

Finally we conclude that regardless of the measurement tool used, this typical informal settlement we surveyed has a problem of subclinical vitamin A deficiency and we recommend that some intervention measure, such as nutritional education, vitamin A supplementation or fortification of foodstuffs, is vital. As already mentioned under section 4.2, the community is obviously poor therefore an intervention measure which would improve the general nutritional status and would also be income generating would probably be the best solution. In addition the provision of services like clean water, sanitation and electricity are vital for the improvement of the general health of the population which together with an improved diet would improve the nutritional status of vitamin A and other nutrients as well.

4.6 SUMMARY

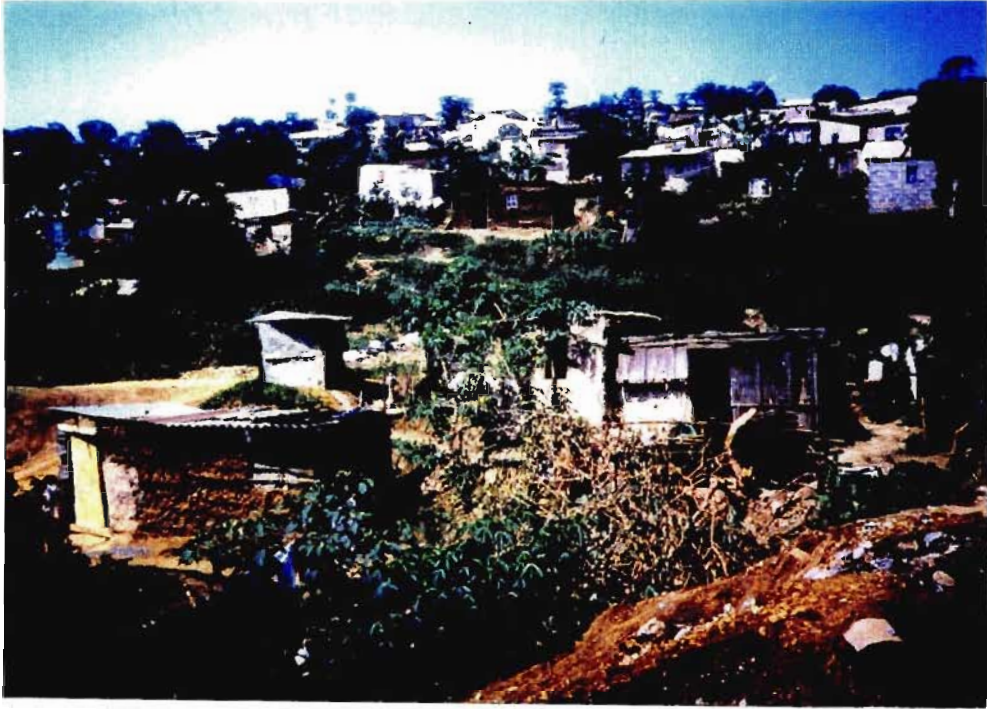
Increased urbanisation of rural populations has led to approximately 7 million South Africans living under appalling conditions on the fringes of the major cities. It is likely that the vitamin A status of children in such conditions may be compromised. Accordingly the vitamin A status of children living in a typical peri-urban informal settlement was assessed. A random representative sample of 190 pre-school children (3 - 6 years of age) was selected from Besters, an informal settlement within metropolitan Durban, South Africa. The following investigations were performed; anthropometry, serum retinol and conjunctival impression cytology (CIC). The mean serum retinol of 169 children tested was $20.8 \pm 7.4 \mu\text{g/dL}$ (mean \pm SD). Nine children (5%) had vitamin A deficiency ($< 10 \mu\text{g/dL}$) and 75 children (44%) had low vitamin A levels ($< 20 \mu\text{g/dL}$). CIC was performed in 185 children and revealed 64 children (34%) with at least one of two abnormal conjunctival specimens and 18% with two abnormal CIC specimens (indicating poor vitamin A status). In conclusion this survey has demonstrated that subclinical vitamin A deficiency is prevalent in this typical peri-urban informal settlement and accordingly we suggest that children living in such informal settlements should be included as one of the selected targets for vitamin A intervention strategies.

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Appendix iii. Photographs taken in Besters Farm



Appendix iv. Photograph showing conjunctival sampling in Besters Farm



CHAPTER 5

Effect of Vitamin A supplementation on morbidity in African children with measles

CHAPTER 5

EFFECT OF VITAMIN A SUPPLEMENTATION ON MORBIDITY IN AFRICAN CHILDREN WITH MEASLES

5.1 INTRODUCTION

Vitamin A deficiency, recognised for its ocular complications has been shown to have systemic effects which increase mortality (Sommer et al. 1983) and morbidity (Sommer et al. 1984, Milton et al. 1986). There is some evidence that this effect on mortality may be reversed by supplementation (Sommer et al. 1986, Rahmathullah et al. 1990). The results of some intervention studies are unfortunately clouded by design and measurement problems (Martorell 1989, Forman 1989).

Serum retinol levels have been shown to be reduced during measles (Bhaskaram et al. 1984, Reddy et al. 1986, Varavithya et al. 1986) and these may be partly responsible for increased mortality from the disease (Markowitz et al. 1989). At the time of commencement of this study, suggestive, though inconclusive evidence, indicated that measles mortality may be reduced by vitamin A supplementation (Ellison 1932, Barclay et al. 1987). Based on this evidence the World Health Organisation (WHO) recommended that in areas where the measles fatality rate is > 1%, children with measles should be supplemented with vitamin A (WHO, 1987). Because measles remains one of the most severe infectious diseases among children in poor communities (Ferrinho 1989) there are profound public health implications in such a policy. However there is no good evidence that morbidity can be reduced by vitamin A supplementation in measles. A major factor which will affect the strength of any vitamin A intervention trial is comparability between the vitamin A treated and control groups as demographic, nutritional, immunological and clinical indices affect outcome (Aaby et al. 1985, Coovadia et al. 1977). A criticism of previous studies has been imprecise recording of morbidity

data, in particular little assessment of the severity of respiratory infection. The detrimental effects of severe measles are maximum within 3 months but may be evident for up to 6 months (Bhaskaram et al. 1984) or even one year (Koster et al. 1981) after the acute attack and therefore any trial designed to assess the effects of vitamin A supplementation on measles should include follow-up of the children. Taking cognisance of some of the problems of other intervention trials this study was designed as a randomized, double blind, placebo controlled vitamin A intervention trial which paid particular attention to many of the known covariants of measles severity and vitamin A status, and in which children were followed up for 6 weeks to 6 months.

5.2 METHODS

5.2.1 Patients

These were sixty African children (4-24 months of age) of poor socio-economic background presenting at Durban's King Edward VIII Hospital (KEH) with measles complicated by pneumonia and diarrhoea severe enough to warrant admission to the Fevers Hospital (Clairwood Hospital). The diagnosis of measles was made on clinical grounds. Dietary and socioeconomic information was obtained from the mother/guardian after entering the child on the trial. The pre-measles vitamin A status of our patients was unclear as there are no good data in South Africa on the prevalence of vitamin A deficiency, but reports suggest that xerophthalmia is most often detected in association with protein energy malnutrition (Konno et al. 1968, Kuming and Politzer 1976) and that there also appears to be a moderate to high prevalence of sub-clinical deficiency in some rural African communities (Argent et al. 1989). Patients were admitted to the trial on weekday mornings between April and October 1989.

5.2.2 Exclusion criteria

Children with rash duration > 5 days; > 24 months of age and those who had received vitamin supplements in the month prior to admission were excluded from the trial. Children who did not have both pneumonia and diarrhoea complications were excluded as were any children with laryngo-tracheo-bronchitis (LTB). Restriction of the trial to those < 24 months was dictated by the fact that this age group is at greatest risk for morbidity and mortality and that this infection is commonest between 4 and 24 months in the third world (Coovadia 1988). Any child with clinical signs of vitamin A deficiency was excluded; such a child would immediately be given a dose of vitamin A. At completion of the study it was noted that no child had been excluded for this reason.

5.2.3 Hospital management of patients

Both groups received standard therapy (antibiotics, antipyretics, oral rehydration) and monitoring for measles and its complications. The trial group received vitamin A (retinyl palmitate, "Arovit Drops", Roche) either 100 000 IU (under 12 months) or 200 000 IU (12 months and over). The control group received a similar placebo syrup. Vitamin A or placebo was administered by the author on admission, days 2 and 8. The above dosage of vitamin A which was given to the experiment group was that recommended by WHO (1987).

5.2.4 Study design

The study was a randomized, placebo controlled, double blind intervention trial. The patients were allocated to treatment or placebo groups according to a table of random numbers. The treatment and placebo dropper bottles were number coded. Only one person who was not involved in the study held the code for the trial and this person was responsible for

dispensing placebo or vitamin A into the correctly number dropper bottles.

5.2.5 Sample size calculation

The assumption used in determining sample size was that the recovery rate (within 7 days) from complicated measles under standard treatment is 50%. Assuming that with vitamin A supplementation 80% of the patients would recover within 7 days and choosing an alpha error of 0.05 and a beta error of 0.5 it was estimated that 29 patients would be needed in each group in order to detect an absolute increase in recovery of 30%.

5.2.6 Determinants of measles severity which were considered

Apart from Vitamin A status many other factors have been suggested as determinants of severity of measles. We therefore recorded these other factors to ensure that when the code was broken the comparability of the two groups could be considered viz: lymphocyte counts less than 2×10^9 /L (Wesley et al. 1982); objective analysis of extent of pneumonia on chest radiograph(CXR) (Wesley et al. 1982); serum complement(C_3) (Coovadia et al. 1977); protein-energy-malnutrition determined by weight/age centiles according to National Center for Health Statistics (NCHS), clinical features and serum albumin levels; extent of rash (Morley 1973) and degree of overcrowding at home (Aaby 1988). Information was taken from the child's parent/guardian in order to derive an overcrowding index (number of persons/number of rooms) which Aaby has suggested is an even more important index of severity than poor nutrition. A higher overcrowding index could, however, also be associated with decreased vitamin A intake (Solon et al. 1978). Rash was graded according to the following: face = 1, 1 + trunk = 2, 2 + limbs = 3.

The CXR score was determined by Prof A. Wesley who was unaware of the treatment groups. The severity of the abnormalities in the lung fields was graded by awarding points for areas of patchy consolidation to a maximum of four, one point being given for each of the following: atelectasis, involvement of the peripheral lung fields, either by emphysema or consolidation, and effusion. Each child was given a score from 0 - 7 for their CXR at day 1 and 42 and at 6 months. A score above 3 was considered as an index for severe measles. The department of Paediatrics and Child Health, University of Natal, have successfully used many of these criteria over the last 15 years to assess severity (Wesley et al. 1982).

Vitamin A is stored in the liver but is dependent on retinol binding protein (RBP) for mobilisation into the plasma where it travels as a RBP complex in association with pre-albumin to target tissues. RBP synthesis appears to be dependent on Zn levels (Solomons and Russel 1980) - therefore Zn, retinol binding protein (RBP) and pre-albumin levels were determined. As there appears to be an interaction between vitamins A and E (Jagadeesan and Reddy 1978), vitamin E concentrations were determined simultaneously with vitamin A concentrations.

5.2.7 Morbidity assessment

Patients were followed up daily in hospital and assessed for duration of fever; diarrhoea (3 or more loose or watery stools a day); and pneumonia (presence of tachypnoea with retractions, crackles or wheezes and other less common complications). Incidence of herpes stomatitis and LTB were recorded. Complete clinical recovery was the stage at which fever, diarrhoea, pneumonia and other complications were clinically resolved.

On discharge each mother was given a plastic laminated appointment card requesting her to bring the child to KEH (for a follow-up appointment on day 42). The card had space for the mother to record any illnesses the child might have had in the interim between hospital discharge and the follow up appointment. The mother was also told to carry this card with her if she ever took the child to a clinic so that the clinic diagnosis could be recorded. Incentives were provided to encourage follow-up. At the six week appointment the children were given another dose of vitamin A/placebo and were given a date for a six month follow up appointment. At the 6 week and 6 month follow-up appointments the child had a CXR taken and a full clinical examination done by the same paediatrician (Dr Mick Broughton, Department of Paediatrics and Child Health, University of Natal).

An integrated morbidity score was calculated for each child based on the following rating system:-

	Score	Weighting for 6 week and 6 month score
Diarrhoea	0 - 4 *	-
URTI	0 - 4 *	-
Pneumonia	0 - 4 *	2 x
LTB	0 - 4 *	2 x
Herpes	0 - 2 +	-
CXR rating	3 - 7	-

* 0 = not present

1 = reported by mother or guardian

2 = documented mild condition

3 = documented severe condition not requiring hospitalisation

4 = condition requiring hospitalisation

+ 0 = absent; 1 = localised; 2 = disseminated.

Each child was assigned a score on day 1 and 8 and immediately after the 6 week and 6 month appointment, prior to the code being broken.

5.2.8 Biochemical assessment procedures

On admission to the trial a blood sample was taken by venipuncture before any vitamin A or placebo had been administered. Serum was separated within 5 hours, aliquoted and stored at -70°C until analysed for zinc, albumin, pre-albumin, vitamins A and E. Serum had been stored between 1 and 10 months before analysis. During all procedures, blood samples were protected from sunlight to prevent photodestruction of vitamin A. Approximately equal numbers of placebo and treated patients were analysed in the same run/plate. All analyses were done in duplicate and a coefficient of variation of $> 5\%$ between duplicates resulted in the analysis being repeated. Vitamin A (as retinol) and vitamin E (as alpha-tocopherol) were simultaneously measured by normal phase high pressure liquid chromatography (HPLC) using fluorescence detection. The method is described in detail in Chapter 2.1. Zinc was measured by atomic absorption spectrophotometry (Varian Atomic Absorption Spectrophotometer - AA-1275 Series). Albumin and pre-albumin were determined by laser nephelometry in the routine biochemistry laboratory attached to King Edward Hospital. RBP levels were determined by commercially available radial immunodiffusion kits LC-Partigen Plates (Behringwerke AG, Marburg). Details of the method are described in Chapter 2.2.

Full and differential blood counts were carried out in the routine KEH haematology laboratory.

5.2.9 Ethical approval

Written informed consent was obtained from the parent or guardian before a child was admitted to the trial. The study protocol was approved by the University of Natal, Faculty of Medicine, Ethics and Research Committee.

5.2.10 Statistical methods

All continuous variables are presented as mean \pm SEM (standard error of the mean). Differences in proportions in the data were examined by the Chi-Square Test. Differences in the means were examined by the students T test (Tables XIII-XVII). The level of significance used was 5%. The software used for the computerised analysis of results was Statistical Analysis System (SAS) - Release 6.03 edition, 1988 (SAS Institute Inc, Cary, NC, USA).

5.3 RESULTS

5.3.1 Baseline comparability

Baseline characteristics of the vitamin A and placebo groups were comparable (see Tables XIII and XIV). There were no significant differences between the two groups in all the covariants considered.

5.3.2 Hospital, acute morbidity outcome

Vitamin A treated children as compared to those given placebo recovered more rapidly from pneumonia ($3.8 \pm .40$ days vs $5.7 \pm .79$ days, $p < 0.05$) diarrhoea and fever, though the latter two were not significantly different (see Figure 12, Table XV). In addition, 28 of the 29 vitamin A treated children (96%) had recovered fully by clinical criteria within 7 days, compared to 11 of the 31 placebo patients (65%), $p = 0.05$. The one death in the placebo group was due to pneumonia; he was lymphopenic on admission. The Integrated Morbidity Score on day 8 was significantly lower in the vitamin A group ($.24 \pm .15$ vs $1.37 \pm .40$, $p = .006$) representing a 82% reduction in the IMS (see Figure 13 and Table XV).

Table XIII. Baseline characteristics of children with measles, by treatment group*+

	Placebo (n = 13 M, 18 F)	Vitamin A (n = 16 M, 13 F)
Age (months)	10.42 ± 0.70	12.17 ± 1.00
Wt/age <80% of standard†	9	9
CXR score > 3	5	7
Lymphocytes <2 X 10 ⁹ /L	6	4
Lymphocytes <2 X 10 ⁹ /L and CXR score > 3	1	2
C ₃ (g/L)	1.02 ± 0.03	1.14 ± 0.06
Overcrowding index§	2.68 ± 0.44	2.65 ± 0.35
Rash grade¶	2.13 ± 0.12	2.00 ± 0.12
IMS**	9.82 ± 1.80	10.56 ± 1.96

* continuous variables presented as mean ± standard error of the mean (SEM)

+ there were no significant differences between the groups for any of the characteristics.

† 50th National Center for Health Statistics percentile; no child had clinical features of protein-energy malnutrition.

§ number of persons per room

¶ 1 = face; 2 = face, trunk; 3 = face, trunk, limbs.

** Integrated morbidity score

Table XIV. Baseline haematological and serum micronutrient concentrations in children with measles, according to treatment group*+

	Placebo (n = 31)	Vitamin A (n = 29)
Hemoglobin (g/L)	110.5 ± 3.5	108.6 ± 3.1
Lymphocytes (x10 ⁹ /L)	3.80 ± 0.42	3.70 ± 0.37
Albumin (g/L)	31.84 ± 0.65	30.10 ± 1.05
Zinc (µmol/L)	13.46 ± 0.43	12.55 ± 0.43
Prealbumin (mg/L)	92.6 ± 7.0	94.5 ± 4.6
Retinol-binding protein (mg/L)	14.6 ± 1.4	14.9 ± 1.2
Vitamin A (µg/dL)	11.73 ± 1.05	12.22 ± 1.05
Vitamin E (mg/L)	4.9 ± 0.4	4.9 ± 0.4

* continuous variables presented as mean ± standard error of the mean (SEM)

+ there were no significant differences between the groups for any of the characteristics.

Table XV. Morbidity outcome in children hospitalised with measles, according to treatment group*

	Placebo (n = 31)	Vitamin A (n = 29)
Clinical-pneumonia duration (days)	5.7 ± 0.79	3.8 ± 0.40+
Diarrhoea duration (days)	4.5 ± 0.35	3.2 ± 0.71
Fever duration (days)	4.2 ± 0.50	3.6 ± 0.30
Herpes stomatitis	2 (6%)	3 (10%)
Laryngotracheobronchitis	1	0
Death	1	0
Complete clinical recovery in < 8 days	20 (65%)	28 (96%)†
IMS	1.37 ± 0.40	0.24 ± 0.15§

* mean ± SEM

+ † § significantly different from placebo:

+p = 0.037, †p = 0.002, §p = 0.006

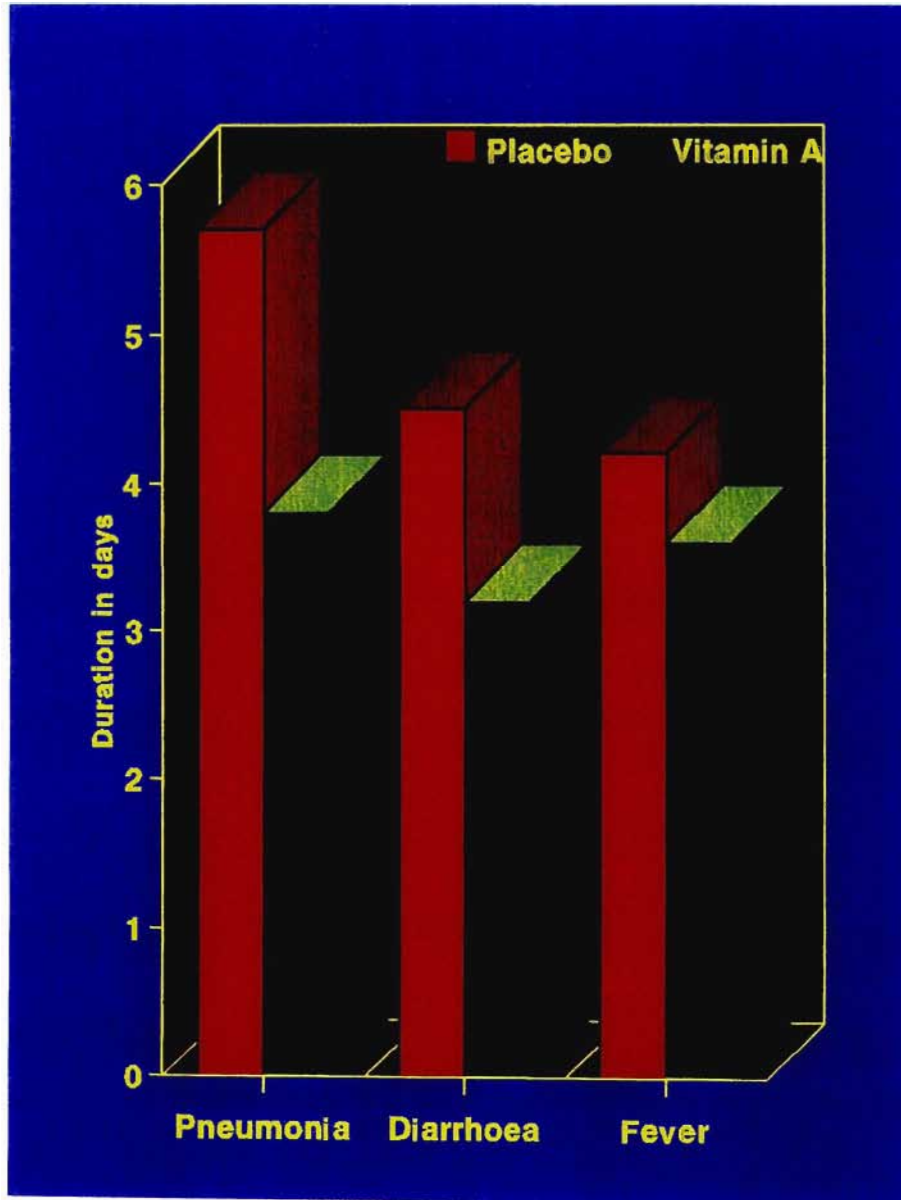


Figure 12. Duration of common measles complications according to treatment group

5.3.3 Six week morbidity outcome

The follow-up was 80%. The six week Integrated Morbidity Score was significantly lower in the vitamin A group ($2.21 \pm .45$ vs 5.74 ± 1.17 , $p=0.02$; see Table XVI and Figure 13). This 61% reduction in the IMS was mainly attributed to more severe episodes of pneumonia in the placebo group (as shown by the significantly higher pneumonia score per episode in the placebo group). Children in the supplemented group had gained significantly more weight over 6 weeks than the placebo group (see Table XVI and Figure 14).

5.3.4 Six month morbidity outcome (Table XVII, figure 13)

There was a poor 6 month follow-up (60%) because of severe political unrest at the time when most of the children were due for their follow-up appointments (see Appendix v and vi, for press cuttings documenting some of the unrest). The six month Integrated Morbidity Score was significantly lower in the vitamin A group compared to the placebo group ($.60 \pm .22$ vs 4.12 ± 1.13 , $p = .002$). The large reduction in the IMS (85%) in the supplemented group was attributed mainly to the increase in frequency and severity (as defined radiologically) of pneumonia episodes in placebo treated children. A significant difference in the number of URTI episodes also contributed to the overall poor morbidity of the placebo group (see Table XVII and Figure 13). Children in the vitamin A group had gained more weight than the placebo group 6 months after being hospitalised but the difference was not significant (see Table XVII and Figure 14).

Table XVI. Morbidity outcome, according to treatment group, in children six weeks after being hospitalised for measles*

	Placebo (n = 24)	Vitamin A (n = 24)
Weight gain (Kg)	0.90 ± 0.14	1.29 ± 0.17+
Diarrhoea episodes	12	6
Score per episode of diarrhoea	2.25 ± 0.25	2.17 ± 0.31
URTI episodes	9	7
Score per episode of URTI	2.66 ± 0.17	1.71 ± 0.28
Pneumonia episodes	6	5
Score per episode of pneumonia	6.67 ± 0.67	4.40 ± 0.98†
CXR score ≥3	6	2
Score per CXR	3.83 ± 0.40	3.00 ± 0
IMS	5.74 ± 1.17	2.21 ± 0.45†

* mean ± SEM

+ † significantly different from placebo:

+p = 0.04, †p = 0.02

Table XVII. Morbidity outcome, according to treatment group, in children six months after being hospitalised with measles*

	Placebo (n = 16)	Vitamin A (n = 20)
Weight gain (kg)	2.37 ± 0.20	2.89 ± 0.23
Diarrhoea episodes	6	3
Score per episode of diarrhoea	2.17 ± 0.31	1.67 ± 0.67
URTI episodes	8	3+
Score per episode of URTI	2.37 ± 0.18	2.00 ± 0.58
Pneumonia episodes	3	0
Score per episode of pneumonia	6.67 ± 0.67	0†
CXR score ≥3	3	0
Score per CXR	3.67 ± 0.67	0§
IMS	4.12 ± 1.13	0.60 ± 0.22¶

* mean ± SEM

+ † § ¶ Significantly different from placebo:

+p = 0.05, †p = 0.005, §p = 0.01, ¶p = 0.002

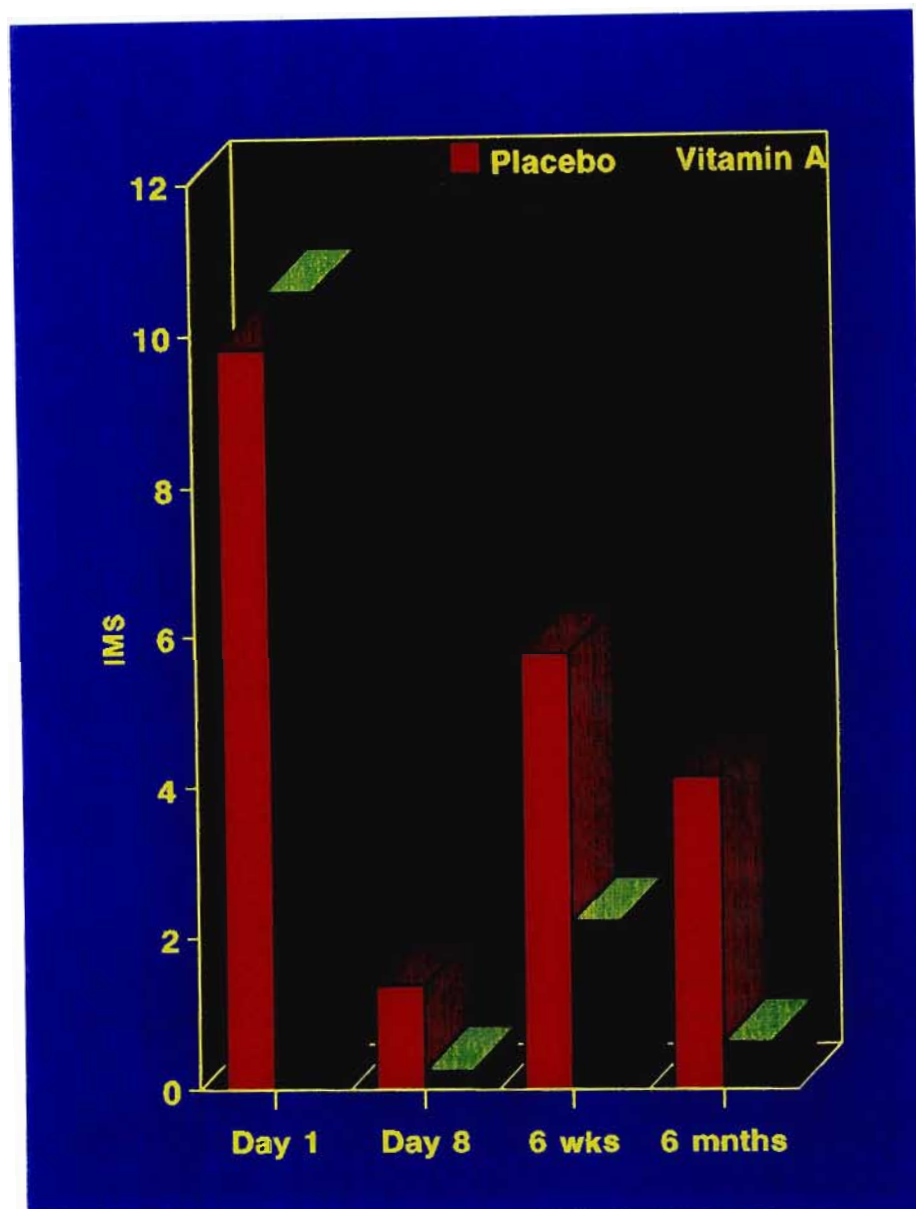


Figure 13. Integrated morbidity scores (IMS) according to treatment group, during and up to 6 months after measles

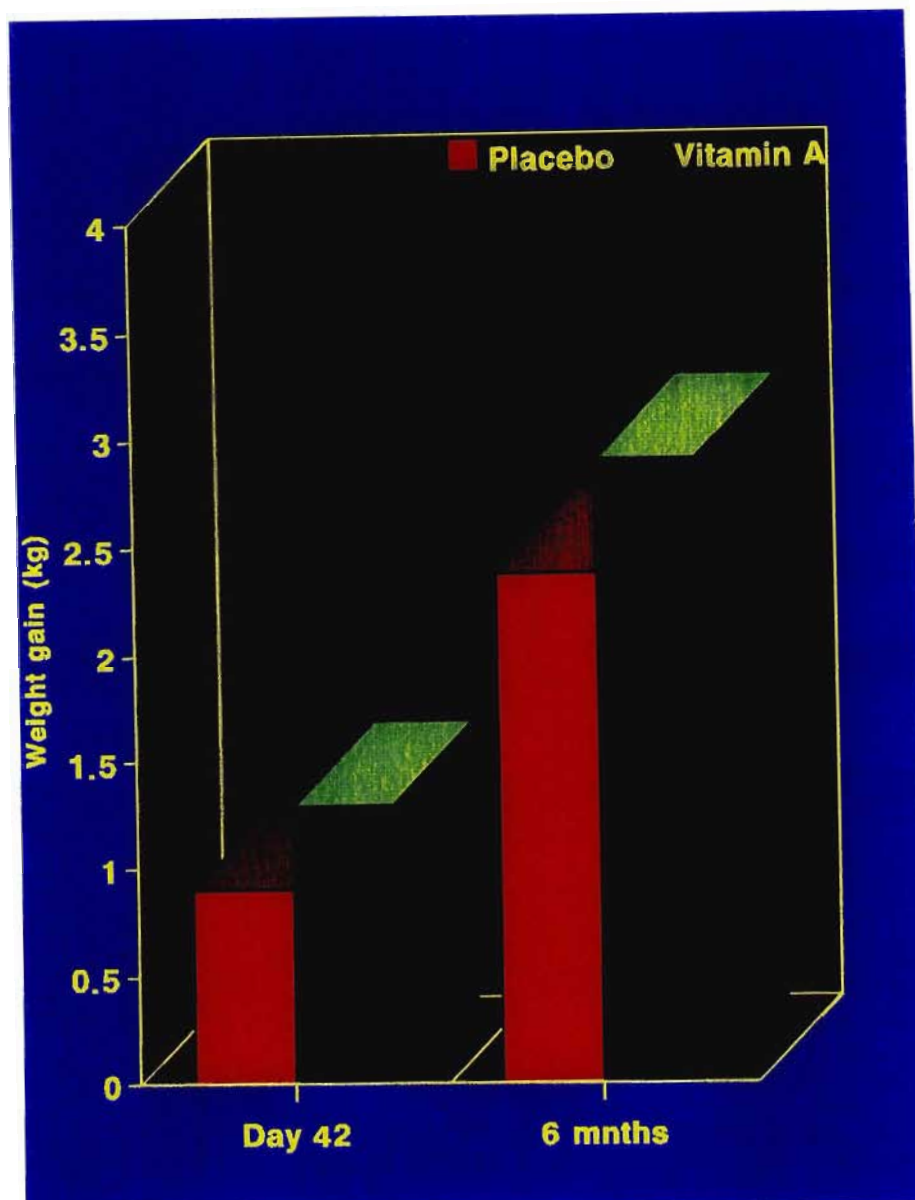


Figure 14. Weight gain according to treatment group, 6 weeks and 6 months after being hospitalised for measles

5.4 DISCUSSION

This study of hospitalised young African children from a community where xerophthalmia is uncommon, but who had reduced levels of serum retinol at onset of measles, has demonstrated that supplementation with vitamin A within 4 days of the rash resulted in speedier recovery from pneumonia during the acute exanthematous phase and produced a significant reduction in the frequency and severity of post-measles respiratory tract complications. Additional vitamin A supplement on day 42 could have contributed to this significant reduction in morbidity which was noted up to 6 months. The diminution of infectious morbidity rates probably accounted for the improved weight gain in the vitamin A treated children during the 6 weeks after the rash. Addition of vitamin A at baseline did not have any significant effect on the frequency or severity of diarrhoea.

Although the prevalence of clinical signs of vitamin A deficiency in Durban appears to be low, our results confirm the findings of other workers that serum retinol levels are lowered during measles (Reddy et al. 1986, Bhaskaram et al. 1984, Markowitz et al. 1989). Hyporetinaemia (serum retinol < 20 $\mu\text{g}/\text{dl}$) was detected in 90% of the patients. Both groups however had a marked increase in serum retinol levels on day 8 with the supplemented group having significantly higher levels than the placebo group (see chapter 7). This finding has recently been confirmed by a study in New York City among children in a community which like ours did not have a prevalence of overt vitamin A deficiency (Frieden et al. 1992). This would imply that the liver stores of these patients were not exhausted but that there was a temporary impaired mobilisation of vitamin A and increased tissue utilisation of vitamin A. This effect of a temporary lowering of serum retinol could be especially serious because of the synergistic deleterious impact of vitamin A deficiency and measles infection. Vitamin A deficiency states have been shown to result in destruction of epithelial tissue and to depress the immune function (Barker 1983). Measles virus produces similar effects (Coovadia 1988).

The exact mechanism by which vitamin A reduces morbidity in measles patients is unknown. Two mechanisms which have been suggested are improvement of immune function and regeneration of damaged epithelial tissue (Barker 1983). It seems likely that the improvement in morbidity in the vitamin A supplemented patients was due to non-specific immune enhancement due to re-establishment of the integrity of the epithelial tissues of the respiratory and gastro-intestinal tracts.

The results in this study on the beneficial effect of vitamin A during measles infection confirms the growing body of information linking vitamin A deficiency with increased susceptibility to infection (Scrimshaw 1968, West et al. 1989) and the potential for reversing this by nutritional intervention. Perplexing evidence however has emerged in recent studies as to which sites of infection are more affected by vitamin A viz. respiratory or enteric. Our study showed that vitamin A supplementation speeded up the recovery from pneumonia and reduced the number and severity of post-measles respiratory infections but it had no significant effect on diarrhoea. Our experience with measles over the last twenty years has shown that measles mortality is mainly due to pneumonia rather than being directly related to diarrhoea. The lower (1.6%) than usual (12-14%) hospital case fatality rate in this study could be accounted for by the exclusion of emergency cases and severe malnutrition.

Although follow-up was unsatisfactory the strength of the study was increased by assessing patients at 6 months. The importance of follow-up of patients is underlined by the observation that measles is followed by an excess of mortality for 12 months after the rash (Bhaskaram et al. 1984, Koster et al. 1981).

The main strength of our study was that the morbidity data were carefully documented (with both clinical and radiological data

for pneumonia) and the Integrated Morbidity Score for each child was determined immediately after the appointment, before the trial code was broken to eliminate any possible bias. Although only sixty children were involved, the power of the study was increased by concentrating on a very small age group (4-24 months) which was the group shown to have the best effect of vitamin A supplementation on mortality (Barclay et al. 1987), to be most at risk for suffering ill effects with hyporetinemia (Markowitz et al. 1989) and the group in which measles is commonest in third world countries (Coovadia 1988).

Our results confirm the findings of a recent trial investigating the effects of vitamin A supplementation on measles mortality and morbidity (Hussey and Klein 1990) which showed that vitamin A supplementation resulted in a significant reduction in mortality and morbidity in the acute phase of measles in children from a community where clinical signs of vitamin A deficiency are uncommon.

The careful consideration of multiple covariants enhances the validity of the data obtained in this study and supports the current WHO recommendations for vitamin A supplementation during measles. Furthermore the study has shown that during measles vitamin A levels are reduced, regardless of pre-measles vitamin A status, and leads us to suggest that the WHO recommendation should be extended to include vitamin A supplementation for all children who are seriously ill with measles and not only for those in areas where vitamin A deficiency is a public health problem or where the measles case fatality rate is $> 1\%$.

5.5 SUMMARY

The effects of vitamin A supplementation on measles morbidity are unclear. Sixty hospitalised children (aged 4-24 months) with complicated measles received a WHO recommended dose of vitamin A or placebo. The two groups were comparable in known covariants of measles severity : weight/age centiles; overcrowding; rash; total lymphocytes; serum levels of Zn, albumin, pre-albumin, RBP, vitamins A and E. 90% of the patients had hyporetinaemia. Integrated Morbidity Scores (IMS) derived from diarrhoea, herpes and respiratory tract infection (radiologically confirmed) were assigned on day 8, at 6 weeks and 6 months - these were reduced by 82%, 61% and 85% respectively in the supplemented group; this was mainly due to reduced respiratory tract infection. There was one death in the placebo group. At 6 weeks weight gain was significant in the supplemented group. Despite the selected sample, attention to multiple covariates enhances the validity of the data obtained and supports the current WHO recommendations for vitamin A supplementation during measles.

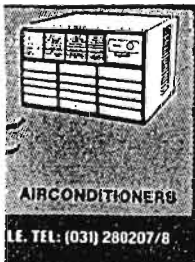
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THE NATAL MERCURY



FREWIN TROPHY WINNER 1989

TUESDAY, DECEMBER 12, 1989

50c (44c + 6c GST) SUBSCRIPTIONS: 37c

Hundreds flee new violence

Necklaced bodies found in township

By Nicola Cunningham-Brown

TWO necklaced corpses are among the score of bodies police have found in the Inanda area since the weekend, after the most recent outbreak of violence which left a pall of black smoke hanging over the township.

A policeman in the area said yesterday they had discovered at least 20 bodies. Two of the victims had been necklaced and one person had been decapitated. He said the violence was the worst the township had seen since August 1984.

Fighting, less severe than that over the weekend, continued sporadically yesterday.

Hundreds of people have fled the strife-torn area, seeking refuge in other areas and in central Durban, with friends, family and employers.

'People here have lost confidence in the ability of the police to protect them, and that is why they are leaving,' a member of the Inanda Youth League told the Mercury, as he stood on the side of the road watching a constant stream of families fleeing one of the worst-hit areas.

'All peace meetings and attempts at peace talks seem to have been a failure. Trouble in our areas has just sky-rocketed during the past three weeks. No one knows where or when it will end.'

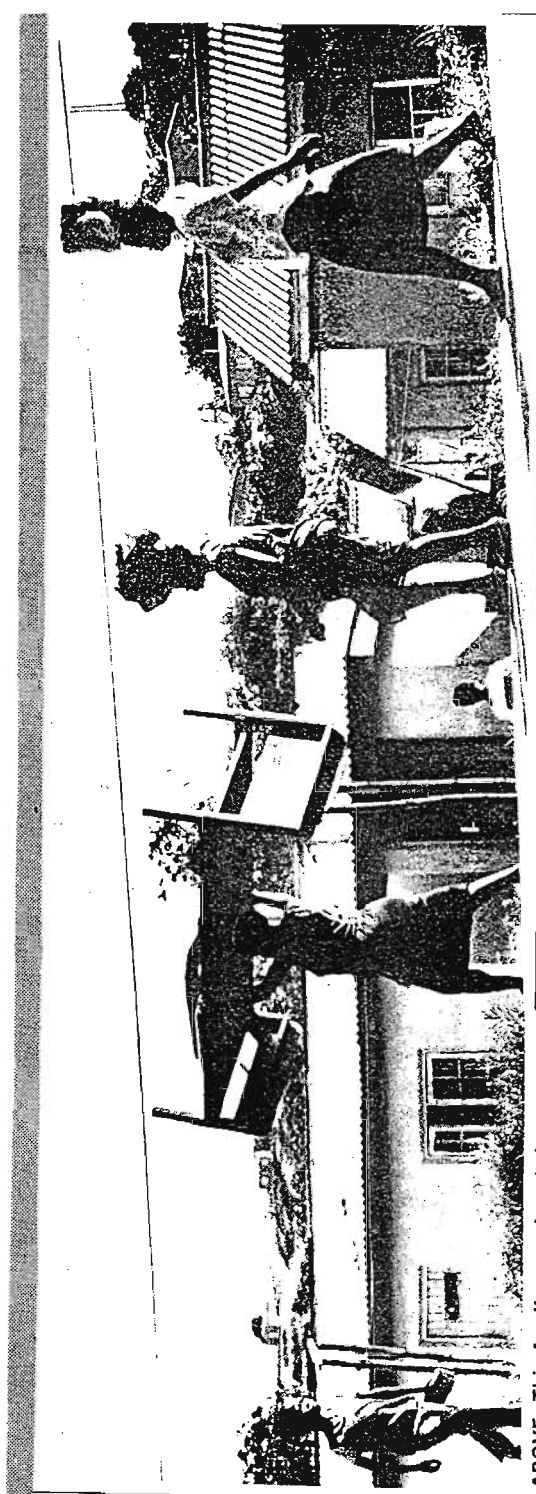
He said although the violence was mainly between groups of Inkatha and UDF supporters, a number of people involved were merely criminals and thugs 'taking this opportunity to loot as much as they can'.

Dozens of Inanda residents, many of whom claimed to be solidly

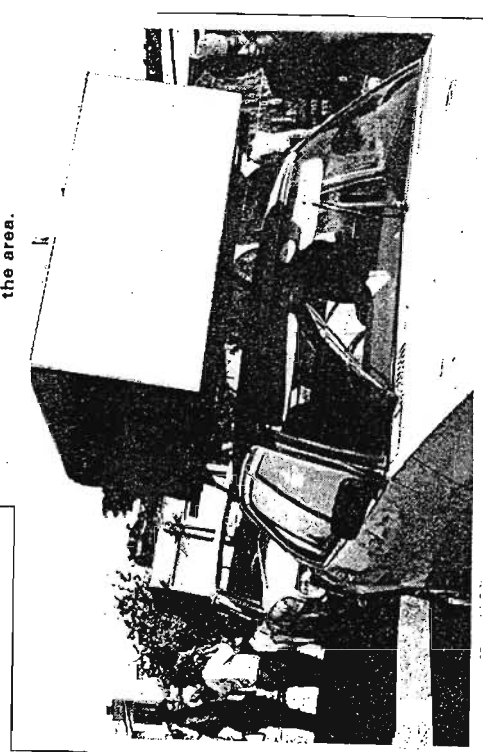
Appendix v. Newspaper cutting documenting political violence which adversely affected the follow-up in this study.

Appendix vi. Newspaper cutting with photographs documenting political violence which adversely affected the follow-up in this study.

THE NATAL MERCURY, TUESDAY DECEMBER 12, 1989



ABOVE: This family, carrying their most valuable household possessions, were among hundreds who fled Inanda township yesterday. BELOW: Small trucks and cars were constantly ferrying families and their possessions out of the area.



CHAPTER 6

Effect of Vitamin A supplementation on immunological parameters in measles Patients

CHAPTER 6

EFFECT OF VITAMIN A SUPPLEMENTATION ON IMMUNOLOGICAL PARAMETERS IN MEASLES PATIENTS

6.1 INTRODUCTION

It has been highlighted in chapter 1 of this thesis that evidence has accumulated over the last 2 decades that vitamin A deficiency (arising from inadequate diets or induced by infections) is associated with increased morbidity and mortality. Measles accentuates or precipitates decreased serum concentrations of vitamin A (see Chapter 7). In Chapter 5 it was documented that significant reductions in measles-specific morbidity were observed after supplementation with vitamin A.

It is not clear how vitamin A deficiency predisposes to infection. The two most likely mechanisms appear to be damage to epithelial membranes and depression of immune responses. Animal studies have shown that vitamin A deficiency results in a wide range of impaired immune responses and that the effect is reversed by vitamin A supplementation (Jurin and Tannock 1972, Cohen and Elin 1974, Nauss et al. 1979). Although much work has been done on animals there is limited information available from human studies, particularly in children who are most at risk for deficiency. Moreover, much of the human data on vitamin A and immunity is confounded by the concomitant presence of protein energy malnutrition, itself a cause of severe immunodeficiency. At the time of commencing this study there was only one published study which specifically looked at the effect of vitamin A supplementation on the immune response in children (Brown et al. 1980) and this study showed no supplementation effect. As this study used a different antibody assay to the one currently used in most studies it was felt that it would be worthwhile to reconsider the role of vitamin A supplementation in enhancing immune functioning particularly in view of the fact that the animal studies which had been

conducted had shown a positive effect.

The existing evidence suggests that the major detrimental effect of inadequate vitamin A is on non-specific defence mechanisms, cell mediated immunity and on T-dependent B cell responses (West et al. 1989, Chandra and Vyas 1989). This study therefore set out to study the effect of vitamin A supplementation on the production of measles IgG antibody and total lymphocyte numbers, IL-2 and C₃ concentrations. These tests were chosen because they represent different components of the immune response and, more importantly because (with the exception of IL-2) they correlate regularly with clinical outcome in measles infection (Coovadia et al. 1977, Wesley et al. 1982).

6.2 METHODS

6.2.1 Subjects

These were sixty African children (4-24 months of age) of poor socioeconomic background presenting at Durban's King Edward VIII Hospital (KEH) with measles complicated by pneumonia and diarrhea severe enough to warrant admission to the Fevers Hospital (Clairwood Hospital). The diagnosis of measles was made on clinical grounds. Dietary and socioeconomic information was obtained from the mother/guardian after entering the child on the trial. Patients were admitted to the trial on weekday mornings between April and October 1989.

Children > 24 months or with rash duration > 5 days and those who had received vitamin A supplements in the month before admission were excluded from the trial. Any child with clinical signs of vitamin A deficiency was excluded; it was decided that such a child would immediately be given a dose of vitamin A. At completion of the study it was noted that no child had been excluded for this reason.

The patients were allocated to treatment or placebo groups according to a table of random numbers. The treatment and placebo dropper bottles were number coded. All investigators and patients involved in the study were blinded as to study group assignment.

Both groups received standard therapy and monitoring for measles and its complications. The trial group received vitamin A (retinyl palmitate, "Arovit Drops", Roche) either 100 000 IU (under 12 months) or 200 000 IU (12 months and over). The control group received an equivalent volume of placebo syrup. Vitamin A or placebo was administered on admission, days 2 and 8. In addition the children were given another dose of vitamin A or placebo at the day 42 follow-up appointment. Details on the methods used for assessing morbidity are given in chapter 5, section 2.7.

6.2.2 Biochemical and immunological investigations

On admission to the trial (day 1), days 8 and 42 a blood sample was taken by venipuncture before any vitamin A or placebo had been administered. Serum was separated within 5 hours, aliquoted and stored at -70°C until analyzed (between 1 and 10 months) for albumin, vitamin A, C_3 , IL-2 and measles IgG antibody. During all procedures, blood samples were protected from sunlight to prevent photodestruction of vitamin A. All analyses were done in duplicate.

Vitamin A (as retinol) was measured by normal phase high pressure liquid chromatography (HPLC) using fluorescence detection as described in chapter 2, section 1. Albumin was determined by laser nephelometry in the routine biochemistry laboratory attached to KEH.

C₃ values were determined by laser nephelometry on days 1, 8 and 42. Total lymphocyte counts were determined in the routine KEH hematology laboratory on days 1 and 42. IL-2 concentrations were determined in serum using a commercial radioimmunoassay kit supplied by Amersham (Aylesbury, Buckinghamshire, UK), code RPA. 531) - the detailed method is described in chapter 2, section 4. The 3 consecutive sera (days 1, 8 and 42) of each patient were tested on the same batch. In addition approximately equal numbers of patients from each group were tested in the same batch. Measles IgG antibody was detected by means of the ELISA assay (Behringwerke AG, Marburg, FRG) - details of the method are provided in chapter 2, section 3.

6.2.3 Controls

These consisted of 20 adequately nourished African children 4 - 24 months of age. Nutritional status was determined by anthropometric measurements and biochemical indicators (serum albumin, vitamin A and E concentrations). These children were in the orthopaedic and surgical wards of KEH for minor operations e.g. hernia, or else they were from the well baby clinic in Kwa Mashu (Durban). All blood samples were taken before any surgical procedures. These controls were used to establish normal values for nutritional parameters, C₃, absolute lymphocyte numbers and IL-2.

6.2.4 Statistical methods

All values are reported as mean \pm SEM (standard error of the mean). Differences in proportions in the data were examined by the Chi-Square Test. Differences in the means were examined by the Student's two-tailed T test. The level of significance used was 5%. In addition because of intrasubject variability in immune factors mean of change from baseline for each group was determined at day 8 and

day 42. Thus each child had a baseline determination on day 1 from which a change in concentration at a specific time could be calculated. Individual changes were summed and a mean value for change in concentration for each group was determined and expressed as a delta (Δ) value. The two groups were compared with relation to these changes using the two sample T test with a Bonferroni adjustment thus the significance level changed to 2.5% ($\alpha/2$) .

The software used for the computerised analysis of results was Statistical Analysis System (SAS) - Release 6.03 edition, 1988 (SAS Institute Inc, Cary, NC, USA).

6.3 RESULTS

6.3.1 Baseline comparability

Baseline characteristics which are covariants of severe disease (age, nutritional status, socioeconomic status, morbidity at presentation and radiologic presentation) of the vitamin A (n = 29) and placebo groups (n = 31) were comparable (Tables XIII and XIV in Chapter 5).

6.3.2 Morbidity outcome

A significantly higher number of vitamin A supplemented children had recovered from complicated measles within 8 days compared with the placebo group (96% vs 65%). The vitamin A group showed a marked reduction in morbidity for up to 6 months as indicated by significantly lower Integrated Morbidity Scores (IMS) than the placebo group on days 8 and 42 and at 6 months. The reduction in morbidity was mainly due to a decrease in incidence and severity of respiratory infections. Details of morbidity outcome are presented in Chapter 5.

6.3.3 Immune factors

On day 1 there was no difference between the two groups in all the immune factors tested (Table XVIII). On day 1 lymphocyte numbers were decreased in placebo and vitamin A treated groups compared with controls ($p = .005$) however they increased within both groups during the next 6 weeks to return to normal values by day 42. On day 42 the vitamin A treated group had higher lymphocyte numbers than the placebo group, $p = .05$ (Table XVIII, Figure 15). When analyzing the changes in lymphocyte numbers (day 42 - day 1) we found the same trend that the rate of change was greater in the treated group ($\Delta = 3.40 \pm 0.74$ vs 1.96 ± 0.51 , $p = .06$), however this was not statistically significant (Figure 16).

The mean concentrations of measles IgG antibody were significantly increased within both groups during the first 6 weeks. On day 8 and day 42 the vitamin A treated groups had higher mean antibody values. This difference was significant on day 8, $p = .01$ (Table XVIII, Figure 17). When analysing the changes in antibody concentrations a similar trend was found with the changes between day 8 and day 1 being significantly higher in the treated group ($\Delta = 340.6 \pm 49.6$ vs 182.3 ± 27.6 , $p = .008$). The changes between day 42 and day 1 were also higher in the treated group ($\Delta = 820.7 \pm 85.5$ vs 617.7 ± 88.5) although this difference did not reach statistical significance (Figure 18).

Table XVIII. Immune factors studied in healthy controls and in placebo and vitamin A treated groups of children with measles (data presented as mean \pm SEM).

	CONTROLS	PLACEBO			VITAMIN A		
DAY		1	8	42	1	8	42
n	20	31	30	20	29	28	21
Lymphocytes $\times 10^9/L$	5.72 \pm 0.61*	3.80 \pm 0.42		6.45 \pm 0.66§	3.75 \pm 0.41		8.10 \pm 0.80§
C3 (g/L)	1.32 \pm 0.07+	1.02 \pm 0.03	1.11 \pm 0.04	1.41 \pm 0.11	1.14 \pm 0.06	1.25 \pm 0.07	1.48 \pm 0.08
IL-2 (IU)/mL	1.79 \pm 0.06†	1.57 \pm 0.08	1.72 \pm 0.08	1.70 \pm 0.08	1.59 \pm 0.06	1.76 \pm 0.08	1.74 \pm 0.08
IgG (mIU)/mL		39.9 \pm 5.5	222.8 \pm 28.7¶	654.8 \pm 91.2	42.6 \pm 5.3	384.2 \pm 52.8¶	869.5 \pm 90.0

*+† controls values significantly higher than day 1 values of measles patients: *p = .005, +p = .003, †p = .02

§ difference between means on day 42, p = .05

¶ significant difference between means on day 8, p = .01

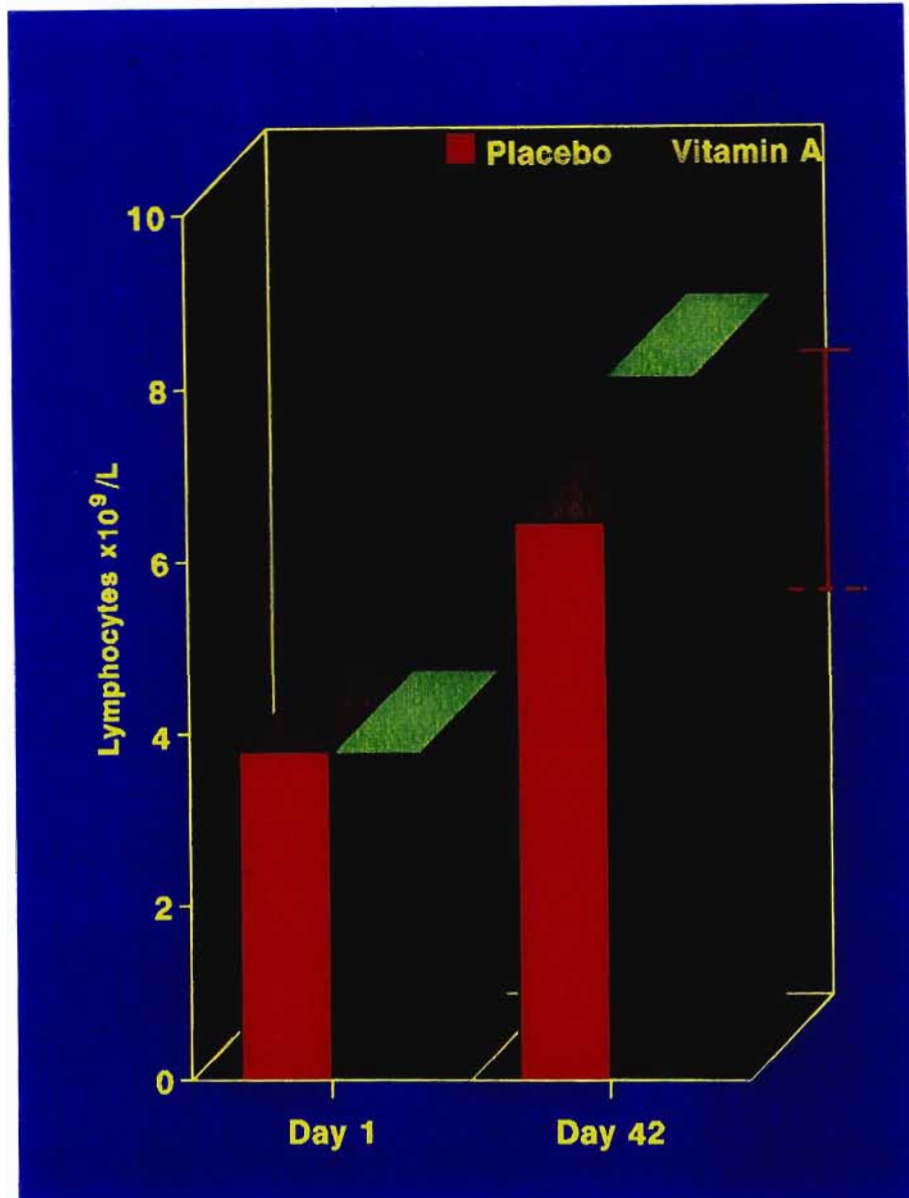


Figure 15. Mean total lymphocyte numbers, in children on days 1 and 42 after being hospitalised for measles, according to treatment group
 Dotted line and T bar represent mean and standard deviation values in healthy controls

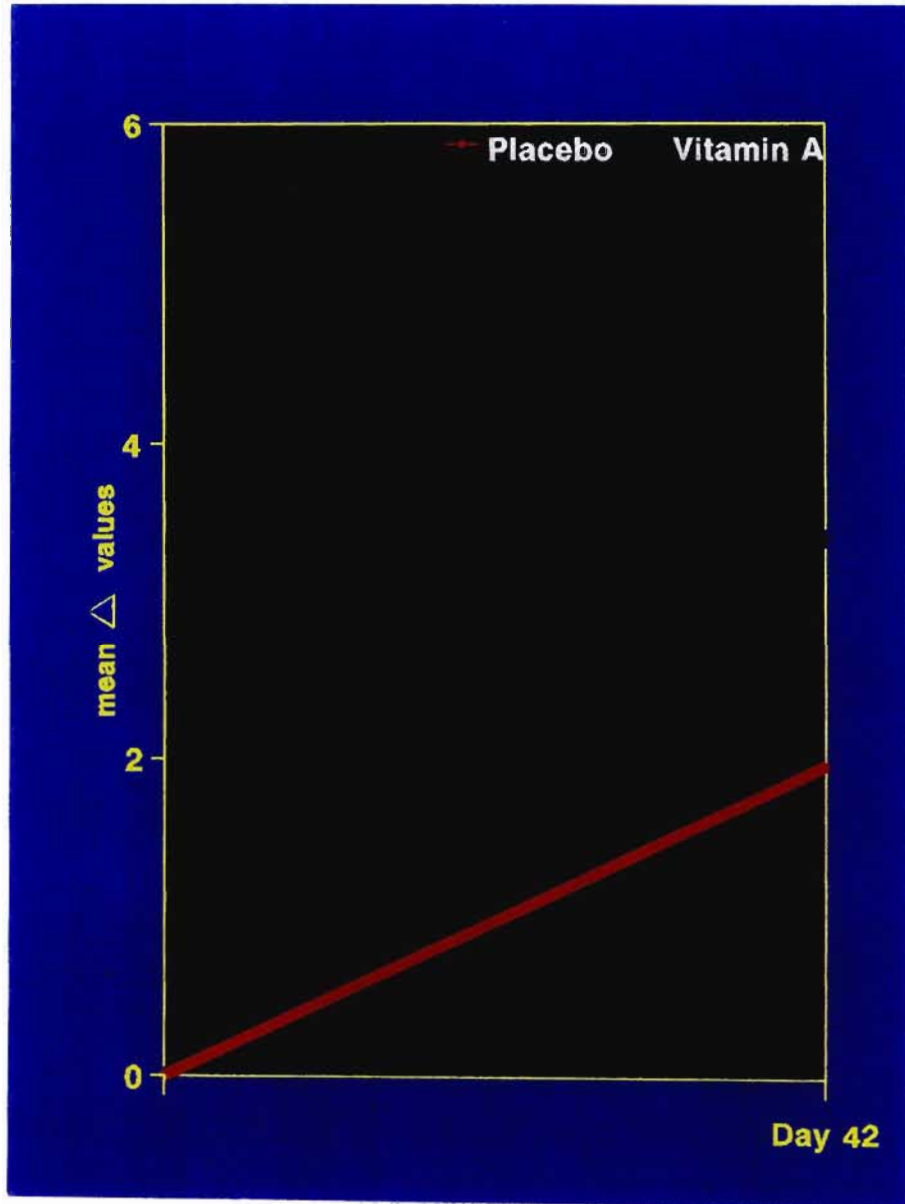


Figure 16. Changes in total lymphocyte numbers, in children on day 42 after being hospitalised for measles, according to treatment group

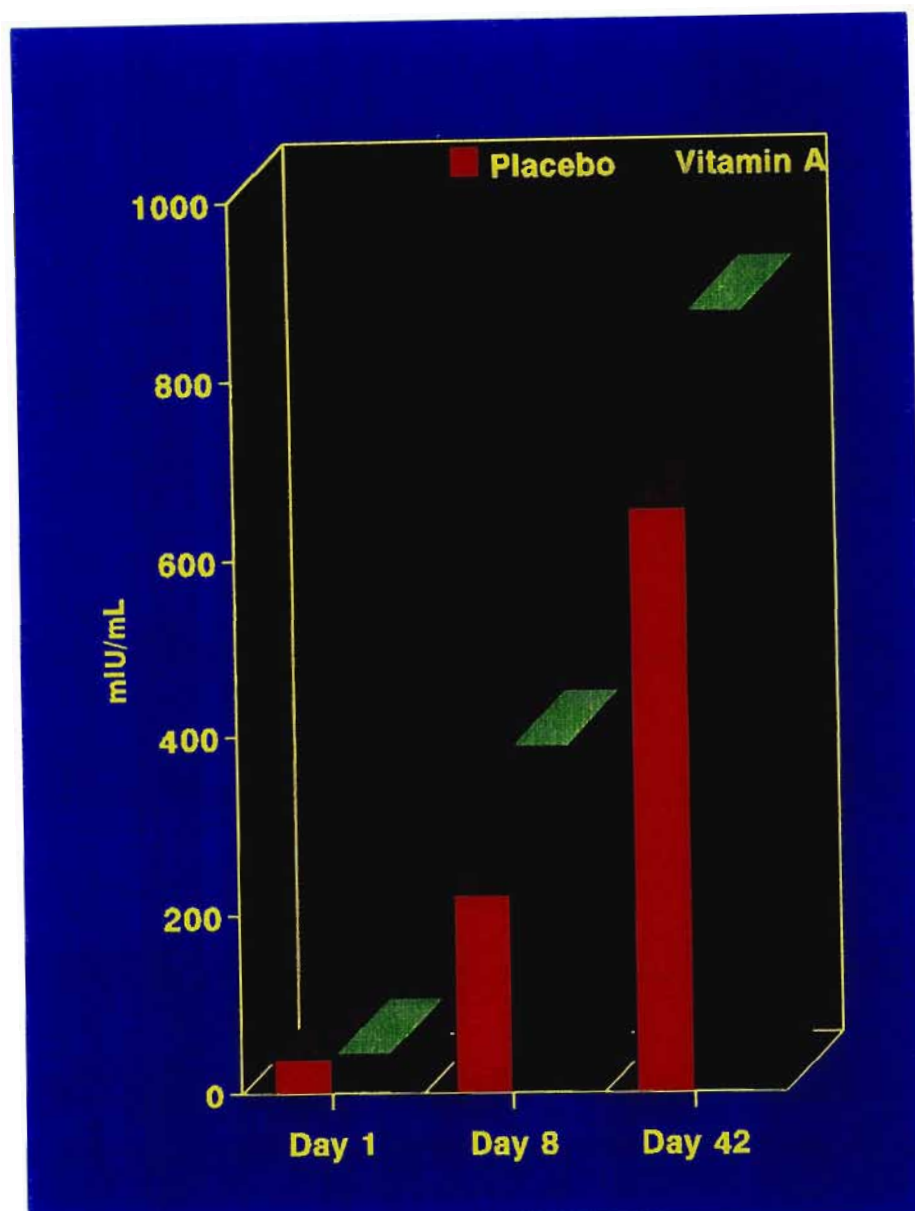


Figure 17. Mean measles IgG antibody levels in children on days 1, 8 and 42 after being hospitalised for measles, according to treatment group

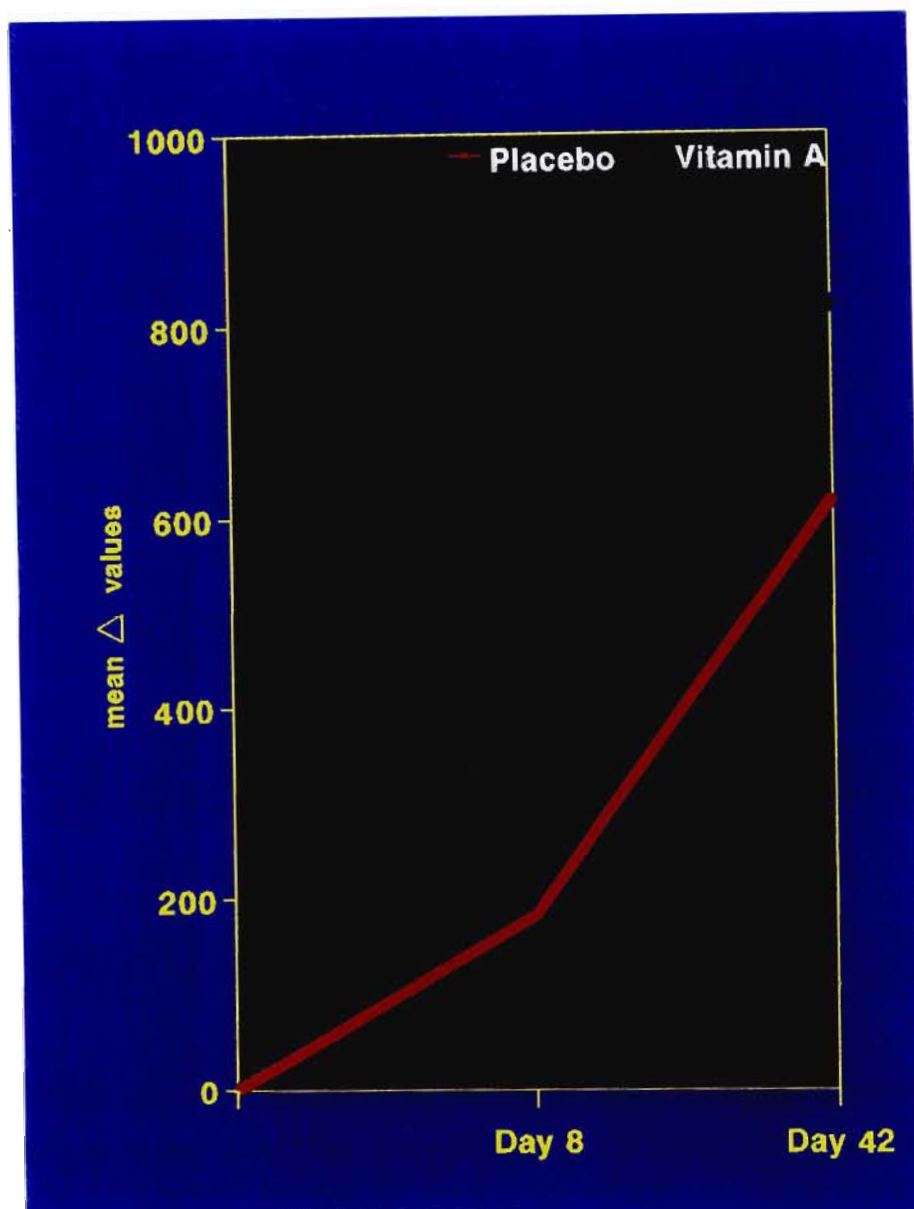


Figure 18. Changes in IgG antibody levels, in children on days 8 and 42 after being hospitalised for measles, according to treatment group

On day 1 C_3 values in both groups were depressed compared with controls ($p = .003$) but by day 42 they had attained control values. C_3 values were not significantly different between the treated and placebo groups on days 1, 8 and 42 (Table XVIII, Figure 19).

IL-2 concentrations on admission were also depressed compared with controls ($p = .02$) but by day 8 they had reached normal control values. IL-2 values were not significantly different between the treated and placebo groups on days 1, 8 and 42 (table XVIII, Figure 20).

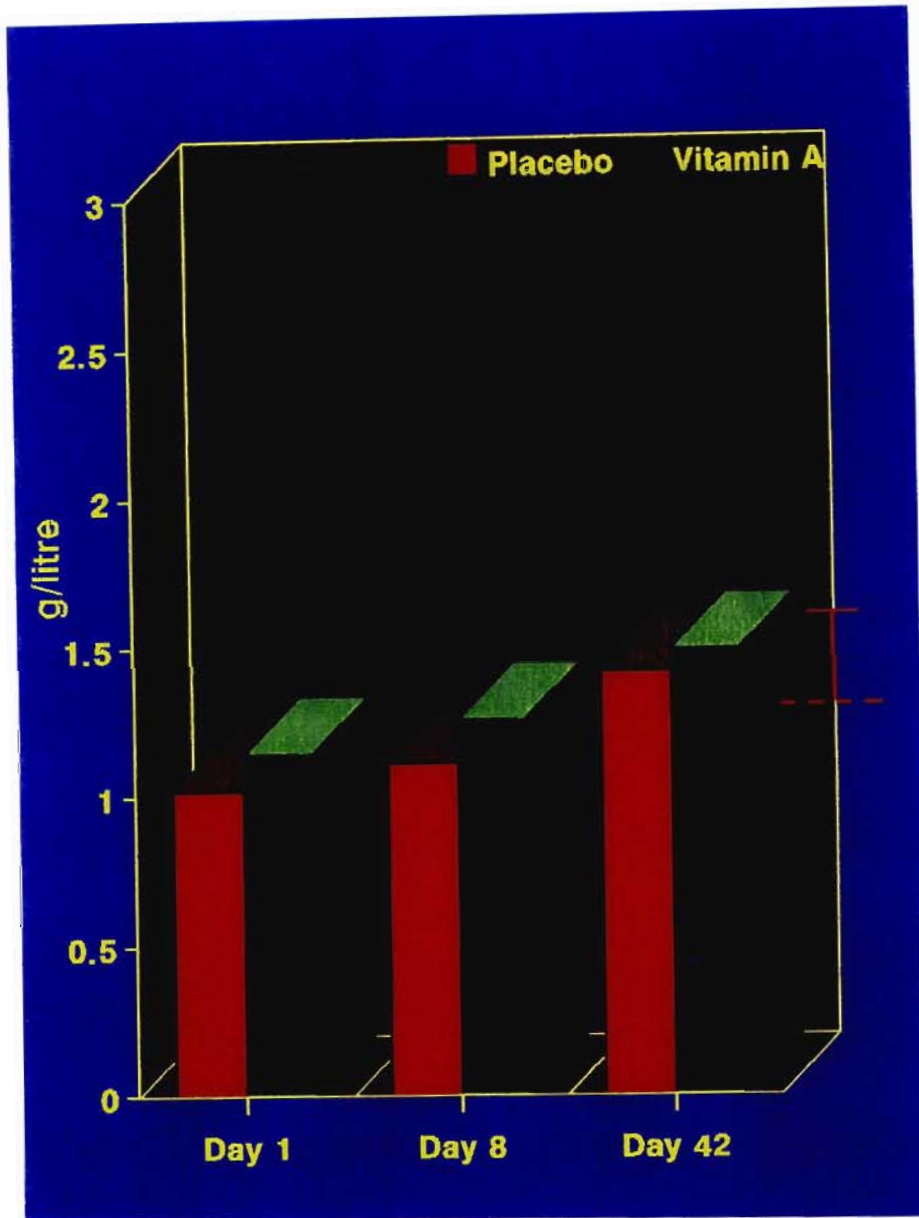


Figure 19. Mean C₃ concentrations, in children on days 1, 8 and 42 after being hospitalised for measles, according to treatment group

Dotted line and T bar represent mean and standard deviation values of healthy controls

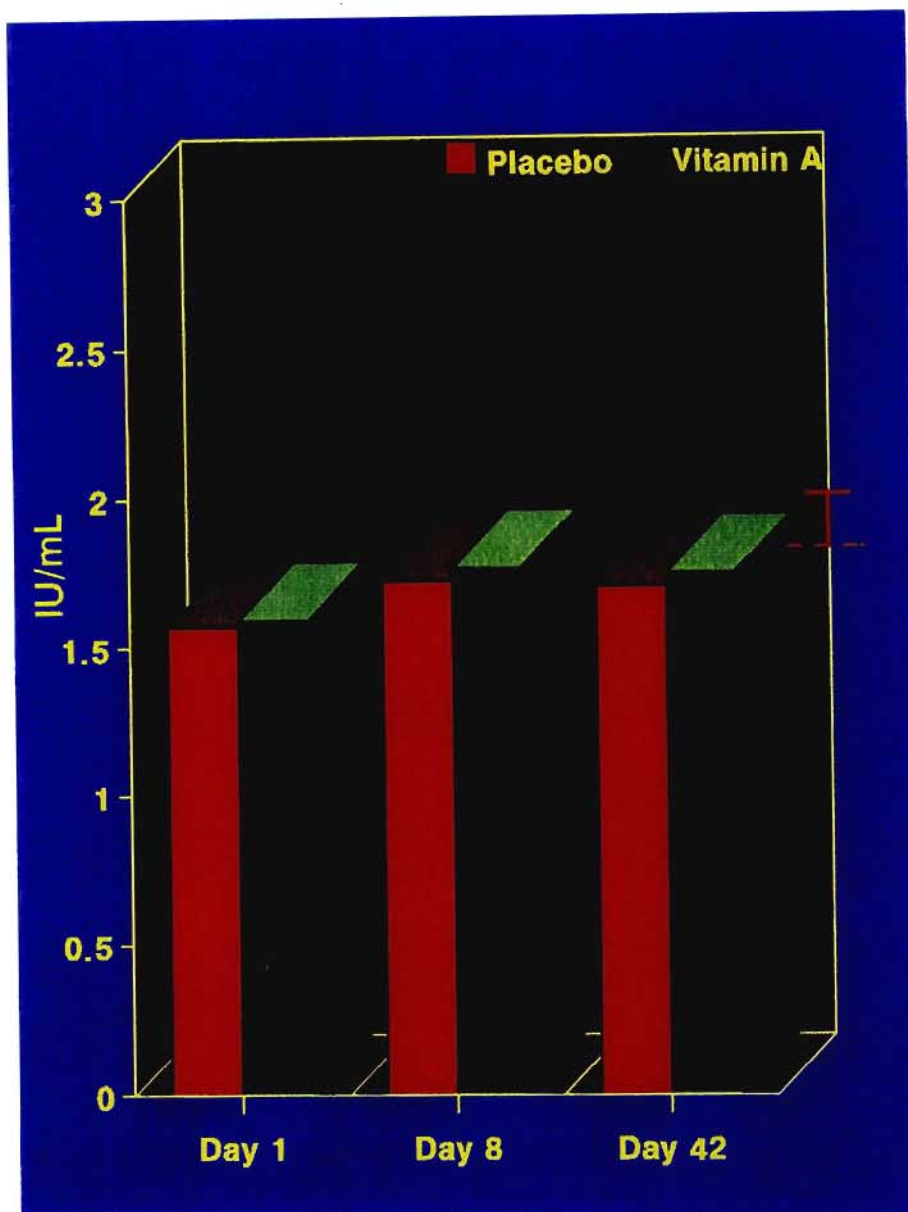


Figure 20. Mean IL-2 concentrations, in children on days 1, 8 and 42 after being hospitalised for measles, according to treatment group
Dotted line and T bar represent mean and standard deviation values in healthy controls

6.4 DISCUSSION

This study has shown that a substantial and sustained reduction in morbidity in vitamin A supplemented African children with measles was paralleled by elements of a more effective immune response. The latter was reflected in higher titers of specific IgG antibodies and a speedier reversal of the lymphopenia induced by virus infection. As there were no significant baseline differences in anthropometric indices and biochemical indicators of protein-energy malnutrition (serum albumin and pre-albumin concentrations), the confounding effect of protein-energy malnutrition on immune responses was limited.

Total lymphocyte numbers and measles IgG antibody concentrations are two factors which are simple measurable indices of a considerably more elaborate network of immune reactions which prevent the initial infection, limit its spread once it has been established and finally eliminate the organism. However, there is clear evidence that specific antibody is of critical importance in measles: it persists for many years and correlates with protection; it is the single most widely utilised parameter of effective vaccination; maternal antibody provides fairly solid immunity to infants and often renders conventional vaccines ineffective; and gamma globulin given during the incubation period aborts measles (Coovadia 1977). Alterations in peripheral blood lymphocyte numbers occur regularly in measles and are due to changes in different sub-populations. The action of these sub-populations, which include HLA restricted cytotoxic T cells, K cells, NK cells and other less clearly defined groups, are believed to be responsible for containing the spread of infection and eliminating the virus. Lymphopenia is not uncommon in severe cases and we have previously shown that this decrease in circulating lymphocytes is mainly due to T helper cells (Kiepiela et al. 1977).

Despite the complexities of the immune response, Coovadia et al. (1978) have shown that in the clinical case of measles, depressed antibody values and lymphopenia are of considerable practical value in predicting outcome; 77% of children who had lymphopenia at onset of rash, died or developed protracted pneumonia, and all who died failed to produce adequate measles antibodies. In addition lymphopenia has been found to correlate strongly with clinical outcome in other infectious diseases besides measles (Barnes et al. 1988).

It has been known for some time that children with secondary immunodeficiency who fail to produce antibody develop fatal measles giant cell pneumonia (Coovadia 1988). As against this C_3 values in measles are much less helpful in making a prognosis of severity (Wesley et al. 1982). Accordingly it is particularly significant that therapy with vitamin A produced an effect on these very components ie. antibodies and peripheral blood lymphocytes.

Measles impairs specific and non-specific immune responses. Accordingly, morbidity and mortality are due to unrestricted measles and to superinfection with opportunistic invaders. Therefore vitamin A supplementation through the augmented immune responses detected here may minimize the harmful effects of measles virus and also of other pathogens. This interpretation would be supported by our findings of the beneficial effect of vitamin A up to 6 months after the rash.

The results of this study suggest that the improvements in morbidity and the changes detected in antibody response and lymphocyte numbers are most likely due to vitamin A supplementation as there were no significant differences between the two groups in all the variables considered.

IL-2 estimations have not been previously employed as a predictive index in measles. However given the central role played by cell mediated immunity (including cytotoxic T cells

and T helper cells and their product IL-2) in controlling measles virus dissemination and killing in the body, we would have expected IL-2 concentrations to accompany improvements in morbidity produced by vitamin A. It is possible that our blood sampling was undertaken at too late a stage (day 8) to detect rapid changes which could possibly have occurred earlier (Griffin 1986). Quantitative values for IL-2 have not been adequately assessed in measles; we have shown here that the IL-2 concentrations in patients were significantly reduced compared with those in healthy controls. This is consistent with our previous observation that T helper cells are depleted during measles. IL-2 is important in the clonal expansion of T helper cells and could therefore play a role in the observed low T helper cell numbers in measles.

With regard to the value of vitamin A intervention in reducing child population morbidity and mortality in regions where xerophthalmia is common, it is pertinent to point out that the beneficial effects on morbidity and aspects of the immune response shown here were produced in an infectious disease which is powerfully immunosuppressive and a major cause of sickness and death in the Third World. It is reasonable to expect more pronounced effects of vitamin A supplementation on immunity associated with improvements in morbidity for the common and less damaging respiratory tract infections and diarrheal diseases of infancy and childhood in developing countries.

The results reported here are consistent with animal studies on the effects of vitamin A on immune function (Jurin and Tannock 1972, Cohen and Elin 1974, Nauss et al. 1979). However one clinical trial among Bangladeshi children failed to show any effect of vitamin A supplementation on antitetanus toxoid antibody titers (Brown et al, 1980). Subsequent to the results of this study being published (Coutsoudis et al. 1992) other studies have confirmed the positive effect of vitamin A supplementation on immune responsiveness in children; Semba et

al. (1992) showed that vitamin A deficient children had smaller IgG responses to tetanus toxoid compared to children who had been supplemented with vitamin A; in addition Semba et al. (1993) also showed in a randomized double-masked, placebo controlled trial that vitamin A deficient children had underlying abnormalities in T-cell subsets and that these abnormalities were reversed with vitamin A supplementation.

The exact pathway by which vitamin A might improve aspects of the immune response noted in this study are unclear: likely mechanisms are more effective specific immunity due to improved T helper cell-mediated antibody synthesis (West et al. 1989) and adjuvanticity (Dresser 1968). This present study and Semba et al.'s study (1993) seem to suggest that a possible mechanism for the action of vitamin A is through enhancement of lymphopoeisis, as suggested in a review by Ross (1992).

This study confirms and extends previous observations which have shown that the immunoparesis secondary to measles is transient and that normal values for most tests of immune function are attained by about 6 weeks after the exanthematous phase of the disease (Wesley et al. 1978).

In conclusion vitamin A supplementation had an effect on immune function as shown by speedier reversal of measles induced lymphopenia and by improvement of the measles IgG antibody response during the acute phase. The effect of supplementation on measles antibody is relevant to the WHO Expanded Programme for Immunisation, which recommends that children in vitamin A deficient communities should be given vitamin A at the time of immunisation. A heightened antibody response could lead to a reduction in the number of vaccine failures which occur in developing countries.

6.5 SUMMARY

The effect of vitamin A supplementation on selected factors of immunity was tested in African children (aged 4 to 24 months with complicated measles) during a randomized double-blind intervention trial. Placebo (n = 31) and treated groups (n = 29) had similar baseline characteristics. The supplemented group had significant reductions in morbidity (expressed as Integrated Morbidity Scores - IMS) during the acute (day 8, p = .006) and chronic phases (day 42, p = .02; 6 months, p = .002). In the treated group there was an increase in total number of lymphocytes (day 42, p = .05) and measles IgG antibody concentrations (day 8, p = .02), both of which have been consistently shown to correlate more closely with outcome in measles than other immunological, clinical and radiological factors. Interleukin-2 (IL-2) and plasma complement (C₃) values were unaffected by vitamin A supplementation. These findings reinforce results from animal studies which show that the pathways of vitamin A activity in decreasing morbidity and mortality are partly founded on selective immunopotentialiation.

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CHAPTER 7

Interrelationships between Vitamin A and other micronutrients in measles patients

CHAPTER 7

INTERRELATIONSHIPS BETWEEN VITAMIN A AND OTHER MICRONUTRIENTS
IN MEASLES PATIENTS

7.1 INTRODUCTION

Vitamin A supplementation was shown in Chapter 5 to reduce the severity of measles complications and to reduce post-measles morbidity for up to 6 months. What was surprising was the fact that the children involved in the study were drawn from a background where overt vitamin A deficiency is not prevalent. It was therefore decided to investigate what effect measles infection was having on the vitamin A levels in blood.

Vitamin A is stored in the liver but is dependent on hepatic retinol binding protein (RBP) for mobilisation into the plasma where it travels as a RBP complex in association with prealbumin to target tissues (Solomons et al. 1980). RBP synthesis in turn appears to be dependent on an adequate zinc (Zn) intake (Solomons et al. 1980). Zinc deficiency is often associated with low concentrations of circulating vitamin A (retinol) despite normal stores, suggesting the defect is not in the absorption or transport of vitamin A to the liver but in its mobilisation (Smith et al. 1973, Solomons et al. 1980). Hustead et al (1988) showed that supplementing pre-term infants with zinc led to an increase in serum retinol levels which they hypothesised was mediated by increased production of RBP and thus enhanced mobilisation of retinol. There also appears to be a parallel but tenuous relationship between vitamins E and A (Jagadeesan and Reddy 1978). Therefore in addition to investigating the effect of measles infection on the vitamin A levels in the blood this study also set about to investigate the micronutrients which could affect vitamin A levels during and after measles infection and also the changes induced in them by vitamin A supplementation.

7.2 METHODS

7.2.1 Patients

60 measles patients (4-24 months of age) with diarrhoea and pneumonia complications severe enough to warrant admission to hospital. The children were part of a vitamin A intervention trial discussed in chapter 6. The children were randomly allocated to receive placebo syrup (31 patients) or vitamin A supplementation (29 patients). The vitamin A dosage which was administered was the dose recommended by the World Health Organisation (Expanded Programme of Immunization 1987).

7.2.2 Controls

These were 20 healthy and adequately nourished children from the same age range some of whom were part of an immunisation study and some were due for minor surgery eg circumcision or hernia. All blood samples were taken before any surgical procedures and after consent had been obtained.

7.2.3 Serological investigations

On admission, day 8 and day 42, a blood sample was taken by venipuncture. Serum was separated within 5 hours, aliquoted and stored at -70°C until analysed. Serum had been stored between 1 and 10 months before analysis. During all procedures, blood samples were protected from sunlight to prevent photodecomposition of vitamin A. The three consecutive samples of each patient were analyzed on the same day. Approximately equal numbers of placebo and treated patients and controls were also analysed on the same day.

Zinc was measured by atomic absorption spectrophotometry (Varian Atomic Absorption Spectrophotometer - AA-1275 Series) in the routine King Edward Hospital Pharmacology laboratory. Zinc was not measured in all the children because of insufficient serum.

Vitamin A (as retinol) and vitamin E (as alpha-tocopherol) were simultaneously measured by normal phase high pressure liquid chromatography (HPLC) using fluorescence detection. The method has been described in detail in chapter 2, section 1.

Albumin and prealbumin were determined by laser nephelometry in the routine biochemistry laboratory attached to King Edward Hospital.

RBP levels were determined by commercially available radial immunodiffusion kits LC-Partigen Plates (Behringwerke AG, Marburg). The method has been described in detail in chapter 2, section 2.

7.2.4 Ethical approval

The study protocol was approved by the University of Natal Medical Faculty's Ethics and Research Committee.

7.2.5 Statistical methods

Differences in the means were examined by the Student's two-tailed T test (Tables XIX and XX) except for the difference in the mean levels of zinc on day 42 which was examined by means of a nonparametric test (Wilcoxon 2 Sample Test) because of small numbers. Because of intrasubject variability in serum micronutrient levels it was decided that in addition to comparing mean values for each group at specific times changes in concentration for each patient would also be analyzed. Thus each child had

a baseline determination on day 1 (admission day) from which a change in concentration at a specific time could be calculated. Individual changes in concentration were summed and a mean value for change in concentration for each group was determined and expressed as a delta (Δ) value. All values are reported as mean \pm standard error of the mean (SEM). The level of significance used was 5%. Spearman correlation coefficients were used to perform a correlation analysis between different serum levels. The software used for the computerised analysis of results was Statistical Analysis System (SAS) - Release 6.03 edition, 1988 SAS Institute Inc, Cary, NC, USA).

7.3 RESULTS

7.3.1 Measles patients compared to controls

The serum levels of zinc, vitamins A and E, albumin, prealbumin and RBP on admission were all significantly lower in the children with measles (both groups) as compared to healthy controls (Table XIX, Figure 21).

Table XIX. Serum micronutrient levels in healthy controls and in measles patients (on admission)*

	Measles Patients	Controls	Significance
n	60	20	
Vit A $\mu\text{g/dL}$	11.96 \pm .74	24.33 \pm 1.74	p = .0001
RBP md/dL	1.48 \pm .09	3.61 \pm .12	p = .0001
Prealbumin mg/dL	9.35 \pm .42	14.80 \pm .53	p = .0001
Albumin g/l	30.90 \pm .85	34.07 \pm .89	p = .006
Zn $\mu\text{mol/L}$	13.02 \pm .31	19.45 \pm .77	p = .0001
Vit E mg/dL	.49 \pm .02	.63 \pm .16	p = .004

* Data presented as mean \pm SEM

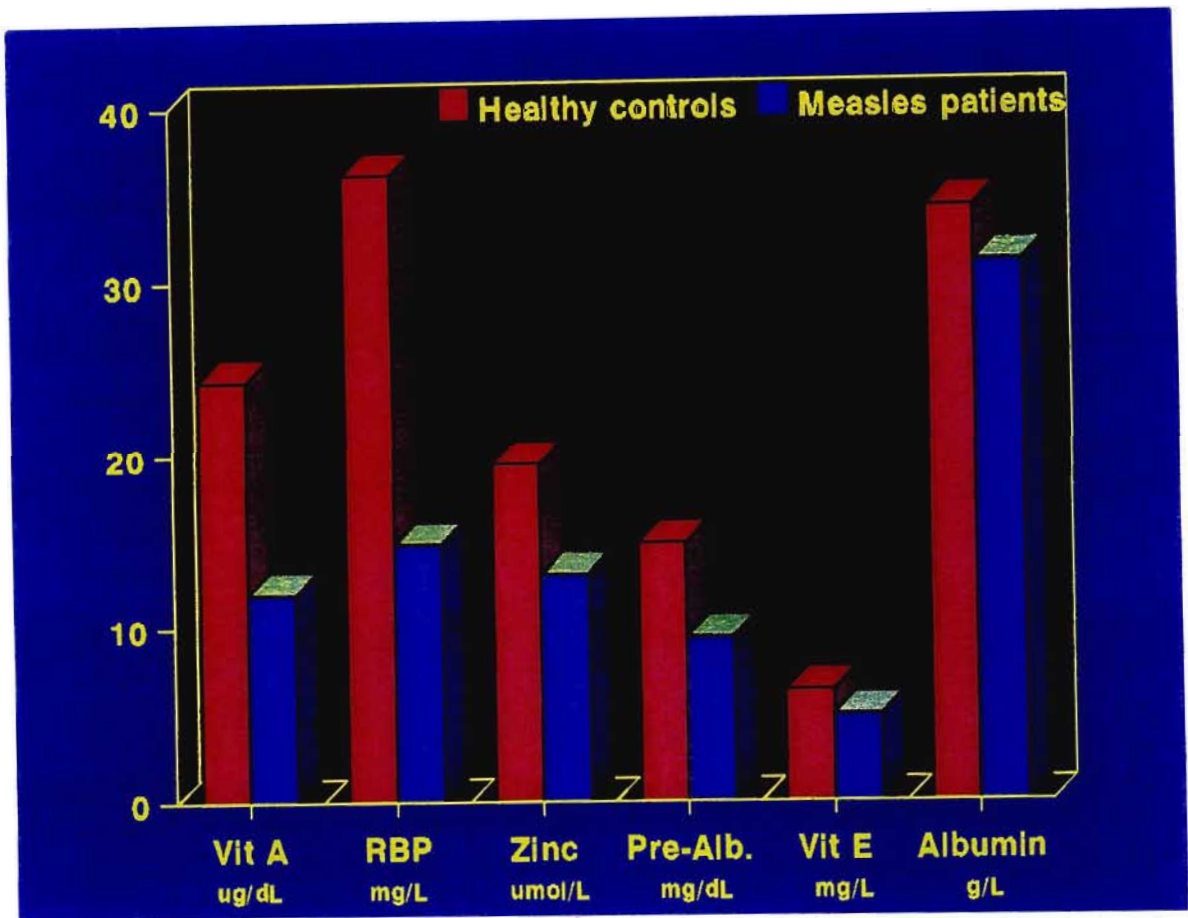


Figure 21. Mean serum micronutrient concentrations in healthy controls and in measles patients on hospital admission (day 1 of the study)

7.3.2 Comparison between vitamin A and placebo treated measles patients

On admission the baseline characteristics (age, sex and socioeconomic data) and serum micronutrient levels of the placebo and vitamin A treated groups were similar (see chapter 5, section 3.1).

7.3.3 Sequential changes in micronutrients in measles patients

The levels of the micronutrients investigated had increased significantly in both groups to reach control values by day 8 except for albumin which attained these levels by day 42 (Table XX, Figure 22). The Zn levels were similar on day 8 in both groups but on day 42 the Zn levels were significantly lowered in the vitamin A group ($14.14 \pm .46$ vs $16.17 \pm .86$ $\mu\text{mol/L}$, $p = .047$). However the mean values for day 42 - day 1 were similar in the two groups ($p = .16$). On admission 90% of the measles patients had hyporetinemia (serum retinol < 20 $\mu\text{g/dl}$). On day 8 the supplemented group had a significantly higher level of vitamin A than the placebo group (38.49 ± 2.99 vs 29.04 ± 2.18 $\mu\text{g/dL}$, $p = .007$). This difference was still observed when we considered the mean values for day 8 - day 1 (25.96 ± 3.27 vs 17.34 ± 1.87 $\mu\text{g/dL}$, $p = .03$). However by day 42 there was no significant difference between placebo and vitamin A treated group. On day 8 prealbumin levels were significantly higher in the vitamin A group compared to the placebo group (18.57 ± 1.34 vs 14.90 ± 1.07 mg/dL , $p = .037$). However when we considered the mean values for day 8 - day 1 (9.07 ± 1.45 vs 5.53 ± 1.16 mg/dL) the difference escaped significance ($p = .06$). By day 42 there was no longer any significant difference between the groups.

Table XX. Serum levels in children on days 1, 8 and 42 after being hospitalised for measles (according to treatment group)*

	PLACEBO GROUP			VITAMIN A GROUP		
	DAY 1	DAY 8	DAY 42	DAY 1	DAY 8	DAY 42
n	31	10	18	29	7	21
Zn ($\mu\text{mol/L}$)	13.44 \pm .43	17.35 \pm 1.42	16.17 \pm .86+	12.55 \pm .43	17.64 \pm 1.55	14.14 \pm .46+
n	31	30	21	29	28	21
Vitamin E mg/dL	.49 \pm .04	.62 \pm .04	.68 \pm .07	.49 \pm .04	.71 \pm .05	.60 \pm .06
Vitamin A $\mu\text{d/dL}$	11.73 \pm 1.05	29.04 \pm 2.18†	28.49 \pm 2.36	12.22 \pm 1.05	38.49 \pm 2.99†	31.05 \pm 3.23
RBP mg/dL	1.46 \pm .14	3.27 \pm .21	2.74 \pm .16	1.49 \pm .12	3.83 \pm .27	3.05 \pm .23
Prealbumin mg/dL	9.26 \pm .70	14.90 \pm 1.07§	13.85 \pm .91	9.45 \pm .46	18.57 \pm 1.34§	14.65 \pm 1.30
Albumin g/L	31.84 \pm .65	29.24 \pm .79	35.10 \pm 1.32	30.10 \pm 1.05	30.11 \pm 1.15	36.50 \pm 1.52

- * Data presented as mean \pm SEM
+ Significant difference between day 42 means, p = .047
† Significant difference between day 8 means, p = .007
§ Significant difference between day 8 means, p = .037

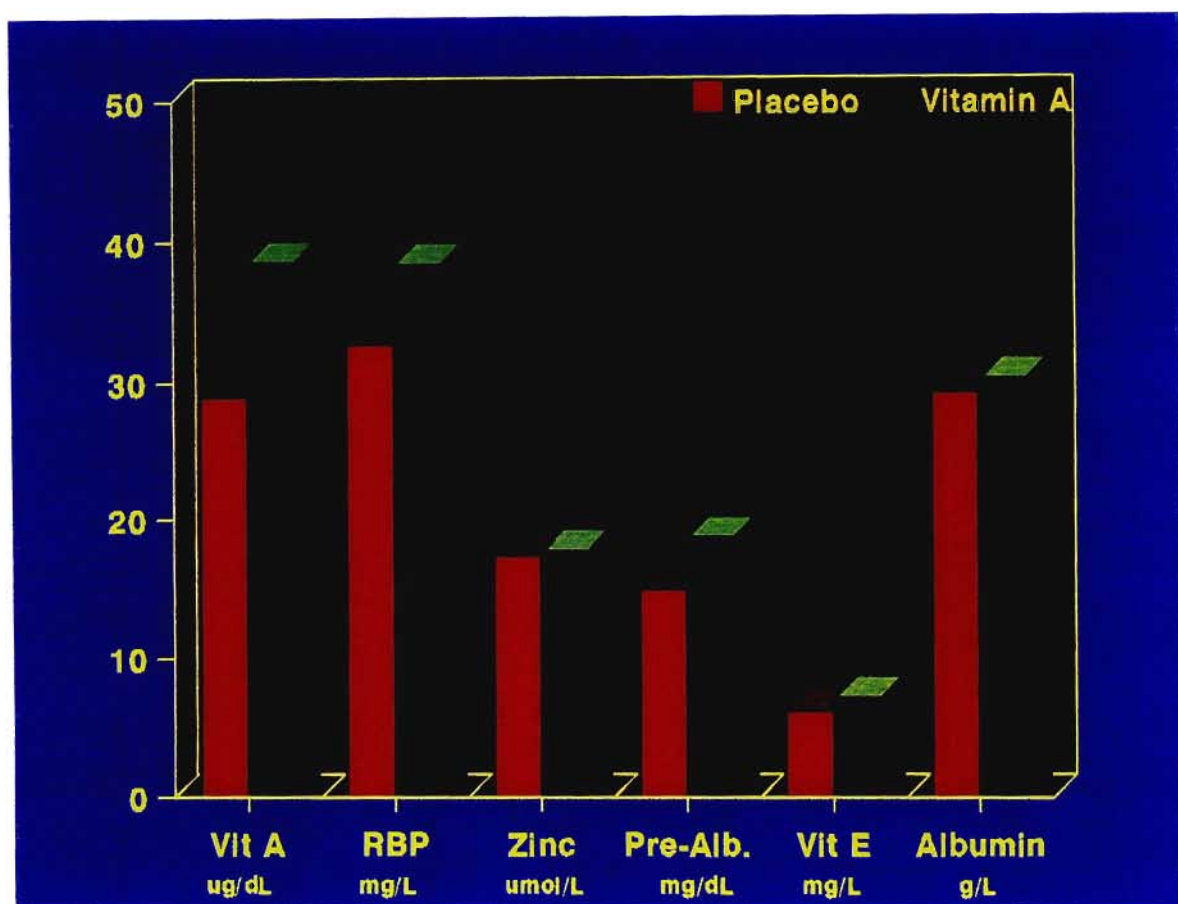


Figure 22. Mean serum micronutrient concentrations in children 8 days after being hospitalised for measles (according to treatment group)

7.3.4 Correlations between micronutrients in measles patients and controls

Examining the serum levels of the measles patients before intervention, and those of the healthy controls it was seen that vitamin A correlated with RBP ($r = .8$, $p = .0001$), with prealbumin ($r = .6$, $p = .0001$) and with zinc ($r = .6$, $p = .0001$). The correlation between vitamin A and prealbumin was even closer on day 8 ($r = .85$, $p = .0001$). RBP correlated with zinc ($r = .7$, $p = .0001$) and prealbumin ($r = .6$, $p = .0001$); the latter correlation was better ($r = .7$, $p = .0001$) at higher concentrations of prealbumin (viz. on day 8). There were no other significant correlations between the micronutrients studied.

7.4 DISCUSSION

The results verify the work of others that during stress of infection the levels of serum micronutrients are depressed due to lowered food intake (Duggan et al. 1986) and alterations in their metabolism (Coovadia 1985). All the micronutrients we tested were significantly reduced compared to healthy control values. As infection resolves these micronutrients return to normal levels. By day 8, when the majority (81%) of the children had recovered clinically, all the micronutrients had returned to normal values except for albumin which only did so by day 42. A study on Ugandan children with measles also showed reduced levels of serum albumin during measles and a return to normal levels only six weeks later (Poskitt 1971).

During measles there is an increased production of protein containing immune complexes which increases the body's requirement for zinc (for protein synthesis) at a time when the dietary intake has been shown to be lowered. In addition there is evidence that during stress Interleukin-1 appears to stimulate a redistribution of plasma zinc to the liver

resulting in a sequestration of some of the circulating zinc (Anon 1989, Cannon and Kluger 1983). This temporary lowering of plasma zinc could explain the lowered levels of RBP and prealbumin as zinc is essential for their synthesis. This in turn would affect the mobilisation of retinol from the liver. The importance of RBP for retinol transport is illustrated by the case report of a child with familial hypo-RBP who presented with clinical signs of vitamin A deficiency. Despite adequate intake of vitamin A the child had consistently low serum levels of RBP and consequently of vitamin A (Matsuo et al. 1988). Furthermore studies have shown that zinc supplementation (apart from increasing serum zinc levels) is effective in increasing serum retinol levels (Hustead et al. 1988).

The relationship between zinc, vitamin A, RBP and prealbumin which has been hypothesised by several workers is borne out by the correlations we found amongst these nutrients. The relationship between vitamin A and prealbumin is further strengthened by the fact that both vitamin A and prealbumin serum levels were significantly increased in the treated group on day 8.

Vitamin E levels were also significantly reduced in the measles patients on admission. However using the generally accepted cut off point for inadequate nutriture (.5 mg/dL) we found that 50% of the children had low vitamin E levels compared to 90% who had low vitamin A levels. It appears that measles has a more profound effect on vitamin A which would explain the therapeutic effect of this vitamin which was discussed in chapter 5. There was no significant difference between the two groups in vitamin E levels at any stage.

The low serum retinol levels which we observed on admission of the measles patients confirms the findings of other workers (Inua et al. 1983, Bhaskaram et al. 1984, Reddy et al. 1986, Frieden et al. 1992). By day 8 the serum retinol levels in both groups had returned to normal values and these levels were

still maintained by day 42. The fact that even the unsupplemented group had normal serum retinol levels by day 8 implies that the low serum retinol levels observed during the acute phase of the infection (day 1 of the study) were transient and due to a mobilisation defect, possibly mediated through a temporary lowering of zinc and consequently of RBP as discussed earlier and not due to pre-existing deficiency resulting from low liver stores.

On admission the serum retinol levels of the two groups of measles patients were similar, however by day 8 the vitamin A treated group had the expected increased levels compared to the placebo group. This significant difference between the groups was maintained when we compared the mean change in serum retinol from baseline to day 8. This confirms the finding that vitamin A supplementation increases the serum retinol levels in children who have low levels prior to supplementation (James et al. 1984, Bergen et al. 1988). On day 42 in the vitamin A treated children there was no further increase in the serum retinol levels (in fact there was a decrease). This probably resulted from excess vitamin A being stored in the liver as a result of homeostatic control. Presumably when preexisting serum levels are normal supplementation will not cause an increase. This supports the results of a study amongst well nourished adults which showed that vitamin A supplementation did not cause an increase in serum retinol levels (Willett et al. 1983). The fact that the serum levels are lowered during measles regardless of adequate liver stores explains why vitamin A supplementation is effective in reducing mortality and morbidity from measles even in areas where the incidence of clinical vitamin A deficiency is not high (Hussey and Klein, 1990). Our own study, in an area where xerophthalmia is also not frequent, also showed that vitamin A supplementation was effective in significantly speeding up recovery from measles and in reducing morbidity for up to six months (details presented in chapter 5).

This present study therefore suggests that although there is now sufficient evidence to justify vitamin A supplementation during measles, it would not be useful (and in fact could be harmful) to give excessive doses of vitamin A prior to illness, in vitamin A replete individuals, in the hope of preventing hyporetinaemia during measles.

7.5 SUMMARY

Micronutrients (zinc, vitamins A and E) and related proteins (retinol binding protein, prealbumin, albumin) were measured in the serum of African children with measles, and the changes induced in these by vitamin A supplementation (offered in a randomised, double blind, placebo controlled trial) were studied. All these substances were significantly reduced early in the exanthem in measles patients as compared to controls; they attained control values by day 8 after the rash, except for serum albumin which became normal by day 42. Vitamin A and prealbumin levels on day 8 were significantly increased in the supplemented over the placebo group. Vitamin A levels in serum correlated with those of RBP, prealbumin and zinc. These findings strengthen the hypothesis that hyporetinemia during measles is the consequence of impaired mobilisation. The results indicate that the measles patients in this study did not have pre-existing low liver stores.

This present study therefore suggests that although there is now sufficient evidence to justify vitamin A supplementation during measles it would not be useful (and in fact could be harmful) to give excessive doses of vitamin A prior to illness in vitamin A replete individuals in the hope of preventing hyporetinaemia during measles.

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CHAPTER 8

Interrelationships between
Vitamin A
and other factors
in children
living in an informal settlement

CHAPTER 8

INTERRELATIONSHIPS BETWEEN VITAMIN A AND OTHER FACTORS IN CHILDREN LIVING IN AN INFORMAL SETTLEMENT

8.1 INTRODUCTION

In Chapter 4 a study assessing the vitamin A status of children living in an informal settlement was described. However assessing single nutrient deficiency can be misleading. Implementing an intervention strategy requires additional information about other factors which are involved in order to formulate an appropriate strategy for a particular setting (Rifkin and Walt 1986, Pinstrup-Andersen 1991). Therefore a supplementary study was set up to investigate other nutritional factors which could have an interrelationship/interaction with the vitamin A status.

8.2 METHODS

8.2.1 Subjects

The subjects included randomly selected preschool children living in Besters Farm, a typical peri-urban informal settlement in South Africa. Full details of the 190 preschool children (3 - 6 years of age) who were included in this study are to be found in Chapter 4, section 2.1.

8.2.2 Anthropometry

All children were brought to the newly constructed clinic in the community, on a pre-decided date. Each child was weighed without shoes to the nearest 0,5 kg on a bathroom scale which was calibrated using a known weight. Height was measured to the nearest 0,5 cm by means of an anthropometer comprising a wooden platform with a scale of 2 metres and a sliding head piece.

Weight-for-age (WA), height-for-age (HA) and weight-for-height (WH) percentiles were determined from the National Center for Health Statistics (NCHS) tables (World Health Organization 1983). Standard deviation scores (Z-scores) were calculated for each child from the NCHS tables. A ZWA score of 2.00 or -2.00 means that the child is 2 SD above or below the median WA respectively, while a ZHA refers to the Z-score for HA and ZWH refers to the Z-score for WH.

8.2.3 Investigations

Blood samples were taken by ante-cubital venipuncture. These samples were used to determine haematological factors and various micronutrients viz. vitamins A and E, calcium (Ca), magnesium (Mg), phosphorus (Ph), albumin, haemoglobin, serum iron (Fe), serum ferritin, percent transferrin saturation (% TS) and mean cell volume (MCV). The sera were kept frozen at -20°C until they were analysed. All samples were analysed within 3 months of collection.

Serum samples for vitamin A & E analysis were posted on dry ice to Cape Town, South Africa where the analysis was performed in the Department of Human Nutrition, University of Stellenbosch, using a modified version of the method of Catigniani et al (1983). During processing and analysis, precautions were taken to protect the serum from light as vitamin A is sensitive to photodestruction.

Haematological factors were determined in the routine Haematology laboratory attached to King Edward VIII Hospital, Durban. Plasma Ca, Mg and Ph were measured by the standard technique on a Beckman Synchron CX5 (USA). Plasma albumin was measured by the bromocresol purple method. Serum ferritin was measured by radioimmunoassay (Amersham, UK). Anaemia was defined by haemoglobin < 11

g/dL according to the WHO criteria for children up to 6 years of age at sea level. Serum ferritin levels < 10 μ g/dL and transferrin saturation values < 10% were regarded as indicative of exhausted iron stores (Milman and Cohen 1984, Dallman 1980).

The normal reference values for Ca, Mg, Ph and albumin were taken from a South African paediatric reference population (Pettifor et al. 1978) and are as follows: Ca 2.25 - 2.62 mmol/L; Mg 0.75 - 1.05 mmol/L; Ph 1.30 - 1.80 mmol/L; albumin > 35 g/L. The generally accepted cut off values for inadequate nutrition of vitamins A and E are 20 μ g/dL and 5 mg/L respectively (ICNND 1963).

Stool samples were collected from a small subsample (75) of children and tested for presence of parasites.

8.2.4 Statistical methods

Continuous variables are presented as mean \pm SD (standard deviation). Descriptive statistics were used and Pearson correlation coefficients were employed to examine the relationships between all the variables considered. The software used for the computerized analysis of results was the Statistical Analysis System (SAS), release 6.03 edition, 1988 (SAS Institute Inc, Cary, NC).

8.3 RESULTS

Demographic and anthropometric data on the 190 children in the study are presented in Table XXI. Anthropometric data are also depicted in Figure 23.

Table XXI. Demographic profile of subjects (n = 190)

AGE (years)	No.	%
3	47	25
4	61	32
5	40	21
6	42	22
SEX		
Male	91	48
Female	99	52
ANTHROPOMETRY		
Weight/Age < 3rd centile*	24	13
Height/Age < 3rd centile*	51	27
Weight/Height < 3rd centile*	7	4

* National Center for Health Statistics (NCHS) standard

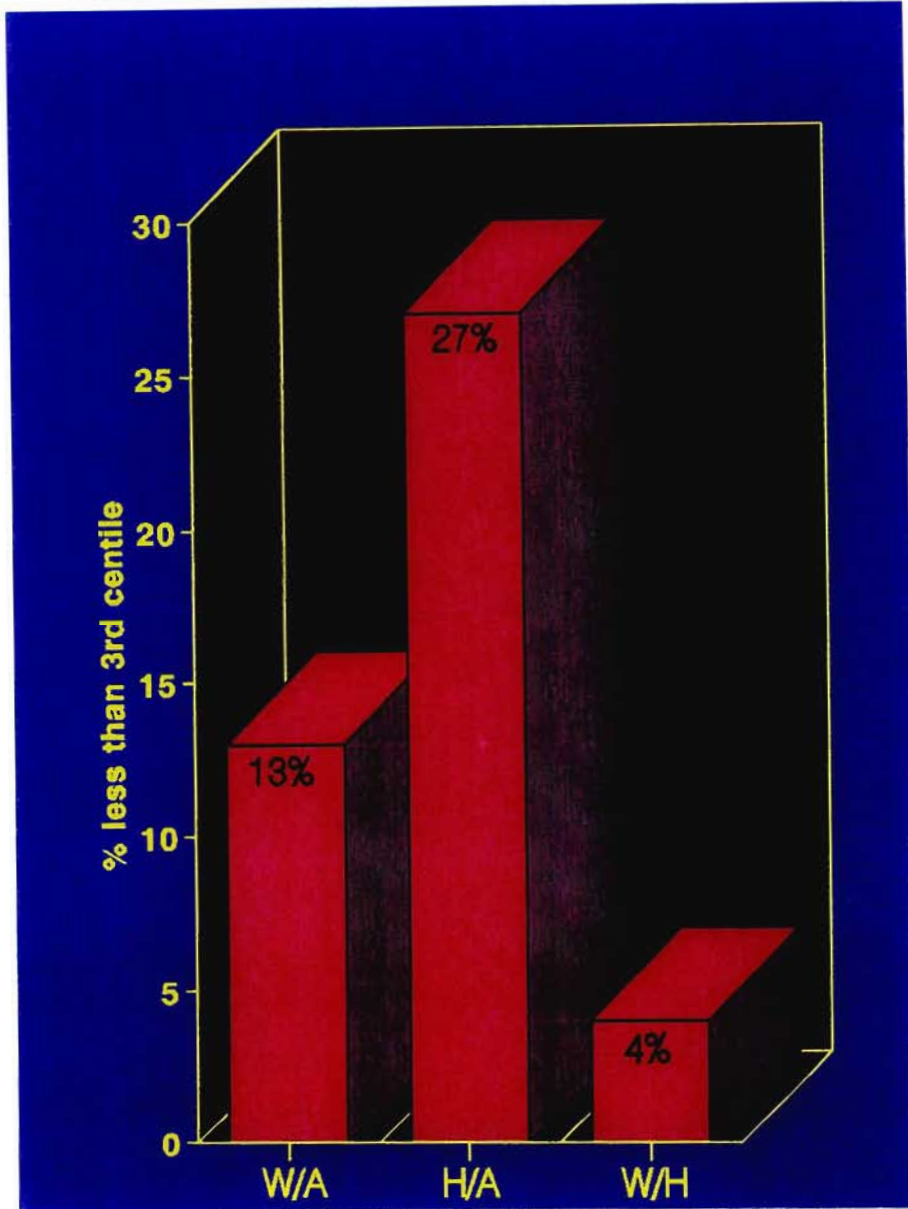


Figure 23. Percentage of children < 3rd NCHS weight for age (WA); height for age (HA); and weight for height (WH) centiles

Five of the 190 children refused to co-operate for venipuncture and in 8 children a blood sample was not obtained in the first attempt and no repeat attempts were made. Not all the children had sufficient unclotted blood and/or serum for all the biochemical investigations - the numbers sampled for each investigation are shown in Table XXII.

The mean values for all the biochemical parameters are shown in Table XXII. Concentrations of albumin, vitamin E, Ca, Mg, and Ph were close to the normal values with not more than 10% of the sample having values outside the normal range. The micronutrients which were out of the normal range were vitamin A and Fe. The mean serum retinol for the whole population was $20.8 \pm 7.4 \mu\text{g/dl}$ (mean \pm SD). Nine children (5%) had vitamin A deficiency as assessed by serum retinol levels $< 10 \mu\text{g/dl}$ and 75 children (44%) had low levels i.e. $< 20 \mu\text{g/dl}$ (IVACG 1977). 21% of the children had anaemia as classified by Hgb levels $< 11 \text{ g/dL}$. Parameters which confirm that the anaemia could be due to iron deficiency are the MCV, serum iron and % transferrin saturation all of which were low in a relatively large percent of the population. In addition there were positive correlations between these parameters; MCV correlated with % transferrin saturation ($r = .25, p < .01$) and with haemoglobin ($r = .47, p < .0001$). Haemoglobin correlated with % transferrin saturation ($r = .31, p < .001$). However a much smaller proportion of the children had low serum ferritin levels.

The interrelationships between the different variables investigated is shown in Table XXIII. There was a significant positive correlation between serum retinol and all the biochemical indicators of Fe status except for serum ferritin.

Stool examination of a subsample of 75 children showed that 91% had parasites; the majority of them having multiple parasites with the most common ones being ascaris (59% frequency), trichuris (61% frequency), and Giardia lamblia (31% frequency).

Table XXII. Definitions of some nutrients in blood and serum of children living in an informal settlement

	n	Mean \pm SD	Normal threshold	% below threshold
Calcium (mmol/L)	95	2.47 \pm 0.09	\geq 2.25	1%
Magnesium (mmol/L)	95	0.87 \pm 0.06	\geq 0.75	1%
Phosphorus (mmol/L)	95	1.45 \pm 0.19	\geq 1.3	10%
Albumin (g/L)	95	39.8 \pm 4.0	\geq 35	6%
Retinol (μ g/dL)	169	20.8 \pm 7.4	\geq 20	44%
Vitamin E (mg/L)	169	8.1 \pm 2.3	\geq 5	5%
Haemoglobin (g/dL)	168	11.7 \pm 1.2	\geq 11	21%
MCV (fL)	168	80.5 \pm 4.9	\geq 77	14%
Ferritin (μ g/dL)	138	30.7 \pm 20.4	\geq 10	8%
Serum iron (μ mol/L)	137	11.2 \pm 5.6	\geq 9	36%
% Transferrin Sat.	114	16.6 \pm 8.9	\geq 10	27%

Table XXIII. Coefficient of correlation (r) between biochemical and anthropometric parameters.

	1	2	3	4	5	6	7	8	9	10
1. Haemoglobin										
2. Mean cell volume	.47§									
3. Haematocrit	.96§	.42§								
4. Serum ferritin	-.07	.12	-.02							
5. Transferrin saturation	.31†	.25+	.30†	.01						
6. Serum iron	.30†	.21+	.28†	-.07	.96§					
7. Serum retinol	.25†	.18*	.21+	-.09	.34†	.32†				
8. Vitamin E	.11	-.01	.10	-.08	.25+	.26+	.28†			
9. ZWA	.01	-.03	.03	.17	.19	.15	.13	-.12		
10. ZHA	.00	-.06	.00	.14	.10	.13	.07	.01	.65§	
11. ZWH	.02	.02	.03	.08	.14	.06	.14	-.15	.68§	-.09

* p < .05

+ p < .01

† p ≤ .001

§ p = .0001

8.4 DISCUSSION

Acute malnutrition (< 3rd NCHS WA) was found in 12.6% of the children, although acute severe malnutrition was not a problem with only 3.6% below the 3rd NCHS WH. However the figure of 26.8% below the 3rd NCHS HA indicates fairly high levels of chronic malnutrition. This finding is not surprising given the general acceptance that stunting is an indicator of environmental conditions in which food security is constantly threatened and where there are frequent infections. The consequences of unchecked rapid urbanisation are in fact the establishment of informal settlements with poor socio-economic and environmental conditions. The percentage of underweight and wasted preschool children in our study is similar to that found presently in other informal settlements in South Africa. In a Cape Town squatter settlement (Hugo-Hamman et al. 1987) these were 13.7% and 1.0% respectively and in a Durban informal settlement (see number 2 on the map provided in Appendix i, Chapter 3) these were 14% and 0% respectively (Peberdy 1991). Stunting was also much more frequent in these studies. These findings are in keeping with the national data for South Africa which reveal a high level of chronic malnutrition among black children. Current understanding of the causes of PEM suggests that it is the end result of a number of macro-economic, social and political causes. The proximate causes however are inadequate dietary intake and frequent infections (United Nations 1991).

The results reveal poor vitamin A status, with 44% of the population having serum retinol levels below 20 $\mu\text{g}/\text{dL}$. Although none of the children had obvious clinical signs of vitamin A deficiency this large percentage with low serum retinol levels implies that a sizeable proportion of the population has marginal vitamin A deficiency which places them at risk for respiratory and diarrhoeal infections (Sommer et al. 1984). It is likely that this poor vitamin A status is partly explained by inadequate dietary intake and partly by the frequent

infections experienced by the children due to the poor sanitary conditions.

The iron status of the children was poor with relatively large numbers of the children having low Hgb, MCV, serum Fe and % transferrin saturation. Given the prevalence of PEM and single nutrient deficiencies it is likely that one of the causes of the poor iron status is inadequate iron in the diet. This poor iron status could however be exacerbated by factors other than nutritional deficiencies, such as the high prevalence of parasites especially trichuris in this situation. It was surprising that no children had hookworm infestation (the more common parasite associated with Fe deficiencies) even though it is prevalent in Natal/KwaZulu. The high prevalence of parasites could be another factor which affected the vitamin A status of the children; worm infestation has been shown to impair absorption of vitamin A (Sivakumar and Reddy 1975; Mahalanabis et al. 1976, 1979; Tomkins 1979); particularly in patients with a high parasite load (Brown et al. 1980). In addition Sturchler et al. (1987) found that following treatment for Giardia lamblia, the average retinol levels increased in those with confirmed infections.

Several epidemiological studies have indicated that vitamin A deficiency and anaemia often coexist and that there are significant associations between serum retinol and biochemical indicators of Fe status (Moharam et al. 1977, Meija et al. 1977, Meija and Arroyave 1982). Our results seem to confirm these findings and imply that the relatively high prevalence of anaemia could also be vitamin A related. The exact mechanism by which vitamin A influences Fe status is not clear although vitamin A seems to be essential for the differentiation of red blood cells, which is suggested by the association between retinol and haematocrit. It is also likely that the increased susceptibility to infections due to vitamin A deficiency could affect haematopoiesis. This association is of great public health significance because vitamin A and Fe deficiencies are 2 of the 3 important micronutrient deficiencies affecting

children of developing nations; such data highlight the importance of ensuring children have an adequate vitamin A status.

This study highlights the interrelationship among some nutrient deficiencies and emphasizes the fact that they are interwoven with other factors in a seamless fabric of deprivation. Thus attempts at correcting the poor vitamin A status in isolation should take this into account and corrective measures should be located within a broad multifaceted comprehensive health and social intervention programme (Rifkin and Walt 1986) else they run the risk of being unsuccessful (Fauveau 1992).

8.5 SUMMARY

The rapid rate of urbanisation in South Africa has led to the creation of informal shack settlements where the health status of children is in jeopardy and needs to be monitored so that appropriate intervention strategies can be formulated.

Accordingly, the nutritional status of 190 children (3-6 years of age) living in Besters, a typical urban shack settlement north of Durban, was assessed by anthropometry. In addition the following biochemical parameters were determined; vitamins A and E, calcium, magnesium, phosphorus, albumin, haemoglobin, serum iron and ferritin and percent transferrin saturation.

Thirteen percent of the children were underweight (below the NCHS (National Center for Health Statistics) third weight-for-age percentile) and 27% were stunted (below the NCHS third height-for-age percentile). Concentrations of albumin, calcium, magnesium, phosphorous and vitamin E were close to normal values with not more than 10% of the sample having values outside the normal range. However 44% of the children had low serum retinol levels ($<20 \mu\text{g/dL}$) and 21% of the children had anaemia ($\text{Hgb} < 11 \mu\text{g/dL}$). Iron status results suggest that most of this anaemia is due to iron deficiency. Significant positive correlations were found between serum retinol and all the biochemical indicators of iron status except for serum ferritin. 91% of a subsample of children had intestinal parasites which could affect the absorption of vitamin A.

This study highlights the interrelationship among some nutrient deficiencies and emphasizes the fact that they are interwoven with other factors in a seamless fabric of deprivation. Thus attempts at correcting the poor vitamin A status in isolation should take this into account and corrective measures should be located within a broad multifaceted health intervention programme.

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