A STUDY OF

IRON NUTRITION AND IMMUNITY IN INFANCY

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ABSTRACT

Motivation and study design

Iron deficiency is a common condition in infancy, particularly in lower socio-economic groups. In Cape Town it remains a problem in spite of public health measures taken against it: a recent survey found a prevalence of iron deficiency anaemia of 34% in healthy 1 year old term infants who had ready access to a municipal health clinic where iron fortified milk formula is sold at subsidized prices.

The consequences of iron deficiency extend beyond anaemia to involve all organ systems including the immune system. Since Helen Mackay's report in 1928 of a striking decrease in incidence of infection in infants treated with iron, clinicians have assumed that iron deficiency predisposes to infection. Despite a sound theoretical basis for this belief, the clinical evidence for the assumption is poor as studies to date have displayed methodological deficiencies.

On the other hand, iron is also essential for the growth of micro-organisms. As such, supplemental iron may predispose to infection. Indeed there is much laboratory and clinical evidence to show that excess iron can result in the recrudescence of quiescent infections and increase the virulence of newly acquired infections. Thus, the competition between host and parasite may sometimes hinge on the relative availability of iron and it has been speculated that excess iron in infant milk formula may increase susceptibility to infectious diarrhoeal disease.

The problem addressed by this thesis was to determine the utility of increasing the level of iron fortification of infant milk formula. Three questions were posed:

Does increasing the level of iron fortification of conventional infant milk formula improve the iron nutrition of normal infants fed on the formula?

Does increased iron fortification of infant milk formula alter immunity as reflected by incidence of infection and laboratory tests of immune function?

Are there any harmful effects of increasing the quantity of iron in conventional infant milk formula?

A double blind randomized trial was carried out in 1983 and 1984 to answer these questions. A group of 149 healthy, well nourished infants from a lower socio-economic community of so called Cape Coloureds were followed from the age of 3 months to 1 year. Half of the infants, the Control group,

were given a commercially available infant milk formula (Lactogen Full Protein) which has 8.3 mg Fe/ 100 g formula and 37 mg ascorbic acid/ 100 g. The other half of subjects, the Test group, were given the same milk formula but fortified with iron to a concentration of 40 mg Fe/ 100 g*. The children were examined every 3 or 4 weeks and any infection or history of infection was noted. Laboratory tests were done at the start of the trial and again on completion. During the trial, laboratory tests were performed only if clinically indicated. The tests included full blood count and differential analysis, red cell zinc protoporphyrin, plasma ferritin, plasma and hair zinc and lymphocyte subtyping with monoclonal antibodies. Within each group, half of the infants were randomly selected for assay of neutrophil bactericidal activity. The other half were assayed for lymphocyte blastogenic response to stimulation with phytohaemagglutinin. Tests of delayed cutaneous hypersensitivity to Candida antigen and PPD were done and all children and their mothers had antibodies to tetanus and polio determined.

Results

74 infants in the Control group started the trial and 62 completed it. In the Test group, 75 infants began and 70 completed the study.

Intake of milk and solid foods was not quantified, but the ages of weaning and of introduction of new foods were determined. The Control and Test groups did not differ significantly on any test item. The mean age of completion of weaning was 3.60 months for the Control group and 4.04 months for the Test group. The Control group was first given meat or fish at a mean age of 5.19 months; the Test group had meat or fish introduced to their diets at a mean age of 4.36 months. These differences were not statistically significant.

The children in the Control group were lighter and shorter than the Test group at the end of the year. Mean standard deviation scores for weight were 0.23 and 0.48 respectively (P = 20%), while for length the SD scores were -0.13 and 0.06 (P = 20%).

Effect of iron fortification on iron status

After 9 months on the milk formulas, the iron nutrition of the Control group was significantly poorer than that of the Test group for several indicators. At the age of 1 year, mean haemoglobin concentrations were 11.49 g/dl and 11.85 g/dl for the Control and Test groups respectively (P = 4%). The red cell width distributions were 15.53% and 14.44% (P = 0.05%). Red cell zinc protoporphyrin

* The milk formulas were made up and donated by Food and Nutritional Products (Pty) Ltd.

levels were 3.95 ug/dl and 3.41 ug/dl (P = 4%) and the geometric mean ferritin levels were 17.3 ug/dl and 29.0 ug/dl for the 2 groups respectively (P = 0.04%).

Effect of iron fortification on incidence of infection and immune function

The results of the individual immune function tests did not differ significantly between the 2 groups. Forty eight hours after the delayed cutaneous hypersensitivity tests the mean diameters of induration were 8.05 mm and 7.03 mm (P = 43%) in the Mantoux test and 8.88 mm and 9.75 mm (P = 56%) in the Candida test for the Control and Test groups respectively.

The geometric mean tetanus and polio antibody titres were 93 and 115 (P = 44%) and 35 and 47 (P = 16%).

Lymphocyte subtypes, *ie* B cells, total T cells, helper-inducer cells, suppressor-killer cells and helper:suppressor cell ratio did not differ between the 2 groups.

The Control group responded less well than the Test group in the lymphocyte phytohaemagglutinin stimulation test and in the bactericidal assay but the difference did not reach statistical significance. At one year of age the PHA stimulation indices were 140% and 142% for the Control and Test groups respectively. In the bactericidal assay, the percentage of the initial inoculum that survived at 1 hour was 105% for the Control infants and 94% for the Test group.

The statistical power of the immune function tests was not higher than 24% and usually well below that level.

The Control group had more infections than the Test group; an average of 6.42 infections per child per year compared with 5.86. When incidence was considered in sub-categories, the Control infants had more infections than the Test group for *Minor*, *Moderate*, and *Severe* infections as well as for gastrointestinal infections, oral thrush, pyoderma, conjunctivitis and *"other"* infections. Only for infections of the respiratory tract did the Control group have a lower incidence than the Test group. No difference came close to reaching statistical significance and the power of the study to detect a real difference equal to that observed was 18%. The 95% confidence interval for the difference in incidence of all infections ranged from -1.61 infections per child per year to 0.49 infections per child per year.

Risks of iron fortification

As zinc and iron compete for absorption it was expected that the Test group would have lower zinc levels than the Control group. This was corroborated by both plasma and hair analyses. Plasma zinc was 90.61 ug/dl in the Control group and 83.53 ug/dl in the Test group (P = 5%). Hair zinc concentrations were 142 ug/g and 129 ug/g respectively (P = 54%).

Apart from the lower mean zinc levels in plasma and hair, the study found no evidence of possibly harmful effects of iron supplementation. In particular, the Test group experienced a lower incidence of diarrhoeal disease than the Control group.

Discussion and Recommendations

Possible confounding factors

The trial of the infant milk formula controlled rigorously for factors which have confounded the interpretation of most previous studies of the role of iron in infection and immunity. The children were selected on the grounds of good health and excellent nutrition. That they maintained this throughout the study may be partly due to the milk formula which was granted to the families, and partly due to the selection of children from better home environments.

Possible confounding factors which may not have been controlled by the stratified allocation procedure were indicated by non-significant trends in completion rate, weaning, and introduction of solid foods. All these factors could weigh in the Test group's favour when considering indices of iron nutrition, measures of immune function or risks of increased iron fortification.

However the weight that should be assigned to these possible confounding influences is limited by several methodological weaknesses. In the subsidiary surveys, ages were calculated from the mother's recollection of events and recorded in months rather than decimals of a year. Also, age of introduction of a food item is likely to be a poor proxy for quantity of food ingested and it gives no indication whether or not the food was consumed with milk formula.

Effect of iron fortification on iron status

The increased iron fortification was shown to improve the iron status of the Test group with statistically significant differences for haemoglobin, red cell distribution width, plasma ferritin and red cell zinc protoporphyrin. The absolute magnitude of the improvement in iron status was small, and the effect may not be clinically important.

Two related questions for further research arise from this aspect of the trial. Why did the Control group not achieve an iron status equivalent to infants in similar trials overseas? And, why did the extra iron fortification not produce a greater disparity in iron status between the 2 groups?

With regard to the first question it may be noted that nadequate intake of formula is unlikely as the families were given generous quantities of milk and the children maintained excellent growth. Increased loss of iron from chronic blood loss is an unlikely cause of the suboptimal iron status as hookworm is uncommon in infants in the Western Cape and other causes of chronic blood loss are rare. The most promising avenue for future research is the determination of the balance in the diet between inhibitors and facilitators of iron absorption.

With regard to the second question, *ie* the failure of the Test group to achieve a large improvement in iron status, one may speculate that similar factors were partly responsible. However, from previous studies of iron absorption and the effect of ascorbic acid, it would have been expected that the fortification program would have had a greater effect on the Test group than that observed.

Further studies are required to answer these questions and to provide quantitative data for planning future trials. Specifically, the effect of ascorbic acid on the absorption of iron from cow's milk based infant formula needs to be measured at levels of iron fortification up to 40 mg Fe/100 g formula, and measurements of percentage absorption from test meals need to be correlated in the same subjects with response to long term programs of iron fortification.

Efforts to improve the iron status of normal infants in the community by fortification of infant formulas should be based on results from such studies since the availability of extra iron added to conventional infant formulas is so low. Certainly, cow's milk formulas should be fortified to a level of at least 8.3 mg Fe/100 g and they should contain ascorbic acid in a molar ratio to iron of at least 1.4:1, and possibly as high as 4:1.

Effect of iron fortification on incidence of infection and immune function

The results of the laboratory tests of immune function showed no statistically significant effect of the increased quantity of iron in the milk. Correlations with iron status *per se* were inconclusive because of the small difference in iron status between the Test and Control groups.

The results of the study of incidence of infection were similar. The increased iron fortification caused no statistically significant change and correlations of iron status with infection rates were inconclusive

due to low statistical power. However, an intriguingly consistent pattern was apparent: children in the Test group had fewer infections than those in the Control group in 8 of 9 categories of infections. The Test group had almost 10% fewer infections than the Control group. This translates to a potential saving of 56 infections per 100 infants per year.

Clearly then, trials to define the relation between iron status and immune function more precisely are required since a possible public health benefit of this magnitude can not be ignored. The difficulties such studies will have in attaining adequate statistical power must be noted. The present study was designed to detect a difference in infection rates of 1 infection per child per year. The sample size calculations yielded 65 as the minimum number required in each group, assuming that the standard deviation of infection rate was 2.5. With the data from the present study it can be estimated that a minimum of 230 infants would be required in each group to detect a difference of 0.59 in infection rates with standard deviations of 2.8 and 3.3.

Besides increasing the number of subjects, steps to increase statistical power might include extending the period of observation and increasing the disparity in iron status between control and test groups. It will be particularly important to learn if there is a threshold level of iron status below which susceptibility to infection is increased. The present study provides a hint that, if such a threshold exists, it could be relatively high.

The mechanisms by which iron influences immune function require further clarification. It is unlikely that studies utilizing current immune function tests and the design of the present trial can economically reach adequate statistical power. New methods must be sought to address this question. For example, the time course of repair of iron deficiency in moderate to severe cases could be correlated with the change in immune function tests. But, measures to control for the confounding effects of infection and malnutrition would need to be included.

Risks of iron fortification

The increased iron fortification was shown to be safe and, in particular, not to increase susceptibility to diarrhoeal disease. It was associated with lower zinc levels, but there was no evidence that this was harmful.

Nevertheless, future studies of iron fortification should take care not to interfere with zinc absorption since zinc deficiency has well known deleterious effects. The molar ratio of iron to zinc should be

maintained at about 2.8 mol Fe/mol Zn. And the effect of iron fortification on the absorption of other divalent metallic ions should be monitored.

Conclusion

In conclusion, it may be stated that increasing the level of iron fortification of cow's milk infant milk formula is not sufficiently effective in improving iron status to warrant a change in commercial practice. But, the potential rewards of improved iron status are such that further studies should be undertaken with increased fortification of iron, ascorbic acid and zinc.

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Prof H de V Heese, head of the Division of Paediatrics and Child Health of the University of Cape Town, conceived the initial idea for the project and supervised its execution.

The Medical Superintendent of the Red Cross War Memorial Childrens Hospital, initially Dr JGL Strauss, and now Dr RO Simpson, gave permission to conduct and publish this research.

Dr R Coogan, Cape Town's Medical Officer of Health, authorized the use of the facilities of the municipal health clinic at Bonteheuwel. Dr NM Durcan, Deputy Medical Officer of Health, ensured that our requirements could be met from the facilities at the clinic.

Sister M Brand and her staff at the clinic cooperated with the study team on a daily basis for almost 2 years.

The private practitioners of Bonteheuwel and nearby areas generously gave of their time to assist me in documenting infections suffered by the study subjects.

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Bacteriological studies were performed in the Bacteriological Laboratory of the Red Cross War Memorial Childrens Hospital with permission from Dr D Hanslo.

Miss R Pearl and the Haematology Laboratory of the Red Cross War Memorial Childrens Hospital processed the haematological analyses.

Biochemical analyses were done in the Research Laboratories of the Institute of Child Health, University of Cape Town. Mr W Dempster, Chief Technician, and Mrs F Pocock performed the tests.

The immunological studies were done in the Research Laboratories of the Institute of Child Health. Prof DW Beatty authorized the work which was performed under the supervision of Miss EJ Hughes.

Earlier drafts of portions of this thesis have been read and criticized by Prof DW Beatty, Prof H de V Heese, Prof P Lanzkowsky, Prof M Mann, Dr CJ Molteno, Dr P Roux and L Vuataz. I am grateful for their advice and suggestions. The flaws in the final document are, of course, my own responsibility.

The secretarial staff on the fifth floor of the Institute of Child Health have all played a willing part in compiling the documentation for this thesis. Mrs L Makepeace ably managed the financial aspects of the project.

In producing the final document I was able to exploit the latest desktop printing technology through the courtesy of Dr JC Stegmann who made available the laser printer in his office.

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My colleagues at work have, for the final stages of the thesis, willingly tolerated me putting their interests below the completion of this document. I must thank them for their patience.

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Dedicated to the 149 children of Bonteheuwel who were my study subjects.

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And as a multitude of laws often only hampers justice, so that a state is best governed when, with few laws, these are rigidly administered; in like manner, instead of the great number of precepts of which logic is composed, I believed that the four following would prove perfectly sufficient for me, provided I took the firm and unwavering resolution never in a single instance to fail in observing them.

The first was never to accept anything for true which I did not clearly know to be such; that is to say, carefully to avoid precipitancy and prejudice, and to comprise nothing more in my judgment than what was presented to my mind so clearly and distinctly as to exclude all grounds of doubt.

The second, to divide each of the difficulties under examination into as many parts as possible, and as might be necessary for its adequate solution.

The third, to conduct my thoughts in such order that, by commencing with objects the simplest and easiest to know, I might ascend little by little, and as it were, step by step, to the knowledge of the more complex; assigning in thought a certain order even to those objects which in their own nature do not stand in a relation of antecedence and sequence.

And the last, in every case to make enumerations so complete, and reviews so general, that I might be assured that nothing was omitted

Rene Descartes

A discourse on method

AD 1637

CHAPTER 1 INTRODUCTION

Prevalence of iron deficiency

Nutritional iron deficiency is common in infancy in both affluent and poor societies. Accurate assessment of the extent of iron deficiency is difficult because diagnostic criteria often differ from study to study. Nevertheless, it is the most common single nutrient deficiency and by far the most common cause of anaemia in infancy.

Iron deficiency in the Western Cape

Surveys of infants in the Western Cape have shown the prevalence of iron deficiency anaemia to range from 55% in the 1960's to 34% in the 1980's. Studies in other countries in similar communities have not differed greatly from this order of magnitude. But the prevalence of the condition does vary from population to population depending on a number of factors. The maximum prevalence is between 10 and 15 months of age when the child is growing most rapidly. Besides direct nutritional influences, the most important correlate with iron deficiency in infancy is socio-economic class; race and sex are much less important determinants.

Consequences of iron deficiency

The consequences of iron deficiency obviously depend on the severity of the disorder and are by no means limited to anaemia and its effects. The systemic manifestations of iron deficiency include: anorexia, malabsorption, loss of weight, impaired physical performance and, in severe anaemia (with haemoglobin less than 4 g/dl), heart failure. Behavioral changes have been attributed to iron deficiency - even in the absence of anaemia.

Iron and immune function

Of special interest for the present study are the observations linking iron deficiency with impaired immune function and increased susceptibility to infection. In 1928 Helen Mackay, a leading London paediatrician, published the first evidence implicating iron deficiency with an increased incidence of infection. Her results were striking, but subject to criticism on methodological grounds. Although several clinical trials have since been made and many laboratory experiments have been performed there is still controversy over the putative link between iron and immunity. And there is also evidence to suggest that iron therapy may exacerbate infections, or increase susceptibility to malaria and perhaps other infections. On balance, the case against iron therapy is not strong and no studies have shown any increased risk of infection from the quantities of iron used to fortify infant milk formulas.

Motivation for the study

In view of its high prevalence and the wide spectrum of its consequences, iron deficiency would seem to be a key target for preventative public health measures.

An effective method of preventing nutritional iron deficiency in infancy is the fortification of infant milk formulas with iron. Infant milk formula is a convenient vehicle for iron fortification in Cape Town since dried milk powder is provided by the City Council at subsidized prices to families with young children. A recent survey in Heideveld, a lower socio-economic community in Cape Town of so called Cape Coloureds, found a prevalence of iron deficiency anaemia of 34%[267]. This was in healthy 1 year old full term infants who had ready access to the municipal clinic where milk formula fortified with iron and ascorbic acid is sold at subsidized prices.

From the foregoing it may be seen that iron deficiency is a common problem in infants in Cape Town and that the efficacy of current public health measures leaves substantial room for improvement.

Aims of the study

1. Trial of increased iron fortification of infant formula

The problem addressed by this thesis was to determine the utility of increasing the level of iron fortification of infant milk formula. Three specific questions were posed:

Does increasing the level of iron fortification of conventional infant milk formula improve the iron nutrition of normal infants fed on the formula?

Does increased iron fortification of infant milk formula alter immunity as reflected by incidence of infection and laboratory tests of immune function?

Are there any harmful effects of increasing the quantity of iron in conventional infant milk formula?

2. Infant Feeding Practices

The principal study provided the opportunity for a survey to be made of feeding practices. The purpose of the feeding survey was to compare practices before and after the trial in order to document any effect that the trial itself may have had on the customs of mothers in the community.

Methods employed

1. Trial of increased iron fortification of infant formula

A group of infants from a lower socio-economic community of so called Cape Coloureds was studied from the age of 3 months to 1 year. Half of the children, the Control group, were given a popular infant milk formula (Lactogen Full Protein) which contains 8.3 mg Fe per 100 g powder and 37 mg ascorbic acid per 100 g powder. The other half, the Test group, were given, in a double blind manner, the same formula fortified with 40 mg Fe per 100 g. At the beginning and at the end of the study tests were made of iron status and immune function. During the study the children were examined every 3 weeks in an attempt to document every infection as accurately as possible. The children were selected from a lower socioeconomic community in Cape Town, and, in order to obviate the confounding effects of malnutrition or severe illness, the infants were carefully screened to ensure that only healthy, well nourished children participated in the trial.

2. Infant Feeding Practices

Infant feeding practices were surveyed in the community before the trial began and again 9 months later when the trial was well under way and every mother attending the clinic was aware of it. The feeding practices of the mothers participating in the trial were also documented for comparative purposes.

Structure of the thesis

The preceding paragraphs have outlined the background and motivation for the study, posed the questions addressed in this thesis and briefly described the protocols of the studies performed. Chapter 2 briefly reviews the physiology and pathophysiology of iron metabolism in order to provide the background necessary for the subsequent detailed and critical surveys of studies that are particularly relevant to the present research.. The review selects from the vast literature on iron and only in the areas related to the questions addressed in this study does it attempt to be comprehensive. Particular emphasis is placed on studies of the role of iron in susceptibility to infection since, not only is this of especial relevance to the present study, but also there has been no comprehensive critical review of such work to date. Present understanding of the association between iron status and behaviour is summarized. A synopsis of the physiology of iron absorption is given, but the issues of iron fortification and supplementation are treated in some detail.

The design of the study is detailed in chapter 3. Ethical considerations in conducting such studies in children are weighed. The protocols for the principal study on iron fortification and immunity and the surveys on feeding practices are presented. This chapter also covers the planning of the project is in detail. Attention is drawn to important features in the design of the study. Comprehensive analyses

support decisions to choose particular features in the design where there were attractive alternatives. Chapter 3 also describes the laboratory methods and materials used and the data processing and statistical procedures employed.

Chapter 4 relates socio-cultural data from the trial in order to portray the community within which the study was undertaken. Much of the material is anecdotal, but it aims to provide the reader with sufficient information to assess the validity of comparisons that he or she might wish to make with other studies.

Results from the surveys of infant feeding habits are presented in chapter 5.

The results from the main study are reported and discussed in chapter 6.

The conclusions and recommendations for further studies are presented in chapter 7 which ends with a summary of the contributions of the present work.

The appendices include the bibliography, as full a tabulation of results and statistics as is practical as well as other material that might need to be referenced such as the composition of the Control and Test infant milk formulas.

With us ther was A DOCTOUR OF PHYSIC,
In al this world ne was ther noon him lik
....
Wel knew he the olde Esculapius,
And Deiscorides, and eek Rufus,
Olde Ypocras, Haly and Galye;
Serapion, Razis and Avicen;
Averrois, Damascien and Constantin;
Bernard and Gatesden and Gilbertin.
Of his diet mesurable was he,
For it was of no superfluitee,
But of greet norissing and digestible.
His studye was but litel on the Bible.

The Canterbury Tales
Geoffrey Chaucer
circa AD 1386

CHAPTER 2 LITERATURE REVIEW

Introduction

The area surveyed

This chapter reviews iron status with particular emphasis on iron deficiency under the rubrics of epidemiology, physiology, pathophysiology, clinical classification and prevention.

The epidemiology of iron deficiency reveals that it is an internationally widespread public health problem in lower socio-economic communities. The Cape peninsula is no exception as iron deficiency is common in infants seen at local health care facilities.

The physiology of iron deficiency is briefly reviewed with particular emphasis on the role of iron in the immune system. The pathophysiology of iron deficiency is broadly sketched in order to show the wide range of changes in this condition. The principal research project was concerned with iron nutrition and immune function; therefore the literature describing effects of iron deficiency on immune function is examined in some detail. Both infection and nutrition are shown to be related to iron status in a complex interdependency. This makes the design and interpretation of scientific studies difficult and prone to confounding variables.

The main aim of this chapter is to review published* studies on the relationship between iron deficiency and infection in order to provide a comprehensive bibliography up to August 1987. Each pertinent study is critically examined for its strength and weakness in testing the hypothesis of a causal relationship of iron deficiency to susceptibility to infection. To place these studies in context it was necessary to review the evidence from studies on laboratory animals and to survey studies on the relationship between iron excess and susceptibility to infection.

The reason that particular emphasis is laid on this aspect of the literature is that, although there have been many reviews of the role of iron in immune function, there has been no comprehensive and critical review on iron deficiency and susceptibility to infection. Also, while laboratory work has established fairly conclusively the changes in tests of immune function in iron deficiency, clinical studies of susceptibility to infection in iron deficient infants have been suggestive, but inconclusive because of methodological problems.

Studies of laboratory tests of immune function in disturbances of iron metabolism are also reviewed, particularly with regard to studies of iron deficiency in infancy.

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* Published in the scientific press, including peer review journals and proceedings or abstracts of scientific congresses. This is a wider field than that covered by Medline, but it excludes newspapers and magazines.

Clinical classification of iron status is discussed, and difficulties in diagnosis highlighted. Public health measures to prevent iron deficiency and its consequences usually rely on the fortification of staple foods and/or the pharmacological supplementation of the diet with iron medications for groups at increased risk for iron deficiency. The relevant literature is surveyed in order to ascertain the viability of the intervention on which this study is based.

Finally, as iron is but one of a number of nutritional factors that affect health, the importance and possible confounding effects of imbalance in other elements, such as zinc, is summarized.

The goals of the review

This survey of the literature aims to provide the theoretical framework for understanding both the motivation for the project and the design of the study. The literature on iron is vast. This survey therefore relies on secondary sources, such as authoritative reviews in books and journals, for most of the subject matter covered. Only with respect to iron deficiency and immune function is an attempt made to review original scientific reports comprehensively. And only those articles that relate to iron deficiency and infection in infancy are critically reviewed in detail.

Criteria for assessment of studies and literature review

A critical review of a study aims to assess the validity of the author's claims and the strength of the conclusions. Evaluation can only be based on the reported design, methods, data and analysis. Guidelines have been taken from two principal sources[137, 6]. Authors should have included information on the *objective* of their research and the *hypotheses tested*. The *eligibility criteria* for admission to the study should have been described and information given on whether the subjects were *assigned* to treatment groups *blindly* and *randomly* in order to minimize selection bias. Other procedures designed to reduce bias include the *patients' blindness to treatment*, and the *investigators' blindness* when assessing outcome. The *methods* section of the report should have explained the *randomization procedure*, and *laboratory* and *clinical methodologies*. *Treatment compliance* and *complications* should have been described and reasons and numbers of subjects *lost to follow-up* should have been given. The *statistical analyses* should have been described and the *power* of the study should have been estimated. Finally, potential *sources of bias* or confounding factors should have been identified and controlled for, either in the design or in the analysis of the study. Few studies explicitly identified these criteria, but in most it was possible to infer much of the information and to discriminate between mere assertions and factual argument.

The methods used in the literature survey

Most references were obtained from citations in other review and original articles. The Institute of Child Health subscribes to the Medical Research Council's MIDS (Medical Information Dissemination System). This service provides a computer search each month of most recent additions to the Medlars/Medline database of medical literature. The search strategy is set to include publications on iron and immune function, but it excludes most work performed on animals and articles not in English, Afrikaans, Dutch or German. An additional search of the Medline/Medlars database was performed on 4 August 1986 using the "Paperchase" system at the Beth Israel Hospital in Boston. This identified 22410 articles with the keyword "iron". Of these, 297 also included the keyword "infection". No exclusions were made on the basis of language or because a study was performed with animals.

Evaluation of methods

The bibliography for immune function and iron status aims to be complete. It is, of course, not possible to know what has been missed, but the results of the "Paperchase" search do give an indication of how closely the goals are met.

It is probably safe to assume that any serious scientific work of importance before 1970 would have been cited in one of the articles reviewed, and would therefore have been identified. Since 1970, the Medlars/Medline database would have indexed any article of interest in this review. Although the keywords assigned to an article may not accurately reflect its contents, the concepts of "iron", "immune function" and "infection" are broad enough in their scope to have been included in most articles pertinent to this research.

The "Paperchase" search was performed after an extensive period of collecting references. While it located 297 articles of potential interest, many were irrelevant. Of these, 102 had not been identified before, mainly because they related to studies in animals or were published in a foreign language. (This was not unexpected as this type of reference had previously been specifically excluded.) Only one (Russian) article[320] was of possible direct importance to the question of the relation between iron status and immune function in infancy.

It would seem therefore, that the literature review is likely to be as complete as could be hoped for with respect to articles published on susceptibility to infection in iron deficient infants, and comprehensive in respect of studies relating immune function to iron status in general.

Epidemiology of iron deficiency

Difficulties in comparing studies

A digression is here necessary to indicate a preliminary difficulty in determining the incidence of anaemia in infancy, namely the lack of any standard of comparison.

HMM Mackay, 1928[314]

The difficulties that Helen Mackay recognized in 1928 are still with us today, better defined but not yet resolved. The following list details important factors that need to be evaluated when studies of iron status are compared.

Intrinsic biological variability

As with all biological variables, the measures of iron status have a degree of intrinsic variability so that a level that is normal for one individual may be high or low for another[356]. A diurnal cycle may compound the intrinsic variability. Serum iron and transferrin saturation exhibit pronounced diurnal variation, and even when measurements are made at the same time of day the individual variability is of the order of 35 per cent[103].

Variation between and within laboratory methods

Modern studies often employ the Coulter counter to measure the haematological indices of iron status. The consequent standardization of equipment, methodology and calibration techniques has made reliable comparisons possible between studies of different laboratories[117, 120]. Older studies have to be interpreted in the light of the methods employed. For example, the studies of Dr Mackay in London between 1925 and 1927 measured haemoglobin according to oxygen combining power and reported results as a percentage[314], but later work showed that the laboratory calibrations resulted in haemoglobin levels that are 7 per cent lower than they ought to be[315].

Erythrocyte protoporphyrin is sometimes measured as FEP (free erythrocyte protoporphyrin) in ug/dl red blood cells and sometimes as ZPP (zinc protoporphyrin) in ug/g haemoglobin. FEP and ZPP results can be compared, with a simple calculation, as there is a linear relationship between the two measurements:

$$ZPP = 0.83*FEP - 8.4$$[324].

The coefficient of variation of erythrocyte protoporphyrin is about 9% (between laboratories). This is about double the coefficient of variation between laboratories for red cell indices measured on the Coulter counter[120].

The method of specimen collection

The manner in which blood specimens are obtained (*eg* arterial, capillary, or venous bleeding) can influence laboratory test results, although if care is taken the differences are negligible[117].

Developmental changes

During the first 2 years of life, developmental changes take place in the indices of iron status[435]. Rapid growth often requires more iron than is available in the diet and iron stores are diminished, if not depleted. The consequent difficulty in distinguishing pathological from physiological changes has led to the concept of a *physiological* anaemia of infancy[162].

Sex

Although indicators of iron status vary markedly with gender in adults, the differences are minimal in infancy[527].

Race

Racial differences in haematological indices and other measures of iron status have often been noted. But, when the confounding effects of genetic conditions (*eg* thalassemias and haemoglobinopathies), socio-economic class and nutrition are controlled there seems to be no intrinsic racial difference in respect of haematologic tests[527, 531].

Nutrition

Inadequate nutrition is directly reflected in changes in haematologic variables[314, 75].

Infection and inflammation

Acute and chronic infections and inflammatory diseases alter the metabolism of iron and hence the indicators of iron status. Chronic infections and inflammatory diseases lead to a microcytic anaemia. The response to any inflammatory process includes a drop in serum iron and rise in serum ferritin. The highest levels of serum ferritin are found in hepatitis as liver cell damage releases iron stores into the circulation[524].

Socio-economic class

When differences in nutrition and exposure to infection are controlled there seems to be no significant difference between socio-economic classes[290].

Altitude

Haemoglobin concentration rises in response to the decrease in oxygen at high altitudes. Haematologic studies conducted at a significant elevation need to be adjusted for altitude effects for meaningful comparisons to be made. Approximate comparability of haemoglobin can be obtained by adding 1g Hb/dl per 3000 meter elevation[135].

Diagnostic criteria

A fundamental problem in comparing studies of iron status is that there is no universally accepted method for grading iron status. The principles are not controversial; iron deficiency manifests itself with decreased haemoglobin, decreased haematocrit, decreased mean cell volume, decreased plasma ferritin and increased plasma transferrin. The problem is that each of these indices has a wide variability, largely independent of the other indices. Any single measure used as a test for iron status has poor discriminatory power. Such qualitative statements ought to be quantified by data on sensitivities, specificities and predictive values for diagnostic tests, but there are few studies that begin to reach this basic level. Test results may be combined in classification schemes to increase discriminatory power, but the selection of measures and choice of cut-off levels between normal and abnormal are often arbitrary. The problem is difficult enough to be the subject of a book length treatise itself and is further analyzed below.

Prevalence of iron deficiency - International studies

In a recent review of iron deficiency Lanzkowsky[527] concludes that the prevalence of iron deficiency anaemia (in lower socio-economic communities) varies from 17 to 44 per cent, while that in white middle class communities ranges from 1.4 to 6.3 per cent.

Table 2.1 below contains results from a selection of surveys of anaemia. While the table shows that direct comparison between almost any two of the studies has to be qualified with details of methodology, the table also demonstrates that anaemia is a common problem, particularly in poorer communities. The following section shows that these surveys of anaemia can be taken, for practical purposes, to indicate the extent of iron deficiency in the populations studied.

Estimating iron deficiency from low haemoglobin levels

In the absence of any better single measure, haemoglobin has often been used as a screening instrument to estimate the extent of iron deficiency in populations*. This is justified on two accounts: In a population of otherwise healthy infants, iron deficiency is the most common cause of anaemia, and the prevalence of anaemia is greatly reduced by programs of iron fortification and supplementation.

* The issues of diagnosis and classification are discussed in more detail below.

Drawbacks in using haemoglobin as an indicator of iron deficiency include those discussed above, the fact that decreased haemoglobin levels are a late manifestation of iron deficiency and that specificity, sensitivity and predictive value of the test have not been published for infants.

Factors that would cause haemoglobin to over-estimate the prevalence of iron deficiency include all the other causes of anaemia. Factors that would cause haemoglobin to under-estimate the prevalence of iron deficiency include cases of latent iron deficiency and cases with haemoglobin levels higher than that chosen as the lower limit of "normal" but which would respond to extra dietary iron with a rise in haemoglobin.

Percentile curves for haemoglobin have been published by Dallman *et al*[124]. These were established by excluding subjects with laboratory evidence of iron deficiency, thalassemia minor and/or haemoglobinopathy. The lower limit of haemoglobin in 1 year old children was found to be 11.0 g/dl. Bird *et al*[41] found a prevalence of 10.4% of thalassemia and/or haemoglobinopathy in so called Cape Coloured infants with mean cell volume less than 60 fl. It can thus be inferred that the prevalence of thalassemia and/or haemoglobinopathy in children selected on the basis of a haemoglobin level less than 11.0 g/dl is likely to be well under 10% in Cape Coloured infants. For haemoglobin levels greater than 11.0 g/dl the proportion of children with thalassemia and/or haemoglobinopathy is likely to be even less. Use of a haemoglobin level of 11.0 g/dl to diagnose iron deficiency anaemia is thus unlikely to include more than 10% iron replete individuals in otherwise healthy infants.

The false negative rate is difficult to estimate as no published studies address this issue. Dallman *et al*[123] found that 35% of 1 year old infants with haemoglobins less than 11.5 g/dl responded to iron treatment with a rise in haemoglobin of at least 1 g/dl. It is unlikely that as many as 35% of infants with haemoglobins above 11.0 would respond to iron treatment so this can be taken as an upper limit, but 10% would perhaps be a reasonable estimate.

The argument can be extended to show that at values of haemoglobin level less than 11.0 g/dl the false positive rate in diagnosing iron deficiency anaemia is likely to remain around 10% while the false negative rate would increase.

Population studies have used haemoglobin in 2 ways to estimate iron deficiency. Some have reported the mean haemoglobin while others have reported the prevalence of anaemia based on a "cutoff" level of haemoglobin. The mean allows a more precise comparison between studies and but the prevalence of anaemia is of more direct interest to the clinician. As there is no generally accepted diagnostic level of low haemoglobin, different workers have used different criteria and published prevalences of anaemia are not directly comparable.

Table 2.1 summarizes the results from a number of studies. The prevalence of anaemia has varied from absent[387, 52] in well off communities to 60% in lower income groups[135]. The mean haemoglobin varied from 12.5 g/dl[387] to 11.1 g/dl[261]. The noteworthy feature of table 2.1 is the demonstration of the relation between haemoglobin and economic status. The conclusion is that iron deficiency is a universal problem in poor societies.

Table 2.1 Surveys of anaemia in infancy

Ref. No.	Author	Date	Age (months)	Mean Hb (g/dl)	Prevalence of anaemia	Hb "cutoff"	Comments
185	Fuerth	1959	12	11.8	6%	10	White, middle class
		1969	9	11.9	3%	10	White, middle class
162	Farquhar	1963	12	12.1	2%	10	White, well off
7	Andelman	1966	12	-	76%	10	Non-white, poor
302	Lovric	1970	6-36	12.2	3%	10	* All classes
							no change with SEC
387	Owen	1971	12-33	11.6	14%	10	Lower-lower class
				12.0	8%		Upper-lower class
				12.4	1%		Lower-middle class
				12.5	0%		Upper-middle class
502	Vacquez-Seone	1971	9-36	11.1	23%	9.8	Non-white, poor
		1984	9-36	11.8	1%	9.8	Non-white, poor
74	Burman	1972	12	11.75	?		White, middle class
261	Katzman	1972	10-36	11.1	18%	9.8	Lower SEC
135	Derman	1978	13-24	?	60%	11.5	Black, 1200m
					53%		"Coloured", 1700m
141	Drigger	1981	12	?	22%	11.5	White, middle class
					38%	11.5	Black, poor
					18%	11.5	Asian
					28%	11.5	Other
440	Sadowitz	1983	9-12	?	8%	11	Lower SEC
52	Brault-Dubuc	1983	12	12.3	0%	10	White, well off
					3.3%	11	
316	Madanat	1984	6-12	?	37%	10.5	** ?lower SEC
			12-18	?	38%		

KEY		
	Ref. No.	Number of reference in bibliography
	Author	First author of article
	Date	Date study was published or conducted
	Mean Hb	Mean haemoglobin concentration in g/dl
	Prevalence of Fe def	Prevalence of iron deficiency anaemia
	Hb "cutoff"	Lowest level of haemoglobin regarded as normal
	SEC	Socio-economic class
	1200m, 1700m	Altitude at which study subjects resided
	?	No data published
	*	Diagnosis on Hb, and blood smear
	**	Diagnosis on Hb and transferrin saturation at least 16%

Prevalence of iron deficiency - Cape Peninsula

Lanzkowsky (1960)

The first survey of anaemia in infants in Cape Town was published in 1960 by Lanzkowsky[288]. Fifty five per cent of "Coloured" infants between 10 & 11 months and 11 & 12 months had a haemoglobin concentration of less than 10 g/dl. The lowest haemoglobin levels were found in infants between 1 and 2 years of age. As capillary blood specimens were taken with adequate precautions and haemoglobins were measured with the oxyhaemoglobin method the results are comparable with those obtained in present day laboratories. As was argued above, the prevalence of anaemia can be taken to indicate a reasonable approximation of the prevalence of iron deficiency.

Robertson and Sundgren (1972)

In 1972 Robertson and Sundgren[420] reported on a survey conducted in Cape Town City child Welfare Clinics. Apparently healthy, thriving children in the age group between 7 and 11 months had a prevalence of 42% of anaemia defined as a haemoglobin concentration less than 10 g/dl. For the age group 12 to 24 months the prevalence of anaemia rose to 60%. Capillary blood was taken and a direct reading hand-held haemoglobinometer used to make the measurements of haemoglobin. This method is accurate to 0.5 g/dl with careful calibration. A direct quantitative comparison with other studies would be invalid, but the extent of anaemia is readily apparent.

Kirsten et al (1984)

The most recent study, by Kirsten *et al*[267], examined 240 infants from a lower socio-economic Cape Coloured community. There were 20 infants in each of 12 age groups ranging from 1 to 12 months. This was the first study that attempted to classify the iron status of the children using multiple criteria in an attempt to be more specific and sensitive. Iron deficiency anaemia was found in 34 per cent, and haematological evidence of iron deficiency without anaemia was present in a further 19 per cent. Making the inference from low serum ferritin levels, 27 per cent had diminished iron stores.

A sophisticated classification scheme was employed to take into account developmental changes in the first year of life. The reference values used in the diagnostic protocol are given in table 2.2 below. Iron deficiency anaemia was diagnosed when the haemoglobin was below the reference level together with at least one low result for mean cell volume and mean cell haemoglobin.

Table 2.2 Haematological reference values for infants

Age (mo.)	Hb (g/dl)	MCV (fl)	MCH (pg)	Ferritin(ug/dl) Low	Very low
1	11.1	93	27	150-215	<150
2	9.5	81	27	64-98	<64
3	10.0	80	24	21-36	<21
4	10.0	74	24	13-21	<13
5-12	10	74	24	8-12	<8

Notes From Kirsten *et al*[267]

A recent study of the prevalence of beta thalassaemia and abnormal haemoglobins[41] allows the above results to be adjusted for the possible inclusion of haemoglobinopathies in cases categorized as iron deficient anaemia. This survey found 10 per cent of children with a mean cell volume less than 60 fl were either carrying the beta thalassaemia gene or had an abnormal haemoglobin. The study by Kirsten *et al* found 131 children with a low mean cell volume. (The reference volumes for each age group are listed in table 2.2.) As the reference mean cell volume was never as low as 65 fl, a conservative estimate of the number of children with a haemoglobinopathy is 10 per cent of 131, which equals 13. Subtracting this from the 81 children diagnosed as having iron deficient anaemia, the prevalence can be recalculated: (81-13)/240 = 28 per cent. This conservative estimate may well be lower than the true figure as the estimated number of haemoglobinopathies surely errs on the side of too many. The point is that iron deficiency is very common in this socio-economic group.

Venous blood was taken and full blood counts made on a Coulter Counter model S. The results are therefore comparable with studies from most modern laboratories.

Summary

The conclusion to be drawn from these three surveys of iron deficiency is that, in lower socio-economic Cape coloured communities, iron deficiency anaemia affects at least one quarter of infants under the age of one year. Iron deficiency without anaemia affects about the same proportion.

Physiology of iron

Iron balance

The following paragraphs give a schematic account of the physiology of iron balance. Details on the complex and sometimes controversial mechanisms are not given as this is beyond the scope of this thesis.

Absorption

Iron is one of the most abundant elements in the earth's crust. However, all cellular organisms find its availability low as both the ferrous (Fe^{2+}) and ferric (Fe^{3+}) ions are highly insoluble at neutral pH. Special systems are required by all forms of life to absorb and transport iron[326]. Bacteria synthesize and excrete high-affinity iron chelating agents[368]. The roots of plants secrete substances that augment iron absorption[58]. Mammals have several mechanisms for the absorption of iron: haem iron is absorbed intact by the intestinal mucosal cell[326] and non-haem iron is absorbed in the proximal small intestine whose mucosal cells contain ferritin and transferrin[251]. The availability of dietary iron is highly variable and dependent on the content of the diet. Non-haem iron is generally poorly absorbed - less than 10 per cent is taken up by the subject. The absorption is inhibited (less than 5 per cent) by tannates and phosphates found in cereals and augmented (10 to 30 per cent) by fish, meat and ascorbic acid[356]. Haem iron is highly available - between 20 and 40 per cent being absorbed in the iron depleted subject and this fraction is not affected by the composition of the diet[164]. About 50 per cent of breast milk iron is absorbed, but only 5 to 10 per cent of iron in infant milk formulas is taken up[438, 434], and as the iron content of food increases, the percentage of iron absorbed decreases[48]. The amount of milk fat, the addition of carbohydrates and acidification of milk do not influence the absorption of iron[482].

Transport and storage

Iron is transported to sites of storage and utilization bound to transferrin - a 77 000 MW protein that can bind 2 atoms of iron. Cells acquire iron by highly selective receptor-mediated endocytosis of transferrin[127]. Excess iron is stored as soluble ferritin or as insoluble haemosiderin[524]. In the formation of haemosiderin part of the protein shell of ferritin is removed and the molecules are aggregated. Ferritin has been detected in every type of human cell so examined, but particularly high concentrations are found in liver, spleen and bone marrow where it is continually degraded and resynthesized. The average ferritin molecule stores about 2000 iron atoms and has a life span of a few days[164].

The amount of ferritin circulating in the blood parallels the concentration of storage iron in the body: 1 *ug* of ferritin per litre of serum is equivalent to about 140 *ug* of storage iron per kilogram body

weight[164] Tissue ferritin differs from serum ferritin which is partly glycosylated and almost entirely free of iron[164].

Losses

In the absence of bleeding, iron losses are very small; basal physiological losses, mainly from skin, the gastrointestinal tract and urinary epithelium, have been calculated to be 0.014 mg/Kg body mass[204] per day for the non-menstruating adult. In infancy, the daily loss has been calculated to be between 0.4 and 1.2 mg iron/Kg body weight[529]. The higher rate of loss in infancy reflects the larger epithelial surface area (skin, gastrointestinal and urinary tracts) relative to body weight. The losses are increased if pasteurized cow's milk is used rather than formula or breast milk and in the presence of diarrhoea.

Growth

The normal infant is born with sufficient iron stores to meet the needs of the first four to six months. But, as the average infant triples its body weight and doubles its body iron in the first year, the nutritional needs for iron at 0.5 - 0.8 mg iron/day are very high in relation to the energy intake[204]. In later childhood, the daily iron requirement for growth falls to 0.2 - 0.3 mg iron/day, but rises again during the adolescent growth spurt.

Homeostasis

Iron absorption is increased in the presence of iron depletion and decreased in the face of iron overload.

A specific storage protein, ferritin, regulates the supply of iron in response to acute or excessive demands for the element.

Iron is highly conserved; losses are minimal and most iron from catabolized cells is circulated for re-utilization.

As the life of a red cell is 110 days on average, the total turnover of iron greatly exceeds the rate at which it is absorbed and lost. Senescent red cells are trapped in the spleen and phagocytosed by reticuloendothelial cells. Iron is released from haemoglobin, taken up by plasma transferrin and transported to the bone marrow for incorporation into the haemoglobin of new red blood cells. About 80 per cent of internal iron exchange follows this cycle through the erythron and macrophage[164].

In the normal situation, iron balance is precarious; the dietary intake of iron in humans is 1 to 2 per cent of that in other mammals[164] and the diet of the poorer segment of the world's population

contains very little available iron. At times of high physiological demands, such as pregnancy and infancy, the requirements are often not met and the consequences of iron deficiency become manifest.

Biochemical functions of iron - The immune system

Polymorphonuclear leukocytes and phagocytes

The polymorphonucleocyte is of particular interest with respect to iron deficiency since the cell has many iron-containing constituents. The primary granule is formed in early maturation of the polymorphonucleocyte. This contains myeloperoxidase, an iron-containing enzyme, which may contribute to the antimicrobial function of the cell. Its clinical importance is uncertain however, since congenital deficiency of myeloperoxidase does not seem to increase susceptibility to infection[355, 206].

The so called specific granule is formed during and subsequent to the myelocyte stage. The specific granule contains cytochrome B, an iron-containing enzyme, that is believed to be required for the oxidative burst that occurs following phagocytosis[355]. It seems that the respiratory burst plays a key role in the killing of some, but not all, bacteria by neutrophils since patients with chronic granulomatous disease (in whom the oxidative burst is absent) are markedly susceptible to certain infections[206, 355].

It has been proposed that oxygen free radicals produced in iron-catalyzed reactions are responsible for the killing of phagocytosed bacteria[352]. The biochemistry of oxygen free radical production and subsequent reactions is complex and only partly understood. The conclusions of Halliwell and Gutteridge in a recent review[206] may be briefly summarized by the following statements. It has been clearly established that superoxide radical is produced *in vivo* during the respiratory burst of phagocytic cells. Superoxide is highly reactive in hydrophobic environments, but poorly reactive in bulk aqueous solution. Similarly, hydrogen peroxide is produced *in vivo* and is poorly reactive in aqueous solutions under physiological conditions. But, unlike superoxide, it can cross biological membranes. The extent of the participation of the cytochromes and other iron containing complexes in the production of oxygen free radicals has not yet been fully delineated, but the potential for iron deficiency to disturb the functioning of the polymorphonucleocyte is clear.

In iron overload, the toxic metabolites of oxygen damage polymorphonucleocytes and cells of normal tissues[501].

B and T lymphocytes

B and T lymphocytes depend on iron for the normal function of many subcellular systems, but the biochemical roles of iron compounds and enzymes have not been as clearly defined for these cells as for phagocytic cells.

In the mouse, the response of lymphocytes to stimulation with concanavalin A has been shown to depend on transferrin-bound iron[318]. No defect was noted in the cells of iron-deficient mice in the response to the mitogen, but the serum of the iron-deficient mice was less able to support thymidine incorporation by stimulated lymphocytes*.

Biochemical functions of iron - Other systems

All cells require iron for their growth. (This fact has prompted the suggestion that tumour growth might be able to be controlled by limiting the supply of iron by blocking transferrin receptors[354].) Tables 2.3 and 2.4 summarize the distribution and function of iron containing compounds in the normal human.

Table 2.3 Distribution of iron compounds in normal adult humans**

Per cent normal body iron	Compound	Amount of iron (Grams)
65%	Haemoglobin	2.600
10%	Ferritin	0.400
10%	Myoglobin	0.400
9%	Haemosiderin	0.360
5%	Unknown	0.200
<1%	Transferrin	0.007
<1%	Cytochrome c	0 004
<1%	Cytochromes a, a_3, b	?
<1%	Peroxidase	?
<1%	Catalase	?
<1%	Flavoproteins	?
<1%	Hydroxylases	?
<1%	Oxidases	?

Notes ** Modified from (460)

* Author's tables 2 and 4

Table 2.4 Function of iron compounds in normal humans*

Class	Compound	Primary function
1 Proteins which store & transport iron	a Ferritin &	Iron storage
	b Haemosiderin	Iron storage
	c Transferrin	Iron transport
	d Lactoferrin	Bactericidal/regulation of bone marrow cell differentiation
2 Haemoproteins which bind oxygen reversibly	a Haemoglobin	Oxygen transport to tissues
3 Iron and molecular oxygen	a Oxygenase	Controlled catalysis of reactions involving molecular oxygen
	b Oxidase	
4 Enzymes containing iron:		
a Haemoproteins which react with molecular oxygen	a Cytochrome p450	Mono-oxygenase: one oxygen atom incorporated in the substrate
	b Tryptophan oxygenase	Catalyzes oxidation of tryptophan to N-formyl-kynurenine
	c Cytochrome oxidase	Reduces molecular oxygen to water
b Haemoproteins which react with peroxides	a Hydroper-oxidase	Oxidize water molecules at the expense of hydrogen peroxide
c Iron-sulfur proteins	a Ferredoxins	Electron transfer mediators in the more negative regions of electron transport
	b Flavoproteins aldehyde oxidase sulfite oxidase xanthine oxidase xanthine dehydrogenase	Liver enzymes of wide substrate specificity
	c Aconitase	Conversion of citric acid to cis-aconitate

Table 2.4 (continued)
Function of iron compounds in normal humans*

Class	Compound	Primary function
d Non-haem oxygenases	a Prolyl hydroxylase	Collagen triple helix stabilization
	b Lysyl hydroxylase	
	c Phenyl alanine hydroxylase	Amino acid metabolism (pteridine dependent)
	d Tyrosine hydroxylase	
	e Tryptophan hydroxylase	

Note * Modified from (324)

Iron dependency of micro-organisms

Introduction

Iron is a nutrient required by most, if not all, living cells[516, 155]: *"If life without iron exists it is probably to be found among certain members of the lactobacilli"*[368]. Pathogenic bacteria, fungi and protozoa all have an absolute requirement for iron[368, 295]. Bacteria whose virulence has been shown to be enhanced by exogenous iron include *Escherichia coli*, *Klebsiella Pneumoniae*, *Pseudomonas aeruginosa*, *Listeria monocytogenes*, *Staphylococcus aureus*, *Bacillis anthracis*, *Yersinia pestis*, *Mycobacterium tuberculosis*, *Neisseria gonorrhoeae*, *Shigella flexneri*, and *Vibrio cholerae*[391, 342, 67, 70].

This requirement for iron by micro-organisms has important implications, both theoretical and practical, on iron supplementation, fortification and therapy. This is because administration of iron may facilitate infection by overloading the iron sequestrating capacity of the host and thereby make iron accessible to the invading organism[102].

Parenteral administration of iron-dextran has been associated with increased infectious morbidity and mortality[31, 376, 19, 17]. Oral iron therapy has been associated with recrudescence of malaria, brucellosis and tuberculosis[361].

Macfarlane *et al*[313] have speculated that iron treatment in kwashiorkor may be associated with increased mortality from infection. However, there is no evidence that iron supplementation in infant milk formulas at the levels commonly used today has any adverse effects[47].

The evidence linking iron administration in humans to increased bacterial virulence is comprehensively reviewed by Stockman[484]. Kochan[270] summarizes studies in animals which have shown that the administration of iron promotes the development of bacterial infection. In conditions of iron overload such as haemochromatosis, siderosis and repeated blood transfusions, patients show a greatly increased susceptibility to infectious disease[295, 512].

Bacterial growth - Iron & iron binding products

The history of the discovery of the iron binding factors in tissue fluids which provide their bacteriostatic properties is reviewed by Kochan[270]. Key observations were that the bacteriostatic nature of serum is neutralized by iron or by heating to 65°C. Transferrin, ferritin and lactoferrin all bind iron so effectively that most micro-organisms are not able to utilize the iron bound to these proteins. Serum from iron deficient patients is less able to support the growth of bacteria *in vitro*[328]. The mechanisms by which iron binding proteins inhibit microbial growth are reviewed in greater detail by Bullen[69].

Bacterial virulence - Effect on pathogen

Pathogenic bacteria often have unusually efficient means of acquiring and utilizing iron under the restricted conditions in physiological fluids and tissues[457, 81].

According to their fate in serum, bacteria can be divided into serum-sensitive and serum-resistant groups. The serum-sensitive bacteria are dependent on siderophores or exogenous iron to meet their requirements, while the serum-resistant bacteria can use their stored iron to meet growth needs[500].

Bacteria are able to utilize iron bound to haemoglobin, haem and haematin except when these molecules are bound to haptoglobin, haemopexin and/or albumin[508].

Griffiths *et al*[198] review the experimental evidence implicating plasmids as the determinants for certain virulence factors. *Vibrio anguillarum* is a fish pathogen whose virulence is dependent upon its ability to sequester iron. Upon losing the plasmid that carries the genetic code for the iron sequestering system, the bacterium loses its virulence. More pertinent for humans is the plasmid Col V which seems to provide *E. coli* with a mechanism which allows it to grow significantly faster in the presence of transferrin.

Escherichia coli acquires iron by specialized systems, including the aerobactin system which has a markedly higher prevalence in pathogenic strains than in isolates from faeces[81].

Bacterial virulence - Effect on host

Idiopathic haemochromatosis has been associated in a case report with *Listeria monocytogenes* meningitis and reduced phagocytic activity which recovered after a course of phlebotomy to reduce iron overload to normal[500]. (*Yersinia enterocolitica* is more commonly associated with idiopathic haemochromatosis and the evidence for this and other effects of iron overload are reviewed in more detail below.)

Bacterial virulence - Effect on bacterial secondary metabolism

Barclay[16] tabulates and describes in some detail toxic factors produced by *Pseudomonas aeruginosa*, *Clostridium perfringens*, *Clostridium tetani*, *Corynebacterium diphtheriae* and *Shigella shigae* when they are subject to an environment with low levels of available iron. He also describes 6 outer membrane proteins of *Escherichia coli* produced under limitation of iron. These are all involved in the acquisition of iron and are potential determinants of virulence.

The iron concentration in culture medium controlled the production of two siderophores, toxin A, proteases and membrane proteins that bind the siderophore-iron complex[523].

"Small quantities of iron bound specifically to human transferrin were found to stimulate infection with Neisseria meningitidis *strain M1011 in mice"*[231].

Fungal requirements for iron

Neilands[368] has reviewed the data on iron assimilation systems in fungi. Siderophores supply fungi with iron via a shuttle mechanism. Their production is increased in response to iron starvation. Iron plays a role in germination and sporulation in some species.

Protozoal requirements for iron

Desferrioxamine has been shown *in vitro* to inhibit the growth of *Plasmodium falciparum* and Murray *et al*[360, 361] have found a lower prevalence of malaria infestation in iron deficient Somali nomads.

The mechanism of immunity to malaria may be dependent upon iron containing enzymes. Allison and Eugui[5], and Clark and Hunt[97] have proposed that asexual forms of malaria parasites may be killed within the erythrocyte by "oxidant stress". *Ie* H_2O_2, O_2^- and other reactive oxygen intermediates.

Summary

In summary it can be simply stated that iron is an essential nutrient for all microbial forms of life (with the possible exception of the non-pathogenic lactobacilli). Organisms often have specialized systems

for procuring iron because its salts are so insoluble. Availability of the element is often rate limiting for growth of microbes.

Pathophysiology of iron deficiency

Non-immunological effects

A number of textbooks and half a dozen recent reviews summarize the effects of iron deficiency[241, 290, 119, 381, 484, 441, 118]. When Lanzkowsky called iron deficiency a *"systemic disease"*[290] his words may have been selected for their dramatic spotlighting of the non-haematological consequences, but they were well chosen. For no physiological system is spared although the haematologic consequences are most prominent. The following paragraphs list, in point format, the major consequences of iron deficiency. Original references are not cited except for immunologic and behavioural effects. If more information is required, the interested reader may use one of the previously cited review articles as a starting point in locating more information.

Iron deficiency - Blood and bone marrow

BLOOD

Haemoglobin	decreased
Haematocrit	decreased
Mean cell volume	decreased
Mean cell haemoglobin	decreased
Mean cell haemoglobin concentration	decreased
Red cell distribution width	increased
Erythrocyte protoporphyrin	increased
Ferritin	decreased
Transferrin/Total Iron Binding Capacity	increased
Transferrin saturation	decreased
Iron	decreased
Blood smear microscopy	hypochromia microcytosis

Red cell biochemistry

Free protoporphyrin	increased
Red cell membrane stiffness	increased (alpha globin monomers associated with membrane)
Red cell life span	decreased
Autohaemolysis	increased
Susceptibility to sulfhydryl inhibitors	increased
Red cell hexokinase	increased
Globin synthesis	decreased (alpha chain decreased more than beta)
Glycine incorporation into haem	decreased
Adenosine triphosphate (ATP)	normal or decreased
ATP stability	decreased
2,3-diphosphoglycerate (2,3-DPG)	decreased
Catalase	decreased
Susceptibility to H_2O_2	increased
Glutathione peroxidase	decreased
Glutamic oxaloacetic transaminase (EGOT)	increased
Glycolytic enzymes	increased
Potassium	increased
Lactate production	increased
NADH-Methaemoglobin reductase activity	increased

Bone-marrow

Stainable iron	decreased
Sideroblast count	decreased
Erythropoiesis	ineffective
DNA synthesis	decreased
RNA synthesis	decreased

Response to treatment

When iron deficiency is treated with adequate doses of iron, haemoglobin rises about 1 g/dl per week and there is a modest reticulocytosis of up to 10 per cent. This is slow compared to the response to treatment of megaloblastic anaemia which may produce a reticulocytosis of up to 50 per cent. The reason for this difference may be that the marrow in iron deficiency is hypoplastic with a defect in cellular proliferation as well as a defect in cellular maturation.

Iron deficiency - Gastrointestinal tract

Symptoms and signs

Anorexia
Pica
Failure to thrive
Exudative enteropathy

Laboratory tests

Gastric acidity		reduced
Absorption:	Xylose	reduced
	Fat	reduced
	Vitamin A	reduced
Biopsy histology		**normal**
Disaccharidase activity		reduced
Cytochrome oxidase activity		reduced
Succinic dehydrogenase		reduced

Iron deficiency - Cardiovascular system

Symptoms and signs	
Heart rate	increased
Cardiac output	increased
with severe anaemia	decompensation
Cardiac development	hypertrophy
Tolerance to digitalis	increased

Iron deficiency - Skin and mucous membranes

Laboratory tests	
Iron content of skin, nails, hair	decreased
Cytochrome oxidase in buccal mucosa	decreased

Iron deficiency - Musculo-skeletal system

Symptoms and signs	
Growth indices (weight, height)	decreased
Work performance	decreased
X ray bones (notably skull)	"hair on end"
Myoglobin	decreased
Cytochrome C	decreased

Iron deficiency - Behaviour

Introduction

The possible importance of the behavioural effects of iron deficiency is becoming widely recognized. Much recent research has sought evidence that iron deficiency may impair cognitive function, affect and activity. Such abnormalities are clearly unfavourable at any stage of life, but in infancy they pose the threat of permanent impairment by interfering with intellectual and emotional development.

There have recently been several comprehensive reviews[130, 157, 292, 403]. This section briefly summarizes results from biochemical and animal research; original works are not cited. Human studies are categorized according to age and only those involving the use of the Bayley Scales of Infant Development are discussed in detail. For a full bibliography and exhaustive analysis of the strengths and weaknesses of studies published between 1974 and 1985 the reader is referred to the authoritative review by Lozoff and Brittenham[304]

Note on the definition of behaviour

Behaviour may be quantified by measures of physical activity, by procedures for rating non-cognitive constructs such as affect, arousal, motivation and fearfulness, and by performance on tests of cognition such as learning and problem solving. Although it is often convenient to treat these categories as

independent they are often closely dependent on each other. For example, if arousal is impaired, spontaneous activity and problem solving will also suffer. In the infant, the distinction between the cognitive, non-cognitive and motor aspects of behaviour is less clear, and, in the laboratory animal, the distinction is even more difficult.

The Bayley Scales of Infant Development[23] (BSID) have been used in most studies of the role of iron status in infant behaviour. The BSID evaluate infant development using three sets of scales.

> *(1) The Mental Scale is designed to assess sensory-perceptual acuities, discriminations, and the ability to respond to these; the early acquisition of "object constancy" and memory, learning and problem solving ability; vocalizations and the beginnings of verbal communication; and early evidence of the ability to form generalizations and classifications, which is the basis for abstract thinking. Results of the administration of the Mental Scale are expressed as a standard score, the MDI, or Mental Development Index.*
>
> *(2) The Motor Scale is designed to provide a measure of the degree of control of the body, coordination of the large muscles and finer manipulatory skills of the hands and fingers. As the Motor Scale is specifically directed towards behaviours reflecting motor coordination and skills, it is not concerned with functions that are commonly thought of as "mental" or "intelligent" in nature. Results of the administration of the Motor Scale are expressed as a standard score, The PDI, or Psychomotor Development Index. ...*
>
> *(3) The Infant Behaviour Record is completed after the Mental and Motor Scales have been administered. The IBR helps the clinician assess the nature of the child's social and objective orientations towards his environment as expressed in attitudes, interests, emotions, energy, activity, and tendencies to approach or withdraw from stimulation.*[23]

The Mental and Motor Scales yield summary scores, the MDI and PDI, which are adjusted for age so that the norm is 100 with a standard deviation of 16 for all age groups between 2 and 30 months. The IBR consists of 30 scales on which the tester rates the infant's behaviour and, in contrast to the MDI and PDI, generates no natural summary measure. Matheny[331] has suggested a weighted linear combination of items of the IBR to give 7 or 8 summary factors. Matheny's method was employed in the analysis of results in the present research and is discussed in more detail below.

Biochemical studies

Metabolic processes which are dependent upon iron include oxygen transport and storage (haemoglobin, myoglobin), oxidative phosphorylation, neurotransmitter metabolism and DNA

synthesis*. Alterations in any of these processes could have major effects on the functioning of the nervous system but there is not much detailed information available about the metabolic role of iron in human brain and neural tissues. In the adult, the basal ganglia have concentrations of iron comparable to those found in the liver, spleen and bone marrow - the major storage sites for iron. Brain iron concentrations rise gradually from birth, increasing tenfold by adulthood. Early iron depletion in the rat leads to a depletion of iron in the brain which is not repaired by treatment even though anaemia and liver non-haem iron are promptly restored to normal.

Defects in thermoregulation, conversion of thyroxine to thyronine and increased urinary excretion of catecholamines have been noted.

Animal studies of behaviour

Studies of behaviour in iron deficient rats have shown deficits in reactivity, responsiveness, level of arousal, attentiveness to environmental stimuli and spontaneous activity**. The diurnal pattern of spontaneous activity is also disturbed. However, no evidence has been found for derangements of basic cognitive performance.

Studies of behaviour in adults, adolescents & children

Pica has long been recognized as a consequence of iron deficiency although the reason remains unclear[349, 332, 287, 201, 428, 416, 29].

Iron deficiency anaemia has been clearly shown to limit peak physical performance and field work suggests that it also limits economic productivity[146, 22].

A number of studies have found associations between iron deficiency and measures of cognition such as IQ tests and scholastic achievement but all these studies suffer from methodologic inadequacies that limit the conclusions that can be drawn.

Studies of behaviour in infants

Infancy is a particularly important period in which to study the effects of iron deficiency on behaviour. This is because iron status reaches a nadir between 12 and 24 months of age and the effects of iron deficiency on the brain and behaviour may be prolonged in spite of treatment. The published studies of behaviour in infancy that have attempted to define the impact of iron deficiency are reviewed in greater detail than the other work which does not directly relate to the present research.

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* Lozoff[130] is a good starting point for a wider review and a fairly extensive bibliography.

** Lozoff[130] is also a fine source for references to original work in this field.

Oski and Honig[383, 233] were the first to study the effects of treatment of iron deficiency on infant behaviour. They administered the Bayley Scales of Infant Development to 24 iron deficient anaemic infants whose ages ranged from 9 to 26 months. Half were treated with intramuscular iron dextran and the other half were given placebo. The BSID were administered a second time 5 to 10 days after treatment. The treated group had a significant rise in the MDI score, but the *change* was not significantly different from the *change* in the placebo treated group. There was a similar non-significant trend towards improvement in the PDI. While showing some intriguing tendencies this study was unable to answer two critical questions: *Is the behaviour of iron deficient infants impaired with respect to iron sufficient controls?* and, *How much of the change in the second administration of the BSID is due to a training effect rather than a specific effect of iron treatment?*

At least five subsequent studies and the present project have attempted to replicate these results and avoid at least some of the methodologic problems inherent in Oski and Honig's design.

Lozoff *et al*[305] studied the acute effects of oral iron treatment on the behaviour of iron deficient anaemic infants aged 6 to 24 months. The MDI showed a trend towards improvement when the 15 treated iron deficient infants were compared with the 12 untreated iron deficient infants or the 40 iron replete controls. The PDI showed no definite tendency in a similar comparison. Examining the pre-treatment results for the effects of anaemia revealed significantly lower MDI and PDI scores in the iron deficient group. To assess the influence of age and iron status the MDI score was subjected to a correlation analysis with a rating of iron status in three age groups, 6 - 12 months, 13 - 18 months and 19 - 24 months[306]. The MDI scores were normal for the two younger groups, but in the 19 - 24 months bracket the MDI scores were lower than average and correlated strikingly with iron status.

In Santiago, Chile, Walter *et al*[507] tested 37 infants at the age of 15 months with the BSID before and after an 11 day regimen of oral iron therapy. The 10 babies with iron deficiency anaemia had MDI scores that averaged 10 points lower than those of the iron depleted and iron replete babies. The MDI scores of the iron deficient babies improved with treatment by 10 points, whereas the iron sufficient group improved by one point.

A second study by Oski and Honig[384] sought to define the effects of iron treatment on non-anaemic iron deficient infants. These workers selected 38 infants between 9 and 12 months of age, all of whom had haemoglobin concentrations greater than 11.0 g/dl. All were treated with intramuscular iron dextran and the Bayley scales were administered immediately before and 7 days after the injection. The infants were placed into one of four graded categories according to their serum ferritin, erythrocyte protoporphyrin, and mean cell volume. The pretreatment MDI scores were lowest for the iron deficient group, but the normal group's mean MDI fell between those of the iron depleted group and the iron deficient group. After treatment the iron deficient group had a clear rise, statistically

significantly different from those of the iron replete and iron depleted groups. The authors speculated that the reason for the similar results in the iron depleted and iron replete groups might be that neural function is not affected by mere depletion of iron stores but is affected by tissue deficiency.

Deinard *et al*[130] and Johnson and McGowan[251] in two observational studies tested 1 year old infants with the BSID. Deinard *et al* used serum ferritin to categorize the iron status of their 34 subjects who all had haematocrits greater than 33%. Johnson and McGowan used haemoglobin levels of 10.5 and 11.5 g/dl as the upper and lower limits in the selection criteria for their iron deficient and iron sufficient groups. Neither study found remarkable differences in PDI or MDI.

The non-cognitive aspects of behaviour are less easily quantified. Lozoff and Brittenham[304] have suggested that analysis of the IBR shows abnormal ratings for iron deficient infants in two classes of summary scores. One class characterizes affect and the other describes orientation to tasks. Iron deficient babies also improved with treatment.

Other investigators have found isolated items on the IBR that are altered in iron deficiency and its treatment. Oski and Honig[383] reported that infants became more alert, responsive and better coordinated. Walter *et al*[507] noted an improvement after treatment in co-operativeness and listening to sounds, while Deinard *et al*[130] found the iron deficient infants to be more fearful, more vocal, less visually and auditorally attentive and less likely to mouth toys. Johnson and McGowan reported no difference in their study[251]. The IBR has 30 items that are scored. No study took account of the relatively high probability of finding "significant" differences purely by chance when making so many comparisons.

Lozoff *et al*[307] analyzed the interaction between mothers and infants at play and found that anaemic babies were not more irritable nor more distractible but that they did maintain closer contact with their mothers. This was interpreted as a manifestation of disturbance in affect. In a similar study Johnson and McGowan[251] found no differences.

Summary

None of these 6 projects completely satisfied the methodologic requirements for unambiguous demonstration of a behavioural effect of iron deficiency and the specific findings of one study are not directly comparable to those of any other study due to differences in ages of the children, criteria for iron status, treatment of iron deficiency and timing of administration of the BSID. Nonetheless, the pattern of results clearly suggests an impairment of mental developmental test scores in iron deficiency anaemia, especially in older infants, and that treatment with oral or intramuscular iron improves the scores before a clinically important response in haemoglobin has taken place. Table 2.5 summarizes the results of these studies.

Table 2.5 Behavioural associations with iron status.

AUTHOR	REF No.	AGE GROUP	NUMBER IN STUDY	IRON STATUS	INTERVENTION	RESULTS (Test group compared with Control group)
	ADULT					
Elwood	154	> 20 yr	47	Hb < 10.5 g/dl	Oral iron	Psychomotor performance: No significant difference
	ADOLESCENT					
Webb	513	12 - 14 yr	T 92 C 101	Hb < 11.5 g/dl Hb > 14.0 g/dl	Observation	Iowa Test of Basic Skills : (T - C) : worse p < 0.025
Webb	513	12 - 14 yr	T 92 C 101	Hb < 11.5 g/dl	Observation	Latency of visualization of after image: Increased
Webb	514	12 - 14 yr	T 74 C 36	Hb < 11.5 g/dl	Observation	Behavior Problem Checklist: Disruptive, irritable, restless
Tucker	498	Youths	A 69	Regression on SF	Observation	Asymmetric EEG, greater verbal fluency, poorer nonverbal test
	CHILD					
Sulzer	488	4 - 5 yr	A 230	Hb < 10.5 g/dl	Observation	Battery incl vocabulary, IQ, association: worse
Pollitt	404	10.8 y	Tr 43 Tp 35 Cr 16 Cp 25	Hb < 11, TRF < 16% " Hb > 11.9, TRF > 19% "	Oral iron, 5m Placebo Oral iron, 5m Placebo	School achievement test: (T1 - C1) : worse P < 0.001 Placebo treatment : no change Iron treatment: (Tr2 - Tr1) : Improved, P < 0.001 (Tr2 - Cr2) : Lower, P < 0.005
Pollitt	383	9.5 y	Tr 18 Tp 10 Cr 19 Cp 21	Hb < 11.5, etc " Hb > 13, etc "	Oral iron, 4m Placebo Oral iron, 4m Placebo	Matching familiar figure test: (T1 - C1) : worse p < 0.05 Placebo treatment : no change Iron treatment: (Tr2 - Cr2) : No difference (Tr2 - Tr1) : Improved, P < 0.05

IRON AND BEHAVIOUR/COGNITIVE FUNCTION

AUTHOR	REF No.	AGE GROUP	NUMBER IN STUDY	IRON STATUS	INTERVENTION	RESULTS (Test group compared with Control group)
		INFANT				
Oski	383	9 - 26 m	T 12	Hb < 10.5 g/dl etc	IM Imferon	Bayley Scales (T2 - T1) - (C2 - C1): PDI = + 6.83
			C 12	Hb < 10.5 g/dl etc	Placebo	MDI = + 7.5
						P < 5% for IBR: 15, 26, 27
Oski	384	9 - 12m	C 10	Hb >10.9, Normal	IM Imferon	Bayley scales (1, 2, 2-1): MDI = 90.8, 97.0, 6.2
			Ta 10	Hb >10.9, FRT < 12	"	MDI = 94.6, 100.2, 5.6
			Tb 10	Hb >10.9, FEP > 30	"	MDI = 83.9, 104.4, 20.1
			Tc 8	Hb >10.9, MCV < 70	"	MDI = 85.5, 109.1, 23.6
						IBR: unremarkable, no trends
Walter	507	15 m	C 15	Hb > 10.9, etc	Oral iron	Bayley scales (1, 2, 2-1): MDI = 113, 112, 1
			Ta 12	Hb > 10.9, etc	"	MDI = 108, 113, -5
			Tb 10	Hb < 11.0	"	MDI = 98, 108, 10
						IBR: 4, 17 improved
Lozoff	305	6 - 24 m	Tr 15	Hb < 10.6 g/dl etc	Oral iron	Bayley scales (1, 2, 2-1): MDI = 82.7, 89.8, 7.1
			Tp 12	"	Placebo	MDI = 91.5, 97.0, 5.5
			Cr 19	Hb > 12.0 g/dl	Oral iron	MDI = 98.6, 104.1, 5.5
			Cp 21	"	Placebo	MDI = 102.0, 107.1, 5.1
						Bayley scales (1, 2, 2-1): PDI = No change
						Bayley scales (1, 2, 2-1): IBR not reported

I R O N A N D B E H A V I O U R / C O G N I T I V E F U N C T I O N

AUTHOR	REF No.	AGE GROUP	NUMBER IN STUDY	IRON STATUS	INTERVENTION	RESULTS (Test group compared with Control group)
		I N F A N T (continued)				
Lozoff	306	6 - 24 m	C 11	Hb > 12.0 g/dl	Observation	
			Ta 3	Hb > 12.0, FRT < 13	"	Bayley Scales (MDI): 120.7 MLR -> 56% Fe status,
			Tb 7	Hb > 12.0, etc	"	: 91.6 12% for age etc
			Tc 4	Hb < 12, etc	"	: 82
						: 73.4
						Bayley Scales (PDI): No correlation
Deinard	130	11 - 13 m	Ta 34	HCT > 33, FRT < 10	Observation	Bayley Scales (PDI, MDI) : 109.8, 120,8
			Tb 21	HCT > 33, FRT 10 - 19	"	: 103.8, 116.3
			C 157	HCT > 33, FRT > 19	"	: 109.1, 121.5
						IBR : 5, 16, 17, 24 : Increased fearfulness, Others less
						Uzgiris & Hunt Ordinal Scales : No differences
Cantwell	80	6 -18m	T 32	Hb < 9.6	Nil	At 7 years of age : Clumsiness increased, IQ decreased
			C 29	Hb > 11.4	Imferon	

Iron deficiency - Other tissues

Tissues other than those of the immune system and systems covered above

Cytochrome C (haem containing)	reduced
Cytochrome oxidase (haem containing)	reduced
Succinic dehydrogenase (iron dependent)	reduced
Aconitase (iron dependent)	reduced
Monoamine oxidase	reduced
Urinary noradrenaline	increased
Tyrosine hydroxylase	reduced

Pathophysiology of iron deficiency

Immunological effects

The literature review up to this point has tried to provide the background necessary for understanding the research reported in this thesis. Review articles have been cited whenever possible although, on occasion, original articles have been selected for their ability to document a point. The following section attempts to be as comprehensive as possible in covering the literature on iron and immune function in infancy.

Interactions - Nutrition, infection, immunity

Nutrition and immune function

In endeavouring to determine the effect of iron deficiency on immune function it is important to be able to control for the effects of other nutritional deficits. If the diet is inadequate as far as iron is concerned, it is also likely to be inadequate in trace elements, proteins, fats, vitamins and energy. Iron deficiency itself may also lead to anorexia, malnutrition and disturbances in metabolism that result in impaired growth[355]. In clinical studies, unless the iron deficiency is clearly due to blood loss, it is not possible to attribute variations in immunological

tests to one specific dietary deficiency such as iron[63].

Kwashiorkor, marasmus, and deficiencies of specific nutrients impair immune responses. These include reduced antibody response to antigenic stimulation, depressed delayed cutaneous hypersensitivity, and impaired *in vitro* tests of T lymphocytes, B lymphocytes and phagocytes[388, 33, 112, 407, 9, 57]

Experiments with animals control for these confounding effects by comparing animals on an iron deficient diet with 2 sets of controls on an adequate diet. One set of controls is fed *ad lib*, and the other set is *pair-fed* the exact quantity consumed by the iron deficient animals[283]. Inferences from human observations, which cannot be as carefully controlled, should be appropriately qualified.

Infection and immune function

Infections with a number of bacterial, viral and protozoal agents have been shown to lead to anergy[63]. These organisms include *Mycobacterium tuberculosis*, measles[112], LAV-III, infectious mononucleosis, influenza, hepatitis B, polio, rubella, and chicken pox. Changes in tests of immune function include an impairment in delayed skin reactions to tuberculin and candida antigen, and in mitogenic lymphocyte transformation *in vitro*[370, 165, 321, 117]. In no clinical study is it possible to exclude intercurrent infection as a cause of immunologic abnormalities[63], although certain experimental designs are better controlled than others for such confounding factors.

Another complicating factor is that leukocytosis itself, whether accompanying infection or not, is associated with a significant degree of anergy[216].

Infection and nutrition

The relationship between iron, nutrition and immune function is complicated by the fact that infection may precipitate malnutrition and malnourished persons are more susceptible to infection[90].

These effects of infection on immune function make it difficult to interpret studies of immune function performed in iron deficient subjects who are also infected.

In addition to its effects on nutrition in general, infection may interfere with absorption, transport to and release from storage of iron[278, 279, 280, 297].

Host control of available iron

Part of the host's defense mechanism against infecting micro-organisms is control of available iron[518, 16, 197]. This is accomplished by:

1 The chelation of iron, thus removing it from the environment. Chelators such as transferrin in serum, lactoferrin in phagocytes and both transferrin and lactoferrin in breast milk and cow's milk exhibit bacteriostatic properties[66, 102, 270]. Lactoferrin-iron complexes are more stable than transferrin-iron complexes at the low pH levels within lysozymes in phagocytes. Lactoferrin is also found in other secretions such as semen, tears and cervical mucus.

2 The reduction of the concentration of iron in the blood[15, 270, 149]. Mechanisms include reducing the absorption of iron from the gut[36], blocking the return of iron from the reticuloendothelial system to transferrin[296], increasing sequestration of iron in tissue stores[32], and accelerated removal of iron from the plasma transferrin pool[295, 296].

3 Raising the body temperature; bacterial siderophore production is critically sensitive to temperature in some species. Injection of *Pasteurella multocida* into rabbits was followed by fever and a marked fall in plasma iron; the inhibitory effect of low iron on bacterial growth *in vitro* was dependent on febrile temperatures[268]. Similar results have been obtained for *Salmonella typhimurium*[189] but do not seem to have been extended to *in vivo* models of infection.

Possible mechanisms for the control of the hypoferraemic response are more extensively reviewed by Beisel [32], Letendre[311] and Ballentyne[15].

Iron deficiency and laboratory tests of immune function

The following sections review the evidence from laboratory tests for the role of iron deficiency in causing immune dysfunction. The tests are grouped according to the part of the immune system that they measure. The grouping is somewhat artificial in that few parts of the immune system can be considered in isolation. For example, the production of antibodies by the B lymphocyte is dependent upon a complex interaction with macrophages and T lymphocytes. The oversimplification that is necessary for structuring the presentation of the laboratory data should not be allowed to contaminate the interpretation of the results in terms of basic mechanisms.

Iron deficiency and humoral immunity

It is convenient to consider the function of the B lymphocyte under this heading as well as complement. The B lymphocyte produces antibody in response to an antigenic stimulus. There is a complex dependency of B cells on macrophages, T helper/inducer cells and T suppressor cells. Laboratory tests of B cell function in iron deficiency have included measurement of immunoglobulin levels, determination of isohaemagglutinin concentrations, the response to immunization with specific agents, (in animals) the distribution of B lymphocytes in splenic tissue and the differentiation response *in vitro* to stimulation with lipopolysaccharide.

Immunoglobulin production - Non-specific

Serum IgG and IgA have been reported to be normal in iron deficiency[93, 92, 88, 311, 445]. Serum IgM was reported in the same studies to be normal or increased[88, 311]. Where it was increased, there is suspicion that this may have been due to recent infection.

Salivary IgA[311] and isohaemagglutinins[312] were also within normal limits in two studies.

Secretory IgA in the gastrointestinal tract, however, has been reported to be increased in healthy infants who had been fed iron-fortified milk for the first 8 weeks of life[274].

Immunoglobulin production - Specific

The results of tests of response to immune function are not always consistent. Chandra[93] documented normal responses to immunization with tetanus toxoid and typhoid O and H antigens in children with iron deficiency anaemia. The control group used for comparison was not described.

Macdougall[312] found that response to immunization against diphtheria was diminished, but that response to typhoid O and H antigens was comparable to the control group. The numbers of subjects was small, and the iron deficient group may have been suffering from the confounding effects of infection and/or malnutrition.

Nalder *et al*[364] found a clear dose-response relation between the degree of iron deficiency and the depression of response to immunization with tetanus toxoid in rats. However, as iron deficiency induces malnutrition, the depressed antibody response may have been due to the effects of malnutrition. This supposition is contradicted by the study of Kuvibidila[281] who reported a depression of response to immunization with sheep red blood cells in mice fed an iron deficient diet. No such depression was found in pair-fed controls.

Malakhovsky[320] reported similar immune responses to natural infections in anaemic children and in non-anaemic children. The micro-organisms included staphylococcus, *Shigella dysenteriae*, *Salmonella typhimurium*, *Escherichia coli*, Adenovirus and parainfluenzavirus*.

B cell function

In a study of B cell stimulation with bacterial lipopolysaccharide, Kuvibidila[283] found that cells from iron deficient rats had a diminished response. *In vitro* repletion by mouse transferrin, haemin or ferric chloride did not restore responsiveness to bacterial lipopolysaccharide. The percentage of B cells in spleen cell suspension was statistically lower and morphologic changes were noted in electron microscopy of treated splenic cells in culture. Iron deficient mice[281] sensitized *in vivo* with sheep erythrocytes generated fewer plaque forming cells per 10^6 spleen cells or 10^6 B lymphocytes.

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* Evaluation of this report is difficult because it is not easy to understand the serological methods in the translation from the Russian.

The roles of T cells and macrophages in antibody production

The roles of the macrophage, T helper cell and T suppressor cell in the production of antibodies in iron deficiency have not been studied[281].

Complement

Tests of complement levels in iron deficiency have shown CH50 and C4 to be normal[311, 92] and C3 to be decreased[93], normal[92] and increased[311]. In no study was malnutrition and/or infection satisfactorily excluded. However, it would seem that complement levels are not changed in any important way in iron deficiency.

Summary and conclusions

In summary, the best scientific evidence for an effect of iron deficiency on humoral immunity is the work of Kuvibidila with laboratory mice. The clinical studies have involved small numbers of patients and none excluded confounding effects of infection and malnutrition, but it would seem that antibody response is depressed, at least to certain classes of antigens, although gross immunoglobulin and complement levels are normal.

Table 2.6 Iron status and immune function
Immunoglobulin and complement levels in iron deficiency. The table indicates the directions in which iron deficient groups differed from control groups. Non-significant trends are shown by enclosing them in parentheses. Where there is no clear trend an N is placed in the table.

REF Number	Author	SERUM IMMUNOGLOBULINS			HETEROHAEM-AGGLUTININS	ISOHAEMAGGLUTININS		SALIVA	SERUM COMPLEMENT		
		IgG	IgM	IgA		anti A	anti B	IgA	CH50	C3	C4
93	Chandra	(I)	(D)	(D)						(D)	
92	Chandra	N	N	N						N	N
275	Krantman	(D)	(I)	(D)							
311	MacDougall	N	(I)	N				N	N	I	
312	MacDougall					N	N				
320	Malakhovsky				N	N	N				
445	Sawitsky	N	N	N							

Notes: TEST RESULTS
D Decreased N No change I Increased

Table 2.7 **Response to immunization in iron deficiency. The table indicates the directions in which iron deficient groups differed from control groups. Non-significant trends are shown by enclosing them in parentheses. Where there is no clear trend an N is placed in the table.**

REF Number	Author	IMMUNIZATION AGENT						B CELL STIMULATION WITH BACTERIAL LIPOPOLYSACCHARIDE
		TETANUS TOXOID	MISCELLANEOUS (see note)	DIPHTHERIA TOXOID	TYPHOID O ag	TYPHOID H ag	SHEEP RBC	
93	Chandra	N			N	N		
281	Kuvibidila						N	
283	Kuvibidila							D
312	MacDougall			(D)	(D)	N		
320	Malakhovsky		N					
364	Nalder	D						

NOTE

Miscellaneous: Staphylococcal antigen, *Shigella dysenteriae*, *Salmonella typhi* (Widal), Adenovirus, Parainfluenzavirus all normal seroconversion rates or titre

TEST RESULTS

D Decreased N No change I Increased

Iron deficiency and T cell function

Tests of T cell lymphocyte function in iron deficiency have included absolute counts and relative proportions of T cells, cytolytic activity and blastogenic response to mitogens. The results of all such studies found in the literature search are summarized in tables 2.8, 2.9 and 2.11 and are discussed below.

T cell absolute number & proportion of lymphocytes

Table 2.11 shows that 4 studies of iron deficiency have found a decrease in the percentage of T cells[39, 93, 275, 473] and one study has reported a normal proportion of T cells[92]. The absolute number of T cells was only reported by one group[275] who found it to be depressed. No fundamental mechanisms have been proposed to account for these findings but Kuvibidila *et al*[283] have suggested that there may be a block in haemopoietic cell differentiation.

Lymphocyte blastogenic stimulation

The response of T lymphocytes to stimulation with a wide variety of mitogens has been reported to be depressed in 11 studies listed in table 2.8*. Only two reports of unchanged or increased blastogenic response of lymphocytes from iron deficient patients were located in the literature search[277, 243]. Neither report includes an estimation of the probability of **not** detecting a real change (*ie* the type II error).

Many of the clinical studies do not allow the confounding effects of over- or under-nutrition, vitamin or zinc deficiency and infection to be ruled out. The most sophisticated experiments (as is to be expected) were performed on laboratory mice[281, 283]. The test group, which was fed an iron deficient diet was compared to:

a **control** group, fed *ad libitum* an iron sufficient diet

a **pair-fed** group, fed the iron sufficient diet, but limited to the quantity consumed by the iron deficient group.

a **repleted** group which had been fed the iron deficient diet until they were anaemic and then fed the iron sufficient diet (for 7 - 14 days) until their haemoglobins had been restored to normal

The results showed a marked and consistent depression of response to PHA and to Con A in iron deficiency with restoration to normal levels in the iron repleted mice.

The restoration of blastogenic responsiveness contradicts the hypothesis proposed by Joynson *et al*[243] and Bhaskaram *et al*[39] to explain their failure to demonstrate such a return to normality. Their theory was that response to mitogens is delayed after repletion of iron status.

Kuvibidila[281] reported that *in vitro* repletion by mouse transferrin (10 - 30 ug/ml) and ferric chloride (0.25 - 1 ug/ml) partially restored responsiveness to stimulation by PHA, but not Con A and also decreased the responsiveness in the two control groups. Haemin (0.5 - 5 uM) partially restored responsiveness to PHA and Con A stimulation in T lymphocytes, and decreased the response of the controls to both mitogens. *In vivo* repletion restored mitogenic response after 10 days of feeding on an iron supplemented diet.

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* Srikantia[473] reported a decreased response to PHA stimulation but the statements made in the text do not agree with the data tabulated. I have assumed that the statements are correct and that there is a typographical error in the table.

Macdougall *et al* 1975[311] studied lymphocyte transformation in response to stimulation by phyto-haemagglutinin and candida antigen in 15 iron deficient infants, 11 of whom were anaemic. Both the latent iron deficiency group and the anaemic group had significantly lower incorporation of 3H-thymidine than the controls. Ten patients were retested two to three months after treatment with intramuscular iron dextran. No statistical tests were published, but the published data were sufficient to allow a paired t test to be made. This yielded significance levels of 0.002 and 0.02 for phytohaemagglutinin and candida respectively. Unfortunately, no controls were retested so the reasons for the improvement can not be assigned with confidence to the treatment.

The results of tests of T cell response to mitogens in the face of iron deficiency are not entirely consistent, but the best controlled study and overall impression indicate that iron deficiency is associated with a reduction in response.

Possible mechanisms for this reduction have been suggested by Kuvibidila *et al*[283]. They assert that decreased mitogenic responses may be partly, but not entirely, due to decreased percentages of mature T cells (assessed by density of anti- antigen on the cell surface). The reason for this assertion was that they failed to reverse mitogenic responses by enriching the T cell fraction by using nylon wool to remove null cells. These authors further hypothesize an increasing effect of iron deficiency on less mature T cells (Con A responsive) compared to the more mature PHA responsive cells. An alternative mechanism could be an increase in null cells resulting in interference with PHA responsiveness. More likely alternatives are the possibility that lymphocytes from iron deficient subjects interact poorly with mitogens and/or have a decreased survival *in vitro*.

Lymphokine production

MIF (migration inhibitory factor) production in response to Candida antigen and to PPD was depressed in iron deficient patients[255]

Cytolysis

Cytolysis* of tumour cells was depressed in iron deficient mice[277].

Several possible mechanisms for this defect in T cell function are proposed by the authors. Iron deficiency might lead to a decrease in the proportion of mature lymphocytes which are sensitized and become cytotoxic or respond to non-specific mitogens. Or, it might lead to a defect in the sensitization process itself. A third possibility is that the killing capacity of each cell might be reduced. Or, there may be some combination of the hypothesized mechanisms. Qualitative impressions of microscopical examination of sensitized splenic cells incubated with tumour cells were that iron deficiency did not impair the ability of attacker cells to contact and adhere to target cells. This suggests that iron deficiency is most important at the level of cell lysis.

* The experiment employed cytolytic cells harvested from the spleen and peritoneal cavity. The authors cite a previous study in which the percentage of T lymphocytes in spleen cell suspension was found to be approximately 50, 44 and 27% in the control, pair-fed, and iron deficient groups respectively. The authors state, without data, that the peritoneal cells included macrophages as well as lymphocytes. The study is included in this section on the assumption that T cells would have been central to the cytolytic function even for the peritoneal cells.

Delayed cutaneous hypersensitivity

Table 2.9 summarizes the results of 10 studies of delayed cutaneous hypersensitivity in iron deficiency. Nine authors report diminished responses to 23 tests of 9 different sensitizing agents. Two authors report 2 tests which showed a normal response to PPD[93] and to DNFB[366]. These 2 "negative" reports contrast with the 23 "positive" reports. The evidence for an impaired delayed cutaneous hypersensitivity is thus fairly strong, although it must be stated again that the results of clinical studies are open to potential confounding influences from malnutrition and infection. The one carefully controlled study on laboratory animals confirms the clinical investigations[284].

Kuvibidila[281] reported that a single injection of parenteral iron dextran 24 hours before the recall dose of DNFB restored responsiveness to normal.

No mechanisms have been proposed to account for this effect of iron deficiency although Kuvibidila[281] noted that iron deficiency did not impair sensitization, and both Kuvibidila *et al*[284] and Krantman *et al*[275] theorized that it may involve factors such as monocyte chemotaxis, local inflammatory response by mast cells and lymphokine production. The latter perhaps due to decreased ribonucleotide reductase activity.

Summary and conclusions

Laboratory tests of immune function are particularly sensitive to methodology. The results of lymphocyte blastogenic stimulation depend on duration of incubation at each stage as well as culture media and other factors. For this reason comparisons of results between different laboratories should not be made without careful validation of the procedures employed[63].

In summary, the clinical evidence is suggestive of a decrease in the proportion of T cells, impairment of delayed cutaneous hypersensitivity and lymphocyte blastogenic response to mitogens including phytohaemagglutinin, candida antigen, concanavalin A, pokeweed mitogen, tetanus antigen and purified protein derivative of *Mycobacterium tuberculosis*. The studies on laboratory animals confirm the clinical observations and show that cytolytic activity is also impaired. In addition, the sensitization step does not seem to be affected. Although these laboratory studies seem conclusive, they have all been performed in one laboratory and independent confirmation by other workers would substantially strengthen the hypothesis of iron deficiency causing a defect in T cell function.

The biochemical mechanisms for these effects of iron deficiency remain to be elucidated although many papers note the impairment in DNA synthesis found by Hershko[223] and the inhibition of ribonucleotide reductase found by Hoffbrand[229]. An interesting surmise was made by Gross *et al*[199], namely that iron deficiency may interfere with folate metabolism since folate deficiency adversely affects cellular immunity.

Table 2.8 T Lymphocyte stimulation tests in iron deficiency. The table indicates the directions in which iron deficient groups differed from control groups. Non-significant trends are shown by enclosing them in parentheses.

REF Number	Author	LYMPHOCYTE STIMULATION MITOGEN						MISCELLANEOUS
		PHA	CANDIDA	CON A	PWM	PPD	TETANUS	
39	Bhaskaram	D						
92	Chandra	D						
93	Chandra	D						
169	Fletcher	D						
199	Gross	(D)						
255	Joynson		(D)			D		Lymphokine production: Decreased
275	Krantman	(D)	(D)				(D)	
277	Kulapongs	(I)						
285	Kuvibidila							Cytolysis of tumour cells: Decreased
283	Kuvibidila	D		D				
311	MacDougall	D	D					
445	Sawitsky	D			(D)			
473	Srikantia	D						

TEST RESULTS
D Decreased I Increased

Table 2.9 Delayed cutaneous hypersensitivity in iron deficiency. The table indicates the directions in which iron deficient groups differed from control groups. Non-significant trends are shown by enclosing them in parentheses. Where there is no clear trend an N is placed in the table.

REF Number	Author	AGENT									
		DNCB	PHA	CANDIDA	PPD	Mumps	Trichophyton	SK/SD	Diphtheria toxoid	Tetanus toxoid	
39	Bhaskaram		N								
93	Chandra			(D)	N	(D)	(D)	D			
92	Chandra			D	(D)	D	D	D			
91	Chandra			D	(D)	D	D	D			
199	Gross	N									
255	Joynson			D	D						
275	Krantman			D						D	
284	Kuvibidila	D									
311	Macdougall			D				D	D		

Test results
D Decreased N No change I Increased

Abbreviations:
DNCB Dinitrochlorobenzene PHA Phytohaemagglutinin SK/SD Streptokinase/Streptodornase
PPD Purified protein derivative of *Mycobacterium tuberculosis*

Iron deficiency and polymorphonuclear leukocyte function

Neutrophil and macrophage function has been measured in iron deficiency by several tests summarized in tables 2.10 and 2.12. The tests include reduction of nitroblue tetrazolium (NBT), chemotaxis, opsonic activity, phagocytic activity, bactericidal activity and activity of the iron containing oxidative enzymes NADPH-oxidase, myeloperoxidase (MPO) and catalase.

Opsonic activity

The neutrophil has surface receptors for the Fc portion of IgG and for complement C3b. If a bacterium has been coated with either of these substances its contact with a neutrophil and subsequent phagocytosis is facilitated. This process of rendering bacteria more "digestible" is not considered under the, perhaps more appropriate heading of humoral immunity because it (and chemotactic activity) are, in the literature, usually grouped together with more direct tests of polymorphonucleocyte (PMN) function.

Chandra and Saraya[93] reported that opsonic activity of plasma for *Candida albicans* was normal in iron deficient children. Foroozanfar *et al*[174] found it to be diminished in *"professional blood donors"* who were *"profoundly iron deficient, anaemic and malnourished"*, often selling up to 900 ml blood a week.

For *Staphylococcus aureus*, opsonic activity of the serum or plasma from iron deficient individuals was reported to be normal[88].

Opsonic activity does not appear to be severely affected in iron deficiency. This is compatible with observations of normal levels of IgG and complement in iron deficiency.

Phagocytosis

The phagocytic activity of PMNs has been reported to be normal in iron deficiency for *Candida albicans* by Chandra and Saraya[93], for *Staphylococcus aureus* by Chandra[88] and for *Staphylococcus aureus*, *Streptococcus pneumoniae* and *Salmonella typhimurium* by Moore and Humbert[352]. Moore and Humbert also report a decreased phagocytic activity for *Candida albicans* which returned to normal on restoration of iron status.

The latter authors speculated that there might be a reversible, Candida-specific membrane defect in PMNs in iron deficiency.

These studies lead to the conclusion that there may be a minor impairment of phagocytosis in iron deficiency.

Killing activity

Polymorphonuclear leukocytes contain lactoferrin which appears to play an essential role in killing phagocytosed bacteria. If the intra-cellular iron-binding protein is saturated with iron, by exposing the cell to ferritin-antibody complex for example, the bactericidal power of the cell is greatly reduced[65]. The reason may be that lactoferrin withholds iron from intra-cellular micro-organisms

Rohrer *et al*[424] reported that macrophage mediated tumouricidal activity is decreased in iron deficient mice compared to both pair-fed and *ad libitum* fed mice.

Bactericidal activity has been found to be decreased for *Staphylococcus aureus* in 8 studies (see table 2.10). It has also been reported to be decreased for *Candida albicans* in 2 studies and *Escherichia coli* in 2. In one study each it has been normal for *Escherichia coli*, *Streptococcus pneumoniae* and *Salmonella typhimurium*.

Staphylococcus aureus, *Candida albicans*, *Escherichia coli* and *Salmonella typhimurium* are catalase positive organisms, while *Streptococcus pneumoniae* is catalase negative. Catalase negative organisms lack the protective effects of catalase and thus can be killed after phagocytosis by self-generated H_2O_2. In contrast, catalase positive organisms, once ingested by the phagocyte must be attacked by the H_2O_2 originating from the PMN in conjunction with the peroxidase-halide system[352].

Moore and Humbert[352] point out that if neutrophil oxidation is defective in iron deficiency then there would be a defect analogous to that in chronic granulomatous disease. The normal killing of *Streptococcus pneumoniae* and decreased killing of *Staphylococcus aureus*, *Candida albicans* and *Escherichia coli* in iron deficiency are consistent with this hypothesis. *Salmonella typhimurium* is catalase positive, and it might have been expected that it too would not be killed efficiently in iron deficiency. Moore and Humbert propound the hypothesis of Okamura and Spitznagel[374] that *Salmonella typhimurium* (which has a peculiarly long intra-cellular survival) is normally killed by non-oxidative mechanisms dependent on the bacterium's lipopolysaccharide structure.

In summary, iron deficiency seems to produce a microbicidal defect similar to, but less severe than, that of chronic granulomatous disease. The defect manifests itself towards catalase positive micro-organisms such as *Staphylococcus aureus* and *Candida albicans*. The possible biochemical mechanisms are discussed in detail below.

Chemotaxis

Macdougall[311] reported normal chemotactic activity of *Escherichia coli* endotoxin activated serum in iron deficient children. The chemotaxis of their PMNs was also within normal limits. Foroozanfar *et al*[174] in *"professional blood donors"* found that chemotactic activity and chemotaxis were reduced. Their subjects were malnourished as well as iron deficient. The evidence for a depression in chemotactic activity and chemotaxis is thus no more than suggestive.

No mechanism was proposed to explain the defect in chemotaxis and chemotactic activity.

Leukocyte alkaline phosphatase

Celada *et al*[85] found a direct relationship of haemoglobin to leukocyte alkaline phosphatase in neutrophils as iron status was manipulated in rabbits by bleeding them and then treating them with iron dextran.

The authors noted that leukocyte alkaline phosphatase may have different functions in the rabbit and in man as it is located in different sub-cellular structures. They also noted that the enzyme has no known role in bactericidal activity, but that it is elevated in response to bacterial infection. The authors cite a

case report of a patient with selective leukocyte alkaline phosphatase deficiency who presented with recurrent infections and was found to have granulocytes with reduced bactericidal activity[86].

Hexose monophosphate shunt activity

Latex particle hexose monophosphate (HMP) shunt activity was measured in granulocytes by Yetgin *et al*[526]. The iron deficient group had a mean stimulation index significantly lower than that of the healthy control group. The patients were malnourished as well as suffering from iron deficiency anaemia. The authors did not hypothesize a cause for the reduction in HMP shunt activity, but did discuss its significance.

The authors imply that it may play a similar role in the polymorphonucleocyte to its function in the erythrocyte. In the red cell, the hexose monophosphate shunt provides NADPH for the reduction of oxidized glutathione (G-S-S-G) to reduced glutathione (2G-SH)[326]. This reaction is catalyzed by glutathione reductase. In turn, reduced glutathione removes H_2O_2 from the erythrocyte in a reaction catalyzed by glutathione peroxidase:

$$2G\text{-}SH + H_2O_2 \text{ ----> } G\text{-}S\text{-}SG + 2H_2O$$

The authors did not explain how this might affect the bactericidal capacity of the granulocyte. As H_2O_2 is important in killing bacteria it would seem that if its degradation was decreased, bactericidal capacity might be increased (if the polymorphonucleocyte was not damaged itself). Also, in favism where glutathione peroxidase is deficient, the affected individuals do not suffer from increased susceptibility to infection[239] as might be expected if decreased degradation of H_2O_2 impaired the phagocyte.

A second function of the hexose monophosphate shunt, but not discussed by these authors, is the provision of pentoses for nucleotide and nucleic acid synthesis[326]. It is tempting to speculate that it may be the depressed hexose monophosphate shunt activity that is responsible for the decreased synthesis of DNA that has been observed in iron deficiency. Or, perhaps, the situation is *vice versa*, as the enzymes of the hexose monophosphate shunt have not been reported to be dependent on iron.

NBT reduction

Table 2.10 shows that nitroblue tetrazolium reduction has been found to be diminished in 4 studies and within normal limits in 4 other studies. NBT dye is reduced to a visible blue colour by superoxide produced by NADPH-oxidase in the PMN during the phagocytic-induced metabolic burst. Infection increases NBT reduction activity and may be the reason for the normal test reported in 4 of the clinical studies. In one of the studies[226], the NBT reduction test was used only as a qualitative screen to exclude patients with chronic granulomatous disease.

The qualitative nature of the NBT test is unfortunate because it allows no more than a crude estimate of oxidative activity to be made. NADPH-oxidase includes a moiety of cytochrome b which contains iron. Iron deficiency might decrease the quantity or activity of cytochrome b[352].

Myeloperoxidase activity

In the search for metabolic mechanisms for the defect in killing activity noted in iron deficiency, intracellular myeloperoxidase has been measured in tissue macrophages and circulating neutrophils. Table 2.12 summarizes these studies (and others). Myeloperoxidase was reduced according to 6 reports. (Higgs and Wells used a qualitative screen of myeloperoxidase activity to exclude its deficiency in their study of iron deficiency in chronic muco-cutaneous candidiasis[226].)

The defect in bactericidal activity found in iron deficiency would seem to be, at least in part, due to myeloperoxidase deficiency.

Catalase activity

Sagone *et al*[441] found a trend towards decreased levels of catalase activity (and myeloperoxidase) in granulocytes from iron deficient patients. These authors noted that catalase contains iron and plays an important role in oxidative killing by phagocytes.

Table 2.10 Neutrophil and macrophage stimulation tests in iron deficiency. The table indicates the directions in which iron deficient groups differed from control groups. Non-significant trends are shown by enclosing them in parentheses. Where there is no clear trend an N is placed in the table.

REF	Author	Opsonic	Phagocytic	Bactericidal	Chemotaxis: PLASMA		Chemotaxis: CELLS		NBT	Myelo-	HMP	Catalase
Number		Activity	Activity	Activity	Class.	Alt.	Class.	Alt.	Reduct.	peroxidase		
9	Arbeter			(D): Sa						D		
11	Baggs			N:St						D		
85	Celada								D			
93	Chandra	N: Ca	N: Ca	D: Sa					D			
88	Chandra	N: Sa	N: Sa	D: Sa					D			
92	Chandra			D: Sa								
174	Foroozanfar	D: Ca		D :Ca	D	D	D		N			
259	Katsushima									(D)		
226	Higgs			.					N	N		
225	Higashi									D		
277	Kulapongs			N: Ec								
311	Macdougall			D: Sa		N		I	N			
328	Masawe			(D): Sa								
352	Moore		N:Sa,Sp,St	N: Sp, St					D	D		
"	"		D:Ca	D: Sa, Ca								
424	Rohrer			D: tumour								
441	Sagone									(D)		(D)
473	Srikantia			D: Ec								
506	Walter		N: Sa	D: Sa								
526	Yetgin	N: Sa		D: Sa					N	N	D	

Notes:

Sa *Staphylococcus aureus* Sp *Streptococcus pneumoniae* St *Salmonella typhimurium*
Ec *Escherichia coli* Ca *Candida albicans*

D Decreased N Normal I Increased

Chemotaxis was measured using either the subject's plasma or cells, and employed either the classical or the alternate pathway for activation (with Ag/Ab complex or endotoxin).

Summary and conclusions

The work reviewed above provides fairly strong evidence for reduced phagocyte killing in iron deficiency, similar to, but less severe than the defect in chronic granulomatous disease. The source of the defect seems not to reside to any important degree in chemotaxis, opsonic activity or phagocytosis,

but to stem from decreased activity of iron containing oxidative enzymes *viz* myeloperoxidase, NADPH-oxidase and catalase. The hexose monophosphate shunt may also be ineffective in polymorphonucleocytes in iron deficiency, but the significance of this is not clear.

Iron deficiency and lymphoid tissue

White blood cells

Table 2.11 summarizes the results reported from microscopic studies of white blood cells in iron deficiency and table 2.12 summarizes results from biochemical studies.

The results from clinical studies may include confounding effects of infection and/or malnutrition. Giving more weight therefore to the laboratory animal studies leads to the conclusion that total white blood cell count, lymphocyte count, neutrophil count and T and B lymphocyte number and proportion may all be decreased in iron deficiency. Kuvibidila[281] reported that null lymphocytes were increased in iron deficient mice.

Monocytes, basophils and eosinophil counts seen to be unaffected by iron deficiency.

Morphological abnormalities have been reported by Beard *et al*[25] and Chanarin *et al*[86] for neutrophils, by Jarvis *et al*[249] and Kuvibidila[281] for lymphocytes in iron deficiency.

Bone marrow

Chronic iron deficiency often results in hypercellularity of the bone marrow. The studies on bone marrow changes in iron deficiency that are discussed below did not characterize the cellular distribution. It would be interesting to know how the erythroid and myeloid lines are affected. Is there a delay at a specific stage in development, or are all stages from the common stem cell equally affected?

Kuvibidila[281] has presented evidence that iron deficiency results in a delay in the maturation of monocytes in mice. Tavasolli[493] described delayed marrow regeneration following correction of iron deficiency in chronic iron deficiency in rats.

Sawitsky[445] reported that granulocyte proliferation in 1 of 5 patients with iron deficiency was depressed in *in vitro* bone marrow culture as measured by tritiated thymidine labelling.

DNA synthesis in bone marrow has been noted to be diminished in iron deficiency by Hoffbrand *et al*[229], Hershko *et al*[223] and Kuvibidila[281]. The rate of RNA synthesis was not affected although the total content of RNA/10^9 nucleated cells was decreased[223].

Spleen

The changes to splenic histology induced by iron deficiency in rabbits were described by Rodvien *et al*[423]. The white pulp was enlarged and the red pulp congested with red cells. Ultrastructural changes to macrophages, lymphocytes and reticular cells included abnormalities in mitochondria, endoplasmic reticulum and membrane structure.

Rothenbacher *et al*[430] studied histopathological changes induced by chronic severe iron deficiency in rats. Both the white pulp and the red pulp were markedly decreased. The spleens were half the size of those of the controls and constituted a smaller percentage of body weight. Although it was cited, The authors did not discuss the discrepancies from the study of Rodvien *et al.*

Kuvibidila and co-workers[282, 281, 430] reported a decreased percentage of T and B lymphocytes in splenic tissue. The same laboratory also observed that the weight of the spleen in relation to the total body weight increased in laboratory mice.

No reports have been published of studies in human of the pathophysiology of the spleen in iron deficiency.

Thymus

The weight of the thymus was decreased in iron deficient mice[281] and rats[430]. The T lymphocyte component was greatly decreased in the iron deficient rats, while the epithelial cell component was relatively increased[430].

Table 2.11 Leukocyte counts and morphology in iron deficiency. The table indicates the directions in which iron deficient groups differed from control groups. Non-significant trends are shown by enclosing them in parentheses. Where there is no clear trend an N is placed in the table.

REF Number	Author	WBC 109/l	Lymphocyte 109/l	Monocyte Count	Basophil Count	Eosinophil Count	Polymorph Count	Nucleated Spleen cells	T Cells No.	T Cells %	B cells SMIg
39	Bhaskaram									D	
93	Chandra		>1.89							D	
92	Chandra		3.0				N			N	N
169	Fletcher		D								
275	Krantman	11.5							(I)	(D)	(I)
283	Kuvibidila	7.8	N	N	(D)	N	N	D			
473	Srikantia									D	

Table 2.12 **Biochemical and morphological studies in iron deficiency. The table indicates the directions in which iron deficient groups differed from control groups.**

REF Number	Author	STUDY AND RESULTS OF IRON DEFICIENCY
12	Baggs	Decreased myeloperoxidase in macrophages & neutrophils
11	Baggs	Decreased myeloperoxidase positive cells in lamina propria & submucosa
25	Beard	Neutrophil nuclei hypersegmented, giant metamyelocytes
85	Celada	Leukocyte alkaline phosphatase decreased and rose with haemoglobin
86	Chanarin	Increased segmentation of neutrophils
223	Hershko	Decreased nucleic acid synthesis in bone marrow
225	Higashi	Myeloperoxidase reduced in monocytes and neutrophils
226	Higgs	Myeloperoxidase normal in chronic mucocutaneous candidiasis with iron deficiency
229	Hoffbrand	Desferrioxamine inhibited DNA synthesis (? inhibited ribonucleotide reductase)
249	Jarvis	Lymphocyte mitochondria were swollen, and vacuolated
259	Katsushima	Decreased leukocyte peroxidase in a case of hypochromic anaemia
283	Kuvibidila	Decreased nucleated cells in spleen
282	Kuvibidila	Decreased T lymphocytes in spleen
"	"	Weight of spleen/100g body weight increased; Ratio of dry to wet weight increased
"	"	Weight of thymus and liver decreased
354	Munn	Decreased DNA synthesis in lymphocytes
"	"	Decreased plaque forming cells (B lymphocytes) after sensitization by sheep red blood cell
352	Moore	Myeloperoxidase reduced in granulocytes
423	Rodvien	Spleen: enlarged white pulp and congested red pulp. Splenic macrophages, lymphocytes &
"	"	reticular cells: changes in mitochondria, endoplasmic reticulum and membrane structure
430	Rothenbacher	Impaired T & B cell lymphopoiesis
441	Sagone	Trend towards decreased granulocyte catalase & myeloperoxidase
445	Sawitsky	Labelling index of granulocyte proliferative compartment of bone marrow depressed
493	Tavasolli	Impaired regeneration of marrow tissue

Summary and conclusions - Lymphoid tissue in iron deficiency

Total white cell count, lymphocyte count, neutrophil count, and T and B lymphocyte number and proportion seem to be depressed in iron deficiency. The spleen and thymus may be both relatively and absolutely diminished in weight, and their histology indicates disturbed production of lymphocytes. Bone marrow growth and metabolic activity are diminished with decreased nucleic acid content and DNA synthesis. The evidence is stronger for some effects than others and is broadly consistent.

Iron as a modulator of immune function

The regulation of the immune system is pivotal in understanding the defense against infection and the role of iron and associated molecules (citrate, lactoferrin, isoferritins (acidic and basic), and other uncharacterized factors) is gradually being worked out. There is a growing body of literature in this complex area and this section does not attempt to review this comprehensively. The aim is rather to highlight a few studies in order to emphasize the importance of the concept of regulation.

The role of iron as a possible modulator of the immune system was investigated by Munn[354]. *In vitro* assays of human lymphocyte function were made where the concentration of ferric citrate, transferrin, lactoferrin, ferritin or haemin was varied at differing stages of the response to various mitogens. Iron modulated the immune response of B cells, enhancing or depressing the assay depending on the concentration. Munn did not attempt to relate the findings to the physiological response *in vivo* other than to speculate that iron may play a regulatory role in modulating immune function.

The uptake of tritiated thymidine and uridine was increased in PHA stimulated lymphocytes by iron-transferrin in serum free medium, and not by iron or apoferritin[396]. Similar results were obtained for concanavalin A stimulated lymphocytes[318].

Iron bound to transferrin enhances lymphocyte response to stimulation with PHA[396]. Interleukin-2 stimulates T lymphocyte production, at least in part, by induction of transferrin receptors on these cells[367].

Ferric citrate enhanced the proliferative response of peripheral blood lymphocytes to PWM and suppressed the response to PHA and Con A. Ferritin (iron content not stated) had no effect on PWM stimulation, but suppressed the response to PHA and Con A stimulation[61].

Ferric citrate significantly suppressed the expression of the OKT3 and OKT4 molecules in PWM stimulated lymphocytes. The helper/suppressor ratio was decreased and the transferrin receptor (OKT9) was significantly enhanced. Three other activation-associated markers were not changed by iron, *viz* OKIa, OKT10 and the receptor that forms thermostable erythrocyte-rosettes[62].

Transferrin, in the absence of serum, augmented proliferation of human lymphocytes to phytohaemagglutinin, concanavalin A and pokeweed mitogen[8]. The response appeared to be independent of the metal content.

It has been suggested that lactoferrin, transferrin and acidic iso-ferritins may act as regulatory molecules in suppressing myelopoiesis, the production of granulocytes and macrophages by controlling

the availability of iron to specific target cells[59]. This hypothesis is very controversial and the interested reader is referred to Broxmayer *et al*[59], Munn[354] and to Nishiya *et al*[369] for detailed discussion on the apparently conflicting nature of experiments in this area and more extensive guides to the literature than can be provided here.

Iron status and infection - Animal studies

The intention in this thesis is to concentrate on clinical studies of the relationship between iron status and infection. Animal studies could not be ignored, however, as they allow more precise testing of hypotheses in experiments that could not be conducted in humans.

Table 2.13 summarizes a representative, but incomplete, selection of such studies. As all the studies involved intentional infection of the experimental animal with a specific organism it seemed logical to classify the reports according to the type of organism. To allow this body of work to be placed in perspective, the table also shows classes of organism which have not been studied. Some major gaps in our knowledge are thus made evident.

The investigation with the broadest spectrum seems to be that of Payne[391] who examined the dependency on iron of various strains of several species of gram-negative bacteria. The results are summarized in table 2.13 part 6. From this work, Payne derived a classification scheme that reflects the efficiency of iron acquisition by bacteria and the clinical consequences.

Bacteria were categorized according to their virulence in the face of altered iron status in the chick embryo.

Class I bacteria remained equally virulent in spite of increased iron or the presence of iron binding proteins. These organisms have particularly effective iron acquisition systems that are not limited by physiological concentrations of iron or by iron binding proteins such as conalbumin.

Class II bacteria are normally virulent, but cannot obtain iron from iron binding proteins which thus render them avirulent.

Class III bacteria are normally relatively avirulent but are enhanced in the presence of available iron. These organisms have less effective systems for the acquisition of iron.

Class IV bacteria are avirulent irrespective of iron status.

Classes I, II, and III have iron acquisition systems of decreasing efficiency. The summary in Table 2.13 does not show that the iron acquisition systems may be strain specific. A particular species may have strains of differing Payne classes.

These notions are useful in interpreting the studies listed in Table 2.13. They concentrate attention on the effects of iron on the pathogen. But, the virulence of an organism depends on its invasiveness in relation to the host's immunity. In Payne's model the host's immune function was held constant (apart from acute changes in iron status). Many of the studies in Table 2.13 do not do this and hence Payne's scheme could be misleading if applied without due consideration of the above factors.

The pattern shown by the results of the tabulated studies is that many bacteria, fungi and protozoa are reduced in virulence by iron restriction in the host and enhanced by readily available iron.

One study that allows effects of iron status to be estimated for both host and pathogen is that of Hart[209] who found in the rat that moderate iron deficiency protected against induced pyelonephritis but that severe iron deficiency reduced resistance. The numbers of test animals in this experiment were small, and it would have enabled much more confidence to be placed in the results if 4 or 5 different degrees of iron deficiency had been employed and had shown the U-shaped trend expected from the conclusion drawn by the author.

The studies on nematode helminths are interesting. All showed increased virulence with iron deficiency. The most likely reason for this is that the parasites are able to obtain sufficient iron for their needs from the host irrespective of the host's iron status and that iron deficiency renders immune function less effective.

The studies on the effects of iron status on resistance to viral infection should be interpreted cautiously. The most likely reason for ferric ammonium citrate enhancing virulence of mouse hepatitis virus is that it is directly toxic to the liver[510]. Not much confidence can be placed in the "negative" results of the study on the effects of iron deficiency on viral infection since this had only 4 calves in the test and control groups and employed an attenuated strain of parainfluenza virus[350]. Clearly, larger studies with virulent viruses from the entire spectrum of types of viruses are required.

TABLE 2.13 IRON STATUS AND INFECTION: ANIMAL STUDIES: EFFECTS OF IRON DEFICIENCY

Table 2.13 Iron status and infection - Animal Studies

Ref Number	Author	DATE	ANIMAL	PATHOGEN	RESULTS Effects of iron deficiency/chelation
BACTERIA: gram-positive cocci					
BACTERIA: gram-negative cocci					
392	Payne	1978	Chick embryo	Neisseria gonorrhoeae	Strains from disseminated infections not inhibited by conalbumin
390	Payne	1975	Chick embryo	Neisseria gonorrhoeae	Relatively avirulent strains were inhibited by conalbumin
231	Holbein	1981	Mouse	Neisseria meningitidis	Iron bound to transferrin enhanced infection
298	Letendre	1984	Mouse	Neisseria meningitidis	Increased resistance to infection
BACTERIA: enteric gram-negative bacilli					
66	Bullen	1972	Guinea pig	Escherichia coli	Lactoferrin & transferrin were bacteriostatic
65	Bullen	1979	Guinea-pig	Escherichia coli	Unsaturated oral lactoferrin reduced gut colony counts
64	Bullen	1975	Guinea-pig	Escherichia coli	Less bacterial growth with G.-pig milk (lactoferrin, transferrin)
209	Hart	1982	Rat	Proteus mirabilis	Moderate iron deficiency protected against pyelonephritis
257	Kampschmidt	1974	Rat	Salmonella species	LEM induced hypoferraemia and protected against infection
12	Baggs	1974	Rat	Salmonella typhimurium	Less resistant to challenge
11	Baggs	1973	Rat	Salmonella typhimurium	Increased susceptibility to infection
254	Jones	1977	Mouse	Salmonella typhimurium	Desferrioxamine decreased survival with wild type infection
271	Kochan	1978	Mouse	Salmonella typhimurium	Less resistant to challenge
296	Puschmann	1977	Mouse	Salmonella typhimurium	Increased resistance to intraperitoneal infection
448	Schade	1944	Mouse	Shigella dysenteriae, Staphylococcus	Increased susceptibility to infection
268	Kluger	1979	Rabbit	Yersinia multocida	Growth inhibited by fever & hypoferraemia
BACTERIA: other aerobic bacteria					
490	Sword	1966	Mouse	Listeria monocytogenes	Desferrioxamine increased resistance

TABLE 2.13 IRON STATUS AND INFECTION: ANIMAL STUDIES: EFFECTS OF IRON DEFICIENCY

Ref Number	Author	DATE	ANIMAL	PATHOGEN	RESULTS Effects of iron deficiency/chelation
BACTERIA: anaerobic bacteria					
228	Hill	1978	Mouse	"anaerobes"	Increased susceptibility to infection
PAYNE'S CLASSIFICATION OF GRAM NEGATIVE BACTERIA					
					CLASS
391	Payne	1977	Chick embryo	Neisseria gonorrhoeae	II,III
				Shigella flexneri	II,III
				Shigella dysenteriae	II,III
				Vibrio cholerae	II,III
				Neisseria meningitidis	III
				Escherichia coli	III
BACTERIA: Mycobacteria, Spirochetes, higher bacteria: actinomyces, nocardia					
FUNGI					
153	Elin	1974	Mouse	Candida albicans	Positive correlation between infection and transferrin sat.
468	Sofaer	1982	Mouse	Candida albicans	Anaemic mice tended to have increased infection & colonization

KEY
Class I: Virulent: unaffected by iron status
Class II: Virulence inhibited by iron-binding proteins
Class III: Virulence enhanced by added iron
Class IV: Avirulent even with iron supplementation
NB Classification is strain specific, but is not indicated in the tabulation

TABLE 2.13 IRON STATUS AND INFECTION: ANIMAL STUDIES: EFFECTS OF IRON DEFICIENCY

Ref Number	Author	DATE	ANIMAL	PATHOGEN	RESULTS Effects of iron deficiency/chelation
RICKETTSIACEAE, MYCOPLASMA, CHLAMYDIA					
PROTOZOA					
144	Duncombe	1980	Mouse	Giardia muris	Decreased infestation
286	Lalonde	1984	Mouse	Trypanosoma cruzi	Desferrioxamine and iron deficiency decreased mortality
HELMINTHS: intestinal nematodes					
179	Foster	1936	Dog, cat	Ancylostoma caninum	Increased infestation
116	Cummins	1978	Rat	Nippostrongylus brasiliensis	Delayed worm expulsion due to block in lymphocyte function
142	Duncombe	1977	Rat	Nippostrongylus brasiliensis	Decreased efficacy of mebendazole and fenbendazole
143	Duncombe	1979	Rat	Nippostrongylus brasiliensis	Decreased efficacy of mebendazole
405	Porter	1935	Rat	Nippostrongylus muris	Increased infestation
HELMINTHS: tissue nematodes, cestodes, trematodes					
VIRUSES					
350	Mollerberg	1975	Calf	Parainfluenza-3 virus	No difference in infection rate or virulence in 8 calves

TABLE 2.13 IRON STATUS AND INFECTION: ANIMAL STUDIES: EFFECT OF IRON EXCESS/SUPPLEMENTATION

Ref Number	Author	DATE	ANIMAL	PATHOGEN	RESULTS Effects of iron excess/supplementation
BACTERIA: gram-positive cocci					
186	Fusillo	1974	-	Staphylococcus aureus	Enhanced infection
131	de Maria	1978	Mouse	Staphylococcus aureus	FAC & iron-dextran had no effect on pulmonary infection
BACTERIA: gram-negative cocci					
392	Payne	1978	Chick embryo	Neisseria gonorrhoeae	
390	Payne	1975	Chick embryo	Neisseria gonorrhoeae	Mortality of relatively avirulent strains was enhanced
78	Calver	1976	Mouse	Neisseria meningitidis	Iron increased mortality to challenge dose of bacteria
145	Dupuy	1983	Mouse	Neisseria meningitidis	Mucin plus iron abrogated resistance to infection
230	Holbein	1980	Mouse	Neisseria meningitidis	Iron dextran increased virulence of pathogenic strains
231	Holbein	1981	Mouse	Neisseria meningitidis	High serum, but not tissue, iron-dextran promoted infection
298	Letendre	1984	Mouse	Neisseria meningitidis	Iron dextran injection increased infection rate
BACTERIA: enteric gram-negative bacilli					
197	Greiger	1973	Iguana	Aeromonas hydrophila	Increased mortality from Fe injection
509	Ward	1983	Mouse	Bacteroides fragilis + E. coli	Ferric ammonium citrate decreased survival
45	Bornside	1970	Mouse	Escherichia coli	Virulence promoted by ferric ammonium citrate & haemoglobin
66	Bullen	1972	Guinea pig	Escherichia coli	Iron & haematin abolished bacteriostasis
65	Bullen	1979	Guinea-pig	Escherichia coli	
64	Bullen	1975	Guinea-pig	Escherichia coli	Guinea-pig milk + haematin enhanced bacterial growth
168	Fletcher	1969	Rat, Mouse	Escherichia coli	Parenteral iron salts enhanced renal abscess formation
256	Kadis	1984	Pig	Escherichia coli	Mortality to oral challenge enhanced by oral but not IM iron
380	Osborne	1968	Pig	Escherichia coli	Enhanced virulence
399	Polk	1971	Mouse	Escherichia coli	Ferric ammonium citrate enhanced virulence
400	Polk	1973	Guinea-pig	Escherichia coli	Enhanced infection
53	Brewer	1982	Mouse	Klebsiella pneumoniae	Ferric ammonium citrate (FAC) increased mortality
325	Martin	1963	Mouse & rat	Klebsiella pneumoniae	Intraperitoneal ferrous ammonium citrate enhanced virulence

TABLE 2.13 IRON STATUS AND INFECTION: ANIMAL STUDIES: EFFECT OF IRON EXCESS/SUPPLEMENTATION

Ref Number	Author	DATE	ANIMAL	PATHOGEN	RESULTS Effects of iron excess/supplementation
BACTERIA: enteric gram-negative bacilli (continued)					
264	Khimji	1978	Guinea-pig	Klebsiella species	Iron chelators enhanced skin infectivity
343	Miles	1976	Guinea-pig	Klebsiella species	Ferric iron enhanced infection by 11 of 16 bacterial strains
87	Chandlee	1965	Mouse, g.-pig	K. pneumoniae, S. typhimurium	Enhanced virulence
209	Hart	1982	Rat	Proteus mirabilis	
87	Chandlee	-		Ps aeruginosa, S typhi, streptococci	Virulence not enhanced
71	Bullen	1974	Mouse, Rabbit	Pseudomonas aeruginosa	FAC, haemoglobin & haematin increased mortality
175	Forsberg	1972	-	Pseudomonas aeruginosa	Enhanced infection
325	Martin	1963		Pseudomonas aeruginosa	Intraperitoneal ferrous ammonium citrate had no ill effect
227	Hill	1979	Chicken	Salmonella gallinarum	Oral parenteral iron decreased mortality and morbidity
466	Smith	1977	Chicken	Salmonella gallinarum	Fe-EDTA, but not Fe sulfate or chloride, reduced mortality
373	O'Brien	1982	Mouse	Salmonella typhi	Ferric ammonium chloride increased virulence
254	Jones	1977	Mouse	Salmonella typhimurium	Iron overload decreased survival after infection
271	Kochan	1978	Mouse	Salmonella typhimurium	FAC and desferrioxamine increased virulence
406	Puschmann	1977	Mouse	Salmonella typhimurium	Decreased resistance to intraperitoneal infection
446	Sawatzki	1983	Mouse	Salmonella typhimurium	Parenteral iron chelates abolished resistance to infection
422	Robins-Browne	1979	Mouse	Yersinia enterocolitica	Parenteral iron increased mortality
421	Robins-Browne	1985	Mouse	Yersinia enterocolitica	Iron-dextran & desferrioxamine increased virulence
240	Jackson	1956	Mouse	Yersinia pestis	Ferrous sulfate enhanced virulence
68	Bullen	1967	Guinea-pig	Yersinia septica	Haematin & FAC abolished passive immunity
72	Bullen	1968	-	Yersinia septica	Enhanced infection
BACTERIA: other aerobic bacteria					
96	Clark	1985	Chicken egg	Campylobacter jejuni	Iron increased rate of infection
490	Sword	1966	Mouse	Listeria monocytogenes	Ferric and ferrous salts increased virulence
173	Ford	1976	-	Vibrio cholerae	Enhanced infection
525	Wright	1981	Mouse	Vibrio vulnificans	Virulence was increased by desferrioxamine, FAC & CCl

TABLE 2.13 IRON STATUS AND INFECTION: ANIMAL STUDIES: EFFECT OF IRON EXCESS/SUPPLEMENTATION

Ref Number	Author	DATE	ANIMAL	PATHOGEN	RESULTS Effects of iron excess/supplementation
BACTERIA: anaerobic bacteria					
68	Bullen	1967	Guinea-pig	Clostridium welchii	Haematin & FAC abolished passive immunity
BACTERIA: Mycobacteria, Spirochetes, higher bacteria: actinomyces, nocardia					
FUNGI					
153	Elin	1974	Mouse	Candida albicans	FAC reversed non-specific protection induced by endotoxin
RICKETTSIACEAE, MYCOPLASMA, CHLAMYDIA					
PROTOZOA					
362	Murray	1975	Rat	Plasmodium berghei	Virulence increased with iron-dextran
286	Lalonde	1984	Mouse	Trypanosoma cruzi	Virulence increased with iron-dextran
HELMINTHS: intestinal nematodes					
452	Scott	1970	Sheep	Haemonchus contortus	Oral iron protected lambs
HELMINTHS: tissue nematodes, cestodes, trematodes					
VIRUSES					
510	Warren	1968	Mouse	Mouse hepatitis virus	Ferric ammonium citrate (FAC) enhanced virulence

Iron status and infection - Clinical studies

Introduction

The research conducted by Dr Helen Mackay between 1925 and 1927 provided the first evidence for a causal relationship between iron deficiency and susceptibility to infection[74]. In spite of methodologic difficulties, her report[314] still remains the best evidence for a deleterious effect of iron deficiency on propensity to infection.

Many of the subsequent positive reports in the literature are anecdotal and have little scientific weight, and the negative reports do not, as a rule, give enough information to enable the studies to be evaluated. In addition, some of the observations have been made as a "by-product" of studies with aims other than to clarify the relationship between iron status and infection*.

The following sections analyze the published evidence relating iron status and propensity to infection in humans. Iron excess (or increased iron availability) and its role in promoting infection is examined. Iron deficiency and its role in either promoting or preventing infection is then explored. Research reports are classified and discussed according to the manner in which their studies are presented since this corresponds with the confidence that can be placed in their results.

Most reports on the relation of iron deficiency to infection can be classified as anecdotal as they do not present details of methods and data. These studies are reviewed, principally because they have often been quoted in support of the hypothesis that iron deficiency lowers resistance to infection. While such observations may be allowed to raise one's curiosity, no weight should be attached to their implications until their methods and data have been subjected to the scientific review process.

Helpful, but inconclusive are the studies of prevalence of infection which are covered in the next section of the review. The studies which employ multiple dietary interventions, including iron, are of the same methodologic standing as prevalence studies and are included in the same section.

Lastly reviewed are those studies whose reports contain enough detail about the methods and results to enable the reader to evaluate the conclusions. Prospective studies of incidence of infection provide a study design which effectively tests the hypothesis that iron deficiency predisposes to infection. The greatest weight should be attached to the conclusions drawn from these studies.

This system for categorizing studies is convenient for classifying their results and assessing the importance of their conclusions. However, an inconvenience manifests itself when a research project involves the testing of several hypotheses. While these hypotheses may be closely related conceptually,

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* An intent expressed by the investigator before the study can add to its strength[13].

the sub-projects often involve radically different research designs. It is thus sometimes necessary to consider one report from the literature under several headings. While this detracts from the evaluation of such studies in themselves, it facilitates the synthesis of results from the entire body of literature in what it is now fashionable to call a *meta-analysis*[439].

The following two sections group reports according to the relation of infection to iron excess or iron deficiency. This dichotomy is sometimes difficult to apply. In such cases the decision on where to place the study has been made on the likely availability of iron to the micro-organism. The case report that begins the next section illustrates this rule as well as adding an historical dimension to the survey.

Iron excess and susceptibility to infection

When a very young physician, I was called to see the wife of an architect suffering from neuralgia, a pale woman, presenting every appearance of chlorosis: I prescribed large preparations of iron, according to Hutchinson's method of treating neuralgia. In less than a fortnight, there was a complete change: the young woman acquired a ravenous appetite, and an unwonted vivacity: but her gratitude and my delight did not last long. The excitement soon became fever: and the restored color of the cheek became every evening more ardent than it had been when she was in good health. A short cough supervened; and in less than a month from the commencement of the treatment, there appeared signs of phthisis which nothing could impede...

I do not blame the iron for having caused this calamity; but I do blame myself for having cured a condition, perhaps, favorable to the maintenance of the tuberculous affection in a latent state.

A Trousseau 1868
Lectures on Clinical Medicine
Translated by Sir John Rose Cormack
New Sydenham Society 1872, Vol 5 p 97*

* I am grateful to Murray *et al*[361] for drawing my attention to Armand Trousseau's illuminating lecture on *True and false chlorosis.*

Introduction

In conditions associated with systemic overload or increased intravascular availability of iron, such as haemochromatosis, sickle cell anaemia[262], thalassaemia major, siderosis, malaria, bartonellosis, hepatitis, louse-borne relapsing fever and multiple blood transfusions, patients show an increased susceptibility to infectious disease. Pearson and Robinson review this body of work critically[394]. There are so many potentially confounding factors in these observations that a causative role for iron status in the susceptibility to infection can remain no more than an attractive hypothesis. For this reason only clinical studies of the effects of iron excess (principally through administration of iron treatments) are individually reviewed in any detail in this section.

Kwashiorkor

McFarlane et al (1970)

McFarlane *et al*[313] studied 40 patients with kwashiorkor. Of the 13 patients who were known to have died, only 2 had serum transferrin greater than 0.4 mg/ml. The initial mean serum transferrin level was three times as high in the 16 patients who were known to have survived at least two weeks. And, two weeks after hospitalization the difference between the 2 groups was even greater - 1.30 mg/dl compared to 0.33 mg/dl.

The authors state that the patients were treated with oral ferrous sulfate or intramuscular iron-dextran but give no details of doses or time relationships with respect to treatments, test measurements and death. No details of infections in these patients are presented.

The authors calculated that by day 4 the serum transferrin level in survivors was sufficient to bind a total of 3.4 ug of iron which *"far exceeds the concentration of iron found in their serum"*. The serum transferrin in those who died was sufficient to bind only 0.55 ug of iron and this would *"leave an appreciable concentration of free iron in the plasma"*.

The study suggests that serum transferrin may be an accurate prognostic factor for death in kwashiorkor. It also suggests that it would be unwise to overwhelm iron transport mechanisms in severely malnourished children. But in this retrospective study the evidence to implicate iron overload as a causative factor in susceptibility to infection is too incomplete to warrant the certainty attributed to it by some authors*

Ethical considerations aside, a scientific study of the efficacy and side effects of iron in severe kwashiorkor would employ a clinical trial to compare outcomes in groups of children treated

* For example 32

identically, except for iron therapy. This would not be possible in the clinical situation, but it should be possible to test this conclusion with an animal model.

Urinary tract infection

Briggs (1963)

Iron-sorbitol-citric acid complex was used as a pyrogen in 10 healthy subjects, 9 patients with chronic urinary tract infection and 5 patients with structural disorders of the kidney[55]. No change in pyuria was found in subjects without infection, but in 7 of the 9 patients with renal tract infection 25 mg of iron-sorbitol produced more than 100% rise in white cell excretion. No patient suffered a clinical exacerbation of disease.

The authors conclude by stating that, although there was no proof of iron-sorbitol provoking infection, *"it may be a wise precaution to avoid the use of iron-sorbitol in patients with an active infection of the urinary tract".*

Scott (1962)

Scott[453], in a case report, described the development of a urinary tract infection in a patient being treated with both oral iron and parenteral iron-sorbitol-citric acid complex for iron deficiency anaemia of pregnancy.

Scott (1963)

Iron-sorbitol-citric acid complex was used to treat 80 antenatal patients for anaemia[457]. Pyelitis developed in 5 of 62 patients with simple iron deficiency anaemia and in 7 of 18 patients who had concomitant folic acid deficiency.

The author speculated that the urinary tract infections were caused by increased iron levels in the urine - 30% of the injection being excreted by the kidneys[55]. In the face of concomitant folic acid deficiency, patients are less able to utilize the iron and more will be available to pathogens.

This study provides a caveat for physicians, but the scientist would have preferred a control group for comparison.

Neonatal septicaemia

Barry, Reeve (1973, 1976, 1977)

In an epidemiological study of neonatal sepsis, Barry and Reeve[20, 19, 17, 18] observed a significant increase in the incidence of Gram-negative sepsis in Polynesian infants given iron-dextran within the first seven days of life. Comparisons could be made to the rate of infection in untreated European infants born at the same time and to untreated Polynesian infants born at a later date.

This evidence that iron-dextran increases susceptibility to severe Gram-negative infection in the neonatal period is corroborated by the results of Becroft, Dix and Farmer[31] in studies on the phagocytic and antibacterial functions of blood from 7 neonates, median age 5 days, before and after intramuscular iron-dextran. Bacteriostasis of serum was lost post treatment, and leukocyte chemotaxis was inhibited by post-treatment serum. The bactericidal capacity of neutrophils, opsonizing capacity of serum and generation of chemotactic stimulus showed no change with treatment.

Because it has been reported that iron-dextran does not bind with transferrin[218], Barry and Reeve suggested that *Escherichia coli* may be capable of utilizing the iron bound to dextran[20].

Anecdotal reports of observations in the United States[293], South Africa[212] and Scandinavia[442] have not associated iron-dextran injections in infants with increased infections. This may be because the practice outside New Zealand was to administer the iron later in life when transferrin levels and production are higher or because of other differences such as dose or exposure to infection.

Tropical infections

Byles, D'Sa (1970)

The response to total dose infusion of iron-dextran was studied in 1000 women who were pregnant or in the puerperium[77]. The iron dextran was administered in 4 regimes with and without promethazine and/or chloroquine. In comparison with the other regimes, treatment including chloroquine was associated with reductions in:

Local phlebitic reactions (as might be consistent with an anti-inflammatory effect).

Plasmodium falciparum in blood films of those with generalized reactions.

Non-allergic generalized reactions.

The results are compatible with iron-dextran aggravating subclinical malaria infection. It would have enabled more weight to have been put on this conclusion if blood films had been screened for malaria before as well as after treatment with iron dextran. The conclusions presented by the authors require the assumption that the reduction in generalized reaction in the chloroquine treated group was due to

an anti-plasmodium effect and not the anti-inflammatory effect (which it displays in rheumatoid arthritis and discoid lupus erythematosus).

Murray et al (1975)

Murray *et al*[362] observed the attack rate for malaria in patients and their relatives (who, in accordance with local custom, had accompanied them to hospital). The study took place during the Sahelian drought in the early 1970-s and all subjects were underweight. Patients and their relatives were given skim milk, grains and multivitamins without iron.

Mean transferrin saturations on arrival, on day 2 and on day 5 were 54%, 96% and 76%. Mean serum iron levels were 143 ug/dl, 250 ug/dl and 222 ug/dl on the same days. Attacks of malaria occurred in 74 of 181 subjects, with a peak on the fifth day after arrival.

The authors speculated that the food supplements may have caused the abnormal rise in serum iron and saturation of transferrin and that this made iron more readily available for plasmodia.

To test this hypothesis the authors infected 40 rats with *Plasmodium berghei*. Half of the rats had been injected with iron-dextran two weeks previously in order to increase their iron stores. The treated rats had higher percentage saturation of transferrin, higher serum iron and shorter latencies to peak parasitaemia and death than the control rats.

Murray et al (1978_1)

The Murrays[361, 360] observed a group of 137 iron deficient Somali nomads who were being treated for starvation. The 66 nomads in the control group had 7 episodes of infection, while the 77 individuals in the test group who were given 900 mg ferrous sulfate daily as well as the food supplement had 36 episodes of infection. The infections included malaria, tuberculosis, brucellosis, schistosomiasis, and pyogenic skin infections. The absence of mosquitoes and milk products make it likely that these infections were the result of recrudescence of suppressed disease[361]. However, as Keusch and Farthing[263] note: *"It is somewhat strange that schistosoma ova were found in 11 of 71 untreated individuals compared to only 2 of 66 placebo-treated subjects*. Since it is biologically impossible for this to reflect new infection during the 30-day observation period, the findings raise the question of some hidden sample selection. At the very least, these data are in need of independent confirmation and careful reinterpretation"*.

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* Chi square analysis with Yates continuity correction yields $P < 0.05$. This was not noted by the authors.

Murray et al (1978_2)

Murray *et al*[363] reported on the attack rates for cerebral malaria in 99 children admitted with a diagnosis of malaria. The children came from an area in Niger where all were suffering from famine malnutrition. The 23 children who were treated for cerebral malaria had higher serum iron levels, greater saturation of transferrin and lower serum folate levels than those who did not. The cerebral malaria was restricted to children being re-fed on grain. The milk diet of nomads seemed to protect them against cerebral malaria.

The attacks of malaria were almost certainly due to a recrudescence of the disease as there had been no rain or mosquitoes for eight months.

The authors discuss a number of possible mechanisms for this phenomenon**. Of interest in the present context is their remark that the changes in iron metabolism were most marked in the children with cerebral malaria, but that their experimental design did not allow cause to be distinguished from effect.

Oppenheimer et al (1986)

Oppenheimer *et al*[376] in a placebo controlled trial of prophylaxis of iron deficiency in infancy found that infants who had been treated with iron dextran had a higher incidence of malaria. This rigorous study provides sound evidence for iron dextran promoting malarial infestation. Because of its importance this work is discussed in detail below in the section where the role of iron deficiency in protecting against or promoting infection is examined.

Yersinia enterocolitica

Melby et al (1982)

Melby *et al*[337] reported two cases of *Yersinia enterocolitica* septicaemia in young children who had accidentally ingested excessive amounts of iron medication. Both children were treated with oral and parenteral desferrioxamine and at least one child had evidence of pre-existing colonization by *Yersinia enterocolitica*.

The authors theorized that iron utilization by the *Yersinia* may have been facilitated by the iron and desferrioxamine since desferrioxamine is a siderophore (produced by *Streptomyces griseus*) and has been shown to enhance growth of *Yersinia enterocolitica in vivo*[497].

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* See also 359

This kind of case report serves as a clear warning to clinicians, but provides little scientific evidence for a causal relationship without conjunction with evidence from the laboratory where the critical variables can be manipulated independently.

Mofenson (1987)

In a letter to the New England Journal of Medicine, Mofenson *et al*[348] report on a case of *Yersinia enterocolitica* septicaemia in a 15 month old boy who presented to hospital with bloody diarrhoea 10 hours after ingestion of approximately 30 tablets of ferrous sulfate. He was treated with syrup of ipecac and intramuscular desferrioxamine. His twin sister, who did not ingest ferrous sulfate, had mild diarrhoea and *Y. enterocolitica* was isolated from her stools.

The authors drew attention to the experimental situations in which desferrioxamine enhances the virulence of *Y. enterocolitica.*

Robins-Browne et al (1983)

Robins-Browne *et al*[422, 76] found marked deposits of haemosiderin in the viscera of 3 patients who died from *Yersinia enterocolitica* infection. Although the patients had *"an underlying illness, the presence of which may have predisposed to infection"*, the authors felt that the siderosis was *"evidence that iron overload increases susceptibility to infection"*. The clinical evidence is circumstantial and based upon a series of three patients. On its own, this evidence would carry little weight, but the authors corroborated it with observations on the effect of intra-peritoneal ferric ammonium citrate on susceptibility to infection by *Y. enterocolitica* in mice. Six of nine mice that received 10^9 organisms and 200 ug of iron died, but only 3 of 32 mice died when they were given the same number of bacteria and 100 ug or less of iron.

Summary

Indirect evidence of the effect of excess iron on host defense mechanism has been adduced from clinical observations of associations of infection with intravascular haemolysis. Systemic salmonellosis is associated with malaria and bartonellosis[518].

Host Associated Iron Transfer Factor (HAITF) is a low molecular weight iron binding protein found in many tissues including normal serum. *In vitro* HAITF is capable of increasing iron uptake and multiplication of Gram negative bacteria and capable of increasing the virulence of *Salmonella typhimurium*. The serum levels of HAITF are increased by a factor of approximately 3 in thalassaemia and it has been suggested that this may be the reason for the increased susceptibility of these patients to infection[253]. Studies of iron absorption have shown that transferrin is a passive

recipient of released iron and that intracellular events control the release of iron. Intestinal absorption of iron is not suppressed in normal human volunteers whose transferrin has been completely saturated by infusion of ferric ammonium citrate and in patients with thalassaemia major it is increased despite very high transferrin saturation[224]. Iron bound to transferrin is protected from forming harmful oxygen free radicals and is generally unavailable to micro-organisms. Non-transferrin plasma iron may thus escape the protective mechanism afforded by transferrin[224].

Iron overload in haemodialysis seems to impair phagocytic activity of neutrophils[512]. Sound theoretical reasons and circumstantial clinical evidence point to iron as a causative factor in the infections to which patients with kwashiorkor so often succumb. Clinical evidence also implicates iron dextran treatment as a factor in neonatal sepsis, urinary tract infection and malaria. Oral iron preparations have been associated with *Yersinia enterocolitica* and "tropical" diseases such as malaria, tuberculosis, brucellosis, and pyogenic skin infections.

Although most studies suffer from methodological inadequacies, the sum of the evidence is strongly suggestive of a deleterious effect of iron on susceptibility to sepsis in neonates treated with iron dextran and to malaria and perhaps other infections in all age groups treated with either oral or parenteral iron.

Before prescribing iron the clinician would do well to follow Trousseau and distinguish true from false chlorosis!

Iron deficiency and susceptibility to infection

Introduction

The literature on iron deficiency and its role in infection is reviewed in 4 sections. The anecdotal reports in the first section are scientifically unimportant, but have been uncritically cited in the literature*. The second and third sections discuss studies with confounding factors and retrospective studies. Such research designs may suggest an association (or lack of association) but cannot distinguish cause and effect. The fourth section reviews prospective controlled studies. The results from these studies deserve to be taken seriously.

* A citation analysis was not made but a number of instances of such citations are documented in order to validate this statement.

Evidence for iron deficiency protecting against infection

Malaria and other Tropical infections

Retrospective or prevalence studies

Masawe et al (1974)

Purpose: The study aimed to determine the prevalence of infection in adult patients with various anaemias[330].

Ascertainment: Consecutive admissions to an adult medical ward with haemoglobin less than 10 g/dl.

Laboratory methods: Haematologic profiles were obtained with a Coulter counter model-S.

Study grouping and design: Patients were classified into 5 classes of anaemia and were screened for the presence of infection, viral, bacterial and protozoal.

Results: Most of the protozoal and helminthic infestations occurred in the iron deficient group and most of the bacterial infections were in the megaloblastic, haemolytic and refractory anaemias.

Interpretation of results: An observational study such as this can draw no conclusions about causal relationships. In addition, with no information as to the prevalence of infections and anaemias in the general population, no valid inferences can be drawn from the association of certain anaemias.

The causes of admission to hospital may determine the patterns of association of one factor with another without one factor being causally dependent on the other; they might have a common source. Stein[478] exemplified this fallacy by pointing out that the reasons for admission to a medical ward in a general hospital in Milwaukee were such that iron deficiency anaemia would be associated with a lower prevalence of infection because the cause was often occult bleeding, whereas other anaemias were associated with debilitating disease and infections.

Conclusion: The study shows that, in Dar es Salaam, iron deficiency is often associated with malaria and helminthic infestations while bacterial infections are associated with other anaemias[147]. Although this association is strong, no conclusions about the causal influence of iron deficiency on susceptibility to infection can be drawn.

Masawe and Swai (1975)

In a letter to the Lancet, Masawe and Swai[329] report on results of a survey of anaemia and infection in 42 children and young adults. The study was an extension of that discussed in the previous section.

The prevalence of bacterial infection was 10% in the group with no iron stores shown on bone marrow examination and 50% in those with positive iron stores. For malaria, the prevalences were 30% and 50% respectively. The caveats on conclusions apply as for the previous study.

Murray et al **(1978)**

The Murrays[361], in a pilot study*, observed 19 infections in 64 Somali nomads who had normal iron status but 0 infections in 26 nomads who were iron deficient.

This kind of clinical anecdote would certainly justify further epidemiological studies, but, in itself cannot carry much weight as a scientific argument for the hypothesis that iron deficiency is protective against infections in general. The two groups may not have been comparable in terms of ascertainment and exposure to infection. Future studies should have at least a case-control design if they are not prospective in nature.

Prospective, controlled studies

Murray et al (1980)

A group of 35 Maasai** given one 300 mg tablet of ferrous sulfate weekly for one year had significantly more cysts, amoebae and sera positive for *Entamoeba histolytica* than 75 tribesmen not given the iron[358]. Six of the iron-treated group had attacks of malaria, but none of the 35 control group were affected. These findings are suggestive of a protective effect of iron deficiency and a deleterious effect of oral iron-treatment on resistance to infection by *Entamoeba histolytica* and malaria.

Oppenheimer et al **(1986)**

Purpose: The object of the study by Oppenheimer *et al*[377, 379, 378, 376] was to examine the effects of iron therapy on infectious morbidity.

Ascertainment: The subjects were healthy infants recruited at birth from infants born in hospital to mothers resident nearby.

Grouping: The infants were paired according to sex, domicile and birth weight.

Iron intake: At two months of age 3 ml of either iron-dextran or placebo was given intra-muscularly in a double blind fashion.

Laboratory methods: Haemoglobin was measured by the cyanmethaemoglobin method with a Corning colorimeter. Thin and thick blood films were examined for malaria.

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* See study 2 above, under the section on iron excess and susceptibility to infection

** The authors use this spelling

Study design: The infants were followed up 1 week after the injection and then at the ages of 6 and 12 months. At the follow-up visits a history of infections during the previous two weeks was taken. The infants were weighed and examined for signs of infection.

An increased prevalence of malaria was found in the iron treated group. This difference was statistically significant for parasitaemia at both 6 months and 12 months. It was also statistically significant for splenomegally at 12 months (and almost significant at 6 months). The iron treated group had a higher prevalence of *Plasmodium falciparum* , *P. vivax* and *P. malariae*.

Malaria could not be incriminated in any of the 12 deaths that occurred. There were five deaths in the iron group and 7 in the placebo group; no further details on the causes of death are given, but it is likely that most of them were due to infections.

Interpretation of results: 1 exposure to infection: By matching infants for domicile and following the two groups simultaneously the experimental design minimized unequal exposure to infection in the two groups.

Interpretation of results: 2 blinding: The study was double blind.

Interpretation of results: 3 haematology: No haematological results were given, but it seems reasonable to assume that the markers of iron status were "better" in the group treated with iron-dextran.

Conclusion This is probably the best designed study in the literature to test the hypothesis that iron status is associated with susceptibility to infection. The one drawback is that the incidence of infection could not be obtained as follow-up took place at 6 and 12 months. This allowed a point-prevalence of infection to be determined at these two ages.

This might not be a serious drawback in determining susceptibility to chronic infections such as malaria, but it would lead to an important loss of discriminatory power in determining the difference in morbidity caused by acute illnesses of short duration such as the common respiratory and diarrhoeal infections of infancy.

Ethical considerations will prevent similar studies being undertaken to corroborate the effect of iron-dextran in infancy on susceptibility to malaria. It would however be useful to repeat this sort of study outside the tropics where protozoal diseases are not a problem as resistance to such diseases differs in many important ways from resistance to viral, bacterial, fungal and helminthic infections.

In short, the administration of intra-muscular iron-dextran in early infancy seems to increase susceptibility to malaria. No conclusions are possible about the risk of other infections, although the mortality data would argue against a similar increased risk for other kinds of infection.

Evidence against the promotion of infection by iron deficiency

Candidiasis

Studies with anecdotal reports

Jacobs et al **(1973)**

In a brief abstract, Jacobs *et al*[242] reported that oral candida counts were not increased in patients with iron deficiency anaemia and did not change with treatment. The one patient who had oral candidiasis did not respond to iron therapy either. If they had stated the number of patients with lesions of the oral mucosa it would have facilitated comparison with the paper by Higgs and Wells[226].

Retrospective or prevalence studies

Walker et al **(1973)**

Walker *et al*[505] compared the prevalence of oral candidiasis in 12 iron deficient anaemic individuals with that in 12 matched controls. This was done before and after iron treatment. Mycological examinations were not significantly different from the controls and did not alter with iron treatment. Clinical candidiasis was present in 1 iron deficient patient and did respond to iron treatment whereas 4 controls had clinical infection with Candida. The authors did not calculate the probability of not detecting a difference between the iron deficient group and the controls. This study offers weak evidence against iron deficiency predisposing to candidiasis.

Adults

Prospective, controlled studies

Vellar et al **(1974)**

Purpose: The object of the study by Vellar *et al*[503] was *"to examine if iron in therapeutic doses might have an effect on the prevalence of upper respiratory tract infections in healthy young adult men and women"*.

Ascertainment: The subjects were 97 physical education students in generally healthy condition. No further details of selection or exclusion criteria are given.

Grouping and iron intake: The 81 students with normal haemoglobin concentrations were allocated to one of two groups by an undocumented method. One group was treated with a placebo while the other group was treated with ferrofumarate to provide 60 mg elemental iron per day. The 5 males with haemoglobin levels less than 14 mg/dl and 11 females with haemoglobin concentrations less than 12.5 mg/dl were allocated to the group to receive iron. At the completion of the study the actual consumption of iron and placebo tablets was recorded but not reported.

Laboratory methods: The methods by which the haematologic parameters were measured were not reported.

Study design: A history of upper respiratory tract symptoms was taken, a physical examination performed and blood tests made on six occasions about eight weeks apart. The number of upper respiratory infections thus diagnosed was recorded for the 5 follow-up examinations.

Results: After 2 months there was no difference in the mean haemoglobin levels of the iron and treatment groups.

The authors present their data in enough detail for the following table to be compiled.

Table 2.14 Iron treatment and upper respiratory infections. Number of upper respiratory infections diagnosed in 5 examinations of 97 healthy young men and women over a period of about 40 weeks.

	IRON	PLACEBO	TOTAL
INFECTION	93	77	170
NO INFECTION	137	95	232
TOTAL	230	172	402

The proportion of infections in the placebo group averaged 44% per examination, while the corresponding figure for the iron treated group was 40%. This difference is not significant (chi square equal 0.59, $p > 0.1$).

Interpretation of results: 1 exposure to infection: There seems little possibility of any bias in exposure to infection with this experimental design.

Interpretation of results: 2 blinding: The study was certainly blinded for the subjects, but no mention is made of blinding for the investigators.

Interpretation of results: 3 haematology: Inclusion of the subjects with low haemoglobin concentrations in the iron-treatment group may have led to a small bias towards incorporating subjects more susceptible to infection. Since there was no difference in the mean haemoglobins of the iron and placebo groups from the time of the first re-examination, this bias is likely to be small.

There was little difference between the haematological parameters of individuals with a history of infection and those without. This mitigates against acute upper respiratory infections as being a significant cause of anaemia.

Conclusion: *"It is necessary to be cautious in the interpretation of the iron/placebo comparison with regard to the prevalence of infection: the iron-treated group was not strictly comparable at the start of the experiment"*[*]. If the infections are counted from the second re-examination period, the iron-group had an average of 32% infections at each point while the placebo-group had a mean of 40% infections. This trend in favour of the iron-treated group was not significant and is not consistent when the infections are analyzed according to sex or for individual examination periods. The authors conclude that *"iron medication has no particular pharmacological effect"* on the resistance to acute upper respiratory infections in otherwise healthy young adults.

The study does not warrant so strong a conclusion, particularly without an analysis of the type II error. It would have been helpful if the authors had calculated confidence limits for the effect of iron treatment.

The conclusion that may be validly drawn from this study is that iron supplementation may have a small effect in reducing susceptibility to acute infection of the upper respiratory tract in healthy young adults.

Infants

Studies with anecdotal reports

Marsh et al **(1959)**

Marsh *et al*[323] in a study involving 74 infants, followed three groups of children from birth to 9 months of age. One group was given an iron-fortified milk formula and the other two groups were given different formulas without extra iron. Mean haemoglobin levels in the three groups at 9 months of age were 12.69, 10.46 and 9.67 g/dl. No data on infection were presented, but the authors stated *"While this study was not designed to measure accurately differences in growth or relative susceptibility to illness, gross observation revealed no significant differences"*[**]. These conclusions can have little

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* page 779

** page 409

weight in refuting the existence of an association of iron deficiency with susceptibility to infection, but they may be taken to indicate that such an association can not be large.

Farquhar (1963)

Farquhar[162] studied 44 infants who were given either a daily supplement of multi-vitamins or multi-vitamins with iron from the age of one month to one year*. The author stated that the *"iron supplement produced a statistically significant increase in haemoglobin and haematocrit levels at three, six, and nine months of age, but the difference was not statistically significant at one year. This difference did not reflect in the height and weight measurements or in the general well-being of these well-nourished, healthy infants."*

No further descriptions of illness or data are given to support this anecdotal conclusion.

Malakhovsky Yu E et al (1983)

Malakhovsky *et al*[320] followed 103 infants for 9 to 12 months. The children were examined weekly and 79 of them were given an iron tonic in a double blind fashion. The study has been included together with the anecdotal reports because many critical issues were not defined in the report. The critical factors include details of the ages at which children entered and completed the study, from what age and for how long the iron supplement was administered, the ages at which iron status was determined and the periods over which infections were monitored. The difficulty in ascertaining these facts from the report may be partly due to the problems inherent in working with a translation; the original is in Russian.

The authors stated that susceptibility to infection was not related to haemoglobin status. Importantly, they qualified this statement by saying that it might not be true for severe iron deficiency (defined by haemoglobin concentrations below 9.0 g/dl).

The authors also stated, somewhat at variance with their first conclusion, that repeated infections were associated with the development of anaemia.

This study can be taken as suggestive evidence against a large effect of iron deficiency on the susceptibility to infection.

Prospective, controlled studies

Burman (1972)

Purpose: The purpose of this study[74] was to determine the normal range for haemoglobin in healthy infants during the first 2 years of life and to observe the effects of iron supplementation.

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* Cited as evidence that iron status does not relate to susceptibility to infection[289].

Ascertainment: Parents living within a prescribed area of Northwest Bristol whose children were born between June 1965 and February 1966 were invited to participate in the survey. Reasons for exclusion included illegitimacy and low birth-weight, but many parents refused to co-operate.

Nutrition: Only well nourished infants were entered into the study.

Socio-economic class: The *"numbers in social classes IV and V were low"* and as a whole the group was relatively affluent.

Feeding and iron intake: No feeding intervention was made, but, from the age of 3 months, children were given either placebo or 10 mg elemental iron daily as ferric hydroxide.

Laboratory methods: Toe or finger prick capillary blood samples were taken and analyzed for haemoglobin with the cyanmethaemoglobin technique.

Study grouping and design: Children were allocated alternately by birth order to receive either placebo or iron tonic. Blood samples were made at intervals of three months. In the intervening months a health visitor went to the home to collect data about illnesses and checked on the supply of medicine.

Results: *"The administration of iron made no difference to the incidence of infection in this study"*.

Interpretation of results: 1 diagnosis of infection: Infections were diagnosed on history at a monthly follow up by health visitors. The author acknowledged that recall of illnesses is unreliable, even from conscientious parents. The statistics of morbidity can therefore not lay claim to much accuracy or precision, and in fact, no statistics are given.

Interpretation of results: 2 exposure to infection: The two groups seem comparable with respect to exposure to infection, although only qualitative comments on socio-economic class supplement the statement which implies that the two groups were studied contemporaneously.

Interpretation of results: 3 blinding: The study was possibly blinded with respect to the subjects, but the study team would surely have known the difference between *mist. rubra* and *mist. nigra*.

Interpretation of results: 4 haematology: Standard laboratory methods were used. The author states that the haemoglobin vs age curves for the *"iron"* and *"no-iron"* group differ at the 5% significance level, and that most of the difference occurred during the second year of life, particularly towards the end.

Two comments may be made on the author's observations. Firstly, the haemoglobin level of the *"iron"* group was higher at the start of the study than that of the *"no-iron"* group. This was a constant trend - as might be expected. The author's unusual statistical method may merely be confirming that this initial bias was maintained at each subsequent test of haemoglobin. The analysis would have been more meaningful had the initial haemoglobin been controlled for. The interpretation of the effect of the iron supplementation should take this imbalance between the two groups into account.

At 12 months of age, the mean increase in haemoglobin was 0.68 g/dl for the *"iron"* group and 0.46 g/dl for the *"no-iron"* group. An optimistic estimate of the result of a t test gives p=13%. The reason for the iron supplementation making so little difference to the haemoglobin is probably due to the poor absorption of iron in the form of ferric hydroxide[134].

Conclusion: The author concludes that iron supplementation had no effect on morbidity. As the regime employed had little effect on haemoglobin it is hardly surprising that no reduction in morbidity was observed. Furthermore, while the author states that there was no difference in observed morbidity, he does not calculate the probability of a type II error[210]. In other words, the chance of the study missing a real difference in infection rate is not given. In addition, with the questionable precision in diagnosis by history up to 4 weeks after an illness, there is no doubt that little weight should be put on the author's conclusions with regard to morbidity and iron status. Unfortunately this study is often uncritically cited in review articles to refute the hypothesis that iron deficiency predisposes towards infection*.

Evidence for the promotion of infection by iron deficiency

Herpes labialis

Studies with anecdotal reports

Chandra et al **(1977)**

In a conference report Chandra *et al*[91] observed that patients with recurrent herpes labialis had lower percentage saturations of transferrin than healthy controls; no data were given.

* See for example, 241, 290, 308, 275, 485, 102, 89, 91

Candidiasis

Studies with anecdotal reports

Cawson (1963)

Two cases with oral candidiasis were reported by Cawson to have responded to treatment with ferrous gluconate for their iron deficiency anaemia[84].

Retrospective or prevalence studies

Rose (1968)

Rose[426] studied 25 consecutively referred patients with angular cheilitis and 25 controls matched for age, sex and use of dentures. The premise for the study was that *Candida albicans* is an important aetiological agent*, but *"since candida infections are regarded as 'diseases of the diseased', a search for other factors capable either of assisting the fungus or acting 'in their own right' to produce the disease suggests itself".*

The group with cheilitis had lower mean haemoglobin levels, lower plasma iron, increased total iron binding capacity and decreased saturation of transferrin and more cases of iron deficiency anaemia.

Taken in conjunction with previous evidence that *Candida albicans* is implicated in angular cheilitis this study provides evidence for an association between iron deficiency and fungal infection of the oral mucosa. In this design cause and effect are impossible to separate.

Higgs and Wells (1972)

In a survey of 31 patients with chronic mucocutaneous candidiasis Higgs and Wells[226] found 23 with iron deficiency anaemia. The criteria for diagnosis of anaemia were not presented and there was no control group against which comparisons could be made**.

Davidson et al (1977)

Haemoglobin, serum ferritin and serum iron were measured in 43 patients with recurrent genital thrush, 31 patients with non-recurrent candidiasis and in 26 controls by Davidson *et al*[129]. The mean haemoglobin level was significantly lower in the first group, but the authors comment that this result was biased by two "outliers". Serum ferritin levels were greatest in group 1 and serum iron levels were lowest in that group. The differences were small and not statistically significant.

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* The author cites several studies to support this assertion

** The second part of their report deals with the effects of iron treatment and is discussed below.

Jenkins et al **(1977)**

Oral candidiasis was studied in 108 patients by Jenkins *et al*[250]. The lesions were classified as either chronic hyperplastic (21 patients) or atrophic candidiasis (87 patients) according to clinical, microbiological and histological criteria. Patients and healthy controls were classified as iron deficient if the percentage saturation of transferrin was less than 16%.

Iron deficiency was found in 33% of patients with hyperplastic lesions, in 14% of patients with atrophic lesions and 14% of controls. The association was significant at the 10% level for hyperplasia. The authors discuss the pathogenesis of the two types of lesions and speculate on the differing roles that iron deficiency might play. Of note is their recognition that cause and effect relationships would be elucidated if treatment with iron could be shown to effect a cure. As this was a retrospective study they were not able to do this.

Prospective, controlled studies

Fletcher (1975)

Fletcher *et al*[169] studied 29 adult patients with chronic iron deficiency anaemia caused by blood loss. Matched controls were found in the same hospital practice. Mouth lesions were present in 16 of the anaemic patients and *Candida albicans* was cultured from the saliva of all patients with mouth lesions and 8 of the 13 without lesions. None of the controls had mouth lesions and 13 of the 29 patients had *Candida albicans* isolated from saliva. The mouth lesions and candida counts improved within a month of starting 200 mg ferrous sulfate bd. The authors comment that the susceptibility to candidiasis may be due to alterations in the oral mucosa. They noted a decreased lymphocyte count and a depressed response of lymphocytes to stimulation with PHA. On treatment with oral iron, the lesions, candida infection and lymphocyte count returned to normal as the haemoglobin level was restored, but before iron stores (as measured by serum iron and total iron binding capacity) had been repleted. The response to PHA stimulation did not return to normal until iron stores were replenished.

Conclusion This study supports the hypothesis of a defect in cell mediated immunity in iron deficiency predisposing to candidiasis, but the weight that can be attached to this conclusion is limited by the small numbers involved.

Higgs and Wells (1972)

Purpose: The purpose of this study[226] was to characterize chronic mucocutaneous candidiasis and to investigate its association with iron status.

Ascertainment: The 46 patients were located as the result of an *"extensive search"* and 31 were investigated for iron status.

Study grouping and design: Eight patients had reduced or absent bone marrow iron stores and haemoglobin concentration greater than 11.7 g/dl. Four of these were randomly assigned to receive a total dose infusion of iron-dextran and 4 were given placebo tablets for 2 months.

Results: Three test patients improved but none of the patients on placebo improved.

The authors remark that it was possible to assess 11 patients in total who had been treated with iron and that 9 had responded to treatment.

Interpretation: The numbers are small and details of ascertainment and selection are not reported. Although the results support the hypothesis that iron deficiency impairs immune function, the mechanism proposed, *viz* an epithelial defect, may not account for the hypothesized susceptibility to other infections with other classes of organisms. It may be that a different mechanism is operative in these patients.

A second difficulty in interpretation arises in determining the cause of iron deficiency. Did the chronic mucocutaneous candidiasis perhaps cause the iron deficiency? Or was there another factor that was responsible for both the candidiasis and the difficulty in maintaining normal iron stores in these patients?

Conclusion: This small study is relevant to a specific and unusual infection but it would be difficult to justify extrapolation from the results to the thesis of a generalized susceptibility to infection in iron deficiency.

Adults

Studies with anecdotal reports

Basta and Churchill (1974)

Chandra *et al*[91] state that Basta and Churchill[21] *"reported a higher prevalence of acute and chronic infections in the iron-deficient anaemic group compared with the non-anaemic group"*. Assessment is not possible since the inter-library loan service was not able to retrieve this report.

Retrospective or prevalence studies

Basta et al (1979)

Basta *et al*[22] compared morbidity scores and period prevalence of infection between anaemic (haematocrit < 38%) and non-anaemic workers on an Indonesian rubber plantation.

"Period prevalence of systemic or local infection was almost twice as high in the anaemic men as in the non-anaemic". The differences in bronchitis, influenza and diarrhoea were notable. *"Disease morbidity was scored and showed a significant correlation ($P < 0.01$) with haemoglobin"*.

Apart from the usual difficulties in interpreting retrospective and prevalence studies, evaluation is hampered by the lack of control for nutritional status. This particular group were severely nutritionally restricted and it is possible that the anaemic members were suffering from deficiencies other than iron that would have confounded interpretation of the results. Nutritional indices were not compared between the two groups.

It seems strange that the anaemic group had a higher period prevalence of reported infections than the non-anaemic group but had a lower morbidity score. The authors state that the morbidity score was significantly correlated with haemoglobin levels. One wonders whether the correlation was positive. *ie* whether lower morbidity scores tended to be associated with lower haemoglobins. The authors stated that episodes of illness were scored from 0 to 3 according to duration and severity but did not state whether the higher scores were assigned to longer and more severe illnesses. If this was not the case, the apparently contradictory results would be resolved.

It is difficult to draw a conclusion from the conflicting results of this study.

Giles et al (1962)

Giles and Brown[193] found an association between the prevalence of bacteriuria and haemoglobin concentration in pregnant women; urinary infection was less than half as common in 447 non-anaemic pregnant controls as in 463 anaemic pregnant women. The authors argued that it was more likely that the anaemia was the result of urinary tract infection, partly because the haemoglobin responded to antibacterial treatment in some patients and partly because no plausible mechanism has been proposed by which iron deficiency might predispose to infection.

Abramson et al (1971)

In a survey of 652 women attending an antenatal clinic Abramson *et al*[2] found haemoglobin levels of 12.10 g/dl and 12.48 g/dl in patients with and without bacteriuria in the first and second trimesters. In the third trimesters the haemoglobin levels were 11.70 and 12.22 respectively. The authors failed to find an association with age, parity, social class, date of immigration or diet. This makes it more likely that the low haemoglobin concentration was a result of infection than of primary iron deficiency which would correlate with several of the above variables.

Savage et al (1967)

Savage *et al*[444] surveyed more than 5000 pregnant women and found no association between haemoglobin level and bacteriuria.

Prospective, controlled studies

Basta et al **(1979)**

Purpose: The purpose of the study of Basta *et al*[22] was *"to determine: (1) whether anemia in low-income workers affected physical endurance, their actual productivity, and their resistance to infection, and (2) whether iron supplementation could diminish iron deficiency anemia and raise work output in this population".*

Ascertainment: "*A total of 302 male plantation workers, 16 to 40 years old, [were] selected randomly from a list of 400 workers*".

Nutrition and diet: The workers ate a restricted diet. Rice was rationed and extra income was spent on edible leaves and fruits. Mean height for the sample was 157.4 cm and mean weight was 46.9 kg. These are both less than the third centile for 18 year old males on the NCHS growth charts. The authors remark that the bench height of the Harvard Step Test was reduced in order to make it more suitable for Indonesians.

Iron intake: The Iron group took, under supervision, 100 mg of ferrous sulfate daily.

Laboratory methods: Blood samples were obtained by finger prick for determination of haematocrit and haemoglobin and for microscopical examination for malarial parasites and red cell morphology. Haemoglobin was measured with the cyanmethaemoglobin method.

Study grouping and design: The trial consisted of 2 groups: a Placebo group who received a daily placebo tablet and a small income supplement to encourage them to continue to participate for the 60 days of intervention and an Iron group who received a daily ferrous sulfate table and the income supplement. (A third group who received neither placebo tablet nor financial inducement was tested haematologically but data on infectious morbidity in this group were not given). Examinations were conducted before and immediately after the intervention study. Diseases reported to have occurred in the 4 weeks preceding the first examination were recorded.

Results: Pre-intervention: These are discussed in the section above where studies of prevalence are reviewed.

Results: Post-intervention: *"Morbidity scores decreased in both anaemic groups, but more significantly in that receiving iron and payment ($P < 0.001$) than in that receiving placebo and payment ($P < 0.02$)... There was a dramatic decrease in the prevalence of infections in both the anemic and non-anaemic iron-payment groups, whereas little change was observed in placebo-payment groups".*

Interpretation of results: 1 nutrition: The subjects in this study were nutritionally deprived. It is possible that deficiencies other than iron were also present in the anaemic group. It is thus not legitimate to assume that iron treatment was the only factor in decreasing morbidity.

Interpretation of results: 2 analysis: The authors' statement of their results is potentially misleading and does not agree with the tabulated data.

It is tempting to conclude that the iron supplement was efficacious because the decrease in morbidity score was more significant ($P < 0.001$) for the iron treated anaemic group than for the placebo treated anaemic group ($P < 0.02$)*. The comparison should not be between the P values but between the changes in morbidity scores (0.48 and 0.41) and a statistical test of significance should be made on this. Figure 2.1 (which was compiled from the authors' table 5) should illustrate the fallacy and that there was, for practical purposes, no difference between the changes in the placebo and iron treated anaemic groups.

* At least one reviewer seems to have fallen into this trap[108].

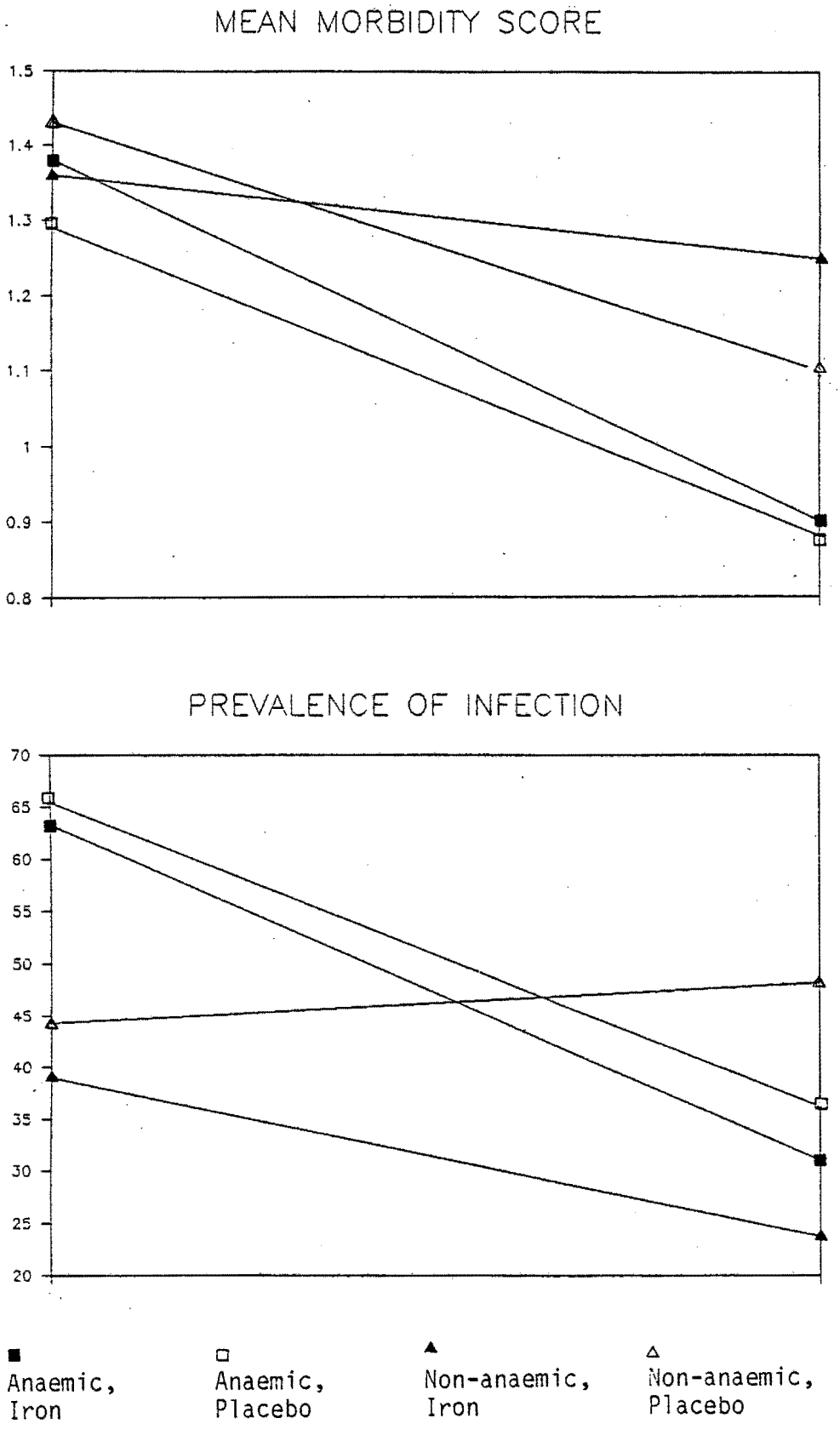

Figure 2.1 **Mean morbidity scores and prevalence of infection in Indonesian workers before and after treatment with placebo or 100 mg ferrous sulfate daily for 60 days.**

The decrease in infection rate experienced by the anaemic group was 0.51 while for the non-anaemic group it was 0.09. The corresponding figures for the iron treated and placebo treated groups are 0.38 and 0.21. It would have been interesting to have seen an analysis of covariance to estimate the relative contribution of temporal effects and iron treatment. From the above analysis one might estimate that the iron had about half the influence of the other, undefined interventions.

Conclusion: Figure 2.1 is a nice demonstration of the Hawthorne effect. *ie* that the study itself had an impressive result on the morbidity of the workers (assuming that the exposure to infection remained constant). The interventions responsible may have been the extra attention given to the workers, their improved nutrition as a consequence of the higher wages they received, or other factors. The study does show a small, (and probably non-significant) trend supporting the hypothesis that iron deficiency predisposes to infection.

Infants

Studies with anecdotal reports

Tonkin (1970)

Tonkin[496, 497] conducted a trial of intramuscular iron-dextran in the prevention of iron deficiency anaemia of infancy in which full term, appropriate for gestational age, babies were either offered oral iron supplementation for one year or given two injections; each equivalent to 50 mg elemental iron. The intramuscular iron prevented the development of anaemia and at one year the mean haemoglobin levels in the two groups were 11.96 mg/dl and 10.98 mg/dl respectively.

The author stated that the *"two groups, test and control, did not show any marked differences in morbidity either of reported illnesses or of defects found at examination."*

The author then presents the results of an analysis of the association of haemoglobin level with morbidity. Respiratory tract illness found on examination was significantly more common in the groups with lower haemoglobin. The results for reported respiratory tract conditions and for other infections, both on examination and by history, were variable in trend.

The conclusion drawn from this is that *"the advantages of an improved haemoglobin became evident"*. This inference is invalid. If iron treatment improves iron status and, concomitantly, resistance to infection, then one would have expected the test group to have had fewer infections than the control group. The numbers of children were small and they were examined only at intervals of three months during the year they were followed. This would be adequate reason for missing a protective effect of iron treatment, and it would have been helpful if the author had calculated the probability of doing so. More importantly, if infection depresses haemoglobin levels (as we know it does[270]), then it would be

expected that lower haemoglobin levels would be associated with increased numbers of infections. The author's results can validly support this latter, weaker, hypothesis.

Howell (1972)

This study has been cited by several authors[308, 91] in favour of iron deficiency increasing susceptibility to infection. The inter-library loan service was unable to provide me with a copy of Howell's report.

Dallman (1981)

It was reported in a conference[126] that the US Public Health Service had in the 1960-s conducted a study in which iron-dextran or placebo was given to about 200 new born infants. The incidence of infections was *"about equal"* in the two groups but more deaths occurred in the placebo group. No data were given.

Heimendinger and Undritz (1955)

In a study of the effects of ferrous gluconate supplementation in children in institutions, Heimendinger and Undritz[215] remarked that infections were less frequent in children with improved haemoglobin levels*. No data were given to support this assertion.

Lovric (1970)

Lovric[302] stated that 458 children in hospital had a mean haemoglobin level of 10.6 g/dl while a matched** control group of healthy children had a mean of 12.0 g/dl. Although he stated that there was a higher prevalence of infection in the hospitalized children than in the controls, no data were given and no association was sought with infection and haemoglobin level. Little weight can be attached to this study in testing the hypothesis of a causal relationship of iron deficiency with susceptibility to infection.

Moe (1963)

In support of the contention that iron deficiency predisposes to infection, Moe[347] offered the observation that 5 of 7 infants admitted to hospital with anaemia (Hb < 8 g/dl) developed signs of infection during their stay. No mention is made of the reasons for admission, and no control cases were used as a comparison.

Salmi et al **(1963)**

Salmi *et al*[442] stated in an abstract that a group of about 30 premature infants had twice the *"incidence of infections"* experienced by a group of about 60 infants who were given iron supplementation. No further data are given#.

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* Cited in 503

** Unstated, but presumably matched for age and sex

Cited in support of the hypothesis that iron deficiency predisposes to infection[484, 102].

Shaw and Robertson (1964)

In a retrospective survey of 797 admission to Columbus Children's Hospital in Ohio, Shaw and Robertson[459] found *"a lower percentage of infants admitted to the surgical service were anaemic than of those admitted to the medical service"*. Two thirds of the 194 admissions with Hb < 10.0 g/dl were prompted by an infectious process.

This study's results may be added to the body of circumstantial evidence in favour of an increased susceptibility to infection in iron deficiency**.

Stekel (1981)

A similar anecdote reported in a conference[126] relates that infants on an iron fortified milk formula, acidified with ascorbic acid, had a lower incidence of diarrhoea than the controls. No data are given, and it seems that the two groups may not have been subject to the same exposure to infection as the report could be taken to indicate that they were in separate parts of the city of Santiago. No weight can be attached to the conclusions made from these observations without a full report on the methods, materials and results of the study.

Werkman et al (1964)

This study[520] has been (mis)used to support the assertion that iron deficiency is associated with increased susceptibility to infection*. The authors interviewed 28 mothers at least 1 year after their children had been treated in hospital for iron deficiency anaemia and compared them with the mothers of 28 control children. Little data is offered by the authors, but they do state that on follow-up the previously iron deficient children had a mean haemoglobin of 11.52 g/dl and that they had more illnesses than the control group. The mean haemoglobin level of the control group is not stated nor is the nature and number of illnesses further defined. There is nothing to support (or to reject) an association of iron deficiency with susceptibility to infection.

Retrospective or prevalence studies

Fortuine (1966)

Fortuine[176] studied the association between outcome in acute purulent meningitis and initial haemoglobin levels in 69 Alaskan natives. A fatal outcome was more likely the lower the initial haemoglobin. The author recommended that preventive measures include treatment of anaemia.

The cautions detailed in the section above apply equally well to the implied conclusion that iron deficiency predisposed to infection.

** Cited by 89

* *"a group of infants and toddlers with iron deficiency anemia incurred more illness, among other behavioural manifestations, than a control group of non-deficient children"*[7].

Fuerth (1971)

In a brief report published in 1971, Fuerth[185] reported on a study undertaken in 1959 in which the haemoglobin levels of 1 year old children were analyzed for correlation with the number of illnesses per child during the first two years of life. No statistically significant correlation was found although there was a trend in favour of the children with higher haemoglobin levels.

No analysis of the types of anaemia or illnesses was reported, but iron deficiency would be the most common cause of anaemia and infection the most common cause of illness. These uncertainties make it difficult to conclude from this study that iron deficiency either does or does not have an association with susceptibility to infection*.

Oppenheimer (1980)

A retrospective study[375] of the prevalence of anaemia in infants admitted with bacterial meningitis or pneumonia found mean haemoglobins well below the local normal values**.

The author contends that, since the weight for age of the children indicated normal nutrition, there was no increased susceptibility to infection caused by generalized malnutrition and that it was more likely that pre-existing anaemia was an aetiological factor in the infections than *vice versa*. The facts that most patients were seen within 36 hours of development of symptoms and that there was a significant variation in haemoglobin between the aetiological groups were offered as further reasons for the putative role of iron deficiency in the infections.

This study provides circumstantial evidence for the theory of increased susceptibility to infection in iron deficiency and suffers from the defects of retrospective studies. As the author concludes: *"These questions could be answered only by a controlled prospective trial of iron administration to infants in this community"*

Reeves et al (1984)

Most of the authors reviewed in this chapter argue either for or against a causal role for iron deficiency in increasing susceptibility to infection. In contrast, Reeves, Yip, Kiley and Dallman[412] concluded from an interesting study of unique design that *"upper respiratory and other mild antecedent infections commonly predispose to iron deficiency (probably because of a decrease in iron absorption)"*.

These authors estimated the rate of infection retrospectively in a group of 291 well one year old infants attending a routine health maintenance clinic by questioning the parents of the children. Half the

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* This has been cited as evidence against such an association[489]

** Necessary because of altitude greater than 1500 m for 1 hospital.

children were treated for three months with an iron supplement providing 3 mg iron/kg/day while the remainder were given a placebo.

Prior infection was significantly associated with lower initial haemoglobin levels.

Children who did not have a history of prior infection did not respond (haematologically) to treatment with iron or placebo. Children who did have a history of prior infection showed a haemoglobin response to treatment with placebo or with iron. The response was greater in those who had more infections and was greater in the iron treated group than in the placebo group.

The authors speculate that iron absorption is sufficiently depressed during infection to affect haematological and other indicators of iron status. In addition they hypothesize that the higher haemoglobin response in infants with prior infections *"suggests that both iron deficiency and inflammatory disease played interactive roles"*.

This seems the most parsimonious explanation for these results. It would have been interesting to know the response of the other measures of iron status to the treatments. More interesting would have been the test of this theory by performing a similar analysis of the relationship of rates of infection between the ages of 12 and 15 months and haemoglobin levels. One would have expected a similar relationship to have been found with little or no effect by treatment with placebo or iron. This seems such an obvious observation to make that one wonders if the result was not perhaps negative.

Bondestam et al (1985)

Serum iron and other trace elements were measured in 28 children, median age 3.5 years, admitted to hospital for investigation of recurrent infections[44]. Thirteen healthy children, median age 15.5 years served as controls. Statistically significant decreases were found for serum iron and zinc, but not for copper and magnesium. Mean serum transferrin and caeruloplasmin levels were almost identical in the two groups. Ferritin and red cell indices were not reported.

The authors postulated that *"the changes in the trace element status observed in this study are the combined effects of infection and malnutrition"*, and remarked that *"the possible role of trace element deficiency in predisposing to or perpetuating undue susceptibility to infections in children remains to be elucidated"*. Their proper caution in interpretation is not matched by reviewers who find in this study evidence for a causal role of iron deficiency in infection or evidence for a lack of protection of iron deficiency against infection*. The data do not contradict this hypothesis, but they are equally consistent with the converse interpretation of a causal influence of infection on iron deficiency.

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* See for example 152

Studies with multiple independent variables

Arbeter et al (1971)

This study[9] is mentioned only because it has been cited by reviewers in support of the hypothesis that iron deficiency predisposes to infection: *"Arbeter et al found that malnourished Colombian children showed a remarkable decrease in infections (particularly gastro-enteritis) following correction of their iron deficiency and, incidentally, of their malnutrition as well"*[235]*.

In fact, the report by Arbeter *et al* is difficult to interpret and does not support the conclusions cited above. Families in a remote Colombian village were provided with free medical care for their children. In addition, panela, the local staple of unrefined sugar, was sold in two forms: either unmodified, or fortified with protein, vitamins and minerals, including iron and calcium. The group receiving the fortified panela showed no difference in height or weight from the control group, but did have a decreased incidence of clinically overt malnutrition and of infectious diseases. No haematological data are presented. The tabulation of infections diagnosed omits the period of observation and (for most age groups) omits the number of children observed. It is therefore not possible to calculate rates of infection**. Thus it would not be valid to attribute any changes to the fortification program, let alone to any single constituent such as iron.

Jacobs and George (1952)

Purpose: *"The purpose of the study was the careful clinical evaluation of a group of young infants, half of whom, were to be fed the standard hospital diet, the other half to be fed the same diet to which strained meat was added as a supplement, in such a quantity as to increase the daily protein intake approximately 25%"*[244].

Ascertainment: Healthy children were alternately assigned to the test and control groups.

Nutrition: Birth weight was greater than 2.3 kg for all infants.

Socio-economic class: The children were housed in an institution and most were awaiting adoption or foster home care.

Feeding and iron intake: Strained meat was added to the milk of test infants until spoon feeding was possible at about three months of age.

Laboratory methods: Blood samples were obtained from heel pricks and haemoglobin concentrations were determined with an Evelyn photoelectric colorimeter.

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* Other reviewers have also misquoted this report, *eg* 152, 91

** For example, per year per 100 children

Study grouping and design: Infants were admitted alternately to the test and control groups. The examining physician and laboratory staff were not informed of the allocation. Examinations were performed monthly.

Results: The only illnesses which occurred during the period of study were upper respiratory infections and gastrointestinal disturbances. Results are presented as a comparison of morbidity rates between the test and control group, with the test group having 60% of the morbidity rate of the control group in aggregate. When compared in age groupings, the test group had lower morbidity rates in 8 of 9 age categories.

Interpretation of results: 1 diagnosis of infection: This is not discussed, but presumably, children in an institution would not have had a significant illness without the study team being notified. One would assume that recording of infections was therefore reasonably complete and accurate.

Interpretation of results: 2 exposure to infection: *"Analysis of morbidity rates according to ward was not possible because of the regular, periodic change in ward assignments with increasing age. No major outbreaks of infection occurred during the study and minor seasonal outbreaks in the respective wards offered equal exposure to both groups, since alternate bed assignment was maintained within the wards".*

Interpretation of results: 3 blinding: The study was blinded to investigators.

Interpretation of results: 4 Nutrition: Weight gain in the meat fed group was significantly increased.

Interpretation of results: 5 haematology: The test group had higher median haemoglobin levels with less variation than the control group.

Conclusion: This study suggests that meat favorably influences resistance to infection. The intention of the study was to increase the protein intake of the infants by a adding meat to the diet. It is not possible to ascribe the results of the intervention to a single nutrient, such as iron, in the face of so many other associated nutritional differences.

Karp and Merz (1986)

Karp and Merz[258] have documented from a study of 70 adults that chemotherapy for acute leukemia is associated with increased serum iron levels and decreased total iron binding capacity (and thus also increased percentage saturation of transferrin) as compared with pre-treatment values. Fungal infections occurred in those patients with the earliest and/or greatest reduction in total iron binding capacity. Effective anti-fungal therapy was associated with a return to normal of total iron binding capacity.

The alteration in iron metabolism may contribute to the susceptibility to fungal infections or, less probably, may merely be a marker of such vulnerability. The study design does not allow the determination of the relative contributions to altered resistance to infection by leukemia, chemotherapy and altered iron metabolism.

Prospective, controlled studies

Mackay (1928)

Purpose: The purpose of Dr Mackay's study[314] was to define the prevalence of anaemia in infancy, to observe its ill-effects and to test prophylactic and therapeutic regimens.

Ascertainment: Infants were referred to the Queen's hospital for Children, London, *"for consultation or light treatment"*[*]. All infants were less than or equal to 18 months of age on admission.

Nutrition: *"The great majority were under normal weight as judged for instance by Griffith's standard weight curve."*

Socio-economic class: The infants came from *"a poor and overcrowded district in the East end of London"*.

Feeding: All infants were given full cream dried milk sweetened with cane sugar, sufficient to provide about half a litre a day to a baby weighing 5Kg. Cod-liver oil and either orange or tomato juice were ordered as a regular supplement. Weaning off milk began from the age of 8 months.

Iron intake: The infants admitted to the study in 1926 and 1927 were given iron in the form of *"iron and ammonium citrate"* - either as a sweetened syrup or as a fortification to the dried milk. The daily dose was adjusted to between 4.5 and 9 grains. Since 1 grain of ammonium ferric citrate contains 80 mg of iron, the daily iron intake can be calculated to be between 360 and 720 mg.

Laboratory methods: Blood samples were obtained by heel or finger prick. Haemoglobin was measured with Haldane's haemoglobinometer which gives results expressed as a percentage of 13.8 g Hb/dl. In a subsequent report it was stated that the haemoglobin results were consistently underestimated by 7%[315].

Study grouping and design: The Control group consisted of infants admitted to the study in 1925/1926 and the Test group consisted of infants who were admitted in 1926/1927 and given iron

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* One of the therapies being evaluated was the mercury vapour quartz lamp which had been found useful for the management of rickets. Needless to say, it had no effect on haemoglobin level.

supplementation. Attendance at the follow up clinic varied from several times a week to once a month. The period of observation varied from 1 to 12 months and averaged 2.98 months for the Control group and 3.31 months for the Test group.

Results: The Control group experienced about twice as many infections as the Test group in both summer and winter. When the diseases were grouped into *"specific fevers"*, diseases of the respiratory tract, diseases of the digestive tract and other infections, a similar relation held*.

Interpretation of results: 1 exposure to infection: Dr Mackay recognized the occurrence of an epidemic of diarrhoeal disease in the Control period and, taking this into account, disclaimed the validity of inferences drawn from comparison of the statistics of digestive tract disease. As the average weekly mortality in London for respiratory disease was comparable for the two groups, in contradistinction to that for diarrhoeal disease**, she claimed that the incidence statistics for respiratory illness and specific fevers could be used to infer a causal relationship between iron deficiency and infection.

Equal mortality rates from one period to another do not imply equal morbidity rates, (although they would be compatible). To compare two groups of infants for infection rates, both groups should be of similar age distribution, and exposed to the same opportunities for infection; the groups should be studied over the same period, in the same location, and their social contacts should be similar in frequency and type.

Interpretation of results: 2 demographic comparability: The report does not give precise age details or compare variables such as number of siblings and socio-economic class which might act as a proxy for exposure to infection. Few details of ascertainment are given; it is possible that children with infections were selected for the Control part of the study and that healthier children were referred during the Test part of the study.

Interpretation of results: 3 blinding: The study was open to both investigator and subject and this may have led to (unintentional) bias in ascertainment of subjects, in collection of data, or in interpretation of results.

Interpretation of results: 4 nutrition: The average weights at the beginning and at the end of observation of a group of Control cases are compared with a group of Test cases. The numbers in these groups, 176 and 62, are very different from the numbers of infants whose morbidity was observed, 235 and 87. As no explanation is given of the derivation of these groups, extrapolation to the morbidity

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* Author's tables D and E and Chart XVI

** Author's table C

study groups may not be valid. The Test cases seemed to start the study with age and weight very similar to the Control cases but complete the trial with an improved weight gain*. However, in both the full morbidity study and in the smaller weight study, the Test cases were observed for longer than the Control cases (3.31 *cf* 2.98 months and 21.7 *cf* 14.4 weeks). The sources of these differences would be expected to bias the groups with respect to both nutrition and morbidity and, as Dr Mackay recognized, this *"makes a fair comparison difficult"*** .

Interpretation of results: 5 haematology: The graphically presented data show a striking difference in cases given iron, prophylactically or therapeutically, compared to Control infants. Reading from the graphs, converting from the units employed (100% = 13.8g Hb/dl blood), and multiplying by 1.07 to correct for calibration errors[74] one may estimate that the averaged haemoglobin of the Test cases was 11.3 g/dl and that of the Controls was 10 g/dl on completion of the trial.

Conclusion: No statistical tests were made, and the data presented is not complete enough to enable such analyses to be made now. However, formal statistics would not be necessary to convince of the effectiveness of iron therapy and they would not obviate the methodological problems in interpreting the morbidity study.

Taking all these objections into consideration one is still left with a strong impression that the iron fortification may have increased the resistance to infection of the Test group infants.

James and Combes (1960)

Purpose: The purpose of the study of James and Combes[247] was to *"study the health record of a group of premature infants maintained in a state of abundant iron nutrition"*.

Ascertainment, Grouping, Nutrition: Two hundred and five consecutive prematurely born babies, who weighed 2000g or less and had survived at least 24 hours were allocated by random selection to either a test group to receive iron-dextran or to a control group who received standard care. Of the 181 who survived the neonatal period, 84 received iron-dextran and 97 did not.

Socio-economic class: The socio-economic class of the families was not reported.

Feeding: The feeding practices were not discussed other than that mothers were advised on milk formula usage and introduction of solids.

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* Author's table F

** page 140

Iron intake: When infants in the test group reached 2000g weight they were given 1ml of iron-dextran daily for 5 days to provide a total of 250 mg of elemental iron. Babies in the control group received supplemental oral iron preparations if their haemoglobin declined below 7 g/dl.

Laboratory methods: Haemoglobin was determined by the oxyhaemoglobin method.

Study design: After discharge from the nursery, babies were seen monthly. *"Additional data covering morbidity and mortality in both groups of infants were obtained from study of the hospital records and from the vital statistics of the Dallas City and County Health Departments."*

Results: The test group had, on average, 1.14 respiratory and diarrhoeal infections in the first year, and 1.83 illnesses per infant attending the outpatient department. The corresponding figures for the control group were 1.20 and 1.85. The differences were not statistically significant.

Interpretation of results: 1 exposure to infection: Random allocation of infants to the study groups would have tended to minimize bias in exposure to infection that might be caused by geographic or demographic bias. The method of randomization is not explained however, and it may itself have been open to (unconscious) bias.

The groups were studied simultaneously, but follow-up was better in the control group. The longer period of observation and the suggestion that there was *"a greater maternal desire to provide good care"*, may have tended to increase the number of infections recorded for the control group.

Interpretation of results: 2 demographic comparability: Apart from the caveat noted in the previous paragraph, demographic comparability is not discussed.

Interpretation of results: 3 blinding: Presumably the study was not blinded as blinding was not reported.

Interpretation of results: 4 nutrition: The weight gain in the two groups of infants was very similar over the first year. A difficulty in accepting this statement at face value is that the evidence for this conclusion is a comparison between two smaller sub-groups whose selection (or exclusion) criteria are not reported*.

Interpretation of results: haematology: An unstated number of the test group had a mean haemoglobin of 11.5 g/dl at one year of age and none had *"significant anaemia"*. The mean haemoglobin of the control group was not stated, but of 48 infants, *"only 5 infants reached the age of*

* Authors' figure 1

one year with a haemoglobin level greater than 9.5 g/dl without having supplemental iron, and almost all developed a significant degree of anaemia during the first year of life".

Conclusion: Although the intervention employed ensured a large difference in iron nutrition between the two groups, the results are difficult to interpret as:

1 The drop-out rate was more than 50%, with a large difference between the two groups.

2 The number of infections was not related to the precise number of infants followed or to the precise period for which they were followed.

The first problem is a potentially important source of bias and the second is an important source of error in calculating morbidity rates.

Taking these two factors into account, one may note the trend in favour of the group who had iron-dextran. There is no justification in using this study as unqualified evidence against a relationship between iron status and susceptibility to infection*.

Andelman and Sered (1965)

Purpose: The study was *"designed to determine the effects of feeding an iron-containing milk formula for a period of 6 to 9 months to an infant population manifesting a high incidence of iron-deficiency anaemia and a high rate of infectious morbidity"*[7].

Ascertainment: Term infants under 4 weeks of age whose birth weight had been greater than 2.5 Kg were randomly selected from those destined to attend Child Welfare Stations in the Eighth Health District of Chicago. The method of randomization is not explained nor is the allocation to Test and Control groups discussed. Some bias may have been present in the selection as 37% of the Test group were enrolled on discharge from hospital, while all the Control infants were enrolled at the Welfare Station.

Nutrition: The mean birth weights of the two groups of infants were 7.1 lb and 7.0 lb.

Socio-economic class: The study population was considered to be the lowest socio-economic of the city.

Feeding and iron intake: The Control group received an evaporated milk formula with separate vitamin supplementation but no extra iron. The Test group were given a milk formula containing all essential vitamins and 12 mg of iron per quart of reconstituted formula. Strained foods were initiated

* As for example in 308

at 3 months of age and by the time the infants were 6 months old, full diets of formula, cereal, egg, meat, vegetable, and fruit had been prescribed.

Laboratory methods: Blood samples were obtained by heel prick. Haemoglobin values were determined by the cyanmethaemoglobin method.

Study grouping and design: A Test group of 603 infants and a Control group of 445 infants were given their respective formulas from entry till the age of 6 to 9 months and periodically examined till they were 18 months old. The frequency varied from every 4 weeks for the first 6 months (the paper is not clear on this point) to every 12 weeks for the last 6 months. The overall dropout rate was 30% by one year of age and 95% by 18 months of age. Infants for whom the haemoglobin fell below 10 g/dl were removed from further observation. This criterion resulted in 76% of the Control children and 9% of the Test infants being prematurely excluded from the study.

Results: The Test group had significantly fewer respiratory infections than the Control group during the period of iron supplementation and the six months thereafter*.

Interpretation of results: 1 diagnosis of infection: No information is given on how infections were diagnosed**, nor on the incidence of infections other than those of the respiratory tract. The intervals between visits were longer than the period for reliable recall of illness[74].

Interpretation of results: 2 exposure to infection: While the Test and Control groups came from similar families, mean maternal parity being 3.3 and 3.2 respectively#, no information is given as to whether the two groups were followed simultaneously.

Interpretation of results: 3 blinding: The study was not blinded and no reason is given for the discrepancy in the numbers allocated to the two groups.

Interpretation of results: 4 Nutrition: No difference in weights or lengths was apparent during the study##. At 12 months the average weight was 9.71 Kg for both groups. This is 96% of the expected weight at that age; presumably therefore, both groups were well nourished.

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* Author's figure 10

** Although Pearson[394] states that this was by questioning the parents

Author's table 1

Author's tables 2 and 3

Interpretation of results: 5 haematology: Differences in haemoglobin concentration became statistically significant at 9 to 12 weeks and at one year the average haemoglobin concentrations were 11.9 g/dl and 10.4 g/dl in the Test and Control groups respectively.

Conclusion: The pronounced difference in respiratory tract infections may have been due to the difference in iron status but the problems in evaluating ascertainment, allocation, blinding, and diagnosis make this inference uncertain. It would have lent strength to the study if the incidence of other infections had been discussed.

Cantwell (1972)

Purpose: The purpose of Cantwell's study[79] was to test the efficacy of iron-dextran in preventing anaemia in late infancy.

Ascertainment: All Maori mothers who delivered in Hawkes Bay Hospital in 1965 were invited to join the study.

Exclusions: Neither criteria for exclusion nor numbers of children excluded were specifically discussed. It can be inferred from the report that infants were excluded from the trial if their parents declined to participate or if they were premature. Children in the control group who became severely anaemic were treated with parenteral iron. The author states that they *"then ceased to be controls"*. Other reasons for dropping out of the trial were not mentioned.

Socio-economic class: *"The majority of Maori families in the Hawkes Bay area belong to the lower socio-economic groups"*.

Feeding: The justification for employing parenteral iron-dextran as the source of iron in this study was that the *"Maori mother tends to prolong bottle feeding with her infant until the age of two years. She is slow in introducing foods rich in iron"*.

Iron intake: Iron-dextran injections were begun on the second day of life and continued daily for a total of 5 injections. The total dose of elemental iron was 250 mg. The author calculated the total requirement for iron in the first year of life to be 284 mg in a typical infant growing from 3.5 kg at birth to 10.5 kg at one year.

Laboratory methods: Blood samples were obtained by finger prick. Haemoglobin was measured with a standardized photocolorimeter using the cyanmethaemoglobin method.

Study grouping and design: Babies born on the odd days of the month were treated. Haemoglobins and haematocrits were measured serially at approximately three month intervals*. The rate of hospitalization for the infants was calculated for the two year period of observation.

Results: The Control group experienced about 35% more admissions to hospital for infections than the Test group. When acute wheezy bronchitis and asthma are excluded from the calculations, the Control group had twice the admission rate of the Test group.

Interpretation of results: 1 exposure to infection: The criterion of admission on alternate days would have ensured equal temporal exposure to infection.

Interpretation of results: 2 demographic comparability: The infants were all resident in the Hawkes bay area, so the two groups were probably demographically similar. It would have been useful if the report had tested some measure of expected similarity between groups such as birth order or gender.

Interpretation of results: 3 blinding: The study was open to both investigator and subject and this may have led to (unintentional) bias in collection of data, or in interpretation of results.

Interpretation of results: 4 nutrition: Measures of nutrition such as weight and height were not reported.

Interpretation of results: 5 haematology: The iron-treated group had statistically significantly higher haemoglobin levels than the untreated controls for all the age groupings from the age of 3 months to 27 months. In contrast to the Test group, the control group had a striking decline in haemoglobin to a nadir of 8.9 mg/dl at 15 to 18 months.

Interpretation of results: 6 calculation of admission rates: The author does not elaborate on the definition of *admission*. It would seem that it refers to both attendance at an outpatient clinic and to admission to a hospital ward since infections include impetigo and meningitis and the admission rate is extraordinarily high.

In general, the calculation of admission rates is subject to several potential errors. When comparing rates for two groups, both the denominator and the numerator should be comparable.

The data on which the calculations of admission rate are made are not described in this study with sufficient clarity to enable the validity of the admission rate to be confirmed. But unclear details in

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* Not stated by the author, but inferred from his 3.

determination of the admission rate arouse the suspicion that the rates may not be strictly comparable as the following analysis demonstrates.

The size of the control and test groups on admission to the study is 144 and 94 respectively. The author states that 21 children were removed from the control group after treatment for severe anaemia. Yet the admission rate is calculated from 61 admissions in 144 children over two years. The admission rate should be calculated for those children who were followed for the full two year period.

The author does not discuss the reasons for excluding treated children, but there is an argument against such exclusions. If the study is regarded as a trial of the efficacy of iron-dextran in the neonatal period in preventing infections in the first two years of life, the approach should be that all children ought to be included, regardless of treatment for medical reasons after allocation to their study grouping. It is difficult to estimate the size of the potential error, but it is probably not large.

In a second type of test of the effects of iron-dextran, the author compares total hospital admissions for Maori infants under the age of two years in the Hawkes bay and Gisborne area for the years 1964 to 1967. Admissions declined in the Hawkes bay area, but not the Gisborne area in 1965 and 1966. In 1967 there was an epidemic of viral respiratory infections in Hawkes Bay, but not in Gisborne.

On inspection it would appear that the iron-dextran might have been responsible for the decline. The author implies that the children in Hawkes Bay had an increased exposure to infection in 1967 and that this year was not strictly comparable. The argument could equally well be applied to the results for Gisborne in the two previous years.

No details on population size, growth and economy are given. It would have made direct comparison simpler if *rates* rather than *admissions* had been given.

Conclusion: This study provides fairly strongly suggestive evidence for a loss of resistance to infection in iron deficiency in infancy. One wonders why it seems to have been ignored in review articles on the subject*.

Summary and Conclusions

The above review shows that, although much has been written about the relation of iron status to infection, there have been few controlled trials, and no study has been free of methodologic defects in design or omissions in reporting.

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* As for example in 235

The body of evidence is strong enough to warrant clinical zeal in treating and preventing iron deficiency, but with due caution to risks of increasing susceptibility to (new or recrudescent) infection. The risks are especially great in patients who might become hyperferraemic (as for example in kwashiorkor or in neonates) or in those living in tropical areas where malaria is endemic.

There is reason to believe that iron deficiency is causally related to increased susceptibility to infection, but this association has not been properly established. It is important that this be done since iron deficiency is common and easily treated.

TABLE 2.15 IRON STATUS AND INFECTION

CLASSIFICATION OF REPORTS AND STUDIES ON HYPOTHESES THAT RELATE IRON STATUS TO A PREDISPOSITON TO INFECTION

REF NUMBER	AUTHOR (first)	TEST OF HYPOTHESIS	INTERVENTION	RESULTS effect of intervention on infection
HYPOTHESIS 1 : IRON EXCESS INCREASES SUSCEPTIBILITY TO INFECTION				
19	Barry	Support	Iron-dextran	Increased incidence of Gram-negative septicaemia
348	Mofenson	Support	Oral iron overdose + desferrioxamine	Yersinia enterocolitica septicaemia
453	Scott	? Support	Iron-sorbitol-citrate	Urinary tract infection precipitated in 1 patient with IDA
SS	Briggs	? Support	Iron-sorbitol-citrate	Increased pyuria in patients with chronic renal tract infection
454	Scott	? Support	Iron-sorbitol-citrate + oral iron	Urinary tract infection precipitated
313	MacFarlane	? Support	Iron-dextran + refeeding in kwashiorkor	Death was associated with low initial transferrin
422	Robins-Browne	? Support	Observation: histology at post mortem	3 patients dying from Yersinia entercolitica septicaemia had siderosis
76	Butzler	? Support	Observation: case report	Child with thalassemia had Yersinia enterocolitica septicaemia
337	Melby	? Support	Oral iron overdose + desferrioxamine	Yersinia enterocolitica septicaemia
HYPOTHESIS 2 : IRON DEFICIENCY INCREASES SUSCEPTIBILITY TO INFECTION				
Prospective controlled trials				
314	Mackay	Support	Fortification of milk formula	Decreased infections: Respiratory, gastrointestinal, "specific", other
7	Andelman	Support	Fortification of milk formula	Decreased respiratory infections
226	Higgs	Support	Iron-dextran plus oral iron fumarate	Chronic mucocutaneous candidiasis improved in 3 of 4 pts & 0 of 4 ctls
79	Cantwell	Support	Iron-dextran	Fewer admissions to hospital
169	Fletcher	Support	Ferrous sulfate	Oral candida counts fell and mouth lesions improved
247	James	? Support	Iron-dextran	Trend towards fewer respiratory and diarrhoeal infections
503	Vellar	? Support	Ferrofumarate (60mg Fe)	Trend towards fewer respiratory infections (40% vs 44%)
22	Basta	? Support	Ferrous sulfate	Decreased infections and morbidity score
74	Burman	? No support	Fortification of milk formula	No difference in infections (but, also little change in Hb)
242	Jacobs	? No support	"Iron therapy"	Oral Candida albicans colony counts were unchanged after treatment

Table 2.15 part 2
Iron status and infection - Human Studies

TABLE 2.15 IRON STATUS AND INFECTION
CLASSIFICATION OF REPORTS AND STUDIES ON HYPOTHESES THAT RELATE IRON STATUS TO A PREDISPOSITON TO INFECTION

REF NUMBER	AUTHOR (first)	TEST OF HYPOTHESIS	INTERVENTION	RESULTS effect of intervention on infection
Studies of prevalence				
176	Fortuine	Support	Observation	Low Hb associated with a poor prognosis in purulent meningitis
226	Higgs	Support	Observation	IDA in 23 of 31 chronic mucocutaneous candidiasis
169	Fletcher	Support	Observation	Oral mucosal lesions and candida colony counts increased
426	Rose	? Support	Observation	Mean SI in patients with angular cheilitis decreased
185	Fuerth	? Support	Observation	Trend: number of illnesses negatively correlated with Hb
91	Chandra	? Support	Observation: serum transferrin saturation	Patients with recurrent herpes labialis had lower % Sat
250	Jenkins	? Support	Observation	Hyperplastic oral candidosis more common in ID
375	Oppenheimer	? Support	Observation	Prevalance of ID increased in infants with meningitis or pneumonia
44	Bondestam	? Support	Observation: serum iron, transferrin	SI, but not TF, depressed in children with recurrent infections
232	Jacobs	? No support	Observation	Oral Candida albicans colony counts same as controls
320	Malakhovsky	? No support	Observation	Incidence of infection not increased in IDA
412	Reeves	? No support	Oral iron supplement	Haemoglobin response associated with number of previous infections
Prospective, controlled studies. Multiple variables including iron status				
244	Jacobs	? Support	Strained meat	Significantly fewer respiratory and gastrointestinal infections
258	Karp	? Support	Observation: serum iron, transferrin sat.	Patients with AML & fungal infection had reduced transferrin saturation

TABLE 2.15 IRON STATUS AND INFECTION
CLASSIFICATION OF REPORTS AND STUDIES ON HYPOTHESES THAT RELATE IRON STATUS TO A PREDISPOSITON TO INFECTION

REF NUMBER	AUTHOR (first)	TEST OF HYPOTHESIS	INTERVENTION	RESULTS effect of intervention on infection
Anecdotal Reports				
215	Heimendinger	? Support	Ferrous gluconate	Children in institutions had fewer infections
442	Salmi	? Support	Observation: infection rate	Decreased rate of infection experienced by test group
84	Cawson	? Support	Iron replacement therapy	Two patients with oral candiasis were cured
347	Moe	? Support	Observation:incidence of infection	5 Of 7 children with Hb < 8g/dl developed infection in hospital
459	Shaw	? Support	Observation	Hb < 10g/dl present in 29% medical and 15% surgical admissions
126	Dallman	? Support	Iron dextran	Treatment associated with fewer deaths, but equal infection rate
302	Lovric	? Support	Observation: haemoglobin	Children in hospital had lower Hb and more infections
21	Basta	? Support	Observation	Acute and chronic infections more prevalent in IDA
126	Stekel	? Support	Fortification of infant milk formula	Decreased infantile diarrhoea
323	Marsh	? No support	Fortification of infant milk formula	30 children in test group had no more infections than 44 controls
162	Farquar	? No support	Supplementation with oral iron	No difference in "well being"
520	Werkman	? No support	Observation: post treatment for ID	Children continued to have more infections AFTER treatment
497	Tonkin	? No support	Iron dextran	No marked difference in morbidity between Test & Control groups
HYPOTHESIS 3 : IRON DEFICIENCY PROTECTS AGAINST INFECTION				
361	Murray	Support	Observation	Malaria, brucellosis, tuberculosis & other infections decreased in ID
358	Murray	Support	Ferrous sulfate 300 mg weekly	Increased attack rate of Entamoeba histolytica
376	Oppenheimer	Support	Iron-dextran	Increased incidence of malaria and splenomegaly
361	Murray	Support	Refeeding after famine + FeSO4	Recrudescence of malaria, brucellosis, tuberculosis, other infections
77	Byles	Support	Iron-dextran	Chloroquine prevented recrudescence of malaria
330	Masawe	? Support	Observation	Protozoal and helminthic infections less prevalent in IDA
"	"	"	"	Bacterial infections more common in IDA
362	Murray	? Support	Refeeding after famine	Transferrin saturation, serum iron & plasmodial parasitaemia increased
329	Masawe	? Support	Observation	Malaria and bacterial infections less common in iron deficiency
332	Murray	? Support	Feeding: grain or milk	Cerebral malaria in grain-fed children, but not in milk fed children

Abbreviations:
Hb Haemoglobin
ID Iron Deficiency
IDA Iron Deficiency Anemia
SI Serum Iron
% Sat Percentage saturation of transferrin
TF Transferrin

Classification and diagnosis of iron deficiency

The effects of iron deficiency are well described and include decreases in haemoglobin, haematocrit, mean cell volume, mean cell haemoglobin, red cell distribution width, serum iron, serum ferritin, percentage saturation of serum transferrin and increased red cell protoporphyrin. The accepted patho-physiological model of the consequences of decreasing iron status is attractively clear and explanatory: As an iron sufficient individual becomes iron deficient the first manifestation is a reduction in iron stores. After depletion of iron stores, haematopoiesis is affected, the red cells become smaller and hypochromic and erythrocyte protoporphyrin rises. Finally, the haemoglobin falls. The "natural" grouping, according to this model, would be into the following classes with the associated test results*:

IRON STATUS	TEST RESULT
Replete	All normal
Depletion of stores	Decreased serum ferritin
Impaired haematopoiesis	Increased red cell ZPP
	Decreased MCV and MCH
Iron deficiency anaemia	Decreased Hb, HCT

These principles of classification are clear and widely accepted. But, their application to determine an individual's iron status is problematic. Hence there is an extensive literature on the practical problems of diagnosis and classification**. The problems arise because the above classification scheme does not allow each individual to be put into one and only one class. The reason for this is that each class above has different tests constituting the decision criteria, and the test results are not simply dependent on each other as assumed in the pathophysiological model. For example, a particular individual may have a low haemoglobin level, but the serum ferritin could be within the normal range. There are three reasons for this uncomfortable state of affairs.

Firstly, the pathophysiological model represents a trend rather than absolute constraints on behaviour. The model "expects" each test to behave as well as the proverbial "gold standard" in measuring the condition with which it has been associated. In actuality, each test is a rather indirect measure of the particular level of iron status with which it is associated.

Secondly, the biological range of "normal" values is wide, and often age and sex specific. This makes the setting of upper or lower limits difficult and arbitrary even when a particular multiple of the standard deviation is taken from the mean or a specific percentile range is calculated from the distribution of results.

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* The selection of tests is for illustrative purposes and far from complete

** See for example 29, 34, 46, 56, 75, 94, 103, 105, 106, 120, 123, 124, 125, 135, 141, 148, 163, 200, 213, 222, 245, 260, 269, 272, 273, 316, 322, 351, 397, 408, 417, 435, 436, 440, 527, 430, 531

And thirdly, the results of tests are often affected by confounding factors. For example, infection can increase serum ferritin and decrease haemoglobin concentration resulting in the awkward case used as an illustration above. The classification scheme assumes the absence of disease or variations from normal. Clinical judgement is required to apply the classification rules for individuals with inflammation, haemoglobinopathies etc. Studies of populations either exclude such cases or assume that their effect is negligible.

The literature on classification and diagnosis contains didactic and instructional articles, assessment of new tests or refinement of established tests (for populations or for individuals), and review articles.

The only article that is reviewed here is chosen because it describes the classification scheme used in the work reported in this thesis, and because it is practical and used in the Department of Paediatrics and Child Health, University of Cape Town. The reader who is interested in other practical aspects of this fascinating and frustrating problem is referred to the articles cited above. The theoretical aspects are discussed by Blois[43], Kraupel-Taylor[276] and Murphy[357].

Kirsten *et al*[267] used haemoglobin concentration, mean cell volume, mean cell haemoglobin and ferritin to classify infants between the ages of 1 and 12 months. A table of limiting values dividing the normal from the abnormal for each test and for 6 different age groups was derived from published data*. These reference values were then used in the decision criteria for their classification of iron status. A child is *normal* if all tests have normal results. *Diminished iron stores* was diagnosed if the ferritin was low. *Haematological iron deficiency* categorizes those children with low mean cell haemoglobin and mean cell volume. The criterion for diagnosis of *iron deficiency anaemia* is a low haemoglobin together with at least one of mean cell haemoglobin and mean cell volume below the normal limit.

Like other classification schemes of iron deficiency, this does not unambiguously assign all possible combinations of test values into one diagnostic class. The example of the child with normal ferritin and low haemoglobin illustrates this.

Like other classification schemes, it has tried to improve specificity and sensitivity by including multiple criteria using the inclusive or to conjoin potential sub-categories. The price to be paid for this is the (unlikely) confusion in classifying a case with low haemoglobin, but normal mean cell volume and normal mean cell haemoglobin. The nosologist usually will establish a catch-all category of *other* to tidy away this sort of non-conforming case.

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* The table is reproduced as 2 above.

Prevention of iron deficiency

Iron supplementation and fortification

Public health programs to combat iron deficiency

With iron deficiency being the most common single nutrient deficiency much effort has been devoted to combating this disorder. This section discusses strategies for developing public health programs to reduce the prevalence of iron deficiency in a population. The following sections review the technology of food fortification with particular reference to infant milk formulas, factors influencing the absorption of iron, studies on iron absorption, trials of iron fortified infant milk formulas and risks of iron fortification.

The World Health Organization has published a useful model of the development of national public health programs aimed at nutritional anaemias[415]. The first step is to define the problem of iron deficiency *ie* its extent and severity. This is done by determining the status of the population, identifying areas and groups at risk and by setting goals for acceptable levels of indicators of iron status such as mean haemoglobin.

The second step entails a pilot trial of iron supplementation or fortification, depending on the prevalence and severity of iron deficiency. In areas with a moderate prevalence of iron deficiency, food fortification may be the best approach in increasing dietary intake since programs may be directed at whole communities or at particular vulnerable groups such as infants.

With the experience gained from the pilot trial, large field trials may be implemented to test the feasibility of national programs.

Technological issues in fortification of food with iron

In implementing a program of iron fortification the food and the additive must satisfy certain requirements to be acceptable. The food must be widely consumed in adequate amounts and must be processed with strict attention to quality control. A suitable additive must be compatible with its food vehicle, readily assimilated and stable under locally available storage conditions.

Vehicles for iron fortification in infancy

Selection of a food for fortification with iron is a relatively simple task for infants. In fact most infant cereals and milk powders are routinely fortified with iron in various forms[310].

Sources of iron for fortification

Hurrell has provided a thorough overview of the technology of iron fortification[237]. The most common form of iron added to infant milk formulas is ferrous sulfate, although ferrous ammonium citrate, ferrous citrate, ferrous gluconate and ferrous lactate are sometimes employed. The iron in ferrous sulfate is as least as well absorbed as iron in the other salts.

Sources of iron commonly used for cereals are elemental iron, ferric pyrophosphate and ferric orthophosphate. These sources of iron are considerably less bioavailable than iron from ferrous sulfate, but the ferrous salts reduce product quality and shelf life unless it is kept in an airtight container.

Factors influencing the absorption of iron

Factors influencing the bioavailability of iron include its chemical properties and the presence of inhibitors and/or facilitators of iron absorption.

Chemical properties of iron that affect its bioavailability

Oxidation states

Iron is added to food as elemental iron or as ferrous (Fe^{2+}) or ferric (Fe^{3+}) salts. Ferrous iron is rapidly oxidized to ferric iron in the presence of oxygen. In a suitable chemical environment, ferrous iron may be formed by the oxidation of elemental iron or the reduction of ferric iron.

Solubility

The solubilities of ferrous iron at pH 7 and pH 8 are 10^{-1} M and 10^{-3} M. Ferric iron has a solubility of 10^{-3} M at pH 2, but only 10^{-18} at pH 7. Thus, although both ferric and ferrous ions are readily soluble in the acid condition of the stomach, the ferric ion is insoluble in the slightly alkaline conditions of the small bowel where iron absorption occurs. In alkaline solution both ferric and ferrous iron tend to form their respective hydroxides which are insoluble.

With few exceptions (*eg ferric* hydroxide, ferric orthophosphate and ferritin) all non-haem iron in a meal exchange with a common pool of ionized iron, and it is from this pool that absorption takes place. Haem is absorbed intact and the iron freed after it has been taken up from the lumen into the mucosal cell.

Formation of complexes

Iron can react to form up to 6 coordinate bonds with ligands. Such a complex is termed monodentate if 6 ligands each form 1 bond with an iron atom; bidentate if 3 ligands each form 2 bonds with an iron atom and so on. A ligand is called a chelate when 2 or more atoms from the same ligand participate in the bonding of a complex. Different ligands may bond with the same atom to form mixed complexes. Binding to complexes is often highly pH dependent.

In the acid conditions of the stomach most iron is released from complexes into a common pool and ferric iron is reduced to ferrous iron in the presence of reducing agents such as ascorbic acid. In the small intestine the pH rises and this favours the reformation of complexes. Bioavailability of iron is determined by the solubility of the complexes, their affinity for iron and the extent to which insoluble hydroxides form. In general, ferric iron binds more strongly with ligands than ferrous iron. Ligands that chelate iron to form insoluble complexes or complexes with very high affinity for iron inhibit its absorption. Examples include phosphates, oxalates, dietary fiber, tannins and phytate. Ligands that form soluble chelates with iron enhance its absorption. Amino acids, citrate and ascorbate are in this category.

Haem iron is not susceptible to binding by ligands.

The single most important determinant of bioavailability of iron is the quantity of animal tissue in the meal. The factors responsible have not been conclusively identified, but certain amino acids may act as ligands and cysteine certainly is important.

In summary, the effect of a given ligand on iron availability depends on its concentration, its chelating efficiency (which is often highly pH dependent) and the types and concentrations of competing ligands.

The interaction of the factors that influence the bioavailabilty of iron is so complex that it is not yet possible from chemical properties to predict quantitatively either the percentage absorption or the effect on iron status. Such effects must be determined by direct measurement.

Facilitators and inhibitors of iron absorption

Factors that enhance the absorption of iron from a meal have been reviewed by Charlton and Bothwell[97]. Animal tissue, ascorbic acid and other organic acids such as lactic, citric, malic and tartaric acids promote iron absorption.

Absorption of iron is inhibited by cereals, soya bean and bran; the responsible agents include polyphenols, tannins (present in tea, coffee and other foodstuffs) and phytates[97]. A common preservative, EDTA, may decrease the availability of iron, depending on the presence of other ligands,

but it has been used to fortify refined sugar with iron. The major components of an infants diet that may contain inhibitors are cereals and soya bean based infant formulas.

Rios *et al*[419] found similar values for the absorption of iron from cow's milk based formula and soya bean based formula, 3.9% and 3.4% compared with 5.4%. Interpretation of this study is difficult because they did not report the levels of ascorbic acid in the formulas. Other workers have reported a markedly lower bioavailability of iron in soya based infant formulas[133]. Gillooly *et al*[194] found that the geometric mean absorption of iron from soya based formulas rose from 1.8% with no ascorbic acid to 7.7% with a molar ratio of ascorbic acid to iron of approximately 8:1. A direct comparison was made with a similar cow's milk formula at two concentrations of ascorbic acid. At a molar ratio of ascorbic acid to iron of approximately 2 the geometric mean absorption from the soya formula was 2.4% and from the cow's milk formula 5.3%. Doubling the ascorbic acid content raised the respective absorptions to 7.2% and 19.5%. When the individual measurements were adjusted to a reference absorption of 40% the difference between the cow's milk and soya formulas was even more striking. Cook and Bothwell[104] review a number of other studies on iron absorption from soya products and conclude that most recent studies suggest that soya impairs the absorption of non-haem iron. Varied explanations have been offered for the conflicting results, but these require confirmation.

Iron absorption from breast milk is considerably more efficient than that from cow's milk or infant formulas. The reason for this is unknown but may be due to increased concentrations of ascorbic acid, cysteine, inosine and taurosine or decreased levels of phosphate[104].

In summary, the bioavailability of iron in a meal depends on the form of the iron and the presence of inhibitors and facilitators of absorption. The presence of meat in a meal or a glass of orange juice after the meal can greatly increase the absorption of iron, while a cup of tea after the meal can largely suppress its assimilation.

Iron absorption from infant milk formulas

This section considers firstly some technical issues in the measurement of absorption of iron from food and then reviews studies of iron assimilation from infant milk formulas. The following section considers reports on the efficacy of programs of fortification of infant milk formulas in improving iron status.

Measurement of food iron absorption

Iron absorption from foods has been measured in several different ways[104]. It can be estimated from the change in iron status after introduction of a particular dietary regime. It may be calculated from

metabolic balance studies using chemical measurements of iron intake, excretion and losses. Or it may be deduced from the retention of radioactive iron ingested in a test meal.

The first method, while being the "bottom line" in measuring the efficacy of a food fortification program, is a crude and ineffective method of determining the availability of iron from a particular compound in a particular food. The reasons for the unsuitability of this method include the assumptions that must be made in calculating total body iron and total iron intake and, more importantly, the fact that assimilation of iron is regulated according to iron status.

Chemical methods of quantifying metabolic balance studies of iron are too imprecise for use in infancy.

The most accurate method of measuring iron absorption in infancy has been to determine the incorporation of radioiron in circulating blood 10 to 14 days after a test meal. This method has been validated in adults by comparison with measurements of total body radio-activity. The method depends upon the existence of 2 common pools of iron in the gut *viz* haem iron and non-haem iron. *Ie* the proportion of iron absorbed from a test meal can be equated to the proportion of added radioiron that is absorbed (with a few exceptions that were noted above).

A major methodologic problem in iron absorption studies is the enormous variation in percentage absorption which makes it difficult to compare results. The effect of the large day-to-day variation in absorption for any particular subject has been reduced by administering the test dose of iron over several days. The variability in biological response may be reduced by expressing absorption of iron as a percentage of the absorption from a "reference" dose of ferrous sulfate and ascorbic acid given in the fasting state. In studies designed to assess iron bioavailability the optimal approach has been to administer 2 (or more in adults) meals to the same individual. The best statistical summary measure of absorption is the geometric mean since this is less affected by "outliers" than the arithmetic mean. The geometric mean is lower than the arithmetic mean (with data in the range obtained in absorption studies).

Studies of iron availability in infant foods are often performed in iron replete adults. Extrapolation of the results to infants depends on a number of assumptions which have not yet been validated, but are probably methodologically sound[104].

Studies of iron absorption from cow's milk infant formulas

The bioavailability of iron in infant milk formulas is highly dependent on the presence of ascorbic acid. This was shown by Derman *et al*[136] who conducted studies in adult women of iron absorption from cow's milk based infant formula. When the molar ratio of ascorbic acid to iron was increased from 0 to

2 and from 2 to 6.3 the (arithmetic) mean percentage absorption rose from 7.2% to 19.6% and from 21.9% to 35.7%.

In a series of similar studies conducted in infants aged between 5 and 18 months Stekel *et al*[482, 125] found geometric mean absorption of iron from formulas without added ascorbic acid to range from 2.9% to 5.1%. Higher mean absorption values, 5.9% to 11.3% were obtained from formulas with supplementary ascorbic acid.

Gillooly *et al*[194] reported geometric mean absorption of iron by adult women from cow's milk based infant formula. These workers found that doubling the ascorbic acid content raised the percentage absorption of iron from 5.3% to 19.5%.

The percentage absorption of iron from cow's milk based formulas that have not had added ascorbic acid has ranged from 2.9% to 19%[125, 136, 334, 418, 434, 438, 482]. In view of these findings it has been accepted that, while cow's milk formulas do not promote the absorption of iron, they have substantially less inhibitory effects than infant cereals or solid foods[104].

These studies are analyzed in more detail in the following chapter.

Trials of iron fortified infant milk formulas

The effectiveness of iron fortification of infant foods in improving the iron status of a population has been shown in a number of studies[7, 125, 323, 346, 347, 432, 502]. These studies are summarized in table 2.16 and discussed below.

Marsh *et al*[323] compared a cow's milk based formula containing 12 mg Fe per litre and 55 mg ascorbic acid per litre with the same formula without iron and with evaporated milk. The milks were given to 3 small groups of term and preterm infants from birth to 9 months of age. The group receiving the iron fortified milk had a mean haemoglobin of 12.69 g/dl at 9 months compared with 10.46 g/dl and 9.67 g/dl in the other 2 groups.

Andelman and Sered[7] compared the same iron fortified milk with evaporated milk in infants from a low socio-economic population. The milk was given from discharge or the first clinic visit until 6 to 9 months of age. At 1 year of age the mean haemoglobin level in the group receiving the iron fortified formula was 11.9 g/dl, while the control group had a mean haemoglobin of 10.4 g/dl and about 76% of these infants were considered to be anaemic at some stage during the study. In contrast, only 15% of the infants receiving iron fortification were considered anaemic at 1 year.

In a small study Saarinen compared the haemoglobin, mean cell volume, percentage saturation of transferrin and serum ferritin levels in infants who received 1 of 3 different milks. The mean haemoglobin level at 1 year of age in the group who were fed an iron fortified cow's milk formula was 12.9.* and none were classified as iron deficient. In the group who received an unfortified infant milk formula the haemoglobin level was 12.7 g/dl and 4% were diagnosed as iron deficient. The group whose source of milk was the breast had a mean haemoglobin level of 12.4 g/dl and 7% were classed as iron deficient. Table 2.16 gives the criteria for classification of iron deficiency employed in this report.

Dallman *et al*[125] and Stekel[479] reported on similar trials conducted in a low socio-economic group in Chile. In a pilot study, cow's milk formula fortified with iron was compared to the same milk without iron. At 9 months of age 14.8% of the infants on the fortified milk had haemoglobin levels below 11 g/dl. The prevalence of anaemia among infants fed the unfortified formula 27.7%. In an effort to improve the bioavailability of the iron in the milk a second pilot study was performed with a group of infants who were given cow's milk formula with 15 mg Fe and 100 mg ascorbic per litre. This group had mean haemoglobin levels of 12.2 g/dl and 12.5 g/dl at 9 and 15 months of age compared with 11.1 g/dl and 11.4 g/dl in the control group who received unfortified formula*. The prevalence of anaemia at 9 months of age was about 7.5% and 34.7% in the respective groups. A large field trial was then implemented and reduced the prevalence of anaemia at 15 months of age to 5.5% from 29.9%. Mean haemoglobin levels improved to 12.2 g/dl from 11.2 g/dl at 15 months.

These results are summarized in table 2.16 below. It is important to note that the results are directly comparable for neither the mean haemoglobin levels nor the proportion of children classed as iron deficient. Since each study used different criteria to diagnose iron deficiency, the diagnosis rate of iron deficiency differs. Also, since the studies removed children from further observation who turned out to be iron deficient during the study, both the mean haemoglobin levels and the prevalence of iron deficiency will be improved in the remaining infants. This effect is largest for the groups with poorest iron nutrition and thus tends to diminish the difference between test and control groups. Nevertheless, the table does provide a clear indication of important trends.

It should also be noted that none of these trials reported the methods of ascertainment and group assignment and that none of the studies were blinded. This leaves open the possibility that subjective factors may have introduced a bias into the composition of the groups.

With these caveats in mind, comparison of the studies leads to some interesting conclusions.

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* The haemoglobin levels were read from figure 1 in the author's paper

* The values for mean haemoglobin were obtained from the graphs in figure 2 of Stekel's paper[479]. The same paper contains the ascorbic acid concentrations of the milk formulas used by Marsh *et al*[323] and Andelman and Sered[7].

The infants in the study of Saarinen who were given cow's milk formula without added iron in fact achieved a higher mean haemoglobin and a lower prevalence of iron deficiency than infants in the other studies who received milk with iron, and in some cases, ascorbic acid as well. The same milk formula fortified with both iron and ascorbic acid was associated with mean haemoglobin levels of 12.69 g/dl and 11.9 g/dl in the studies of Marsh *et al* and Andelman and Sered. It thus seems that excellent iron status may be achieved in infants who are fed an adequate solid diet, irrespective of the composition of the milk formula. It also seems that iron in a formula is of most benefit to those infants who have the least adequate solid diet.

A corollary to this conclusion is that infants in a lower socio-economic community may benefit from infant milk formulas with more bioavailable iron than has been provided in the studies cited.

Table 2.16 Trials of iron fortified infant milk formula

STUDY CHARACTERISTIC	Marsh[1] Term	Marsh[1] Premature	Andelman[2]	Saarinen[3]	Dallman[4] Pilot 1	Dallman[4] Pilot 2	Stekel[5] Field
Criteria for iron deficiency							
Haemoglobin below	8 g/dl[6]	8 g/dl[6]	10 g/dl	11 g/dl[7]	11 g/dl	11 g/dl	11 g/dl
Beikost	Nil	Nil	Advised	Vits + advice	Advised	Advised	Advised
MILKS COMPARED							
1 Type	Formula	Formula	Formula	Formula	Formula	Formula	Formula
Fe (mg/dl)	12	12	12	11	15	15	15
Vit C (mg/dl)	55	55	55			100	100
2 Type	Formula	Formula	Evaporated	Formula	Formula	Formula	Formula
Fe (mg/dl)	1	1	1	1	1	1	1
Vit C (mg/dl)	55	55					
3 Type	Evaporated	Evaporated		Breast milk			
Fe (mg/dl)	1	1		1			
Vit C (mg/dl)							
RESULTS: Mean Haemoglobin (mg/dl) [Age (months)][8]							
Group 1	12.69 [9]	12.49 [9]	11.9 [9]	12.9 [12]	N/A [9]	12.3 [9]	12.2 [15]
Group 2	10.46	9.40	10.4	12.7	N/A	11.3	11.2
Group 3	9.67	8.55		12.4			
RESULTS: Proportion Iron Deficient							
Group 1	N/A	N/A	N/A	0%	14.8%	7.5% approx	5.5%
Group 2	N/A	N/A	N/A	4%	27.7%	34.7%	29.9%
Group 3				7%			

This page reserved for table 2.16

NOTES: N/A Not Available

1 Marsh A, Long H, Stierwalt RN. Comparative hematologic response to iron fortification of a milk formula for infants. Pediatrics 1959; 24: 404-412(323)

2 Andelman MB, Sered BR. Utilization of dietary iron by term infants. A study of 1048 infants from a low socioeconomic population. American Journal of Diseases of Children 1966; 111: 45-55(7)

3 Saarinen UM. Need for iron supplementation in infants on prolonged breast feeding. Journal of Pediatrics 1978; 93: 177-180(432)

4 Dallman PR, Siimes MA, Stekel A. Iron deficiency in infancy and childhood. American Journal of Clinical Nutrition 1980; 33: 86-118(125)

5 Stekel A. Prevention of iron deficiency. in: Iron nutrition in infancy and childhood. ed Stekel A. Raven Press. New York 1984: 179-194(479)

6 Criteria for iron deficiency also include serum iron < 50 ug/dl

7 Criteria for iron deficiency are at least 2 of the following criteria: haemoglobin < 11 g/dl, mean cell volume < 70 fl, percentage saturation < 10%, serum ferritin < 10 ug/l.

8 Haemoglobin levels were read from graphs in the papers of Saarinen, Dallman and Stekel

Risks of iron fortification

Risk of infection

Hegenauer and Saltman[214] and Fomon *et al*[171] reviewed the literature and concluded that the clinical and experimental evidence suggests that oral iron supplements and iron fortification present a minimal public health risk of increased susceptibility to infection. However, the European Society of Paediatric Gastroenterology and Nutrition (ESPGAN) in their guidelines on infant nutrition[156] warned that iron in infant formula which exceeded the binding capacity of lactoferrin might increase the susceptibility of infants to infection with *Escherichia coli*. The source of their concern was the study by Bullen *et al*[66] which measured the effect of haematin on bacterial colonization after challenge with *Escherichia coli* in new born, suckled guinea pigs. This was found to increase bacterial counts. In the same report it was noted that the bacteriostatic effect of human milk on *Escherichia coli* was abolished by the addition of sufficient ferric ammonium citrate to saturate iron binding. The ESPGAN paper noted that the clinical relevance of these findings was questionable for milk formula stored and prepared under hygienic conditions.

Risk of decreased absorption of zinc and other metals

Dietary iron (organic and inorganic) has been shown in many studies to inhibit the absorption of zinc[499, 470, 98, 418, 438, 317, 236, 150, 114, 529, 183, 211, 238, 335]. However, at least one study has not confirmed the competition for absorption between iron and zinc[443]. Most studies have used isotope studies to determine absorption and plasma zinc to determine zinc status but hair zinc levels were shown to be lower in malnourished infants than in normal children[139].

The evidence for a competitive interaction between iron and zinc in the diet is reviewed by Solomons[469] who also considers the consequences for human nutrition and nutritional programs. He concludes that *"conscious adjustment of the Fe/Zn ratio in human diets, foods and therapeutic nutrients should become a priority"*.

The reason for this concern is that zinc deficiency includes amongst its many manifestations growth retardation[195] and impairment of the immune system[211, 33, 32].

Hurley *et al*[236] in a review of trace metal interactions state that, as for iron and zinc, there is a similar inverse relationship between the absorption of iron and manganese and their concentrations in the diet but that the dependency of copper absorption on iron concentration in the diet was unimportant. Other interactions were not discussed.

Risk of iron overload

Bothwell and Charlton[47] have suggested that the risk of increasing the prevalence of iron overload in the population does not outweigh the benefits offered by programs of fortification of infant foods. The group at particular risk for early iron overload from fortification programs are idiopathic haemochromatosis homozygotes. The overall prevalence of this disorder is not known, but there are areas in North America and Europe where it is as high as 1 in 300 to 500 of the population[47].

Risk of anaemia in vitamin E deficiency

Williams *et al*[522] have suggested that in small premature infants the development of vitamin E deficiency may lead to anaemia if they are given iron fortified milk formulas. The mechanism is postulated by the authors to be loss of protection by vitamin E against lipid peroxidation.

Risk of gastrointestinal symptoms

In a summary of feeding recommendations for normal infants Fomon *et al*[171] observed that: *Some physicians are opposed to the use of iron-fortified formulas because of the belief that such feeding results in fussiness, colic, spitting-up, diarrhea, or constipation. Other physicians, including the authors, use iron-fortified formulas regularly and consider such manifestations relatively uncommon. Unfortunately, no study that satisfactorily resolves this controversy has been reported in the literature.*

Conclusions

The literature review revealed some useful guidelines for planning a fortification program and outlined factors influencing the bioavailability of iron. With reference to infant milk formulas it was noted that ascorbic acid is an important facilitator of iron absorption and that iron is less available from formulas based on soya bean. It was shown that iron status in infants depends on both the source of milk and additional foods taken. In upper socio-economic communities the level of iron fortification in infant milk formula is less important than it is in lower socio-economic communities where the beikost is of a poorer quality. In lower socio-economic communities optimal iron nutrition has not been achieved with infant milk formulas fortified at levels employed in studies reported to date.

That we must examine, not the final, but the efficient, causes of created things.

Likewise, finally, we will not seek reasons of natural things from the end which God or nature proposed to himself in their creation (i.e. final causes), for we ought not to presume so far as to think that we are sharers in the counsels of Deity, but, considering him as the efficient cause of all things, let us endeavour to discover by the natural light which he has planted in us, applied to those of his attributes of which he has been willing we should have some knowledge, what must be concluded regarding those effects we perceive by our senses; bearing in mind, however, what has been already said, that we must only confide in this natural light so long as nothing contrary to its dictates is revealed by God himself.

Rene Descartes
The principles of philosophy
AD 1646

CHAPTER 3 STUDY DESIGN, METHODS AND MATERIALS

Introduction

This chapter describes the problem addressed by the thesis, outlines the design and protocol, details the planning of the trial and documents the methods and materials employed.

The problem addressed by the study

In Cape Town the municipal health clinics provide at subsidized prices cow's milk formula fortified with iron and ascorbic acid to families with young children. Yet surveys such as that of Kirsten *et al*[267] have found a disturbingly high prevalence of iron deficiency in otherwise normal infants. The review of the literature in chapter 2 showed that infants in lower socio-economic communities certainly benefit from the fortification of infant milk formulas with iron but that this often does not achieve optimal iron nutrition in the population. From 5 to 10 percent of the infants may still be considered iron deficient in spite of being fed with a formula containing 25% more iron and 100% more ascorbic acid than that employed in a popular commercial product.

The primary aim of the study thus was to see if increased iron fortification of a conventional infant milk formula would result in improved iron nutrition in healthy full term infants.

The literature review in chapter 2 showed that iron deficiency impairs a number of laboratory tests of immune function and that it may increase susceptibility to infection. The second aim of the study thus was to document any changes in immune function tests and susceptibility to infection that might follow from increasing the quantity of iron in the milk formula.

The third aim of the study was to determine if the additional iron in the milk formula was associated with any risks. In chapter 2 a number of potential risks of iron fortification were identified and discussed. It was noted that authorities considered the risks to be minimal but that data establishing the safety of high levels of iron fortification of infant milk formula have not been published. The risks include an increased susceptibility to infection, particularly infectious diarrhoeal disease, decreased zinc absorption and gastrointestinal upsets.

The following section describes the design of the clinical trial that was made of an infant milk formula with the concentration of iron substantially increased. Advantage was taken of the study infrastructure to conduct surveys of the infant feeding habits in the community and of the socio-economic characteristics of the participating families. These surveys not only provided independently valuable descriptive data but allow the main study to be placed in its social and economic context. Their protocols are described at the end of this chapter.

Study design - Iron fortification trial

Acknowledgements

The protocol for this study was drawn up with the assistance of Prof H de V Heese and in consultation with Prof DW Beatty.

Protocol

The study employed a prospective, stratified, double blind, controlled trial of iron fortified milk formula in which a group of infants were studied from the age of 3 months to 1 year. Half of the children, the Control group, were given a standard milk formula (Lactogen Full Protein) which contains 8.3 mg Fe/100 g formula and 37 mg ascorbic acid/100g formula. The other half of the subjects, ie the Test group, were given the same milk powder supplemented with extra iron to give 40 mg Fe/ 100g formula. Mothers were provided with sufficient milk formula to give their child the lesser of 180 ml/kg/day or 1.5 liters/day.

The data collected on entry to and exit from the study included biographical details, the weight, length and skull circumference, evidence for possible infection (if any), full blood count with differential enumeration, either leukocyte phytohaemagglutinin stimulation response or neutrophil bactericidal index, lymphocyte subtyping and estimation of T and B cell numbers and proportions, delayed cutaneous hypersensitivity to PPD and candida antigen, and plasma antibody levels to polio and tetanus. Plasma antibody levels to polio and tetanus were also determined in the mother when the child was 3 months old. On entry, a sample of faeces was cultured for pathogenic viruses, and on exit each child had swabs for viral culture taken from the nasopharynx and rectum. Zinc concentrations were determined in plasma and hair at the start and on completion of the trial.

Each child was seen every 3 or 4 weeks and subjected to a clinical examination with recording of weight, length, head circumference and any features suggestive of infection. The child's caretaker was specifically asked if the child had had symptoms of infection or had been taken to a doctor, hospital or clinic since the previous examination.

The data collection form is reproduced in appendix 6.

Children excluded for reasons of iron deficiency and infants found to be iron deficient at the end of the study were treated with oral iron supplementation for 3 months.

Care was taken to avoid ethical problems that might have arisen from using an infant milk formula in a setting where breast feeding is actively encouraged.

Important design features and the reasons for the decisions and choices that were made in designing this experiment are detailed in the following sections.

Eligibility criteria

As ill-health and malnutrition adversely affect the immune system the eligibility criteria were designed to select a group of healthy, well-nourished children in order to avoid confounding factors. The selection criteria included the following:

Birth weight at least 3000 g

Weight at 3 months at least 5000 g for girls and 5500 g for boys

No serious illness before entry to the study

No blood transfusions before entry to study

The prospective controlled trial is such a well accepted paradigm that it does not in itself require explanation. It is however worthwhile elaborating briefly on the care with which the Control group was designed to be comparable to the Test group at the start of the trial and to be exposed to equivalent environmental influences. For these epidemiological reasons as well as logistic constraints the children had to come from a circumscribed area in a relatively homogeneous lower socio- economic group. Bonteheuwel, a city council housing estate, was chosen because it fulfilled these criteria, was close to the Red Cross War Memorial Childrens Hospital, and had Cape Town City Health Department clinic facilities within easy walking distance of all residents.

If children from disparate social backgrounds and residential areas had been studied, their exposure to sources of infection and perhaps their susceptibility to infection would have been heterogeneous. It was hoped that the choice of Bonteheuwel would minimize the differences in susceptibility and exposure to infection between children in the study.

Although enrollment took place over an extended period, approximately equal numbers of infants from each group were entered into the study each month. The intention was to avoid the bias that differential enrollment would have had on the exposure to infection experienced by the two groups.

Ascertainment

Mothers in Cape Town use a number of hospitals and maternity units to deliver their children. These facilities mail notification of birth to the primary health clinic serving the the area in which the mother resides. In Bonteheuwel, virtually all mothers deliver their children in one of the recognized health care facilities. Since the notification system works efficiently, the Bonteheuwel municipal health clinic has an accurate record of births in the area it serves. These notifications of birth were used to ascertain the children in the study.

Control and Test group allocation and "blinding"

Each month, from April 1983 to August 1983, the birth notifications for the previous month were obtained for Bonteheuwel and the name and address of the mother, her parity, any complications during pregnancy, the date of birth and sex and weight of the child were extracted.

Children were admitted monthly to the study and were allocated to their group, Test or Control, by a computer program that discarded children with a birth weight less than 3000 g and then performed a stratified match on two potentially confounding variables. These were the number of previous pregnancies of the mother, and birth weight. The reason for stratifying the infants according to their weight and the parity of the mother was that it was hoped that this would control, in the best available way, for susceptibility and exposure to infection. Weight was used as a proxy for nutritional and socio-economic status and parity as a proxy for number of possible infectious contacts.

The allocations were made before the children had been seen or the parents contacted. The parents, the study team and the laboratory staff were unaware of the group allocations until the end of the study and all laboratory investigations had been completed. Each child was allocated a unique code number by a computer program. The list of names, code numbers and Test/Control group assignment was kept by staff of Food and Nutritional Products so they could label the correct tins of milk formula for each child with his or her name.

Exclusions and drop-outs

Of the first 157 children allocated to the study, 2 were excluded because their haemoglobin was less than 9.0 g/dl and 6 parents declined the offer to participate. No children had medical reasons for exclusion such as a blood transfusion or serious illness. Of the 149 children that began in the trial, 10 mothers withdrew for personal and domestic reasons and 7 families moved away from the study area; 132 children completed the study. Details of the children that dropped out are tabulated in appendix 1 and completion rates for the Test and Control Groups are presented with the other results for the study.

Study size

Estimation of the number of subjects required to reach a meaningful conclusion from the study proved a difficult task. As will be shown below it was expected that a conclusive difference in iron status would be shown with a relatively small number of subjects. The possible benefits and risks of increased iron fortification that the study aimed to determine centered on immune function and susceptibility to infection. Since infection is the important factor from the point of view of the child, parent and health care system, the number of subjects was established so that a clinically important difference in infection rate would be detected with a high degree of probability.

There was little assistance to be gained from the literature in estimating the "normal" number of infections experienced by a group of normal infants. This is partly because there are few published studies[354, 472, 475] with adequate data, and partly because infectious morbidity is so dependent on social, economic and geographic factors that generalization from one community to another is often not valid.

The study with the best data for our planning purposes was found in the study of Mackay[314] made in the nineteen twenties. From the data given in her report it is possible to calculate that the incidence of infection in the *control* group was 4.16 per child per year and in the *iron* group it was 1.99 cases per year, the difference being 2.17 infections per child per annum. No study was found where the standard deviation of incidence of infection of normal infants could be estimated.

As a rough guide the following calculation was performed:

> If the trial intervention resulted in a "saving" of one infection per child per year, and the standard deviation of the incidence of infection was 2.5, the number required in each group to reach a statistical significance level of 5% with a type II error of 10% is:
> $N = ((1.96 + 1.28)*2.5/1)^2 = 65.6$

Ie the total sample size should be at least 132 subjects. It was decided to attempt to enroll 150 subjects, 75 in each group, as the errors in the above calculation could not be estimated. Given the rate at which subjects could be enrolled (10 per week), the duration over which the study could be held (2 years), and the capacities of the laboratories and study team, the target of 150 subjects was the practical limit within these resources.

Reason for fortification as an intervention strategy

The intervention strategies that were considered in the design of the study were:

i The use of intra-muscular iron dextran to prevent iron deficiency from occurring in a group of children

ii The use of supplementation with oral iron preparations

iii The use of fortification of a common food

The first strategy was rejected because intra-muscular iron dextran has not found general favour as an agent in preventing the development of iron deficiency in groups at risk. The reason for this general reluctance may be due to the nature of administration (it is a painful injection) and to fears about possible untoward side-effects of the drug. These include lingering doubts about the danger of inducing sarcomas, concern about the risk of increasing susceptibility to infection and concern about the possibility of idiosyncratic reactions[529]. The risks are small and these concerns are not sufficient to inhibit the use of intra-muscular iron dextran in treating established iron deficiency, but, where the

alternative exists of a painless, safe, effective and "transparent" method of prophylaxis of iron deficiency, the latter must be chosen[14].

The second strategy considered was that of iron supplementation. Oral iron supplementation is effective in preventing iron deficiency but poor compliance with prescribed drug regimes is a ubiquitous problem in medical practice. In view of previous experience in Cape Town it was considered that compliance with a prophylactic medicine would be low in spite of steps that could be taken to persuade mothers to administer the iron preparation as advised*.

The final strategy considered for the experiment was to fortify a common infant food with iron. This has the advantages of safety, ease of delivery and increased compliance[479].

One doubt that remained was of the efficacy of the intervention. The analysis that provided the decision in favour of this strategy is given in the following three sections.

It may be asked why, given the not inconsiderable problems involved in conducting this sort of field trial, an animal model was not used instead. There are 2 principal reasons. Firstly, since the effectiveness of a food fortification program depends on the delivery system, acceptability of the product and other cultural and logistic factors, such programs will always require verification by means of field trials. Secondly, experiments on animals have provided convincing evidence for disturbances of laboratory tests of immune function in severely iron deficient rats[281, 121]. It has not been possible however, to develop a suitable animal model for the incidence of common infections in infancy.

Reason for infant milk formula as vehicle

Various foods have been used as vehicles for fortification of iron and infant cereals and milk powders are routinely fortified. Extensive reviews of the subject have been published *eg* references (14, 94). Criteria identified by the World Health Organization for practical vehicles for iron fortification are that *"The vehicle should be one that is already consumed in adequate amounts by the people in need; one that is available for fortification in relatively few centres so that quality can be adequately controlled and monitored; one that is suitable for fortification on a large scale; and one that results in a product which is stable under extreme conditions of storage and does not alter the palatability of the food"*[415].

Infant milk formula meets these conditions since the health clinics of the City of Cape Town provide a milk powder at subsidized prices to families with infants and young children at a time when milk forms a major part of their diet.

* Kirsten G and Heese H de V, personal communication. Also see the comments of Stekel on page 189 of reference 479

Lactogen Full Protein was used for the trial as Food and Nutritional Products (Nestl) offered to supply standard and modified formulas free of charge and to assist in implementing the double blind protocol. The one important criterion in selecting the milk formula was that it should be based on cow's milk protein and not soya bean since such such preparations may have decreased iron absorption[104].

The commercial infant milk formula employed in the study for the Control group contains 37 mg of ascorbic acid per 100 g formula and 8.3 mg Fe (as ferrous sulfate) per 100 g of powder. The reconstituted formula contained 12 mg Fe/ l and 53 mg ascorbic acid /l. The molar ratio of ascorbic acid to iron is 1.41.

With 40 mg Fe/ 100 g, the Test formula contained 58 mg Fe /l of reconstituted milk and the molar ratio of ascorbic acid to iron was 0.29.

The reasons for using ferrous sulfate as fortificant, for choosing the fortification levels of 8.3 and 40 mg Fe/100 g, and for not increasing the ascorbic acid fortification in the Test formula are given in the following paragraphs.

Reason for ferrous sulfate as fortificant

Forms of iron employed for fortification of infant milk formula include ferrous sulfate, ferrous fumarate, ferrous gluconate and ferrous lactate. Ferrous sulfate is cheap and relatively easily absorbed (compared to other non-haem iron products), but is quite chemically reactive and can impair storage of formula which is not in a sealed container[237]. However, ferrous sulfate is successfully used in many commercially available infant milk formulas and most studies of absorption of iron from infant milk formulas have employed it. The decision to employ it in the trial was not difficult.

Reason for choosing 8.3 mg Fe/100g as the level of fortification for the Control group

(a) Ethical considerations

Having decided on the structure of the experiment, the levels of iron in the milk offered to the Control and Test Groups had to be set. The statistical power of the study to detect differences in iron status, immune function and incidence of infection would have been improved if a (third) group had been given unfortified formula, but this would not have been ethical in view of the known risks of iron deficiency.

(b) Considerations of ability to generalize conclusions

To assess the relative risks and benefits of increasing the concentration of iron in the Test group, the Control group had to have a conventional formula. The milk offered to the Control group therefore was fortified with the amount of iron present in commercially available Lactogen Full Protein. An additional reason for using a commercially available milk formula for the Control group was that such formulas are sold at subsidized prices at municipal health clinics. The results of the study would therefore be readily generalizable to the community.

(c) Estimations of effect on iron status

(i) Experience in Cape Town

In Heideveld, a similar community to that chosen for the present study, Kirsten *et al*[241] found a mean haemoglobin level of 10.5 g/dl in 1 year old infants. These were all healthy infants who regularly attended a municipal primary health care clinic where infant milk formula (fortified with iron and ascorbic acid) was available at a subsidized price. With the present study's protocol it was expected that the selection criteria would exclude a certain proportion of children at higher risk for developing iron deficiency. In addition, making fortified milk freely available would tend to raise the average level of iron nutrition in the Control group from what it would have been with no intervention. These 2 effects could not be quantified, but it was expected that the mean haemoglobin of the Control group would be about 1 g/dl higher than that found in Heideveld by Kirsten *et al*.

In effect, a "target" level of haemoglobin for the Control group at 1 year was set at 11.5 g/dl. Some uncertainties in predicting this target are discussed in the following 2 sections.

(ii) Similar international studies

The results of several studies of iron fortification are discussed in chapter 2 and summarized in table 2.16. This shows that infants fed on similar milk formulas had mean haemoglobin levels that varied from 11.9 g/dl to about 12.9 g/dl. Infants from Finland had higher mean haemoglobin levels and a lower incidence of iron deficiency even when the group given unfortified formula is compared with other groups given the formula with the largest amount of iron and highest ratio of ascorbic acid to iron*. Prediction of the results of a program of iron fortification may thus be made in qualitative rather than quantitative terms. It was further concluded in chapter 2 that infants in lower socio-economic communities might benefit from increases in the iron fortification of infant milk formula.

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* This statement assumes the qualifications expressed in the review in chapter 2. Some mean haemoglobin levels were obtained from graphs rather than from text in the papers cited and criteria for defining iron deficiency differ. These reservations notwithstanding, the data are adequate to make the point that iron nutrition depends on the total diet rather than just one component.

(iii) Effects of genetic, socio-economic class and cultural factors

The effect of the prevalence of individuals with thalassemia or an haemoglobinopathy on the means of measures of iron status in populations is likely to be small and can be disregarded in the calculations used in planning studies of iron fortification. Justification for this assertion rests on studies of patients at the Red Cross War Memorial Childrens Hospital by Bird *et al*[41]. These workers found that 10.4% of "coloured" children with a mean cell volume less than 60% had a haemoglobinopathy or a thalassemia. The order of magnitude of the effect that such conditions might have on measures of iron status in the population may be estimated. If 1% of 1 year old children have a mean cell volume less than 60 fl, then 1 in 10 of these infants might be misdiagnosed as iron deficient. Ie about 0.1% might have either a thalassaemia syndrome or an haemoglobinopathy but be misclassified as iron deficient. It can thus be concluded that measures of the iron status of a population should not be affected to a significant extent by such conditions.

Reasons for choosing 40 mg Fe/100g as the level of fortification for the Test group and not altering the concentration of ascorbic acid.

(a) Optimal iron status

The mean haemoglobin level of a well nourished group of Canadian infants was 12.2 g/dl[52] and the mean haemoglobin level of one year old infants who do not have evidence of iron deficiency has been reported to be 12.5 g/dl by Dallman *et al*[124] and 12.7 g/dl by Saarinen and Siimes[435]. These results indicate the mean haemoglobin level in communities with optimal iron status. It was felt that, with excellent iron fortification, the Test group could achieve a mean haemoglobin concentration of 12.0 to 12.5 g/dl.

(b) Considerations of iron absorption and ascorbic acid

(i) Recommendations of the World Health Organization

The decision to set the level of fortification of iron at 40 mg Fe/100 formula was made on the basis of an "educated guess" since the literature had no specific guidelines. Dallman *et al*[125] have shown that the percentage absorption of iron from infant milk formula decreases as the dose increases, but that total absorption continues to increase, albeit at a diminishing rate. It was also known that iron absorption is enhanced by ascorbic acid and that the World Health Organization had recommended that "*it would seem advisable for infant formulas to include both an absorbable iron salt and ascorbic acid with an iron:ascorbic acid ratio of at least 1:10*"[415]. Assuming this ratio is mass:mass it is equivalent to a molar ratio of ascorbic acid to iron of 3.17. The WHO recommendation was based on data from absorption studies on cereals which have inhibitory effects on iron absorption. The recommended ratio of ascorbic acid to iron is therefore probably excessive for cow's milk based formulas.

(ii) Data on the association of ascorbic acid with iron absorption

Chapter 2 reviewed several studies which have shown that ascorbic acid improves the absorption of iron from infant milk formula. In 3 studies of a formula with 15 mg iron per litre Dallman *et al*[125] found that 100 mg ascorbic acid improved the absorption of iron from 5.5%, 4.0% and 4.9% to 12.0%, 10.7% and 11.3% (geometric means). Subsequent work has confirmed this and their results are summarized in table 3.1 and figure 3.1. (This does not include the data from Dallman *et al* since it seems that Stekel included it in his report.) The table and figure show the relationship between the molar ratio of ascorbic acid to iron and percentage absorption of iron. Each study confirms the increase in iron absorption with increased ascorbic acid:iron ratio.

The next section presents an analysis of an attempt to predict the percentage iron absorption from lines fitted by the least squares technique to the published data.

Table 3.1 Iron absorption from infant milk formulas

STUDY	IRON (mg/l)	ASCORBIC ACID (mg/l)	[Fe]/[AA] mol/mol	ABSORPTION %	Std Dev	N
2	15.0	0	0.00	2.9	4.3	23
2	15.0	0	0.00	2.9	3.6	12
2	15.0	0	0.00	2.9	3.2	12
2	15.0	0	0.00	3.0	4.7	10
2	10.0	0	0.00	3.1	5.3	23
2	15.0	0	0.00	3.4	3.4	13
2	15.0	0	0.00	3.6	3.8	13
2	10.0	0	0.00	3.7	6.9	35
2	10.0	0	0.00	4.0	6.7	15
2	15.0	0	0.00	4.4	5.2	13
2	15.0	0	0.00	4.4	5.7	39
2	15.0	0	0.00	4.7	4.5	11
2	15.0	0	0.00	4.7	4.3	12
2	10.0	0	0.00	5.1	6.2	16
2	12.7	0	0.00	7.2	9.4	12
2	19.0	10	0.17	3.4	4.2	19
2	15.0	25	0.53	3.4	3.9	12
2	19.0	40	0.67	8.8	8.1	12
2	15.0	50	1.06	5.5	6.3	12
2	12.0	55	1.45	10.3	12.2	22
2	15.0	75	1.59	8.3	6.1	20
2	12.7	80	2.00	19.6	14.5	12
2	12.7	80	2.00	21.9	28.9	10
2	20.0	133	2.11	5.3	8.25	12
2	20.0	133	2.11	6.9	13.6	13
2	15.0	100	2.11	7.9	8.8	13
2	15.0	100	2.11	10.2	10.1	39
2	15.0	100	2.11	11.3	9.1	13
2	10.0	100	3.17	5.9	12.7	14
2	15.0	200	4.23	7.9	9.7	12
2	20.0	267	4.23	19.5	27.9	7
2	12.7	254	6.34	55.7	30.9	10
2	15.0	400	8.45	6.1	5.9	10
2	15.0	800	16.91	11.8	11.8	11

NOTES

1: Derman DP, Bothwell TH, Torrance JD, Bezwoda WR, Charlton RW, Mayet FGH. Importance of ascorbic acid in the absorption of iron from infant foods. Scandinavian Journal of Haematology 1980; 25: 193-201
Arithmetic means and standard deviations reported

2: Stekel A, Olivares M, Pizarro F, Chadud P, Lopez I, Amar M. Absorption of fortification iron from milk formulas in infants. American Journal of Clinical Nutrition 1986; 43: 917-922
Geometric means and standard deviations reported

3: Gillooly M, Torrance JD, MacPhail AP, Mills W, Mayet F. The relative effect of ascorbic acid on iron absorption from soy-based and milk based infant formulas. American Journal of Clinical Nutrition 1984; 40: 522-527
Geometric means and ranges reported; the mean of the range is tabulated in the Std Dev column

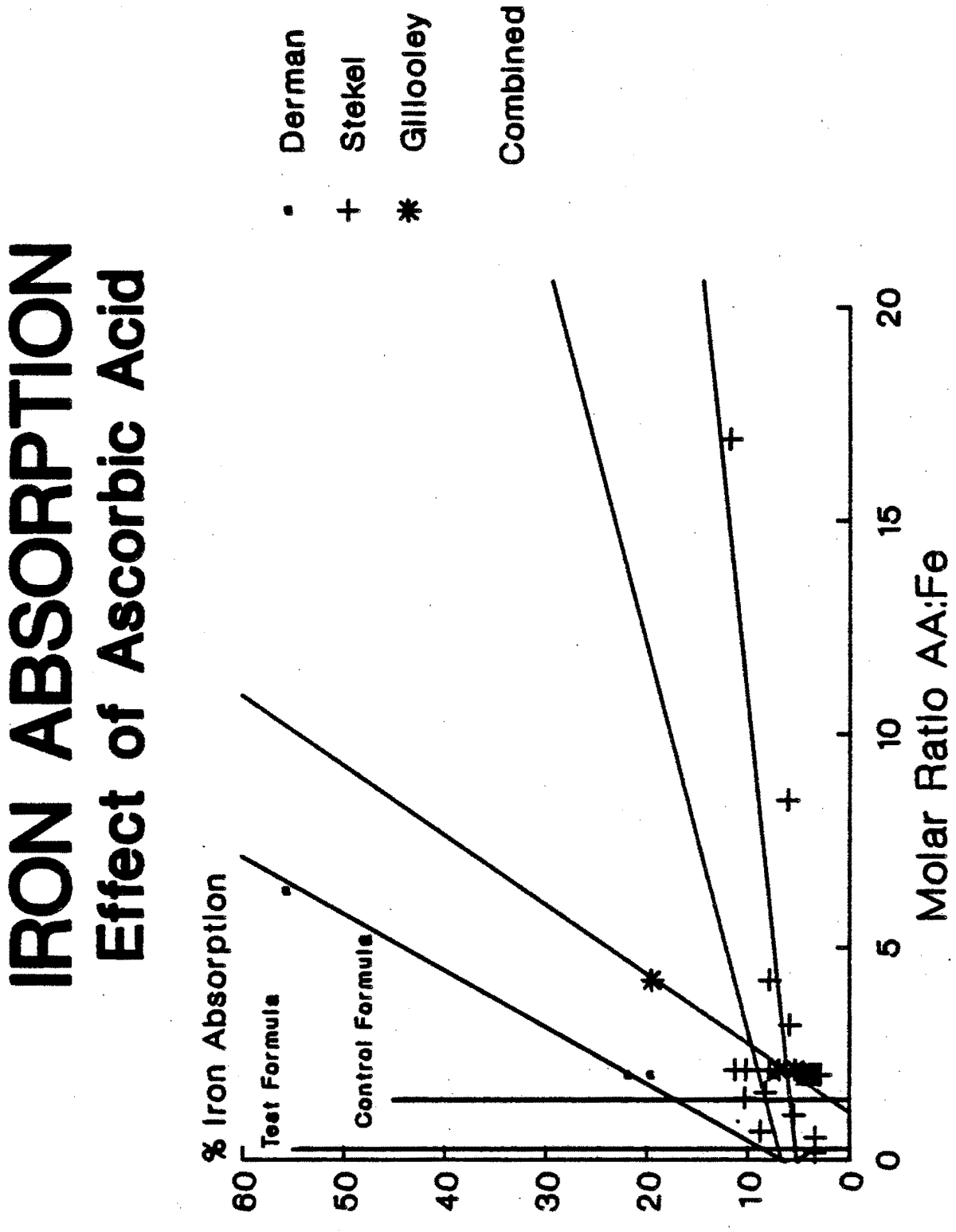

Figure 3.1 The relation between percentage iron absorption and molar ratio of ascorbic acid to iron in infant milk formulas from the studies of Derman *et al*, Stekel *et al* and Gillooly *et al*.

(iii) Prediction of % iron absorption from regression analysis

Linear regression analysis was performed on the data in table 3.1 in order to estimate the bioavailability of iron in the infant milk formulas employed in the present study. The results are summarized in table 3.2 and figure 3.1. Figure 3.1 shows the regression lines that were fitted by least squares to the 3 sets of investigations. To facilitate relating this analysis to the present study vertical lines in figure 3.1 indicate the ratio of ascorbic acid to iron in the Control and Test formulas.

For example, from the data of Derman *et al* it was calculated by this technique that the Control group would absorb 17% of the iron present in the milk and the Test group would absorb 8.37%. If the infants ingested on average 500 ml of formula per day (a conservative amount), they would be receiving 5.98 mg and 28.83 mg iron respectively. The predicted amounts absorbed are 1.02 mg per day and 2.41 mg per day.

This analysis applied to the data from the other studies gave estimates of total iron absorption varying from an impossible -1.57 mg/day to 1.94 mg/day for all studies combined. It may be concluded that this technique does not allow an accurate prediction to be made of the amount of available iron in the Test formula. This is not surprising since the simple linear regression analysis ignores factors such as the iron status and age of the subjects, the presence or absence of other facilitators and inhibitors of iron absorption and the size of the meal. Linear regression also implies a simple (and improbable) chemical relationship between ascorbic acid and bioavailable iron in a complex environment.

Table 3.2 Prediction of iron absorption from regression analysis

			ABSORPTION Percentage		ABSORPTION from 500 ml formula (mg)	
STUDY	Const	Coeff	Control	Test	Control	Test
Derman	6.09	7.74	17.00	8.37	1.02	2.41
Stekel	4.92	0.46	5.58	5.06	0.33	1.46
Gillooly	-7.30	6.34	1.63	-5.44	0.10	-1.57
Combined	6.40	1.14	8.00	6.74	0.48	1.94

NOTE Least squares analysis was applied to data in the published studies (see text for references). Percentage absorption of iron was modeled as a linear function of the molar ratio of ascorbic acid to iron. The linear regression coefficients were then used to predict the percentage and actual amounts of iron absorbed from 500 ml of the Test and Control infant milk formulas. The Control formula had a molar ratio of ascorbic acid to iron of 1.41 and contained 11.96 mg Fe per liter when reconstituted. The values for the Test formula were 0.29 and 57.66 respectively.

(iv) Estimation of iron absorption from comparison with therapy

The quantity of iron offered to the Test group was therefore assessed in a different way. The accepted dose for therapeutic administration of ferrous sulfate in clinical iron deficiency is 6 mg/kg/day at the Red Cross War Memorial Childrens Hospital[111]. Since the Test milk had 40 mg Fe /100 g, an infant would be provided with 5.8 mg/100 ml of reconstituted formula. If the infant ingests 100 ml formula/kg body mass, he or she is provided with 100% of the therapeutic dose of iron, and with an intake of 50 ml/kg, 50% of the therapeutic dose of iron is provided. Thus, a conservative estimate of the amount of iron provided by the Test formula was at least 50% of the therapeutic dose.

From these considerations it was felt that infants in the Test group would have sufficient absorbable iron to ensure that their iron status would be replete even if they were taking less than half the formula prescribed.

(v) Calculation of % iron absorption from target iron status

A third way of assessing the suitability of the level of 40mg of Fe/100 g is to estimate the extra iron required by the Test group to increase their average haemoglobin by 1 g/dl but maintain equal iron stores. Assuming that the average well nourished infant weighs 10 kg at the age of 1 year, has a blood volume of 80 ml/kg and has a haemoglobin level 1 g/dl greater than a similar control infant, then he or she will have an additional 30 mg of iron in haemoglobin. Averaged over 9 months the extra daily absorption is 0.11 mg Fe/day. If the child is taking (a conservative estimate of) 500 ml formula per day this extra iron should come from the 58/2 = 28.5 mg Fe/day in the milk formula. The increase in total absorption required is therefore 0.11/28.5 = 0.38%. It seemed not unreasonable to expect a 382% increase in iron fortification to cause at least a 0.4% increase in absorption*.

A similar calculation may be made for changes in total body iron by using plasma ferritin to estimate iron stores. This would confirm the point that a small increase in iron absorption was expected from a very large increase in iron fortification. The method is based on such controversial assumptions about the quantitative relationship of plasma ferritin to iron stores in infancy that the exercise is not not worth repeating here.

(vi) Consideration of effect of ferrous sulfate on milk formula

It may be asked why the level of fortification was set at 40 mg Fe/100 g and not 30 mg Fe/100 g or 50 mg/100 g. The decision was essentially arbitrary, but was based on discussions with the manufacturer and was as high as was felt possible without compromising the quality of the milk with respect to colour, taste and shelf life. It is of interest to note that a survey of 53 infant milk formulas in the USA,

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* Calculated from: 100*(40-8.3)/8.3 = 382.

Japan and Europe found the iron content to range from 0.06 mg Fe/l to 58.5 mg Fe/l[310]. The latter concentration was found in 6 formulas for sale in the USA and is essentially equivalent to the 57.7 mg Fe/l in the Test formula.

(vii) Number of experimental variables and interpretation of results

A final remark on the decision not to increase ascorbic acid is necessary. The analysis above provided grounds for expecting that manipulation of the level of iron in the milk formula would result in a sufficiently large differentiation between the iron status of the Control and Test groups.

An additional factor that argued against the simultaneous manipulation of iron and ascorbic acid was the desire for a clear interpretation of results. It was felt that interpretation of results from the experiment would be clearer if the only experimental variable was the level of iron fortification. If the Test milk had differed from the Control formula with respect to both iron and ascorbic acid there would have been 2 experimental variables and it would have been difficult to assign outcome effects to the one or the other.

Quantity of milk provided for infants

In order to ensure that all infants ingested their full milk dietary requirements from the age of three months to one year, parents were given a generous supply of the milk formula *viz* 180 ml/kg/day or a maximum of 1.5 ml per day. To discourage family members from consuming infant formula some studies have used acidified milk powder[479]. The feasibility of this strategy was explored with the help of health care workers familiar with the community. The common consensus was that acidified milk would be unpopular with mothers. Consequently, a conventional infant milk formula was selected and mothers were given more than the average infant could have ingested.

The milk was issued at the regular examination that each child underwent every 3 weeks. Mothers who were breast feeding (partly or wholly) were encouraged to continue nursing their infants, but were given the appropriate milk formula if they asked for it.

The study team attempted to promote the practice of breast feeding in the community and it was felt that, if breast feeding mothers were not issued with the milk formula, this might discourage mothers from nursing and correctly reporting their infant's feeding habits. Similar reasoning led to the decision not to disqualify breast fed infants from participation in the study.

Reasons for entry at 3 months

The greatest demand for iron is between 3 and 24 months and the highest prevalence of iron deficiency anaemia in term infants is in the second year of life. Many programs of iron supplementation or fortification for normal infants begin around the third month and continue until 9 to 15 months of age. Three months was a convenient time for entry into the study since simple criteria could be employed to select the healthy, well nourished infants required for the study and this time marked the start of increased iron requirements.

Reasons for follow up period of 9 months

The period between 3 and 12 months is a time when infant milk formula provides the bulk of many childrens' diets and thus provides an excellent opportunity for food fortification programs. A prime requirement of the study was that children be followed for a sufficient length of time for the incidence of infection to be determined with confidence. Follow up to the age of 1 year satisfied the requirements of being able to determine the effect of the increased iron on both iron status and incidence of infection.

Selection of laboratory tests

(i) Volume of blood

For ethical reasons it was decided that not more than 10 ml of blood would be taken from the children on each of the 2 occasions they were to be tested. This constrained the number of laboratory tests that could be performed on each sample and the reasons for excluding certain desirable tests are discussed in the following sections.

(ii) Measures of iron status

The study employed the full blood count (with differential count), plasma ferritin* and red cell zinc protoporphyrin as indicators of iron status. Since micro methods were not available at the time, it was decided that plasma iron, total iron binding capacity and percentage saturation of transferrin would not be measured. Characterization of the iron status of the subjects in the study would have been more complete had these variables been measured. Cook and Finch[105] have recommended that the iron status of populations be assessed by means of the haemoglobin, percentage saturation of transferrin, free erythrocyte protoporphyrin and serum ferritin. However, plasma iron, total iron binding capacity and percentage saturation of transferrin have not proved to be very useful diagnostic tools in infants*.

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* For reasons explained below blood was collected into heparinized tubes and measurements were thus made on plasma rather than serum.

* Heese H de V.: personal communication

Both transferrin and serum iron have wide normal ranges in 1 year old infants with no other evidence of iron deficiency[124, 527]. Dallman[120] has discussed the analytical and biological variations of laboratory tests and the implications for diagnosis of anaemia and iron deficiency. Conceptually, the transferrin saturation would seem to be a most useful indicator of iron deficiency. However, since it is calculated by dividing the concentration of serum iron by total iron binding capacity and multiplying by 100, it will reflect the laboratory and biological variations of both serum iron and total iron binding capacity. (Unfortunately, Dallman did not report the coefficient of variation for transferrin saturation.) According to Dallman, serum iron has a biological coefficient of variation of about 28% while the coefficient of variation for total iron binding capacity is from 5 to 14%. The analytic and biological variations are generally lower for the other indicators of iron status.

Dallman *et al*[125] advised that haemoglobin, mean cell volume and serum ferritin or free erythrocyte protoporphyrin be used in population surveys of iron status. The relative utility of percentage saturation of transferrin was not discussed in this paper but presumably was felt to be lower than that of serum ferritin or free erythrocyte protoporphyrin.

Cook *et al*[107] studied 326 adults to establish normal ranges for serum ferritin. They found the within-subject variation in serum iron and percentage saturation of transferrin to be as great as the variation within the group. The total iron binding capacity and serum ferritin were more consistent.

Derman *et al*[135] studied children between the age of 1 and 6 years of age and reported statistically significant correlations of serum ferritin with age, haemoglobin, percentage saturation of transferrin and serum iron. The low correlation coefficients for serum iron and percentage saturation of transferrin (all were less than 45%) indicate that these measure are largely independent of serum ferritin. The authors concluded that *"the accuracy of detection of individuals with Fe-deficiency anaemia in a population is substantially improved if two independent measures of Fe deficiency are used in combination".*

Hershko *et al*[197] assessed the iron status of a group of rural children and reported the sensitivities of serum ferritin and percentage saturation of transferrin in detecting individuals with iron deficiency anaemia defined as haemoglobin less than 11.0 g/dl and red cell protoporphyrin greater than 40 ug/dl. Of 23 individuals with iron deficiency anaemia, serum ferritin was less than 16 g/l in 19 (83%) and saturation of transferrin was less than 16% in 20 (91%). It would have been useful if the sensitivity of serum ferritin combined with percentage saturation of transferrin had been reported. It would then have been possible to assess if the 2 tests are equivalent or complementary in diagnosing iron deficiency anaemia.

Dallman *et al*[123] studied the ability of several tests to predict a response of at least 1g/dl in the haemoglobin level of 1 year old infants after a therapeutic trial of iron. The tests were the mean cell volume, percentage saturation of transferrin, free erythrocyte protoporphyrin and serum ferritin. None could reliably predict a response in haemoglobin levels and the tests were partly complementary. (It is of interest that about 10% of the responders were not identified by any test.) The authors did not compare the relative utility of the tests with each other or discuss what would have been lost if 1 test had been omitted.

Cook and Finch[105] have stated that the percentage saturation of transferrin is roughly equivalent to the free erythrocyte protoporphyrin in assessing iron status.

It thus seemed that there would be a minimal opportunity cost in foregoing the iron, total iron binding capacity and transferrin saturation tests in the present study and that iron status would be fairly well characterized by the use of the full blood count, plasma ferritin and red cell zinc protoporphyrin.

(iii) Measures of immune function

In order to characterize the immune status of the subjects a battery of immune function tests were applied.

The numbers and proportions of B lymphocytes and subtypes of T lymphocytes were determined with antibody techniques. An incidental benefit of the study was the ability to determine normal ranges for the laboratory.

Leukocyte function was measured in half of the children with the response of lymphocytes to stimulation with phytohaemaglutinin. Samples from the other children were subjected to a neutrophil bactericidal assay. Each child had the same test at both 3 months and 12 months in order to minimize the effects of inter-subject variation. A side benefit again of the study was the ability to describe normal ranges for these tests in infants.

The response to immunization with polio and tetanus was determined. These measured different arms of the immune system since the polio vaccine was live virus administered orally and the tetanus vaccine was a toxiod administered intramuscularly.

Delayed cutaneous hypersensitivity was measured with the Mantoux test and candida antigen test. These tests indicate both exposure to the agent inducing immunity and the ability of cell mediated immunity to respond to a challenge.

(iv) Measures of viral carriage rate

If iron status influences immune function or if iron fortification affects bowel flora it might be expected that the carriage rate of viruses might differ between the 2 study groups. For this reason fecal material was tested at both 3 and 12 months and nasopharyngeal swabs were tested at 12 months. An incidental benefit was the ability to describe the pattern of viral infection in healthy infants in the community; an opportunity that does not present itself often to laboratories which receive most of their specimens from hospital patients.

(v) Sociological characterization

Data was collected to define certain social and economic characteristics of the families in order to place the study into its social context. This included information on age, education marital status and occupation of parents, living conditions and numbers of children in the family.

Project team

The project was conducted by myself, then a registrar in the Department of Paediatrics and Child Health. I was fortunate to have the assistance of Mrs D Phillips, a health educator, as my assistant for the duration of the trial. Mrs Phillips traced subjects, coordinated the project with the operations of the Bonteheuwel Health Clinic, ensured that subjects kept their appointments, assisted in the examination room, made innumerable home visits to participating families, helped trace patient files in hospital record departments, administered the questionnaires, and assisted in the entry of data and its preliminary analysis.

The entire project was directed by Professor H de V Heese who initiated the study. Professor DW Beatty provided guidance and advice at all stages. Where I have been especially indebted to others for particular parts of this undertaking I have acknowledged this in the text. The assistance of many other individuals and institutions is acknowledged at the beginning of this thesis.

Study Design - Social survey of participating families

Introduction

In order to place the main study in its social context, a survey was made of the participating families to supplement the limited demographic data gathered at the examinations of each child. Restricted resources did not allow the detailed investigation that a sociologist would have recommended, but the survey did allow the community of Bonteheuwel to be described with a measure of objectivity.

Acknowledgements

The questionnaire was designed in consultation with Mrs D Lynch, social worker.

Protocol

Each family was visited by the research assistant and asked to complete a questionnaire. The visits took place after the family had been participating for at least 3 months. The data collected was:

Date of each immunization

Result of WR test on mother during pregnancy

Place of birth

The names of all hospitals and doctors from whom health care had been sought

The caretaker of the child during the day and at night

The names of all siblings

The type of housing: council house or shack

Whether the house belonged to the family or was rented

Whether the family was lodging with in-laws, or with others

The mother's religion

Whether there was a television set in the house

The ages of the child's parents

The names of any household members with pica

The names of household members with tuberculosis

The data that was collected on entry to the study included:

The marital status of the mother

The educational attainments of the mother and of the father

The occupations of the parents

The principal source of income for the family and whether this was regular or irregular

Whether the family was living alone or with others*

Whether the child was cared for by one or both parents

The number of adults in the house

The number of siblings

The number of rooms used for sleeping

Study Design - Survey of infant feeding practices

Mothers attending the immunization clinic at Bonteheuwel were questioned on their infant feeding practices. The survey was performed over three weeks on two occasions; in April 1983 before the principal study commenced and again in October 1983 when the study was well under way. The data collection sheets are shown in appendix 6. The study design and results are given in chapter 5.

Ethical Considerations

Introduction

Research involving children and infants often poses difficult ethical problems of consent, risk versus benefit, rights of the individual and society, review and approval by independent committees, and the distinction between and specific difficulties of therapeutic and non-therapeutic research. As medical ethics has become a topical subject in recent years, the researcher can find in the literature much of the guidance required [393, 248, 294, 178, 4, 10, 303, 1, 82, 411, 467, 464, 495].

The Medical Research Council stipulates that any research worker who receives its support should adhere to its document *"The Ethical Code and Practice for Clinical Investigations on Human Beings"*[471]. The University of Cape Town has requirements for review of projects as described in the next section.

The non-therapeutic aspects of the research reported in this thesis involved no risk or discomfort to subjects or their families. The research that involved injections and blood sampling was justified by the therapeutic benefits obtained in comparison with the minimal risks and minor procedures involved. Written consent was obtained from parents before admission to the trial, but parents were free to withdraw at any stage from the study. Two did in fact do so shortly before their children were due for their second blood sample and delayed hypersensitivity skin test. Bonteheuwel is a small community and every family in the trial was friendly with several other participants. One may speculate that if there had been general dissatisfaction with the study methods, the dropout rate would have been much greater.

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* This question was poorly worded in the entry examination and was rephrased for the social survey

The project thus complied with the formal ethical requirements and attempted to meet the informal ethical requirements of professional peers and the local community.

Research and Ethics Committee approval

A requirement of the University of Cape Town for all studies under its auspices which involve human subjects is that the protocols should be approved by the independent Ethical Review and Research Committee in accordance with the Helsinki and Tokyo Declarations of the World Medical Assembly. One requirement of the committee is that fully informed consent be obtained from the subjects, or, as in this case, from their legal guardians. The present study adhered to these requirements.

Copies of the authorizations from the Ethics and Research Committee are included in appendix 2.

Parental consent

Signed consent was obtained from the mother or father of each child. There was one exception in the case of an orphan where consent was obtained from the legal guardian and foster mother. The form that parents were asked to sign is included in appendix 6

The promotion of breast feeding

It was recognized that there was a possibility that the design of the study might create the impression in the community that formula feeding was to be preferred to breast feeding. The precautions that were taken to avoid this are detailed in the section on the design of the main study.

It was considered important to document possible effects of the study on infant feeding practices in the community. The surveys that were made in order to do this are described in chapter 5.

Potential side effects of iron fortification

Increased susceptibility to infection

The literature review in chapter 2 cited a number of studies that have suggested a role for excess iron in promoting infection. The conclusion was that, although there was a theoretical risk, there is no clinical or experimental evidence to implicate iron fortification of infant milk formula in increasing susceptibility to infection. However, no studies have conclusively documented the safety of such formulas. It was hoped that the study would provide such evidence. The steps taken to accomplish are described in the clinical methods section below.

Decreased zinc absorption

The zinc status of the infants was determined because iron has been reported to interfere with zinc absorption[469] and zinc deficiency can impair growth[183] and immune function[33].

Gastrointestinal symptoms

It was noted in chapter 2 that there was a lingering concern that iron fortification might be a cause of diarrhoea, constipation, colic and fussiness. For this reason, the caretakers of the infants were specifically asked at their first 3 or 4 visits if they had noticed any such symptoms.

Anaemia

Iron fortified milk has been thought to lead to anaemia in small premature infants who are deficient in vitamin E[522]. The postulated mechanism is promotion of lipid peroxidation by iron in the face of diminished protection of cell membranes by vitamin E leading to increased haemolysis. This phenomenon has not been described in full term infants.

As premature infants are particularly at risk for vitamin E deficiency for the first few weeks of life, it was considered unlikely that the levels of iron fortification used in the present trial would constitute any risk of haemolysis in well nourished infants at the age of three months.

Iron overload

The risk of increasing the prevalence of iron overload was reviewed in chapter 2. The consensus in the literature is that iron fortification poses little or no risk to infants.

Painful procedures and blood sampling in infants

The main study involved the taking of a blood specimen and the administration of two intracutaneous injections to healthy infants at the ages of 3 and 12 months. It was decided on ethical grounds to limit attempts at venesection to 2 skin punctures.

In the main study, the tests that were made, particularly the full blood count, were judged to be beneficial to the infants in that they provided a screening for disease. In an ideal situation with unlimited resources it would not be unreasonable to do such screening routinely.

Useful guidelines were found in a statement by the Medical Research Council of Great Britain[393]. The authors state:

> *"... it is clearly within the competence of a parent or guardian of a child to give permission for procedures intended to benefit that child when he is not old or intelligent enough to be able himself to give a valid consent. ... preventives are given to people who are not, at the moment, suffering from the relevant illness. But the ethical and legal considerations are the same as those that govern the introduction of a new treatment."*

Treatment of Iron Deficient Infants

Infants discovered to be iron deficient on entry to the study (2 infants), incidentally during the study (2 infants) and on discharge from the study (57 infants) were treated with oral iron supplementation for 3 months.

Clinical methods and data collection

The data collection forms used during the study are shown in appendix 6. These and the full tabulation of statistical analyses list the clinical and laboratory variables studied for each child. The sections below note methods used where this is important to establish the validity, accuracy or precision of the variable concerned.

Age

At each examination the age of each child was recorded in decimals of a year as recommended by Tanner *et al*[491], using their table to calculate the ages. For analysis of ages at entry to and exit from the study the age was calculated from the appropriate dates with an accuracy of one day. These dates were also used to determine the precise duration each child was under observation, and hence, an accurate measure of the incidence of infection.

Weight

Each occasion an infant was examined he or she was weighed on an Seca infant scale. The same scale was employed throughout the study and it was periodically checked with a standard weight. For analytic purposes the first and last recordings were used to establish weight gain during the study period.

Standard deviation score for weight

A computer program performed a linear interpolation for both weight and age to estimate the standard deviation score for weight (SD-W score) from the NCHS growth tables.

Length

The length of an infant was measured on an "infantometer", a board with a fixed foot-stop and a headboard that moved along a scale marked in centimeters and millimeters. To obtain a reproducible result it was necessary for both team members together to position the infant and adjust the headboard.

Standard deviation score for length

The standard deviation score for length (SD-L score) was measured from the NCHS tables by a method similar to that used for SD-W score.

Skull circumference

Skull circumference was measured with a tape measure around the forehead and over the inion.

Diagnosis of infection

The incidence of infection in the children was probably the most important information to be acquired by the study. Hence, a special effort was made to determine the infection rate as precisely as possible. In order to do this, and to facilitate comparison with other studies an *operational* definition was adopted for infection. A set of criteria were specified from which all diagnostic decisions were made as objectively as possible. For purposes of analysis, infections were graded according to severity and classed in one of eight groups according to organ system affected *viz* conjunctivitis, gastro-enteritis, respiratory tract infection, pyoderma, oral thrush and *"other"*.

This section describes first how episodes of illness were ascertained and then describes the sequence of decisions that had to be reached in order to:

1) Diagnose the reason for a visit to a medical practitioner as an infection.
2) Count the infection as a discrete episode of morbidity.
3) Grade the severity of the infection.
4) Group the infection by organ system.

This section then concludes with a discussion of the difficulties in determining infection rates precisely, accurately and in a manner which facilitates comparison with other studies of incidence of infection.

Ascertainment of episodes of infection

Each child was examined and a pertinent history was taken every three weeks throughout the study period. Any infection discovered was noted in the child's research folder.

A member of the study team was available for consultation by the study families every weekday morning at the Bonteheuwel Health Clinic in addition to their regular three weekly appointment. Parents were also encouraged to use other available health care facilities if they so wished. Some mothers and fathers thus consulted private practitioners, municipal health clinics or provincial hospitals when their child was ill. Parents were encouraged to report each episode of illness as soon as possible after consultation with another source of health care and the details were noted.

The diagnosis of these events was not made from the history obtained from parents but from the original records of the private practitioner, clinic or hospital. At the end of the study, I drew up a list of all clinics, hospitals and private practitioners that had been used as a source of medical care for any subject. At each clinic and hospital thus indentified, the patient index was searched to see if any

subject* had been registered as a patient. I then scrutinized the medical records for all registered subjects and noted any infections. All general practitioners that had treated any subject were contacted by post and by telephone and asked to assist in tracing episodes of infections in all the study patients. I requested that I be given the date, diagnosis and treatment prescribed for all infections suffered by study subjects. From these sources and my own files a single consolidated record of episodes of infection was constructed for each subject.

Diagnosis

Diagnosis of an infection, whether by myself or by another doctor, involved "clinical judgment" applied to the textbook criteria of history and physical examination. When it seemed clinically indicated, I (and some hospital based doctors) sought confirmation of the infection by bacteriological and fungal culture.

Infections were counted according to the following criteria:

Pyoderma (including impetigo, folliculitis and abscesses) was the only type of skin infection counted.

Prolonged infections may have been counted more than once, but only if diagnosed on consultations at least 15 days apart.

Recurrent wheezing was counted as one respiratory tract infection for every 10 episodes of lower airways obstruction.

Concurrent symptoms were counted as multiple infections, except:

> **Diarrhea** with systemic and/or respiratory symptoms was enumerated as one gastro-intestinal infection.
>
> **Conjunctivitis** with respiratory symptoms was counted as one respiratory tract infection.
>
> **Respiratory tract symptoms** together with otitis media was counted as one respiratory tract infection.

When there was more than one pathogenic organism isolated from one specimen, each organism was counted separately. The episode was counted as one infection.

In cases of otorrhoea where multiple bacterial species were isolated, and each was considered to be probably non-pathogenic, the organism was coded as "mixed" but the incident was counted as 1 infection.

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* Including those who had NOT volunteered that they had been there. This was necessary because parents sometimes omitted to tell us that they had consulted other sources of health care.

Grading of infection

Grading of infection was made on "clinical judgment".

Grade 1 Minimal disease; no treatment indicated.

Grade 2 Moderate disease; treatment warranted.

Grade 3 Severe disease; hospital treatment required.

Grouping of infection

Infections were grouped according to the organ systems for final analysis. Only grade 2 and 3 infections were grouped.

Oral candidiasis was the only infection grouped on pathogen. Aphthous ulcers were grouped under respiratory tract infections.

Eye infections were limited to bacterial conjunctivitis. Pharyngoconjunctivitis was included with the respiratory tract infections.

Diarrhoea (with or without vomiting, respiratory symptoms or systemic symptoms) was grouped as gastro-enteritis.

Respiratory tract infections were the most heterogeneous group. This included any combination of signs and symptoms that indicated the presence of an infection of the lower or upper respiratory tract or the middle ear.

Skin infections grouped all forms of pyoderma, but excluded fungal infections.

All other infections were classed as "**other**" diseases.

Potential Problems in determining infection rates

Several factors may lead to over- or underestimation of the rates of infections. The most important and problematic were controlled in the *operational* nature of the definition of infection. The rules for diagnosis were chosen to minimize the possibilities of counting the same infection more than once and of including non-infectious diseases as infections.

Problem - Multiple doctors

Although most episodes of infectious disease were observed by myself personally at some stage in each illness, many were observed only by other doctors. Sometimes a diagnosis had to be made from the

records of doctors who were not aware that they were to be used for the purposes of this study. Care had to be taken to use consistent criteria for diagnosing an infection. And, for those infants who were treated by multiple doctors, care had to be taken to count each individual episode exactly once.

Problem - Non-infectious conditions which may be confused with infectious disease.

Conditions such as wheezing may have an infectious aetiology, or (perhaps more commonly) have some other cause. This could lead to a bias towards the over-diagnosis of infection. The operational definition's rules attempted to avoid this. Diarrhoeal disease was assumed to be infectious in aetiology.

Problem - Multiple consultations for the same infection

Mothers with a sick child would often seek help more than once during the course of an infection, and often from more than one source of health care. Care was taken in analyzing the records to count such episodes as one infectious incident.

Problem - Multiple simultaneous infections

Concurrent infections are sometimes difficult to differentiate from one infection with multiple symptoms and signs. The criteria for diagnosis of infection were designed to err on the conservative side and hence may have slightly underestimated the true rates of infection.

Problem - Prolonged infections and recurrent infections

Diarrhoeal disease sometimes presented a diagnostic challenge in this category as did the child with a wheezing chest. Somewhat arbitrary criteria* were used to separate multiple diagnoses of prolonged infections from multiple diagnoses of recurrent infections. An incident was counted as an infection if it was at least 15 days after a similar infection. And one in ten episodes of wheezing were counted as infections. The error is unlikely to be large**.

Problem - Parents with different care-seeking behaviour

Some parents frequently sought help for many trivial conditions, while other parents had a higher threshold for seeking care for their child. "Clinical judgment" was used to temper decision making, but it is likely that a few infectious episodes were not detected in children of the few less caring mothers.

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* I could find little assistance in the literature

** The most important "outlier" was a child who had recurrent otorrhoea. These episodes were analyzed as both one and many infections, but did not materially affect the results.

Weaning

Weaning was measured with two variables called *"first bottle"* and *"last breast"* in the tables of results. The variables give the age in months attained at which the child was first given milk formula and last offered the breast. (There were a number of working mothers whose infants were breast-fed at night and bottle-fed during the day.)

Laboratory methods

Specimen collection and storage

Blood

Blood was sampled from a vein in the arm; eight ml was taken into a sterile plastic syringe that had been moistened with preservative-free heparin (Pularin, Evans). A further 2 ml was then taken into a new sterile plastic syringe. This portion was decanted into a tube containing K-EDTA as an anticoagulant, and used for the full blood count. The heparinized blood was transported in a plastic tube on ice. In the laboratory the specimens were centrifuged at 400 g for 10 minutes to separate cells from plasma. Plasma that was not used immediately for analysis was stored at -80°C. All blood specimens were collected between 8h00 and 9h30.

Precautions were taken to prevent bacteriological or trace elemental contamination. The skin was swabbed with gauze soaked in 70% alcohol. All needles, syringes and tubes were sterile and disposable. Previously, quality control measures in the laboratory had shown that significant amounts of zinc contaminate specimens if proprietary alcohol swabs are used, but that needles, tubes, syringes and 70% ethanol on gauze do not interfere with zinc analysis*.

Hair

Hair for zinc analysis was taken close to the scalp over the occiput.

Bacterial and fungal cultures

Material for bacterial and fungal cultures was taken on dry sterile cotton swabs and transported in sterile containers to the laboratory.

Viral specimens

Throat and rectal swabs were transported in Hepes buffered Hank's Lactalbumin hydrolysate medium. Faecal specimens were not specifically preserved. Processing of the specimens began within 24 hours of collection.

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* Personal communication, Dempster W, Institute of Child Health, University of Cape Town

Haematology

These tests were performed by the laboratories and staff of the haematology department of the Red Cross War Memorial Childrens Hospital under the direction of Ms R Pearl.

Full blood count

Full blood counts were made on a Coulter Counter model S plus II. The performance of the instrument was monitored for all parameters by frequent regular assays of 4C PLUS cell controls supplied by Coulter Diagnostics.

Differential count

Differential counts were performed manually on blood smears stained with May, Grunewald, Giemsa stain, while reticulocytes were counted from smears stained with brilliant cresyl blue.

Biochemistry

The biochemical measurements were made in the laboratory of the Institute of Child Health of the University of Cape Town under the direction of Mr WB Dempster and supervision of Mrs F Pocock.

Red cell zinc protoporphyrin

Blood zinc protoporphyrin was measured with a haematofluorometer* calibrated in units of ug/g Hb[324]. Standardization of the Aviv haematofluorometer was accomplished by using a calibration slide supplied with the instrument. The slides were impregnated with rhodium B dye and correspond to readings of 15.5 and 3.1 ug of zinc protoporphyrin per gram of haemoglobin. The calibration is set at the factory and any deviation greater than 5% requires the instrument to be returned to factory for adjustment. This has occurred 3 times in 6 years.

Measurements of red cell zinc protoporphyrin in our laboratory have been compared with values of free erythrocyte protoporphyrin measured by the extraction procedure of Piomelli[398]. A correlation coefficient of 0.93 was obtained in 90 paired samples.

Plasma ferritin

The method employed in the assay of plasma ferritin has been described in detail by Dempster *et al*[132]. The immunoradio-metric assay employed anti-human ferritin antibody** labelled with ^{125}I in

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* supplied by Aviv, 810 Towbin Avenue, Lakewood, New Jersey 08701, USA

** Made in the laboratories of the Institute of Child Health[132]

the laboratory[184] and ferritin prepared from human splenic tissue as an antigen and standard[80]. The assay was performed in three stages following the method of Miles *et al*[344].

Stage 1. Polystyrene tubes (Falcon Plastics 2052)# were coated with anti-human ferritin antibody by addition of 500 ul of rabbit anti-serum at a dilution of 1:10 000 in 0.2 M sodium hydrogen carbonate buffer/sodium carbonate buffer pH 9.2 and incubated for 24 hours at 4°C. The contents of the tubes were aspirated and washed twice with 2 ml of 0.1% BSA buffer pH 8.0 and twice with 2 ml distilled water. The anti-human ferritin antibody remains adsorbed to the interior wall of the test tube.

Stage 2. Two hundred ul of a known concentration of ferritin or of plasma with an unknown concentration was added to the coated tubes and incubated for 24 hours at 4°C. The ferritin forms an antigen-antibody complex with the anti-human ferritin on the tube wall.

Stage 3. The contents of the tubes were aspirated and washed once with 2 ml 0.1% BSA buffer. Thereafter 200 ul of ^{125}I-labelled anti-human ferritin antibody (approximately 20 000 counts per minute (CPM)) were added to the tubes and incubated for 48 hours at 4°C; the antibody attached itself to the free antigen binding sites of the ferritin. Excess labelled antigen was removed by aspiration and washing the tubes twice with 2 ml of 0.04 M phosphate buffer pH 7.4. The radioactivity of the remaining antigen/antibody complex was measured in a gamma counter and was proportional to the amount of ferritin present. Standard concentrations of ferritin, ranging from 100 to 0.159 ug/l prepared in 4% BSA buffer pH 8.0 were measured in triplicate. Plasma from subjects was routinely diluted 1:30 in 4% BSA buffer and assayed in triplicate.

Calculation of Results. A standard dose-response curve was constructed by plotting the logarithm of the concentration of ferritin against the logit transformation of the count of radioactivity. *Ie* log(ferritin concentration) was plotted on the X-axis with log(y/(1-y)) on the Y-axis, where y is the radioactivity count. The infinite dose response was estimated by a computerized iterative technique which finds that value which gives the best fit (minimum residual sum of squares) to the straight line fitted to the observed points and the estimated infinite dose response. The final estimate of the infinite dose response was made by quadratic interpolation from the last three sets of observations. For routine analysis, counts in triplicate were observed for each test sample. These were then rescaled and converted to logits. Interpolation on the standard curve then estimated the ferritin concentration in the serum.

Falcon Plastics (Div. Becton Dickson & Co.), 1950 Williams Drive, Oxnard, California 93030, U.S.A.

Plasma zinc

The plasma zinc concentration was measured according to the method of Henry, Cannon and Winkelman[220]. The plasma was deproteinized with trichloroacetic acid, and the supernatent analyzed for zinc by atomic absorption at 214 nm on a Beckman model 1272M spectrophotometer.

Hair zinc

Hair zinc was analyzed according to a method adapted from that of V Yuzbarsiyan, Department of Paediatrics, Istanbul University, Turkey (personal communication). The hair was sequentially washed in hexane, in ethanol, then at least three times in deionized distilled water. It was then dried, weighed and digested in nitric acid before aspiration into a Beckman model 1272m atomic absorption spectrophotometer. (On a practical note, one may remark that the method is labour intensive; one technician was able to perform about 20 analyses per week.)

Microbiology

Bacterial and fungal characterization

Specimens for bacterial and fungal characterization were processed in the microbiology laboratory of the Red Cross War Memorial Childrens Hospital under the direction of Dr D Hanslo.

Viral characterization

These studies were performed by the staff and virology laboratories of the Department of Medical Microbiology of the University of Cape Town under the direction of Professor JW Moodie and supervision of Drs JP MacIntyre and GA Keen.

Immunological methods

The immunological tests were made in the laboratory of the Institute of Child Health of the University of Cape Town under the direction of Professor DW Beatty and supervision of Ms J Hughes. These tests are more fully described than most others in this thesis because immunological methods have not reached the state of standardization that exists in biochemistry and haematology and results may vary greatly with seemingly minor variations in laboratory procedures.

Materials and laboratory reagents used in more than one assay.

Microtiter equipment

U-bottomed microtiter plates were manufactured by Greiner and by Nunc and supplied by Laboratory and Scientific and by the Weil Organization (Distr.) respectively.

Automatic pipettes were manufactured by Gilson, and supplied by Miles Seravac.

Multiple pipettes were manufactured by Flow Titertek, and supplied by Miles Seravac.

Pipette tips were supplied by Laboratory and Scientific.

Reagents

Saline: The salt solution used as a diluent was 0.9% NaCl.

PBS pH 7.2: Phosphate buffered saline at pH 7.2 was prepared from 100 ml saline plus 23.9 ml 0.15M KH_2PO_4 and 76 ml 0.15M Na_2HPO_4.

PBS pH 6.4: Phosphate buffered saline at pH 6.4 was prepared from 100 ml saline plus 67.7 ml 0.15M KH_2PO_4 and 32.2 ml 0.15M Na_2HPO_4.

EDTA-PBS: The composition of this was: NaCl 8.0 g, KCl 0.2 g, Na_2HPO_4 1.15 g, KH_2PO_4 0.2 g made up to 1 litre. Then 0.2 ml EDTA (55.836 g/l) was added to 100 ml of the PBS.

Tris-NH_4Cl buffer: This was made from one part tris ((hydroxy methyl)-aminomethane (Merck)), 20.6 g/l at pH 7.3 and 9 parts NH_4Cl 8.3 g/l.

RPMI-Hepes consisted of RPMI-1640 medium (Gibco, USA) buffered with 25 mM Hepes (Schwarz Mann).

RPMI-Hepes-bicarb was prepared as above with the addition of 2 g $NaHCO_3$/L.

Lymphocytes: Blood mononuclear cells were isolated from freshly drawn heparinized blood by the Ficoll-Isopaque technique of Byum[50] and resuspended in RPMI-HEPES-bicarb at 2x106 cells/ml as described by Beatty and Dowdle[27].

Tetanus antibody titration

The procedure for tetanus antibody determination followed that of Stavitsky[476] and Sewer[456].

Materials

Erythrocytes: Fresh human group O blood (Rh positive or negative) was stored with 1 mg Na EDTA/ 1 ml blood.

Tannic acid: The stock solution was prepared from 0.1 g tannic acid and 10 ml 0.9% NaCl. It was stored at 4°C. The 0.006% tannic acid working solution was made fresh for use from 0.3 ml of the stock solution and 49.7 ml 0.9% saline.

Tanning and coating of red cells

Erythrocytes were washed 3 times in saline and then 0.25 ml red cells were suspended in 10 ml PBS pH 7.2. Storage was at 4°C for up to 7 days. The red cells were tanned after re-washing and re-suspension to 2.5% in PBS pH 7.2. One volume of cell suspension with one volume of 0.006% tannic acid solution was incubated at 37°C for 15 minutes in an agitating water-bath. The suspension was then centrifuged, the supernatent discarded, and the cells washed twice in PBS pH 7.2. To coat tanned cells with tetanus toxoid, they were washed twice and resuspended to 2.5% concentration in PBS pH 6.4. One volume of tanned red cell suspension, 4 volumes of 1/3 dilution tetanus toxoid (obtained from the South African Institute of Medical Research) and 4 volumes of PBS pH 6.4 were incubated for 10 minutes in a water bath at 37°C. The erythrocytes were kept in suspension by shaking the tubes every 30 seconds. After centrifuging, the supernatent was removed and the red cells washed and resuspended to 1% dilution in 1/100 heat inactivated normal rabbit serum.

Titration

Tetanus antibody titration was carried out in U-type microtiter plates. Wells 2 to 12 had 0.025 ml diluent placed in them. The test serum was diluted 1/10 with 1/100 heat inactivated rabbit serum and 0.05 ml placed into well 1. Serial dilutions of serum were made by transferring 0.025 ml from well 1 to well 2, thoroughly mixing the contents of well 2 and then transferring 0.025 ml from well 2 to well 3. The procedure was then repeated for wells 3 to 12 to produce dilutions of the test serum of 1/10, 1/20, 1/40, ..., 1/20480 in wells 1, 2, 3, ..., 12. To the serum was added 0.025 ml of the coated tanned red cells. The plate was read after 3 to 12 hours incubation. Two controls were used on each plate: 1) uncoated tanned cells with serum (to test for non-specific binding factors in the serum), and 2) coated tanned cells with diluent (to provide a negative control). The titre of anti-tetanus antibody present in a serum was the reciprocal of the highest dilution that produced hemagglutination.

Polio antibody titration

The procedure followed that recommended by the Institute Virion for the performance of complement fixation.

Materials

Virion reagents were obtained from Combined Medical Specialities. These were:

Virion 1127 Polio virus complement fixing antigen

Virion 2127	Control antigen for complement fixation
Virion 3127	Human antiserum, positive control
Virion 4127	Human antiserum, negative control

Haemolytic serum: Wellcome UDIS haemolytic serum was obtained from Wellcome Laboratories.

Veronal-buffered saline (VBS) stock solution was prepared from 5.75 g diethylbarbituric acid dissolved in 500 ml hot distilled water. To this 3.75 g sodium diethylbarbituric acid and 85.0 g NaCl was added and the volume made up to 2000 ml with distilled water. Finally, 1.68 g $MnCl_2.6H_2O$ and 0.28 g $CaCl_2$ were added and the stock solution autoclaved at 115°C. It was stored at 4°C. The working solution was prepared by diluting the stock solution 1 : 5 with distilled water just before use.

Haemolysin or haemolytic serum stock solution was prepared from 94 ml VBS thoroughly mixed with 1 ml of haemolytic serum (titre 1 in 6000) and 1 ml glycerol A.R. to which 4 ml of 0.85% NaCl solution containing 5% phenol was added. The stock solution was stored at 4°C and diluted optimally 1 in 25 with VBS just before use.

Sheep Erythrocytes were obtained from the animal unit of the Cape Provincial Administration and stored for up to 6 weeks in Alseever's solution. A working suspension of erythrocytes was prepared by diluting the stock 1 in 10 with VBS and centrifuging for 7 minutes at 600 g. This was repeated 4 times, but centrifuging the cells for 10 minutes on the last occasion. The supernatent was carefully removed and the sedimented cells diluted to 2% in VBS. The erythrocyte concentration was measured with a photometer. The erythrocyte suspension was divided into aliquots and stored for up to 5 days.

Guinea pig serum was obtained from the animal unit of the Cape Provincial Administration and used for its Complement. It was stored at -20°C and diluted optimally 1 in 20 with VBS immediately before use. For each new batch, complement activity was determined by titration according to the following scheme for microtitre plates.

Complement titration

Step 1: complement dilution											
Stabilized C′ (diluted 1 in 2 with VBS)	ml	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
VBS	ml	1.9	2.4	2.9	3.4	3.9	4.4	4.9	5.4	5.9	6.4
Step 2: complement titration											
Diluted C′	ul	25	25	25	25	25	25	25	25	25	25
Diluted antigen	ul	25	25	25	25	25	25	25	25	25	25
VBS	ul	25	25	25	25	25	25	25	25	25	25

The microtitre plates were covered with moistened filter paper and incubated overnight at 4°C and read the following day. The highest dilution causing complete haemolysis was used as the working dilution of complement. (This provided a 100% reserve as the complement was diluted 1 in 2 just before performing the titration.)

Polio virus antibody titration

Specimens: 20 ul of patients plasma or control serum were diluted with 180 ul VBS and inactivated for 30 minutes at 56°C in a water-bath. Paired specimens from the same patient at the ages of 3 and 12 months were tested simultaneously on the same plate.

Antigens: Polio virus antigen (Virion 1127) and control antigen (Virion 2127) were restored with 1 ml distilled water and stored for up to one week in tightly stoppered tubes at 4°C. Working dilutions were 1 : 2 and 1 : 8 respectively, and were prepared on the day of use.

Test: The test was performed in microtitre plates with 96 wells arranged in 12 columns numbered 1 to 12 and 8 rows labelled A to H. The titration of a specimen was made in the wells of one row. As each test employed one positive control serum (Virion 3127) and one negative control serum (Virion 4127), up to 6 specimens could be titrated on one plate. The method was as follows:

1 25 ul of VBS were pipetted into wells 3 to 6 of a row.

2 25 ul of specimen dilution 1 in 10 were pipetted into wells 1, 2, 3 and 8

3 Serial dilutions were made with a 25 ul diluter from well 3 to well 6. This resulted in dilutions from 1 in 10 to 1 in 160 with reciprocal titres of 10, 20, 40, 80 and 160 in wells 2 to 6.

4 25 ul of the working dilution of antigen were pipetted into wells 2 to 6.

5 25 ul VBS were pipetted into well 1 as a negative control of antigen.

6 25 ul of the working dilution of complement were pipetted into wells 1 to 6.

7 Complement controls were prepared according to the following scheme:

Tube No.	1	2	3	4
Working dilution of complement ml	1.2 ml	0.9 ml	0.6 ml	0.3
VBS ml	0.0 ml	0.3 ml	0.6 ml	0.9
Resulting complement units	2	1.5	1	0.5

The complement controls were then pipetted as follows:

Complement units	2	1.5	1	0.5
Complement ul	25 ul	25 ul	25 ul	25
Polio virus antigen ul	25 ul	25 ul	25 ul	25
VBS ul	25 ul	25 ul	25 ul	25

8 A polio virus antigen/SRBC control was made by pipetting 25 ul polio virus antigen, 25 ul 1 in 10 dilution of SRBC, and 25 ul VBS into a well.

Haemolytic system: The haemolytic system to visualize the complement fixation was prepared daily. To 1 ml of haemolysin stock solution 24 ml VBS was added and the working solution prewarmed in a water-bath at 37°C for 10 minutes. The haemolysin was added dropwise to an equal volume of prewarmed 2% erythrocyte suspension and mixed with a magnetic stirrer. Sensitization of the SRBC was accomplished by incubation at room temperature for 15 minutes. In the meanwhile, the microtitre plates were warmed in the incubator at 37°C for 15 minutes.

50 ul of the freshly prepared haemolytic system was pipetted into all wells, including control wells. The microtiter plates were gently shaken and returned to the incubator for a further 20 to 30 minutes until the complement controls showed complete haemolysis in the wells with 2 and 1.5 units and traces of inhibition of haemolysis in the well with 1 unit of complement.

The plates were then centrifuged at 400 rpm for 5 minutes and read within 2 hours. A well was considered positive if it showed from 50 to 100% inhibition of haemolysis.

Lymphocyte subtyping

T cells were counted by rosette formation with AET treated sheep red blood cells[341].

T cells and T cell subsets were enumerated using murine monoclonal antibodies; OKT3 for total T cell count, OKT4 for helper/inducer cells and OKT8 for suppressor/cytotoxic cells (Ortho Diagnostic Systems) as follows: $1x10^6$ mononuclear cells resuspended in 200 ml of phosphate buffered saline (PBS) were incubated for 30 minutes in an ice bath with 10 ul (0.05 ug) of monoclonal antibody. The cells were washed at 4°C, and 20 ul of 1/40 fluorescent labelled rabbit anti-mouse IgG (Miles-Yeda) was added; they were incubated on ice for 30 minutes, washed, resuspended in 30% glycerol in PBS and counted using fluorescent microscopy.

B lymphocytes were counted using a FITC (fluorescein isothiocyanate) conjugated F(ab')2 rabbit anti-human IgG (Miles-Yeda) was employed. This enabled the number of cells with surface membrane immunoglobulin (SMIG) to be counted using fluorescent microscopy.

Lymphocyte transformation by stimulation with PHA

PHA stimulated lymphocyte transformation was performed in triplicate in microplates as described by Beatty and Dowdle[28] and Beatty[26]. $2x10^5$ mononuclear cells, 25 ul of PHA (2.5 ug/ml final concentration) (Wellcome) and 25 ul of serum in a final volume of 200 ul of RPMI-HEPES were placed in a well of a microtitre plate. In order to distinguish between cell and serum factors, the patient's cells were incubated in both autologous and normal sera, and normal cells in normal and patients' sera. The plates were incubated at 37°C for 72 hours in an humid atmosphere of 5% carbon dioxide and 95% air in a Hotpak incubator. Twenty four hours before completion of the incubation period 0.07 uCi ^{14}C-thymidine (Radiochemical centre, Amersham, England, specific activity 60 mCi/mmole) was added to each well. Radioactive lymphocytes were harvested onto glass fiber filter paper sheets (Skatron) using a multiple automatic sample harvester designed and built in the laboratories of the University of Cape Town Medical School[26]. The glass fiber disks were dried, placed in 3 ml of scintillator solution (Instagel, Packard Instrument Co.) and the radioactivity taken up by transformed lymphocytes counted in a Tricarb scintillation counter spectrophotometer (Packard Instrument Co.). The results were expressed as the arithmetic mean of the disintegrations per minute (dpm) of the triplicate cultures after the sample readings had been corrected for background radioactivity and counting efficiency.

Neutrophil phagocytosis and killing activity

The method for determining the phagocytic plus bactericidal efficiency of neutrophils has been described by Haddad, Beatty and Dowdle[202]. Minor modifications were made to accommodate the smaller volume of blood obtainable from infants.

Preparation of neutrophils

All procedures were performed with aseptic techniques at room temperature. Two aliquots of 4 ml of heparinized blood was mixed in 15 ml Falcon tubes (Laboratory and Scientific) with 0.67 ml of 6% dextran (MW 200 000 to 275 000, BDH Biochemicals) in 0.85% NaCl. The cells were allowed to sediment for 30 minutes with the tubes held at a 45 degree angle. The plasma/leukocyte layer was aspirated with a pasteur pipette and centrifuged at 180 g for 10 minutes. After discarding the supernatent fluid, the button of cells was resuspended in 8 ml of Tris-NH_4Cl buffer (to lyse contaminating red cells). The cells were kept in suspension for ten minutes in the buffer. After centrifugation for 10 minutes at 180 g, the pellets of cells were resuspended in 1 ml EDTA-PBS and

pooled in one tube with the volume made up to 8 ml. Centrifugation at 180 g for ten minutes, aspiration of supernatent and re-suspension of the cells was repeated 3 times. The final pellet was resuspended in RPMI-Hepes. Standard methods were used to perform total leukocyte and differential counts, and the number of neutrophils were adjusted to 10^7/ml with medium. The percentage of neutrophils harvested was computed.

The viability of the cells was determined by the vital dye exclusion method[140]. Equal volumes of NaCl (1.8%) and trypan blue (2% in distilled water) were mixed immediately before the test. Equal volumes of this mixture and the cell suspension were then incubated for 10 minutes at room temperature.

Staphylococcus aureus

A strain of *S. aureus* (Oxford) was obtained from the Department of Bacteriology at the University of Cape Town Medical School. After overnight growth in tryptic soy broth (Difco), 0.5 ml of culture material was added to 4.5 ml of fresh warm broth and incubated for 4 hours in a shaking water-bath at 37^{O}C. It had previously been established that the bacteria would now be in the late exponential log growth phase. After centrifugation of 2 ml of this culture at 1300 g for 20 minutes, the pellet was resuspended in 4 ml of RPMI-Hepes medium. The absorbance at 620 nm was measured and the corresponding viable count determined from a calibration curve established for this organism. This was adjusted to 10^8 organisms per ml with medium.

Controls

Neutrophils were prepared from a normal adult. Normal serum was obtained either from the normal cell donor or from a pool of normal human group AB serum.

Phagocytosis and bactericidal assay

The bactericidal assay was modified from the methods of Steibigel *et al*[477]. The following were added to 12 x 75 mm plastic test tubes (Falcon 2054): 250 ul PMN suspension, 50 ul serum, 150 ul RPMI-HEPES medium and 50 ul bacterial suspension, giving a neutrophil to bacteria ratio of 1 : 2. Control tubes without PMNs were also prepared. The tubes were incubated on a tilting mixer at 37^{O}C and 100 ul samples were removed at 0, 60, and 120 minutes. These were added to 900 ul sterile distilled water. The neutrophils were then further disrupted to release live ingested bacteria. This was achieved by sonication for 15 seconds at an amplitude of 6 um with an MSE ultrasonic disintegrator. It had previously been shown that sonication of this intensity and duration disrupted all leukocytes without affecting the viable count of *S. aureus*. The total remaining viable bacterial count was then determined by standard dilution techniques and plating. Samples from control tubes were treated in an identical manner. Colonies growing on blood-agar base were counted the day following plating.

Results at 1 and 2 hours were expressed as:

(i) the bacterial count as a percentage of the initial inoculum

$100*Cs_{60}/Cs_0$ & $100*Cs_{120}/Cs_0$

(ii) the bacterial count as a percentage of that for control cells

$100*Cs_{60}/Cc_{60}$ & $100*Cs_{120}/Cc_{120}$

(iii) the bacterial count as a pereentage of that for control AB serum

$100*Cs_{60}/Cab_{60}$ & $100*Cs_{120}/Cab_{120}$

(iv) the ratio of the percentage change in colony counts for the subject to the percentage change for the adult control.

$(Cs_{60}/Cs_0)/(Cc_0/Cc_{60})$ & $(Cs_{120}/Cs_0)/(Cc_0/Cc_{120})$

Where C indicates the colony count, the suffix indicates the subject (s), the control cells (c) or the control AB serum (ab) and the subscript indicates the time at the start ($_0$), at 1 hour ($_{60}$) or at 2 hours ($_{120}$). Although these methods are conceptually dissimilar, the results turned out to be equivalent. For this reason, the results are presented in chapter 6 in using method 1.

Skin tests of delayed hypersensitivity

The skin tests of delayed hypersensitivity were performed according to the recommendations of the WHO Committee on Primary Immunodeficiency[184]. 100 ul of the appropriate antigen was injected intradermally into the volar aspect of the forearm and the degree of induration read at 48 hours. The transverse diameter was measured and recorded as the response to the antigen. The reagents used were:

Mantoux test

Purified tuberculin, (PPD) 5 T.U./0.1 ml, was obtained from Evans Medical Ltd.

Candida antigen test

Candida albicans 1/10 w/v in 50% glycerin was obtained from Hollister Stier Laboratories. This was further diluted 1:10 in sterile buffered saline to give an antigen concentration of 1/100. The 1/100 dilution is stable for 3 months when kept at 4^oC[26].

Record keeping and statistical analysis

Record keeping

Each child in the study had a folder and a card, both of which were stored in numerical sequence according to the number allocated to the child on entry. The folder was used to store the data collection forms and laboratory test results. The card was used to record the milk issued to the child's caretaker and to note any incidents of morbidity.

Data processing

At the conclusion of the principal study, the data of interest were extracted from the records and entered into a Tektronix micro-computer. After checking twice against the manual record for clerical accuracy, the data were transferred to a Sperry mainframe computer for the statistical analysis.

Sociological data of anecdotal interest were analyzed manually.

Data from the subsidiary studies were stored and processed on a personal computer.

Statistical analysis

Computer programs employed

For the principal study the BMDP suite of statistical programs were employed for analysis[138]. The programs included P2D, for basic data description, P4F for frequency tables and chi square analysis, P6D for bivariate plots and linear regression, P7D for t tests with Bonferroni adjustment for multiple comparisons when appropriate, P1V for analysis of variance and P1R for multiple linear regression.

Some of the analyses for the smaller studies were made on a personal computer using Lotus 123 and included descriptive statistics, chi square analysis and t tests, both paired and two-sample. In these calculations P values were obtained from statistical tables. Since the values obtained are limits rather than exact quantities, these P values are reported as being greater or smaller than some number, typically 5%.

Missing data

When a subject dropped out of the study or a test was not performed for a technical reason, the data collection for that subject would be incomplete. If the data for a subject were missing for one variable then that subject was omitted from all analyses involving that variable; other data from that subject was included in other analyses.

The numbers of subjects who did not complete the study and the reasons for their failure to do so are reported in chapter 6.

Calculation of confidence intervals and type II errors

The programs that were used to calculate the confidence intervals and probability of type II errors are listed in appendix 7. Chapter 6 contains an additional note on the application of confidence intervals and their relation to statistical significance.

Significance levels

The convention is adopted that a P value of 5% is "significant". However, when results are discussed, the actual P value is given in the text.

Two-sided tests are used throughout. There is a case to be made for using one-sided tests in certain instances, but as the conversion is so simple (division by 2 of the stated P value) and almost all the literature uses two-sided tests this convention has been adopted.

Reporting of results

Results are reported as arithmetic means unless otherwise noted in the text. The number of significant figures given is generally to 1 more decimal place than that used in the individual measurements. This convention should not be taken to imply a greater degree of accuracy in the measurements than that afforded by the techniques employed.

CHAPTER 4 THE COMMUNITY OF BONTEHEUWEL

Introduction

This chapter describes the social setting of the principal study. In extrapolating the findings to other groups of children it is necessary to be aware of potentially confounding factors which may invalidate predictions and comparisons. Such factors include unambiguously measured variables such as age, sex, place and time, as well as "soft" data that attempt to quantify concepts such as socio-economic class, overcrowding and standard of home care. This chapter presents the "soft" data that provides the background information necessary for the reader to place the main study in its social context and to judge the validity of comparisons with other studies that he or she may wish to make. Where possible, statements are supported by data collected according to the protocols described in chapter 3. In contrast to the rest of this thesis however, it has sometimes been necessary to make subjective statements based on personal impressions. The personal impressions are based on eighteen months spent in the community, during which time each infant in the trial was examined on 15 to 20 twenty occasions and several visits were paid to each child's home. By the end of the study the families and the study teams knew each other fairly intimately.

I shall begin with some personal impressions and observations that may help the reader to attain an understanding of the community of Bonteheuwel. These impressions try to convey some feeling for the culture and living conditions of the group under study. They may, however, not be applicable to the population of Bonteheuwel as a whole since the selection criteria would have tended to exclude the poorer sections.

Personal impressions

Bonteheuwel is the site of a Cape Town City Council subsidized housing scheme. Most of the dwellings are about 25 years old and consist of rows of semi-detached houses, usually with a single floor, but some are double storied. The houses have 2 or 3 living rooms plus kitchen, bathroom and inside toilet. The council leases the houses, but has little control over subletting by tenants. The shortage of housing has encouraged some people to build shacks in the few hundred square meters of garden behind their house. A typical "hokkie" is a single room with sand floor, no window, walls of old packing cases and roof of corrugated iron. It may house a family of four, who often could afford better quarters if they were available.

The population of Bonteheuwel was 47 060 according to the 1980 population census with a population density of 141.78 persons per hectare*. The community is served by a civic center which has shops, post office, general practitioners in private practice, a cinema and the inevitable liquor store. The nearby

* Technical Management Services Data Report No. 1 of 1984, City Engineer's Department, City of Cape Town.

municipal clinic offers preventative services targeted at children and selected adult groups. The only curative services are for tuberculosis and sexually transmitted diseases. The clinic is within walking distance for all residents. Cars are scarce and most people use the buses, taxis or trains for transport beyond their suburb.

The people with whom I came in contact were friendly and helpful. Alcohol abuse is a long standing problem, particularly amongst the men, and "recreational" drugs are now widely used. Marijuana and Mandrax were in fashion; opiates and cocaine did not seem to be readily available. Litter is everywhere, so it was surprising to see how clean, neat and tidy it was inside homes even in the face of gross overcrowding.

Several observations deserve a full social-anthropological study to probe their deeper significance. Windows and curtains were rarely opened; is this to shut the uncontrollable world out? There is a general deficiency in quantitive reasoning of any kind. Times, dates, frequencies, weights, volumes often seem to have no meaning. Is this a manifestation of a similar culturally felt inability to control destiny? Perhaps these questions are better answered by the poet and novelist.

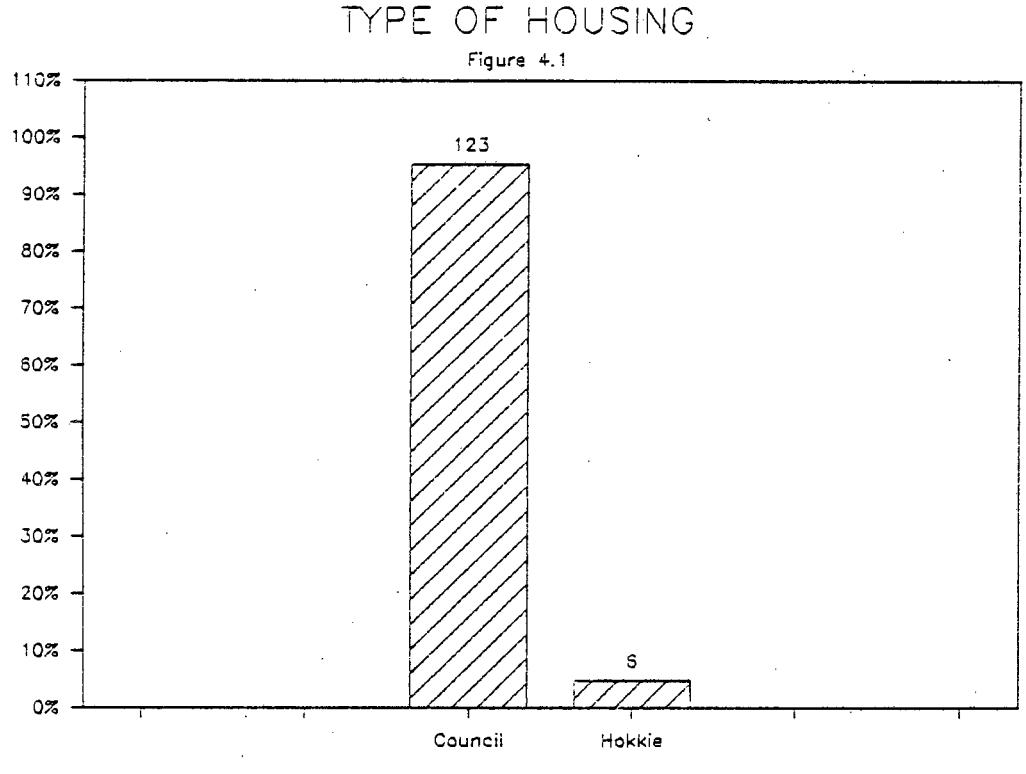
Survey results

Housing

Table 4.1 and figure 4.1 show that 95% of the families lived in council houses and 5% lived in lean-to shacks built in the yards of the council houses.

Table 4.1 Type of housing used by study families. Numbers living in council built houses and owner or lessee constructed shacks or "hokkies".

TYPE	No.	%
Council	123	95%
Hokkie	6	5%
TOTAL	129	100%



Measures of crowding of accommodation

The type of housing available in Bonteheuwel is in itself an indication of the chronic shortage of accommodation. Table 4.2 and figure 4.2 show the number of families considered by the project team to have poor, adequate and good housing. More objective criteria are given in tables 4.3, 4.4, 4.5, 4.6 and figures 4.3, 4.4, 4.5, and 4.6. which show that as many as 75% of study families lived with others. The survey counted those living in "hokkies" as living alone if they did not share the shack with others. As the shack dwellers had to use the kitchen, bathroom and toilet facilities of the main house, a more realistic estimation of the extent of overcrowding might be obtained by adding 5% to the percentage of families sharing accommodation.

The houses are small; only 17% have 3 living rooms and 61% have two rooms used for sleeping as demonstrated in table 4.2 and figure 4.2. In many houses occupants had to sleep on mattresses in the sitting room/dining room and it was often necessary for the beds to be shared.

Tables and figures 4.5 and 4.6 show that the mean number of occupants was at least 7.6 persons per house*. This underestimates the true number because the survey did not count the number of non-sibling children present in the house. At the time the survey was drawn up it was not realized that there would often be more than one mother and her children living in the same abode. A second difficulty in estimating the average occupancy of the houses arose from the large number of "transients". Most houses would have a significant number of people, often young men, who stayed temporarily. The interviewee would often not consider these persons as living in the house and they would therefore not be counted.

In retrospect, ambiguities in the questionnaires introduce an element of uncertainty in the data. For example, the questionnaires should not have attempted an immediate reduction to concepts such as "sibling" and "lodger", but instead should have taken a census of the persons who slept in the house during the previous 24 hours. In any event, the data do show how crowded the houses were and thus allow a qualitative estimate of the exposure of the study children to potential sources of infection (*ie* siblings and adults) in the same dwelling.

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* 5.8 adults + 0.6 siblings + 1 study infant.

Table 4.2 **Living conditions of study families: Numbers of families with poor, barely adequate and good living conditions in terms of crowding in the home. The measure of crowding was made on subjective grounds by the study team after several home visits.**

CONDITIONS	No.	%
Poor	73	47%
Adequate	31	11%
Good	14	42%
TOTAL	144	100%

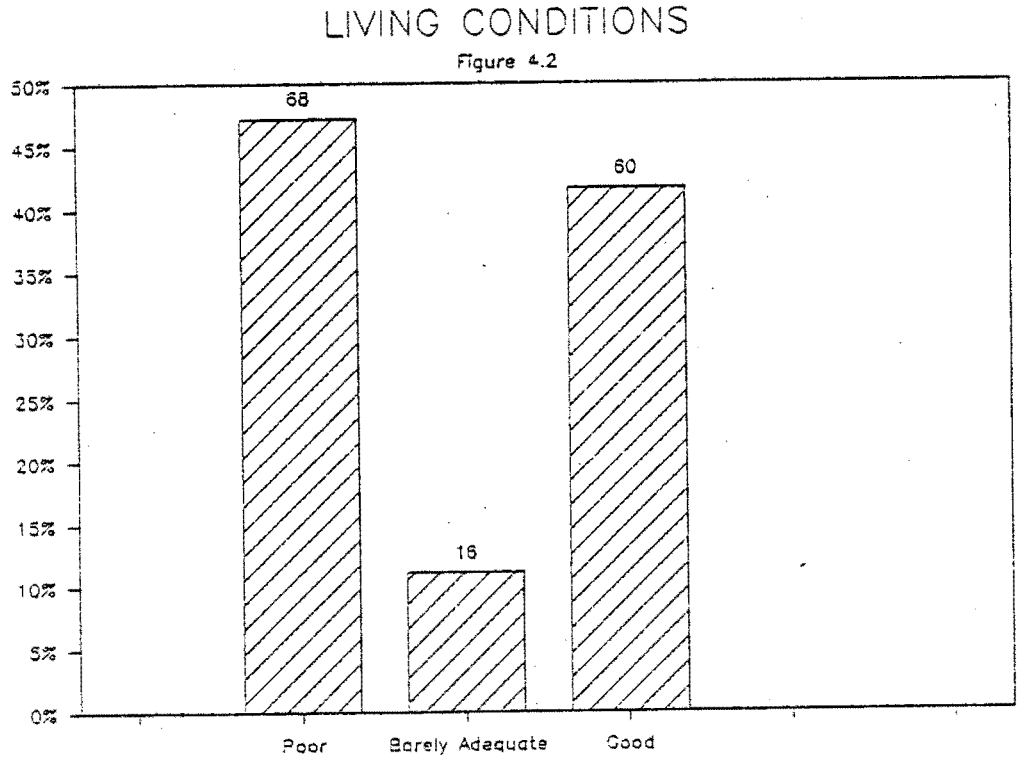


Figure 4.2

Table 4.3 Number of rooms used for sleeping by study families*.

ROOMS	No.	%
1	29	21%
2	83	61%
3	23	17%
TOTAL	135	100%

Note * Rounding is the cause of the failure to add to 100%

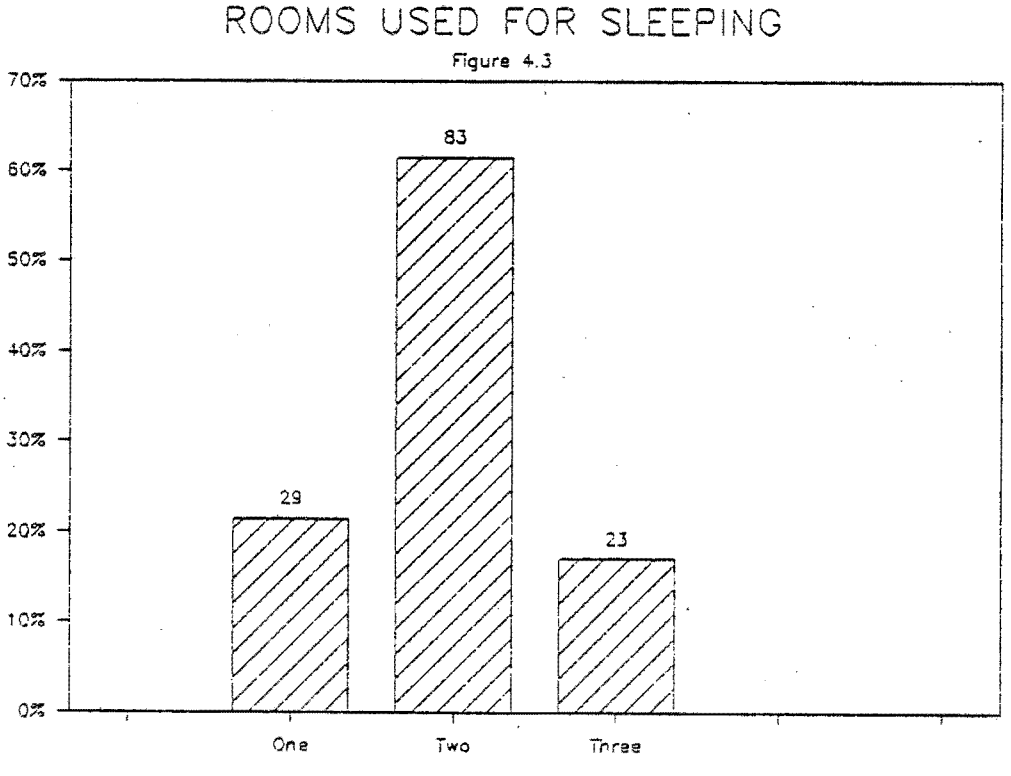


Figure 4.3

Table 4.4 **Lodging arrangements of study families: Numbers lodging with family, or living in their own house (including rented or leased properties) or renting accommodation to tenants.**

TYPE	No.	%
Family	72	56%
Own	43	34%
Tenant	13	10%
TOTAL	128	100%

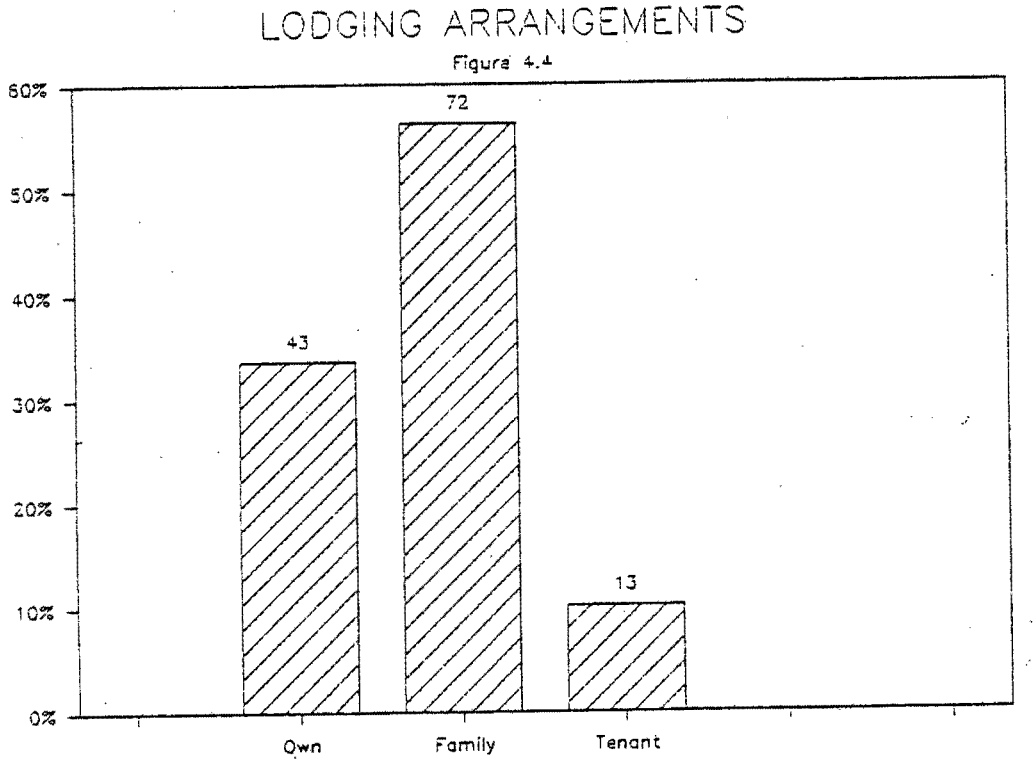


Figure 4.4

Table 4.5 **Number of adults in the houses of the study families*.**

ADULTS	No.	%	
1	1	1%	
2	9	7%	
3	10	7%	
4	23	17%	
5	28	20%	
6	20	15%	
7	15	11%	
8	8	6%	
9	11	8%	
10	8	6%	
11	1	1%	
12	2	1%	
13	1	1%	
TOTAL	137	100%	MEAN = 5.8

Note * Rounding is the cause of the failure to add to 100%

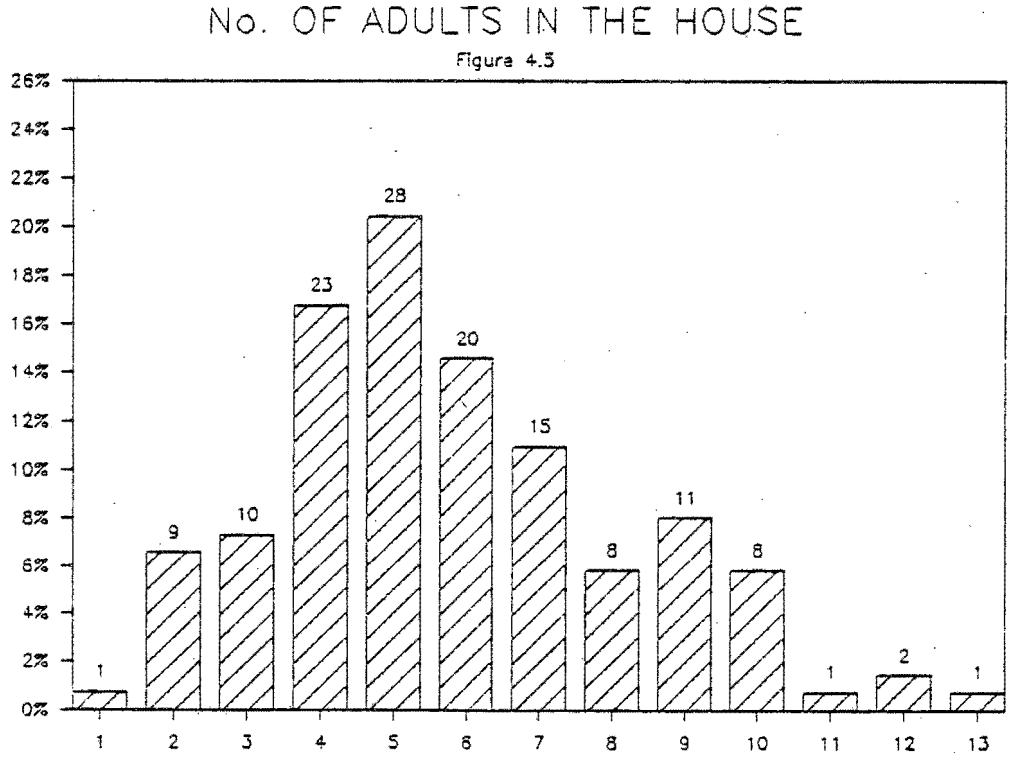


Figure 4.5

Table 4.6 Number of siblings of the study infants*

Sibs	No.	%	
0	69	51%	
1	32	24%	
2	18	13%	
3	11	8%	
4	2	1%	
5	2	1%	
	134	100%	**MEAN**=1.8

Note * Rounding is the cause of the failure to add to 100%

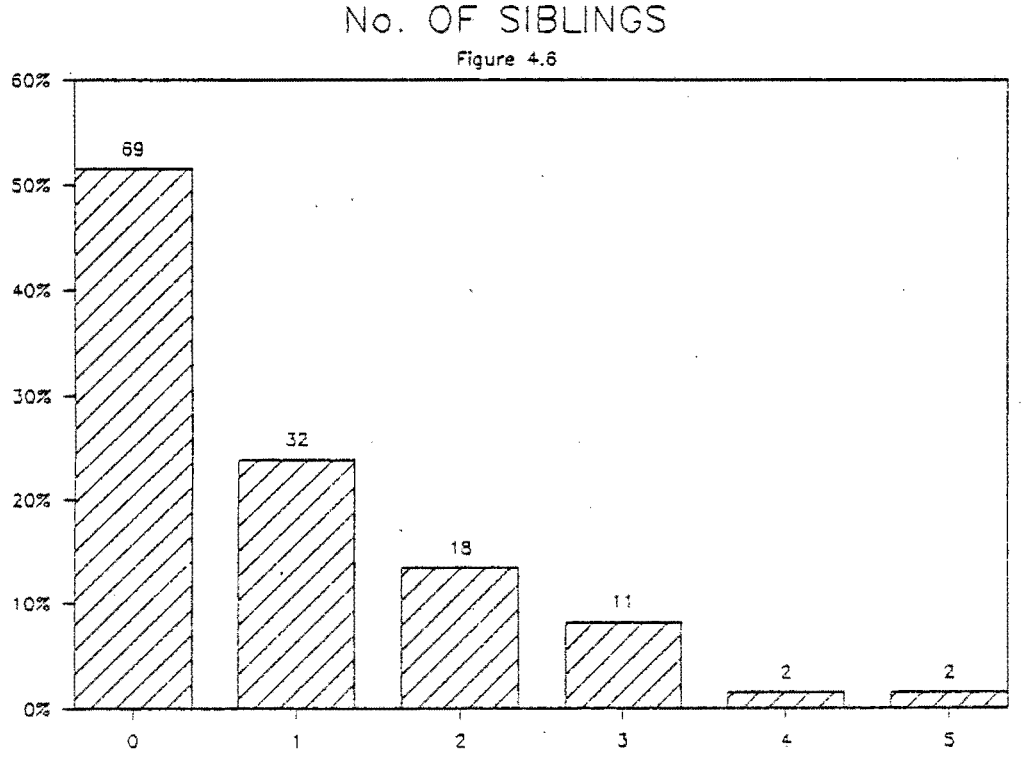


Figure 4.6

Measures of care of study subjects

Table 4.7 shows that 65% of babies were cared for by mothers by day and by night and 11% were entirely in the care of others. Most children were well cared for physically and emotionally. In an attempt to quantify the subjective measure of care, the study team assigned a rating of 1 (poor), 2 (adequate) or 3 (good) to each study child for the standard of hygiene employed in the home. The results are tabulated and graphically displayed in table 4.8 and figure 4.8 from which it can be seen that 87% of the families employed good standards of personal hygiene and 13% tended to neglect their child's health.

As some families tended to under- or over-utilize health care facilities, their care-seeking behaviour was also rated on a scale of 1 to 3 for inadequate, adequate and over-protective care-seeking. These estimates are given in table 4.9 and figure 4.9 which show that 4% of mothers tended to seek professional advice later than appropriate and 3% of mothers were over-protective of their children.

Table 4.7 **Caretaker of the study children by day and by night.**

	DAY		NIGHT	
	No.	%	No.	%
MOTHER	87	65%	119	89%
GRANDMOTHER	22	17%	7	5%
AUNT	8	6%	5	4%
OTHER	16	12%	2	2%
TOTAL	133	100%	133	100%

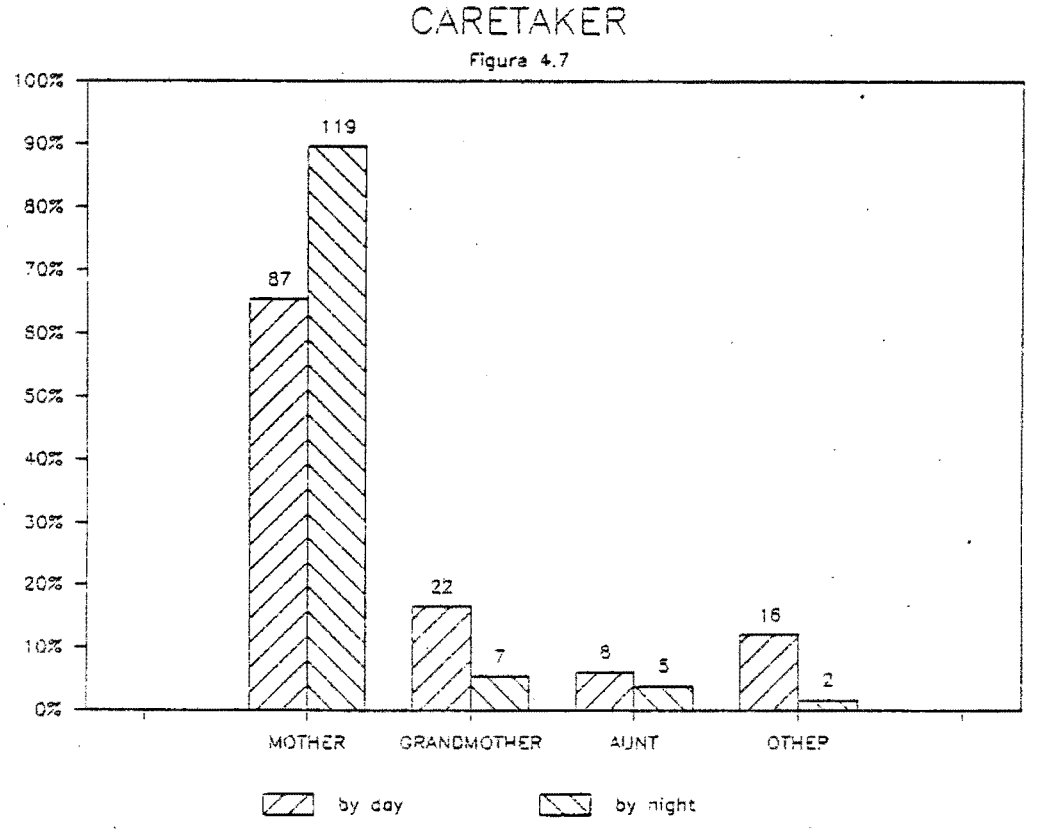


Table 4.8 Standards of hygiene employed in the homes of study families estimated subjectively by the study team after several home visits.

STANDARD	No.	%
Poor	19	13%
Adequate	10	7%
Good	115	80%
TOTAL	144	100%

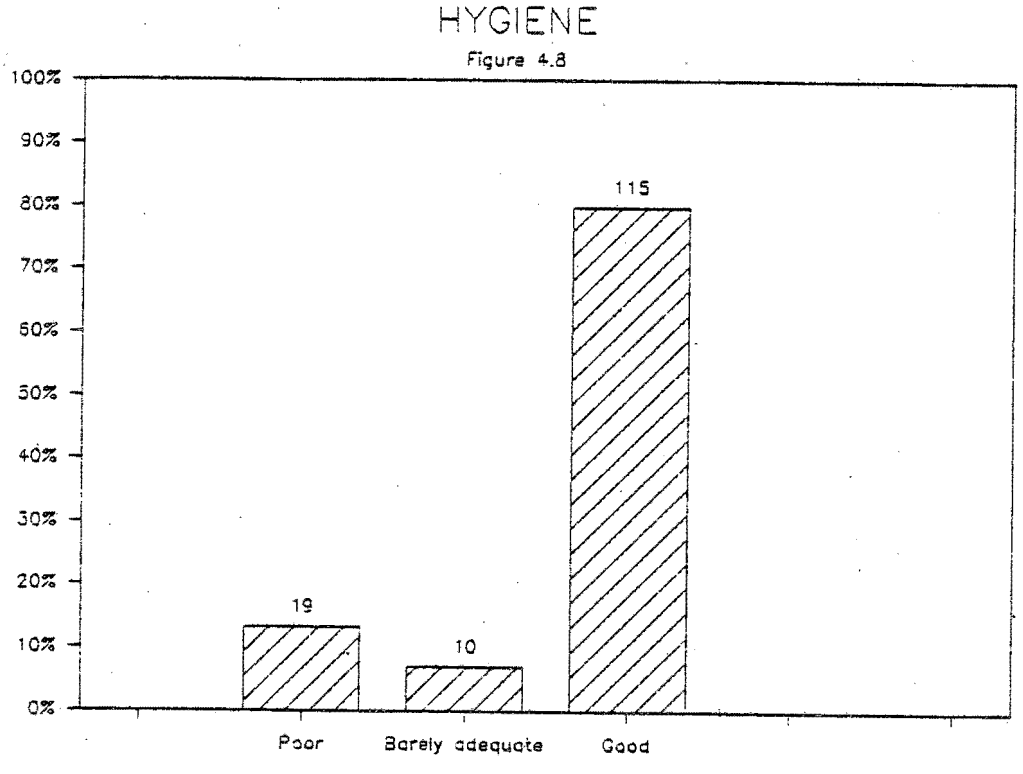


Table 4.9 Care-seeking behaviour of families subjectively ranked as poor, good or over-protective after 9 months of observation*.

RANK	No.	%
Poor	6	4%
Good	133	92%
Over-protective	5	3%
TOTAL	144	100%

Note * Rounding is the cause of the failure to add to 100%

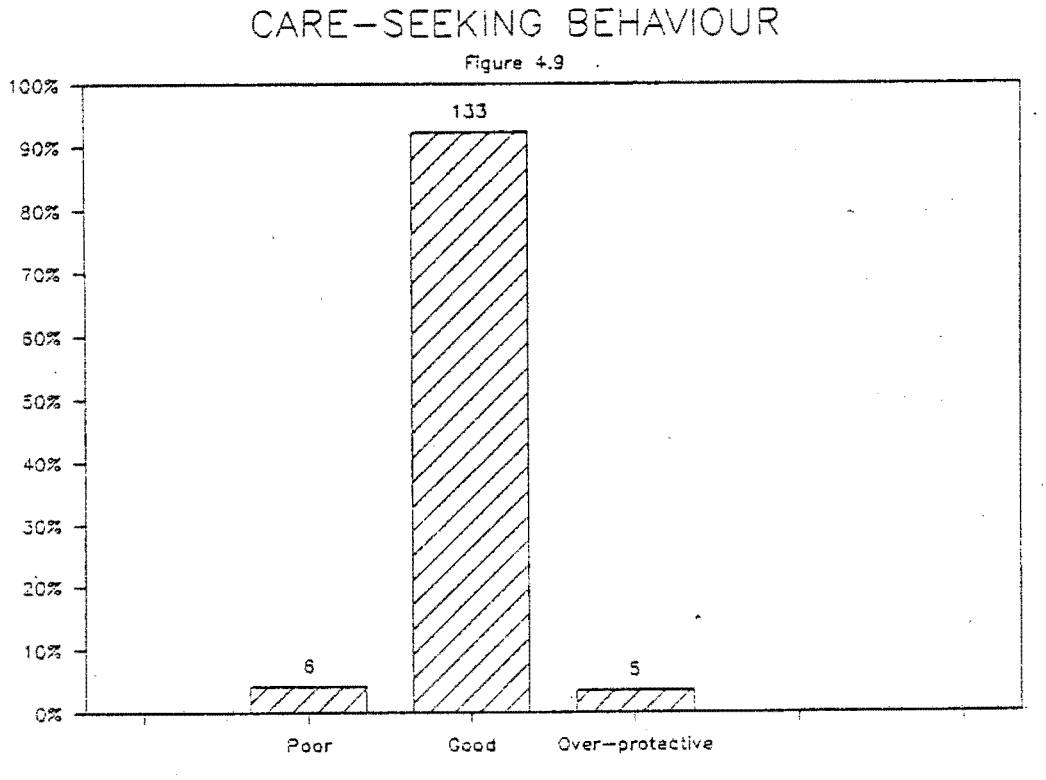


Figure 4.9

Measures of family stability and unity

Tables 4.10 and 4.11 and figures 4.10 and 4.11 show that 52% of mothers were married to the father of their child at the start of the study, but, by the end of the trial, a further 9% had remarried. Table 4.12 shows that at the start of the trial, 51% of the mothers were actually living with the father of their child. The inference is easy to make that the ratio of unstable to stable families is uncomfortably high. The figures do not portray the scope of the extended family that we came to know during the study. However, tables 4.4 and 4.16 do hint at a wider role of the family in accommodation and finance than might be found in white middle class South African families.

Table 4.10 **Marital status of mothers on entry to the study***

Status	No.	%
MARRIED	72	52%
SINGLE	58	42%
SEPARATED	3	2%
COHABITING	3	2%
DIVORCED	2	1%
TOTAL	138	100%

Note * Rounding is the cause of the failure to add to 100%

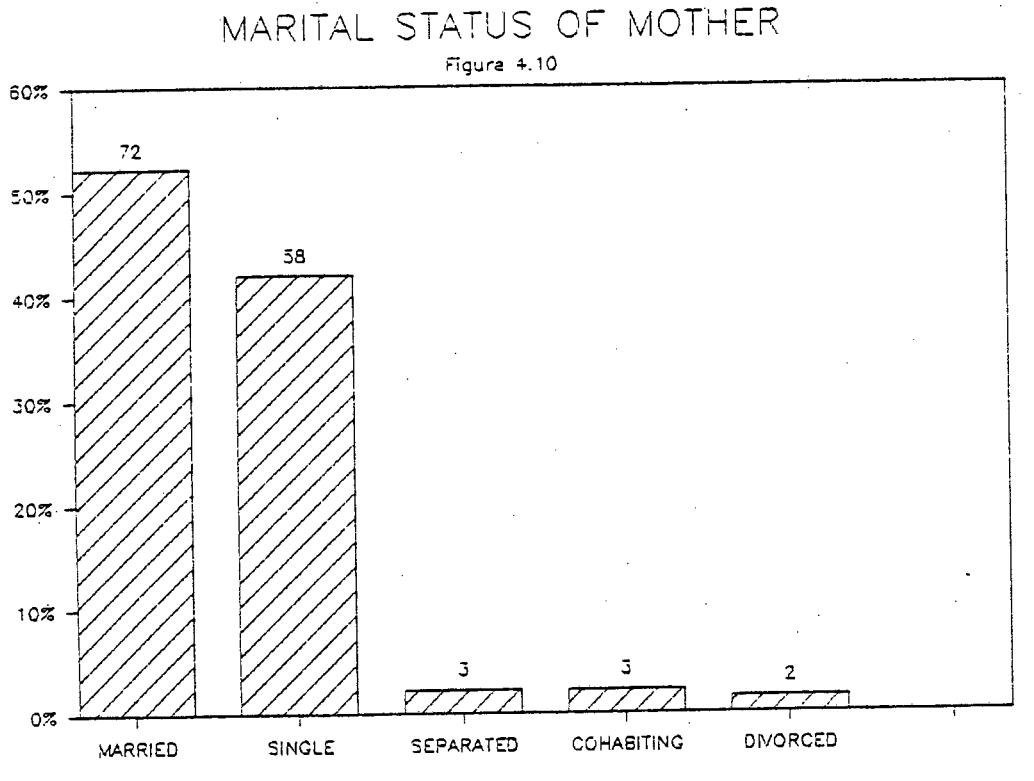


Figure 4.10

Table 4.11 **Changes in mothers' marital status during the study period.**

Status	No.	%
NO CHANGE	125	91%
MARRIED	12	9%
DIVORCED	1	1%
TOTAL	138	100%

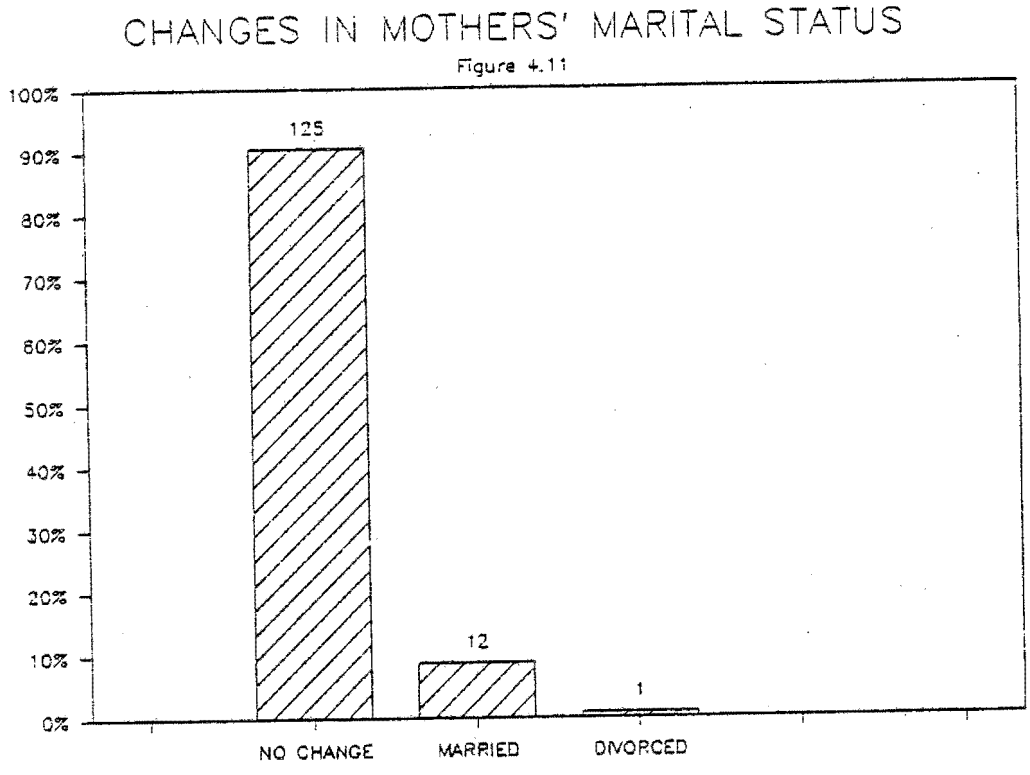
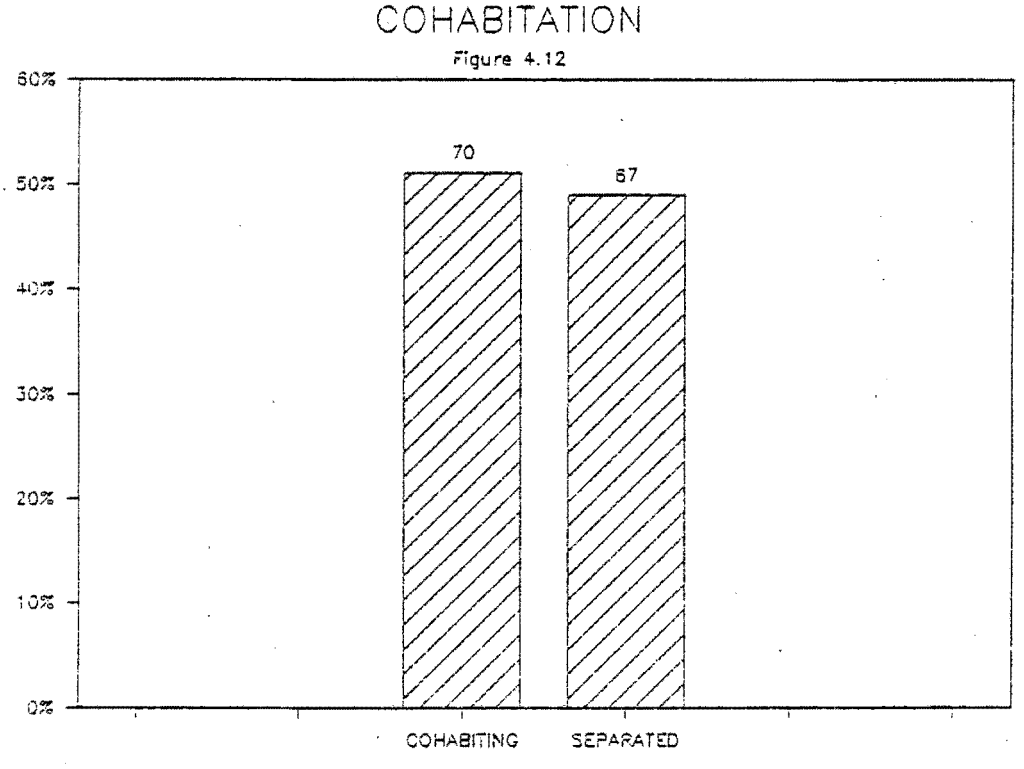


Table 4.12 **Cohabitation - Numbers of mothers (married or single) of study infants who were living with the fathers of their child or were living separately.**

Cohabitation	No.	%
COHABITING	70	51%
SEPARATED	67	49%
TOTAL	137	100%



Measures of economic status

At the start of the study, 3 months post partum, 70% of mothers were housewives. When the survey was conducted, about 6 months post partum, 35% of mothers were in part-time or temporary employment and 20% were in full-time jobs. Tables 4.14 and 4.15 show that 88% of fathers were employed, but that only in only 58% of families did fathers provide the principal source of income. Table 4.16 shows that only 69% of families had a regular source of earnings. In spite of this evidence of restricted financial circumstances, 89% of households had a television set*. Many homes also had a video recorder.

* It would be interesting to know how the television sets were funded, but unfortunately the interview was not structured to obtain this information. For many families the television set was regarded as an absolute necessity, and a few had the attitude that it certainly was not to be relinquished in favour of money to pay rent, water or even electricity accounts!

Table 4.13 **Mothers' occupation 3 months post partum.**

Occupation	No.	%
HOUSEWIFE	72	52%
PART-TIME	47	34%
FULL-TIME	20	14%
TOTAL	139	100%

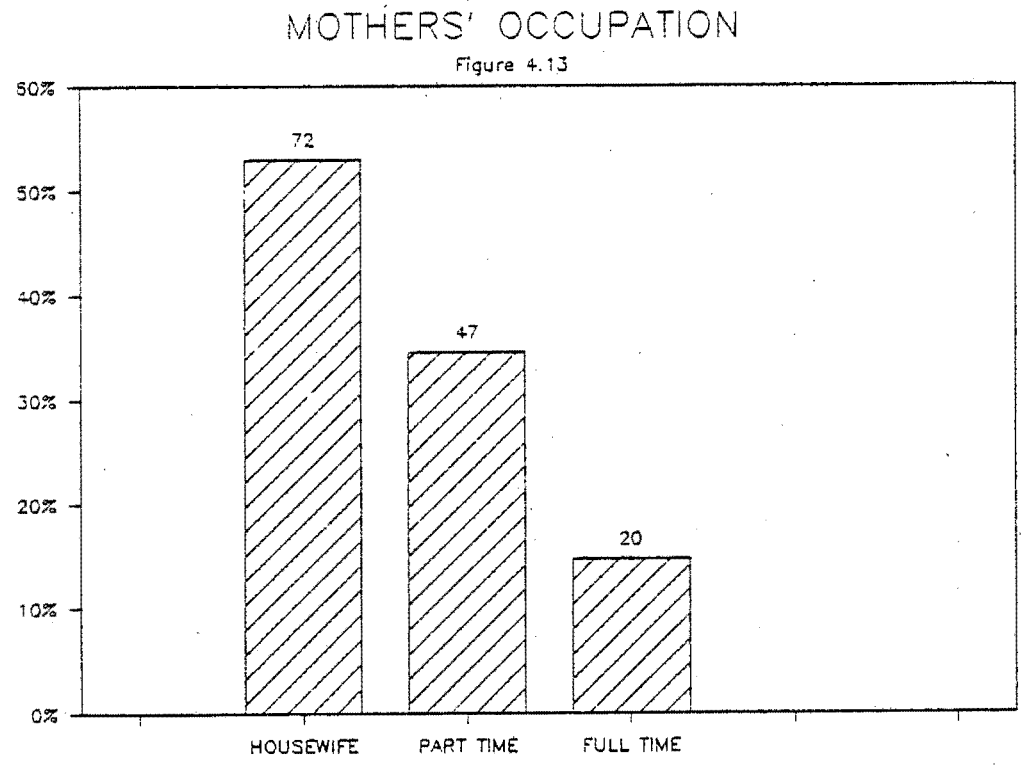


Table 4.14 **Fathers' occupation on entry to the study**

Occupation	No.	%
TEACHER	1	1%
SKILLED	32	24%
SEMI-SKILLED	32	24%
UNSKILLED	52	39%
UNEMPLOYED	16	12%
TOTAL	133	100%

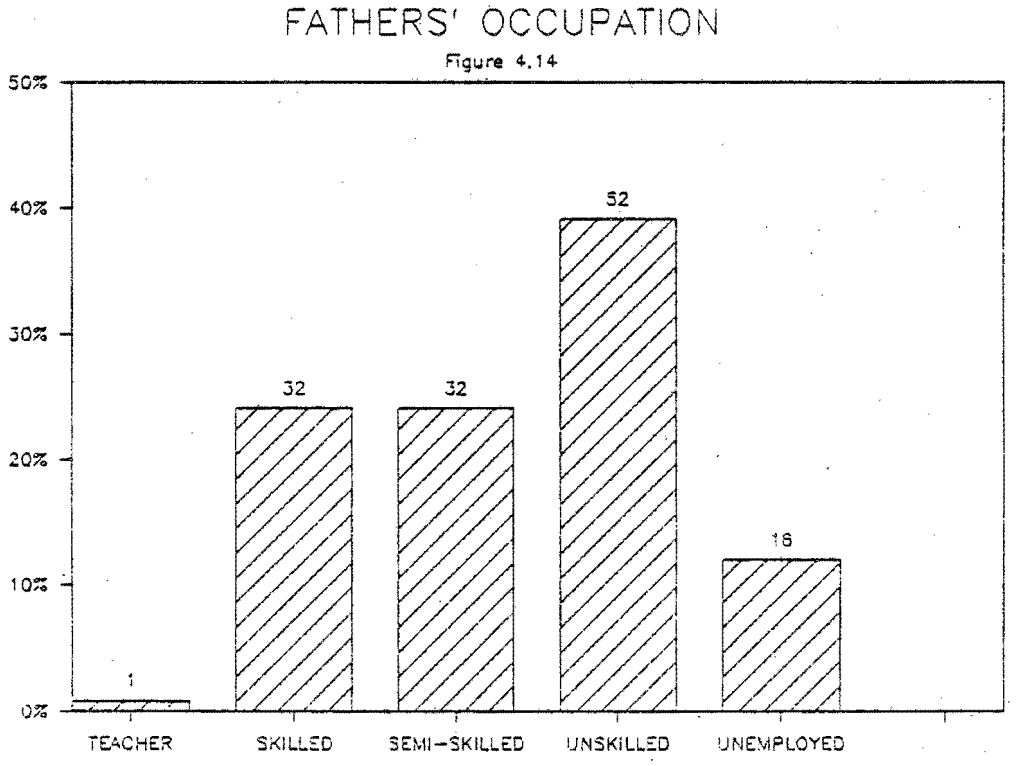


Table 4.15 **Sources of income for the study families*.**

Source	No.	%
FATHER	81	58%
MOTHER	34	24%
FAMILY	8	6%
COMBINATION	6	4%
OTHER	6	4%
STATE GRANT	4	3%
TOTAL	139	100%

Note * Rounding is the cause of the failure to add to 100%

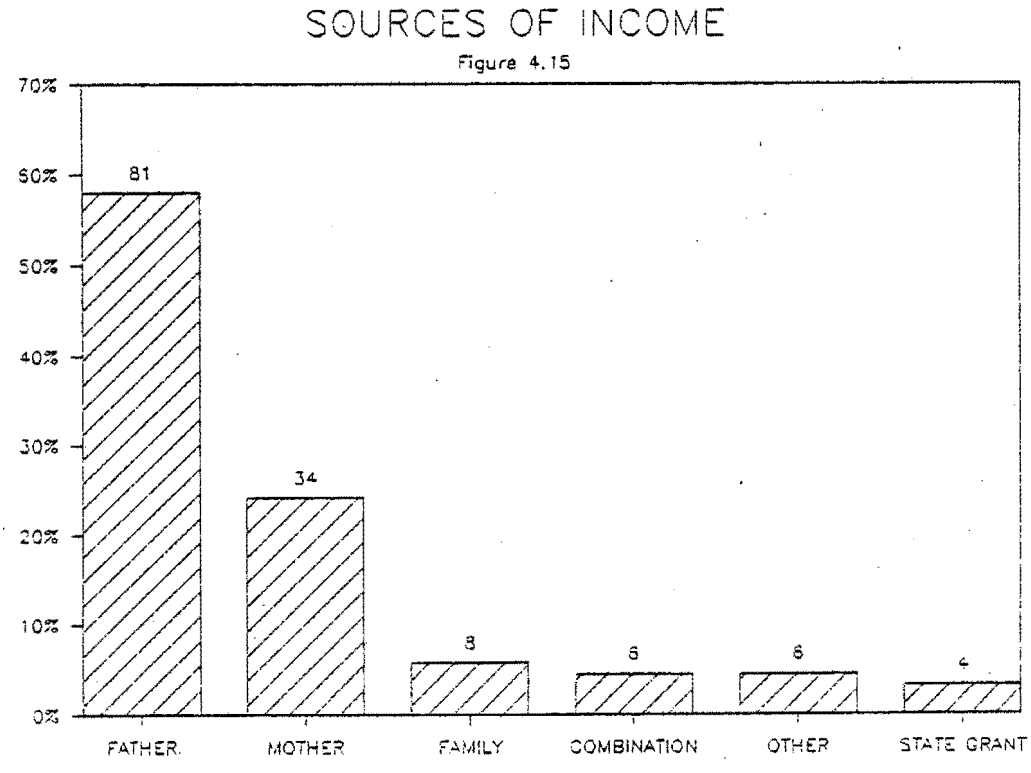


Figure 4.15

Table 4.16 Regularity of income for the study families estimated subjectively by the study team after 9 months observation. The criterion for assigning a subject to the category irregular was that money for food seemed to be a recurring problem.

Regularity	No.	%
REGULAR	89	69%
IRREGULAR	40	31%
TOTAL	129	100%

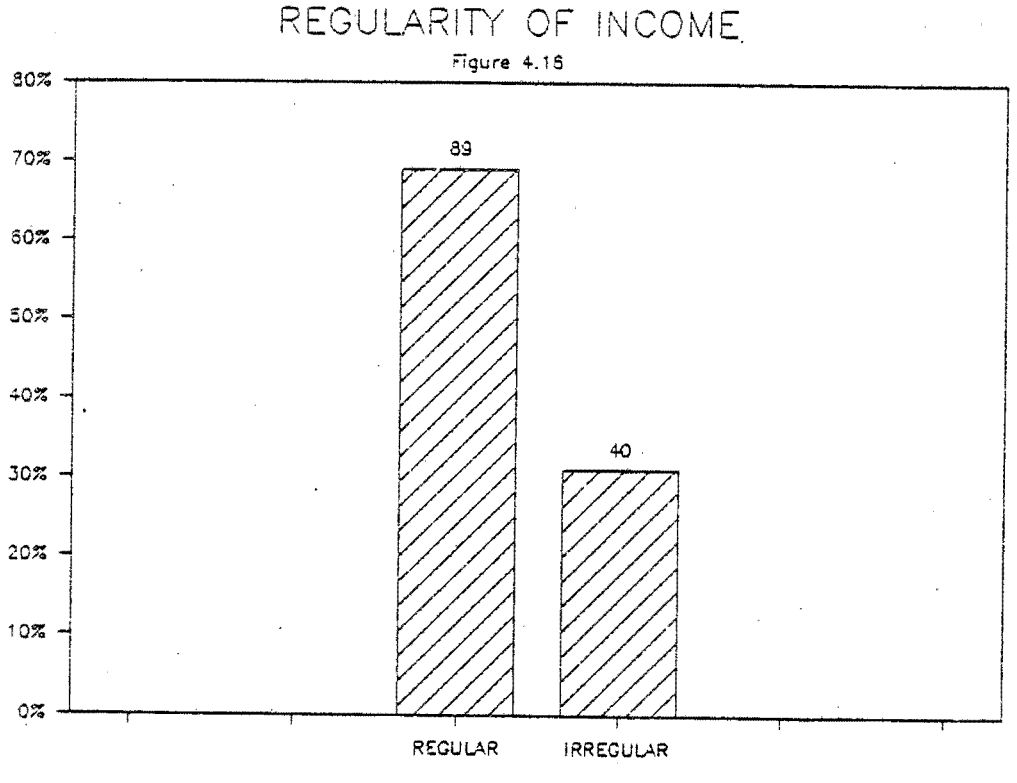


Table 4.17 **Presence of television sets in the homes of families of study infants.**

Television	No.	%
PRESENT	118	89%
ABSENT	14	11%
TOTAL	132	100%

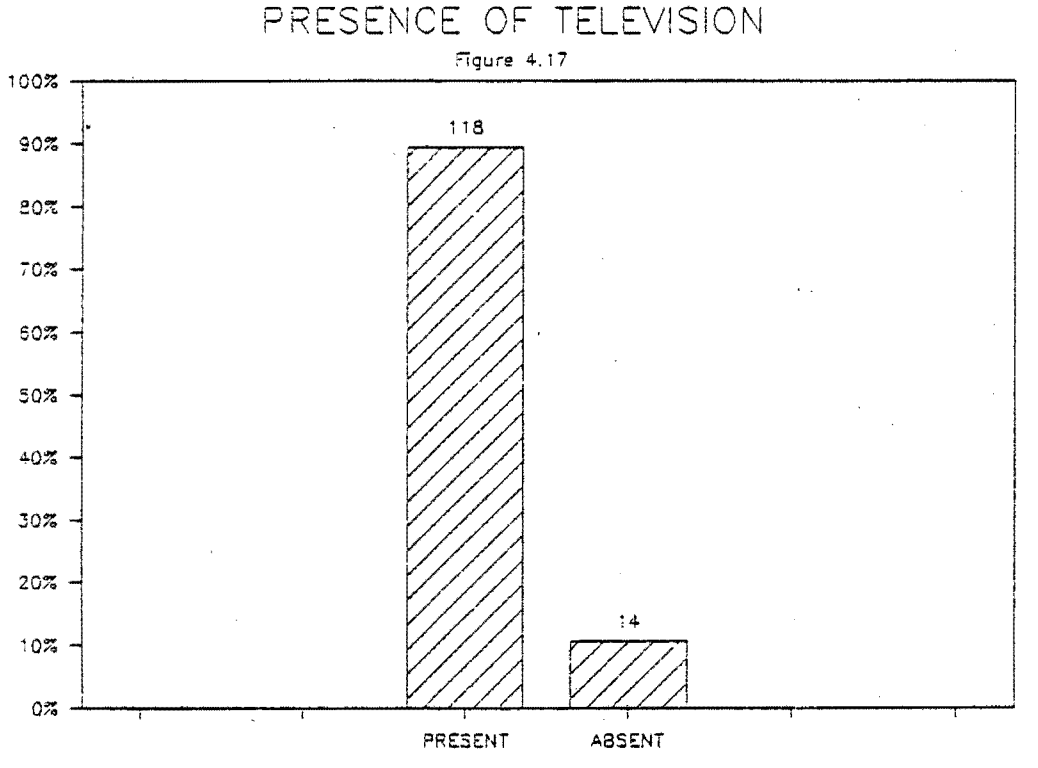


Figure 4.17

Measures of age and educational attainments of parents

Table 4.18 shows that the mean age of fathers of children in the study was 26 years while that of mothers was 24 years. The youngest mother was aged 16, and 27% were under the age of 21 years.

Table 4.18 **Age of parents of study infants when the child was about 6 months of age.**

	MOTHER		FATHER	
AGE	No.	%	No.	%
15-20 yrs	37	27%	13	10%
21-25 yrs	42	31%	51	39%
26-30 yrs	36	26%	41	31%
31-35 yrs	16	12%	18	14%
36-40 yrs	4	3%	7	5%
41-45 yrs	1	1%	1	1%
TOTAL	136	100%	131	100%
	MEAN AGE = 24.		MEAN AGE = 26.	

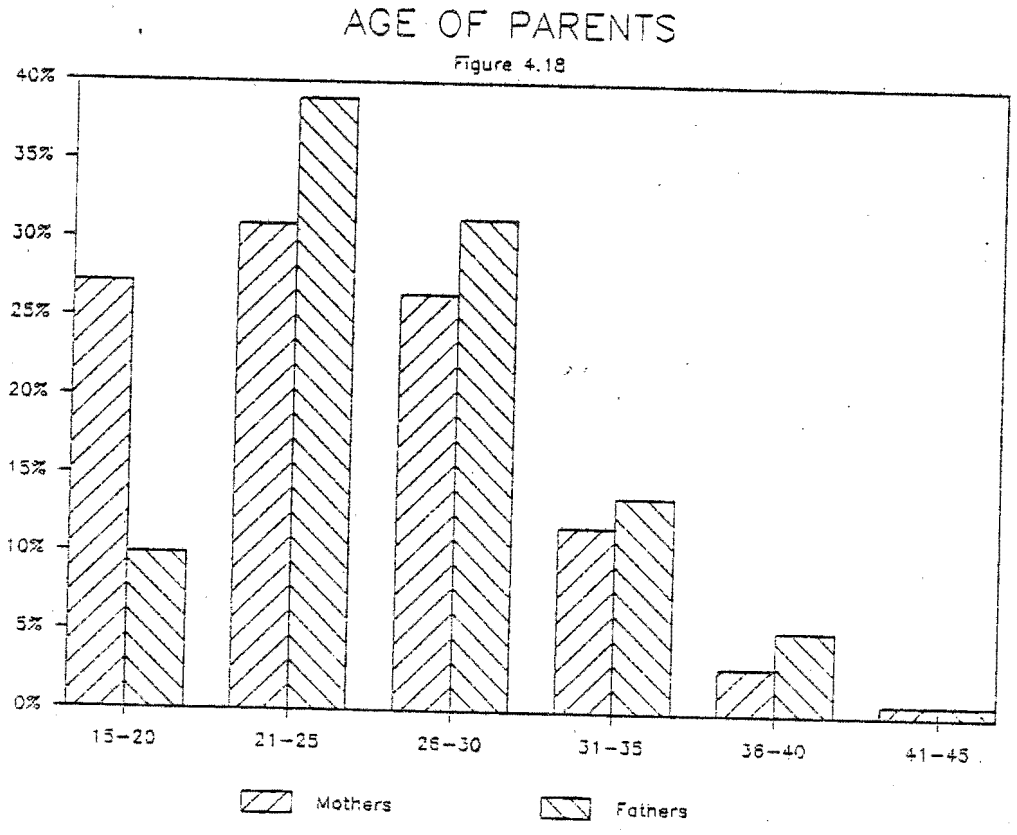
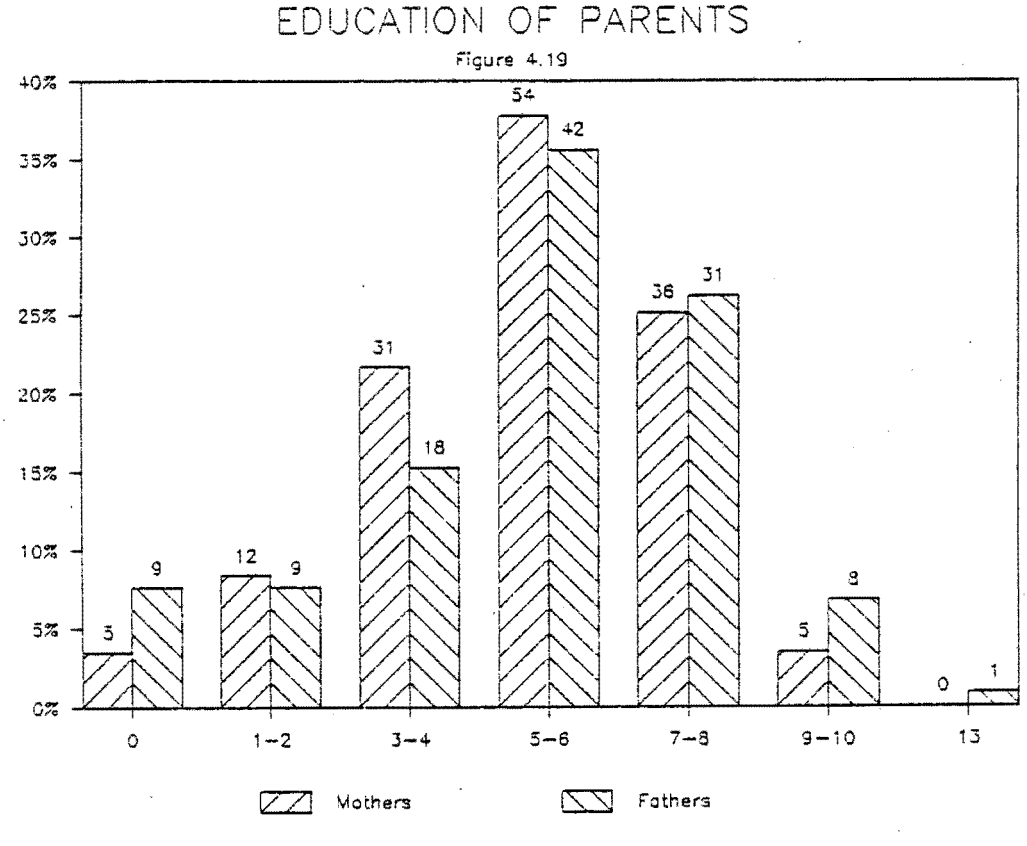


Table 4.19 shows that only one parent had had tertiary education and that 3% of the mothers and 8% of the fathers had had no formal schooling at all. The average educational attainment was standard 5 for both men and women.

Table 4.19 Education of parents measured in years of formal schooling; from 1 to 6 is junior school, from 7 to 10 is high school and 13 is tertiary education*.

Years Education	MOTHER No.	%	FATHER No.	%
0	5	3%	9	8%
1-2	12	8%	9	8%
3-4	31	22%	18	15%
5-6	54	38%	42	36%
7-8	36	25%	31	26%
9-10	5	3%	8	7%
13	0	0%	1	1%
TOTAL	143	100%	118	100%
	MEAN = 5.1		**MEAN = 5.3**	

Note * Rounding is the cause of the failure to add to 100%



Now have I toold you shortly in a clause
The staat, th'array, the nombre and eek the cause
Why that assembled was this compaignye

....

Also I prey you to foryeve it me
Al have I nat set folk in hir degree,
Heere in this tale, as that they sholde stonde.
My wit is short, ye may wel understonde.

The Canterbury Tales
Geoffrey Chaucer
Circa AD 1386

CHAPTER 5 RESULTS: INFANT FEEDING HABITS

Introduction

Infant feeding practices were of interest in the present research for two reasons. Firstly, as the main project was concerned with a trial of a milk formula with increased iron concentration, it would have been useful to have documented the actual quantities of formula (and other foods) ingested. If the diet had been determined it would have been possible to estimate the total intake of iron and its availability.

A second source of interest in feeding practices arose from ethical concerns about a possible conflict between the community's perception of the value of breast feeding and the influence of the trial of the infant milk formula. As was explained in chapter 2, mothers who were breast feeding were included in the trial and were encouraged to continue nursing their infants for as long as they wished. These mothers were also provided with milk formula, if they asked for it, in order to encourage their continued participation and continued breast feeding. The intention was that no mother should feel pressured to remove her child from the breast.

Studies made

Introduction

Studies were made of the age at which children in the trial and children in the community were weaned from the breast; of the prevalence of breast feeding in the community before, during and after the trial; and of the reasons for stopping breast feeding in trial children as well as in children from the community. In addition, a survey was made of the age at which various solid foods were introduced to infants in the trial.

In the planning stages of the project it was imagined that, if each child was to be examined every 3 weeks, it would be possible to gather an accurate history of the changes in the child's diet throughout the study. Two unforeseen factors rendered this plan unworkable. In the first place, if the child was well, he or she was often brought to the examination by a relative or friend of the mother who would not know what feeds the child was taking. With many mothers working, it was often the case that different people cared for the child during the day and during the night.

A second and greater problem was cultural in nature. There was a widespread inability of people in the community to think quantitatively. Even intelligent, caring and cooperative mothers found it impossible to recall with accuracy how much milk the child had taken in the previous 24 hours. However, part of the problem in communicating may also, it seems with hindsight, have been due to a fear on the mother's part that she was being monitored in her use of the milk and that the supply of milk might be reduced if she was not "performing". Obtaining full dietary data would have entailed

several additional time-consuming visits by the study team to each child's home. As these resources were unavailable this part of the project was reluctantly abandoned.

Ascertainment and Survey Construction

Data for the children in the main trial was gathered throughout the study. At every contact, the parents or caretakers were asked the appropriate questions about feeding.

Data for children in the community was acquired during three surveys conducted in the Bonteheuwel Health Clinic during April 1983, September/October 1983 and September/October 1984. The first survey was carried out shortly before the main study commenced, the second survey was made immediately after the last infant had been enrolled in the trial, and the third was performed shortly after the trial had concluded.

The Bonteheuwel Health Clinic provides immunization and other primary health care services. Most of the children attending the clinic are well, but some are brought for the treatment of minor ailments. Infants are weighed before being seen by the clinic staff, and mothers in this queue, with children of 12 months of age or younger, were asked the age, sex, weight, age of first bottle feed, age of last breast feed and, if appropriate, the reason for weaning the child from the breast.

Similar information was gathered in an ongoing fashion from mothers of children in the main study. In addition, they were also asked the age at which they introduced cereal, egg, fruit, vegetables and fish and meat to the child's regular diet.

Acknowledgements

The questionnaires were administered by Mrs D Phillips.

Methodological Cautionary Note.

Some methodological problems inherent in the study need to be borne in mind in evaluating these results.

The body of data from the trial infants constitutes a longitudinal study from a well defined, homogeneous group whereas the surveys at the clinic are cross-sectional studies from an inhomogeneous and poorly defined group. Data from the two sets are not directly comparable. Even within the community group comparisons of one time period with another may be misleading because of the unselective nature of the surveys.

A further problem is that with the intention of improving the precision in collecting the data a small change was made to the form used for the third survey*. In retrospect it is now clear that this imposes an unknown change on the data which reduces the validity of comparisons made with the 2 surveys in 1983.

A third methodologic weakness in the study is the lack of a control group. If the surveys had also been conducted in a similar clinic in a community not subjected to the influences of the trial this would have provided a yardstick against which to evaluate the effects of the trial against the effects of changing customs. Lack of resources prohibited control surveys from being made.

The age of the children was recorded as months attained. The surveys would have been more precise and accurate had the ages been recorded in decimals of a year.

The prime reasons for mothers bringing their children to the clinic are for routine postnatal follow up and for immunization. The common age brackets of infants attending the clinic are thus less than 2 months and between 3 and 6 months. Age groups that are uncommon at the clinic are 2 months and between 7 and 12 months.

Results:

The results of the surveys are shown in tables 5.1, 5.2 and 5.3 and in figures 5.1, 5.2 and 5.3. The data for children over the age of 6 months is not shown because the numbers are small and the results may be confusing.

Age of weaning off breast

Table 5.1 shows the age (in months) at which children were first offered a bottle of milk. The results for the study children are similar to those for the infants from the community. About 15% of babies were first offered milk in the first month of life. A third of infants begin drinking milk in each of the second and third months of life and by the end of the fourth month only 5% were exclusively breast fed.

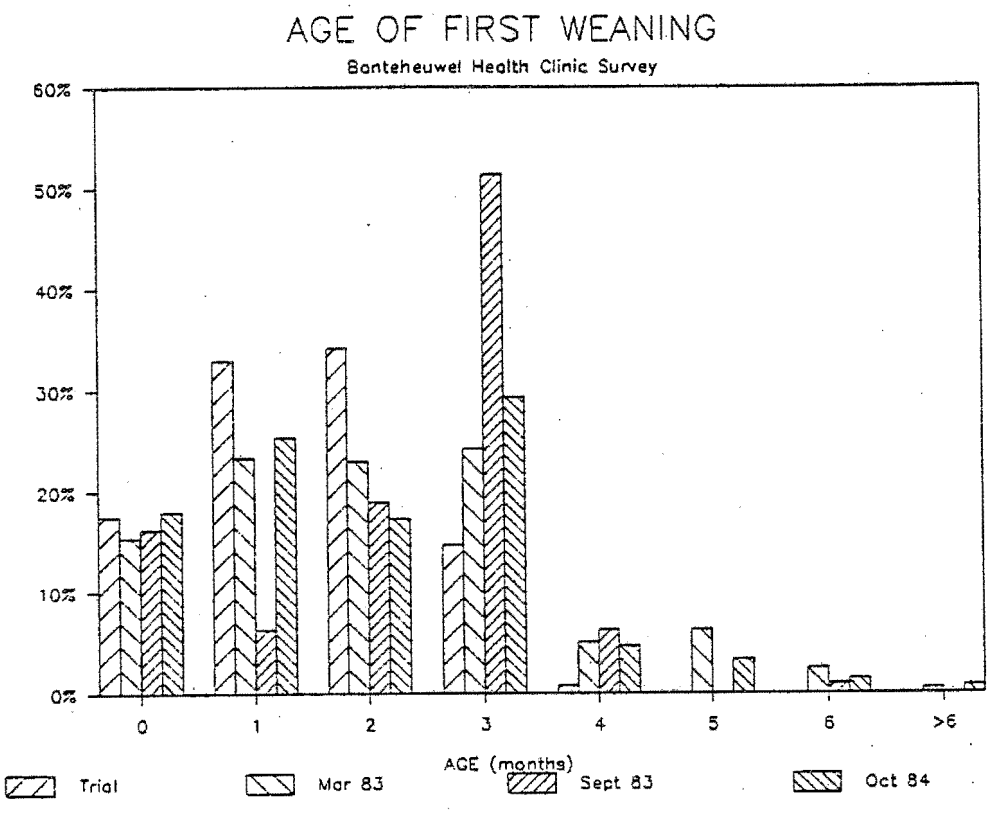
Because of the early introduction of milk feeds, the cross-sectional studies provide a relatively unbiased view and comparison with the trial children is valid. Weaning from the breast is often completed much later. A cross-sectional study with children at different ages will have many in various stages of the weaning process and it is thus not possible to compare the ages at which children are last given the breast.

* The forms are included in appendix 6

Table 5.1 **Age at which infants are first offered milk formula. Comparison between the infants in the trial and infants from the community of Bonteheuwel surveyed April 1983, September/October 1983 and September/October 1984.**

AGE (months)	TRIAL		MAR '83		SEPT '83		OCT '84	
0	26	17%	37	15%	18	16%	27	18%
1	49	33%	56	23%	7	6%	38	26%
2	51	34%	55	23%	21	19%	26	17%
3	22	15%	58	24%	57	51%	44	30%
4	1	1%	12	5%	7	6%	7	5%
5	0	0%	15	6%	0	0%	5	3%
6	0	0%	6	3%	1	1%	2	1%
TOTAL	149		239		111		149	
MEAN AGE	1.5		2.1		2.3		2.0	

Notes. The survey methods differ for the infants from the Trial and the Community. The results are not strictly comparable. Details are given in the text.
Rounding causes some of the columns of percentages not to total 100%.



Prevalence of breast feeding in Bonteheuwel

Table 5.2 shows, for children in their first year of life, the prevalences of breast feeding, formula feeding and combined formula and breast feeding. The data are from surveys made in April 1983, September/October 1983 and September/October 1984.

Figure 5.2 shows a linear trend for the prevalence of breast feeding to fall from around 80% in the first month of life to about 10% by 4 months of age.

The figure shows a tendency for the prevalence of breast feeding to fall with time. As explained in the methods section, interpretation of this is hampered by lack of a control group for comparison and the slightly different data collection procedure adopted for the last survey. The difference between the April 1983 and September/October 1984 surveys (*ie* before and after the trial) is not statistically significant*.

In view of the ethically sensitive nature of trials involving infant feeding formulas, studies similar to the main project should in future include well controlled and standardized surveys of breast feeding practices.

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* Regression analysis of prevalence of breast feeding (y) on age for the first 5 months (x) yielded:
For the April 1983 group $y = -16.14x + 83.0$
For the Sept/Oct 1984 group $y = -14.57x + 73.76$

The F values for the slopes and intercepts are 0.3 and 1.35 respectively. The P values for significance tests of parallel slopes and equal intercepts are greater than 0.05.

Table 5.2 Prevalence of breast, formula and combined breast and formula feeding in Bonteheuwel in April 1983, September/October 1983 and September/October 1984.

AGE (months)	Formula		Breast		Breast & Formula		TOTALS
APRIL 1983							
0	1	1%	4	5%	72	94%	77
1	2	13%	4	25%	10	63%	16
2	1	20%	2	40%	2	40%	5
3	33	35%	29	31%	32	34%	94
4	33	37%	34	38%	22	25%	89
5	35	83%	5	12%	2	5%	42
6	35	47%	33	44%	7	9%	75
SEPTEMBER 1983							
0	3	8%	2	6%	31	86%	36
1	3	33%	0	0%	6	67%	9
2	0	0%	0	0%	4	100%	4
3	18	44%	7	17%	16	39%	41
4	17	63%	4	15%	6	22%	27
5	16	100%	0	0%	0	0%	16
6	15	68%	3	14%	4	18%	22
OCTOBER 1984							
0	2	9%	4	18%	16	73%	22
1	8	36%	2	9%	12	55%	22
2	10	50%	3	15%	7	35%	20
3	18	60%	4	13%	8	27%	30
4	30	88%	2	6%	2	6%	34
5	15	65%	3	13%	5	22%	23
6	20	83%	2	8%	2	8%	24

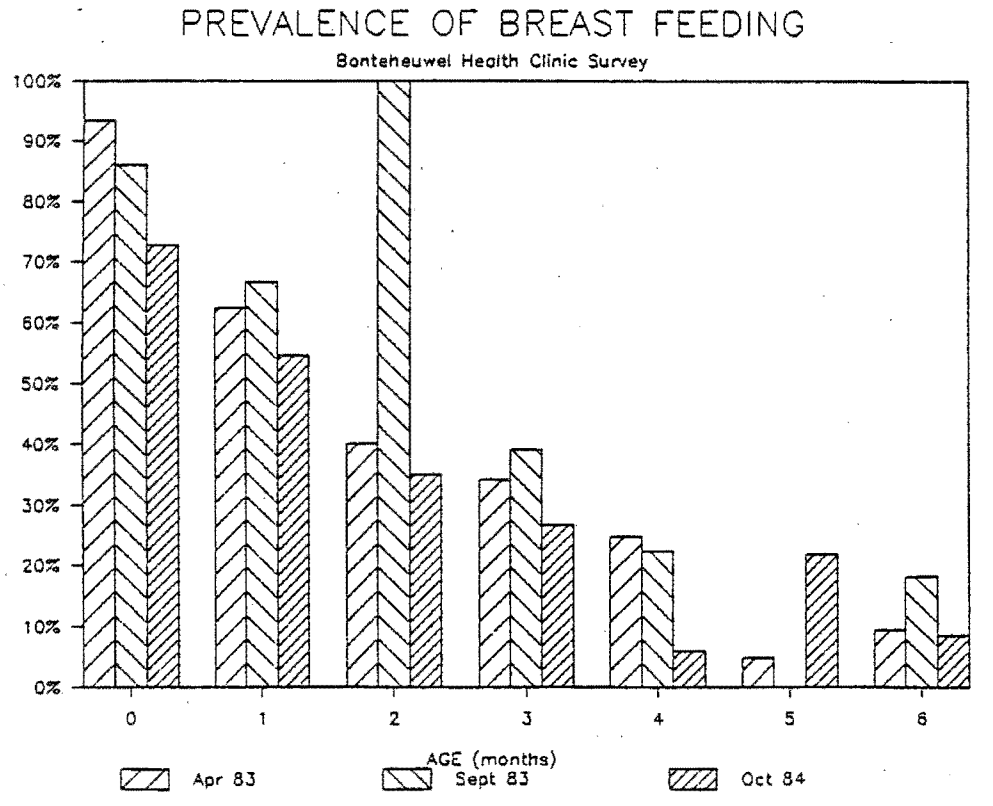

Figure 5.2 **Prevalence of exclusive breast feeding in the first year of life.**

Reasons for cessation of breast feeding

Table 5.3 and figure 5.3 summarize the replies of mothers to the question *Why did you stop breast feeding?* No striking difference is apparent between the Trial group and the three Community surveys. The most common answers were the demands of employment and that the child was not getting enough milk from the breast.

Table 5.3 **Reasons for stopping breast feeding given by mothers of the infants in the main trial and mothers in the community of Bonteheuwel.**

REASONS FOR WEANING

REASON	TRIAL		MAR '83		SEPT '83		OCT '84	
Working	35	23%	96	40%	49	45%	57	36%
Social	20	13%	6	2%	3	3%	5	3%
No milk	80	54%	87	36%	45	42%	58	37%
Breast disease	3	2%	15	6%	3	3%	11	7%
Baby refused	5	3%	30	12%	3	3%	13	8%
Other	6	4%	9	4%	5	5%	14	9%
TOTALS	149		243		108		158	

Note Rounding causes some of the columns of percentages not to total 100%.

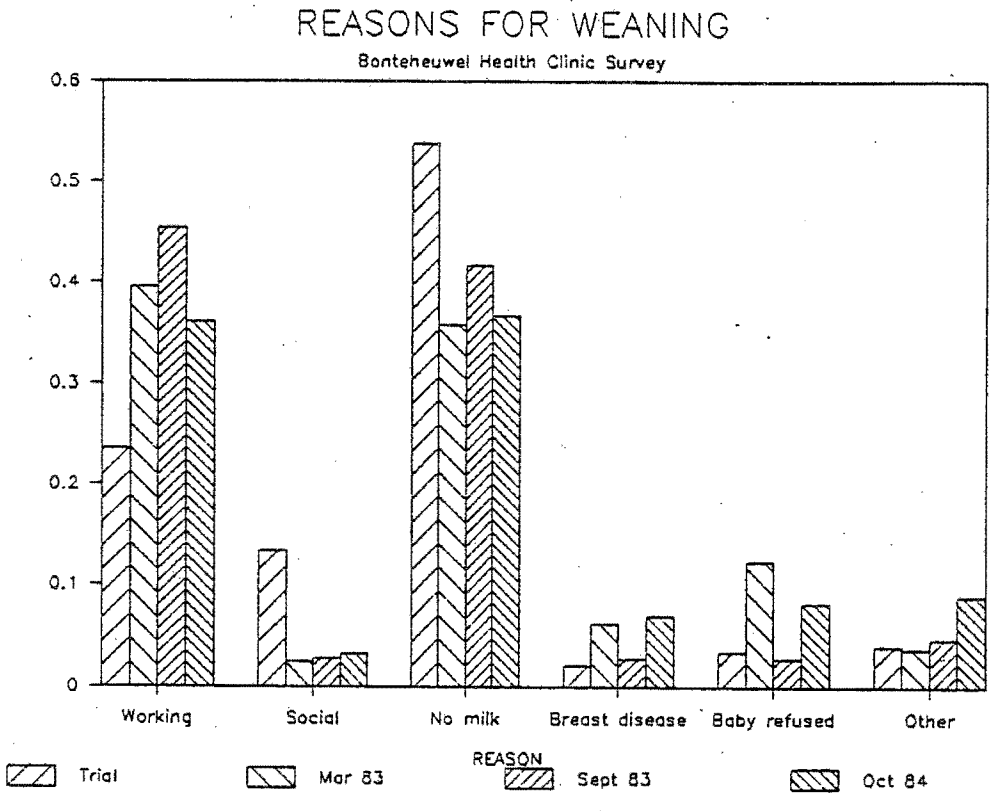

Figure 5.3 **Reasons for stopping breast feeding offered by mothers in the main study and by mothers in the community of Bonteheuwel**

Conclusions

The results of the feeding surveys suggest that the prevalence of breast feeding in Bonteheuwel may have declined during the period of the main study although this was not statistically significant. It is not possible to attribute the change in feeding practices to the influence of the trial itself for various reasons that are detailed above.

Having noted these problems in interpretation, it should also be noted that the present study has gone further than similar previous studies which have not reported their influence on breast feeding[314, 7, 323, 298]. Previous investigators, one may speculate, have operated in times when there was less awareness of the need to protect mothers, particularly in underdeveloped areas, from excessive promotion of infant formulas to the detriment of the health of their children.

Future trials of infant milk formulas should include well controlled surveys of breast feeding practices so that more substantive conclusions can be drawn about their effects on infant feeding practices.

CHAPTER 6 RESULTS: IRON FORTIFICATION

Introduction

The results from the principal study are reported and discussed in this chapter. Appendix 3 contains a more detailed tabulation of the statistical analyses that were made. Results of interest are extracted from the schedules in the appendix and presented in more easily read tables and graphs.

The analysis is presented in three main sections. In the first, the validity of the Control group is established. In the second, the Test group is compared with the Control group in order to define the effects of the increased iron fortification in the milk formula offered to the Test group. In the third section of the analysis, an association is sought between iron status and immune function. Finally, evidence from the trial for the safety of iron fortification is reviewed.

Statistical methods

Introduction

The statistical methods employed are described in chapter 3. This section explains the graphical representations used and some applications of the statistics.

Graphical representation of numerical results

The figures in this chapter use the same graphical conventions to compare results for the Test and Control groups, show the difference between the 2 groups, the confidence limits of the means and statistical significance from a t test. The mean value of a variable is represented by a bar and the 95% confidence interval is depicted by a vertical line. The difference between the two groups is shown by the difference in height between the bars. The difference is also shown graphically by a separate bar with a line representing the 95% confidence interval through it. If this confidence interval line does not cross the x-axis the difference between the two groups is significant at the 5% level. These concepts are illustrated in figure 6.2 which shows a significant difference and in figure 6.22 which shows a non-significant difference.

Comparison of changes

The results of the intervention employed in the trial are often expressed in terms of the values of variables measured at the end of the trial. In many instances however, the effect of the intervention is most rigorously measured by the change in that variable over the course of the study. This takes account of small differences that exist at the start of the trial. For example, the difference in haemoglobin concentrations between the two groups was 0.35 g/dl at the end of the trial. But the difference in the change in haemoglobins was 0.49 g/dl. The figures have all employed absolute values as these result in clear graphs

and the conclusions drawn from them are consistent with those drawn from a comparison of changes. For precision, the tables and text, where appropriate, give the change, increase or decrease as well as the absolute magnitude of results. The paired t test was used to compute P values when changes were compared.

Validity of the Control group
(Comparison of Test and Control groups at 3 months)

Rationale

A key factor in determining the strength of inferences drawn from a controlled trial is the comparability of the control group with the test group. At the start of the trial the two groups should be identical with respect to all the variables to be compared. The care taken in the present study to construct control and test groups without bias was described in chapter 3. An important question remains to be answered: *Were the Control and Test groups actually comparable?*

Methodology

This question is answered by comparing the results of the various tests and measurements made at the age of 3 months. If the selection procedures worked as intended there should be no difference between the test results for the two groups. For this reason, the tables of results in the following sections show the results of tests made at 3 months of age although the discussion is focused on the differences found at 12 months. The statistical tests show that the groups may be assumed to be equivalent at the start of the trial. And, apart from the evaluation below of the results for two classes of variable of particular interest, no further comment is made on the validity of the control group.

Statistical tests for equivalence

Before commenting on the results, a few remarks need to be made on statistical procedures used but not mentioned in the chapter on methods and materials. The appropriate statistical test is a test for *equivalence* rather than the usual test for a *difference*. The test for a difference often results in "P value" of statistical significance. If the P value is not significant, it does not follow that the two groups are necessarily equivalent[411]. The reason for this is that a real difference might be missed by an inappropriate test.

An additional complication in the search for evidence for equivalence is that when many variables are compared, it is to be expected that about 5% will appear to be significantly different purely by chance[70]. Interpretation of the statistical tests therefore involves consideration of factors other than the absolute level of the P value.

The approach to equivalence testing taken in this section is to see if the 95% confidence interval of the difference between the variable under consideration in the Control and Test groups contains a clinically or physiologically important disparity. The determination of what differences are *important* is necessarily arbitrary and subjective to a certain degree. But this should not be seen as a move from the "objective" concept of statistical significance to the subjective concept of importance, but as a step towards the quantification of what was actually determined by the study[170].

Iron status

As iron status was the variable to be manipulated in the study (*ie* the independent or input variable) it is important to confirm the equivalence of the Control and Test groups at the start of the experiment. Table 6.1 lists the major indices of iron status for the Control and Test groups . (Appendix 3 contains a full tabulation of all statistical analyses.)

Table 6.1 Iron status at 3 months of age compared in the Control and Test groups in order to establish equivalence of the two groups.

Index of Iron Status	*Control Mean*	*Test Mean*	*P Value*	*95% CI of diff. of means*		*Smallest Important Diff.*
Haemoglobin (g/dl)	11.28	11.38	51%	-0.19	0.37	+0.5
Haematocrit (%)	32.89	33.27	34%	-0.41	1.17	+1.0
Mean cell volume (ul)	82.06	82.10	97%	-1.80	1.87	+1.0
Red cell ZPP (ug/g Hb)	2.92	2.82	63%	-0.50	0.30	+1.
ferritin[1] (ug/l)	117	120	91%			+10

Notes P values were calculated from student's t test.
95% CI = 95% confidence intervals.

1 The geometric means are shown for ferritin. 95% confidence limits were calculated for log(ferritin) and are shown in appendix 3.

It may be seen from the table that no variable differs significantly between the Control and Test groups; the lowest P value is 34%. The 95% confidence interval includes zero difference for all the measures of iron status. The smallest clinical or physiologically important difference is greater than the measured difference for all the variables and is inside (by a narrow margin) the 95% confidence interval only for haematocrit and mean cell volume.

All the measures of iron status shown in table 6.1 show a slight tendency in favour of the Test group. This trend is far from reaching statistical significance and the differences are all minor. For the purposes of the present research it can thus be concluded that the Control and Test groups had comparable iron status at the start of the trial.

Future studies of a similar nature should consider stratification of the subjects according to their iron status in order to ensure deterministically rather than stochastically that the two groups have equivalent iron nutrition.

Participation rate and gender

Table 6.2 compares the Test and Control groups with respect to completion rate and gender. The table shows that the two groups are equivalent as far as the later two variables are concerned. The proportion of subjects who completed the trial differs between the Test and Control groups and a chi square test of significance yields a P value of 7%.

This poses a question about the equivalence of the two groups: *If the dropout rates are different between the Test and Control groups are the groups still equivalent with respect to other variables?*

One may speculate that the families who dropped out were indeed different in many other respects from the families who remained in the study. The consequence of this would be to reduce inhomogeneities in the remainder and thus the differences between the two groups would be diminished. (With respect to completion rate the differences disappear entirely.) With such small numbers and incomplete data it is not possible to test this hypothesis. Although it seems unlikely that the disparity in completion rates is important, the possibility of some bias should be borne in mind when interpreting the effects of the experiment.

Table 6.2 Participation and Gender compared between the Control and Test groups.

	Control	Test	P
Participation			
Number at start	74	75	
at completion	62	70	7%
Sex			
Male	38	39	
Female	36	36	94%

Notes P values are calculated from the chi square test.

Specimen collection rates

The failure to collect specimens was an unimportant problem, except for the initial full blood counts which were obtained from 122 of 149 subjects. Initial indices were missing in 18 of 74 infants in the Control group and 9 of 75 in the Test group. Chi square analysis of this distribution yields a value of 3.81 which is very close to the value (3.84) for statistical significance at the 5% level. This raises the question whether there may have been some bias in assembling the 2 groups.

This fear is dispelled by similar analyses of the distribution of missing specimens for other parameters. For example, a total of 9 specimens were not obtained for plasma ferritin and 15 for red cell zinc protoporphyrin at 3 months and chi square analysis yielded values of 0.45 and 0.08 which are very far from the value (3.84) denoting statistical significance at the 5% level.

The uneven distribution of full blood counts at 3 months also raises the question of a possible bias in comparisons involving these parameters. Comparisons of changes over the trial period in haemoglobin, mean cell volume and red cell distribution width were made using paired samples from the same child at both 3 and 12 months. These gave essentially equivalent results as when group means were compared. The data are given in table 6.6 and 6.7.

It may therefore be concluded that the uneven distribution in missing specimens neither indicates a bias in assembling the groups, nor affects interpretation of the results of the study.

A number of factors accounted for the missing specimens. Since the infants were to receive 2 painful injections for the delayed cutaneous hypersensitivity tests, it was decided on ethical grounds not to attempt venesection more than twice. There were 9 complete failures to obtain blood specimens at the start of the

trial and 5 at the end. The first portion of blood was decanted into heparinized tubes for the plasma measurements and immune function tests. The later portion of blood was placed into EDTA tubes for the full blood count. In retrospect it seems that it may have been wiser to have collected the full blood counts first since they were more easily invalidated by minor degrees of platelet clumping and clotting than the other tests.

Temporal comparisons

Differences were sought, but were not found, for temporal disparity between the Control and Test groups. The variables that were available for examination were the ages of the children and the dates of entry to and exit from the study. The two groups did not differ significantly with respect to any of the 4 measures and the only trend that might have biased the study was the slightly longer participation period for the Test group *viz* 8.96 months compared with 8.85 months for the Control group. This would have tended to increase the number of infections observed in the Test group*.

Table 6.3 Temporal variations compared between the Test and Control groups. Dates of birth, entry to the study and exit from the trial are compared for the two groups as well as the ages of the subjects on entry and exit.

Variable	*Control Mean*	*Test Mean*	*P Value*	*95% CI of diff. of means*		*Power Obs*	*10%*
DATES (decimals of a year)							
Date of birth	83.30	83.32	29%	-0.02	0.06	19	100
Date of entry	83.54	83.56	33%	-0.02	0.05	17	100
Date of exit	84.27	84.30	14%	-0.01	0.07	31	100
AGES (months)							
Age on entry	2.96	2.90	14%	-0.15	0.02	31	99
Age at exit	11.81	11.86	41%	-0.06	0.15	13	100

Notes P values were calculated from student's t test.
95% CI = 95% confidence intervals.
The statistical power of the study was determined for both the observed difference (*Obs*) and a 10% difference (*10%*) from the combined mean for a type I error probability of 5%.
Dates are given as the decade plus decimal of a year. For example, 83.30 is 20 April 1983.

* Calculation of the rate of infection adjusts for differences in periods of observation.

Age related changes

(Comparison of results at 3 and 12 months)

Between the age of 3 and 12 months, major developmental changes are taking place and the reader will observe differences in many variables comparing the two age groups. It was not the purpose of this study to investigate such changes, and no further remarks will be made on this subject.

Study children - Feeding practices

Before considering the effects of the increased iron fortification it is of interest to examine the feeding practices of the study mothers.

Breast feeding

Results

Table 6.4 compares the extent of breast feeding in the two groups. Mothers in the Control group began bottle feeding their infants at an average age of 1.53 months. This was not significantly different from the mean age of 1.49 months for the Test group. Mothers in the Control and Test groups completed the weaning process when their infants were 3.60 and 4.04 months of age respectively. The difference is far from statistical significance with the 95% confidence interval ranging from -0.94 to 1.83 months.

Table 6.4 Breast feeding compared between the Control and Test groups. The extent of breast feeding is measured by two estimates: the mean age at which milk formula was first given and the mean age at which the breast was last offered to the infant.

Variable	Control Mean	Test Mean	P Value	95% CI of diff. of means		Power Obs	Power 10%
Age weaned (in months)							
First given formula	1.53	1.49	83%	-0.36	0.29	0%	16%
Last given breast	3.60	4.04	52%	-0.94	1.83	0%	0%

Notes P values were calculated from student's t test.
95% CI = 95% confidence interval.
The statistical power of the study was determined for both the observed difference (Obs) and a 10% difference (10%) from the combined mean for a type I error probability of 5%.

Solid foods

Introduction

At every encounter with each child in the main study (*ie* at approximately three week intervals) the mother or caretaker was asked at what age cereal, egg, fruits, vegetables and fish/meat were added to the child's regular diet.

Results

The results are shown in table 6.5. The first solid foods to be given are cereals and vegetables which are introduced from the first to third month. Eggs and fruit are added about a month later and fish and meat are included slightly later still. There is no significant difference between the 2 groups. Interesting trends are the earlier ages at which the Test group were offered egg, meat and fish in comparison with the Control group. The smallest P value, 6.4%, was for meat and fish.

Table 6.5 **Age of introduction to solid foods for the Control and Test groups.**

Food	*Control*	*Test*	*P*	*95% CI of diff.*		*Power*	
	Mean Age	*Mean Age*	*Value*	*of means*		*Obs*	*10%*
Meat/Fish	5.19	4.36	6.4%	-1.04	0.02	46%	44%
Cereal	2.06	1.93	>10%	-0.21	0.31	0%	35%
Egg	4.24	3.49	>10%	-0.95	0.15	29%	30%
Fruit	4.16	4.16	100%	-0.48	0.48	0%	40%
Vegetable	3.40	2.96	>10%	-0.60	0.20	17%	36%

Notes P values were calculated from student's t test.
95% CI = 95% confidence interval.
The statistical power of the study was determined for both the observed difference (Obs) and a 10% difference (10%) from the combined mean for a type I error probability of 5%.

Discussion

The survey of breast feeding practices and of the ages at which solid foods were introduced, showed trends that would have favoured the Test group, as far as iron nutrition is concerned since meat and fish not only contain readily available haem iron, but also promote the absorption of non-haem iron[104, 237]. The trends were not statistically significant.

The date at which a certain feeding practice is initiated, *eg* bottle feeding, provides a crude measure of nutrient intake. A complete dietary survey at various points in the trial would have provided answers to questions about quantity and frequency that the present surveys could not. Cook and Reusser[104] recommend that the development of a strategy for iron fortification include determination of iron intake from dietary surveys as the step following ascertainment of the prevalence of iron deficiency. The present project did not have the resources to attempt such a large project, but, as the present study demonstrates, lack of dietary information can hinder interpretation. None of the trials on which the present study was modeled have reported detailed dietary studies[7, 71, 77, 221, 285, 294].

A weakness in the methodology is that the ages at which events occurred were recorded as months attained. The precision and accuracy of the data would have been improved if decimals of a year had been employed[447].

A weakness in the gathering of the data is that the information was obtained from the caretaker of the infants. If the mother was working, the information provided by the day-time caretaker may not have been as accurate as could have been provided by the mother.

Conclusions and recommendations

The trends shown in the feeding practices may indicate a confounding factor, both in terms of iron nutrition and in terms of immune function. This possible bias in favour of the Test group should be borne in mind in interpreting the results of the trial.

It would be useful if future trials of iron fortification of infant foods include dietary surveys of ingestion of the food vehicle and inhibitors and facilitators of iron absorption[125]. The aim should be to determine both the composition of the diet and the temporal relationship of ingestion of facilitators and/or inhibitors of iron absorption to ingestion of the food vehicle.

The effect of increased iron fortification of milk formula on iron status (Comparison of Test and Control groups at 12 months)

Introduction

The first question to be asked of the trial is whether the increased iron fortification had the desired effect on the iron status of the groups. It was shown above that the Control and Test groups had essentially the same iron status at the start of the trial. Tables 6.7 and 6.8 show that at the end of the trial the Test group had statistically significantly better iron nutrition than the Control group as indicated by mean levels of haemoglobin (11.85 *cf* 11.49 g/dl, P = 4%), red cell distribution width (14.44% *cf* 15.53%, P = 0.05%), red cell zinc protoporphyrin (3.41 *cf* 3.95 ug/g Hb, P = 4%) and plasma ferritin (29.0 *cf* 17.3 ug/l, P = 0.04%).

This pattern was expected but the magnitude of the changes were smaller than expected. To evaluate the results the following 3 sections compare the present study with (a) reference standards, (b) epidemiological surveys of populations, and (c) similar trials of iron fortification. The achievements of the trial are then assessed in terms of its aims.

Table 6.6 **Haematological indicators of iron status at the start of the trial.**

Indicator of Iron Status	*Control Mean*	*Test Mean*	*P Value*	*95% CI of diff. of means*		*Power Obs*	*10%*
AT 3 MONTHS OF AGE							
Red cell count (10^{12}/l)	4.01	4.06	42%	-0.07	0.17	13	99
Haemoglobin (g/dl)	11.28	11.38	51%	-0.19	0.37	0	99
Haematocrit (%)	32.89	33.27	34%	0.38	1.17	16	99
Mean cell volume (fl)	82.06	82.10	97%	-1.80	1.87	0	99
Mean cell Hb (pg)	28.12	28.11	98%	-0.71	0.69	0	99
Mean cell Hb conc. (ug/dl)	34.28	34.21	69%	-0.43	0.28	0	100
RBC width distr. (%)	13.35	13.69	19%	-0.17	0.84	26	99
Reticulocytes (%)	2.01	2.28	22%	-0.16	0.70	23	17

Notes P values were calculated from student's t test.
95% CI = 95% confidence interval.
The statistical power of the study was determined for both the observed difference (Obs) and a 10% difference (10%) from the combined mean for a type I error probability of 5%.

Table 6.8 Non-haematological indicators of iron status compared for the Control and Test groups at the start and end of the trial.

Indicator of Iron Status	*Control Mean*	*Test Mean*	*P Value*	*95% CI of diff. of means*		*Power Obs*	*10%*
AT 3 MONTHS OF AGE							
Red cell ZPP (ug/g Hb)	2.92	2.82	63%	-0.50	0.30	0	29
Ferritin (ug/l)	155.61	157.20	94%	-38.34	41.53	0	12
Log(ferritin)[1] (log(ug/l))	2.07	2.08	91%	-0.11	0.12	0	93
AT 1 YEAR OF AGE							
Red Cell ZPP	3.95	3.41	4%	-1.04	-0.04	56	30
Change in ZPP (ug/g Hb)	1.18	0.51	2%	-1.23	-0.10	63	0
Ferritin	23.80	37.70	1%	3.95	23.84	78	0
Change Ferritin (ug/l)	-137.93	-111.42	24%	-17.70	70.71	22	0
Log(ferritin)[2]	1.24	1.46	0.04%	0.10	0.35	95	58
Chng Log(ferritin) (log(ug/l))	-0.86	-0.59	0.10%	0.11	0.44	91	14

Notes P values were calculated from student's t test.
95% CI = 95% confidence intervals.
Changes are reported as the mean of the variable at 12 months minus the mean value at 3 months.
The statistical power of the study was determined for both the observed difference (Obs) and a 10% difference (10%) from the combined mean for a type I error probability of 5%.

1 Geometric mean ferritin levels for Control and Test groups are 117 and 120 ug/l respectively

2 Geometric mean ferritin levels for Control and Test groups are 17.3 and 29.0 ug/l respectively

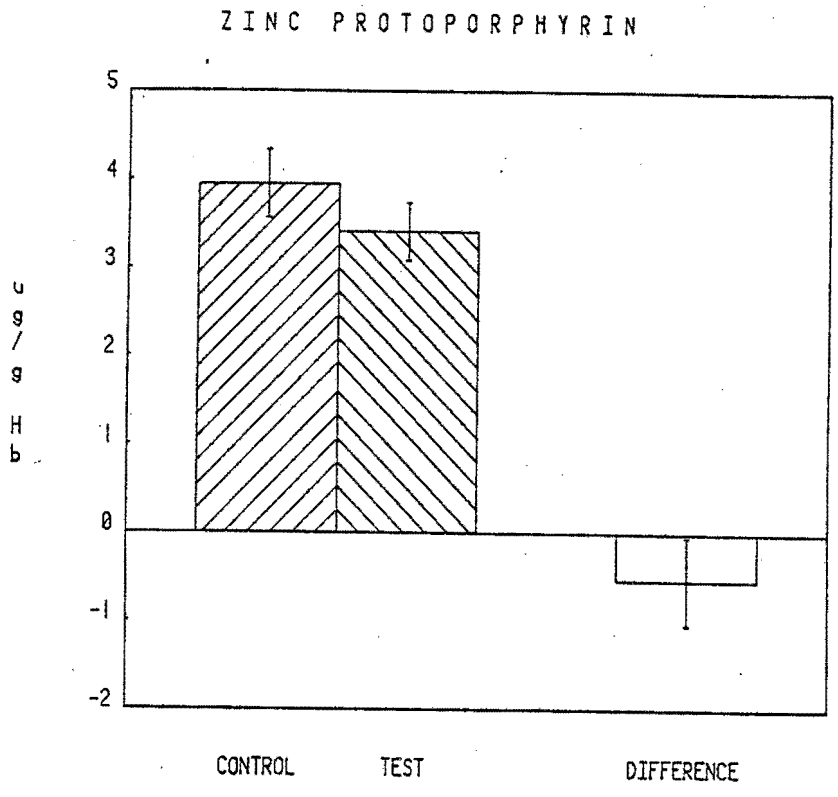

Figure 6.3 **Mean zinc protoporphyrin levels for the Test and Control groups at 12 months of age***

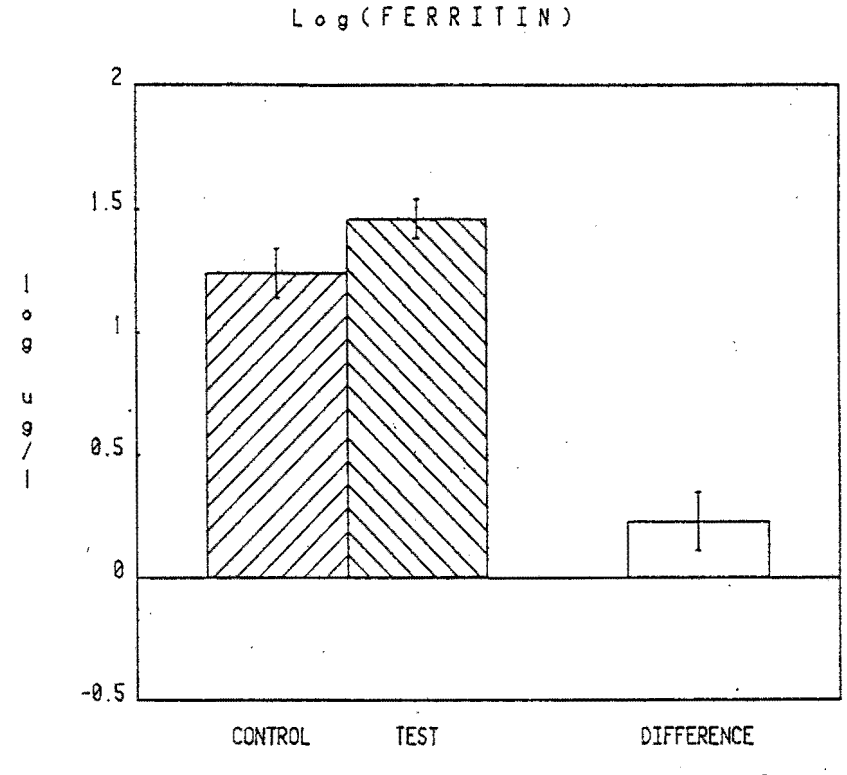

Figure 6.4 **Log(ferritin) for the Test and Control groups at 12 months of age***

* The bars represent the means and the vertical lines through the bars represent the 95% confidence intervals

Comparison with reference levels for haematological indicators of iron status

The haemoglobin levels achieved by the Control and Test groups compare favourably with "normal" levels established by early workers, *eg* Guest and Brown[181] (*Source* 1 in table 6.9) and Smith[423] (*Source* 2), but are slightly lower than that in the later textbook by Dacie and Lewis[110] (*Source* 3).

More recently, efforts have been made to define reference levels for haematological parameters by excluding subjects with evidence of iron deficiency. Employing this principle, Saarinen and Siimes[396] (*Source* 4) proposed ideal iron nutritional objectives for haematological variables. With the exception of the red cell count, all parameters are substantially higher than those found in the trial groups. The ideal haemoglobin according to these standards is 1.2 g/dl greater than that of the Control group and 0.8 g/dl greater than that of the Test group. The ideal mean cell volume at 77 fl is substantially higher than the mean cell volumes of 72 fl and 73 fl in the Control and Test groups.

Reference levels for haematological parameters were published in the form of percentile curves by Dallman and Siimes[116] (*Source* 5). In establishing the reference curves Dallman and Siimes excluded subjects with evidence of iron deficiency in one or more laboratory tests. For haemoglobin in 1 year old children these criteria were serum ferritin less than 10 ng/ml, serum transferrin saturation less than 16% and mean cell volume less than 70 fl. Centiles for mean cell volume were computed after excluding those subjects whose haemoglobin values were more than 2 standard deviations below the mean determined as above or who had serum ferritin below 10 ng/ml, transferrin saturation less than 16% or abnormal haemoglobin electrophoresis. This ensured that only iron sufficient subjects were included, but the authors caution that iron sufficient subjects with lower test values may have been excluded. The standards set by the 50th centiles are similar to those of Saarinen and Siimes.

Yip *et al*[478] (*Source* 5) published similar reference levels for haematological parameters by combining results from several surveys.

While these "reference" centile curves provide useful standards for clinical decisions they are less useful norms for population comparisons since they may be based on data that excluded iron sufficient subjects with parameters in the low range of normal. In addition, the proportions of the various haemoglobinopathies in the reference and study populations differs because of the exclusion criteria and differences in genetic composition of the American and Cape Town populations.

From the foregoing it may be seen that neither of the study groups reached the levels of iron sufficiency indicated by ideal reference standards.

The parameter that deviated most from the ideal was the mean cell volume. It is possible that this may be due to a large proportion of infants in the study with thalassaemia trait or heterozygotes for a

haemoglobinopathy. This is unlikely since Bird *et al*[39] found such conditions in 10.4% of "coloured" infants presenting to the Red Cross War Memorial Childrens Hospital with a mean cell volume less than 60 fl*. The prevalence is likely to be very much less in children with mean cell volumes greater than 60 fl.

It is possible that the laboratory results are systematically low for the mean cell volume but this is unlikely because the laboratory makes regular, frequent quality control assays with an internationally accepted standard.

A more likely reason for the relatively low mean cell volumes in the study children is that their iron status was sub-optimal in spite of the iron fortification.

If so, this might imply that mean cell volume is a more sensitive indicator of minor degrees of suboptimal iron status in a population than haemoglobin. Unfortunately, this parameter was not reported in the studies of Marsh *et al*[323], Andelman and Sered[7] and Stekel[479] so it is not possible to test this hypothesis by reference to the literature.

Since the red cell distribution width showed the smallest P values when the haematological parameters of the Control and Test groups were compared, it will be interesting to relate the results in the present study to reference levels when these are established. It seems that red cell distribution width is a sensitive indicator of suboptimal iron nutrition.

A further conclusion is that the present study may reflect the community's position in a "secular trend" in haematological parameters analogous to the well known secular trend in stature. This speculation arises from the historical comparisons made in table 6.9 and is further confirmed in the next section.

* There was 1 such child in the present study and he turned out on further investigation to have thalassaemia trait.

Table 6.9 **Reference standards for haematological parameters compared with the Control and Test groups.**

Source*	Age month	R/S	Hb g/dl	HCT %	RBC 10^{12}/l	MCV fl	MCH pg	MCHC g/dl	RDW%
Control	12		11.49	34.85	4.85	71.96	23.78	32.98	15.53
Test	12		11.85	35.80	4.90	73.20	24.29	33.14	14.44
1	12		11.3	34.4	4.78	73	23.7	32.4	
2	12		11.6	35.2	4.6	77	25	33	
3	12		12	40	4.4	78	27	32.5	
4	12		12.7	37	4.7	77.7	26.8	34.3	
5	6-24		12.5			70			
6	12-24		12.3	35.9	4.34	79	27.4	34.4	

* Source

1 Guest GM, Brown EW. Erythrocytes and hemoglobin of the blood in infancy and childhood. AMA Journal of Diseases of Children 1957; 93: 486-509[200]

2 Smith CH. Blood diseases of infancy and childhood. 3rd ed Mosby, St Louis 1972[423]

3 Dacie JV, Lewis SM. Practical Haematology 6th ed. Churchill Livingstone, Edinburgh, 1984[110]

4 Saarinen UM, Siimes MA. Developmental changes in red blood cell counts and indices after exclusion of iron deficiency by laboratory criteria and continuous iron supplementation. Pediatrics 1978; 92: 412-416[436]

5 Dallman PR, Siimes MA. Percentile curves for haemoglobin and red cell volume in infancy and childhood. Pediatrics 1979; 94: 26-31[124]

6 Yip R, Johnson C, Dallman PR. Age related changes in laboratory values used in the diagnosis of anaemia and iron deficiency. Am J Clin Nutr 1984; 39: 427-436[827]

Comparison with epidemiological surveys of haematological indicators of iron status

Interesting comparisons may be made with values found in an affluent community (*Source* 1 in table 6.10), with values found in a population survey in the USA (*Source* 4) and with values found in surveys in the Western Cape (*Sources* 2 and 3).

In an affluent community in Canada, children at the age of one year had a mean haemoglobin 0.4 g/dl greater than the Test group and 0.8 g/dl greater than the Control group[49].

In Cape Town, Lanzkowsky[262] showed in 1960 that the mean haemoglobin level was 11.2 g/dl for one year old white children, and 9.57 and 9.84 g/dl for "coloured" and black children. In 1984 Kirsten *et al*[241] showed that the iron nutrition of "coloured" children had improved since the mean haemoglobin level was 10.5 g/dl. His study was conducted in Heideveld, a community similar to that of Bonteheuwel.

The effects of the extra iron employed in the present trial can be compared to the effects of the trial itself by contrasting the difference between the Control and Test groups with the difference between the Control group and the children in Kirsten's study. The Test group had a mean haemoglobin 0.36 g/dl greater than the Control group. In contrast, the Control group had a mean haemoglobin 1 g/dl greater than infants in Kirsten's study.

It may thus be inferred that the present study was effective in ensuring that the iron status of the both groups of children was substantially better than that of similar peers who did not participate in the trial.

Yip *et al*[482] studied haematocrits in white and black American children and found essentially equal mean levels. These are 1.5 and 0.5 percentage points greater than those in the Control and Test groups at 12 months of age.

Table 6.10 **Epidemiological surveys of haematological parameters compared with the Control and Test groups.**

Source*	Age month	R/S	Hb g/dl	HCT %	RBC 10^{12}/l	MCV fl	MCH pg	MCHC g/dl	RDW%
Control	12		11.49	34.85	4.85	71.96	23.78	32.98	15.53
Test	12		11.85	35.80	4.90	73.20	24.29	33.14	14.44
1	12	F	12.29	36.9					
		M	12.24	36.7					
2	12	W	11.2						
		C	9.57						
		B	9.84						
3	12		10.5			67.2	23		
4	11-23	W		36.54					
		B		36.33					

* Source

1 Brault-Dubuc M, Nadeau M, Dickie J. Iron status of French Canadian children: a 3 year follow-up study. Hum Nutr: Appl Nutr 1983; 37A: 210-221(62)

2 Lanzkowsky P. Mean haematological values in healthy infants and pre-school children in Cape Town. S Afr Med J 1960; 34: 469-471(288)

3 Kirsten GF, Heese H de V, de Villiers S, Dempster WS, Varkevisser HE, Hoffman M. The prevalence of iron deficiency in apparently healthy Cape Coloured infants. S Afr Med J 1984; 65: 378-380(241)

4 Yip R, Schwartz S, Deinach AS. Haematocrit values in white, black and American Indian children with comparable iron status. Am J Dis Child 1984; 138: 824-827(631)

Comparison with other trials of iron fortification for haematological indicators of iron status

Table 6.11 below (extracted from table 2.16) lists the mean haemoglobins attained by infants in 3 clinical trials similar to the present study. It also shows that the iron and ascorbic acid levels in those trials was similar to that employed in the present study for the Control group.

In all 3 studies children fed on the iron fortified milk had substantially higher mean haemoglobins than those on unfortified milks. It may be noted that these children's haemoglobin levels were also higher than those in even the Test group of the present study. The lowest was 11.9 g/dl and the highest 12.69g/dl, the Control and Test group's haemoglobins were 11.49 g/dl and 11.85 g/dl.

Table 6.11 Haematological parameters from other trials of fortification of cow's milk based infant formula compared with those of the Test and Control groups.

Source*	AGE months	[IRON] mg Fe/l	[ASCORBIC ACID] mg AA/l	MEAN Hb g/dl
Control	**12**	**11.96**	**53**	**11.49**
Test	**12**	**57.66**	**53**	**11.85**
Marsh[1]	9	12	55	12.69
Unfortified milk		?	?	10.46
Evaporated milk		?	?	9.67
Andelman[2]	12	12	55	11.9
Evaporated milk		?	?	10.4
Stekel[3]	9	15	100	12.2
Unfortified milk		?	?	11.1

Notes

* **Source**

1 Marsh A, Long H, Stierwalt RN. Comparative haematologic response to iron fortification of a milk formula for infants. Pediatrics 1959; 24: 404-412

2 Andelman MB, Sered BR. Utilization of dietary iron by term infants. Am J Dis Child 1966; 111: 45-55(7)

3 The ascorbic acid levels in the formulas used by Marsh et al and Andelman and Sered were not published by these authors but are given in the article by Stekel on pages 181 and 182 of "Prevention of iron deficiency", Stekel A, 179-194 in Iron nutrition in Infancy and Childhood. Edited by Stekel A, Nestl, Vevey/Raven Press, New York 1984. The haemoglobin levels from Stekel's study are read from figure 2 on page 184.

Assessment of achievements of the trial with respect to iron nutrition

The first question addressed by the trial was:

> *Does increasing the level of iron fortification of conventional infant milk formula improve the iron nutrition of normal infants fed on the formula?*

The results presented above provide an affirmative answer to the question, but show that increasing iron alone, even in substantial quantities, is unlikely to make a clinically important difference. It is likely that the availability of iron added to milk formula is more dependent on the quantity of ascorbic acid than the quantity of iron[134, 133, 176, 519]. The present study thus indirectly confirms the importance of ascorbic acid in facilitating the assimilation of iron.

The study suggests that the mean cell volume may be a more sensitive indicator of sub-optimal iron status, since this was the haematological parameter which showed the greatest difference from reference levels.

Table 6.7 shows that the red cell distribution width (RDW%) was the variable with the most statistically significant difference between the Test and Control groups. For the Test group the RDW% was 14.44% while for the Control group it was 15.53% with a P value of 0.05% for a 2 sample t test of the difference. The extra iron was associated with a smaller range of red cell sizes. Recent work[35, 383] shows that the RDW% is a sensitive indicator of the iron status of the population and the present study bears this out.

The study also raises the question of why the iron fortified milk formula was less successful in promoting optimal iron nutrition than similar formulas in similar trials. It is possible that the children ingested small quantities of the milk. Although it was not possible to gather data on this, it was the impression of the study team that the children received adequate amounts in almost all cases. Since the children grew adequately (mean standard deviation scores for weight were above 0 for both groups) their general nutrition was surely good.

It is also possible, but unlikely, that the mothers relied too much on the formula and did not give their children other haematinic foods in sufficient quantities.

More likely is the possibility of a relative increase in inhibitors of iron absorption or the relative decrease in facilitators of iron assimilation with respect to the diets of children in the studies listed in table 6.11.

Another possibility is that the children suffered from increased iron losses. Diet is an unlikely cause and, as hookworm is rare in Cape Town*, it seems that increased iron losses are not probable.

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* Household, KC. Epidemiology, clinical features, aetiology and course of acute infectious diarrhoea in infants. MD thesis. University of Cape Town 1985.

These results lead to the conclusion, articulated by Dallman *et al*[125], that more studies are needed "*to determine the overall influence on iron absorption of various types of fortified and unfortified transitional infant foods*".

The effect of increased iron fortification of milk formula on immune function

Introduction

The second question addressed by the trial was:

Does increased iron fortification of infant milk formula alter immunity as reflected by incidence of infection and laboratory tests of immune function?

This section then, compares the Control and Test groups firstly for laboratory tests of immune function and secondly for incidence of infection in order to determine the effect of the increased iron fortification. The following 2 sections use classification and multivariate analysis to seek evidence for a relationship of iron status *per se* with immune function.

Delayed cutaneous hypersensitivity

Introduction

Delayed cutaneous hypersensitivity is tested by the delayed reaction to an antigen injected intracutaneously. Such a reaction can only occur if the child has been sensitized by natural infection or by immunization. Two antigens were used in this study: PPD (purified protein derivative of *Mycobacterium tuberculosis*), and Candida antigen in the Mantoux and Candida tests. All the results tabulated are for children who had been immunized with BCG shortly after birth.

In healthy children who have been immunized with BCG at birth the Mantoux test would be expected to be reactive (diameter of induration from 6 to 14 mm) or strongly reactive (diameter of induration > 14 mm) in 90% at 3 months and 72% at 18 months[162, 207]. The Mantoux test would also be expected to be reactive in a child infected with tuberculosis, but there was no reason to suspect this in any subject in this study.

Healthy children develop an immunity to Candida at an early age and by the age of 1 year 80% of children would be expected to react to the delayed cutaneous hypersensitivity test[417].

Results

Tables 6.12 and 6.13 show that at one year of age the Test group responded more strongly than the Control group to Candida antigen, but showed lower reactivity in the Mantoux test. The differences were small and far from statistical significance.

The data were also analyzed in order to determine reactivity of those children from whom *Candida albicans* had been isolated from a swab of a clinically suspicious lesion. In this group and in the group from whom

Candida albicans had not been isolated, the mean diameters of induration were 10.1 mm and 9.2 mm respectively and a t test yielded a P value of 70% for the difference between the two groups.

Table 6.13 shows that, at the age of one year, the Candida test elicited 5 mm or more of induration in 55% of Control infants and 63% of the Test group. For the Mantoux test the response rates were 58% and 52% respectively.

Table 6.12 Delayed cutaneous hypersensitivity mean diameters of reaction compared in the Test and Control groups.

Antigen	*Control Mean*	*Test Mean*	*P Value*	*95% CI of diff. of means*		*Power Obs*	*10%*
AT 3 MONTHS OF AGE							
Mantoux (mm induration)	5.96	5.36	53%	-2.50	1.30	0	0
Candida (mm induration)	4.35	4.32	97%	-2.03	1.95	0	0
AT 1 YEAR OF AGE							
Mantoux	8.05	7.03	43%	-3.60	1.56	12	0
Change in Mantoux (mm induration)	2.25	1.47	62%	-3.86	2.30	0	0
Candida	8.88	9.75	56%	-2.10	3.83	0	0
Change in Candida (mm induration)	4.87	5.18	84%	-2.78	3.40	0	0

Notes P values were calculated from student's t test.
95% CI = 95% confidence intervals.
The statistical power of the study was determined for both the observed difference (Obs) and a 10% difference (10%) from the combined mean for a type I error probability of 5%.
Changes are reported as the mean of the variable at 12 months minus the mean value at 3 months.
Antigen, purified tuberculin 5 T.U./0.1 ml for the Mantoux test and 0.1 ml 1/100 Candida antigen, was injected intracutaneously on the volar aspect of the forearms. The transverse width of induration was measured 48 hours later.

Table 6.13 **Delayed cutaneous hypersensitivity response rates compared in the Test and Control groups.**

REACTION		0 - 4mm		5 - 9 mm		10 - 30 mm		
	Group	No	%	No	%	No	%	Chi^2
AT 3 MONTHS OF AGE								
Candida								
	Control	46	65%	19	27%	6	8%	
	Test	50	68%	17	23%	6	8%	0.25
Mantoux								
	Control	33	49%	31	46%	4	6%	
	Test	39	56%	27	39%	4	6%	0.74
AT 1 YEAR OF AGE								
Candida								
	Control	27	45%	14	23%	19	32%	
	Test	26	39%	18	27%	24	36%	0.60
Mantoux								
	Control	25	42%	21	35%	14	23%	
	Test	32	48%	22	33%	13	19%	0.53

Notes P values were calculated from Chi square test; all were more than 10%.
Antigen, purified tuberculin 5 T.U./0.1 ml for the Mantoux test and 0.1 ml 1/100 Candida antigen, was injected intracutaneously on the volar aspect of the forearms. The transverse width of induration was measured 48 hours later.

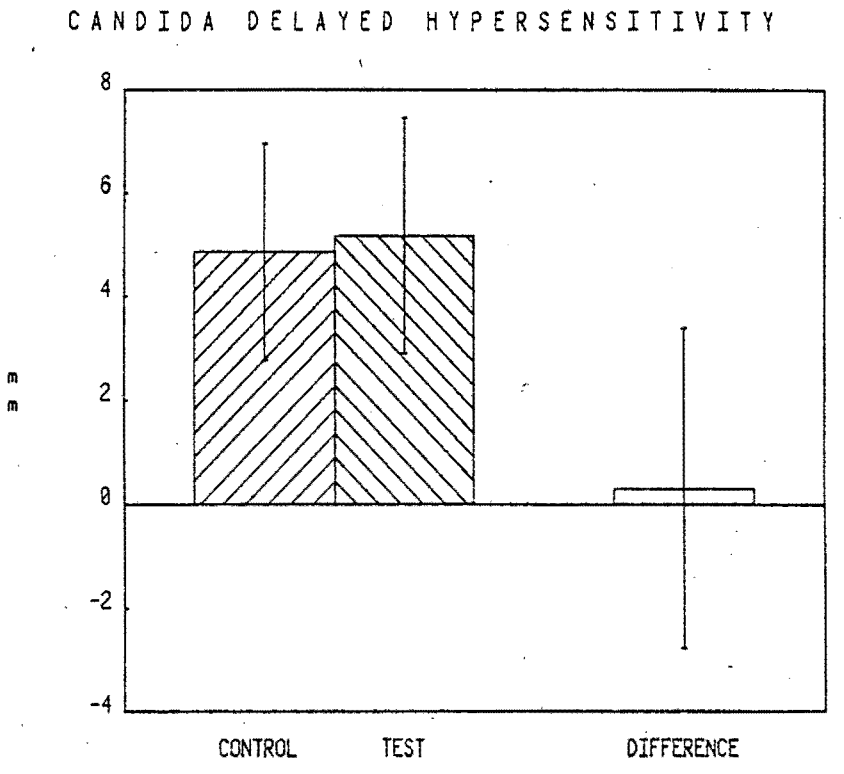

Figure 6.5 **Candida test of delayed cutaneous hypersensitivity for the Test and Control groups at 12 months of age***

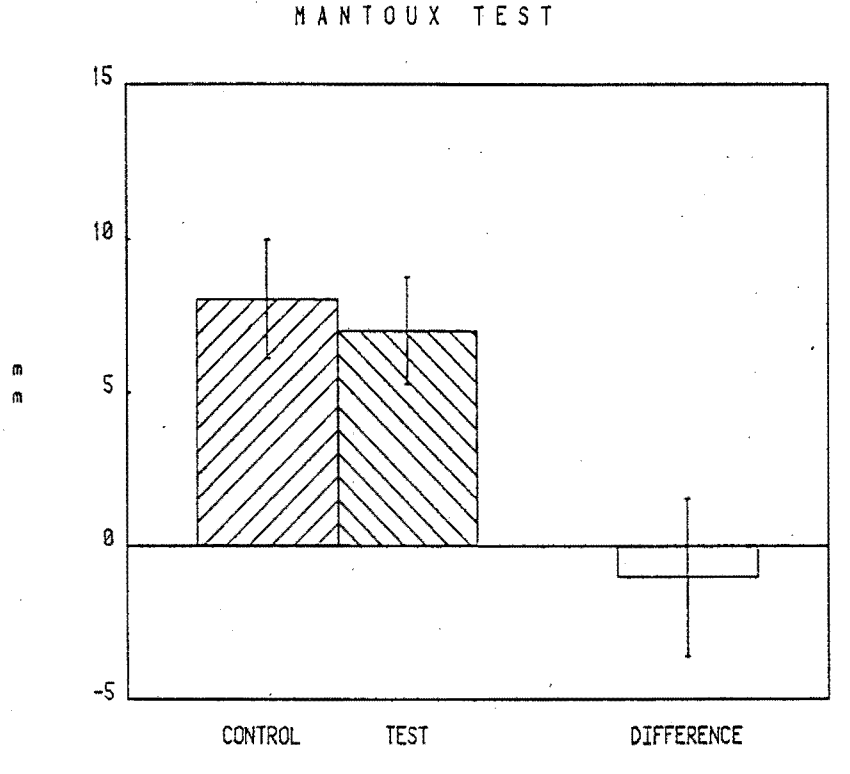

Figure 6.6 **Mantoux test compared for the Test and Control groups at 12 months of age***

* The bars represent the means and the vertical lines through the bars represent the 95% confidence intervals

Discussion

Although microbiological swabs were taken only when Candida infection was clinically expected (there was no survey of all the children), it is to be expected that normal children who had yielded swabs positive for *Candida albicans* would have shown a greater reaction on average than the other children who would presumably include both a subgroup who had experienced subclinical infection and a subgroup who had not yet been exposed to Candida. The small difference that was found between the group who had proven exposure to Candida and the group whose exposure was unknown raises the suspicion that the test has poor discriminatory power for small variations in immune function.

In the present study the Mantoux test was being used in an attempt to detect a difference in immune function between the Test and Control groups. It is also of interest to compare the results in the present study with surveys of reactivity to the Mantoux test in healthy children. This is done in table 6.14 where the present study is contrasted with a study designed to evaluate the effectiveness of locally produced PPD and to determine the "conversion rate" following immunization with BCG. At 3 months of age, the present study yielded a smaller mean diameter of reaction, but at 12 months of age the results are comparable to those from the studies of Fourie and Kibel[162, 207]. The differences between studies are greater than the differences within studies for both the means and the standard deviations. The inference to be drawn from this is that the Mantoux test is more reliable for comparisons within a particular study than for comparisons between studies.

Table 6.14 Mantoux reaction compared between the present study groups and other studies. The reaction was the transverse diameter measured in mm 48 hours after intracutaneous injection of 5TU of PPD (purified protein derivative of Mycobacterium tuberculosis) on the volar aspect of the forearm.

Study	Mean (mm)	Std Dev	N
AT 3 MONTHS OF AGE			
Control[1]	**5.96**	**5.48**	**68**
Test[2]	**5.36**	**5.77**	**71**
Kibel[3]	7.3	4.0	21
Kibel[4]	9.8	4.3	11
AT 6 MONTHS OF AGE			
Kibel[3]	8.1	4.1	16
Kibel[4]	1.7	2.4	3
AT 12 MONTHS OF AGE			
Control[1]	8.05	7.48	60
Test[2]	7.03	7.18	67
AT 18 MONTHS OF AGE			
Kibel[4]	8.1	5.4	13

Notes
1. The Control group in the present study, tested with British PPD
2. The Test group in the present study, tested with British PPD
3. Cape Coloured children tested with South African PPD[162, 207]
4. Cape Coloured children tested with Japanese PPD[162, 207]

Comparison with previous studies

Gross *et al*[180] report that the dinitrochlorobenzene test in 5 iron deficient adults (mean haemoglobin 6.8 g/dl) was similar to that of normal controls and did not change with iron therapy.

Bhaskaram and Reddy[37] reported that the delayed cutaneous hypersensitivity test was positive in 4 of 9 iron deficient children before iron replacement therapy. In 1 child it became positive after treatment. Haemoglobin levels ranged from 4 to 7 g/dl before treatment; responses to treatment were not stated.

Joynson *et al*[229, 217, 216] report that the candida delayed cutaneous hypersensitivity test was positive in all 12 adult controls (mean haemoglobin 14.2 g/dl) but in only 3 of 12 iron deficient adults (mean haemoglobin 9.0 g/dl). For PPD the results were positive in 10 and 5 subjects respectively.

Chandra and and Saraya[91] reported trends towards decreased delayed cutaneous hypersensitivity responses in 20 iron deficient children to candida, PPD, mumps and trichophyton. For streptokinase/streptodornase the response of the iron deficient group was significantly depressed. Mean

ages and haemoglobins were not reported, so comparison with the present study is difficult. In addition, differences between the two groups in prevalence of infection and malnutrition can not be ruled out.

In 28 obese children Chandra and Kutty[90] reported an impairment of iron nutrition as measured by serum ferritin. The obese children had lower serum zinc and significantly fewer individuals with positive delayed cutaneous hypersensitivity test with candida, mumps, trichophyton and streptokinase/streptodornase. Mean ages and haemoglobins were not reported.

Similar results were reported by Chandra, Woodford and Hyam[89] but no details of iron status, ages or selection of their subjects were given.

Kuvibidila[258], in a carefully controlled study showed a significant impairment to dinitrochlorobenzene in mice with severe iron deficient anaemia.

Krantman *et al*[249] tested 5 iron deficient infants and found positive responses to candida and tetanus in 2 and 3 respectively. After treatment the mean haemoglobin rose from 8.36 to 12.04 g/dl and all responded to the delayed cutaneous hypersensitivity tests. Infection could not be entirely discounted as a possible confounding factor as the children had raised IgM concentrations.

Macdougall *et al*[282] tested delayed cutaneous hypersensitivity with diphtheria, candida and streptococcal antigen in 14 control children (Hb=12.8), 7 with latent iron deficiency (Hb=10.8) and 11 with iron deficiency anaemia (Hb=8.1). Compared to controls, the iron deficient patients had significantly fewer positive tests; and after iron therapy they responded normally to the delayed cutaneous hypersensitivity tests. As the iron deficient children were selected from hospital patients, infection is a confounding factor whose influence cannot be evaluated.

Summary and conclusions

In summary, the results of the delayed cutaneous hypersensitivity tests are inconclusive and do not suggest that the Test group had altered immune function. However the statistical power of the Mantoux and Candida tests was low and the difference in iron status was small so it is unlikely that a real difference would have been detected.

Comparison with studies in the literature is difficult since the reported studies have compared groups with much greater differences in iron status. In contrast to the present study, many clinical studies could not rule out the confounding effects of malnutrition and infection as their subjects were hospitalized patients. A further problem that inhibits comparison is that clinical studies have followed clinical practice and reported their results in terms of the numbers of "positive" or "negative" tests. This is, in a sense, discarding data; a more accurate and precise summary measure is the mean diameter of induration.

Specific antibody response to immunization

Introduction

The responses to immunization with live trivalent polio virus vaccine administered orally and tetanus toxoid administered intramuscularly are compared in table 6.15 for the Test and Control groups. The two tests measure specific antibody titre. Although the tests use the same outcome measure they evaluate different parts of the immune system since the antibodies are produced in response to different types of stimulation.

Statistical note

There is a theoretical difficulty in using the t test on data such as antibody titres which are far from normally distributed. The results shown in table 6.15 are from non-parametric statistical tests which avoid this sort of problem.

The non-parametric tests do not readily lend themselves to graphical representation or to the calculation of confidence intervals. Logarithmic transformation of skewed data often brings the data close enough to Gaussian distribution for parametric analysis. This was done for the titres and the analysis, shown in appendix 3, gave similar P values and trends to those obtained from the non-parametric statistics. Since the validity of the log transformation was thus confirmed, figures 6.7 and 6.8 compare the two groups for log(antibody titre).

Results

The Test group had a greater response to immunization than the Control group for polio but a smaller response for tetanus. The differences were far from statistical significance.

Table 6.15 **Specific antibody responses to immunization compared between the Control and Test groups for polio and tetanus. The children were immunized at 6 and 12 weeks with 3 drops of trivalent oral live attenuated polio vaccine and 1 ml intra-muscular tetanus toxoid. Specific antibody titres were measured in the mothers on entry to the study and in the children at 3 and 12 months of age.**

Antibody Titre	Control Rank	Test Rank	P Value
MOTHERS 3 MONTHS POST PARTUM			
Tetanus	55.21	59.33	49%
Polio	56.08	58.65	52%
INFANTS AT 3 MONTHS OF AGE			
Tetanus	60.02	55.46	40%
Polio	54.71	59.76	40%
INFANTS AT 1 YEAR OF AGE			
Tetanus	60.41	55.14	39%
Polio	53.78	60.50	27%

Notes P values were calculated from Kruskal-Wallis and Mann-Whitney test statistics.
The mean rank was computed by dividing the rank sum by the number of subjects.

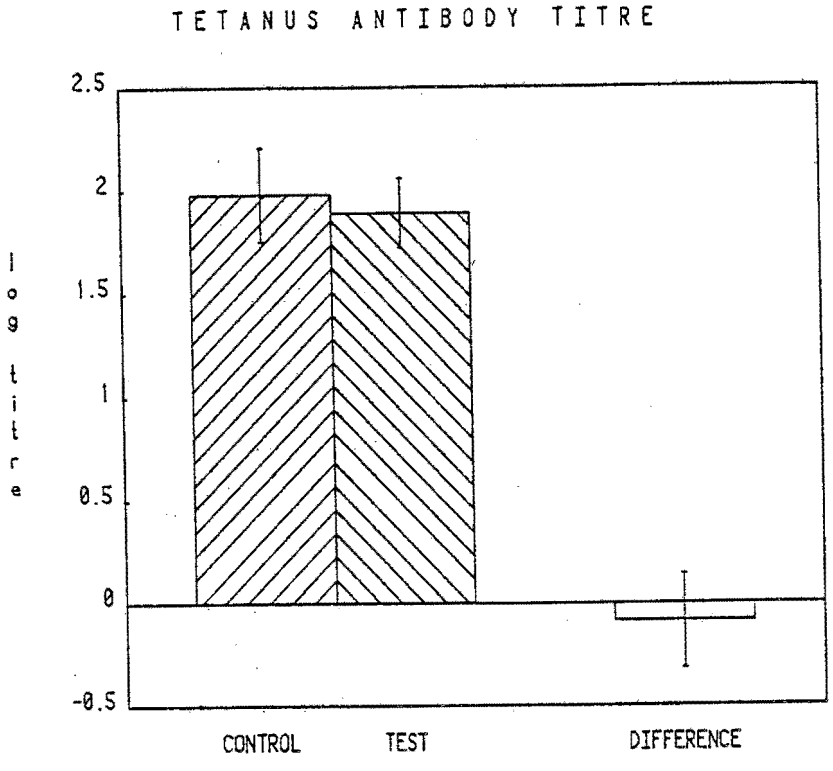

Figure 6.7 **Log(tetanus antibody titre) for the Test and Control groups at 12 months of age***

* The bars represent the means and the vertical lines through the bars represent the 95% confidence intervals

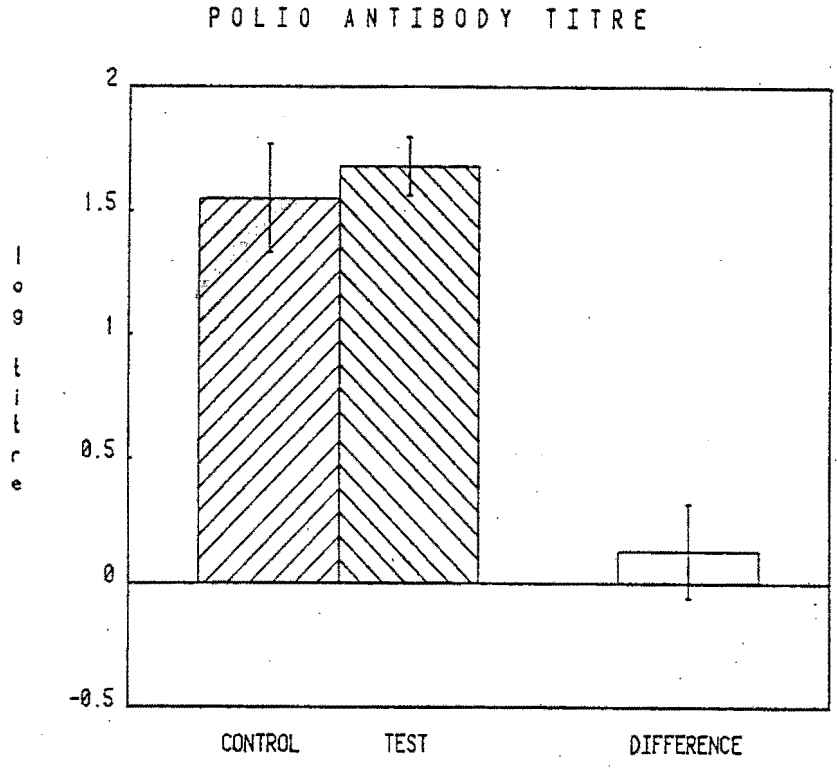

Figure 6.8 Log(polio antibody titre) compared for the Test and Control groups at 12 months of age

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Comparison with previous studies

Chandra and Saraya[91] indicated that there was no difference in response to immunization with tetanus toxoid and *Salmonella typhi* vaccine in iron deficient children and controls. Mean ages and haemoglobins were not reported.

Kuvibidila[255] found that antibody production against sheep red blood cells was severely depressed in iron deficient anaemic mice but not in pair-fed controls. In a related study, Kuvibidila and her colleagues[257] reported that the blastogenic response of B cells to bacterial lipopolysaccharide was significantly impaired in iron deficient mice.

Macdougall and Jacobs[283] found non-significant tendencies for iron deficient children to respond less efficiently to immunization with diphtheria toxoid and *Salmonella typhi* vaccine. Both infection and nutritional factors were poorly controlled and their control group was probably also mildly iron deficient as their mean haemoglobin level was 10.95 g/dl. The test group's haemoglobins ranged from 4.6 to 7.4 g/dl.

Malakhovsky *et al*[291] also reported tendencies for decreased immune responses in iron deficient infants. Their paper does not provide sufficient data to evaluate the methods and results.

The clearest results are from the study of Nalder[333] who found a decreased response to immunization with tetanus toxoid in rats. There was a striking dose/response relationship between the level of iron in the diet and antibody titre. As there was no pair-fed control group, however, the effects may be due to concomitant nutritional deficiencies.

Summary

In summary, the results of the antibody analyses are inconclusive. The differences are not statistically significant and the power of the statistical analysis to detect a real difference is less than 50%. Previous studies have compared groups with much larger differences in iron status than that obtained in the present trial and have often not excluded malnutrition or infection.

Leukocyte counts

Results

The white cell counts for the Test and Control groups are compared in table 6.16. At three months of age there was no difference between the two groups. At 1 year of age the total white cell count was minimally lower in the Test group. The Test group had significantly more monocytes and fewer lymphocytes than the Control group. Smaller differences were manifest in the other classes of lymphocytes.

Table 6.16 White cell counts compared at the start and end of the trial.

Leukocytes	*Control Mean*	*Test Mean*	*P Value*	*95% CI of diff. of means*		*Power Obs*	*10%*
AT 3 MONTHS OF AGE							
Total Leukocytes	11.25	11.12	25%	-0.46	1.76	21	53
Polymorphs (%)	26.98	27.53	80%	-3.78	4.88	0	24
Lymphocytes (%)	64.78	65.06	90%	-4.25	4.81	0	80
Monocytes (%)	5.13	5.02	83%	-1.13	0.90	0	17
Eosinophils (%)	2.89	2.26	14%	-1.48	0.22	31	0
Basophils (%)	0.18	0.14	60%	-0.21	0.12	0	0
AT 1 YEAR OF AGE							
Total leukocytes	10.95	10.35	87%	-0.97	1.15	0	53
Polymorphs (%)	31.32	34.79	11%	-0.74	7.68	35	34
Lymphocytes (%)	60.75	56.39	5%	-8.66	-0.08	51	76
Monocytes (%)	4.63	5.92	3%	0.13	2.45	59	15
Eosinophils (%)	3.22	2.74	40%	-1.59	0.63	14	0
Basophils (%)	0.10	0.11	88%	-0.12	0.14	0	0

Notes P values were calculated from student's t test.
95% CI = 95% confidence intervals.
The statistical power of the study was determined for both the observed difference (Obs) and a 10% difference (10%) from the combined mean for a type I error probability of 5%.

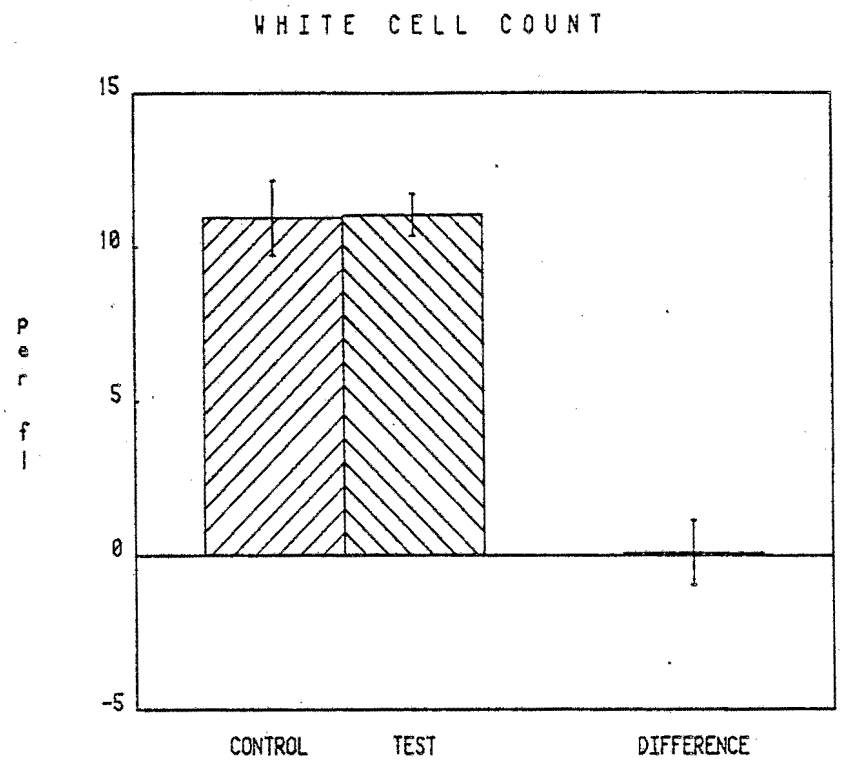

Figure 6.9 **White cell counts for the Test and Control groups at 12 months of age***

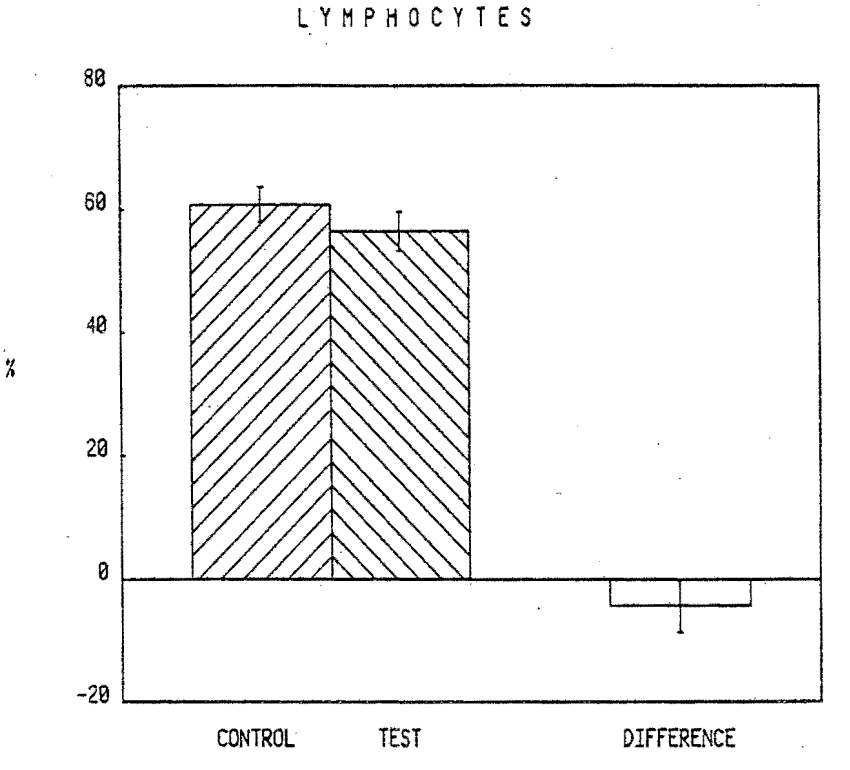

Figure 6.10 **Lymphocyte counts compared for the Test and Control groups at 12 months of age***

* The bars represent the means and the vertical lines through the bars represent the 95% confidence intervals

Discussion

The interpretation of the differences is not clear but the results would be consistent with the Control group having had more infections than the Test group, particularly if the infections were predominantly viral.

The review of the literature in chapter 2 (see table 2.11) found no study that reported a depression of leukocytes or granulocytes in iron deficiency although their subjects had much greater degrees of iron depletion than those in the present study.

Lymphocyte subtypes

Introduction

The many different functions of the immune system are carried out by specialized cells. Prominent among these are the T lymphocytes. These have been characterized by surface antigens which are "recognized" by specific monoclonal antibodies. Virtually all T lymphocytes are identified by the OKT3 antibody. OKT4 and OKT8 antibodies "recognize" helper/inducer and suppressor/killer T cells respectively. Table 6.17 displays the results of lymphocyte subtyping applied to the Test and Control groups at 3 and 12 months of age.

Changes in these markers have been documented in severe immune deficiencies. In the present population, where subtle changes in immune function were hypothesized it was difficult to predict the direction of changes.

Results

The results in table 6.17 show that there was little difference between the Test and Control groups at both 3 and 12 months of age. The statistical power of the test to detect a 10% difference between the Test and Control groups was low.

Discussion

The literature review found no previous study that reported on lymphocyte subtypes in relation to iron nutrition. Decreased proportions of T cells have been reported by Bhaskaram and Reddy[37], Chandra and Saraya[91], Srikantia *et al*[431] and Krantman *et al*[249]. Nutrition and infection were likely confounding factors in the first three investigations. Chandra and Kutty[90] found normal proportions of T cells in obese children with iron deficiency.

Table 6.17 Lymphocyte subtypes compared in the Control and Test groups at the start and completion of the trial. Lymphocytes were subtyped with commercial monoclonal antibodies in the case of T cells and with commercial multivalent anti-gammaglobulin to count B cells.

Lymphocyte Subtype	*Control Mean*	*Test Mean*	*P Value*	*95% CI of diff. of means*		*Power of Test*	
AT 3 MONTHS OF AGE							
Total T cells OKT3	69.47	67.98	39%	-4.87	1.90	14	97
Helper-inducer OKT4	50.42	51.60	59%	-3.11	5.48	0	64
Suppressor-killer OKT8	16.42	15.84	66%	-3.16	2.00	0	24
Helper/suppressor OKT4/OKT8 ratio	3.60	4.16	16%	-0.21	1.33	29	17
B cells SMIG	19.61	17.40	7%	-4.58	0.16	45	33
AT 1 YEAR OF AGE							
Total T cells OKT3	74.14	72.59	44%	-5.49	2.40	12	95
Helper-inducer OKT4	46.90	46.83	98%	-4.87	4.73	0	48
Suppressor-killer OKT8	22.53	22.94	81%	-3.06	3.90	0	25
Helper/suppressor OKT4/OKT8 ratio	2.53	2.41	62%	-0.59	0.35	0	18
Change OKT4/OKT8 OKT4/OKT8 ratio	-0.97	-1.62	10%	-1.44	0.13	38	0
B cells SMIG	18.60	19.19	66%	-2.10	3.28	0	28

Notes P values were calculated from student's t test.
95% CI = 95% confidence intervals.
Changes are reported as the mean of the variable at 12 months minus the mean value at 3 months.
SMIG = surface membrane immunoglobulin positive cells.
The statistical power of the study was determined for both the observed difference (Obs) and a 10% difference (10%) from the combined mean for a type I error probability of 5%.

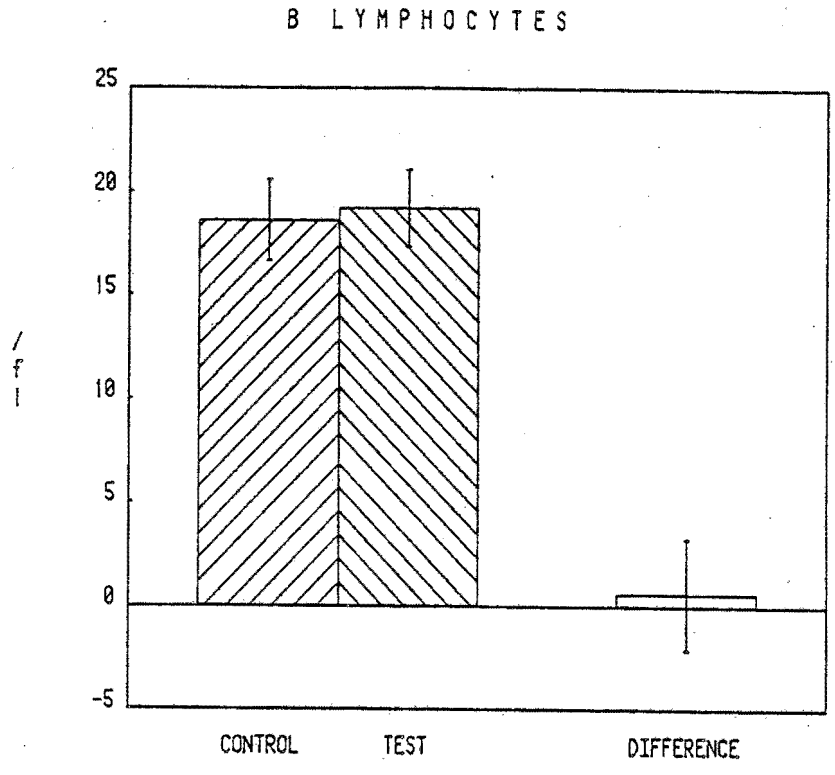

Figure 6.11 **B lymphocyte counts for the Test and Control groups at 12 months of age**

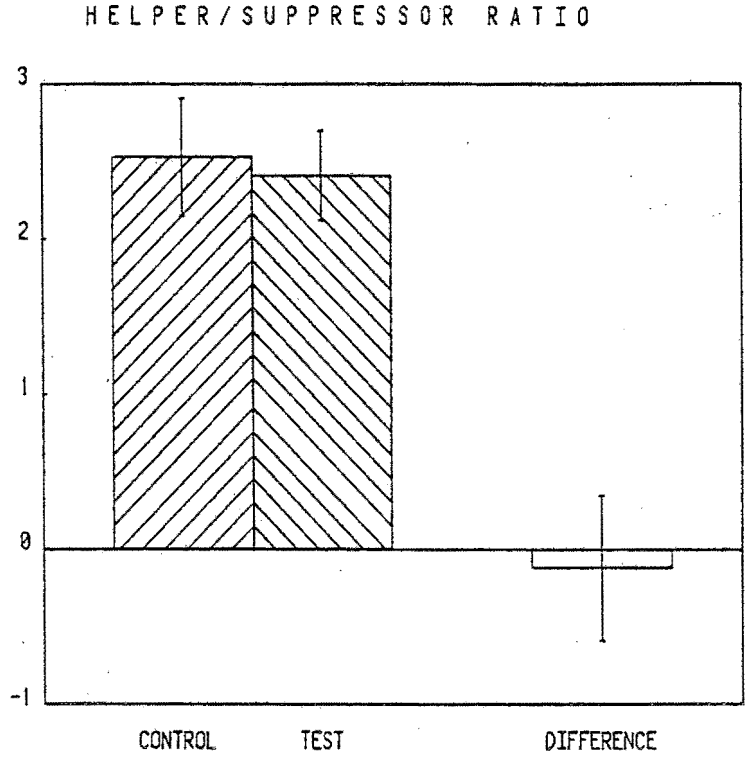

Figure 6.12 **T helper/suppressor ratios compared for the Test and Control groups at 12 months of age***

* The bars represent the means and the vertical lines through the bars represent the 95% confidence intervals

Lymphocyte stimulation with phytohaemagglutinin

Introduction

The phytohaemagglutinin transformation test is a laboratory measure of the ability of lymphocytes to respond to foreign material.

Results

Table 6.18 shows that the Test group, with a mean stimulation index of 140%, responded marginally better than the Control group with a mean index of 142%.

Table 6.18 Lymphocyte stimulation with phytohaemagglutinin (PHA) compared between the Control and Test groups. The infant's lymphocyte response is reported as a percentage of the response of an adult control. In order to distinguish cellular and serum effects the tests were performed with the infant's cells and serum, the infant's cells and serum from an adult control, and adult control cells with serum from the infant.

PHA Test		*Control*	*Test*	*P*	*95% CI of diff.*		*Power*	
Cells	*Serum*	*Mean*	*Mean*	*Value*	*of means*		*Obs*	*10%*
AT 3 MONTHS OF AGE								
Infant	Infant	133.74	137.48	77%	-21.35	28.83	14	29
Infant	Adult	138.78	143.11	72%	-19.36	28.02	15	32
Adult	Infant	97.97	103.80	14%	-1.88	13.53	42	83
Adult	Adult	100.00	100.00	N/A				
AT 1 YEAR OF AGE								
Infant	Infant	140.40	142.06	92%	-29.81	33.13	12	25
Infant	Adult	142.83	143.39	96%	-23.25	24.35	11	32
Adult	Infant	102.14	101.54	90%	-9.87	8.69	12	68
Adult	Adult	100.00	100.00	N/A				
Change in Inf-Inf		15.03	-1.27	37%	-52.40	19.80	15	0

Notes
P values were calculated from student's t test.
95% CI = 95% confidence intervals.
Changes are reported as the mean of the test with cells and serum from the infants at 12 months minus the mean value at 3 months.
The control results are 100% by definition.
The statistical power of the study was determined for both the observed difference (Obs) and a 10% difference (10%) from the combined mean for a type I error probability of 5%.

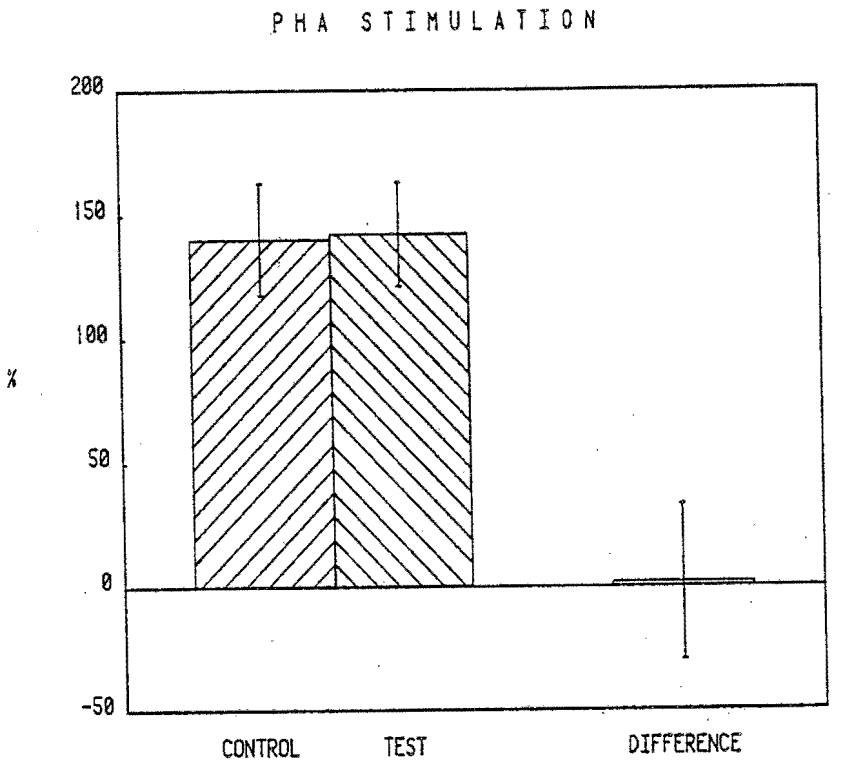

Figure 6.13 Lymphocyte responses to stimulation with phytohaemagglutinin for the Test and Control groups at 12 months of age*

Discussion

The results show that the increased iron fortification had essentially no effect on iron status.

Comparison with previous studies

Table 2.8 in chapter 2 summarizes the results of studies found in the literature survey that reported on lymphocyte stimulation tests in iron deficiency. Of these, 8 documented significantly impaired responses to phytohaemagglutinin, 2 showed similar but non-significant trends and in 1 there was no change in iron deficiency.

In the study with the anomalous results[251], all the children were severely iron deficient (the mean haemoglobin was 3.5 g/dl) and 7 of the 8 had hookworm ova or parasites in the stools.

The other clinical studies derived their subjects from hospital populations where confounding factors of nutrition and infection are prevalent. The only laboratory experiment[257] used pair-fed rats to control for nutritional deficiencies other than iron. These investigations all compared groups with greater differences in iron status than existed in the present project.

* The bars represent the means and the vertical lines through the bars represent the 95% confidence intervals

Neutrophil bactericidal indices

Introduction

The bactericidal test is an *in vitro* test of the ability of cells and serum to kill bacteria (in this case *Staphylococcus aureus*).

Results

Table 6.19 shows that the bactericidal indices at 12 months were slightly better in the Test group than in the Control group; for instance, 105% compared with 94% at one hour. The Test group also manifested a greater improvement in killing ability from that at 3 months to that at 12 months of age. The 95% confidence intervals are relatively wide and the statistical power of the test is low so no definite conclusion can be drawn with respect to the effect of extra iron supplementation on the bactericidal index.

Table 6.19 Neutrophil bactericidal indices compared between the Test and Control groups. The bactericidal index is expressed as the percentage of the initial inoculum of Staphylococcus aureus **surviving after culture with neutrophils from the subject.**

Incubation Period	*Control Mean*	*Test Mean*	*P Value*	*95% CI of diff. of means*		*Power Obs*	*10%*
AT 3 MONTHS OF AGE							
1 hour	108.84	102.52	58%	-29.18	16.52	0	0
2 hours	124.41	111.00	43%	-47.13	20.31	0	0
AT 1 YEAR OF AGE							
1 hour	105.14	94.00	44%	-40.10	17.81	22	20
Change in index	15.05	-11.72	21%	-69.05	15.52	24	24
2 hours	126.75	113.31	57%	-60.53	33.65	18	17
Change in index	23.71	2.36	52%	-88.51	45.80	0	0

Notes P values were calculated from student's t test.
95% CI = 95% confidence intervals.
Changes are reported as the mean of the variable at 12 months minus the mean value at 3 months.
The statistical power of the study was determined for both the observed difference (Obs) and a 10% difference (10%) from the combined mean for a type I error probability of 5%.

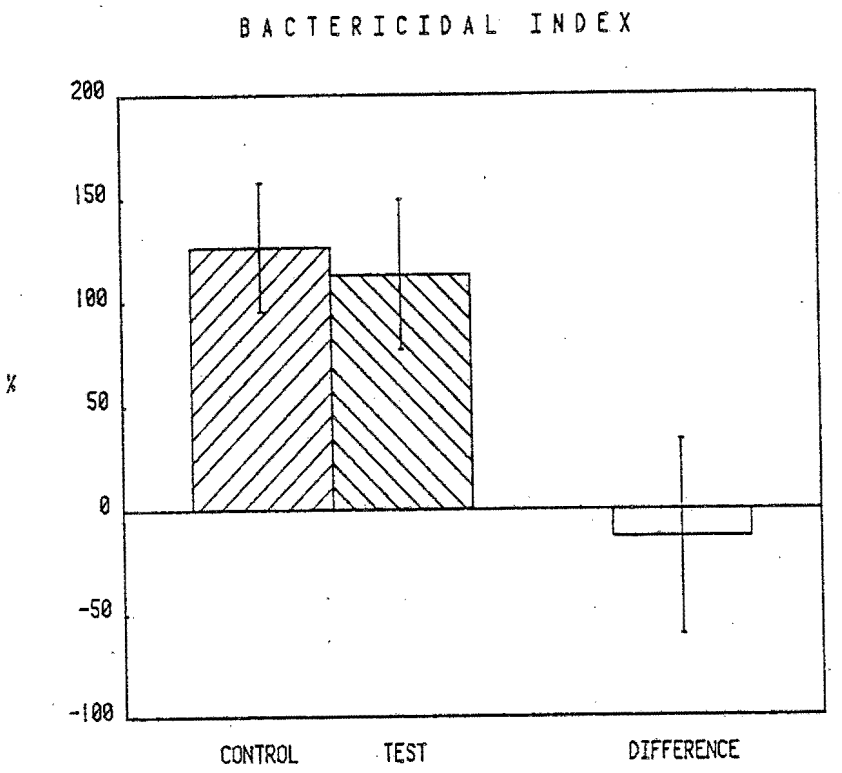

Figure 6.14 **Neutrophil bactericidal indices for the Test and Control groups at 12 months of age***

Discussion

In appendix 3 the bactericidal indices are reported in several different ways. As the computational methods are fundamentally different some comment is indicated. Table 6.19 gives the percentage of the initial bacterial inoculum surviving after 1 and 2 hours. In appendix 3 the subjects are compared to the adult controls by computing the ratio of the colony counts in subject's test to those of the control test at 1 and 2 hours. In a more complex calculation the subjects were compared to the positive and negative controls (adult cells and pooled AB serum). As the various methods produced similar results only the simplest index is reported here.

Comparison with previous studies

Decreased bactericidal activity has been reported in catalase positive organisms in 12 of 13 studies found in the literature survey. Their results are summarized in table 2.10, chapter 2. All these studies compared groups with much larger differences in iron status than in the present study**. With the exception of the

* The bars represent the means and the vertical lines through the bars represent the 95% confidence intervals

** This can be inferred even if it was not explicitly stated as, for example, in 91, 86, 156.

report of Walter *et al*[459], the design of the studies makes it difficult to discriminate between the effects of iron deficiency and coexisting infection or malnutrition or both*.

Walter *et al* conducted an elegant study of the changes in bactericidal activity as iron status was restored to normal. But, even this design does not completely rule out the possibility of infection precipitating the seeking of medical help and thus inclusion in the study. On referral, the mean haemoglobin of 10 infants was 7.9 g/dl. By day 15 it had improved to 9.6 g/dl in response to treatment with oral ferrous sulfate and the bactericidal capacity had risen from 59% to 91% (approximately equivalent to 41% and 9% in terms of the bactericidal index used in this report). From day 15 there was no further improvement in bactericidal capacity.

Conclusion

The bactericidal assay in the present study showed trends that paralleled results from other studies, but the statistical power of the study was low and no effect can be conclusively assigned to the extra iron fortification.

* To be precise, one should also exclude from this judgment the experiment of Arbeter *et al*[9] in which 2 rabbits were made iron deficient by bleeding. And, also, it should be noted that Masawe *et al*[299] tested the bactericidal activity of whole blood.

Viral survey

Introduction

A survey of virus carriage rate was made on entry to the study and on exit. On entry, when the infants were 3 months old, a faecal specimen was sent for viral culture. On exit, at 1 year of age, swabs from the rectum and from the nasopharynx were sent for viral analysis.

Results

The virus isolation rates are given in table 6.20. At 3 months of age, 18% of Control infants were excreting virus in the stool compared with 20% of Test infants. At 1 year of age, the isolation rates from rectal swabs were 36% and 38% respectively, while from nasopharyngeal swabs the rates were 14% for both groups.

The types of viruses isolated are described in appendix 3.

Table 6.20 Virus isolation rates compared for the Control and Test groups. At 3 months of age faecal specimens were cultured for viruses. At 12 months of age swabs were sent for viral culture from the rectum and from the nasopharynx.

Group	3 Months Faeces		1 Year Rectal Swab		1 year Throat Swab	
	No.	%	No.	%	No.	%
Control	11	18	23	36	9	14
(No.)	(61)		(64)		(64)	
Test	12	20	25	38	9	14
(No.)	(60)		(66)		(66)	

Notes P values calculated from the Chi square test were far from significant.

Discussion

The virus carriage rate was essentially equal in the Control and Test groups. This can be taken as evidence against the increased iron fortification having an adverse effect on the bowel and nasopharyngeal viral flora.

Incidence of infection

Introduction

The rate of infection experienced by children in the Test and Control groups is analyzed in tables 6.21 and 6.22. The first table compares the mean numbers of infections experienced in the two groups during the trial period while the second compares the mean incidence rates of infection. The incidence rates are expressed in numbers of infections per child per year and therefore remove the small bias introduced by a slightly longer mean period of observation for children in the Test group - 8.96 months compared with 8.85 months for the Control group.

Analysis of numbers and of rates of infection leads to similar conclusions. Discussion is therefore confined to table 6.22.

Infections were graded according to severity and classed according to clinical type. Grade 1 illnesses were of minor importance and did not warrant treatment. Grade 2 infections were of moderate importance and required treatment as an outpatient. Infections severe enough to require hospitalization were recorded as grade 3.

Infections of grade 2 or 3 were also classed according to their clinical type, *viz* conjunctivitis, pyoderma, thrush, gastroenteritis and infections of the upper or lower respiratory tract (including otitis media). These classes were chosen after the study because they included sufficient numbers for meaningful analysis. Morbidity in infancy is not defined well enough to allow such classes to be defined in the planning stages of this study. Infections that did not fall into one of the above classes are tabulated as "other". Details of the operational definition of infection and the precautions taken to avoid counting infections more than once or missing infections are given in the *Methods and Materials* section of chapter 3.

Results

Table 6.22 shows that the Test group had fewer infections than the Control group for each grade of severity and for 5 of the 6 classes of infection. The Test group also had fewer infections in total than the Control group. No difference is statistically significant and the 95% confidence intervals are relatively wide.

In the interests of planning future studies, a power analysis for each category of infection is presented in table 6.23. The statistical power of the trial to detect a 10% difference in infectious morbidity is less than 25%, to detect a 25% difference in morbidity 81%, and to detect a 50% difference in incidence rates the power is 99%. The difference actually observed between the two groups is 9.12%.

Table 6.21 **Numbers of infections compared between the Test and Control groups. The infants were observed from the age of 3 months to 1 year.**

Infection Category	*Control Mean*	*Test Mean*	*P Value*	*95% CI of diff. of means*		*Power Obs*	*10%*
Severity of infection							
Minor	1.26	1.16	0.64	-0.52	0.32	0	0
Important	3.34	3.13	0.58	-0.96	0.54	0	14
Severe	0.16	0.11	0.44	-0.17	0.07	12	0
TOTAL Infections	4.76	4.40	0.37	-1.15	0.43	15	21
TYPE OF INFECTION							
Conjunctivitis	0.24	0.21	0.72	-0.18	0.12	0	0
Other	0.27	0.14	0.12	-0.30	0.04	35	0
Pyoderma	0.39	0.33	0.70	-0.35	0.24	0	0
Thrush	0.40	0.36	0.72	-0.30	0.21	0	0
Gastroenteritis	0.79	0.71	0.64	-0.40	0.24	0	0
Respiratory	1.43	1.49	0.71	-0.39	0.50	0	0
TOTAL Infections	4.76	4.40	0.37	-1.15	0.43	15	0

Notes P values were calculated from student's t test.
95% CI = 95% confidence intervals.
The statistical power of the study was determined for both the observed difference (Obs) and a 10% difference (10%) from the combined mean for a type I error probability of 5%.

Table 6.22 **Incidence of infection compared between the Test and Control groups. The incidence is expressed as the average number of infections per infant per year. It was determined by dividing the number of infections each infant experienced by the number of days he or she participated in the trial and then calculating the mean rate of infection.**

Infection	*Control*	*Test*	*P*	*95% CI of diff.*		*Power*	
Category	*Mean*	*Mean*	*Value*	*of means*		*Obs*	*10%*
Severity of infection							
Minor	1.69	1.55	62%	-0.70	0.42	0	0
Important	4.51	4.16	48%	-1.35	0.65	10	14
Severe	0.22	0.15	43%	-0.23	0.10	12	0
TOTAL Infections	6.42	5.86	30%	-1.61	0.49	18	21
TYPE OF INFECTION							
Conjunctivitis	0.32	0.29	72%	-0.24	0.17	0	0
Other	0.37	0.19	11%	-0.41	0.04	33	0
Pyoderma	0.52	0.44	66%	-0.49	0.31	0	0
Thrush	0.54	0.48	68%	-0.40	0.27	0	0
Gastroenteritis	1.07	0.95	59%	-0.55	0.31	0	0
Respiratory Infect.	1.90	1.98	80%	-0.52	68	0	0
TOTAL Infections	6.42	5.86	30%	-1.61	0.49	18	21

Notes P values were calculated from student's t test.
95% CI = 95% confidence intervals.
The statistical power of the study was determined for both the observed difference (Obs) and a 10% difference (10%) from the combined mean for a type I error probability of 5%.

Table 6.23 Statistical power of the present study to detect a difference in infection rate between the Test and Control groups with a 5% significance level.

Variable	DIFFERENCE: Actual	10%	25%	50%
SEVERITY OF INFECTION				
Minor	0	0	29	80
Important	10	14	56	99
Severe	12	0	0	20
TOTAL Infections	18	21	81	99
TYPE OF INFECTION				
Conjunctivitis	0	0	11	31
Other	23	0	0	23
Pyoderma	0	0	0	22
Thrush	0	0	12	32
Gastroenteritis	0	0	21	63
Respiratory Infect.	0	0	36	89
TOTAL Infections	18	21	81	99

Notes The differences for which the statistical power was calculated are that difference actually observed between the means and 10%, 25% and 50% of the mean for both groups combined.

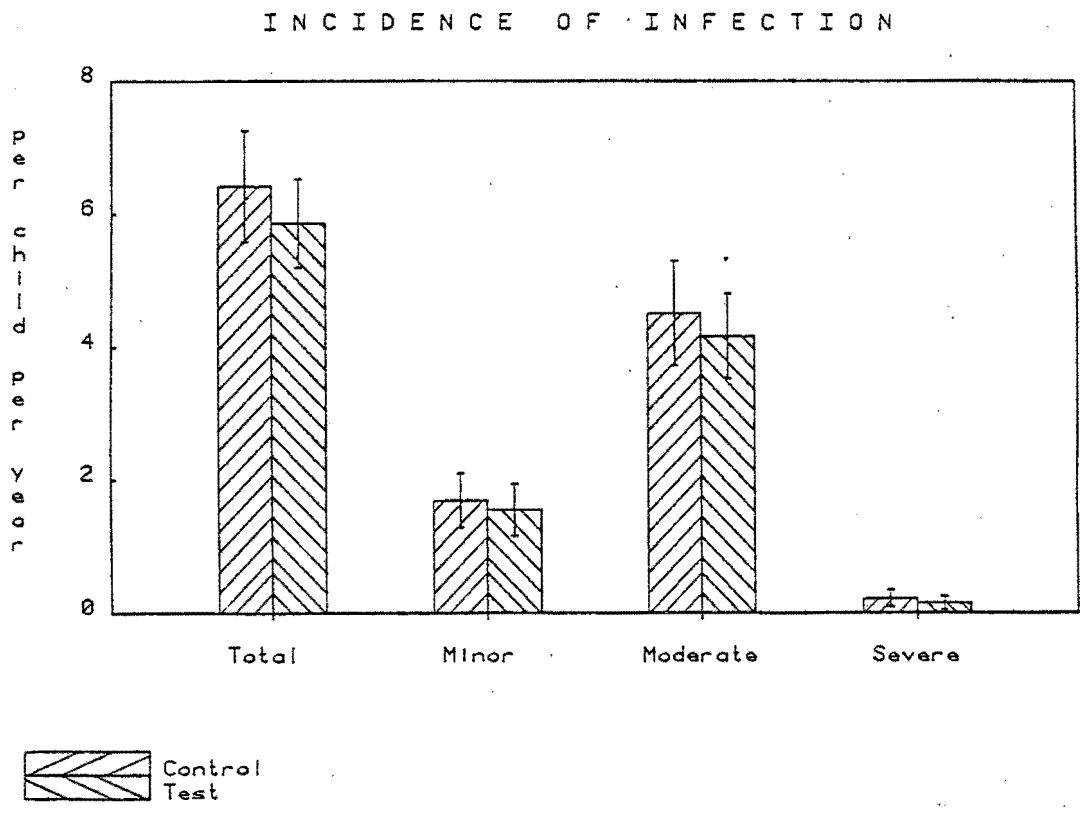

Figure 6.15 **Incidence of grades of infection compared for the Test and Control groups at 12 months of age***

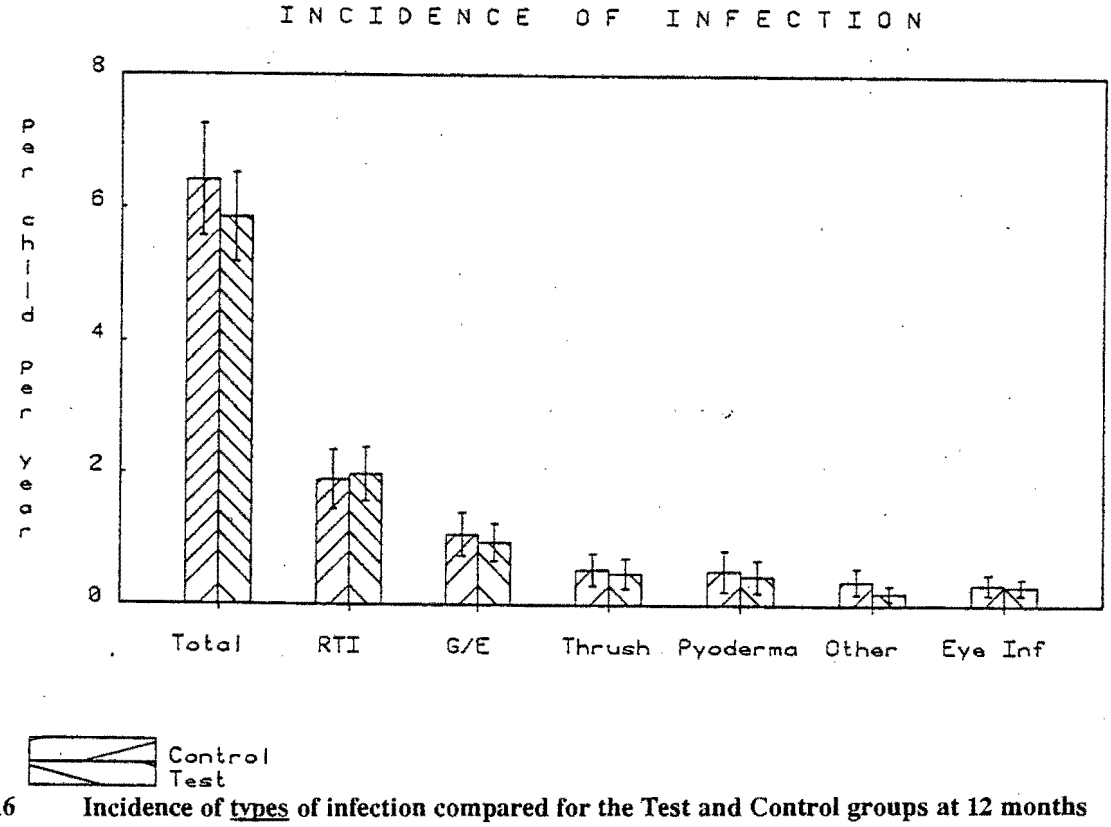

Figure 6.16 **Incidence of types of infection compared for the Test and Control groups at 12 months of age***

* The bars represent the means and the vertical lines through the bars represent the 95% confidence intervals

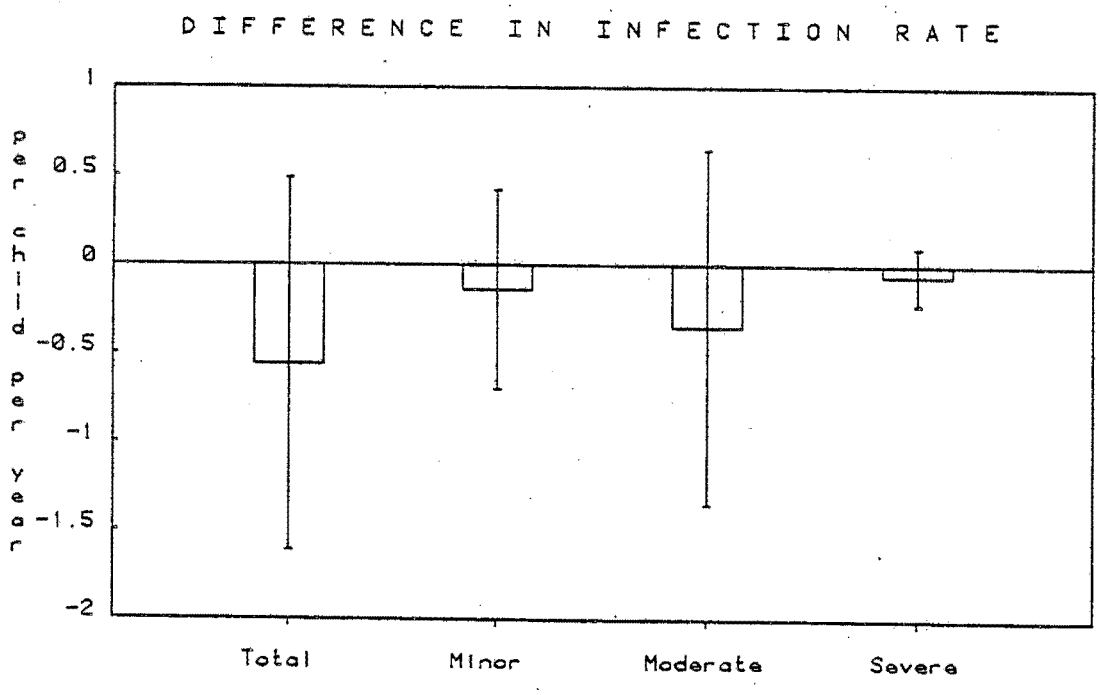

Figure 6.17 **Difference in incidence of grades of infection at 12 months of age*. (Test group mean - Control group mean)**

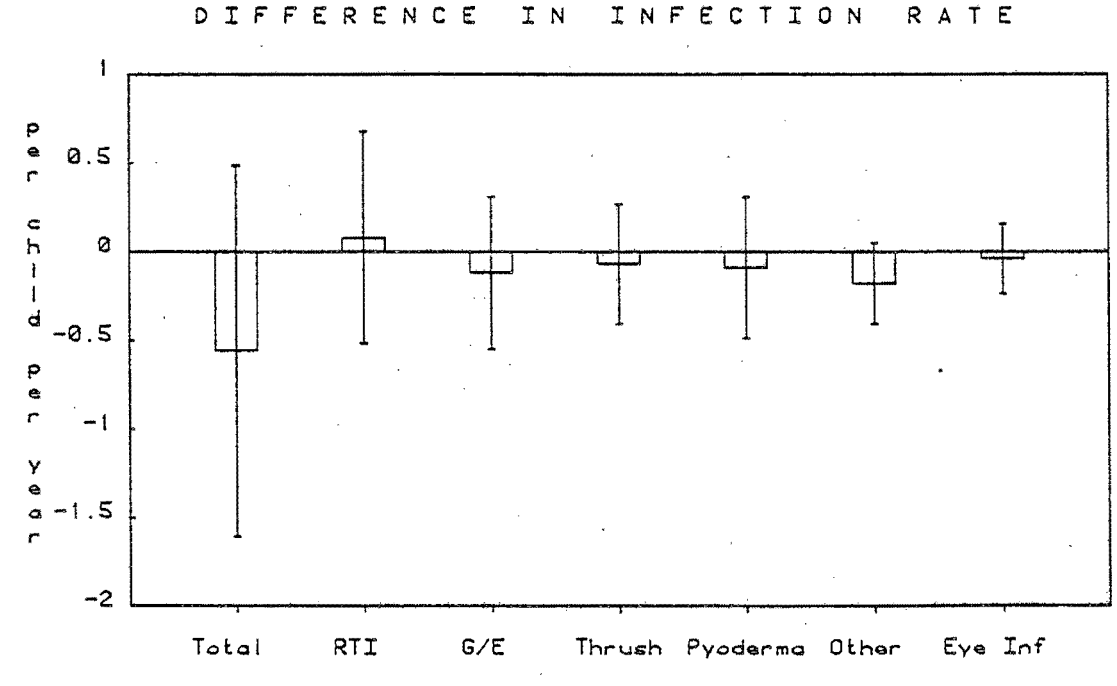

Figure 6.18 **Difference in incidence of types of infection at 12 months of age*. (Test group mean - Control group mean)**

* The bars represent the means and the vertical lines through the bars represent the 95% confidence intervals

Comparison with previous studies

Chapter 2 contains a detailed analytical review of previous studies that have attempted to define the putative association of iron status with susceptibility to infection. Five studies in infants have been reported in sufficient detail for comparison to be made with the present study.

Burman[71] followed a group of infants from the age of 3 months to the age of 18 months. Children were allocated alternately to receive either placebo or an oral iron preparation to be taken daily. At one year of age the mean haemoglobins of the two groups were 11.75 and 11.76 g/dl. There was no difference in incidence of reported infection. The power of the study was not reported, but it is surely small.

In a similar trial to the present one Andelman and Sered[7] compared the effect of fortification of an infant milk formula with 12mg Fe/quart with unfortified formula. At one year of age, the mean haemoglobins of the two groups of infants were 11.9 and 10.4 g/dl respectively and the control group had had significantly more respiratory infection than the test group. Little information is given on how infections were diagnosed and no data are provided for other classes of infection.

Cantwell[77] studied the effects of iron dextran in premature infants. The regimen resulted in the control group having a mean haemoglobin of 9.0 g/dl at 1 year while the iron treated group had a mean haemoglobin of 11.3 g/dl. The control group had 39% more admissions than the test group over a 2 year period of observation. When acute wheezy bronchitis and asthma are excluded, the control group had twice the number of infection of the test group. Some methodologic problems are noted in chapter 2, but the study does suggest an aetiological role for iron deficiency in susceptibility to infection.

Mackay[285] compared infection rates in iron deficient infants treated with an oral iron preparation to infection rates in an untreated control group. The control group's mean haemoglobin at 1 year of age was 10.0 g/dl while the treated group attained a mean haemoglobin of 11.3 g/dl. There was a remarkable relationship between infection rate and iron treatment. In both winter and in summer the test group had about half the number of infections that the controls experienced. The relationship continued to hold when the infections were classed as "specific fevers", respiratory infections, gastro-intestinal infections and "other". A major problem in interpretation was discussed at length by the author *viz* that the two groups were studied over different time periods. Even though she presented evidence that, in London, the infant death rate from infections did not change over that period, this retrospective open study leaves many confounding factors uncontrolled.

The utility of iron dextran in preventing iron deficiency anaemia in the first year of life was studied by Oppenheimer *et al*[343] in Papua New Guinea. The iron-treated infants experienced a significantly increased incidence of malaria in the first year of life. The incidence of other infections was not discussed.

Conclusions

The studies in temperate climates have suggested that iron deficiency predisposes to infection and the present study, while not reaching statistical significance, shows remarkable consistency in the parallel trends revealed. The sole study from the tropics indicates that iron dextran in young infants increases susceptibility to malaria.

Figure 6.18 shows the difference in infection rates between the Control and Test groups. For "total infections" the difference amounts to 56 infections per 100 children per year. These are clinically important differences with substantial implications for public health. It is important that further studies be made in order to determine whether this is a chance association or the consequence of improved iron nutrition.

The difficulties such studies will have in attaining adequate statistical power must be noted. The present study was designed to detect a difference in infection rates of 1 infection per child per year. The sample size calculations yielded 65 as the minimum number required in each group, assuming that the standard deviation of infection rate was 2.5. With the data from the present study it can be estimated that a minimum of 230 infants would be required in each group to detect a difference of 0.59 in infection rates with standard deviations of 2.8 and 3.3.

Besides increasing the number of subjects, steps that future studies could take to increase statistical power are to follow the subjects for a longer period or to increase the disparity in iron nutrition between the two groups or use a combination of these manoeuvres in order to improve the statistical power of the trial. The third option is limited by ethical considerations since gross iron deficiency should be treated and gross iron excess may be dangerous and should be avoided. The first and second options are limited only by availability of resources. In tropical areas a special consideration is the increased risk of malaria in iron sufficient children.

Summary of results of immune function tests

Laboratory and clinical tests of immune function were made in the Test and Control groups. These were delayed cutaneous hypersensitivity with Candida antigen and PPD, specific antibody response to immunization with oral polio vaccine and intramuscular tetanus toxoid, leukocyte counts, lymphocyte subtyping with monoclonal antibodies, lymphocyte stimulation with phytohaemagglutinin, and neutrophil bactericidal index and incidence of infection. The Test and Control groups had similar results with a clear trend emerging only for the incidence of infection; in 9 of 10 categories of infection the Test group had a lower incidence than the Control group.

Each measure of immune function had a relatively wide 95% confidence interval and a low statistical power. However, the study is definitive in the confidence limits set for the means of the various indices of immune function and their differences between the two groups. From this analysis it is possible to state, for example, that the extra iron fortification is unlikely to have reduced the incidence of infection by more than 25% or to have increased it by more than 8%.

In all the immune function tests there is no evidence to suggest that the extra iron was harmful.

The most intriguing results are the rates of infection which consistently favoured the Test group. One may calculate using the binomial function the probability of obtaining by chance this sort of distribution; it is 3.5%*. Although it is tempting to do so, this value of 3.5% may not be taken as equivalent to the "P value" obtained from a significance test because the calculations rely on the assumption that the various categories of infection are statistically independent. Current medical knowledge would contradict this assumption. Defining an association between multiple variables is properly the function of multivariate analysis. The following section reports the multivariate analyses that were made in seeking an association of iron status with immune function.

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* Of the 10 categories of infection, 8 are statistically independent. The probability of at least 7 of 8 independent categories favouring the Test group is $1/2^8 + (8!/7!)*(1/2^8) = 9/256 = 3.52\%$

The association of iron status with immune function tests (Multivariate analyses)

Classification of iron status

Introduction

The section above examined the results of the study in relation to the hypothesis that additional iron supplementation of infant milk formula would lead to improved immune function. The analysis therefore compared the Control group of infants with the Test group for incidence of infection (in several different categories of infection) and for performance in a variety of different laboratory measures of immune function.

This section presents an analysis of immune function tests and the incidence rates of infection when the study subjects are categorized according to iron status. If iron deficiency depresses immune function and predisposes to infection then children classed as "iron deficient" should experience more infections and should have poorer immune function tests than children classed as "iron sufficient".

The statistical procedures involved computing the mean and standard deviation for each variable within each category of iron status and then calculating the significance level for each pair-wise comparison. The Bonferroni procedure was used to adjust the significance level for the multiple comparisons made in this analysis.

The method of classification

There is no scheme of classification of iron status that simply and unambiguously measures the amount (relative to requirements) of iron available to the tissues, cells and enzymes that need it to perform their functions. This study made 10 tests that directly reflect the status of iron nutrition*. Five of these were used to classify (by computer) the status of iron nutrition of each child. The scheme is detailed in the following section.

Categories of iron status

Most children can be categorized with respect to iron status along the spectrum:- iron replete (*Normal*), depletion of iron stores (*Depleted*), biochemical evidence of iron deficiency (*Deficient*), and iron deficiency with anaemia (*Deficient and anaemic*). With this model of iron metabolism in mind, a classification system has been drawn up for use in the Department of Paediatrics and Child Health of the University of Cape Town and is described in tables 6.24 and 6.25. Table 6.24 gives reference values for the variables employed in allocating a set of results to a particular category of iron status. Table 6.25 gives the decision

* These are: RBC, Hb, HCT, MCV, MCH, MCHC, RDW%, Reticulocyte count, plasma ferritin, and red cell ZPP.

criteria to be satisfied for each category. For example, a child classified as *Normal* will have a haemoglobin greater than 11.0 g/dl, plasma ferritin more than 12 ug/dl and at least two of the following criteria true: mean cell volume greater than 70 fl, mean cell haemoglobin greater than 23 pg, and red cell zinc protoporphyrin less than 3.5 ug/dl.

Some children do not fit into the categories of iron status defined above and must be classed as *Normochromic normocytic anaemic* or placed in the *Unclassified* category. This may be because of normal biological variation or because of some abnormal condition affecting the variables used for categorization. For example, a number of infants with normal levels of ferritin have a normochromic normocytic anaemia (one of the causes of which may be the effects of infection[439]). Their iron stores are probably normal, but they need to be kept in a separate group when associations of iron status with immune function tests are sought. A substantial number of children have tests of iron status with conflicting interpretations. There are several reasons for this, for example thalassaemia and haemoglobinopathies that occur in 10% of Cape Coloured children with a mean cell volume less than 65 fl[39], and these will not be further mentioned. This group of children also needs to be kept separate when seeking relationships with iron status.

Table 6.24 Reference values used in the classification of iron status of 1 year old infants.

TEST	REFERENCE VALUE
Haemoglobin(Hb)	>11.0 g/dl
Ferritin(FRTN)	>12.0 ug/l
Mean cell volume (MCV)	>70.0 fl
Mean cell haemoglobin (MCH)	>23.0 pg
Zinc protoporphyrin (ZPP)	<3.5 ug/g

Table 6.25 Criteria for the classification of iron status in 1 year old infants. Reference values for the application of these decision criteria are given in table 6.24.

IRON STATUS	CRITERIA FOR CLASSIFICATION		
	Hb	FRTN	>1 of: MCV, MCH & ZPP
Normal	Y	Y	Y
Depleted	Y	N	Y
Deficient	Y	N	N
Deficient and anaemic	N	N	N
Unclassified 1	Y	Y	N
Unclassified 2	N	Y	Y
Unclassified 3	N	N	Y
Normochromic normocytic Anaemia	N	Y	Y

Notes Y in the table indicates that the criterion in that column has to be satisfied for the category named in that row.
N in the table indicates that the criterion in that column has to be NOT attained for the category named in that row.
For example, a child with *normal* iron status has an Hb at least 11 g/dl, plasma ferritin at least 12 ug/l and at least 2 of MCV, MCH and ZPP satisfying their respective criteria.

Association of iron status with immune function

Introduction

Table 6.26 summarizes the results from computing the mean for each group of the infants according to iron status. Statistical tests included the *t* test. As none of the tests were significant (less than 5%) the P values are not included in the table. The results become difficult to interpret in this type of analysis because the number within a group becomes small. However, trends are apparent that are in keeping with the analysis in the main body of the report and that lend support to the hypothesis that deficient iron status is causally related to poor immune function.

Incidence of infection - Normal iron status

Within the *Normal* group, in 9 of 10 categories of incidence of infection the infants in the Test group had lower rates of infection that those in the Control group - see tables 6.26 and 6.27. Tables 6.29 and 6.30 show that of 10 measures of iron status, all except mean cell haemoglobin and mean cell haemoglobin concentration indicated better iron nutrition in the Test group.

Incidence of infection - Normochromic normocytic group

In 7 of the ten categories of infection, the group categorized as *Normochromic normocytic anaemic* had more infections than the *Normal* group. This group presumably had normal body iron stores as their mean plasma ferritin was (by definition) at least 12 ug/l. The results are consistent with previous observations[439] that chronic infection blocks the transport of iron to erythrocytes*.

Incidence of infection

Comparison between between categories of iron status

The trend of fewer infections with better iron nutrition is best shown when the Total number of infections is considered. For Test and Control groups combined, the class with *Normal* iron status had a lower incidence of infection than each of the categories of iron deficiency. A suggestion of a trend to more infections with increasing iron deficiency is also apparent. The numbers of children classified as *Depleted*, *Deficient* and *Deficient and anaemic* are small, 8, 7 and 8 respectively, compared to the 69 iron sufficient children.

* The results are also consistent with other hypotheses, *eg* that anaemia per se predisposes to infection. But it is not the intention here to become in the philosopher's problem of induction so other theories are not discussed.

Table 6.26 **Incidence of infection by severity according to the category of iron status. Incidence is tabulated as the number of infections per infant per year. The number of infants in each sub-group is shown in parentheses.**

IRON STATUS:	*NORMAL*	*DEPLETED*	*DEFICIENT*	*DEFICIENT & ANAEMIC*	*UNCLASSI-FIED*	*NORMO-CHR/CYT*
SEVERITY OF INFECTION						
MINOR						
Control	2.09	4.76	1.02	1.73	2.65	1.38
	(25)	(6)	(7)	(6)	(13)	(4)
Test	1.82	1.84	-	0	2.20	3.63
	(44)	(2)	(0)	(2)	(13)	(4)
All	1.92	4.03	1.02	1.30	2.42	2.51
MODERATE						
Control	6.02	3.31	10.0	6.01	5.87	8.63
	(25)	(6)	(7)	(6)	(13)	(4)
Test	5.26	6.32	-	7.72	5.18	7.22
	(44)	(2)	(0)	(2)	(13)	(4)
All	5.54	4.06	10.00	6.43	5.52	7.90
SEVERE						
Control	0.15	0.30	0.26	0.58	0.29	1.35
	(25)	(6)	(7)	(6)	(13)	(4)
Test	0.12	0.92	-	0	0.28	0.91
	(44)	(2)	(0)	(2)	(13)	(4)
All	0.13	0.45	0.26	0.43	0.28	1.13
TOTAL INFECTIONS						
Control	8.26	8.37	11.27	8.31	8.81	11.36
	(25)	(6)	(7)	(6)	(13)	(4)
Test	7.20	9.0	-	7.72	7.66	11.77
	(44)	(2)	(0)	(2)	(13)	(4)
All	7.58	8.55	11.27	8.16	8.23	11.56

Notes The t tests for statistical significance employed the Bonferroni adjustment for multiple comparisons. No P values were as small as 5%.

Table 6.27 Incidence of infection by type according to the category of iron status. Incidence is tabulated as the number of infections per infant per year. The number of infants in each sub-group is shown in parentheses.

IRON STATUS:	*NORMAL*	*DEPLETED*	*DEFICIENT*	*DEFICIENT ANAEMIC*	*UNCLASSI -FIED*	*NORMO- CHR/CYT*
TYPE OF INFECTION						
CONJUNCTIVITIS						
Control	0.43	0.30	1.03	0.29	0.27	0.46
	(25)	(6)	(7)	(6)	(13)	(4)
Test	0.29	0.92	-	1.73	0.55	0.0
	(44)	(2)	(0)	(2)	(13)	(4)
All	0.34	0.46	1.03	0.65	0.41	0.23
OTHER INFECTIONS						
Control	0.22	0.58	0.60	0.29	0.42	1.35
	(25)	(6)	(7)	(6)	(13)	(4)
Test	0.20	0	-	0	0.27	0.43
	(44)	(2)	(0)	(2)	(13)	(4)
All	0.21	0.44	0.60	0.22	0.34	0.89
PYODERMA						
Control	0.40	0.0	2.01	0.0	1.16	1.35
	(25)	(6)	(7)	(6)	(13)	(4)
Test	0.73	0.0	-	0.85	0.135	0
	(44)	(2)	(0)	(2)	(13)	(4)
All	0.59	0	2.01	0.21	0.65	0.68
THRUSH						
Control	0.80	0.60	1.76	0.58	0.29	0.46
	(25)	(6)	(7)	(6)	(13)	(4)
Test	0.56	4.50	-	0.85	0.28	0.0
	(44)	(2)	(0)	(2)	(13)	(4)
All	0.65	1.58	1.76	0.65	0.28	0.23
GASTROENTERITIS						
Control	1.52	0.59	0.51	1.46	1.67	3.2
	(25)	(6)	(7)	(6)	(13)	(4)
Test	1.21	0.89	-	2.56	0.95	3.12
	(44)	(2)	(0)	(2)	(13)	(4)
All	1.33	0.67	1.73	1.31	3.16	0.60
RESPIRATORY TRACT INFECTION						
Control	2.76	1.52	1.79	3.96	2.36	3.62
	(25)	(6)	(7)	(6)	(13)	(4)
Test	2.27	0.92	-	1.73	3.0	4.58
	(44)	(2)	(0)	(2)	(13)	(4)
All	2.45	1.37	1.79	3.4	2.68	4.1

Table 6.28 **Immune function indicators compared between categories of iron status. The number of infants in each sub-group is shown in parentheses**

IRON STATUS:	*NORMAL*	*DEPLETED*	*DEFICIENT*	*DEFICIENT ANAEMIC*	*UNCLASSI -FIED*	*NORMO- CHR/CYT*
OKT4/OKT8 RATIO						
Control	2.25	3.71	2.56	2.13	2.66	2.45
	(22)	(6)	(7)	(6)	(11)	(4)
Test	2.52	1.06	-	3.20	2.23	2.07
	(44)	(2)	(0)	(2)	(13)	(4)
All	2.43	3.05	2.56	2.40	2.42	2.26
PHA STIMULATION (Percentage of adult control)						
Control	61	94	162	84	122	106
	(11)	(2)	(3)	(1)	(4)	(2)
Test	153	119	-	-	110	117
	(25)	(2)	(0)	(0)	(7)	(2)
All	155	107	162	84	114	111
BACTERICIDAL INDEX (Percentage of initial inoculum surviving at 1 hour)						
Control	103	115	67	118	100	185
	(9)	(4)	(3)	(4)	(7)	(1)
Test	90	-	-	63	70	98
	(18)	(0)	(0)	(1)	(5)	(2)
All	94	115	67	107	87	127
CANDIDA SKIN TEST (mm induration)						
Control	5.96	11.37	13.0	13.5	10.00	0.25
	(23)	(6)	(7)	(6)	(13)	(4)
Test	13.5	13.50	-	19.0	7.70	4.5
	(42)	(2)	(0)	(2)	(13)	(4)
All	8.45	11.88	13.0	14.88	8.85	2.38
MANTOUX TEST (mm induration)						
Control	6.48	6.67	11.57	6.33	9.46	9.25
	(23)	(6)	(7)	(6)	(13)	(4)
Test	7.81	11.50	-	1.00	3.83	8.25
	(42)	(2)	(0)	(2)	(12)	(4)
All	7.34	7.87	11.57	5.00	6.76	8.75
TETANUS ANTIBODY TITRE (titre)						
Control	2.03	1.87	1.86	2.06	2.05	1.66
	(25)	(6)	(7)	(6)	(11)	(4)
Test	1.98	1.61	-	0.95	1.89	1.54
	(44)	(2)	(0)	(2)	(13)	(4)
All	2.00	1.80	1.86	1.80	1.96	1.60
POLIO ANTIBODY TITRE (titre)						
Control	1.62	0.98	1.66	1.40	1.60	1.71
	(21)	(6)	(7)	(6)	(1)	(3)
Test	1.69	1.61	-	1.76	1.68	1.62
	(40)	(1)	(0)	(2)	(12)	(4)
All	1.66	1.07	1.66	1.50	1.64	1.66

Tests of immune function

Comparison between categories of iron status

Results of the laboratory tests of immune function are summarized in table 6.28 for each category of iron status. The tests of leukocyte function, PHA stimulation and bactericidal assay, both show a trend towards "better" (i.e. more active) performance in the classes with better iron nutrition. The opposite trend is evident in the delayed hypersensitivity test with Candida antigen. The Mantoux test shows no clear trend. The ratio of Helper cells to Suppressor cells (OKT4/OKT8 ratio) does not vary much between the various categories. The specific antibody levels to polio and to tetanus both showed a trend to a "better" (higher) response to immunization in the infants with more satisfactory iron nutrition. Although the trends are not statistically significant, these results not only tend to support the hypotheses of the study, but offer no evidence of harmful effects from iron supplementation of infant milk formulas.

These trends should be interpreted even more cautiously than for the trends indentified in the comparison between the Test and Control groups for not only was no difference statistically significant according to the t test, but the confidence limits are wider and statistical power less than for the corresponding comparison reported in the previous section.

Measures of iron nutrition

Comparison between between categories of iron status

Tables 6.29 and 6.30 give the mean values of those tests that reflect iron status. The results are shown to facilitate comparison with other studies and to illustrate the claims implied by the classification scheme. For example, from the description of the way in which the classification scheme was employed it may not be immediately apparent that the infants falling into the group called *Normochromic normocytic* were indeed normochromic-normocytic anaemic children. Inspection of table 6.29 and comparison with the *Normal* group for MCH, MCV and Hb will confirm this.

From a clinical point of view it is interesting to note the proportions in the various categories of iron status. Table 6.29 shows that only 25% of the Control group were iron replete but that 68% of the Test group fell into this category. The proportions classed as *Normochromic normocytic* or *Unclassified* were similar for the two groups; about 7% and 21% respectively. This analysis clearly demonstrates the efficacy of the extra iron fortification in preventing iron deficiency.

Table 6.29 Mean values of haematologic parameters compared between the categories of iron status.

IRON STATUS:	*NORMAL*	*DEPLETED*	*DEFICIENT*	*DEFICIENT ANAEMIC*	*UNCLASSI -FIED*	*NORMO- CHR/CYT*
RED CELL COUNT (10^{12}/l)						
Control	4.73	5.01	5.23	4.72	4.98	4.42
Test	4.86	4.59	-	4.90	5.28	4.28
All	4.8	4.91	5.23	4.76	5.13	4.35
HAEMOGLOBIN (g/dl)						
Control	11.92	12.70	11.34	10.13	11.04	10.70
Test	12.23	11.85	-	10.85	11.12	10.64
All	12.12	12.49	11.40	10.31	11.08	10.67
HAEMATOCRIT (%)						
Control	35.68	38.43	35.03	31.68	33.70	32.45
Test	36.72	36.10	-	32.80	34.43	31.42
All	36.34	37.85	35.03	31.96	34.07	31.94
MEAN CELL VOLUME (fl)						
Control	75.38	76.52	67.09	67.12	67.73	73.35
Test	75.59	78.56	-	66.90	65.23	73.30
All	75.52	77.02	67.09	67.06	66.48	73.32
MEAN CELL HAEMOGLOBIN (pg)						
Control	25.22	25.35	21.86	21.48	22.24	24.17
Test	25.20	25.85	-	22.23	21.12	24.82
All	25.21	25.47	21.86	21.67	21.68	24.50
MEAN CELL HAEMOGLOBIN CONCENTRATION (ug/dl)						
Control	33.41	33.13	32.54	31.98	32.80	32.95
Test	33.32	32.95	-	33.20	32.37	33.85
All	33.35	33.09	32.54	32.29	32.58	33.40
RED CELL DISTRIBUTION WIDTH (%)						
Control	14.50	14.13	16.59	17.18	16.48	16.67
Test	13.87	14.30	-	14.70	16.20	14.90
All	14.10	14.17	16.59	16.56	16.34	15.79
RETICULOCYTE COUNT (%)						
Control	2.06	1.38	1.30	1.38	1.79	0.72
Test	1.79	1.55	-	1.55	2.09	1.37
All	1.89	1.42	1.30	1.42	1.94	1.05
NUMBER (%)						
Control	25 (41)	6 (10)	7 (11)	6 (10)	13 (21)	4 (7)
Test	44 (68)	2 (3)	0 (0)	2 (3)	13 (20)	4 (6)
All	69 (55)	8 (6)	7 (6)	8 (6)	26 (21)	8 (6)

Table 6.30 Non-haematologic indicators of iron status compared between the categories of iron status.

IRON STATUS:	*NORMAL*	*DEPLETED*	*DEFICIENT*	*DEFICIENT ANAEMIC*	*UNCLASSI -FIED*	*NORMO- CHR/CYT*
FERRITIN[1] (ug/dl)						
Control	28.2	7.6	4.5	6.8	23.4	37.2
Test	31.6	8.5	-	2.63	30.2	41.7
All	30.9	7.8	4.5	6.0	26.9	39.8
RED CELL ZINC PROTOPORPHYRIN (ug/g Hb)						
Control	3.43	3.30	4.69	5.60	4.23	3.45
Test	3.11	2.80	-	4.00	4.35	3.35
All	3.22	3.17	4.69	5.37	4.30	3.40
NUMBER						
Control	25	6	7	6	13	4
Test	44	2	0	2	13	4
All	69	8	7	8	26	8

1 Log(ferritin) was used in the calculations.

Multiple linear regression

The relation of iron status to immune function was analyzed by multiple linear regression (MLR). Multiple linear regression is a mathematical technique for quantifying the "contribution" one variable makes to its association with another parameter. "Input" or predictor parameters are known as *independent variables* and "output" or predicted variables are called *dependent variables*. The multiple linear regression technique allows for more than one input variable and/or more than one dependent variable. The result of an analysis includes a set of coefficients of the dependent variables, a constant and a set of P values from significance tests of the coefficients. The analyses were all made with the BMDP suite of programs[126].

Many analyses were made and two sets were chosen to illustrate the kind of results that were obtained. Table 6.31 shows the coefficients when the independent variables were incidences of the various categories of infection. The dependent variables were study group, haemoglobin and iron status. Each dependent variable was subject to a separate investigation. Haemoglobin was chosen as an indicator of iron status for obvious reasons.

Study group was selected in order to quantify the association with infection. To perform the multiple linear regression a numerical value must be given to categorical variables such as *group*. The value of 0 was assigned to the Control group and 1 to the Test group. The values are arbitrary and unimportant except in their relation to one another. These values were chosen so that a positive coefficient would indicate that its variable is likely to have higher values for the Test group.

For similar reasons, values of 1, 2, 3, and 4 were given to the categories of iron status *Normal*, *Depleted*, *Deficient*, and *Deficient and anaemic*. Again, variables with positive coefficients are more likely to have higher values with iron deficiency.

Table 6.31 is difficult to interpret as the numerical values have no immediate intuitive interpretation. The P values are not shown since no coefficient was statistically significant. To aid in the interpretation of the table the trends shown by the multiple linear regression analyses are summarized in table 6.33. If a variable supports the hypothesis that iron deficiency is associated with increased susceptibility to infection an entry of *Y* is found in the table. If the variable does not support the hypothesis an entry of *N* is made.

A slight tendency for infections to be increased in iron deficiency and decreased with extra iron fortification of infant milk formula is thus revealed by table 6.33.

A similar set of analyses was made for the laboratory indicators of immune function and infection rates as predictors of iron status*. The results are tabulated in table 6.32 and the trends analyzed in table 6.34. No

* These analyses omit PHA and bactericidal indices because they were made for separate halves of the study population. No child had both tests made. It is therefore not possible to include both together in an multiple linear regression analysis.

variable was statistically significantly associated with iron status. Overall, there is a slight tendency for the study's hypotheses to be supported.

Table 6.31 Multiple linear regression (MLR) analysis of association of iron status with incidence of infection. The regression coefficients are tabulated for three separate analyses with incidences of infection as independent variables and with the dependent variables being the group code[1], haemoglobin concentration and code for iron status[2].

INDEPENDENT VARIABLES	DEPENDENT VARIABLES GROUP	HAEMOGLOBIN	FE STATUS
Grade 1	-0.05	0.02	-0.02
Grade 2	-0.05		
Grade 3	-0.09	-0.21	0.45
Thrush	-0.63	-0.09	0.08
Conjunctivitis	-0.004	-0.15	0.20
Gastroenteritis	0.03		-0.01
Respiratory Infection	-0.04	-0.003	0.03
Pyoderma	0.005	0.06	0.01
Other	-0.035	0.001	-0.03

Notes
1 Group codes were: 0 = Control, 1 = Test
2 Iron status codes were: 0 = *Normal*, 1 = *Depleted*, 2 = *Deficient*, 4 = *Deficient anaemic*
3 A blank entry in the table is made for variables whose contribution to the MLR was minimal
4 No coefficient reached statistical significance (P < 5%)

Table 6.32 Multiple linear regression (MLR) analysis of association of iron status with indicators of immune function. The regression coefficients are tabulated for three separate analyses with indicators of immune function as independent variables and with the dependent variables being the group code[1], haemoglobin concentration and code for iron status[2].

INDEPENDENT VARIABLES	DEPENDENT VARIABLES GROUP	HAEMOGLOBIN	FE STATUS
B Cells	0.01	-0.002	-0.001
Mantoux	-0.001	-0.01	0.001
Candida test	0.002	-0.005	0.03
Grade 1	-0.03	0.04	-0.02
Grade 2		-0.07	0.03
Grade 3	-0.08	-0.23	0.43
Thrush	-0.06	-0.07	
Conjunctivitis	-0.01	-0.13	0.10
Gastroenteritis	0.04	0.01	-0.05
Respiratory Infection	-0.03	0.03	0.008
Pyoderma	0.02	0.09	-0.0005
Other	-0.08		-0.07
Tetanus titre	0.0001	-0.0001	-0.0001
Polio titre	0.002	0.002	-0.0008
T4/T8 ratio	0.03	-0.02	0.03

Notes
1 Group codes were: 0 = Control, 1 = Test
2 Iron status codes were: 0 = *Normal*, 1 = *Depleted*, 2 = *Deficient*, 4 = *Deficient anaemic*
3 A blank entry in the table is made for variables whose contribution to the MLR was minimal
4 No coefficient reached statistical significance (P < 5%)

Table 6.33 Trends shown by the multiple linear regression analyses (MLR) in table 6.31. The trend shown by the coefficient of the independent variables is indicated by a Y if it supports the hypothesis of iron deficiency increasing susceptibility to infection. An N indicates that the MLR does not support the hypothesis.

INDEPENDENT VARIABLES	DEPENDENT VARIABLES GROUP		HAEMOGLOBIN		FE STATUS	
Grade 1	Y			N		N
Grade 2			Y			
Grade 3	Y		Y		Y	
Thrush	Y		Y		Y	
Conjunctivitis	Y		Y		Y	
Gastroenteritis		N				N
Respiratory Infection	Y		Y		Y	
Pyoderma		N		N	Y	
Other	Y			N		N
TOTALS	6	2	5	3	5	3

Notes
1 Group codes were: 0 = Control, 1 = Test
2 Iron status codes were: 0 = *Normal*, 1 = *Depleted*, 2 = *Deficient*, 4 = *Deficient anaemic*
3 A blank entry in the table is made for variables whose contribution to the MLR was minimal
4 No coefficient reached statistical significance (P < 5%)

Table 6.34 Trends shown by the multiple linear regression analyses (MLR) in table 6.32. The trend shown by the coefficient of the independent variables in indicated by a Y if it supports the hypothesis of iron deficiency depressing immune function. An N indicates that the MLR does not support the hypothesis.

INDEPENDENT VARIABLES	DEPENDENT VARIABLES GROUP		HAEMOGLOBIN		FE STATUS	
B Cells	Y			N	Y	
Mantoux		N		N		N
Candida	Y			N	Y	
Grade 1	Y			N		N
Grade 2			Y		Y	
Grade 3	Y		Y		Y	
Thrush	Y		Y			
Conjunctivitis	Y		Y		Y	
Gastroenteritis		N		N		N
Respiratory Infection	Y			N	Y	
Pyoderma		N		N		N
Other	Y					N
Tetanus titre	Y			N	Y	
Polio titre	Y		Y		Y	
T4/T8 ratio	Y			N		N
TOTAL	11	2	5	9	8	6

Notes
1 Group codes were: 0 = Control, 1 = Test
2 Iron status codes were: 0 = *Normal*, 1 = *Depleted*, 2 = *Deficient*, 4 = *Deficient anaemic*
3 A blank entry in the table is made for variables whose contribution to the MLR was minimal
4 No coefficient reached statistical significance (P < 5%)

Summary

One hypothesis that motivated the present study was that iron deficiency depresses immune function. Confirmation of this was sought by comparing tests of immune function in infants categorized with respect to iron status as *Normal*, *Depleted*, *Deficient* and *Deficient with anaemia*. Confirmation of the hypothesis was also sought by multiple linear regression analysis. Both analyses identified weak trends that supported the theory.

Discussion and conclusions

The literature review failed to reveal any similar analyses in comparable trials. The design of the study created a relatively homogeneous set of subjects. This ensured that the confounding influences of malnutrition and serious illness were eliminated. It also resulted in most infants having better iron status than their peers as documented by Kirsten *et al*[241] and discussed above. The trial could therefore only test the hypothesis for mild degrees of iron deficiencies. It may be concluded that mild iron deficiency does not have a major detrimental effect on immune function and resistance to infection.

Risks of increased iron fortification of milk formula

Introduction

The third question addressed by the trial was the safety of increased iron fortification of infant milk formula. The evidence for and opinion on untoward effects of iron fortification were reviewed in chapter 2. Although unlikely, the risk most feared is of increasing susceptibility to infection by upsetting the host-parasite competition for iron. It was shown above that the Test group had fewer infections than the Control group in all categories except respiratory illness for which the difference was minimal. In particular, the Test group had fewer episodes of diarrhoeal disease.

It may thus be concluded that increased iron fortification is safe and poses no significant risk of increasing susceptibility to infection.

At the start of the trial mothers were closely questioned about the acceptability of the formula. No mother noticed any abnormal colour or taste in the milk and no mother attributed colic, fussiness or change in bowel habits to the formula. The control and test formulas were indistinguishable by both mothers and the research team.

The increased iron fortification was, it may be concluded, acceptable from the consumer's point of view.

More important effects of iron fortification are on zinc absorption and growth. These are discussed in the following 2 sections.

Indicators of zinc status

Results

The zinc status of the Test and Control groups is compared in table 6.36. Plasma zinc concentration is significantly lower in the Test group than in the Control group at the end of the trial. Hair zinc follows the same trend, but the difference is far from being statistically significant.

Table 6.36 Zinc status compared in the Control and Test groups

Specimen	*Control Mean*	*Test Mean*	*P Value*	*95% CI of diff. of means*		*Power Obs*	*10%*
AT 3 MONTHS OF AGE							
Plasma (ug/l)	91.16	85.64	15%	-13.03	1.99	30	64
Hair (ug/g)	224.02	261.31	32%	-37.61	112.19	0	0
AT 1 YEAR OF AGE							
Plasma	90.61	83.53	5%	-14.27	0.12	49	66
Change in plasma (ug/l)	-0.90	-3.32	64%	-12.50	7.68	0	0
Hair	142.30	129.21	54%	-54.92	28.74	0	0
Change in hair (ug/g)	-93.94	-161.39	27%	-187.81	52.90	30	0

Notes P values were calculated from student's t test.
95% CI = 95% confidence intervals.
Changes are reported as the mean of the variable at 12 months minus the mean value at 3 months.
The statistical power of the study was determined for both the observed difference (Obs) and a 10% difference (10%) from the combined mean for a type I error probability of 5%.

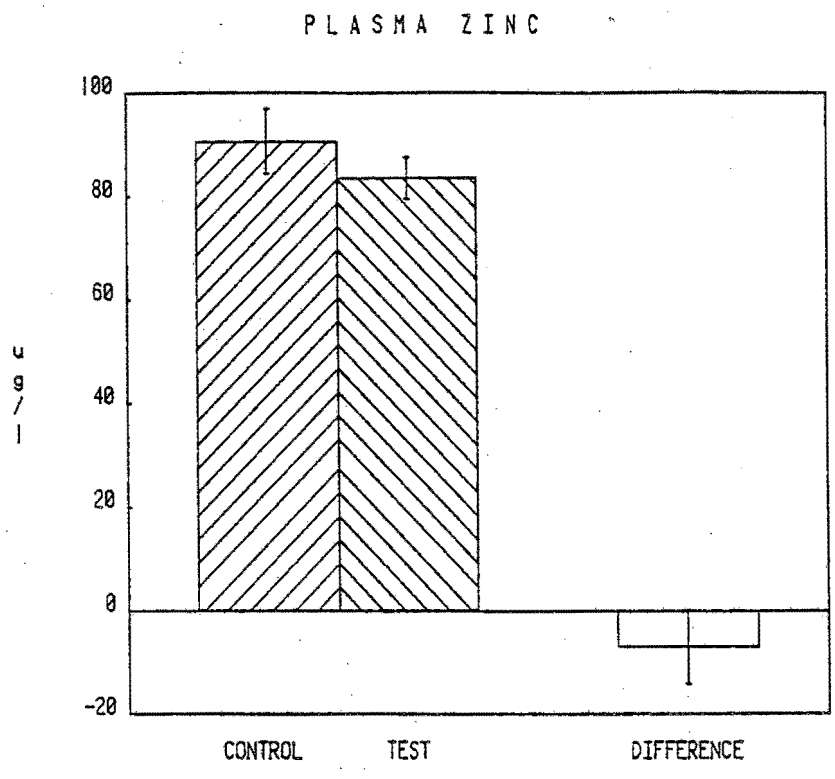

Figure 6.19 **Plasma zinc concentrations for the Test and Control groups at 12 months of age***

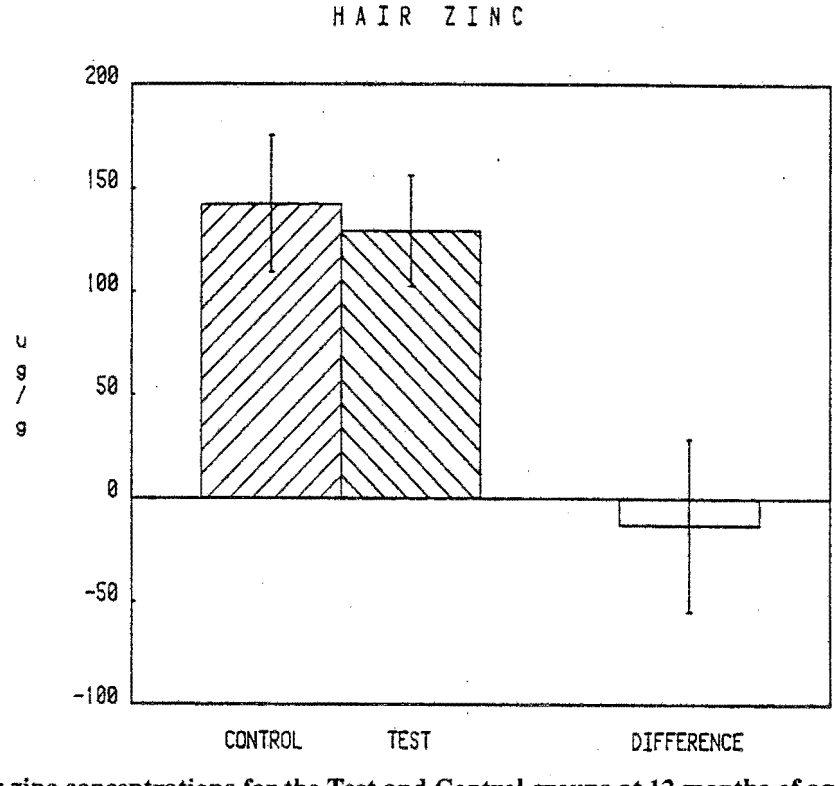

Figure 6.20 **Hair zinc concentrations for the Test and Control groups at 12 months of age**

* The bars represent the means and the vertical lines through the bars represent the 95% confidence intervals

Discussion

These results were anticipated since similar associations with iron fortification have been found in other studies[138], although others have failed to confirm the competition between iron and zinc for absorption[480].

The trend shown by the hair results concurs with the conclusion drawn by Hambidge that hair analyses are of limited worth[186]. It may not be clinically worthwhile to use hair zinc content as an index of body zinc status, but for population studies where blood samples are impractical it might yet prove to be a useful tool to measure long term effects.

A note of caution should be added to the above inferences since the Test group had a substantially lower mean serum zinc at the start of the trial. This may indicate some imbalance in selection of the two groups, but the difference in zinc concentration widened during the study. As similar studies have shown that iron interferes with the absorption of zinc, the likelihood is increased that the initial difference was spurious.

In view of these results, future studies of increased iron fortification should maintain the molar ratio of iron to zinc at the level commonly used in commercial milk formulas, *ie* 2.8 mol Fe/mol Zn.

Measures of growth and nutrition

Results

At 1 year of age the infants of the Test group were taller and heavier than those of the Control group. Table 6.37 shows that this trend is followed by other measures of growth, *viz* increase in weight, increase in length, standard deviation scores for weight and length and the increases in the standard deviation scores. Head circumference alone does not favour the Test group. No differences are statistically significant, but the increase in weight has a P value of 7%.

Table 6.37 Nutritional status and growth compared in the Control and Test groups.

Nutritional Variable	*Control Mean*	*Test Mean*	*P Value*	*95% CI of diff. of means*		*Power Obs*	*10%*
AT BIRTH							
Weight (Kg)	3.41	3.37	40%	-0.14	0.06	14	99
SD score weight	0.36	0.28	51%	-0.31	0.16	0	0
AT 3 MONTHS OF AGE							
Weight (kg)	6.06	6.04	82%	-0.23	0.18	0	99
Length (Cm)	59.13	59.12	97%	-0.67	0.64	0	100
Head circumference (Cm)	40.71	40.36	10%	-0.75	0.07	37	100
SD score weight	0.48	0.51	75%	-0.18	0.25	0	0
SD score length	-0.42	-0.31	31%	-0.11	0.33	18	0
AT 1 YEAR OF AGE							
Weight	10.09	10.33	24%	-0.16	0.64	22	99
Increase in weight (Kg)	4.00	4.29	7%	-0.03	0.62	43	71
Length	74.74	75.23	27%	-0.38	1.37	20	100
Increase in length (Cm)	15.45	16.01	17%	-0.24	1.36	28	96
Head circumference (Cm)	46.71	46.54	44%	-0.60	0.26	12	100
SD score weight	0.23	0.48	20%	-0.13	0.63	26	0
Change in SD score	-0.25	-0.03	16%	-0.09	0.53	29	0
SD score length	-0.13	0.06	20%	-0.11	0.50	25	0
Change in SD score	0.25	0.32	58%	-0.18	0.31	0	0

Notes P values were calculated from student's t test.
95% CI = 95% confidence intervals.
Changes are reported as the mean of the variable at 12 months minus the mean value at 3 months.
The *SD score* is the mean of standard deviation scores calculated from NCHS tables
The statistical power of the study was determined for both the observed difference (Obs) and a 10% difference (10%) from the combined mean for a type I error probability of 5%.

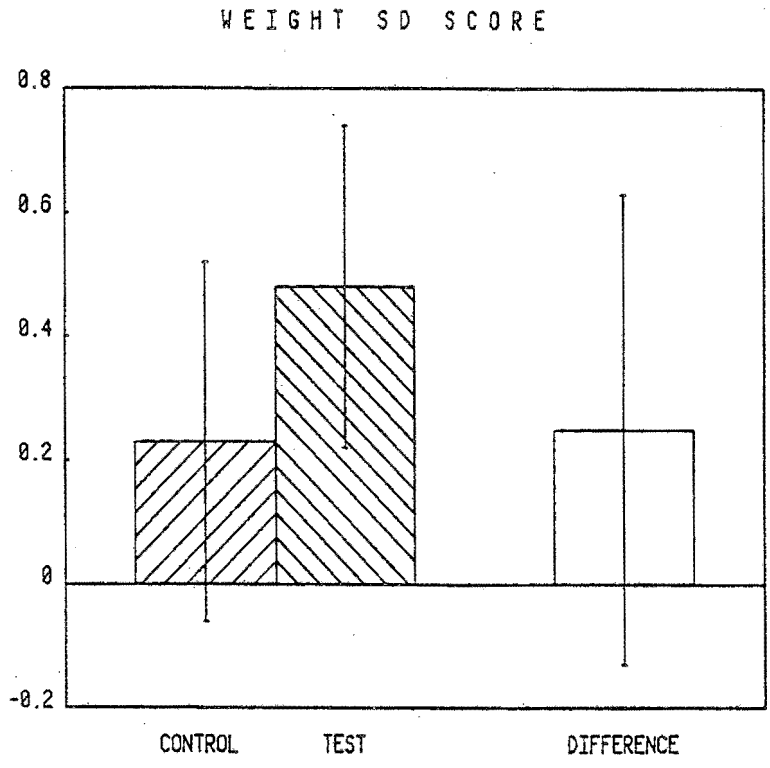

Figure 6.21 **Mean weight standard deviation scores for the Test and Control groups at 12 months of age***

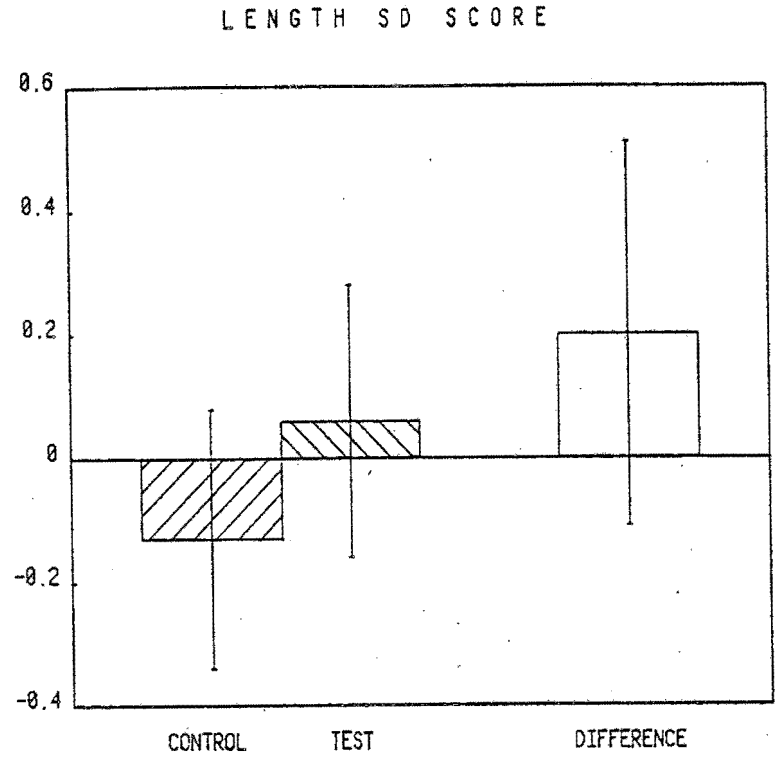

Figure 6.22 **Mean length standard deviation scores for the Test and Control groups at 12 months of age***

* The bars represent the means and the vertical lines through the bars represent the 95% confidence intervals

Discussion

Head growth in infants is preserved in severe malnutrition and is a poor indicator of general nutrition. It is also difficult to measure head circumference as accurately as weight and length. This is reflected in the relatively larger standard deviation, larger P value and lower power of the head measurements compared with those of the other growth indices.

In summary, the administration of extra iron to the Test group was associated with improved growth as measured by length and weight and this almost reached statistical significance.

Discussion and conclusions

The foregoing sections showed that the increased iron fortification was safe and acceptable, but that it was associated with decreased zinc levels in plasma and hair. Future trials of iron fortification should maintain the Fe:Zn molar ratio at about 2.8 and should monitor the effect on the absorption of other divalent metals.

Lessons should be drawn from our experience of what diets are best for men in health

Hippocrates
Regimen in Acute Disease
Circa 400 BC

CHAPTER 7 CONCLUSIONS AND RECOMMENDATIONS

Introduction

This thesis describes a clinical trial of an infant milk formula with 40 mg iron per 100 g which was compared with a conventional formula which had 8.3 mg iron per 100 g. The problem addressed by this study was articulated as 3 questions in the introductory chapter:

Does increasing the level of iron fortification of conventional infant milk formula improve the iron nutrition of normal infants fed on the formula?

Does increased iron fortification of infant milk formula alter immunity as reflected by incidence of infection and laboratory tests of immune function?

Are there any harmful effects of increasing the quantity of iron in conventional infant milk formula?

In short, the present study suggests that the answers to these questions are:

1 Increasing the level of iron fortification does improve the iron nutrition of normal infants fed on the formula. But, this is to such a small amount that increased iron fortification on its own is not a practical method of combating iron deficiency.

2 Increased iron fortification of infant milk formula does not alter immunity. However, this is not surprising in view of the small difference in iron status created by the manipulation of iron fortification.

3 The increased quantity of iron in the milk formula has no harmful effects. In particular, there was no suggestion of an increased susceptibility to infection or to diarrhoeal disease. However, a depression of zinc status was observed in the infants who consumed the milk with increased iron and this is potentially harmful.

These conclusions are amplified in the following sections and a number of recommendations are made for further investigations.

Possible confounding factors

The trial of the infant milk formula controlled rigorously for factors which have confounded the interpretation of most previous studies of the role of iron in infection and immunity. The children were selected on the grounds of good health and excellent nutrition. That they maintained this throughout

the study may be partly due to the milk formula which was granted to the families, and partly due to the selection of children from better home environments.

Possible confounding factors which may not have been controlled by the stratified allocation procedure were indicated by non-significant trends in completion rate, weaning, and introduction of solid foods. All these factors could weigh in the Test group's favour when considering indices of iron nutrition, measures of immune function or risks of increased iron fortification.

However the weight that should be assigned to these possible confounding influences is limited by several methodological weaknesses. In the subsidiary surveys, ages were calculated from the mother's recollection of events and recorded in months rather than decimals of a year. Also, age of introduction of a food item is likely to be a poor proxy for quantity of food ingested and it gives no indication whether or not the food was consumed with milk formula.

The effect of increased iron fortification on iron status

In the planning stage of this project, it seemed from the results of other studies of fortification of infant milk formula that an increase of iron by a factor of almost 5 would have a marked effect on iron status of infants offered the formula. It was known that ascorbic acid was an important factor in enhancing the bioavailability of iron in milk formulas, but it was not suspected that the eventual availability of iron in the Test formula would be of the order of magnitude of 1%. Or, to express this finding more dramatically, the bioavailability of the extra iron in the Test formula was about 0.02%.

The derivation of these estimates of iron absorption is outlined in Table 7.1. Because of the difficulty in estimating storage iron from serum ferritin (or body weight) in infancy these calculations assume that storage iron is constant. This should not, however, alter the conclusions which are drawn about the unexpectedly low bioavailability of iron in the Test formula.

Table 7.1 Absorption of iron in Control and Test groups calculated from the changes in weight and haemoglobin levels at the start and end of the trial. Storage iron is assumed constant.

	CONTROL	TEST	
3 MONTHS			CONVERSION FACTORS
Age (months)	2.96	2.90	80 ml blood/kg body weight
Hb (g/dl)	11.28	11.38	0.03425 mg Fe/g Hb
Wt (kg)	6.06	6.04	
Fe in Hb (mg)	187.35	188.13	
12 MONTHS			
Age (months)	11.81	11.86	
Hb (g/dl)	11.49	11.85	
Wt (kg)	10.09	10.34	
Fe in Hb (mg)	317.86	335.81	
Change in Hb Fe (mg)	130.52	147.69	
Fe absorbed (mg/day)	0.291	0.295	
Fe in 500 ml formula (mg)	5.98	28.83	
% Fe absorbed	4.87	1.02	
% extra Fe absorbed		0.02	100*(0.295-0.291)/(28.83-5.98)

The present study indirectly confirms the importance of the ratio of ascorbic acid to iron in assuring that iron in infant milk formula is available for assimilation. Until further work allows more definitive recommendations to be made it would seem advisable to set the molar ratio of ascorbic acid to iron in cow's milk based infant formulas to at least 1.4:1 (the ratio in the Control formula in the present study), and perhaps as high as 4:1 as recommended by Gillooly *et al*[194].

The low bioavailability of the iron in the fortified milk was a surprising finding in view of previous measurements of absorption of iron from cow's milk formulas without added ascorbic acid. These have ranged from 2.9% to 19%[125, 136, 334, 418, 434, 438, 482]. Until now it has been generally accepted as Cook and Bothwell state *that, while milk does not promote iron absorption, it has significantly less inhibitory effects than infant cereals or solid foods*[104].

The present study did not make a direct measurement of the absorption of iron from the infant milk formulas and it did not determine the quantities of milk and supplementary foods in the diet. These are two investigations that should be made in order to learn the availability of iron in fairly large concentrations in infant milk formula and to determine the effect of supplementary foods on the bioavailability of iron in infant milk formula and eventual iron status. This is particularly important in view of the contrast between the eventual iron status of the infants in the present study with those in similar studies elsewhere. For example, on the one hand, infants in a study in Helsinki who were fed on unfortified infant milk formula attained an excellent state of iron nutriture[432]. While on the other

hand, although the Control infants in the present study were given a formula fortified with both iron and ascorbic acid, they reached substantially lower levels of iron nutrition than infants in the USA who were offered a similar formula[7, 323].

In categorizing the iron status of the infants in the present study it was noted that at the age of 1 year they had unusually low levels of mean red cell volume in comparison with reference standards. It would have been useful in assessing the likely reasons for this discrepancy if the prevalences of thalassaemia, thalassaemia trait and the various haemoglobinopathies had been determined for the community studied. A study has been made of the prevalence of such genetic disorders in patients at the Red Cross War Memorial Childrens Hospital who have a mean cell volume less than 60 fl. This work needs to be extended to an unselected population.

The relative utility of the several indicators of iron status has not been clearly determined. A useful study would be to determine these indicators before and after a therapeutic trial (as Dallman *et al*[123] have done) and then compare the predictive value of each indicator with the predictive value of every other indicator.

The effect of increased iron fortification on immunity

The results of the study on the effect of increased iron fortification on immunity (as measured by several laboratory tests and incidence of infection) was inconclusive because of the small increase in iron status in the Test group.

Although not statistically significant, an intriguing finding was that the infants in the Test group had fewer infections than those in the Control group. This relationship held for 7 of 8 independent categories of infection, and in the sole exception the incidence was essentially equal. This finding has important implications for public health since it implies a "saving" of 56 infections per 100 infants per year.

Clearly then, trials to define the relation between iron status and immune function more precisely are required since a possible public health benefit of this magnitude can not be ignored. The difficulties such studies will have in attaining adequate statistical power must be noted. The present study was designed to detect a difference in infection rates of 1 infection per child per year. The sample size calculations yielded 65 as the minimum number required in each group and assumed that the standard deviation of infection rate was 2.5. With the data from the present study it can be estimated that a minimum of 230 infants would be required in each group to detect a difference of 0.59 in infection rates with standard deviations of 2.8 and 3.3.

Besides increasing the number of subjects, steps to increase statistical power might include extending the period of observation and increasing the disparity in iron status between control and test groups. It will be particularly important to learn if there is a threshold level of iron status below which susceptibility to infection is increased. The present study provides a hint that, if such a threshold exists, it could be relatively high.

The laboratory tests of immune function in themselves give little indication of the direction future research should take. But, taken in conjunction with previous work in the literature, they suggest a need for well designed and reported clinical studies. Such studies should control for confounding factors of infection and malnutrition and report in detail the method of ascertainment of the subjects and their iron status. The time course to restoration of iron status to normal in patients with iron deficiency anaemia should be studied along with parameters of immune function. Careful selection of iron deficient subjects and comparison with the use of a placebo treatment for 1 or 2 weeks would enable the effects of infection and malnutrition to be controlled.

Risks of increased iron fortification of infant milk formula

The study found no evidence that increased iron fortification of infant milk formula was poorly tolerated or associated with increased susceptibility to infection. In particular, the incidence of diarrhoeal disease was not increased. The Test formula was indistinguishable from the Control formula as far as the infants, parents and study team were concerned.

Previous work that has suggested that iron interferes with the absorption of zinc was confirmed. No symptoms or harmful effects could be attributed to the lower plasma and hair zinc levels found in the Test group, but two recommendations can made for future studies of iron fortification of infant milk formula.

Firstly, such studies should maintain the molar ratio of iron to zinc at about 2.8:1. Secondly, since iron may also inhibit the absorption of other divalent metals, such studies should monitor their levels in the subjects.

Epidemiological conclusions

In the course of the study a number of incidental but independently valuable contributions were made in the field of epidemiology.

The data on infectious morbidity in infants between the ages of 3 and 12 months are valuable not only as a characterization of a particular community at a particular point in time, but also as a baseline for

planning health services and for comparing morbidity in other communities or in the same community in the future.

The social data on the study families and the surveys on infant feeding practices are valuable for similar reasons.

A hospital laboratory receives most of its specimens from ill subjects. Assessment of the pathogenicity of a particular isolate requires the carriage rate in normal subjects to be known. The survey of viral carriage rate in faecal specimens from healthy 3 and 12 month old infants and in nasopharyngeal swabs from 12 month old subjects thus provided useful information for the virology laboratory. Such a survey had not been previously made in Cape Town.

The immunology laboratory which also receives most of its specimens from hospital patients has a similar problem in determining normal ranges. The present study helped to establish normal ranges in 3 and 12 month old infants for several tests *viz* OKT3, OKT4, OKT8, surface membrane immunoglobulin, lymphocyte response to stimulation with phytohaemaglutinin and neutrophil bactericidal index.

Valuable epidemiological information with respect to immunization programs was obtained with the surveys of polio and tetanus antibody levels. When the infants were 3 months of age the antibody levels were measured in both mother and child. They were measured again in the infants at the age of 12 months. This confirmed the efficacy of the immunization service (and vaccines) and will be a useful baseline for future surveillance efforts.

Summary of contributions

The contributions made by the present work may be briefly summarized.

Infant milk formula fortified with milk and iron was shown to be less effective than similar formulas overseas. (Future studies will probably find differences in supplementary foods and dietary habits to explain this finding.) Fortification with extra iron resulted in minimally improved iron status and was surprisingly ineffectual. The increased iron fortification was not associated with altered immune function tests nor with changes in the incidence of infection. A relationship was sought between iron status and immune function tests and incidence of infection but the results were inconclusive because of low statistical power. However, concerns that have been raised in the literature about the safety and acceptability of large quantities of iron in infant milk formulas were shown to be unfounded. Reports of laboratory experiments showing that iron interferes with zinc absorption have been extended to the field and confirmed. Finally, a number of independently valuable epidemiological studies were made. These included determination of infectious morbidity, immunization responses and viral carriage rates

in infants, description of infant feeding practices and social characteristics of families in Bonteheuwel, and normal ranges in infancy for a number of immunological tests.

Conclusion

In conclusion, it may be stated that increasing the level of iron fortification of cow's milk infant milk formula is not sufficiently effective in improving iron status to warrant a change in commercial practice. But, the potential rewards of improved iron status are such that further studies should be undertaken with increased fortification of iron, ascorbic acid and zinc.

APPENDICES

APPENDICES

APPENDIX 1 - Dropouts from the study

STUDY No.	NAME	REASON
1	Cynthia Davids	Mother declined participation +
9	Yusuf Jamodien	Mother scared of second blood test
15	Redewaan Benjamin	Mother declined participation *
16	Lee-Ann Windvogel	Mother scared of second blood test
24	Carmelita Fredericks	Moved away from Bonteheuwel
32	Mogamat Hendricks	Mother declined participation *
54	Elroy Snyman	Moved away from Bonteheuwel
56	Ryan Greef	Mother declined participation *
59	Rishana Abrahams	Mother declined participation +
82	Adele Henry	Mother declined participation +
87	Donovan Wessels	Moved away from Bonteheuwel
89	Rosaline Manuel	Mother declined participation *
94	Shireen Jonathan	Moved away from Bonteheuwel
97	Quanita Jacobs	Moved away from Bonteheuwel
101	Nadeem Abrahams	Moved away from Bonteheuwel
120	Badronesa Hendricks	Moved away from Bonteheuwel
131	Feroza Mohammed	Mother declined participation *

NOTE

\+ Mother declined out of concern for her child
* Mother declined out of lack of concern for her child

APPENDIX 2 - Research and Ethics Committee Approval

UNIVERSITY OF CAPE TOWN
(WITH WHICH IS INCORPORATED THE SOUTH AFRICAN COLLEGE)

DEAN & PROFESSOR OF MEDICAL EDUCATION
PROFESSOR D McKENZIE
M.B., Ch.B., M.Med.(Path.) (Cape Town), Dip. Bact. (Lond).
TELEPHONE 47-1250

THE DEAN OF THE FACULTY OF MEDICINE
MEDICAL SCHOOL
OBSERVATORY
7925

17 May 1983

Dr M Power et al
Institute of Child Health
Red Cross Childrens Hospital

Dear Dr Power

Effects of iron deficiency on immune function and development of infants.

I am pleased to inform you that the Ethics and Research Committee has raised no objections to your proposed study.

Yours sincerely

Prof J P deV van Niekerk
CHAIRMAN

APPENDIX 3 - Tables of Statistical analyses

VARIABLE	BOTH GROUPS COMBINED N	Min	Max	SD	95% CI Bottom	Mean	95% CI Top
First bottle	147	0.0000	4.0000	0.9820	1.3499	1.5100	1.6701
Last breast	130	0.0000	13.0000	3.9770	3.1409	3.8310	4.5211
Date of birth	147	83.0920	83.4870	0.1150	83.2863	83.3050	83.3237
Date @ 3/12 entry	147	83.3360	83.7000	0.1050	83.5309	83.5480	83.5651
Date @ 1y exit	134	84.0100	84.4810	0.1210	84.2693	84.2900	84.3107
Age @ entry (months)	147	2.1710	3.9110	0.2580	2.8869	2.9290	2.9711
Age at exit (months)	134	10.6550	12.8870	0.3130	11.7835	11.8370	11.8905
WBC-3/12	122	5.0000	23.1990	3.1000	11.0474	11.6030	12.1586
RBC-3/12	122	3.1390	4.9490	0.3310	3.9817	4.0410	4.1003
Hb-3/12	122	9.0000	12.7990	0.7690	11.1952	11.3330	11.4708
HCT-3/12	122	26.2990	37.7990	2.1910	32.7013	33.0940	33.4867
MCV-3/12	122	57.8990	92.7990	5.0730	81.1708	82.0800	82.9892
MCH-3/12	122	19.2990	33.0000	1.9410	27.7641	28.1120	28.4599
MCHC-3/12	122	31.1990	37.0990	0.9800	34.0684	34.2440	34.4196
RDW%-3/12	122	11.5000	20.7990	1.4040	13.2804	13.5320	13.7836
PLT-3/12	122	221.0000	773.0000	115.3020	445.9330	466.5980	487.2630
RETICS-3/12	117	0.5990	7.2990	1.1820	1.9416	2.1580	2.3744
POLYs-3/12	121	7.0000	73.0000	11.9250	25.1347	27.2810	29.4273
LYMPHs-3/12	121	24.0000	88.0000	12.4880	62.6864	64.9340	67.1816
MONOs-3/12	121	0.0000	14.0000	2.7980	4.5624	5.0660	5.5696
EOSINs-3/12	120	0.0000	11.0000	2.3440	2.1263	2.5500	2.9737
BASOs-3/12	120	0.0000	2.0000	0.4490	0.0768	0.1580	0.2392
WBC-1y	126	5.0990	20.0990	2.9820	10.4673	10.9930	11.5187
RBC-1y	126	4.0690	6.0490	0.4030	4.8049	4.8760	4.9471
RBC *	104	-0.2400	1.7000	0.3640	0.7282	0.7990	0.8698
Hb-1y	126	9.0990	14.0990	0.9870	11.5050	11.6790	11.8530
Hb *	104	-2.3000	2.6000	0.9830	0.0938	0.2850	0.4762
HCT-1y	126	27.0000	43.0000	2.9810	34.8124	35.3380	35.8636
HCT *	104	-6.2000	9.8000	2.9260	1.4010	1.9700	2.5390
MCV-1y	126	53.0000	83.0000	5.4150	71.6473	72.6020	73.5567
MCV *	104	-26.2000	1.3000	4.7480	-10.3533	-9.4300	-8.5067
MCH-1y	126	16.1990	28.2990	2.0890	23.6737	24.0420	24.4103
MCH *	104	-9.3990	-0.2000	1.8440	-4.3486	-3.9900	-3.6314
MCHC-1y	126	30.5000	35.3990	0.9240	32.9031	33.0660	33.2289
MCHC *	104	4.5990	3.4000	1.2550	-1.3480	-1.1040	-0.8600
RDW%-1y	126	12.0000	21.5990	1.7890	14.6516	14.9670	15.2824
RDW% *	104	-5.2990	8.0990	2.1470	0.9755	1.3930	1.8105
PLT-1y	126	82.0000	672.0000	106.9340	330.1072	348.9600	367.8128
RETICs-1y	122	0.2990	6.7990	1.1030	1.5463	1.7440	1.9417
POLYs-1y	125	6.0000	67.0000	11.9670	31.0015	33.1200	35.2385
LYMPHs-1y	125	23.0000	88.0000	12.2540	56.3107	58.4800	60.6493
MONOs-1y	125	0.0000	15.0000	3.3240	4.7156	5.3040	5.8924
EOSINs-1y	125	0.0000	16.0000	3.1310	2.4137	2.9680	3.5223
BASOs-1y	124	0.0000	2.0000	0.3570	0.0415	0.1050	0.1685
ZPP-3/12	133	1.0000	8.7990	1.1570	2.6666	2.8650	3.0634
FERRITIN-3/12	140	3.0000	704.0000	119.0510	136.5001	156.3930	176.2859
Log(ferritin) 3/12	140	0.4770	2.8480	0.3470	2.0160	2.0740	2.1320
ZPP-1y	124	1.5000	11.0990	1.4230	3.4161	3.6690	3.9219
ZPP *	110	-5.1000	5.8990	1.5280	0.5353	0.8240	1.1127
FERRITIN-1y	129	1.0000	244.0000	29.2050	26.1453	31.2330	36.3207
FERRITIN *	121	-677.0000	158.0000	122.7910	-146.0091	-123.9090	-101.8089
Log(ferritin) 1 y	129	0.0000	2.3870	0.3680	1.2929	1.3570	1.4211
Log(ferritin) *	121	-1.9490	0.5400	0.4700	-0.7996	-0.7150	-0.6304
ZINC-PLASMA-3/12	139	46.0000	236.0000	22.4670	84.6492	88.4170	92.1848

	BOTH GROUPS COMBINED				95% CI		95% CI
VARIABLE	N	Min	Max	SD	Bottom	Mean	Top
ZINC-HAIR-3/12	78	54.5990	759.7990	164.6700	207.9373	245.0580	282.1787
ZINC-PLASMA-1y	124	54.0000	204.0000	20.3730	83.1048	86.7260	90.3472
ZINC-PLASMA *	115	-62.0000	62.0000	27.0900	-7.2298	-2.2260	2.7778
ZINC-HAIR-1y	101	16.5990	536.5990	105.5560	114.7217	135.5580	156.3943
ZINC-HAIR *	52	-703.7990	253.1000	215.9730	-190.3598	-130.2590	-70.1582
# Infect:grade 1+2+3	132	0.0000	12.0000	2.2890	4.1739	4.5680	4.9621
# Infect. grade 1	132	0.0000	5.0000	1.2220	0.9946	1.2050	1.4154
# Infect. grade 2	132	0.0000	10.0000	2.1640	2.8544	3.2270	3.5996
# Infect. grade 3	132	0.0000	1.0000	0.3440	0.0768	0.1360	0.1952
# Infect. resp tract	132	0.0000	6.0000	1.2800	1.2266	1.4470	1.6674
# Infect. diarrhoea	132	0.0000	4.0000	0.9280	0.5902	0.7500	0.9098
# Infect. pyoderma	132	0.0000	5.0000	0.8570	0.2084	0.3560	0.5036
# Infect. thrush	132	0.0000	3.0000	0.7260	0.2540	0.3790	0.5040
# Infect other(-max)	131	0.0000	2.0000	0.4260	0.1094	0.1830	0.2566
# infect: other	132	0.0000	4.1450	0.6600	0.1610	0.2746	0.3882
# Infect. other	132	0.0000	3.0000	0.4900	0.1206	0.2050	0.2894
# Infect. conjunct.	132	0.0000	2.0000	0.4380	0.1516	0.2270	0.3024
# Infect:grade 1+2+3	132	0.0000	15.7740	3.0490	5.5950	6.1200	6.6450
# Infect:grade 1	132	0.0000	6.5380	1.6180	1.3344	1.6130	1.8916
# Infect:grade 2	132	0.0000	13.1450	2.8880	3.8268	4.3240	4.8212
# Infect:grade 3	132	0.0000	1.4410	0.4660	0.1038	0.1840	0.2642
# Infect: resp tract	132	0.0000	8.2330	1.7280	1.6455	1.9430	2.2405
# Infect: diarrhoea	132	0.0000	5.4520	1.2410	0.7893	1.0030	1.2167
# Infect: pyoderma	132	0.0000	6.5730	1.1470	0.2795	0.4770	0.6745
# Infect: thrush	132	0.0000	3.9960	0.9700	0.3400	0.5070	0.6740
# Infect:other(-max)	131	0.0000	2.7370	0.5680	0.1458	0.2440	0.3422
# Infect: conjunct	132	0.0000	2.6460	0.5860	0.2031	0.3040	0.4049
Mantoux 3/12	138	0.0000	21.0000	5.6200	4.7060	5.6520	6.5980
Candida 3/12	144	0.0000	20.0000	6.0200	3.3414	4.3330	5.3246
Mantoux 1 y	127	0.0000	26.0000	7.3130	6.2278	7.5120	8.7962
Mantoux *	121	-20.0000	26.0000	8.5130	0.3028	1.8350	3.3672
Candida 1 y	128	0.0000	28.0000	8.4350	7.8688	9.3440	10.8192
Candida *	127	-17.0000	26.0000	8.7500	3.4945	5.0310	6.5675
Antibody tetanus ma	130	0.0000	5120.0000	478.1460	36.1875	119.1540	202.1205
Antibody polio ma	138	0.0000	20.0000	4.7260	1.0885	1.8840	2.6795
log[Tetanus] ma	130	0.0000	3.7090	0.9950	0.9314	1.1040	1.2766
log[Polio] ma	138	0.0000	1.3220	0.3980	0.1150	0.1820	0.2490
Antibody tetanus 3m	139	0.0000	320.0000	35.0160	6.3577	12.2300	18.1023
Antibody polio 3/12	129	0.0000	160.0000	39.5840	30.0032	36.8990	43.7948
log[Tetanus] 3/12	139	0.0000	2.5070	0.6800	0.3830	0.4970	0.6110
log[Polio] 3/12	129	0.0000	2.2070	0.6380	1.1669	1.2780	1.3891
Antibody tetanus 1y	130	0.0000	10240.0000	1025.6580	121.0307	299.0000	476.9693
Antibody tetanus *	122	-280.0000	10230.0000	1059.3630	103.5791	293.4430	483.3069
Antibody polio 1 y	128	0.0000	160.0000	52.7810	55.7689	65.0000	74.2311
Antibody polio *	115	-160.0000	160.0000	66.7620	19.2334	31.5650	43.8966
log[Tetanus] 1 yr	130	0.0000	4.0100	0.6590	1.8177	1.9320	2.0463
log[Tetanus] 1 y *	122	-1.6130	3.7090	1.0550	1.2289	1.4180	1.6071
log[[Polio] 1 yr	128	0.0000	2.2070	0.5330	1.5228	1.6160	1.7092
log[Polio] 1 yr *	115	-2.2070	2.2070	0.8400	0.2208	0.3760	0.5312
OKT3 3/12	127	47.0000	88.0000	9.6260	67.0417	68.7320	70.4223
OKT4 3/12	127	19.0000	87.0000	12.1920	48.8671	51.0080	53.1489
OKT8 3/12	127	5.0000	42.0000	7.3290	14.8470	16.1340	17.4210
SMIG 3/12	127	5.0000	43.0000	6.8030	17.3174	18.5120	19.7066
OKT4/OKT8 3/12	127	0.7620	12.4290	2.2120	3.4916	3.8800	4.2684

	BOTH GROUPS COMBINED				95% CI		95% CI
VARIABLE	N	Min	Max	SD	Bottom	Mean	Top
OKT3 1y	126	40.0000	95.0000	11.1110	71.3351	73.2940	75.2529
OKT4 1y	126	0.0000	75.0000	13.5010	44.4767	46.8570	49.2373
OKT8 1y	126	5.0000	67.0000	9.7870	21.0285	22.7540	24.4795
SMIG 1y	126	4.0000	46.0000	7.5760	17.5853	18.9210	20.2567
OKT4/OKT8 1y	126	0.0000	8.1430	1.3140	2.2323	2.4640	2.6957
OKT4/OKT8 *	109	-9.5270	3.4550	2.0670	-1.7184	-1.3260	-0.9336
pha-pcps-3/12	56	0.0690	285.5790	46.4040	123.2555	135.6780	148.1005
pha-pccs-3/12	56	0.1190	280.1690	43.8440	129.2819	141.0190	152.7561
pha-ccps-3/12	56	62.3790	133.0490	14.5410	97.0973	100.9900	104.8827
pha-pcps-1y	62	43.2190	360.0790	59.8510	126.2204	141.4150	156.6096
pha-pccs-1y	62	63.8590	299.7090	45.2580	131.6822	143.1720	154.6618
pha-ccps-1y	61	50.8290	147.0890	17.4160	95.0780	101.7670	108.4560
pha-pcps *	50	-161.8200	196.6400	62.5530	-12.1907	5.5780	23.3467
Birth weight	147	3.0000	4.3690	0.3060	3.3401	3.3900	3.4399
SD score weight brth	147	-0.7500	2.7500	0.7220	0.1983	0.3160	0.4337
Weight @ 3/12	147	4.8490	8.1590	0.6320	5.9450	6.0480	6.1510
Length @ 3/12	147	52.3990	65.8990	2.0060	58.7990	59.1260	59.4530
Head circ @ 3/12	147	37.5990	44.6990	1.2630	40.3251	40.5310	40.7369
SD score weight 3/12	147	-0.9040	2.6770	0.6650	0.3856	0.4940	0.6024
SD score length 3/12	146	-2.0840	1.6840	0.6660	-0.4719	-0.3630	-0.2541
Weight/(length)**3 3	146	23.4940	36.5870	2.1000	28.7955	29.1390	29.4825
Weight @ 1y	133	7.6590	13.6590	1.1740	10.0186	10.2200	10.4214
Weight *	133	2.0000	6.5300	0.9470	3.9906	4.1530	4.3154
Length @ 1y	134	68.3990	82.0000	2.5500	74.5613	74.9970	75.4327
Length *	134	7.7000	25.9000	2.2349	15.3622	15.7440	16.1258
Head circ @ 1y	134	43.5990	50.5000	1.2650	46.4009	46.6170	46.8331
SD score weight 1 y	133	-2.0990	3.0900	1.1130	0.1721	0.3630	0.5539
SD score length 1 y	134	-2.0370	2.2750	0.8940	-0.1807	-0.0280	0.1247
SD score length *	133	-2.4080	2.4330	0.7130	0.1687	0.2910	0.4133
Weight/(length)**3y	133	19.9290	33.9580	2.0650	23.8208	24.1750	24.5292
Bact % init 1hr 3/12	65	32.0000	224.0000	45.8430	94.2747	105.6310	116.9873
Bact % init 2hr 3/12	65	27.0000	378.0000	67.8150	100.8007	117.6000	134.3993
Bact % ctl 1hr 3/12	65	30.2290	481.8090	101.6010	158.5412	183.7100	208.8788
Bact % ctl 2hr 3/12	65	63.0690	2400.0000	378.7230	267.6379	361.4560	455.2741
Bact % AB 1 hr 3/12	65	13.0490	109.3190	19.5870	43.9939	48.8460	53.6981
Bact % AB 2 hr 3/12	65	8.5190	105.5790	18.3790	28.5761	33.1290	37.6819
SD score weight *	133	-2.0510	2.0630	0.9030	-0.2899	-0.1350	0.0199
Bact norm 1 hr 3/12	65	-142.8600	112.1590	39.3730	12.3734	22.1270	31.8806
Bact norm 2 hr 3/12	65	-6.8600	106.4690	21.1110	17.4763	22.7060	27.9357
Bact % init 1hr 1y	57	20.0000	227.0000	54.3310	85.0634	99.4740	113.8846
Bact % init 1hr *	46	-149.0000	172.0000	71.3750	-20.6831	0.5000	21.6831
Bact % init 2hr 1y	57	14.0000	347.0000	88.1520	96.5308	119.9120	143.2932
Bact % init 2hr *	46	-193.0000	213.0000	111.8260	-21.0794	12.1090	45.2974
Bact % ctl 1 hr 1y	49	28.2290	487.1690	76.2880	107.9688	129.8700	151.7712
Bact % ctl 1 hr *	39	-319.7800	341.7200	110.0500	-87.9522	-52.3080	-16.6638
Bact % ctl 2 hr 1y	57	32.4890	1446.1490	235.5080	142.1944	204.6600	267.1256
Bact % AB 1 hr 1y	50	8.0590	114.1890	27.6950	37.1440	45.0110	52.8780
Bact % AB 2 hr 1y	54	2.6290	91.4790	24.6650	25.8759	32.6050	39.3341
Bact norm 1 hr 1 y	46	-360.0000	142.5890	90.9340	-27.8309	-0.8430	26.1449
Bact norm 2 hr 1 y	54	-63.0900	83.8290	31.2870	3.3253	11.8610	20.3967
hygiene	144	1.0000	3.0000	0.7000	2.5517	2.6670	2.7823
daycare	144	1.0000	3.0000	0.3860	2.7764	2.8400	2.9036
crowding	144	1.0000	3.0000	0.9440	1.7885	1.9440	2.0995
finance	144	1.0000	3.0000	0.7080	2.5084	2.6250	2.7416

VARIABLE	BOTH GROUPS COMBINED N	Min	Max	SD	95% CI Bottom	Mean	95% CI Top
careseeking	144	1.0000	3.0000	0.2640	1.9425	1.9860	2.0295
MDI-pre	31				0.0000		0.0000
PDI-pre	31				0.0000		0.0000
MDI-diff (Post)	22				0.0000		0.0000
PDI-diff (Post)	22				0.0000		0.0000
Meat/Fish	142	2.0000	10.0000	1.6200	4.6513	4.9200	5.1887
Cereal	146	0.0000	5.0000	0.7900	1.9508	2.0800	2.2092
Egg	142	0.0000	9.0000	1.6800	3.7513	4.0300	4.3087
Fruit	137	1.0000	9.0000	1.4200	3.9201	4.1600	4.3999
Vegetable	147	1.0000	8.0000	1.2400	3.0979	3.3000	3.5021

	CONTROL GROUP				95% CI		95% CI
VARIABLE	N	Min	Max	SD	Bottom	Mean	Top
First bottle	72	0.0000	4.0000	1.0210	1.2881	1.5280	1.7679
Last breast	62	0.0000	13.0000	3.7610	2.6422	3.5970	4.5518
Date of birth	72	83.0950	83.4890	0.1180	83.2673	83.2950	83.3227
Date @ 3/12 entry	72	83.3360	83.7000	0.1080	83.5146	83.5400	83.5654
Date @ 1y exit	63	84.0100	84.4810	0.1230	84.2420	84.2730	84.3040
Age @ entry (months)	72	2.5070	3.9110	0.2570	2.9016	2.9620	3.0224
Age at exit (months)	63	10.6550	12.8870	0.3510	11.7246	11.8130	11.9014
WBC-3/12	56	5.0000	18.8000	2.9700	10.4559	11.2510	12.0461
RBC-3/12	56	3.1390	4.6990	0.2990	3.9340	4.0140	4.0940
Hb-3/12	56	9.0000	12.5990	0.7230	11.0895	11.2830	11.4765
HCT-3/12	56	26.2990	36.7990	2.0090	32.3492	32.8870	33.4248
MCV-3/12	56	71.1990	92.7990	4.6550	80.8158	82.0620	83.3082
MCH-3/12	56	23.2990	30.8990	1.8080	27.6330	28.1170	28.6010
MCHC-3/12	56	32.0990	37.0990	1.0290	34.0075	34.2830	34.5585
RDW%-3/12	56	11.6990	20.7990	1.4400	12.9635	13.3490	13.7345
PLT-3/12	56	244.0000	704.0000	111.8390	428.9535	458.8930	488.8325
RETICS-3/12	53	0.5990	7.2990	1.1720	1.6881	2.0110	2.3339
POLYs-3/12	55	8.0000	53.0000	10.7520	24.0765	26.9820	29.8875
LYMPHs-3/12	55	42.0000	87.0000	11.2510	61.7416	64.7820	67.8224
MONOs-3/12	55	0.0000	14.0000	2.9760	4.3228	5.1270	5.9312
EOSINs-3/12	55	0.0000	11.0000	2.8520	2.1203	2.8910	3.6617
BASOs-3/12	55	0.0000	2.0000	0.5120	0.0436	0.1820	0.3204
WBC-1y	61	5.0990	20.0990	3.2020	9.7172	10.9470	12.1768
RBC-1y	61	4.0690	5.6390	0.3920	4.6994	4.8500	5.0006
RBC *	46	0.1100	1.7000	0.3840	0.6610	0.7750	0.8890
Hb-1y	61	9.3990	13.6990	0.9380	11.1337	11.4940	11.8543
Hb *	46	-2.3000	2.6000	1.0380	-0.2951	0.0130	0.3211
HCT-1y	61	29.8990	41.7990	2.8080	33.7695	34.8480	35.9265
HCT *	46	-6.2000	9.8000	3.1120	0.4984	1.4220	2.3456
MCV-1y	61	61.2990	81.3990	5.1020	70.0035	71.9630	73.9225
MCV *	46	-26.2000	-2.0990	5.1370	-11.7636	-10.2390	-8.7144
MCH-1y	61	19.7990	27.0990	1.9670	23.0195	23.7750	24.5305
MCH *	46	-9.3990	-0.2000	2.0020	-5.0242	-4.4300	-3.8358
MCHC-1y	61	31.0000	34.7990	0.9060	32.6350	32.9830	33.3310
MCHC *	46	-4.5990	1.2000	1.2780	-1.7443	-1.3650	-0.9857
RDW%-1y	61	12.5990	21.5990	1.8090	14.8352	15.5300	16.2248
RDW% *	46	-5.2990	8.0990	2.2020	1.6035	2.2570	2.9105
PLT-1y	61	142.0000	672.0000	102.7590	315.2381	354.7050	394.1719
RETICs-1y	58	0.2990	6.7990	1.2540	1.3494	1.6790	2.0086
POLYs-1y	60	7.0000	60.0000	10.8370	28.5185	31.3170	34.1155
LYMPHs-1y	60	37.0000	88.0000	11.0690	57.8916	60.7500	63.6084
MONOs-1y	60	0.0000	14.0000	3.1350	3.8234	4.6330	5.4426
EOSINs-1y	60	0.0000	16.0000	3.6920	2.2636	3.2170	4.1704
BASOs-1y	60	0.0000	2.0000	0.3540	0.0086	0.1000	0.1914
ZPP-3/12	65	1.0000	8.7990	1.2370	2.6086	2.9150	3.2214
FERRITIN-3/12	71	3.0000	704.0000	120.6420	127.0568	155.6060	184.1552
Log(ferritin) 3/12	71	0.4770	2.8480	0.3590	1.9850	2.0700	2.1550
ZPP-1y	59	2.0990	8.3990	1.4890	3.5621	3.9500	4.3379
ZPP *	51	-5.1000	5.8990	1.7970	0.6748	1.1800	1.6852
FERRITIN-1y	60	1.0000	106.0000	19.3290	18.8085	23.8000	28.7915
FERRITIN *	57	-677.0000	3.0000	122.8240	-170.5075	-137.9300	-105.3525
Log(ferritin) 1 y	60	0.0000	2.0250	0.3760	1.1399	1.2370	1.3341
Log(ferritin) *	57	-1.9490	0.3010	0.4430	-0.9795	-0.8620	-0.7445
ZINC-PLASMA-3/12	70	46.0000	263.0000	26.0030	84.9582	91.1570	97.3558

CONTROL GROUP					95% CI		95% CI
VARIABLE	N	Min	Max	SD	Bottom	Mean	Top
ZINC-HAIR-3/12	34	72.0990	661.5990	129.7440	178.8025	224.0230	269.2435
ZINC-PLASMA-1y	56	54.0000	204.0000	23.4890	84.3189	90.6070	96.8951
ZINC-PLASMA *	52	-61.0000	53.0000	28.3830	-8.8024	-0.9040	6.9944
ZINC-HAIR-1y	49	16.5990	536.5990	114.9890	109.2853	142.2970	175.3087
ZINC-HAIR *	24	-588.3000	227.2990	173.4560	-167.0126	-93.9370	-20.8614
# Infect:grade 1+2+3	62	0.0000	12.0000	2.4610	4.1332	4.7580	5.3828
# Infect. grade 1	62	0.0000	5.0000	1.2270	0.9465	1.2580	1.5695
# Infect. grade 2	62	0.0000	10.0000	2.3040	2.7541	3.3390	3.9239
# Infect. grade 3	62	0.0000	1.0000	0.3710	0.0668	0.1610	0.2552
# Infect. resp tract	62	0.0000	5.0000	1.2860	1.1035	1.4300	1.7565
# Infect. diarrhoea	62	0.0000	4.0000	0.9600	0.5463	0.7900	1.0337
# Infect. pyoderma	62	0.0000	5.0000	0.9300	0.1509	0.3870	0.6231
# Infect. thrush	62	0.0000	3.0000	0.7350	0.2164	0.4030	0.5896
# Infect other(-max)	61	0.0000	2.0000	0.4620	0.0526	0.2300	0.4074
# infect: other	62	0.0000	4.1450	0.7870	0.1712	0.3710	0.5708
# Infect. other	62	0.0000	3.0000	0.5770	0.1275	0.2740	0.4205
# Infect. conjunct.	62	0.0000	2.0000	0.4680	0.1232	0.2420	0.3608
# Infect:grade 1+2+3	62	0.0000	15.7740	3.3040	5.5782	6.4170	7.2558
# Infect:grade 1	62	0.0000	6.5380	1.6190	1.2760	1.6870	2.0980
# Infect:grade 2	62	0.0000	13.1450	3.1050	3.7227	4.5110	5.2993
# Infect:grade 3	62	0.0000	1.4410	0.5020	0.0906	0.2180	0.3454
# Infect: resp tract	62	0.0000	7.3560	1.7630	1.4534	1.9010	2.3486
# Infect: diarrhoea	62	0.0000	5.4520	1.2950	0.7362	1.0650	1.3938
# Infect: pyoderma	62	0.0000	6.5730	1.2600	0.2041	0.5240	0.8439
# Infect: thrush	62	0.0000	3.9280	0.9860	0.2937	0.5440	0.7943
# Infect:other(-max)	61	0.0000	2.7370	0.6230	0.0697	0.3090	0.5483
# Infect: conjunct	62	0.0000	2.6460	0.6260	0.1651	0.3240	0.4829
Mantoux 3/12	68	0.0000	18.0000	5.4840	4.6289	5.9560	7.2831
Candida 3/12	71	0.0000	20.0000	6.1440	2.8981	4.3520	5.8059
Mantoux 1 y	60	0.0000	26.0000	7.4840	6.1173	8.0500	9.9827
Mantoux *	57	-13.0000	26.0000	8.6030	-0.0358	2.2460	4.5278
Candida 1 y	60	0.0000	28.0000	8.5910	6.6645	8.8830	11.1015
Candida *	60	-10.0000	26.0000	8.0920	2.7773	4.8670	6.9567
Antibody tetanus ma	62	0.0000	320.0000	99.3110	41.2396	66.4520	91.6644
Antibody polio ma	69	0.0000	20.0000	5.1680	0.7878	2.0290	3.2702
log[Tetanus] ma	62	0.0000	2.5070	0.9870	0.8134	1.0640	1.3146
log[Polio] ma	69	0.0000	1.3220	0.4110	0.0833	0.1820	0.2807
Antibody tetanus 3m	70	0.0000	160.0000	23.3390	5.7223	11.2860	16.8497
Antibody polio 3/12	60	0.0000	160.0000	41.0080	25.5772	36.1670	46.7568
log[Tetanus] 3/12	70	0.0000	2.2070	0.6810	0.3977	0.5600	0.7223
log[Polio] 3/12	60	0.0000	2.2070	0.6680	1.0655	1.2380	1.4105
Antibody tetanus 1y	61	0.0000	1280.0000	233.3070	102.8523	192.4590	282.0657
Antibody tetanus *	58	-140.0000	1280.0000	243.3600	117.5882	181.5520	245.5158
Antibody polio 1 y	61	0.0000	160.0000	49.9060	39.1935	58.3610	77.5285
Antibody polio *	52	-160.0000	160.0000	62.9960	12.0845	29.6150	47.1455
log[Tetanus] 1 yr	61	0.0000	3.1080	0.5890	1.7528	1.9790	2.2052
log[Tetanus] 1 y *	58	-1.3220	3.1080	1.0150	1.1392	1.4060	1.6728
log[[Polio] 1 yr	61	0.0000	2.2070	0.5710	1.3267	1.5460	1.7653
log[Polio] 1 yr *	52	-2.2070	2.2070	0.8750	0.1485	0.3920	0.6355
OKT3 3/12	64	54.0000	87.0000	9.1940	67.1731	69.4690	71.7649
OKT4 3/12	64	24.0000	80.0000	11.6000	47.5253	50.4220	53.3187
OKT8 3/12	64	5.0000	42.0000	6.9390	14.6892	16.4220	18.1548
SMIG 3/12	64	7.0000	33.0000	5.9700	18.1182	19.6090	21.0998
OKT4/OKT8 3/12	64	1.3330	12.0000	1.8160	3.1485	3.6020	4.0555

CONTROL GROUP					95% CI		95% CI
VARIABLE	N	Min	Max	SD	Bottom	Mean	Top
OKT3 1y	57	43.0000	95.0000	10.9270	71.2417	74.1400	77.0383
OKT4 1y	57	0.0000	70.0000	14.8960	42.9440	46.8950	50.8460
OKT8 1y	57	5.0000	55.0000	10.2330	19.8118	22.5260	25.2402
SMIG 1y	57	8.0000	44.0000	7.4470	16.6208	18.5960	20.5712
OKT4/OKT8 1y	57	0.0000	8.1430	1.4440	2.1460	2.5290	2.9120
OKT4/OKT8 *	49	-3.9610	3.4550	1.5630	-1.4137	-0.9650	-0.5163
pha-pcps-3/12	27	0.0690	221.3990	53.5480	112.5975	133.7420	154.8865
pha-pccs-3/12	27	0.1190	255.3990	46.3090	120.4910	138.7770	157.0630
pha-ccps-3/12	27	62.3790	120.5490	14.0440	92.4255	97.9710	103.5165
pha-pcps-1y	24	81.6590	316.0790	53.6140	117.8109	140.3980	162.9851
pha-pccs-1y	24	75.9390	299.7090	47.1900	122.9522	142.8330	162.7138
pha-ccps-1y	23	75.6490	129.6390	12.9770	96.5373	102.1350	107.7327
pha-pcps *	21	-114.9100	196.6400	75.7950	-19.3642	15.0320	49.4282
Birth weight	72	3.0000	4.3690	0.3060	3.3401	3.4120	3.4839
SD score weight brth	72	-0.6250	2.7500	0.6990	0.1918	0.3560	0.5202
Weight @ 3/12	72	4.9590	8.1490	0.6510	5.9071	6.0600	6.2129
Length @ 3/12	72	54.6990	63.1990	1.8710	58.6924	59.1320	59.5716
Head circ @ 3/12	72	38.3990	44.6990	1.3710	40.3829	40.7050	41.0271
SD score weight 3/12	72	-0.9040	2.6770	0.7030	0.3108	0.4760	0.6412
SD score length 3/12	72	-2.0840	1.1250	0.6470	-0.5710	-0.4190	-0.2670
Weight/(length)**3 3	72	24.2430	33.7040	1.8770	28.8120	29.2530	29.6940
Weight @ 1y	63	7.6590	13.5590	1.1890	9.7936	10.0930	10.3924
Weight *	63	2.0000	5.9500	0.9180	3.7659	3.9970	4.2281
Length @ 1y	63	68.3990	78.7990	2.5380	74.0970	74.7360	75.3750
Length *	63	11.8990	20.8000	2.1150	14.9165	15.4490	15.9815
Head circ @ 1y	63	44.1990	50.5000	1.2500	46.3923	46.7070	47.0217
SD score weight 1 y	63	-2.0990	3.0900	1.1510	-0.0598	0.2300	0.5198
SD score length 1 y	63	-2.0090	1.5560	0.8420	-0.3450	-0.1330	0.0790
SD score length *	63	-1.4320	2.0050	0.6830	0.0830	0.2550	0.4270
Weight/(length)**3y	63	20.1160	28.3550	1.6870	23.6943	24.1190	24.5437
Bact % init 1hr 3/12	32	47.0000	224.0000	47.2770	91.8198	108.8440	125.8682
Bact % init 2hr 3/12	32	41.0000	378.0000	80.2400	95.5120	124.4060	153.3000
Bact % ctl 1hr 3/12	32	30.2290	481.8090	117.5690	150.3601	192.6960	235.0319
Bact % ctl 2hr 3/12	32	63.0690	1618.1790	332.0210	257.2392	376.7980	496.3568
Bact % AB 1 hr 3/12	32	13.0490	109.3190	21.9410	42.7622	50.6630	58.5638
Bact % AB 2 hr 3/12	32	8.5190	105.5790	22.0670	27.2258	35.1720	43.1182
SD score weight *	63	-2.0510	1.5320	0.8820	-0.4751	-0.2530	-0.0309
Bact norm 1 hr 3/12	32	-142.8600	112.1590	49.8930	2.9068	20.8730	38.8392
Bact norm 2 hr 3/12	32	-6.8600	106.4690	25.7090	15.8383	25.0960	34.3537
Bact % init 1hr 1y	28	25.0000	222.0000	51.4820	85.2137	105.1430	125.0723
Bact % init 1hr *	21	-80.0000	118.0000	63.3790	-13.7138	15.0480	43.8098
Bact % init 2hr 1y	28	25.0000	291.0000	80.6920	95.5132	126.7500	157.9868
Bact % init 2hr *	21	-193.0000	194.0000	104.8590	-23.8716	23.7140	71.2996
Bact % ctl 1 hr 1y	25	41.6590	315.3790	59.2130	106.3632	130.7530	155.1428
Bact % ctl 1 hr *	18	-319.7800	66.1900	95.9220	-98.1023	-50.6030	-3.1037
Bact % ctl 2 hr 1y	28	32.4890	638.4590	138.3580	146.4691	200.0290	253.5889
Bact % AB 1 hr 1y	22	16.0190	114.1890	27.9830	35.9581	48.3310	60.7039
Bact % AB 2 hr 1y	25	4.1490	81.4390	21.3020	25.0247	33.7990	42.5733
Bact norm 1 hr 1 y	22	-360.0000	142.5890	91.9240	-37.1348	3.5100	44.1548
Bact norm 2 hr 1 y	25	-63.0900	64.9490	27.4270	1.0148	12.3120	23.6092
hygiene	71	1.0000	3.0000	0.6800	2.5571	2.7180	2.8789
daycare	71	2.0000	3.0000	0.4010	2.7091	2.8040	2.8989
crowding	71	1.0000	3.0000	0.9490	1.7894	2.0140	2.2386
finance	71	1.0000	3.0000	0.6750	2.5023	2.6620	2.8217

CONTROL GROUP					95% CI		95% CI
VARIABLE	N	Min	Max	SD	Bottom	Mean	Top
careseeking	71	1.0000	3.0000	0.3160	1.9112	1.9860	2.0608
MDI-pre	9			5.0000	109.2297	113.0000	116.7703
PDI-pre	9			7.2000	98.9707	104.4000	109.8293
MDI-diff (Post)	14			7.5000	-0.2992	4.0000	8.2992
PDI-diff (Post)	14			2.9000	-2.1623	-0.5000	1.1623
Meat/Fish	68	2.0000	10.0000	1.8200	4.7496	5.1900	5.6304
Cereal	71	0.0000	5.0000	0.7900	1.8731	2.0600	2.2469
Egg	68	1.0000	9.0000	1.8200	3.7996	4.2400	4.6804
Fruit	67	2.0000	9.0000	1.4200	3.8137	4.1600	4.5063
Vegetable	72	1.0000	8.0000	1.3900	3.0734	3.4000	3.7266

TEST GROUP					95% CI		95% CI
VARIABLE	N	Min	Max	SD	Bottom	Mean	Top
First bottle	75	0.0000	3.0000	0.9500	1.2745	1.4930	1.7115
Last breast	68	0.0000	13.0000	4.1800	3.0325	4.0440	5.0555
Date of birth	75	83.0920	83.4890	0.1120	83.2892	83.3150	83.3408
Date @ 3/12 entry	75	83.3500	83.7000	0.1020	83.5335	83.5570	83.5805
Date @ 1y exit	71	84.0880	84.4790	0.1180	84.2761	84.3040	84.3319
Age @ entry (months)	75	2.1710	3.4790	0.2580	2.8396	2.8990	2.9584
Age at exit (months)	71	11.0750	12.5270	0.2760	11.7927	11.8580	11.9233
WBC-3/12	66	6.2990	23.1990	3.1980	11.1160	11.9020	12.6880
RBC-3/12	66	3.3090	4.9490	0.3560	3.9755	4.0630	4.1505
Hb-3/12	66	9.5990	12.7990	0.8090	11.1762	11.3750	11.5738
HCT-3/12	66	28.1990	37.7990	2.3350	32.6961	33.2700	33.8439
MCV-3/12	66	57.8990	90.8990	5.4380	80.7595	82.0960	83.4325
MCH-3/12	66	19.2990	33.0000	2.0620	27.6012	28.1080	28.6148
MCHC-3/12	66	31.1990	36.3990	0.9440	33.9790	34.2110	34.4430
RDW%-3/12	66	11.5000	17.3990	1.3640	13.3518	13.6870	14.0222
PLT-3/12	66	221.0000	773.0000	118.6180	443.9839	473.1360	502.2881
RETICS-3/12	64	0.6990	5.7990	1.1850	1.9841	2.2800	2.5759
POLYs-3/12	66	7.0000	73.0000	12.8970	24.3604	27.5300	30.6996
LYMPHs-3/12	66	24.0000	88.0000	13.5170	61.7390	65.0610	68.3830
MONOs-3/12	66	1.0000	14.0000	2.6630	4.3605	5.0150	5.6695
EOSINs-3/12	65	0.0000	7.0000	1.7790	1.8213	2.2620	2.7027
BASOs-3/12	65	0.0000	2.0000	0.3900	0.0414	0.1380	0.2346
WBC-1y	65	5.5000	17.2990	2.7840	10.3463	11.0360	11.7257
RBC-1y	65	4.1490	6.0490	0.4150	4.7972	4.9000	5.0028
RBC *	58	-0.2400	1.4900	0.3490	0.7253	0.8170	0.9087
Hb-1y	65	9.0990	14.0990	1.0080	11.6033	11.8530	12.1027
Hb *	58	-1.6000	2.3990	0.8890	0.2663	0.5000	0.7337
HCT-1y	65	27.0000	43.0000	3.0860	35.0335	35.7980	36.5625
HCT *	58	-5.1990	8.9000	2.7180	1.6906	2.4050	3.1194
MCV-1y	65	53.0000	83.0000	5.6680	71.7979	73.2020	74.6061
MCV *	58	-19.6000	1.3000	4.3530	-9.9321	-8.7880	-7.6439
MCH-1y	65	16.1990	28.2990	2.1830	23.7522	24.2930	24.8338
MCH *	58	-8.3010	-0.2000	1.6430	-4.0728	-3.6410	-3.2092
MCHC-1y	65	30.5000	35.3990	0.9410	32.9109	33.1440	33.3771
MCHC *	58	-2.8990	3.4000	1.2070	-1.2132	-0.8960	-0.5788
RDW%-1y	65	12.0000	21.5000	1.6120	14.0397	14.4390	14.8383
RDW% *	58	-2.5000	5.4000	1.8510	0.2225	0.7090	1.1955
PLT-1y	65	82.0000	623.0000	111.2360	316.0134	343.5690	371.1246
RETICs-1y	64	0.2990	5.3990	0.9520	1.5643	1.8020	2.0397
POLYs-1y	65	6.0000	67.0000	12.7800	31.6191	34.7850	37.9509
LYMPHs-1y	65	23.0000	82.0000	12.9880	53.1676	56.3850	59.6024
MONOs-1y	65	1.0000	15.0000	3.3970	5.0815	5.9230	6.7645
EOSINs-1y	65	0.0000	11.0000	2.5140	2.1152	2.7380	3.3608
BASOs-1y	64	0.0000	2.0000	0.3620	0.0186	0.1090	0.1994
ZPP-3/12	68	1.0990	7.1990	1.0830	2.5559	2.8180	3.0801
FERRITIN-3/12	69	20.0000	565.0000	118.2690	128.7984	157.2030	185.6076
Log(ferritin) 3/12	69	1.3010	2.7520	0.3370	1.9961	2.0770	2.1579
ZPP-1y	65	1.5000	11.0990	1.3220	3.0855	3.4130	3.7405
ZPP *	59	-2.4010	3.9000	1.1810	0.2073	0.5150	0.8227
FERRITIN-1y	69	1.0000	244.0000	34.5000	29.4101	37.6960	45.9819
FERRITIN *	64	-538.0000	158.0000	122.3670	-141.9786	-111.4220	-80.8654
Log(ferritin) 1 y	69	0.0000	2.3870	0.3290	1.3830	1.4620	1.5410
Log(ferritin) *	64	-1.6230	0.5400	0.4570	-0.6991	-0.5850	-0.4709
ZINC-PLASMA-3/12	69	55.0000	134.0000	17.9640	81.3236	85.6380	89.9524

	TEST GROUP				95% CI		95% CI
VARIABLE	N	Min	Max	SD	Bottom	Mean	Top
ZINC-HAIR-3/12	44	54.5990	759.7990	187.1310	204.4564	261.3130	318.1696
ZINC-PLASMA-1y	68	54.0000	142.0000	16.9150	79.4357	83.5290	87.6223
ZINC-PLASMA *	63	-62.0000	62.0000	26.1530	-9.9017	-3.3170	3.2677
ZINC-HAIR-1y	52	18.0000	457.0000	96.5280	102.3453	129.2070	156.0687
ZINC-HAIR *	28	-703.7990	253.1000	245.5570	-256.4509	-161.3930	-66.3351
# Infect:grade 1+2+3	70	0.0000	9.0000	2.1290	3.8925	4.4000	4.9075
# Infect. grade 1	70	0.0000	5.0000	1.2230	0.8655	1.1570	1.4485
# Infect. grade 2	70	0.0000	9.0000	2.0420	2.6422	3.1290	3.6158
# Infect. grade 3	70	0.0000	1.0000	0.3200	0.0377	0.1140	0.1903
# Infect. resp tract	70	0.0000	6.0000	1.2820	1.1804	1.4860	1.7916
# Infect. diarrhoea	70	0.0000	3.0000	0.9030	0.4987	0.7140	0.9293
# Infect. pyoderma	70	0.0000	4.0000	0.7930	0.1400	0.3290	0.5180
# Infect. thrush	70	0.0000	3.0000	0.7230	0.1846	0.3570	0.5294
# Infect other(-max)	70	0.0000	2.0000	0.3910	0.0498	0.1430	0.2362
# infect: other	70	0.0000	2.5520	0.5130	0.0657	0.1880	0.3103
# Infect. other	70	0.0000	2.0000	0.3910	0.0498	0.1430	0.2362
# Infect. conjunct.	70	0.0000	1.0000	0.4130	0.1155	0.2140	0.3125
# Infect:grade 1+2+3	70	0.0000	11.8930	2.8030	5.1918	5.8600	6.5282
# Infect:grade 1	70	0.0000	6.5380	1.6260	1.1604	1.5480	1.9356
# Infect:grade 2	70	0.0000	11.8930	2.6920	3.5163	4.1580	4.7997
# Infect:grade 3	70	0.0000	1.3480	0.4330	0.0508	0.1540	0.2572
# Infect: resp tract	70	0.0000	8.2330	1.7080	1.5718	1.9790	2.3862
# Infect: diarrhoea	70	0.0000	4.1340	1.1980	0.6624	0.9480	1.2336
# Infect: pyoderma	70	0.0000	5.2860	1.0450	0.1859	0.4350	0.6841
# Infect: thrush	70	0.0000	3.9960	0.9610	0.2459	0.4750	0.7041
# Infect:other(-max)	70	0.0000	2.5520	0.5130	0.0657	0.1880	0.3103
# Infect: conjunct	70	0.0000	1.4090	0.5530	0.1542	0.2860	0.4178
Mantoux 3/12	70	0.0000	21.0000	5.7740	3.9805	5.3570	6.7335
Candida 3/12	73	0.0000	20.0000	5.9390	2.9296	4.3150	5.7004
Mantoux 1 y	67	0.0000	22.0000	7.1770	5.2798	7.0300	8.7802
Mantoux *	64	-20.0000	22.0000	8.4830	-0.6493	1.4690	3.5873
Candida 1 y	68	0.0000	27.0000	8.3390	7.7320	9.7500	11.7680
Candida *	67	-17.0000	25.0000	9.3580	2.8969	5.1790	7.4611
Antibody tetanus ma	68	0.0000	5120.0000	652.9100	9.2084	167.2060	325.2036
Antibody polio ma	69	0.0000	20.0000	4.2730	0.7128	1.7390	2.7652
log[Tetanus] ma	68	0.0000	3.7090	1.0080	0.8971	1.1410	1.3849
log[Polio] ma	69	0.0000	1.3220	0.3890	0.0876	0.1810	0.2744
Antibody tetanus 3m	69	0.0000	320.0000	43.9750	2.6265	13.1880	23.7495
Antibody polio 3/12	69	0.0000	160.0000	38.5930	28.2671	37.5360	46.8049
log[Tetanus] 3/12	69	0.0000	2.5070	0.6780	0.2692	0.4320	0.5948
log[Polio] 3/12	69	0.0000	2.2070	0.6140	1.1665	1.3140	1.4615
Antibody tetanus 1y	69	0.0000	10240.0000	1388.6840	59.6681	393.1880	726.7079
Antibody tetanus *	64	-280.0000	10230.0000	1442.1780	34.7141	394.8440	754.9739
Antibody polio 1 y	67	0.0000	160.0000	54.9420	57.6467	71.0450	84.4433
Antibody polio *	63	-140.0000	160.0000	70.1800	15.5054	33.1750	50.8446
log[Tetanus] 1 yr	69	0.0000	4.0100	0.7170	1.7178	1.8900	2.0622
log[Tetanus] 1 y *	64	-1.6130	3.7090	1.0980	1.1558	1.4300	1.7042
log[[Polio] 1 yr	67	0.0000	2.2070	0.4920	1.5590	1.6790	1.7990
log[Polio] 1 yr *	63	-1.6130	2.2070	0.8180	0.1570	0.3630	0.5690
OKT3 3/12	63	47.0000	88.0000	10.0630	65.4504	67.9840	70.5176
OKT4 3/12	63	19.0000	87.0000	12.8310	48.3725	51.6030	54.8335
OKT8 3/12	63	5.0000	42.0000	7.7510	13.8895	15.8410	17.7925
SMIG 3/12	63	5.0000	43.0000	7.4390	15.5240	17.3970	19.2700
OKT4/OKT8 3/12	63	0.7620	12.4290	2.5370	3.5232	4.1620	4.8008

TEST GROUP VARIABLE	N	Min	Max	SD	95% CI Bottom	Mean	95% CI Top
OKT3 1y	69	40.0000	93.0000	11.2920	69.8820	72.5940	75.3060
OKT4 1y	69	16.0000	75.0000	12.3430	43.8616	46.8260	49.7904
OKT8 1y	69	7.0000	67.0000	9.4740	20.6666	22.9420	25.2174
SMIG 1y	69	4.0000	46.0000	7.7260	17.3324	19.1880	21.0436
OKT4/OKT8 1y	69	0.3580	5.9000	1.2040	2.1208	2.4100	2.6992
OKT4/OKT8 *	60	-9.5270	1.6920	2.3750	-2.2343	-1.6210	-1.0077
pha-pcps-3/12	29	89.7990	285.5790	39.4990	122.4799	137.4810	152.4821
pha-pccs-3/12	29	78.4190	280.1690	42.1350	127.1048	143.1070	159.1092
pha-ccps-3/12	29	66.9090	133.0490	14.6710	98.2282	103.8000	109.3718
pha-pcps-1y	38	43.2190	360.0790	64.1700	120.9845	142.0580	163.1315
pha-pccs-1y	38	63.8590	298.2690	44.6370	128.7272	143.3860	158.0448
pha-ccps-1y	38	50.8290	147.0890	19.7890	95.0453	101.5440	108.0427
pha-pcps *	29	-161.8200	108.5100	51.2680	-20.7388	-1.2680	18.2028
Birth weight	75	3.0000	4.2090	0.3060	3.2986	3.3690	3.4394
SD score weight brth	75	-0.7500	2.5240	0.7470	0.1062	0.2780	0.4498
Weight @ 3/12	75	4.8490	8.1590	0.6180	5.8938	6.0360	6.1782
Length @ 3/12	75	52.3990	65.8990	2.1410	58.6275	59.1200	59.6125
Head circ @ 3/12	75	37.5990	43.0990	1.1340	40.1041	40.3650	40.6259
SD score weight 3/12	75	-0.5670	2.4530	0.6300	0.3671	0.5120	0.6569
SD score length 3/12	74	-1.8850	1.6840	0.6850	-0.4657	-0.3070	-0.1483
Weight/(length)**3 3	74	23.4940	36.5870	2.3040	28.4953	29.0290	29.5627
Weight @ 1y	70	8.0090	12.9190	1.1570	10.0582	10.3340	10.6098
Weight *	70	2.5400	6.5300	0.9580	4.0646	4.2930	4.5214
Length @ 1y	71	69.8990	82.0000	2.5560	74.6241	75.2290	75.8339
Length *	71	7.7000	25.9000	2.5250	15.4085	16.0060	16.6035
Head circ @ 1y	71	43.5990	50.0000	1.2820	46.2336	46.5370	46.8404
SD score weight 1 y	70	-1.4610	2.5570	1.0720	0.2254	0.4810	0.7366
SD score length 1 y	71	-2.0370	2.2750	0.9340	-0.1560	0.0650	0.2860
SD score length *	70	-2.4080	2.4330	0.7430	0.1469	0.3240	0.5011
Weight/(length)**3y	70	19.9290	33.9580	2.3630	23.6627	24.2260	24.7893
Bact % init 1hr 3/12	33	32.0000	224.0000	44.9180	86.6068	102.5150	118.4232
Bact % init 2hr 3/12	33	27.0000	237.0000	53.5660	92.0290	111.0000	129.9710
Bact % ctl 1hr 3/12	33	52.9390	427.2690	84.2370	145.1625	174.9960	204.8295
Bact % ctl 2hr 3/12	33	86.4790	2400.0000	423.8000	196.4856	346.5790	496.6724
Bact % AB 1 hr 3/12	33	16.1590	93.3290	17.1590	41.0070	47.0840	53.1610
Bact % AB 2 hr 3/12	33	8.7390	69.4090	13.9850	26.1961	31.1490	36.1019
SD score weight *	70	-1.8290	2.0630	0.9150	-0.2481	-0.0300	0.1881
Bact norm 1 hr 3/12	33	-34.7900	88.3990	26.1900	14.0685	23.3440	32.6195
Bact norm 2 hr 3/12	33	-2.3400	64.9590	15.4850	14.9048	20.3890	25.8732
Bact % init 1hr 1y	29	20.0000	227.0000	57.3120	72.2338	94.0000	115.7662
Bact % init 1hr *	25	-149.0000	172.0000	76.5750	-43.2612	-11.7200	19.8212
Bact % init 2hr 1y	29	14.0000	347.0000	95.7650	76.9400	113.3100	149.6800
Bact % init 2hr *	25	-160.0000	213.0000	118.6110	-46.4959	2.3600	51.2159
Bact % ctl 1 hr 1y	24	28.2290	487.1690	92.1160	90.1423	128.9500	167.7577
Bact % ctl 1 hr *	21	-247.2300	341.7200	123.2280	-109.7096	-53.7880	2.1336
Bact % ctl 2 hr 1y	29	42.5490	1446.1490	304.0170	93.6702	209.1310	324.5918
Bact % AB 1 hr 1y	28	8.0590	100.6090	27.6920	31.6821	42.4020	53.1219
Bact % AB 2 hr 1y	29	2.6290	91.4790	27.5720	21.1036	31.5750	42.0464
Bact norm 1 hr 1 y	24	-286.6700	101.8490	91.8060	-43.5101	-4.8330	33.8441
Bact norm 2 hr 1 y	29	-54.3700	83.8290	34.7530	-1.7266	11.4720	24.6706
hygiene	73	1.0000	3.0000	0.7190	2.4483	2.6160	2.7837
daycare	73	1.0000	3.0000	0.3710	2.7905	2.8770	2.9635
crowding	73	1.0000	3.0000	0.9420	1.6573	1.8770	2.0967
finance	73	1.0000	3.0000	0.7420	2.4159	2.5890	2.7621

TEST GROUP					95% CI		95% CI
VARIABLE	N	Min	Max	SD	Bottom	Mean	Top
careseeking	73	1.0000	3.0000	0.2040	1.9384	1.9860	2.0336
MDI-pre	22			10.7000	105.5689	110.3000	115.0311
PDI-pre	22			7.9000	94.0070	97.5000	100.9930
MDI-diff (Post)	8			5.0000	-7.6765	-3.6000	0.4765
PDI-diff (Post)	8			8.9000	-5.4561	1.8000	9.0561
Meat/Fish	74	2.0000	9.0000	1.3800	4.3603	4.6800	4.9997
Cereal	75	0.0000	4.0000	0.7900	1.9283	2.1100	2.2917
Egg	74	0.0000	7.0000	1.5200	3.4879	3.8400	4.1921
Fruit	70	1.0000	9.0000	1.4100	3.8239	4.1600	4.4961
Vegetable	75	1.0000	6.0000	1.0600	2.9562	3.2000	3.4438

	95% CI of DIFFERENCE of MEANS						POWER for alpha = 5%, & diff :		
VARIABLE	Bottom	Mean	Top	P	N_5_PCT	BETA	Observ	10%	25%	50%
First bottle	-0.0350	-0.3563	0.2863	0.8325	6090	0.9999	0.0001	0.1565	0.6328	0.9902
Last breast	0.4470	-0.9379	1.8319	0.5239	611	0.9999	0.0001	0.0001	0.2753	0.7709
Date of birth	0.0200	-0.0175	0.0575	0.2945	254	0.8149	0.1851	1.0000	1.0000	1.0000
Date @ 3/12 entry	0.0170	-0.0172	0.0512	0.3306	293	0.8315	0.1685	1.0000	1.0000	1.0000
Date @ 1y exit	0.0310	-0.0102	0.0722	0.1359	116	0.6865	0.3135	1.0000	1.0000	1.0000
Age @ entry (months)	-0.0630	-0.1470	0.0210	0.1402	128	0.6883	0.3117	0.9916	0.9982	1.0000
Age at exit (months)	0.0450	-0.0623	0.1523	0.4086	373	0.8660	0.1340	1.0000	1.0000	1.0000
WBC-3/12	0.6510	-0.4625	1.7645	0.2492	174	0.7886	0.2114	0.5332	0.9905	0.9938
RBC-3/12	0.0490	-0.0701	0.1681	0.4184	351	0.8694	0.1306	0.9915	0.9980	1.0000
Hb-3/12	0.0920	-0.1853	0.3693	0.5131	539	0.9999	0.0001	0.9924	1.0000	1.0000
HCT-3/12	0.3830	-0.4054	1.1714	0.3373	252	0.8359	0.1641	0.9925	1.0000	1.0000
MCV-3/12	0.0340	-1.7984	1.8664	0.9703	172471	0.9999	0.0001	0.9929	1.0000	1.0000
MCH-3/12	-0.0090	-0.7103	0.6923	0.9804	360571	0.9999	0.0001	0.9923	0.9999	1.0000
MCHC-3/12	-0.0720	-0.4259	0.2819	0.6883	1435	0.9999	0.0001	0.9995	1.0000	1.0000
RDW%-3/12	0.3380	-0.1654	0.8414	0.1864	132	0.7365	0.2635	0.9906	0.9957	1.0000
PLT-3/12	14.2430	-27.3257	55.8117	0.4988	506	0.9999	0.0001	0.5970	0.9908	0.9943
RETICS-3/12	0.2690	-0.1648	0.7028	0.2204	148	0.7672	0.2328	0.1694	0.6834	0.9904
POLYs-3/12	0.5480	-3.7798	4.8758	0.8023	3667	0.9999	0.0001	0.2388	0.8660	0.9912
LYMPHs-3/12	0.2790	-4.2542	4.8122	0.9033	15520	0.9999	0.0001	0.8014	0.9918	0.9963
MONOs-3/12	-0.1120	-1.1276	0.9036	0.8273	4834	0.9999	0.0001	0.1699	0.6854	0.9904
EOSINs-3/12	-0.6290	-1.4751	0.2171	0.1435	106	0.6921	0.3079	0.0001	0.3150	0.8350
BASOs-3/12	-0.0440	-0.2072	0.1192	0.6003	803	0.9999	0.0001	0.0001	0.0001	0.1633
WBC-1y	0.0890	-0.9672	1.1452	0.8673	8692	0.9999	0.0001	0.5320	0.9905	0.9938
RBC-1y	0.0500	-0.0926	0.1926	0.4892	502	0.8968	0.1032	0.9916	0.9980	1.0000
RBC *	0.0420	-0.1009	0.1849	0.5578	580	0.9999	0.0001	0.1975	0.7822	0.9908
Hb-1y	0.3590	0.0151	0.7029	0.0410	57	0.4656	0.5344	0.9915	0.9980	1.0000
Hb *	0.4870	0.1120	0.8620	0.0114	30	0.2794	0.7206	0.0001	0.0001	0.1168
HCT-1y	0.9500	-0.0925	1.9925	0.0739	74	0.5706	0.4294	0.9915	0.9979	1.0000
HCT *	0.9830	-0.1521	2.1181	0.0886	67	0.6044	0.3956	0.0001	0.1411	0.3969
MCV-1y	1.2390	-0.6669	3.1449	0.2005	146	0.7495	0.2505	0.9920	0.9993	1.0000
MCV *	1.4510	-0.3954	3.2974	0.1221	81	0.6612	0.3388	0.1757	0.7070	0.9905
MCH-1y	0.5180	-0.2164	1.2524	0.1649	124	0.7163	0.2837	0.9914	0.9976	1.0000
MCH *	0.7890	0.0801	1.4979	0.0295	40	0.4089	0.5911	0.1992	0.7878	0.9908
MCHC-1y	0.1610	-0.1651	0.4871	0.3305	253	0.8325	0.1675	1.0000	1.0000	1.0000
MCHC *	0.4690	-0.0161	0.9541	0.0581	54	0.5250	0.4750	0.0001	0.2026	0.6096
RDW%-1y	-1.0910	-1.6944	-0.4876	0.0005	19	0.0583	0.9417	0.9904	0.9951	1.0000
RDW% *	-1.5480	-2.3365	-0.7595	0.0002	13	0.0353	0.9647	0.0001	0.1446	0.4089
PLT-1y	-11.1360	-48.9666	26.6946	0.5612	712	0.9999	0.0001	0.4383	0.9901	0.9931
RETICs-1y	0.1230	-0.2739	0.5199	0.5381	621	0.9999	0.0001	0.1432	0.5780	0.9900
POLYs-1y	3.4680	-0.7448	7.6808	0.1058	90	0.6359	0.3641	0.3379	0.9645	0.9922
LYMPHs-1y	-4.3650	-8.6550	-0.0750	0.0462	59	0.4864	0.5136	0.7589	0.9915	0.9958
MONOs-1y	1.2900	0.1298	2.4502	0.0297	49	0.4116	0.5884	0.1511	0.6115	0.9901
EOSINs-1y	-0.4790	-1.5899	0.6319	0.3958	329	0.8606	0.1394	0.0001	0.2612	0.7420
BASOs-1y	0.0090	-0.1184	0.1364	0.8844	12167	0.9999	0.0001	0.0001	0.0001	0.1308
ZPP-3/12	-0.0970	-0.4953	0.3013	0.6336	1100	0.9999	0.0001	0.2918	0.9388	0.9918
FERRITIN-3/12	1.5970	-38.3398	41.5338	0.9371	43004	0.9999	0.0001	0.1214	0.4823	0.9634
Log(ferritin) 3/12	0.0070	-0.1094	0.1234	0.9053	19025	0.9999	0.0001	0.9341	0.9929	0.9985
ZPP-1y	-0.5370	-1.0367	-0.0373	0.0353	53	0.4411	0.5589	0.3012	0.9491	0.9919
ZPP *	-0.6650	-1.2327	-0.0973	0.0220	39	0.3672	0.6328	0.0001	0.1089	0.2965
FERRITIN-1y	13.8960	3.9490	23.8430	0.0066	32	0.2205	0.7795	0.0001	0.3369	0.8629
FERRITIN *	26.5080	-17.6979	70.7139	0.2374	164	0.7796	0.2204	0.0001	0.2811	0.7828
Log(ferritin) 1 y	0.2250	0.1022	0.3478	0.0004	19	0.0520	0.9480	0.5825	0.9907	0.9942
Log(ferritin) *	0.2770	0.1146	0.4394	0.0010	20	0.0856	0.9144	0.1435	0.5795	0.9900
ZINC-PLASMA-3/12	-5.5190	-13.0257	1.9877	0.1482	126	0.6986	0.3014	0.6354	0.9910	0.9947

	95% CI of DIFFERENCE of MEANS						POWER for alpha = 5%, & diff :		
VARIABLE	Bottom	Mean	Top	P	N_5_PCT	BETA	Observ	10%	25%	50%
ZINC-HAIR-3/12	37.2900	-37.6075	112.1875	0.3245	150	0.9999	0.0001	0.0001	0.0001	0.0001
ZINC-PLASMA-1y	-7.0780	-14.2746	0.1186	0.0538	62	0.5132	0.4868	0.6556	0.9910	0.9948
ZINC-PLASMA *	-2.4130	-12.5029	7.6769	0.6365	975	0.9999	0.0001	0.0001	0.0001	0.0001
ZINC-HAIR-1y	-13.0900	-54.9201	28.7401	0.5361	503	0.9999	0.0001	0.0001	0.0001	0.0001
ZINC-HAIR *	-67.4560	-187.8139	52.9019	0.2657	78	0.7032	0.2968	0.1315	0.1788	0.2882
# Infect:grade 1+2+3	-0.3580	-1.1484	0.4324	0.3718	315	0.8509	0.1491	0.2071	0.8065	0.9909
# Infect. grade 1	-0.1010	-0.5236	0.3216	0.6375	1130	0.9999	0.0001	0.0001	0.2880	0.7970
# Infect. grade 2	-0.2100	-0.9583	0.5383	0.5795	820	0.9999	0.0001	0.1394	0.5611	0.9863
# Infect. grade 3	-0.0470	-0.1660	0.0720	0.4361	414	0.8769	0.1231	0.0001	0.0001	0.2032
# Infect. resp tract	0.0560	-0.3870	0.4990	0.7132	4038	0.9999	0.0001	0.0001	0.3592	0.8912
# Infect. diarrhoea	-0.0760	-0.3969	0.2449	0.6402	1151	0.9999	0.0001	0.0001	0.2109	0.6292
# Infect. pyoderma	-0.0580	-0.3547	0.2387	0.6970	1689	0.9999	0.0001	0.0001	0.0001	0.2202
# Infect. thrush	-0.0460	-0.2974	0.2054	0.7174	1928	0.9999	0.0001	0.0001	0.1149	0.3147
# Infect other(-max)	-0.0870	-0.2345	0.0605	0.2471	184	0.7857	0.2143	0.0001	0.0001	0.2326
# infect: other	-0.1830	-0.4093	0.0433	0.1136	99	0.6466	0.3534	0.0001	0.0001	0.2243
# Infect. other	-0.1310	-0.2991	0.0371	0.1249	106	0.6672	0.3328	0.0001	0.0001	0.2262
# Infect. conjunct.	-0.0280	-0.1797	0.1237	0.7191	1894	0.9999	0.0001	0.0001	0.1137	0.3108
# Infect:grade 1+2+3	-0.5570	-1.6088	0.4948	0.2971	230	0.8164	0.1836	0.2094	0.8109	0.9909
# Infect:grade 1	-0.1390	-0.6989	0.4209	0.6240	1047	0.9999	0.0001	0.0001	0.2924	0.8045
# Infect:grade 2	-0.3530	-1.3512	0.6452	0.4848	516	0.8955	0.1045	0.1402	0.5650	0.9874
# Infect:grade 3	-0.0640	-0.2250	0.0970	0.4339	408	0.8758	0.1242	0.0001	0.0001	0.2032
# Infect: resp tract	0.0780	-0.5203	0.6763	0.7961	3797	0.9999	0.0001	0.0001	0.3558	0.8869
# Infect: diarrhoea	-0.1170	-0.5464	0.3124	0.5906	869	0.9999	0.0001	0.0001	0.2107	0.6288
# Infect: pyoderma	-0.0890	-0.4861	0.3081	0.6568	1285	0.9999	0.0001	0.0001	0.0001	0.2207
# Infect: thrush	-0.0690	-0.4046	0.2666	0.6828	1527	0.9999	0.0001	0.0001	0.1152	0.3158
# Infect:other(-max)	-0.1210	-0.3174	0.0754	0.2277	169	0.7701	0.2299	0.0001	0.0001	0.2330
# Infect: conjunct	-0.0380	-0.2410	0.1650	0.7151	1842	0.9999	0.0001	0.0001	0.1138	0.3112
Mantoux 3/12	-0.5990	-2.4958	1.2978	0.5335	679	0.9999	0.0001	0.0001	0.3083	0.8265
Candida 3/12	-0.0370	-2.0275	1.9535	0.9707	204807	0.9999	0.0001	0.0001	0.1900	0.5686
Mantoux 1 y	-1.0200	-3.5963	1.5563	0.4347	396	0.8765	0.1235	0.0001	0.2978	0.8126
Mantoux *	-0.7770	-3.8566	2.3026	0.6184	928	0.9999	0.0001	0.0001	0.0001	0.0001
Candida 1 y	0.8670	-2.0977	3.8317	0.5639	731	0.9999	0.0001	0.0001	0.3390	0.8656
Candida *	0.3120	-2.7778	3.4018	0.8417	6089	0.9999	0.0001	0.0001	0.1286	0.3576
Antibody tetanus ma	100.7540	-65.0953	266.6033	0.2316	172	0:7751	0.2249	0.0001	0.0001	0.1070
Antibody polio ma	-0.2900	-1.8865	1.3065	0.7201	2054	0.9999	0.0001	0.0001	0.0001	0.2142
log[Tetanus] ma	0.0770	-0.2698	0.4238	0.6580	1291	0.9999	0.0001	0.0001	0.3446	0.8727
log[Polio] ma	-0.0010	-0.1357	0.1337	0.9921	999999	0.9999	0.0001	0.0001	0.0001	0.2654
Antibody tetanus 3m	1.9020	-9.8833	13.6873	0.7500	2621	0.9999	0.0001	0.0001	0.0001	0.1786
Antibody polio 3/12	1.3690	-12.5104	15.2484	0.8455	6472	0.9999	0.0001	0.0001	0.2592	0.7377
log[Tetanus] 3/12	-0.1280	-0.3560	0.1000	0.2675	217	0.8022	0.1978	0.0001	0.1904	0.5703
log[Polio] 3/12	0.0760	-0.1474	0.2994	0.5015	544	0.9999	0.0001	0.2036	0.7999	0.9908
Antibody tetanus 1y	200.7290	-155.6047	557.0627	0.2671	200	0.8012	0.1988	0.0001	0.0001	0.1341
Antibody tetanus *	213.2920	-166.5834	593.1674	0.2685	189	0.8019	0.1981	0.0001	0.0001	0.1193
Antibody polio 1 y	12.6840	-5.7393	31.1073	0.1755	132	0.7265	0.2735	0.1041	0.4062	0.9299
Antibody polio *	3.5600	-21.3225	28.4425	0.7774	2724	0.9999	0.0001	0.0001	0.0001	0.2414
log[Tetanus] 1 yr	-0.0890	-0.3185	0.1405	0.4436	423	0.8802	0.1198	0.3769	0.9803	0.9926
log[Tetanus] 1 y *	0.0240	-0.3563	0.4043	0.9004	14970	0.9999	0.0001	0.1133	0.4463	0.9530
log[[Polio] 1 yr	0.1330	-0.0530	0.3190	0.1589	123	0.7106	0.2894	0.3962	0.9882	0.9927
log[Polio] 1 yr *	-0.0290	-0.3424	0.2844	0.8570	6511	0.9999	0.0001	0.0001	0.0001	0.2208
OKT3 3/12	-1.4850	-4.8693	1.8993	0.3870	323	0.8572	0.1428	0.9720	0.9936	1.0000
OKT4 3/12	1.1810	-3.1136	5.4756	0.5871	823	0.9999	0.0001	0.6431	0.9910	0.9947
OKT8 3/12	-0.5810	-3.1638	2.0018	0.6571	1231	0.9999	0.0001	0.2353	0.8593	0.9912
SMIG 3/12	-2.2120	-4.5791	0.1551	0.0667	71	0.5522	0.4478	0.3349	0.9633	0.9922
OKT4/OKT8 3/12	0.5600	-0.2139	1.3339	0.1553	119	0.7054	0.2946	0.1710	0.6895	0.9904

	95% CI of DIFFERENCE of MEANS						POWER for alpha = 5%, & diff :			
VARIABLE	Bottom	Mean	Top	P	N_5_PCT	BETA	Observ	10%	25%	50%
OKT3 1y	-1.5460	-5.4885	2.3965	0.4391	398	0.8781	0.1219	0.9523	0.9931	0.9990
OKT4 1y	-0.0690	-4.8714	4.7334	0.9775	296540	0.9999	0.0001	0.4805	0.9903	0.9934
OKT8 1y	0.4160	-3.0644	3.8964	0.8135	4285	0.9999	0.0001	0.2527	0.8921	0.9914
SMIG 1y	0.5920	-2.1009	3.2849	0.6643	1267	0.9999	0.0001	0.2820	0.9279	0.9917
OKT4/OKT8 1y	-0.1190	-0.5859	0.3479	0.6156	942	0.9999	0.0001	0.1829	0.7317	0.9906
OKT4/OKT8 *	-0.6560	-1.4388	0.1268	0.1000	75	0.6246	0.3754	0.0001	0.1362	0.3818
pha-pcps-3/12	3.7390	-21.3489	28.8269	0.7663	1203	0.8567	0.1433	0.2876	0.8564	1.0000
pha-pccs-3/12	4.3300	-19.3645	28.0245	0.7155	800	0.8469	0.1531	0.3166	0.9151	1.0000
pha-ccps-3/12	5.8290	-1.8768	13.5348	0.1352	47	0.5791	0.4209	0.8304	1.0000	1.0000
pha-pcps-1y	1.6600	-29.8113	33.1313	0.9163	10152	0.8847	0.1153	0.2464	0.6989	1.0000
pha-pccs-1y	0.5530	-23.2465	24.3525	0.9631	52317	0.8933	0.1067	0.3198	0.9180	1.0000
pha-ccps-1y	-0.5910	-9.8742	8.6922	0.8990	6783	0.8815	0.1185	0.6783	1.0000	1.0000
pha-pcps *	-16.3000	-52.4018	19.8018	0.3685	114	0.8527	0.1473	0.0001	0.0001	0.0001
Birth weight	-0.0430	-0.1428	0.0568	0.3995	389	0.8610	0.1390	0.9915	0.9979	1.0000
SD score weight brth	-0.0780	-0.3141	0.1581	0.5132	662	0.9999	0.0001	0.0001	0.0001	0.2615
Weight @ 3/12	-0.0240	-0.2309	0.1829	0.8211	5368	0.9999	0.0001	0.9909	0.9965	1.0000
Length @ 3/12	-0.0120	-0.6686	0.6446	0.9711	216275	0.9999	0.0001	0.9986	1.0000	1.0000
Head circ @ 3/12	-0.3400	-0.7495	0.0695	0.1027	105	0.6317	0.3683	0.9997	1.0000	1.0000
SD score weight 3/12	0.0360	-0.1814	0.2534	0.7453	2635	0.9999	0.0001	0.0001	0.2008	0.6055
SD score length 3/12	0.1120	-0.1061	0.3301	0.3122	272	0.8238	0.1762	0.0001	0.1324	0.3696
Weight/(length)**3 3	-0.2240	-0.9125	0.4645	0.5211	678	0.9999	0.0001	0.9926	1.0000	1.0000
Weight @ 1y	0.2410	-0.1617	0.6437	0.2391	182	0.7807	0.2193	0.9904	0.9952	1.0000
Weight *	0.2960	-0.0267	0.6187	0.0719	77	0.5661	0.4339	0.7113	0.9913	0.9954
Length @ 1y	0.4930	-0.3792	1.3652	0.2655	205	0.8004	0.1996	0.9981	1.0000	1.0000
Length *	0.5570	-0.2446	1.3586	0.1721	136	0.7229	0.2771	0.9642	0.9934	0.9996
Head circ @ 1y	-0.1700	-0.6038	0.2638	0.4397	427	0.8784	0.1216	1.0000	1.0000	1.0000
SD score weight 1 y	0.2510	-0.1304	0.6324	0.1951	150	0.7449	0.2551	0.0001	0.0001	0.1594
SD score length 1 y	0.1980	-0.1074	0.5034	0.2021	156	0.7508	0.2492	0.0001	0.0001	0.0001
SD score length *	0.0690	-0.1767	0.3147	0.5776	826	0.9999	0.0001	0.0001	0.0001	0.2156
Weight/(length)**3y	0.1070	-0.6044	0.8184	0.7665	2878	0.9999	0.0001	0.9915	0.9980	1.0000
Bact % init 1hr 3/12	-6.3290	-29.1824	16.5244	0.5819	408	0.9999	0.0001	0.0001	0.0001	0.0001
Bact % init 2hr 3/12	-13.4060	-47.1261	20.3141	0.4299	198	0.9999	0.0001	0.0001	0.0001	0.0001
Bact % ctl 1hr 3/12	-17.7000	-68.2760	32.8760	0.4869	255	0.9999	0.0001	0.0001	0.0001	0.0001
Bact % ctl 2hr 3/12	-30.2190	-219.3218	158.8838	0.7505	1224	0.9999	0.0001	0.0001	0.0001	0.0001
Bact % AB 1 hr 3/12	-3.5790	-13.3254	6.1674	0.4658	232	0.9999	0.0001	0.0001	0.0001	0.0001
Bact % AB 2 hr 3/12	-4.0230	-13.1510	5.1050	0.3818	161	0.9999	0.0001	0.0001	0.0001	0.0001
SD score weight *	0.2230	-0.0860	0.5320	0.1550	125	0.7068	0.2932	0.0001	0.0001	0.0001
Bact norm 1 hr 3/12	2.4710	-17.1949	22.1369	0.8026	1980	0.9999	0.0001	0.0001	0.0001	0.0001
Bact norm 2 hr 3/12	-4.7070	-15.1897	5.7757	0.3730	155	0.9999	0.0001	0.0001	0.0001	0.0001
Bact % init 1hr 1y	-11.1430	-40.0963	17.8103	0.4438	184	0.7819	0.2181	0.1998	0.4968	0.9665
Bact % init 1hr *	-26.7680	-69.0540	15.5180	0.2087	54	0.7607	0.2393	0.0001	0.0001	0.0001
Bact % init 2hr 1y	-13.4400	-60.5307	33.6507	0.5697	335	0.8171	0.1829	0.1740	0.3424	0.8068
Bact % init 2hr *	-21.3540	-88.5089	45.8009	0.5249	214	0.9999	0.0001	0.0001	0.0001	0.0001
Bact % ctl 1 hr 1y	-1.8030	-46.1233	42.5173	0.9351	14046	0.9999	0.0001	0.0001	0.3013	0.8172
Bact % ctl 1 hr *	-3.1850	-75.7628	69.3928	0.9296	9419	0.9999	0.0001	0.0001	0.0001	0.1087
Bact % ctl 2 hr 1y	9.1020	-117.0558	135.2598	0.8856	5235	0.8790	0.1210	0.1471	0.2273	0.4613
Bact % AB 1 hr 1y	-5.9290	-21.8648	10.0068	0.4581	169	0.8874	0.1126	0.0001	0.2843	0.7905
Bact % AB 2 hr 1y	-2.2240	-15.8472	11.3992	0.7445	961	0.8525	0.1475	0.1696	0.3190	0.7541
Bact norm 1 hr 1 y	-8.3430	-62.9893	46.3033	0.7598	931	0.9999	0.0001	0.0001	0.0001	0.0001
Bact norm 2 hr 1 y	-0.8400	-18.1371	16.4571	0.9228	10862	0.8859	0.1141	0.1199	0.1499	0.1997
hygiene	-0.1020	-0.3327	0.1287	0.3842	362	0.8559	0.1441	0.6199	0.9909	0.9945
daycare	0.0730	-0.0542	0.2002	0.2524	215	0.7956	0.2044	0.9901	0.9943	1.0000
crowding	-0.1370	-0.4485	0.1745	0.3847	366	0.8570	0.1430	0.2344	0.8577	0.9912
finance	-0.0730	-0.3069	0.1609	0.5386	726	0.9999	0.0001	0.5955	0.9908	0.9943

	95% CI of DIFFERENCE of MEANS					POWER for alpha = 5%, & diff :				
VARIABLE	Bottom	Mean	Top	P	N_5_PCT	BETA	Observ	10%	25%	50%
careseeking	0.0000	-0.0874	0.0874	0.9930	999999	0.9999	0.0001	0.9901	0.9944	1.0000
MDI-pre	-2.7000	-10.3688	4.9688	1.0000	95	0.8956	0.1044	0.0001	0.0001	0.0001
PDI-pre	-6.9000	-13.1420	-0.6580	0.0500	10	0.4112	0.5888	0.0001	0.0001	0.0001
MDI-diff (Post)	-7.6000	-13.8234	-1.3766	0.0200	6	0.3223	0.6777	0.0001	0.0001	0.0001
PDI-diff (Post)	2.3000	-3.0262	7.6262	1.0000	48	0.8585	0.1415	0.0001	0.0001	0.0001
Meat/Fish	-0.5100	-1.0433	0.0233	0.0000	76	0.5357	0.4643	0.4376	0.9901	0.9931
Cereal	0.0500	-0.2086	0.3086	0.0000	1918	0.9999	0.0001	0.3500	0.9694	0.9923
Egg	-0.4000	-0.9548	0.1548	0.0000	134	0.7074	0.2926	0.2958	0.9433	0.9918
Fruit	0.0000	-0.4783	0.4783	0.0000	999999	0.9999	0.0001	0.3965	0.9883	0.9927
Vegetable	-0.2000	-0.6020	0.2020	0.0000	292	0.8311	0.1689	0.3616	0.9741	0.9924

APPENDIX 4 - Composition of Control and Test milk formulas

COMPOSITION OF LACTOGEN FULL PROTEIN

According to the manufacturer's label, Lactogen full protein is made from partially skimmed milk, sucrose, maltodextrin, corn oil, lactose, vitamins, ferrous sulfate, zinc sulfate and copper sulfate. Its average composition is Fat: 19.0% (including 3.8% corn oil, rich in unsaturated fatty acids), Protein: 21.6%, Lactose: 31.6%: Sucrose: 12.0%: Maltodextrin: 8.0%, Ash: 4.8%: Moisture: 3.0%. Energy per 100 g powder = 1940 kj.

Vitamin and mineral content of LACTOGEN FULL PROTEIN

Nutrient	per 100 g	per litre
Vitamin A	1390 IU	2003 IU
Vitamin D3	280 IU	403 IU
Vitamin E	5.5 IU	7.9 IU
Vitamin C	37 mg	53 mg
Vitamin B1	0.28 mg	0.40 mg
Vitamin B2	0.4 mg	0.58 mg
Vitamin B4	0.35 mg	0.50 mg
Vitamin B12	1 ug	1.4 ug
Niacin	3.5 mg	5.1 mg
Pantothenic acid	2 mg	2.9 mg
Vitamin K	38 ug	54.8 ug
Folic acid	42 ug	60.5 ug
Biotin	10 ug	14.4 ug
Choline	35 mg	50.5 mg
Inositol	21 mg	30.3 mg
Iron for Control group	**8.3 mg**	**11.96 mg**
for Test group	**40 mg**	**57.66 mg**
Copper	0.28 mg	0.40 mg
Zinc	3.5 mg	5.1 mg
Manganese	24 ug	34.6 ug
Calcium	770 mg	1109.9 mg
Phosphorus	600 mg	865 mg
Sodium	320 mg	461 mg
Magnesium	70 mg	101 mg
Chloride	725 mg	1045 mg
Potassium	970 mg	1398 mg

Note The concentrations of nutrients per 100 g formula are the manufacturer's specifications. The concentrations of nutrients per litre were calculated according to the manufacturer's recommendation for reconstitution: 8 measures of 4 g for 200 ml water. When reconstituted, the fluid volume was 222 ml. The values in the left hand column were multiplied by 320/222 to obtain the right hand set of figures.

APPENDIX 5 - Programs

Program to Stratify Subjects

This program was written for the Tektronix microcomputer

```
1 REM      S T R A T I F Y
2 REM  Program to stratify subjects according to number of siblings and weight
3 REM
5 GO TO 100
100 INIT
110 P=41
120 DIM A0(2), A$(72), Z$(132)
130 PAGE
140 PRINT "STRATIFY ROUTINE"
150 PRINT "J_J_Enter number of file containing the data"
160 INPUT I0
170 FIND I0
180 READ @33:A0
190 DIM C$(90+8*A0(1)),B0(A0(2),A0(1)),A(30,8),B(30,8),N(8)
200 DIM A1(8),B1(8)
210 READ @33:C$
220 READ @33:B0,Z0
230 N(1)=(I0-INT(I0/2)*2)*2+1
240 FOR I=1 TO 8
250 IF I=1 THEN 290
260 N(I)=3
270 IF N(I-1)=1 THEN 290
280 N(I)=1
290 A(I)=0
300 B1(I)=0
310 FOR J=1 TO 30
320 A(J,I)=0
330 B(J,I)=0
340 NEXT J
350 NEXT I
360 N1=A0(2)
370 FOR I=1 TO N1
380 IF B0(I,3)<3000 OR B0(I,3)>4999 THEN 520
390 J=B0(I,5)
400 PRINT B0(I,3),J
410 IF J>15 OR J<1 THEN 520
420 J=8 MIN J
430 IF N(J)=2 OR N(J)=3 THEN 470
440 A1(J)=A1(J)+1
450 A(A1(J),J)=B0(I,1)
460 GO TO 490
470 B1(J)=B1(J)+1
480 B(B1(J),J)=B0(I,1)
490 N(J)=N(J)+1
500 IF N(J)<5 THEN 520
510 N(J)=1
520 NEXT I
530 A$=SEG(C$,1,72)
540 PRINT @P: USING "P, 4X,72A":A$
```

```
550 PRINT @P: USING "///":
560 PRINT @P: USING "/14A":"Number of sibs"
570 FOR I=1 TO 8
580 PRINT @P: USING 590:I,I;
590 IMAGE 4D,4D,2X,S
600 NEXT I
610 PRINT @P:
620 FOR I=1 TO 8
630 PRINT @P: USING "10A,S":" TST CTL   "
640 NEXT I
650 PRINT @P: USING "//":
660 FOR J=1 TO 30
670 IF A(J,1)+B(J,1)=0 THEN 820
680 FOR I=1 TO 8
690 IF A(J,I)=0 THEN 730
700 PRINT @P: USING 710:A(J,I);
710 IMAGE 4D,S
720 GO TO 750
730 PRINT @P: USING 740:
740 IMAGE 4X,S
750 IF B(J,I)=0 THEN 780
760 PRINT @P: USING 710:B(J,I);
770 GO TO 790
780 PRINT @P: USING 740:
790 PRINT @P: USING "2X,S":
800 NEXT I
810 PRINT @P:
820 NEXT J
830 PRINT @P: USING "P,4X,72A":A$
840 PRINT @P: USING "//4A,36X,7A":"TEST","CONTROL"
850 FOR I=8 TO 1 STEP -1
860 PRINT @P: USING "//15A,1D":"Number of sibs=",I
870 FOR J=1 TO 30
880 IF A(J,I)=0 AND B(J,I)=0 THEN 970
890 IF A(J,I)=0 THEN 920
900 PRINT @P: USING "4D,36X,S":A(J,I);
910 GO TO 940
920 PRINT @P: USING "40X,4D,S":B(J,I);
930 GO TO 960
940 IF B(J,I)=0 THEN 960
950 PRINT @P: USING "4D,S":B(J,I);
960 PRINT @P: USING "//":
970 NEXT J
980 NEXT I
990 PAGE
1000 PRINT "THE END"
1010 END
```

Program to Calculate 95% Confidence Limits and "N5%"

This program was written in dBase III and runs on an IBM compatable PC.

```
Note/*     PROGRAM TO CALCULATE 95% CONFIDENCE LIMITS OF MEANS
Note/*
Note/* REFERENCES:
Note/*     Colton T. Statistics in Medicine. Little Brown & Co. Boston 1974
Note/*
Note/*     Gardner MJ, Altman DG. Confidence limits rather than P values:
Note/*       estimation rather than hypothesis testing.
Note/*       British Medical Journal 1986: 292; 746-750
Note/*
Note/* INPUT VARIABLES:
Note/*     Variable          Name (ie Lotus Lable) of the variable
Note/*     N_ALL, N_CTL, N_TST Numbers in All, Control and Test Groups
Note/*     Mean_ALL, Mean_CTL, Mean_TST
Note/*                  Means in  All, Control and Test Groups
Note/*     SD_ALL, SD_CTL, SD_TST,
Note/*                  Standard Deviations in All, Control and Test
Note/*                     Groups

Note/* CALCULATED VARIABLES:
Note/*     MAF, MCF, MTF      Lower bound (Floor) of 95% confidence limit
Note/*     MAC, MCC, MTC      Upper bound (Ceiling) of 95% confidence limit
Note/*     MD              Difference of Test and Control Means
Note/*     MDF, MDC           95% confidence limits of the Difference
Note/*     N_5_PCT          Number required to reach 5% significance
Note /*                     given beta = 100%
Note/* FILES:
Note/*     STATS.dbf          Contains the statistics
Note/*                     NB Must have above the fields
Note/*     T95TAB.dbf         Table of Significance limits of the t
Note/*                  distribution from Documenta Geigy, Ciba-Geigy
Note/*                  Scientific Tables 7th edition pp 32-35
Note/*
Note/*                  ***** NB t for alpha = 0.025 *****
Note/*
Note/*     N.ndx            Index for above table
Note/*
clear
set echo off
set talk off
set default to c
za=1.96                  &&   5% alpha for N 5%
zb=0                     &&   use 1.645 for 10% beta for N 5%
select 1
use t95tab index n
select 2
use stats
go top
r1=0
do while .not. eof()
 clear
 r1=r1+1
 @1,1 say 'record '
```

```
? r1
@3,1 say variable
na=n_all
if na<1
  na=1
  endif
if na>200
  na=200
  endif
nc=n_ctl
if nc<1
  nc=1
  endif
if nc>200
  nc=200
  endif
nt=n_tst
if nt<1
  nt=1
  endif
if nt>200
  nt=200
  endif
nd=n_all-2
if nd<1
  nd=1
  endif
if nd>200
  nd=200
  endif
select 1
seek na
ta=t95
seek nc
tc=t95
seek nt
tt=t95
seek nd
td=t95
select 2
replace maf with (mean_all - ta*sd_all/sqrt(n_all))
replace mac with (mean_all + ta*sd_all/sqrt(n_all))
replace mcf with (mean_ctl - tc*sd_ctl/sqrt(n_ctl))
replace mcc with (mean_ctl + tc*sd_ctl/sqrt(n_ctl))
replace mtf with (mean_tst - tt*sd_tst/sqrt(n_tst))
replace mtc with (mean_tst + tt*sd_tst/sqrt(n_tst))
replace md  with (mean_tst - mean_ctl)
sd_pooled=sqrt(((n_tst-1)*sd_tst**2 + (n_ctl-1)*sd_ctl**2)/(n_tst+n_ctl-2))
se_diff=sd_pooled*sqrt(1/n_tst +1/n_ctl)
replace mdf with (md-td*se_diff)
replace mdc with (md+td*se_diff)
if mean_ctl < > mean_tst
  replace N_5_PCT with
min(999999,2*(sd_pooled*(za+zb)/(mean_ctl-mean_tst))^2)
else
  replace N_5_PCT with 999999
endif
```

```
 skip
enddo
@ 15,15 say 'd o n e'
```

dBase III Program to Calculate Beta (type II error)

The following program was written in dBase III and runs on an IBM compatible PC.

```
Note/* This program calculates Beta given the numbers, means and
Note/* standard deviations of Control and Tests sets of data.
Note/* Beta is the type II error and is calculated for alpha = 0.025 for a one-
Note/* sided significance test from tables for the noncentral t distribution
Note/*
Note/*
Note/* REFERENCE: Statistical principles in experimental design
Note/*         Winer BJ, McGraw-Hill, 1971 Tokyo
Note/*         table C.13 page 884,  and pages 33-35.
Note/*
Note/*

Note/* VARIABLES:
Note/*     Variable          Name (eg Lotus Lable) of the variable
Note/*     N_CTL, N_TST      Numbers in Control and Test Groups
Note/*     Mean_CTL, Mean_TST  Means in  Control and Test Groups
Note/*     Mean_ALL, N_ALL   Mean and Number for both groups combined
Note/*     SD_CTL, SD_TST    Standard Deviations in Control and Test Groups
Note/*     Beta              Type II error for Alpha = 5%, observed diff.
Note/*     Power             1. - beta
Note/*     Beta10pc          Type II error for Alpha = 5%, diff = 10% mean
Note/*     Power10pc         1. - beta10pc
Note/*     Power25pc         Power for 25% difference
Note/*     Power50%          Power for 50% difference
Note/*
Note/*
Note/* FILES:
Note/*     STATS.dbf         Contains the statistics
Note/*                  NB Must have above the fields
Note/*     NONCENTR.dbf      Contains the areas for calculating Beta from
Note/*                       the noncentral t distribution.
Note/*     F.ndx             Index for NONCENTR
Note/*
Note/*     BETAPROC.PRG      Procedure file for interpolating Beta
Note/*                       from the tables
Note/*
Note/*
Note/*
Note/*
CLEAR
set default to c
set console on
set talk off
set echo off
SET PROCEDURE TO BETAPROC
bell = chr(7)
b=0
select 1
use noncentr index f
select 2
use stats
```

```
go top
clear
r1=0
DO WHILE .not. eof()
  clear
  r1=r1+1
  @ 1,1 say 'record # '
  ?? r1
  ? variable
  @ 4,1 say mean_all
  IF MEAN_ctl < mean_TST
    V1=MEAN_CTL
    V2=MEAN_TST
    S1=SD_CTL
    S2=SD_TST
    N1=N_CTL
    N2=N_TST
    ELSE
      V1=MEAN_TST
      V2=MEAN_CTL
      S1=SD_TST
      S2=SD_CTL
      N1=N_TST
      N2=N_CTL
    ENDIF
  FF = N1 + N2 - 2
  VAR = ((N1-1)*S1*S1 + (N2-1)*S2*S2)/FF
  D = (V2-V1)/SQRT(VAR/N1 + VAR/N2)
  DO TYPEII WITH FF,D,B
  SELECT 2
  replace beta with b
  REPLACE POWER WITH 1. - B
NOTE/* CALCULATE BETA & POWER FOR 10% DIFFERENCE
  D = abs((0.1*mean_all)/SQRT(VAR/N1 + VAR/N2))
  DO TYPEII WITH FF,D,B
  SELECT 2
  replace beta10pc with b
  REPLACE POWER10pc WITH 1. - B
Note/* calculate power for a 25% difference
  D = abs((0.25*mean_all)/sqrt(var/n1 + var/n2))
  do typeii with ff,d,b
  select 2
  replace power25pc with 1. - b
Note/* calculate power for a 50% difference
  d = abs((0.5*mean_all)/sqrt(var/n1 + var/n2))
  do typeii with ff, d, b
  select 2
  replace power50pc with 1. - b
  SKIP
  ENDDO
set console on
LIST OFF VARIABLE,BETA
CLEAR
close all
@ 1,1 SAY 'DONE'
RETURN
```

```
NOTE/*  THIS PROCEDURE FILE 'BETAPROC.PRG' CONTAINS ROUTINES TO CALCULATE
NOTE/*  THE TYPE II OR BETA ERROR
NOTE/*  IT IS CALLED FROM THE PROGRAM 'BETA.PRG'
NOTE/*  THE FILES AND VARIABLES USED ARE DOCUMENTED IN THAT FILE
NOTE/*
NOTE/*
NOTE/*
PROCEDURE TYPEII
PARAMETERS FF,D,B
b=0
IF FF>30 .AND. FF<40
  FF=30 + INT(FF/34.5)*10
  ENDIF
IF FF>40 .AND. FF<60
  FF=INT(40 + INT(FF/49.5)*20 + 0.01)
  ENDIF
IF FF>100
  FF=INT(100 + INT(FF/149)*100 +.01)
  FF=MIN(200,FF)
  ENDIF
SELECT 1
SEEK FF
DO FINDBETA WITH D,B
B=MAX(0, MIN(0.9999, B))
RETURN
NOTE/*
NOTE/*
NOTE/*
PROCEDURE FINDBETA
PARAMETERS D,B
IF D >= BETA01
  B = 0.01 + (0.01 - 0.00)*(BETA01 - D)/(BETA00 - BETA01)
  RETURN
  ENDIF
*
IF D >= BETA05
  B = 0.05 + (0.05 - 0.01)*(BETA05 - D)/(BETA01 - BETA05)
  RETURN
  ENDIF
*
IF D >= BETA10
  B = 0.10 + (0.10 - 0.05)*(BETA10 - D)/(BETA05 - BETA10)
  RETURN
  ENDIF
*
IF D >= BETA20
  B = 0.20 + (0.20 - 0.10)*(BETA20 - D)/(BETA10 - BETA20)
  RETURN
  ENDIF
*
IF D >= BETA30
  B = 0.30 + (0.30 - 0.20)*(BETA30 - D)/(BETA20 - BETA30)
  RETURN
  ENDIF
*
IF D >= BETA40
  B = 0.40 + (0.40 - 0.30)*(BETA40 - D)/(BETA30 - BETA40)
```

```
  RETURN
  ENDIF
*
IF D > = BETA50
  B = 0.50 + (0.50 - 0.40)*(BETA50 - D)/(BETA40 - BETA50)
  RETURN
  ENDIF
*
IF D > = BETA60
  B = 0.60 + (0.60 - 0.50)*(BETA60 - D)/(BETA50 - BETA60)
  RETURN
  ENDIF
*
IF D > = BETA70
  B = 0.70 + (0.70 - 0.60)*(BETA70 - D)/(BETA60 - BETA70)
  RETURN
  ENDIF
*
IF D > = BETA80
  B = 0.80 + (0.80 - 0.70)*(BETA80 - D)/(BETA70 - BETA80)
  RETURN
  ENDIF
*
IF D > = BETA90
  B = 0.90 + (0.90 - 0.80)*(BETA90 - D)/(BETA80 - BETA90)
  RETURN
  ENDIF
*
IF D > = BETA999
  B = 1 + (1 - 0.90)*(BETA999 - D)/(BETA999 - BETA10)
  RETURN
  ENDIF
*
If D < beta999
  b=.9999
? bell
? 'DELTA = ', D, ', but should be > 0!!!!!!!!!!!!'
? bell
? bell
? bell
? bell
return
```

APPENDIX 6 - Forms for consent and data collection

Consent to participate

UNIVERSITEIT VAN KAAPSTAD

DEPARTEMENT VAN PEDIATRIE EN KINDERGESONDHEID

DATUM: _____

NAAM: _____

MOEDER VAN: _____

Ek gee my toestemming dat my kind deur middel van bloed en veltoetse ondersoek word. Ek besef dat my kind nie noodwendig direk baat sal vind by hierdie toetse nie, maar dat sulke ondersoeke mag bydra tot ons kennis van ander kinders se siektes.

Die aard en gevare van die toetse is deur die betrokke dokter aan my verduidelik.

GETEKEN: _____

GETUIES: 1. _____ (Dokter)

2. _____

UNIVERSITY OF CAPE TOWN

DEPARTMENT OF PAEDIATRICS AND CHILD HEALTH

DATE: _____

NAME: _____

MOTHER OF: _____

I hereby consent to my child being investigated by means of blood and skin tests.

I realise these tests will not necessarily benefit my child directly, but that they may lead to a better understanding of other children's illnesses.

The nature and risks of the test have been explained to me by the doctor concerned.

SIGNED: _____

WITNESSES: 1. _____ (Doctor)

2. _____

Iron and immune function

IRON AND IMMUNITY - DATA PROFORMA - FIRST EXAMINATION

NUMBER: _____

DATE: _____

GROUP: (Control = 1, Test = 2) _____

NAME OF MOTHER: _____

NAME OF CHILD: _____

DATE OF BIRTH: _____

AGE: _____

SEX: Male = 1 Female = 2

ADDRESS: _____

_____

_____

ANTE NATAL: Normal = 1 Abnormal = 2

BIRTH: NVD = 1 Assisted = 2 C/S = 3

BIRTHWEIGHT:

POST NATAL: Well = 1 Neonatal Jaundice (Not treated) = 2

Other, specify = 3 _____

ANY HOSPITAL TREATMENT SINCE BIRTH? YES = 1 NO = 2

CURRENT FEEDING: Breast = 1 Breast & bottle = 2

Bottle = 3

Brand of milk if on formula _____

(Klim, Nespray = 1; SMA, S26 = 2; Lactogen, Nan = 3; Pelargon = 4;

Infasoy, Isomil = 5; Skim = 6; Other = 7)

SOCIAL DATA:

MARITAL:

CHILDREN:

FINANCIAL/EMPLOYMENT:

HOUSING:

LEGAL:

ALCOHOL:

HEALTH:

OTHER:

(TO BE COMPLETED DURING STUDY)

Age first given bottle (months)

Age last given breast

Reason for weaning: Working = 1 Other Social = 2

Not enough milk = 3 Breast Disease = 4

Other, specify = 5 Refused Breast = 6

Age at introduction of cereals _____

Age at introduction of eggs _____

Age at introduction of vegetables _____

Age at introduction of fruit _____

Age at introduction of meat and fish _____

SOCIAL DATA

Marital Status : Married = 1 Living together = 2

Widowed = 3 Separated = 4

Single = 5 Divorced = 6

Education of Mother: Standard passed _____

Post-School qualifications: Degree = 1 Diploma = 2 Other = 3

(Mother) Nil = 4

Education of Father: Standard passed _____

Post-School qualifications: Degree = 1 Diploma = 2 Other = 3

(Father) Nil = 4

Occupation of of Mother _____

Occupation of Breadwinner: _____

Source of income: Mother = 1 Father = 2 Extended family = 3

State Grant = 4 Other = 5 Combination = 6

Is financial support regular? Yes = 1 No = 2 N/A = 3

Family living: Alone = 1 Extended Family = 2

Friends = 3 With others = 4

Baby lives with: Both parents = 1 One parent = 2

Neither parent = 3

Number of Adults: _____

Number of Siblings: _____

Number of Children less than 10 years: _____

Number of rooms used for sleeping: _____

Social Stability Score

Weight: _____ kg Height: _____ cm

Head Circumference: _____ cm

EVIDENCE OF INFECTION:

Fever		Skin	_____
Skin	rash, impetigo, ...	U.R.T.I.	_____
Ears:	otitis externa, otitis media	L.R.T.I.	_____
Nose:	coryza, purulent	G.I.Inf.	_____
Throat:	tonsillitis, pharyngitis	Systemic:	_____
Mouth:	gum sepsis, herpes, candida		
Resp:	croup, lower airway infection		
Abdo:	diarrhoea, worms		
Other:	specify		

Clinical impression: Well = 0 Viral = 1 Bacterial = 2 Other = 3

Swabs sent: specify site(s) _____

bacterial pathogens _____

viral pathogens _____

Viral Survey:

Stool Virus Isolates

Maternal Antibody Titers:

Tetanus

Polio

Study Number:

Special Investigations:

	IgG
	IgM
	IgA
Mantoux	Sm Ig
Candida	OKT3
WBC	OKT4
RBC	OKT8
Hb	
HCT	
MCV	PHA Stim
MCH	
MCHC	Bactericidal Index
PLT	
% retics	Antitetanus-toxoid
% neutrophils	Anti-polio
% lymphs	
% monocytes	
% eosinophils	
% basophils	
Ferritin	
FEP	

INTERMEDIATE FOLLOW-UP

V	S No	Date	Feeds	Sx	Hx No Infect R	C	S	O	O	H	Etc	Rx	Mx Weight	Length	Head
03															
04															
05															
06															
07															
08															
09															
10															
11															
12															
13															
14															
15															
16															
17															
18															
19															
20															
21															
22															
23															
24															
25															

IRON AND IMMUNITY - DATA PROFORMA - FINAL EXAMINATION

Study Number:

Date:

(Fill in intermediate follow-up sheet)

Viral Survey:

Isolates throat

Isolates stool

Special Investigations:

	IgG
	IgM
	IgA
Mantoux	Sm Ig
Candida	OKT3
WBC	OKT4
RBC	OKT8
Hb	
HCT	
MCV	PHA Stim
MCH	
MCHC	Bactericidal Index
PLT	
% retics	Antitetanus-toxoid
% neutrophils	Anti-polio
% lymphs	
% monocytes	Autologous rosettes
% eosinophils	RBC C3b-receptors
% basophils	
Ferritin	

INTERMEDIATE FOLLOW-UP

No Infects on Ex						SWAB	Dx					GRADING			
C	U	L	G	S	TYPE	SITE	Vi	Ba	Fu	Pr	He	Hx	Ex	RxO	RxH

APPENDIX A

DATA COLLECTION PROFORMA

DATE: _____

AGE (MONTHS)	SEX	WEIGHT	FEEDING	AGE OFF BREAST AND REASON MONTHS	AGE AT INTRODUCTION OF SOLIDS (MONTHS)			
					CEREAL	EGG	VEG & FRUIT	FISH & MEAT

CODES: Sex: Male = 1 Female = 2

Feeding: Breast = 1; Breast & Bottle = 2; Bottle = 3; No milk = 4;
(Cow's milk = 3.1; SMA, S26 = 3.2; Lactogen, Nespray = 3.3; Pelargon = 3.4 Infasoy, Isomil = 3.5;
Skim milk = 3.6; Other = 3.7)

Reasons for stopping breast feeding: Working = 1; Other social reasons = 2;
"Too little milk" = 3; Breast disease = 4; Other = 5

INFANT FEEDING SURVEY

DATE: / /1984

Number	Age (months)	Sex	Weight	Feeding Formula	Feeding Measures/ 25ml	Age off Breast First bottle	Age off Breast Last breast	Reason for weaning
1								
2								
3								
4								
5								
6								
7								
8								
9								
10								
11								
12								
13								
14								
15								
16								

Infant Feeding Survey

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