**An investigation of the relationship between plasma, erythrocyte and tissue trace element concentrations**

Dr Jennifer Logue

$BMedSci_{(Hons)}$ MBChB MRCP

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Department of Surgery

College of Medical, Veterinary and Life Sciences

University of Glasgow

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**Abstract**

**Introduction** Trace element status may be important in acutely-inflamed patients. Plasma concentrations of trace elements are known to alter during the evolution of the acute phase response, however, erythrocyte trace element concentrations do not. It is not known whether either erythrocyte or plasma concentrations reflect the status of the tissues where trace elements are utilised. Therefore trace element concentrations were examined in tissues and blood from non-inflamed patients, with plasma and erythrocyte concentration changes studied during the evolution of the acute phase response.

**Methods** 31 patients undergoing liver resection had liver, rectus muscle, and blood samples obtained pre-operatively, and blood sampling for 3 days post-operatively. Se, Cu and Zn concentrations were obtained by inductively coupled mass spectrometry after nitric acid digestion. Erythrocyte glutathione peroxidase (GPx) was measured by spectrophotometry. C-reactive protein and albumin concentration were measured on each day.

**Results** C-reactive protein increased and albumin concentration decreased over the 3 days postoperatively. Plasma Zn and Se concentration changed in the 3 days post-operatively ($p<0.001$); erythrocyte Cu, Zn and Se concentration, GPx activity and plasma Cu concentration did not change. Preoperatively, liver Cu concentration was associated with erythrocyte Cu concentration ($r^2$ 15.9%; $p=0.036$) but not plasma Cu concentration ($r^2$ 4.3%; $p=0.264$); plasma Zn concentration was associated with liver Zn concentration ($r^2$ 14.4%; $p=0.046$) but erythrocyte Zn concentration was not ($r^2$ 0.1%; $p=0.896$); and liver Se concentration was associated with erythrocyte Se concentration ($r^2$ 17.1%; $p=0.023$), erythrocyte glutathione peroxidase ($r^2$ 22.6%; 0.008) and plasma Se concentration ($r^2$ 43.1%; $p<0.001$).

**Conclusions** Erythrocyte Cu and Se concentration, and GPx activity are associated with liver Cu and Se concentration respectively, and do not change during the evolution of the acute phase response. They should be considered as potential markers of Cu and Se status. Plasma Zn is associated with liver Zn concentration but the concentration changes during the acute phase response; caution should be taken interpreting results in patients with inflammation, and further work is required to find a suitable alternative marker of Zn status.

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**Declaration**

I declare that the work in this thesis is original and has not been presented by anyone else for a higher degree.

The original idea for the work in this thesis was conceived by Dr Denis O'Reilly.

I designed the study process and study paperwork and liaised with the Department of Surgery to coordinate the process. I applied for the ethical and management approvals.

Mr Campbell Roxburgh, Clinical Fellow, recruited the patients, gained consent and ensured the samples were taken and delivered to the laboratory.

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I performed the statistical analysis and wrote all that is in this thesis.

# Chapter 1

# Introduction

## 1.1 Background

The Scottish Trace Element and Micronutrient Laboratory is based at Glasgow Royal Infirmary. It is funded by the National Health Service (NHS) and provides a supra-regional assay service for trace elements and micronutrients for all of the NHS in Scotland, as well as receiving samples from the rest of the UK, overseas and industry. During the year 2009-2010, the laboratory received 5073 requests for plasma copper measurement, 3459 requests for plasma zinc measurement and 1726 requests for plasma selenium measurement from hospitals within Scotland alone.

It is now well established that plasma concentrations of trace element micronutrients can be affected by the acute phase response seen during acute and chronic disease[1]; however these are exactly the types of patients that clinicians wish to know the micronutrient status of. The Scottish Trace Element and Micronutrient laboratory only has access to information about the inflammatory state of patients in a small percentage of the samples which have been sent from Glasgow Hospitals, and which the laboratory has access to other blood results from. The remainder of the samples will be analysed with no knowledge of whether the result will have been potentially invalidated by an inflammatory response within the patient.

The purpose of this thesis is to find a potential solution to this problem which will be applicable to an NHS laboratory with a high through-put of samples. Previous work carried out in the Scottish Trace Element and Micronutrient Laboratory found that erythrocyte trace element micronutrients did not alter with the acute phase response[2], making them a potentially suitable marker for trace element micronutrient status in patients with inflammation. Therefore, this work concentrates on plasma and erythrocyte trace element micronutrient concentrations: developing methods for their measurement that are robust

enough for use in a routine laboratory, establishing whether they are an accurate reflection of whole body trace element status, while also examining the changes seen during the acute phase response to elucidate the potential predictors of the magnitude of the concentration changes seen.

## 1.2 Trace Elements

A trace element is an inorganic micronutrient which is present in humans at very low concentrations: μg/L in body fluids and μg/g in tissue. Trace elements include iron, iodine, cobalt, selenium, copper, zinc, manganese, chromium, molybdenum, vanadium, bromine, fluorine, cadmium, lead, strontium, lithium and tin[3].

An essential trace element is one is in which dietary deficiency can lead to physical effects which improve upon dietary supplementation. An example of this is zinc deficiency resulting in skin lesions which improve significantly with oral zinc supplementation. In most cases a biochemical basis for the elements essential function is known, such as an enzyme containing the trace element (a metallo-enzyme) in which the element is essential for the enzyme's function or activation. The essential trace elements include iron, zinc, copper, cobalt, iodine, molybdenum and selenium. Essential functions have been found for Manganese but a physiologically detrimental deficiency state has never been identified in humans[4].

## 1.3 The acute phase response

Both acute and chronic inflammation result in systemic changes that are known as the acute phase response[5]. The acute phase response involves changes in the concentrations of many plasma proteins which are known as acute phase proteins; an acute phase protein is defined as one whose plasma concentration increases (positive acute phase reactant) or decreases (negative acute phase reactant) by at least 25 percent during inflammatory disorders[6]. Changes are most likely due to increased or decreased hepatic synthesis. A substantial acute phase response with large changes in acute phase proteins is seen in infection, post-trauma, in autoimmune inflammatory diseases (e.g. rheumatoid arthritis) and advanced cancer.

C-reactive protein is the archetypal positive acute phase reactant. A protein with a physiological role to bind to phosphocholine expressed on the surface of bacteria and dead or dying cells in order to activate the complement system, it can increase by up to a 1000 percent in response in inflammation. Other positive acute phase reactants include haptoglobin, fibrinogen, plasminogen, ferritin and serum amyloid A. Negative acute phase reactants include albumin, transferrin and thyroxine binding globulin[7]. Albumin is the most abundant protein in blood and maintains the colloid osmotic pressure along with acting as the major transport protein for many molecules, including drugs and micronutrients. An advantageous reason for decreasing concentrations of negative acute phase reactants has yet to be found; it may simply be that available amino acids are diverted to the production of positive acute phase proteins which have an immunological role to fulfil during inflammation[7].

### 1.4 The importance of trace element status during the acute phase response

Patient who are unwell are at higher risk of trace element deficiencies than the general population. This is due to a reduced nutritional intake, increased losses and possible increased requirements[8].

Reduced appetite is common in patients who are unwell with an inflammatory response. Abdominal surgery and mechanical ventilation both cause patients to be physically unable to eat for several days. Patients who are chronically malnourished, for example those in nursing homes and chronic alcoholics are also more susceptible to acute illness and make up a disproportionate amount of the hospital population. If patients receive artificial nutrition, such as enteral or parenteral nutrition, monitoring is required to ensure trace elements and other micronutrients are adequate replaced[9].

Increased loss of trace elements is especially common in surgical and burns patients. Losses include those from diarrhoea or gastric fluid and loss from burns exudates. Treatments such as dialysis can also lead to micronutrient depletion[10].

Moreover, while having a higher risk for nutritional deficiency, unwell inflamed patients may also have higher requirements for trace element micronutrients. Requirements for trace elements may increase during the catabolism associated with inflammation as they have a role as coenzymes for catabolic pathways[11]. There is also the role of trace elements, such as selenium[12], in anti-oxidant pathways which will be required to manage the increased oxidative stress found with inflammation. Once the illness has resolved the patient will need increased micronutrients both to compensate for increased consumption during illness and also when regaining weight[13].

### 1.4.1 Critical illness

Trace elements are of interest in critical illness because they are thought to have a role in the reduction of oxidative damage from free radical production. The major complication of critical illness is systemic inflammatory response syndrome (SIRS) which includes the production of free radicals, cytokines and other mediators; associated conditions include sepsis, pancreatitis, major trauma and burns[14]. Critical illness and SIRS is associated with a redistribution of selenium and zinc from the circulation to tissues and organs[1]. It has been hypothesised that the redistribution of trace elements during the acute phase response may be detrimental if prolonged due to depletion of antioxidants from the circulation[14].

However, if trace element micronutrients and their relative deficiency were an important factor in morbidity and mortality from critical illness, then supplementation during critical illness would seem appropriate. Selenium has been of particular interest and there have been many small studies of selenium supplementation in critically ill patients. A Cochrane systematic review of selenium supplementation in the critically ill was inconclusive[15]. It examined five trials of selenium selenite supplementation in critically ill patients; there was no reduction in mortality (RR 0.71, 95% CI 0.43 to 1.17). However, when general intensive care patients were examined alone there was a suggestion of benefit (RR 0.75, 95% CI 0.59 to 0.96). However the main problems were that these were small studies with a short follow up period and statistical heterogeneity was high.

A recent Scottish Trial (SIGNET) attempted to overcome some of the methodological problems of previous studies by enrolling 500 patients in a 2 x 2 factorial randomised controlled trial. This compared 4 groups of differing supplementation of parenteral nutrition: selenium, glutamine, selenium and glutamine and no supplementation, with a primary outcome of episodes of infection in the first 14 days after starting treatment[16]. This showed no

difference in rate of infections in any group; subgroup analysis did show a significant reduction in infections in those receiving selenium for greater than 5 days duration[17].

Studies of zinc supplementation in critical illness have had similar problems of small sample sizes and poorly defined outcomes making meta-analysis difficult. Heyland and colleagues performed a systematic review of 4 randomised controlled trials of zinc supplementation in critical illness which showed no effect on mortality (RR 0.63, 95%CI 0.25 to 1.69)[18].

While trials to date have been inconclusive for the need for trace element supplementation in critical illness this does not necessarily mean that trace element status during critical illness is not important. Further work is required to examine the links between trace element status and outcome in critical care. As plasma trace elements are redistributed during the acute phase response an accurate measure of trace element status during the acute phase response is required. Having an assessment of trace element status during critical illness may also be helpful for guiding the need for supplementation; perhaps if robust supplementation trials were carried out on patients known to be deficient in trace elements positive results on morbidity and mortality would be more forthcoming.

### 1.4.2 Chronic inflammatory conditions

If trace element status is important in critical illness due to increased free radicals and a relative decrease in circulating trace elements, them the same could be said for chronic inflammatory conditions such as inflammatory bowel disease, rheumatoid arthritis and chronic infections. These conditions are far more complex with the potential for a remitting course over many years, as opposed to an acute critical illness in a previously well individual. How this affects trace element status and whether supplementation would be beneficial is unknown.

Chronic inflammatory conditions have additional potential risk factors for trace element deficiencies. While perhaps less likely to cause immediate problems in a previously well individual, decreased appetite, cachexia and malabsorption (especially in gastrointestinal inflammation) over a long period of time makes the patient with chronic inflammation at greater risk of trace element deficiency.

As for critical illness, a robust measure of trace element status during inflammation would help establish the incidence of trace element deficiencies, risk factors and how quickly deficiency develops and if supplementation has benefit on long term outcomes.

## 1.5 Copper

### *1.5.1 Copper and health*

Copper is an essential trace element because it is crucial for the activity of many enzymes including those involved in iron metabolism, antioxidant defenses, neuropeptide synthesis and immune function[19]. These include:

- Ceruloplasmin – a ferroxidase necessary for the conversion of iron II to iron III in the plasma
- cytochrome c oxidase – the terminal step in the electron transport chain
- lysyl oxidase – essential for crosslinking between the polypeptide chains in collagen and elastase
- tyrosinase – involved in the synthesis of melanin
- dopamine hydroxylase – converts dopamine to noradrenaline
- superoxide dismutase – destroys free radicals[20]

### *1.5.2 Copper homeostasis*

Copper is absorbed in the duodenum. Copper is transported from the intestine to the liver, via the portal vein. Copper is stored in the liver and then redistributed to other organs. Copper status is regulated via the intestine by duodenal absorption and biliary excretion. High copper exposure will result in down-regulation of duodenal absorption and increased biliary excretion[21].

In plasma copper is mainly (90%) bound to the protein ceruloplasmin, with one molecule of ceruloplasmin containing six copper ions[20]. Hepatic synthesis of ceruloplasmin is dependent on adequate copper intake. As the majority of copper is bound to ceruloplasmin, plasma copper is in turn dependent on ceruloplasmin synthesis[22].

Copper concentration is highest in the brain, heart, liver and kidneys. Liver contains 10% of total body copper and muscle 30% [23].

*1.5.3 Effects of copper deficiency*

Copper deficiency is rare and is usually a result of severe malnutrition and/or malabsorption. Another possible cause is a high zinc intake which prevents the absorption of copper. The clinical features of copper deficiency include microcytic anaemia, neutropenia and osteoporosis[20].

Copper deficiency in the population has been related to cardiovascular disease. The original link was made as cattle with very low copper intake were dying from sudden cardiac death. Later studies examining the hearts of people who had died from ischaemic heart disease found a low concentration of copper in their cardiac tissue at autopsy[24]. Total cholesterol does increase in men fed a copper-deficient diet[25] and it is also linked to impaired glucose tolerance[26]. Conversely, there are several epidemiological studies linking higher copper status with an increased incidence of risk factors for ischaemic heart disease[27;28].

## 1.6 Zinc

### *1.6.1 Zinc and health*

Zinc is an essential trace metal micronutrient as it is a constituent for more than 150 enzymes, functioning at the active site, as a structural component or both. It is the most common catalytic metal ion in the cytoplasm of cells. Zinc metalloenzymes include carbonic anhydrase, alkaline phosphatase, carboxypeptidase, transferases, DNA/RNA polymerase, reverse transcriptase and superoxide dismutase[20]. Genes have recently been identified which are potentially regulated by zinc, some positively, some negatively. These include genes that regulate the redox state, signal transduction, growth factor activity and fatty acid synthesis and degradation. Zinc is necessary for insulin synthesis and action and also helps the stability of proinsulin and insulin by the formation of complexes[29].

### *1.6.2 Zinc homeostasis*

Zinc is absorbed in the proximal small intestine. It is then bound to (metallo)thionein, a protein which acts as a control over the supply of zinc to the blood stream by having an increased synthesis when zinc intake is high and decreased synthesis when zinc intake is low. Metallothionein binds copper with a higher affinity than zinc which is why a high zinc intake can lead to copper deficiency[20]. Zinc is then transferred to the liver and released into the blood stream where is bound mainly to albumin (90 percent) and $a_2$-macroglobulin (10 percent)[30].

### *1.6.3 Effects of zinc deficiency*

Symptomatic zinc deficiency is rare in developed countries and mainly seen in patients with malabsorption due to surgical resection of the small intestine without adequate parenteral supplementation[20]. However, it is a large problem in some developing countries where a combination of helmintic infestations, high phytate content in unleavened breads and high prevalence of diarrhoeal disease leads to a far greater number of severe deficiency states[29].

Features of zinc deficiency include poor wound healing, immune deficiency, decreased taste and a variety of dermatitis[20]. In severe cases, particularly chronic zinc deficiency, there can be stunted growth in children, hypopigmented hair, hypogondadism and delayed sexual maturation. Night blindness can occur due to secondary vitamin A deficiency as zinc is a coenzyme for the conversion of retinol to retinaldehyde by retinol dehydrogenase[29].

## 1.7 Selenium

### *1.7.1 Selenium and health*

Selenium, as selenocysteine, is a component of many important selenoproteins which function as enzymes. The three most studied classes of selenoproteins are the glutathione peroxidases, the thioredoxin reductases and the deiodinases; in all of which selenium functions as a redox centre.

Glutathione peroxidases are antioxidant enzymes. They reduce hydrogen peroxide, lipid and phospholipid hyproperoxides to water and alcohols[12].

Thioredoxin reductases regulate transcription factors through redox control, regulate apoptosis, acts as a cytokine, a chemokine and stimulate eosinophils[31].

Deiodinases are required for the formation of tri-iodothyronine (T3) as they catalyse the deiodination of T4, thus generating T3, reverse T2 and T2[32].

As such, selenium is felt to be an essential component of the immune system and antioxidant defence.

### *1.7.2 Selenium homeostasis*

The two most common forms of selenium that are absorbed from the diet are selenomethionine from plants and selenocysteine from meat. Absorption takes place primarily in the duodenum with close to 100% of selenomethionine absorbed. Absorption appears to be independent of selenium status[33].

The primary tissues for uptake of selenium are heart, kidney, lung, liver, pancreas and muscle. Except for the lens of the eyes, the liver has the highest concentration of selenium in the body[34].

Selenium homeostasis is regulated by urinary (50-67%) and faecal (40-50%) excretion, with urinary excretion being the main system under physiological control[33].

### *1.7.3 Effects of Selenium deficiency*

The archetypical selenium deficiency state in humans is Keshan disease, a cardiomyopathy, possibly with coxsackie virus as a cofactor in its development. It has been seen in areas of rural China with very low levels of soil selenium with selenium supplementation virtually eliminating the disease[12]. Selenium

deficiency is a costly problem in farm animals grazing in selenium poor fields, known as white muscle disease as skeletal and cardiac muscle gets abnormal calcium deposition[32].

However, given its role in the immune system and antioxidant defences, selenium deficiency is hypothesised to be causally linked in a number of other conditions. Selenium appears to be important in HIV, with in vitro studies showing it can inhibit viral replication and selenium status being a strong predictor of outcome[35]. A recent randomised selenium supplementation study in patients with HIV helped support this theory by showing a decreased progression of viral burden and improvement in CD4 count with selenium supplementation[36].

The effect of selenium deficiency on other conditions has a far poorer evidence base, with associations seen in epidemiological analysis, not being backed up by consistent positive outcomes from supplementation studies; these conditions include cardiovascular disease, male infertility, and cancer. It is however possible that the reason that these trials have been inconclusive so far is that they have primarily been carried out in selenium replete areas of the world and positive results would be more likely in area with a selenium deplete population[12].

## 1.8 Biomarkers of trace element status

There are many potential blood components and tissues which can act as biomarkers for trace element status including plasma, erythrocytes, hair and measuring the activity of metal containing enzymes.

### *1.8.1 Plasma*

Plasma concentration has been used in both clinical practice and in research as a biomarker for trace element status. It is easy to sample blood and is a sample type that patients are comfortable and familiar with. It requires little sample processing with centrifugation to separate the plasma, which is a familiar process in routine clinical biochemistry laboratories.

There are however few studies showing that plasma is a reliable biomarker for any trace metal micronutrient[22]. For many trace elements, concentrations in plasma are low and dependent on protein binding. However, as homeostatic mechanisms may exist which maintain plasma levels within set concentrations except in severe deficiency, plasma trace elements may be more useful in identifying severe deficiency states. Plasma levels are generally presented in either simple mass or molar units.

### *1.8.2 Erythrocytes*

Erythrocyte trace element concentrations are of interest as potential biomarkers of trace element status because, as circulating cells, they may reflect other cellular levels and act as a surrogate for tissue concentrations. As erythrocytes have a lifespan of 120 days they may be a marker of longer term status, however this may also have the disadvantage of changes in status not being seen for

several weeks. They may also be affected by blood transfusion. As per plasma, erythrocytes are easy to obtain, however they do require greater processing by the laboratory than many routine clinical samples[23].

Erythrocytes are separated by centrifugation of the sample; however this should happen quickly after sampling so as to avoid ion exchange. The plasma along with the leucocytes and platelets (buffy coat) need to be removed so as to avoid contamination. After centrifugation, the cells will be in a gradient from young to old and samples will either require homogenization or standardisation of the height of sampling[23]. Accurate pipetting of red cells is very difficult given their viscosity. Some feel that washing of the red blood cells in trace element-free isotonic saline solution is required to avoid contamination from plasma. All these steps and potential sources of inaccuracy make the use of erythrocytes in clinical practice challenging.

Results from erythrocytes can be expressed in a number of ways with per litre of packed cells, per gram of haemoglobin, per number of cells and per gram of dry material most commonly used. However, expressing the results by a further measurement adds an additional analytical step and potential for yet more error. The haemoglobin concentration of red blood cells obtained via a standard haematology analyser and the iron concentration measured by ICP-MS have been found to correlate ($r=0.93$, $p<0.001$) over a range of whole blood haemoglobin concentrations including anaemia (5.6-18.2 g/dL)[2] providing an alternative way to express results. If iron can be measured simultaneously with trace elements on erythrocyte samples, some of the potential additional error would be reduced and analysis would be simplified.

### *1.8.3 Hair*

Body hair is a potential marker for long term trace element status. It is easy to obtain and can be sent over long distances using conventional post with no concerns about sample deterioration, but there is little evidence for its use in assessing nutritional status[22]. There are also many potential contaminants most notable shampoos and hair dyes which contain many trace elements. Using hair for monitoring for toxic trace elements is better established, with hair mercury felt to be a marker of chronic mercury exposure[37].

### *1.8.4 Other cell types*

Platelets, mononuclear and polynuclear cells can be used for trace element determination. These cells require larger sample sizes due to low concentrations of cells in whole blood, complicated separation techniques and fast processing times, making their use in routine practice limited. They also need to be expressed by either cell count or protein concentration adding a further analytical step (at low concentrations) with the potential for high error rates.

### *1.8.5 Functional indices*

As part of the essentiality of trace elements is their incorporation into metallo-enzyme, in which the trace element is essential for the enzyme's function or activation, the measurement of metallo-enzyme activity may be an accurate measure of clinically significant deficiency. Examples of functional indices include superoxide dismutase, glutathione peroxidase and alkaline phosphatase. However, many of these enzymes are so vital that during deficiency the body will channel all of the available trace element to their production; resultantly the enzyme activity may not decrease until there is a very severe deficiency.

### 1.9 Study designs to assess biomarkers

A useful biomarker of trace element status is one which reflects tissue status, decreases in deficiency states and increases with supplementation. There are many studies that look at changes in biomarker concentrations or activity in patients who are given trace element supplements; however these studies are complicated as the trace element needs to be supplemented in a bio-available form. Also, as volunteers for such studies will be healthy and so be unlikely to have any trace element deficiencies, the response measured may be blunted compared to that of the more clinically relevant deficient patient. Inducing deficiency is also very difficult, not least due to ethical regulations; subjects would have to be supplied with a specially made diet deficient in the element of interest for several weeks, and comply fully in order to detect any change in status.

Several studies have looked at the trace element concentrations in the organs of the deceased undergoing autopsy. This of course does not allow for comparison with blood concentrations.

## 1.10 Assessing copper status

### *1.10.1 Plasma copper*

Plasma copper is the most frequently used biomarker of copper status. While plasma copper does decrease in copper deficiency states, it plateaus when adequate intake is reached and no longer reflects intake. [22].

A meta-analysis of copper supplementation studies with plasma copper as the outcome measure, including 2 supplementation studies and 2 depletion studies (total of 35 participants), showed no response of plasma copper to supplementation; copper supplementation/depletion altered the mean plasma copper concentration by 1.02 μmol/L (95% CI -0.92, 2.96)[38]. However in a study of 17 children recovering from malnutrition and copper deficiency, copper supplementation resulted in a mean increase in plasma copper of 10.48 μmol/L (95% CI 2.35, 18.61)[39].

The evidence for plasma copper to reflect copper status marked by a response to copper supplementation or depletion is very limited; however, there is some evidence of its use in monitoring copper repletion in previously deplete patients.

Plasma copper can be affected by the sex of the patient and especially the use of hormone treatments and pregnancy. This is because ceruloplasmin concentrations increase in the presence of oestrogens with women resultantly having higher plasma copper than men, and hormone treatments and pregnancy resulting in large increases in plasma copper, often above the population reference interval[40].

*1.10.2 Ceruloplasmin*

A similar meta-analysis of copper supplementation studies with plasma ceruloplasmin as the outcome measure included 8 supplementation and 2 depletion studies (total of 100 participants)[38]. In contrast to plasma copper, ceruloplasmin responded to changes in copper intake with a mean increase of 0.035 g/L (95% CI -2.08, 3.77) (p=0.03).

When the data was split into those who were either copper replete or deplete at baseline, ceruloplasmin only increased significantly in those studies where the participants were copper deplete at baseline. In those who were copper replete the mean difference was -0.009 g/L (95% CI -0.03, 0.011) but in those deplete at baseline it was 0.258 g/L (95% CI 0.225, 0.29) ($p<0.001$)[38].

So, as with plasma copper, while the volume of evidence is low, ceruloplasmin appears to respond to copper supplementation when the patient is deplete at baseline.

*1.10.3 Erythrocyte Copper/Zinc Superoxide Dismutase*

Erythrocyte Copper/Zinc Superoxide Dismutase (SOD) is a copper containing enzyme which has been assessed as a potential biomarker of copper status. A meta-analysis of the results of 6 copper supplementation and 2 copper depletion studies (97 participants in total) showed no effect of the intervention on erythrocyte SOD activity; mean increase 51.05 U/g Hb (95% CI -54.96, 157.06)[38]. However it should be noted that the time scale of these studies ranges from 30-91 days which is within the lifespan of erythrocytes so may not have been sufficiently long enough to see a difference in an erythrocyte measurement. Also, the meta-analysis took no account for methodological validity of the assays used in the studies.

### *1.10.4 Erythrocyte Copper*

There are no studies evaluating the use of erythrocyte copper as a marker of copper status. However, compared to plasma, red blood cell copper does not increase with oral contraceptive use[41].

### *1.10.5 Liver copper*

As the liver is one of the main stores of copper, liver copper concentration is felt to be the only precise indicator of copper load[42]. A study of autopsy material from healthy young adults who were accident victims showed that the liver had the highest concentration of copper of all the available tissues[43].

The decrease in liver copper during copper deficiency was shown through autopsy results of children dying of malnutrition compared to children dying of other causes; controls had a higher mean liver copper concentration (22.4 μg/ g dry weight) versus children who had died from kwashiorkor (3.9 μg/ g dry weight) or marasmus (12.8 μg/ g dry weight)[44].

### *1.10.6 Effects of the acute phase response*

Ceruloplasmin is an acute phase reactant and therefore its concentration is increased during the acute phase response due to increased hepatic synthesis; as ceruloplasmin is the main copper carrying protein in plasma, plasma copper concentration also increases[45]. As such, changes in copper seen during the acute phase response are of a similar magnitude to changes seen in ceruloplasmin concentration[46].

The rise in ceruloplasmin concentration, and therefore the rise in plasma copper concentration, is late in the acute phase response, with the maximum rise seen after 7 days. In studies following patients with an acute phase response to surgery, major surgery resulted in copper concentrations that were 30% higher[46] while with minor surgery they were 12% higher[47].

A study which followed erythrocyte copper both immediately before and 3 days after elective knee arthroplasty showed no difference in erythrocyte copper concentration despite the evolution of the acute phase response[2].

## 1.11 Assessing zinc status

### *1.11.1 Plasma zinc*

Plasma zinc is the most widely used biomarker of zinc status despite being acknowledged to have poor sensitivity and specificity[22]. It is the only biochemical measure recommended by the World Health Organisation for the assessment of the zinc status of populations[48].

A meta-analysis of zinc supplementation studies with plasma zinc as the outcome measure, including 35 supplementation studies and 10 depletion studies (total of 1454 participants), showed a significant response of plasma zinc to dietary zinc intake; zinc supplementation/depletion altered the mean plasma zinc concentration by 2.9 μmol/L (95% CI 2.2, 3.5)[49].

The number of participants in this meta-analysis allowed subgroup analysis to assess the validity of plasma zinc as a biomarker of dietary zinc intake in a variety of age groups and baseline zinc statuses. The usefulness of zinc as a biomarker of zinc intake was unclear in children and adolescents, post-

menopausal women and those with high-normal status at baseline, however this may have been confounded by very small numbers of studies in these group[49].

### *1.11.2 Erythrocyte Zinc*

In a meta-analysis of studies of erythrocyte zinc as a marker of dietary intake, 5 supplementation and 2 depletion studies were included. Neither the meta-analysis nor any individual study suggested a significant change in red blood cell zinc in response to changes in dietary intake. Importantly, the majority of these studies were of >20 weeks duration so far greater than the lifespan of red blood cells, and changes would have be expected if erythrocyte zinc was an accurate biomarker of intake[49].

### *1.11.3 Mononuclear cell, polynuclear cell and platelet zinc*

Meta-analysis of 5 supplementation studies suggested that mononuclear cell zinc is not a useful biomarker with a mean difference in concentration of -0.05 μmol/$10^{10}$ cells (95% CI -0.21, 0.11)[49].

Pooling of results from 5 supplementation and 1 depletion study evaluating polynuclear cell zinc as a biomarker of zinc intake revealed no significant response to changes in zinc intake[49].

Platelet zinc was examined in 2 supplementation and 3 depletion studies; meta-analysis did not show a significant response to zinc supplementation or depletion with a mean difference of 0.09 nmol/$10^{9}$ cells (95% CI -1.12, 1.30)[49].

### *1.11.4 Hair Zinc*

Hair zinc was examined in 3 supplementation studies which included a total of 93 participants with either low or moderate zinc intakes. Hair zinc was found to increase significantly after supplementation with a mean difference of 13.24 ppm (95% CI 11.91, 14.56)[49].

### *1.11.5 Plasma alkaline phosphatase activity*

Alkaline phosphatase is a zinc dependent metalloenzyme[20]. 6 studies were pooled in a meta-analysis; 3 supplementation and 3 depletion studies. No significant change in activity was found with changes in zinc intake with a mean difference of 4.14 IU/L (95% CI -2.38, 10.66)[49].

### *1.11.6 Liver zinc*

5% of zinc is stored in the liver, 30% in bone and 60% in skeletal muscle with the majority of the remainder intra-cellular or bound to protein in plasma[23]. A study of autopsy material from healthy young adults who were accident victims showed that the liver had the highest concentration of zinc of all the available tissues[43].

Liver zinc has been shown to be lower in cases of malnutrition in children. Zinc deficiency was shown through autopsy results of children dying of malnutrition compared to children dying of other causes; controls had a higher mean liver zinc concentration (163.0 µg/ g dry weight) versus children who had died from kwashiorkor (31.4 µg/ g dry weight) or marasmus (102.5 µg/ g dry weight)[44].

### *1.11.7 Effects of acute phase response*

Plasma zinc is a negative acute phase reactant. After major surgery, serum zinc falls by 40-50% within 6 hours[46]. This fall is medicated by cytokines which promote the uptake of zinc by the liver where it is used for the synthesis of new proteins[50]. There is also a local role around the site of inflammation, possibly immunological or tissue regeneration[51]. The majority of the decrease in zinc can be related to the decrease in albumin, it main carrier protein, but there is a drop in zinc further to the decrease in albumin seen through a decrease in the zinc: albumin ratio[46].

Erythrocyte zinc was studied in patients undergoing elective knee arthroplasty. There was no difference in erythrocyte zinc concentration 3 days after surgery compared to values from immediately before surgery, despite the evolution of the acute phase response[2].

## 1.12 Assessing Selenium status

### *1.12.1 Plasma selenium*

Plasma selenium is the most commonly used biomarker of selenium status. Its usefulness as a biomarker was confirmed in a meta-analysis of selenium supplementation studies with plasma selenium as the outcome measure, including 9 randomised supplementation studies (total of 512 participants). Selenium supplementation significantly increased plasma selenium concentration with a mean difference in plasma selenium concentration of 0.9 µmol/L (95% CI 0.67, 1.14)[52]. Subgroup analyses confirmed the usefulness of plasma selenium as a biomarker of selenium intake in participants with low, medium or high status at baseline and for a wide variety of dosing regimes.

### *1.12.2 Erythrocyte selenium*

In a meta-analysis of studies investigating the use of erythrocyte selenium as a marker of dietary intake, 6 supplementation studies were included. Two studies reported results in micromoles per litre and four in nanomoles per gram of haemoglobin so the results could not be combined. However, using both measures erythrocyte selenium increased significantly with supplementation. Mean increases in erthryocyte selenium were 1.4 μmol/L (95% CI 1.16, 1.64) and 1.48 nmol/g Hb (95% CI 0.45, 2.52)[52]. All these studies gave the supplement for at least 8 weeks allowing time for erythrogensis.

### *1.12.3 Whole blood selenium*

Four supplementation studies were assessed in a meta-analysis to examine the usefulness of whole blood selenium as a marker of dietary intake. Whole blood selenium increased significantly with supplementation with a mean increase in whole blood selenium of 1.07 μmol/L (95% CI 0.39, 1.76)[52].

### *1.12.4 Plasma glutathione peroxidase activity*

Glutathione peroxidase (GPx) is a selenium containing metallo-enzyme. The usefulness of plasma GPx activity as a marker of dietary intake was examined in a meta-analysis of 8 supplementation studies (319 participants). Plasma GPx was found to be a useful marker with a mean increase of 0.37 μmol NADPH oxidised.$min^{-1}$.g $protein^{-1}$ (95% CI 0.15, 0.60)[52].

### *1.12.5 Platelet glutathione peroxidase activity*

In a meta-analysis of four supplementation studies (109 participants) platelet GPx was found to increase significantly with platelet supplementation; mean difference 69.4 U/g protein (95% CI 12.6, 126.2)[52].

### *1.12.6 Whole blood glutathione peroxidase activity*

Four studies, which included 73 participants, of selenium supplementation were included in a meta-analysis. Whole blood GPx increased significantly with selenium supplementation with a mean difference of 3.18 U/g Hb (95% CI 0.07, 6.29)[52].

### *1.12.7 Erythrocyte glutathione peroxidase activity*

Three randomised trials of selenium supplementation measuring erythrocyte GPx activity were combined in a meta-analysis. This only included 23 participants receiving supplementation and no significant difference in erythrocyte GPx activity was seen with a mean difference of 3.37 μmol NADPH oxidised.$min^{-1}$.g $haemoglobin^{-1}$ (95% CI -0.99, 7.74)[52]. All these studies gave the supplement for at least 8 weeks allowing time for erythrogenesis.

### *1.12.8 Plasma Selenoprotein P*

Selenoprotein P is the most common selenoprotein found in the plasma. A meta-analysis of three selenium supplementation studies (68 participants) showed a statistically significant increase in plasma selenoprotein P concentration with selenium supplementation; mean increase 2.19 μg/mL (95% CI 0.25, 4.12)[52].

### *1.12.9 Liver selenium*

Liver selenium has been relatively poorly studied with no report of liver concentrations from patient likely to have severe nutritional deficiencies. There are however several autopsies studies that have reported liver selenium concentrations; these results range from a mean of 0.3 µg/g wet weight in patients from New Zealand[53], an area known to have low selenium levels, to 0.54 µg/g wet weight in patients from the USA where soil selenium is far higher[54].

### *1.12.10 Effects of acute phase response*

Selenium is a negative acute phase reactant; patients in an intensive care unit were found to have plasma selenium concentrations 40-60% lower than the population reference interval[55]. Plasma selenium was also found to fall after minor surgery, with a 10% decrease after 24 hours[47]. One case report showed a reciprocal relationship between the fall and rise in plasma selenium and the rise and fall of C-reactive protein during the acute phase response in a patient with a constant selenium intake due to total parenteral nutrition[56].

Erythrocyte selenium has been studied in patients undergoing elective knee arthroplasty. No difference was found in erythrocyte selenium concentration 3 days after surgery compared to values from immediately before surgery, despite the evolution of the acute phase response[2].

## 1.13 Methods for the measurement of trace element concentrations

### *1.13.1 Pre-analytical considerations, sample collection and preparation*

As trace elements are at low concentrations, they are very susceptible to contamination. Sources of contamination include needles and surgical blades, where stainless steel has been found to increase the concentration of cobalt and nickel. Powder in surgical gloves can also cause contamination, as can skin, sweat and dust on glassware. Recommendations for sample collection and handling therefore include powder-free plastic gloves, ultra-pure reagents and water and acid washing of laboratory containers[57].

For the collection of blood in clinical setting, special tubes are available which are contaminant free. They contain sodium heparin as the anticoagulant as it is does not contain potentially contaminating trace elements; EDTA can contain both chromium and zinc.

Tissue samples will require cutting and trimming; while the use of stainless steel knives is a potential source of contamination, studies have not shown any effect on the measurement of aluminium, cadmium, chromium, copper, mercury, nickel, lead or selenium. However, it is considered good practice to use a titanium knife for the preparation of samples for trace element analysis[58].

### *1.13.2 Analytical techniques for the measurement of trace elements*

There are four main spectrochemical techniques for the measurement of trace element concentration: flame atomic absorption spectrometry (FAAS), graphite furnace atomic absorption spectrometry (GFAAS), inductively coupled plasma atomic emission spectrometry (ICP-AES) and inductively coupled plasma mass spectrometry (ICP-MS)[57].

#### *1.13.2.1 Flame atomic absorption spectrometry*

The element is dissociated from its chemical bonds by a flame but it left in a neutral state. A beam is produced by a hollow cathode lamp with a cathode made from the element under study. The ground-state atoms in the flame will absorb the light thus decreasing the light detected at the detector[4].

This technique is limited by requirements for an aqueous sample with a volume of several millilitres and high limits of detection making precision very poor for samples with concentrations at µg/L levels and below[57].

#### *1.13.2.2 Graphite furnace atomic absorption spectrometry*

GFAAS is a similar technique to FAAS, however instead of a flame the atomisation occurs in a graphite furnace heated by an electrical current. A small sample size is required (5-100µL) and the sample is dried, charred and atomized in the furnace. Absorption using a hollow cathode lamp is identical to FAAS[57].

There are many advantages to GFAAS over FAAS. These include a far lower limit of detection (as low as 0.01 µg/L) and smaller sample size. The technique also

allows for correction of background signal (the Zeeman Effect): the analyte is placed in a pulsed magnetic field, splitting the absorption signal into high and low wavelengths that can be detected separately to background absorption. The graphite furnace allows a variety of solid and slurry sample types to be used, rather than limiting to aqueous samples. GFAAS does take longer than FAAS but multi-element technology is becoming available[59].

*1.13.2.3 Inductively coupled plasma atomic emission spectrometry*

Inductively coupled plasma is a mix of argon, argon ions and electrons and can conduct electricity; it is produced by atomisation and ionisation of argon gas by a high-energy, radio-frequency field and reaches an excitation temperature of 7000-10,000˚K. A sample is sprayed into the plasma as an aerosol mixed with argon gas and the sample is excited, atomised and ionised. The electrons in the sample are unstable in a high-energy state and release their excess energy as photons of a certain wavelength; in atomic emission spectrometry this light is detected. Line spectra of wavelengths of light emitted are specific for each element[60].

ICP-AES does have multi-element capability, either simultaneously or sequentially, but the limit of detection is high and similar to FAAS. ICP has a wide linear dynamic range allowing analysis of several elements covering a range of concentrations in the same sample[57].

*1.13.2.4 Inductively coupled plasma mass spectrometry (ICP-MS)*

ICP-MS combined inductively coupled plasma as an ion source and a mass spectrometer. Mass spectrometers separate the sample on the basis of mass: charge ratio by using one of a variety of techniques to ensure only ions with the mass: charge ratio of interest reaches the detector; one way to do this is a

system of rods with rapidly charging voltage and polarity that cause all other ions except for those of interest to follow a corkscrew trajectory, while the ions of interest continue in a stable path[4].

The ICP-MS offers all the advantages of inductively coupled plasma (high linear dynamic range) with the extremely low detection limits of mass spectrometry. Multi-element analysis screening is simple and efficient with low sample volumes required and allowance for low concentration samples[61].

A diagram summarising the principles of spectrochemical techniques can be seen in figure 1.1

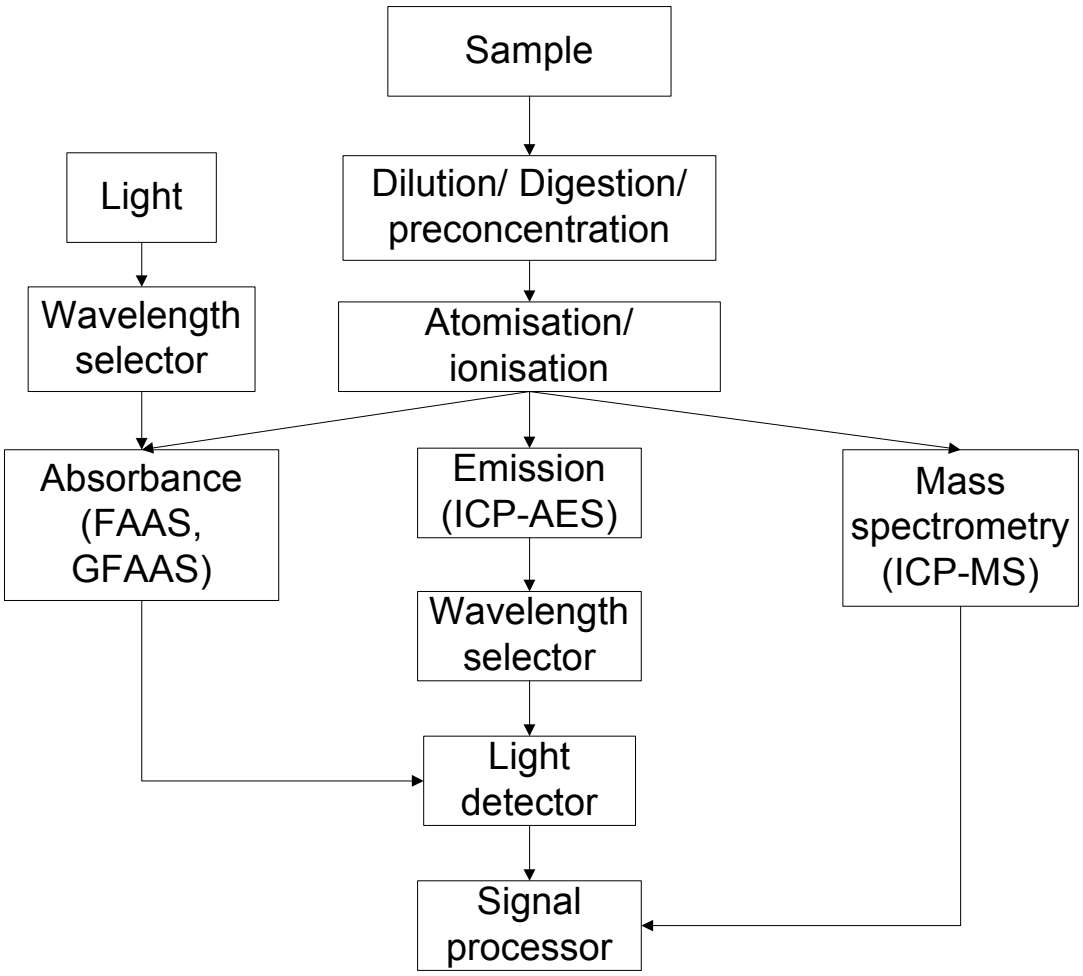



**Fig 1.1** Summary of spectrochemical techniques. Reproduced from Bolann et al, Scan J Clin Lab Invest 2007[57], with permission.

*1.13.3 Interferences*

Spectrochemical methods are subject to interference which can be divided into matrix and spectral interferences.

Matrix interferences are either physical interferences from dissolved solids or chemical interferences from chemical reactions that happen during the atomisation process. The physical interferences can occur in all methods but are more severe in ICP-MS; methods to minimise these interferences include matrix

matching of standards and blanks to the sample, chemical separation methods, standard additions or sample dilution[62]. Chemical interferences are common in FAAS and GFAAS when the analyte can form new compounds that act differently to the standards use; background correction methods and standard addition can help to control these problems[62].

Spectral interferences can occur in ICP-AES when there is wavelength overlap from an interfering element; the use of high resolution spectrometers, or selection of an alternative wavelength, are possible methods to reduce spectral interferences[63]. In ICP-MS spectral interferences can be causes by the isotope of another element (isobaric interference), doubly charge ions or polyatomic ions such as those formed with argon (polyatomic interference) having the same mass number as the analyte; these interferences are predictable and the solution is to measure an isotope of the analyte that is less abundant but less prone to interferences[64].

## 1.14 Magnesium

Magnesium is not a trace element; it is the fourth most abundant mineral in the body[65]. Magnesium is the main intracellular divalent cation and is essential for all energy-dependent transport systems, glycolysis, and oxidative energy metabolism as well as being an integral part of intracellular signalling pathways, receptor activity and phosphorylation pathways[66].

Despite its importance to normal physiology, a suitable biomarker of tissue status has not yet been found[67]. As it can be measured using the same analytical techniques as those used for trace element analysis it was included within this study.

### *1.14.1 Magnesium and health*

There has been increasing interest in magnesium status and the effects on human health. This is particularly true of cardiovascular disease and diabetes and is due to magnesium's role in pathways for glucose metabolism, anti-arrhythmic effects, role in platelet aggregation and relaxation of vascular smooth muscle tone[66]. Magnesium is a vital component of muscle insulin tyrosine kinase and hypomagnesaemia can result in impaired glucose uptake[68].

The effects of magnesium are utilised through its role as a therapeutic agent. Intravenous magnesium therapy is now established as part of the treatment of acute severe asthma[69]; in women with pre-eclampsia, intravenous magnesium was effective in prophylaxis of seizures[70]. High doses of intravenous magnesium are given during cardiac surgery to stabilise the myocardium and it is also used in the management of arrhythmias[71].

There is however less evidence for the use of magnesium supplementation in chronic conditions. Patients with type 1 and type 2 diabetes have been shown to have lower serum magnesium concentrations than non-diabetic controls[72;73], and the magnesium concentration appears to be inversely correlated with glycaemia and complications[74], however there have been no successful supplementation studies showing an improvement in these factors with increasing magnesium concentration, suggesting that the link may not be causal[75].

### *1.14.2 Magnesium homeostasis*

Magnesium is distributed between bone (65%), the intracellular space (34%) and the extracellular fluid (1%)[66]. Magnesium is absorbed in the small intestine, in part under the influence of vitamin D, with 30-50% of dietary intake absorbed[76]; the percentage of the dietary intake that is absorbed decreases with increased intake, aging and chronic renal impairment. Magnesium absorption can be up-regulated by as much as 40% during deficiency[77].

Magnesium is excreted in the urine, determined by the rate of filtration and tubular reabsorption. Urinary magnesium excretion is increased during a high natriuresis, high osmotic load and metabolic acidosis[77].

### *1.14.3 Effects of Magnesium deficiency*

Magnesium deficiency can present as muscle weakness, convulsions, tetany or cardiac arrhythmias. As magnesium is required for parathyroid hormone secretion, the major symptoms can be related to hypocalcaemia. Refractory hypokalaemia, due to the renal tubules selectively reabsorbing magnesium ahead of potassium, is often the main issue raising concern. Both hypokalaemia and hypocalcaemia will improve on magnesium supplementation[78].

### *1.14.4 Assessing Magnesium status*

Despite magnesium having many vital physiological functions, biomarkers of tissue status have been poorly investigated, with serum/plasma magnesium being the most commonly used marker.

#### *1.14.4.1 Magnesium retention test*

The magnesium retention test is a physiological balance study which examines magnesium retention post magnesium infusion. It does this through measurement of urinary magnesium excretion in the 24 hours after an oral or intravenous magnesium load; obviously it relies on normal renal function[79]. Retention of magnesium is thought to be higher in magnesium deficient patients and it has been used to investigate the validity of other magnesium biomarkers.

The major problem with the magnesium loading test is the lack of a standard protocol leading to variation in the result achieved, and lack of any evidence that the deficits measured are proportional to the total body magnesium deficit[67].

#### *1.14.4.2 Serum magnesium*

Serum magnesium is the most commonly measured biomarker of magnesium status. However, there is very little evidence that is reflects total body status. Patients with critical illness who had high magnesium retention on retention testing did not have a serum magnesium level that would have allowed them to be differentiated from those with normal magnesium retention[80], though whether this reflects the lack of usefulness of serum magnesium, magnesium retention testing or both is unclear.

Certainly serum magnesium concentrations do increase with oral magnesium supplementation. In a study where patients with type 2 diabetes were given 30 mmol oral magnesium per day for three months, their serum magnesium increased to that of a non-diabetic control[72].

The main problem with serum magnesium as biomarker of magnesium status is that, as it is has such a vital physiological role, the serum concentration will be maintained via homeostasis for as long as the total body magnesium allows; by the time it is depleted, body stores will be very low.

*1.14.4.3 Ionised Magnesium*

Ionised magnesium is fraction of plasma magnesium that is not bound to albumin or other proteins, and is felt to be the biologically active fraction of total plasma magnesium; it is measured using an ion-selective electrode. There are very few studies including ionised magnesium, but those that have investigated it have found strong correlation with total serum magnesium questioning its advantage. One possible advantage may be in critical illness where albumin concentration decrease may affect the total serum magnesium but leave the ionised fraction within the normal range[81].

*1.14.4.4 Erythrocyte Magnesium*

It is hypothesised that erythrocyte magnesium is an indicator of long-term magnesium status and deficiency[82]. In a study of 12 post-menopausal women who were placed on a magnesium deficient diet for 93 days, followed by a magnesium repletion diet for 49 days, erythrocyte magnesium concentration decreased significantly from baseline during the depletion phase and increased again during the repletion phase. In a study of 34 children recently diagnosed with type 1 diabetes, erythrocyte magnesium was found to negatively correlate

with magnesium retention after a loading test ($r=-0.44$; $p<0.01$) when serum magnesium did not, leading the authors to suggest that erythrocyte magnesium concentration may be a more sensitive test[83].

*1.14.4.5 Leukocyte Magnesium*

Lymphocytes have been suggested as a potentially useful biomarker of tissue magnesium deficiency as studies of induced magnesium deficiency in rats have shown that the lymphocyte magnesium concentration decreases at a similar rate to that of cardiac and skeletal muscle[84].

One study in 30 patients with Crohn's disease showed a significant increase in mononuclear cell magnesium concentration after an intravenous infusion of 60 mmol magnesium[85]. However, a study of patients in intensive care who were receiving intravenous magnesium infusions for hypomagnesaemia (diagnosed by serum concentration), showed no increase in intracellular magnesium concentration[86]; this may be due to the diagnosis being made via serum concentration and thus the patients may not have had an intracellular magnesium deficiency at baseline.

*1.14.4.6 Muscle Magnesium*

Given that skeletal muscle is a major store and utiliser of magnesium, muscle seems a sensible tissue to use as a surrogate of total body status. Muscle magnesium is an indicator of long-term magnesium status and deficiency[82]. In a study of 12 post-menopausal women who were placed on a magnesium deficient diet for 93 days, followed by a magnesium repletion diet for 49 days, muscle magnesium concentration decreased significantly from baseline and increased again during the repletion phase[87]. This correlated with erythrocyte magnesium concentration and negatively correlated with magnesium retention.

One study compared serum magnesium and muscle magnesium concentrations in patient with chronic obstructive pulmonary disease and acute respiratory failure: low muscle magnesium concentration was found in 47% of the patients with normal serum magnesium concentration[88].

*1.14.4.7 Effects of acute phase response*

There are no studies which have followed changes in magnesium status throughout the development and resolution of the acute phase response. The changes in magnesium concentration during the acute phase response are of interest given the increased medical attention given to magnesium replacement during acute illness. Albumin, which decreases during the acute phase response, has a linear relationship with serum magnesium concentration at concentrations below the reference interval, due to around 25% of circulating magnesium being bound to albumin[89].

Total and ionised serum magnesium concentrations were compared in patients in intensive care units; 51.3% of patients had a total serum magnesium concentration below the reference interval, however, only 29% of these patients had an ionised magnesium concentration below the reference interval. This led the authors to conclude that the low total serum magnesium concentrations seen were a result of the acute phase response and not a true representation of body status, and the ionised magnesium should be used in critically ill patients.

### 1.15 Suitable tissues to act as a maker for whole body trace element status

In order to identify suitable biomarkers of trace element status, a suitable surrogate for whole body status is required for comparison. Tissue would seen a suitable material for this and it would have to have a high concentration of the trace element of interest, preferably a role in metabolism and homeostasis and be known to vary it's concentration in response to depletion and repletion. It also has to be a tissue that can be easily and ethically sampled. Liver and skeletal muscle would fulfil these roles.

As outlined in the preceding sections:

The liver is one of the main stores of copper[42] and decreases in copper deficiency[44].

5% of zinc is stored in the liver[23]. Liver zinc has been shown to be lower in cases of malnutrition in children[44].

Except for the lens of the eyes, the liver has the highest concentration of selenium in the body[34].

Skeletal muscle is a major store and utiliser of magnesium with evidence of change in magnesium concentration with depletion and repletion[87].

## 1.16 Summary

Trace elements and magnesium have an important role in human health. The availability of simple and reliable biomarkers for their status is invaluable for further clinical research into the efficacy of supplementation regimes. There is a particular problem of measuring trace element status during acute and chronic illnesses, when changes in binding proteins due the acute-phase response lead to changes in plasma levels of trace elements.

There have been limited studies of biomarkers during depletion and supplementation, but these have only studied blood markers and not the concentrations in tissues where the trace elements and magnesium are stored and utilised.

To further the evidence base in this area the trace element and magnesium concentration in human liver and skeletal muscle will be compared to plasma and erythrocyte concentrations to ascertain if they are a suitable biomarker for tissue status. The effects of the acute phase response on trace element and magnesium concentration in plasma and erythrocytes will also be studied and related to the initial tissue concentrations.

## 1.17 Thesis aims

1. To establish the relationship between liver, muscle, plasma and erythrocyte selenium (including glutathione peroxidase activity), zinc, copper (including ferroxidase activity and ceruloplasmin mass) and magnesium concentrations in individuals with no systemic inflammation.

2. To determine the changes in plasma and erythrocyte selenium (+ glutathione peroxidase), zinc, copper (+ ferroxidase activity and ceruloplasmin mass) and magnesium concentrations during the evolution of the acute phase response.

3. To investigate factors that affect the magnitude of any change in plasma and erythrocyte selenium (+ glutathione peroxidase), zinc, copper (+ ferroxidase and Ceruloplasmin mass) and magnesium concentrations during the evolution of the acute phase response; these include baseline tissue concentration, change in albumin and other binding protein concentrations and baseline plasma and erythrocyte concentration.

## 1.18 Thesis objectives

1. To establish methods for the measurement of plasma and erythrocyte selenium, zinc, copper and magnesium that will be simple to perform in the routine clinical biochemistry laboratory and reproducible to a standard acceptable for a clinical assay.

2. To establish methods for whole blood and erythrocyte glutathione peroxidase that will be simple to perform in the routine clinical biochemistry laboratory and reproducible to a standard acceptable for a clinical assay.

3. To develop a laboratory reference interval for whole blood and erythrocyte glutathione peroxidase.

4. To design a study using a suitable patient cohort that will allow paired blood and tissue samples (liver and skeletal muscle) to be taken when there is no systemic inflammation (low C-reactive protein) and also allow blood sampling during the evolution of the acute phase response.

5. To question how the results will affect clinical practice and suggest what further studies would be required in this area.

# Chapter 2

## Trace Element and Magnesium Methods:

## Acid digestion and inductively coupled plasma mass spectrometry

### 2.1 Principle of the method

Samples (tissue, cells or plasma) were digested in nitric acid therefore breaking down all protein structures but leaving the trace element micronutrients unchanged, thus removing any potential matrix interference in the method.

The inductively coupled plasma mass spectrometer (ICP-MS) ionises trace elements in high temperature plasma. The ions produced are separated by mass spectrometry and the response on the detector system is proportional to their concentrations.

### 2.2 General points

All plastic and Pyrex test tubes used in these methods were pre-washed with 20% nitric acid prior to use to decrease the incidence of trace metal contamination.

All experimental results were performed in duplicate and the mean result is presented in the results section.

### 2.3 Specimen Requirements

#### *2.3.1 Human tissue samples*

Approximately 75mg of human liver or muscle sample was used. Samples were stored at -50˚C and defrosted before use. The samples were trimmed using a titanium knife to avoid metal contamination.

### *2.3.2 Red blood cells*

100µl of red blood cells were required. Red blood cells are obtained from blood collected in specialist sodium heparin trace element-free blood tubes. The samples were separated by centrifugation within 4 hours of collection. The plasma was removed along with careful removal of the buffy coat by pipette. Red blood cells were stored at -50˚C and defrosted before use.

### *2.3.3 Plasma*

100µl of plasma was required. Plasma was obtained from blood collected in specialist sodium heparin trace element-free blood tubes. The samples were separated by centrifugation within 4 hours of collection. The plasma was removed by pipette, carefully avoiding contamination with the buffy coat. Plasma was stored at -50˚C and defrosted before use.

## 2.4 Reagents

Nitric Acid. 60% UltraPur Grade (McQuilken, East Kilbride, UK)

Copper Standard 1000mg/L Spectrosol Grade (BDH Prolabo, Lutterworth, UK)

Zinc Standard 1000mg/L Spectrosol Grade (BDH Prolabo, Lutterworth, UK)

Selenium Standard 1000mg/L Spectrosol Grade (BDH Prolabo, Lutterworth, UK)

Magnesium Standard 1000mg/L Spectrosol Grade (BDH Prolabo, Lutterworth, UK)

Iron Standard 1000mg/L Spectrosol Grade (BDH Prolabo, Lutterworth, UK)

Scandium Standard 1000mg/L Spectrosol Grade (BDH Prolabo, Lutterworth, UK)

Germanium ICP Standard 1000mg/L (Merck Chemical, Darmstadt, Germany)

All reagents were stored at room temperature and were stable until expiry date.

## 2.5 Standards

### *2.5.1 Human Tissue Samples*

A stock standard was made containing:

3.5mL of 1000mg/L Magnesium Standard

1mL of 1000mg/L Copper Standard

700µL of 1000mg/L Zinc Standard

20µL of 1000mg/L Selenium Standard

This was made up to 100mL with 1% UltraPur nitric acid.

Working standards were made, and stored at room temperature, using dilutions outlined in table 2.1a, which resulted in standards with concentrations as seen in table 2.1b.

| Standard | Stock Standard | 1% UltrPur nitric acid |
|---|---|---|
| 1 | 0 µL | 20 mL |
| 2 | 25 µL | 19.975 mL |
| 3 | 50 µL | 19.95 mL |
| 4 | 200 µL | 19.8 mL |
| 5 | 1 mL | 19 mL |
| 6 | 4 mL | 16 mL |
| 7 | 20 mL | 0 mL |

**Table 2.1a** Dilutions required for stock standard for tissue trace element analysis

| | Conc. of Std 1 (µg/L) | Conc. of Std 2 (µg/L) | Conc. of Std 3 (µg/L) | Conc. of Std 4 (µg/L) | Conc. of Std 5 (µg/L) | Conc. of Std 6 (µg/L) | Conc. of Std 7 (µg/L) |
|---|---|---|---|---|---|---|---|
| Mg | 0 | 43.75 | 87.5 | 350 | 1750 | 7000 | 35000 |
| Cu | 0 | 12.50 | 25 | 100 | 500 | 2000 | 10000 |
| Zn | 0 | 8.75 | 17.5 | 70 | 350 | 1400 | 7000 |
| Se | 0 | 0.25 | 0.5 | 2 | 10 | 40 | 200 |

**Table 2.1b** Concentrations of working standards for tissue trace element analysis

*2.5.2 Red blood cell and plasma samples*

A stock standard was made containing:

10mL of 1000mg/L Iron Standard

5mL of 1000mg/L Magnesium Standard

400µL of 1000mg/L Copper Standard

1.4ml of 1000mg/L Zinc Standard

40µl of 1000mg/L Selenium Standard

This was made up to 100ml with 1% UltraPur nitric acid.

Working standards were made for each run using dilutions outlined in table 2.2a, which resulted in standards with concentrations as seen in table 2.2b.

| Standard | Stock Standard | 1% UltraPur nitric acid |
|---|---|---|
| 1 | 0 µL | 500 µL |
| 2 | 50 µL | 450 µL |
| 3 | 100 µL | 400 µL |
| 4 | 300 µL | 200 µL |
| 5 | 500 µL | 0 µL |

**Table 2.2a** Dilutions required for stock standard for red blood cell and plasma trace element analysis

| | Conc. of Std 1 (µg/L) | Conc. of Std 2 (µg/L) | Conc. of Std 3 (µg/L) | Conc. of Std 4 (µg/L) | Conc. of Std 5 (µg/L) |
|---|---|---|---|---|---|
| Fe | 0 | 10000 | 20000 | 60000 | 100000 |
| Mg | 0 | 5000 | 10000 | 30000 | 50000 |
| Cu | 0 | 400 | 800 | 2400 | 4000 |
| Zn | 0 | 1400 | 2800 | 8400 | 14000 |
| Se | 0 | 40 | 80 | 240 | 400 |

**Table 2.2b** Concentrations of working standards for red blood cell and plasma trace element analysis

*2.5.3 Internal Standards*

A diluting reagent was made which contained the internal standards; 50 µg/L Germanium and 50 µg/L Scandium. 25 µL of 1000mg/L Scandium standard and 25 µL of 1000mg/L Germanium standard were added to 500ml of 1% UltraPur nitric acid. Scandium acted as the internal standard for Magnesium whereas Germanium was used for all other elements, as recommended by the manufacturer.

## 2.6 Pre analytical specimen preparation

### *2.6.1 Human tissue samples*

Samples were placed in a Pyrex test tube and put in an oven at 90˚C for 12 hours until dried to a constant weight. Once dried, samples were carefully weighed using a second acid-washed Pyrex test tube to avoid contamination. Once cooled, 3mL of 60% UltraPur nitric acid was added and the test tube was covered with 2 layers of parafilm and left at room temperature for 24 hours. It was then placed in an oven at 70˚C for 12 hours. After this time the sample was a clear yellow liquid with no particulate matter.

For analysis, 200µl of sample (or standard) was added to 2300µl of diluting reagent (which included internal standards).

### *2.6.2 Red blood cell and plasma samples*

100µl of red blood cells or plasma were placed in a plastic test tube and dried in a heating block at 70˚C for 3 hours. 500µl of 60% UltraPur nitric acid was added and the tubes were capped and heated in a heating block for at 85˚C for 4 hours. After this time the samples were a clear straw-coloured liquid with no particulate matter.

For analysis, 4500µl of diluting reagent (which included internal standards) was added to the sample tubes or standards.

## 2.7 Analysis

The ICP-MS used was an Agilent 7500ce ICP-MS, with operational control using Agilent ChemStation Windows software. All analyses were performed in helium gas mode except Selenium which was measured using hydrogen gas mode, as recommended by the manufacturer. The machine was rinsed between samples with 1% UltraPur nitric acid.

Iron was measured using the less abundant isotope at 54 g/ mol (rather than 56 g/ mol) as it was at far higher concentration than the rest of the analytes. Magnesium (24 g/ mol), selenium (78 g/ mol), copper (63 g/ mol) and zinc (66g/ mol), where measured using their most abundant isotopes.

## 2.8 Calculation of results

### *2.8.1 Human tissue samples*

Human tissue sample results were presented as µg/g dry weight.

As they were diluted 1:3 for acid digestion results achieved were multiplied by 3 (this was done via the ICP-MS software).

Trace element concentration (µg/g dry weight) = (µg/L x 3)/ dry weight (g)

### *2.8.2 Red blood cells*

Red blood cell results were presented as µg/µg Fe:

Trace element concentration (µg/µg Fe) = (µg/L trace element) / (µg/L Fe)

*2.8.3 Plasma*

Plasma results were presented as µg/L and required no calculation.

They were also converted to molar units:

Mg mmol/L = (µg/L / 24.3)/1000

Cu µmol/L = (µg/L / 63.55)

Se µmol/L = (µg/L / 78.96)

Zn µmol/L = (µg/L / 65.38)

## 2.9 Method development

For liver and tissue method development bovine liver and muscle obtained from a local butcher were used. For red blood cell and plasma method development the remainder of samples from the routine laboratory were used.

*2.9.1 Choice of standard concentration*

*2.9.1.1 Human tissue samples*

As the concentrations of trace element micronutrients in liver and muscle samples were not available from published literature a set of standards were used that simply had 400 µg/L, 80 µg/L, 20 µg/L, 4 µg/L, 1 µg/L and 0.5 µg/L of each element. Results obtained on runs of bovine liver and muscle were then used to alter the concentrations of the standards so that they covered the appropriate concentration range.

*2.9.1.2 Red blood cells*

Ranges of red blood cell concentrations of some trace elements were available from the handbook of the SAS Trace Elements Laboratories (Ed. Andrew Taylor. 4th Edition, Guildford 2006) and the concentration calculated for a high iron or haemoglobin level. The upper ranges were:

Zinc up to 12420 µg/L

Copper up to 800 µg/L

Selenium up to 181 µg/L

Iron up to 950 mg/L

A concentration range for red blood cell magnesium was not available, but a previous study was found with a mean red blood cell magnesium concentration of 150 µg/ g Hb[82]. This was then calculated for a maximum haemoglobin of 34 g/ dL which calculated as 51 mg/L.

*2.9.1.3 Plasma*

Plasma standard concentrations were decided upon by use of the upper limit of the laboratory reference intervals; zinc 1175 µg/L, copper 1398 µg/L, magnesium 24.3mg/L, and selenium 158 µg/L.

*2.9.2 Precision Studies*

*2.9.2.1 Liver*

Intra-assay precision was assessed using 10 pieces of one bovine liver, which had gone through all pre-analytical preparation as separate samples, assayed in one batch. Results can be seen in table 2.3a.

| Sample | Mg (µg/g dry weight) | Cu (µg/g dry weight) | Zn (µg/g dry weight) | Se (µg/g dry weight) |
|---|---|---|---|---|
| 1 | 552.28 | 237.16 | 100.83 | 1.27 |
| 2 | 578.89 | 243.65 | 102.65 | 1.29 |
| 3 | 569.42 | 219.50 | 102.86 | 1.14 |
| 4 | 575.59 | 233.74 | 103.91 | 1.29 |
| 5 | 575.17 | 206.01 | 102.71 | 1.20 |
| 6 | 610.63 | 250.42 | 110.21 | 1.27 |
| 7 | 591.76 | 244.60 | 101.13 | 1.18 |
| 8 | 597.21 | 242.08 | 109.37 | 1.28 |
| 9 | 589.51 | 230.69 | 101.84 | 1.18 |
| 10 | 578.16 | 249.11 | 101.04 | 1.21 |
| Mean | 581.86 | 235.70 | 103.66 | 1.23 |
| SD | 16.2 | 14.0 | 3.4 | 0.05 |
| CV(%) | 2.8 | 5.9 | 3.3 | 4.4 |

**Table 2.3a** Mg, Zn, Cu and Se concentration (µg/g dry weight) for 10 samples of one piece of bovine liver performed in one batch. Mean, standard deviation, and co-efficient of variation (CV) values are given for each.

Intra-assay co-efficient of variation was satisfactory for zinc (3.3%), copper (5.9%), magnesium (2.8%) and selenium (4.4%).

Inter-assay precision was assessed using a 10 pieces of one bovine liver, which had gone through all pre-analytical preparation as separate samples, assayed each on separate days using new standards each time. Results can be seen in table 2.3b.

| Sample | Mg (µg/g dry weight) | Cu (µg/g dry weight) | Zn (µg/g dry weight) | Se (µg/g dry weight) |
|---|---|---|---|---|
| 1 | 571.81 | 231.77 | 100.73 | 1.28 |
| 2 | 552.28 | 237.16 | 100.83 | 1.27 |
| 3 | 584.36 | 239.51 | 105.83 | 1.23 |
| 4 | 561.02 | 218.42 | 107.29 | 1.19 |
| 5 | 554.06 | 212.25 | 108.01 | 1.31 |
| 6 | 641.97 | 225.82 | 98.76 | 1.31 |
| 7 | 554.66 | 228.54 | 112.35 | 1.27 |
| 8 | 584.67 | 236.51 | 108.33 | 1.12 |
| 9 | 572.90 | 233.98 | 101.24 | 1.31 |
| 10 | 543.85 | 200.28 | 101.66 | 1.23 |
| Mean | 572.16 | 226.42 | 104.50 | 1.25 |
| SD | 28.2 | 12.6 | 4.4 | 0.06 |
| CV(%) | 4.9 | 5.6 | 4.3 | 4.9 |

**Table 2.3b** Mg, Zn, Cu and Se concentration (µg/g dry weight) for 10 samples of one piece of bovine liver each performed on separate days using new standards. Mean, standard deviation, and CV values are given for each.

Inter-assay co-efficient of variation was satisfactory for zinc (4.3%), copper (5.6%), magnesium (4.9%) and selenium (4.9%).

*2.9.2.2 Muscle*

Intra-assay precision was assessed using a 10 pieces of piece of bovine muscle, which had gone through all the pre-analytical preparation as separate samples, assayed in one batch. Results can be seen in table 2.4a.

| Sample | Mg (µg/g dry weight) | Cu (µg/g dry weight) | Zn (µg/g dry weight) | Se (µg/g dry weight) |
|---|---|---|---|---|
| 1 | 924.68 | 2.83 | 160.51 | 0.24 |
| 2 | 907.32 | 4.06 | 140.73 | 0.20 |
| 3 | 932.08 | 2.91 | 156.10 | 0.24 |
| 4 | 842.31 | 2.70 | 143.16 | 0.26 |
| 5 | 878.02 | 3.34 | 158.73 | 0.21 |
| 6 | 878.46 | 3.85 | 136.59 | 0.22 |
| 7 | 933.40 | 2.89 | 163.44 | 0.21 |
| 8 | 918.27 | 4.00 | 148.70 | 0.22 |
| 9 | 921.03 | 3.24 | 155.91 | 0.25 |
| 10 | 920.39 | 11.28 | 151.32 | 0.22 |
| Mean | 905.60 | 4.11 | 151.52 | 0.23 |
| SD | 29.7 | 2.6 | 9.0 | 0.02 |
| CV(%) | 3.3 | 62.5 | 6.0 | 8.8 |

**Table 2.4a** Mg, Zn, Cu and Se concentration (µg/g dry weight) for 10 samples of one piece of bovine muscle performed in one batch. Mean, standard deviation, and CV values are given for each.

Intra-assay co-efficient of variation was satisfactory for zinc (6.0%), magnesium (3.3%) and selenium (8.8%) but not for copper (62.5%).

Inter-assay precision was assessed using a 10 pieces of one bovine muscle, which had gone through all pre-analytical preparation as separate samples, assayed each on separate days using new standards each time. Results can be seen in table 2.4b.

| Sample | Mg (µg/g dry weight) | Cu (µg/g dry weight) | Zn (µg/g dry weight) | Se (µg/g dry weight) |
|---|---|---|---|---|
| 1 | 897.44 | 4.11 | 145.44 | 0.36 |
| 2 | 932.08 | 2.91 | 156.10 | 0.24 |
| 3 | 907.47 | 3.08 | 165.49 | 0.40 |
| 4 | 911.26 | 0.00 | 139.73 | 0.01 |
| 5 | 890.46 | 2.63 | 172.35 | 0.31 |
| 6 | 941.54 | 3.31 | 148.72 | 0.28 |
| 7 | 919.88 | 4.81 | 155.69 | 0.31 |
| 8 | 933.45 | 10.08 | 155.62 | 0.13 |
| 9 | 865.61 | 3.48 | 152.51 | 0.18 |
| 10 | 902.08 | 5.84 | 139.97 | 0.30 |
| Mean | 910.13 | 4.03 | 153.16 | 0.25 |
| SD | 22.9 | 2.6 | 10.4 | 0.12 |
| CV(%) | 2.5 | 65.0 | 6.8 | 46.8 |

**Table 2.4b** Mg, Zn, Cu and Se concentration (µg/g dry weight) for 10 samples of one piece of bovine muscle each performed on separate days using new standards. Mean, standard deviation, and CV values are given for each.

Intra-assay co-efficient of variation was satisfactory for zinc (6.8%) and magnesium (2.5%). They were not satisfactory for copper (65.0%) or selenium (46.8%). Due to the low concentration of selenium and copper found in muscle, the measurement of these cannot be done precisely. Therefore, muscle selenium and copper were not reported in this study.

*2.9.2.3 Plasma*

Intra-assay precision was assessed using one plasma sample in 10 aliquots which had gone through all the pre-analytical preparation as separate samples, assayed in one batch. Results can be seen in table 2.5a.

| Sample | Mg (µg/L) | Cu (µg/L) | Zn (µg/L) | Se (µg/L) |
|---|---|---|---|---|
| 1 | 20610 | 2004 | 809.4 | 130.8 |
| 2 | 19540 | 2101 | 875.0 | 130.3 |
| 3 | 19210 | 1941 | 785.4 | 129.3 |
| 4 | 19360 | 1953 | 792.0 | 127.3 |
| 5 | 19540 | 1966 | 793.3 | 128.9 |
| 6 | 19410 | 1952 | 777.4 | 128.1 |
| 7 | 19570 | 1981 | 794.2 | 129.4 |
| 8 | 19240 | 1968 | 778.4 | 128.0 |
| 9 | 20390 | 2037 | 816.4 | 129.6 |
| 10 | 19430 | 1968 | 818.7 | 127.9 |
| Mean | 19630 | 1987 | 804.0 | 129.0 |
| SD | 477.0 | 48.9 | 28.9 | 1.1 |
| CV(%) | 2.4 | 2.5 | 3.6 | 0.9 |

**Table 2.5a** Mg, Zn, Cu and Se concentration (µg/L) for 10 aliquots of one plasma sample performed in one batch. Mean, standard deviation, and CV values are given for each.

Intra-assay co-efficient of variation was satisfactory for zinc (3.6%), copper (2.5%), magnesium (2.4%) and selenium (0.9%).

Inter-assay precision was assessed using a one plasma sample in 10 aliquots which had gone through all the pre-analytical preparation as separate samples, assayed each on separate days using new standards each time. Results can be seen in table 2.5b.

| Sample | Mg (µg/L) | Cu (µg/L) | Zn (µg/L) | Se (µg/L) |
|---|---|---|---|---|
| 1 | 20610 | 2004 | 809.4 | 130.8 |
| 2 | 20190 | 2024 | 826.1 | 135.2 |
| 3 | 21400 | 2106 | 898.5 | 142.5 |
| 4 | 19530 | 1963 | 845.5 | 135.6 |
| 5 | 21640 | 2136 | 910.1 | 143.8 |
| 6 | 21470 | 2092 | 911.1 | 142.9 |
| 7 | 20940 | 2111 | 921.8 | 143.6 |
| 8 | 18660 | 1825 | 827.5 | 125.2 |
| 9 | 18660 | 1867 | 831.8 | 127.5 |
| 10 | 19390 | 2195 | 879.3 | 133.7 |
| Mean | 20249 | 2032 | 866.1 | 136.1 |
| SD | 1138.5 | 119.5 | 42.4 | 6.9 |
| CV(%) | 5.6 | 5.9 | 4.9 | 5.1 |

**Table 2.5b** Mg, Zn, Cu and Se concentration (µg/g dry weight) for 10 aliquots of one plasma sample each performed on separate days using new standards. Mean, standard deviation, and CV values are given for each.

Intra-assay co-efficient of variation was satisfactory for zinc (4.9%), copper (5.9%), magnesium (5.6%) and selenium (5.1%).

*2.9.2.4 Red blood cells*

Intra-assay precision was assessed using one red blood cell sample in 10 aliquots which had gone through all the pre-analytical preparation as separate samples, assayed in one batch. Results can be seen in table 2.6a.

| Sample | Mg (µg/ µg Fe) | Cu (µg/ µg Fe) | Zn (µg/ µg Fe) | Se (µg/ µg Fe) |
|---|---|---|---|---|
| 1 | 0.0561 | 0.00070 | 0.01246 | 0.000264 |
| 2 | 0.0561 | 0.00071 | 0.01247 | 0.000259 |
| 3 | 0.0561 | 0.00073 | 0.01266 | 0.000256 |
| 4 | 0.0565 | 0.00068 | 0.01262 | 0.000259 |
| 5 | 0.0541 | 0.00068 | 0.01260 | 0.000256 |
| 6 | 0.0589 | 0.00072 | 0.01260 | 0.000263 |
| 7 | 0.0521 | 0.00074 | 0.01261 | 0.000259 |
| 8 | 0.0500 | 0.00065 | 0.01255 | 0.000252 |
| 9 | 0.0506 | 0.00065 | 0.01260 | 0.000254 |
| 10 | 0.0513 | 0.00068 | 0.01264 | 0.000257 |
| Mean | 0.0542 | 0.00069 | 0.01258 | 0.000258 |
| SD | 0.0030 | 0.00003 | 0.00007 | 0.0000036 |
| CV(%) | 5.6 | 4.4 | 0.6 | 1.4 |

**Table 2.6a** Mg, Zn, Cu and Se concentration (µg/ µg Fe) for 10 aliquots of one red blood cell sample performed in one batch. Mean, standard deviation, and CV values are given for each.

Intra-assay co-efficient of variation was satisfactory for zinc (0.6%), copper (4.4%), magnesium (5.6%) and selenium (1.4%).

Inter-assay precision was assessed using a one plasma sample in 10 aliquots which had gone through all the pre-analytical preparation as separate samples, assayed each on separate days using new standards each time. Results can be seen in table 2.6b.

| Sample | Mg (µg/ µg Fe) | Cu (µg/ µg Fe) | Zn (µg/ µg Fe) | Se (µg/ µg Fe) |
|---|---|---|---|---|
| 1 | 0.0561 | 0.00070 | 0.01246 | 0.000264 |
| 2 | 0.0474 | 0.00069 | 0.01263 | 0.000270 |
| 3 | 0.0484 | 0.00067 | 0.01236 | 0.000261 |
| 4 | 0.0492 | 0.00066 | 0.01240 | 0.000265 |
| 5 | 0.0527 | 0.00071 | 0.01252 | 0.000266 |
| 6 | 0.0537 | 0.00076 | 0.01249 | 0.000269 |
| 7 | 0.0560 | 0.00069 | 0.01250 | 0.000263 |
| 8 | 0.0697 | 0.00076 | 0.01238 | 0.000270 |
| 9 | 0.0537 | 0.00076 | 0.01288 | 0.000272 |
| 10 | 0.0521 | 0.00069 | 0.01239 | 0.000263 |
| Mean | 0.0539 | 0.00071 | 0.01250 | 0.000266 |
| SD | 0.0063 | 0.00004 | 0.00016 | 0.000004 |
| CV(%) | 11.7 | 5.3 | 1.3 | 1.4 |

**Table 2.6b** Mg, Zn, Cu and Se concentration (µg/ µg Fe) for 10 aliquots of one red blood cell sample each performed on separate days using new standards. Mean, standard deviation, and CV values are given for each.

Intra-assay co-efficient of variation was satisfactory for zinc (1.3%), copper (5.3%), magnesium (11.7%) and selenium (1.4%).

*2.9.3 Accuracy*

*2.9.3.1 Tissue samples*

The accuracy of the tissue trace metal methods was assessed by running certified reference materials: Bovine Liver 1577a and H4 Animal Muscle. The aim was to achieve results similar to the reference values, though the reference materials were all >20 years old and the values would have been assigned by methods that are less accurate than ICP-MS.

Bovine Liver was analysed 4 times and Animal Muscle 5 times; results, including reference values, can be seen in table 2.7.

| | Reference value (µg/g dry weight) | Run 1 (µg/g dry weight) | Run 2 (µg/g dry weight) | Run 3 (µg/g dry weight) | Run 4 (µg/g dry weight) | Run 5 (µg/g dry weight) | Mean (µg/g dry weight) |
|---|---|---|---|---|---|---|---|
| Bovine Liver | | | | | | | |
| Zn | 142 | 140.07 | 136.85 | 122.99 | 134.17 | | 133.52 |
| Cu | 189 | 206.23 | 204.19 | 183.43 | 197.19 | | 197.75 |
| Se | 0.45 | 0.71 | 0.69 | 0.59 | 0.52 | | 0.63 |
| Mg | N/A | 723.23 | 706.49 | 635.31 | 616.49 | | 670.38 |
| Animal Muscle | | | | | | | |
| Zn | 82.54 | 81.01 | 79.34 | 79.04 | 80.69 | 77.49 | 79.51 |
| Cu | 3.95 | 3.83 | 3.80 | 3.88 | 3.69 | 3.75 | 3.79 |
| Se | 0.34 | 0.35 | 0.37 | 0.33 | 0.34 | 0.32 | 0.34 |
| Mg | 1019.73 | 1102.32 | 1080.04 | 1073.63 | 1060.20 | 1043.05 | 1071.85 |

**Table 2.7** Reference and obtained values for bovine liver and animal muscle reference material

Mean values obtained for reference materials are similar to the assigned reference values.

*2.9.3.2 Red blood cell and plasma samples*

No certified reference materials were available for red blood cell or plasma trace elements. There was however a preparation of lyophilised whole blood which had been assigned an average value from measurements by several laboratories (Seronorm), and this was used for comparison. The values had been assigned using different methods to ICP-MS (namely ICP-sector field mass spectrometry and electrothermal atomic absorption spectroscopy).

Three levels of sample were available and each were re-constituted as per the instructions and run as normal samples 5 times; results, including laboratory mean values, can be seen in table 2.8.

| | Assigned value (µg/L) | Run 1 (µg/L) | Run 2 (µg/L) | Run 3 (µg/L) | Run 4 (µg/L) | Run 5 (µg/L) | Mean (µg/L) |
|---|---|---|---|---|---|---|---|
| Seronorm Level 1 | | | | | | | |
| Zn | 5500 | 4793 | 4790 | 4739 | 4657 | 4705 | 4737 |
| Cu | 564 | 547 | 560.4 | 554.6 | 544.0 | 560.0 | 553 |
| Se | 79.8 | 111.5 | 111.7 | 111.7 | 110.4 | 110.9 | 111.2 |
| Mg | 19600 | 19100 | 20380 | 20220 | 19220 | 19920 | 19768 |
| Fe | 432000 | 417300 | 411600 | 405300 | 412100 | 407000 | 410660 |
| Seronorm level 2 | | | | | | | |
| Zn | 5038 | 4620 | 4947 | 4654 | 4636 | 4435 | 4658 |
| Cu | 604.6 | 591.9 | 643.6 | 604.3 | 598.9 | 665.4 | 621 |
| Se | 123 | 163.3 | 168.6 | 167.4 | 166.8 | 164.2 | 166.1 |
| Mg | 20100 | 17780 | 20320 | 18900 | 19390 | 17620 | 18802 |
| Fe | 433000 | 392800 | 396300 | 395400 | 395700 | 380200 | 392080 |
| Seronorm level 3 | | | | | | | |
| Zn | 8032 | 7424 | 7211 | 7439 | 7069 | 7481 | 7325 |
| Cu | 1741 | 1542 | 1508 | 1547 | 1461 | 1558 | 1523 |
| Se | 146 | 243.6 | 237.5 | 240.6 | 229.6 | 247.3 | 239.7 |
| Mg | 21700 | 21680 | 21600 | 22270 | 20180 | 22510 | 21648 |
| Fe | 471000 | 447700 | 434300 | 446800 | 430900 | 449200 | 441780 |

**Table 2.8** Assigned and obtained values for Seronorm whole blood level 1-3

Values obtained for iron and magnesium concentration were broadly comparable with slightly lower values obtained for copper and zinc. Selenium values obtained were high compared to the assigned values: standards had been checked and this was not an analytical error (it is not high by a given percentage at each level). This difference may be due to matrix effects in the laboratories assigning the values; they are very unlikely to have used an acid digestion method for whole blood measurement.

Differences in these values do not affect the use of this methods within the study; the values are not reference values (reference values in the tissue reference materials were far closer), and the precision of the methods is excellent which is the important factor in a study where the main outcome is correlation and change, rather than exact values.

*2.9.4 Limit of Detection*

The limit if detection was calculated by running 10 blank samples which went through all the pre-analytical steps, including heating in a heating block. Limit of detection was calculated as the mean of 10 blank samples plus 3 standard deviations. Results can be seen in table 2.9.

| Sample | Mg (µg/L) | Cu (µg/L) | Zn (µg/L) | Se (µg/L) |
|---|---|---|---|---|
| 1 | 0 | 0 | 0 | 0 |
| 2 | 0 | 5.35 | 0 | 0 |
| 3 | 0 | 0 | 0 | 0 |
| 4 | 0 | 0 | 0 | 0 |
| 5 | 0 | 0 | 0 | 0 |
| 6 | 0 | 0 | 0 | 0 |
| 7 | 0 | 0 | 0 | 0 |
| 8 | 0 | 5.17 | 0 | 0 |
| 9 | 0 | 0 | 0 | 0 |
| 10 | 0 | 0 | 0 | 0 |
| Mean | 0 | 1.05 | 0 | 0 |
| SD | 0 | 2.22 | 0 | 0 |
| LoD | 0 | 7.70 | 0 | 0 |

**Table 2.9** Mg, Zn, Cu and Se concentration (µg/L) for 10 blank samples performed in one analytical run. Mean, standard deviation, and limit of detection (LoD) values are given for each.

Only copper was detected in the blank samples, giving it a limit of detection of 7.7 µg/L.

*2.9.5 Recovery*

A recovery experiment was performed using 3 bovine liver and 3 bovine muscle samples.

200 µl of sample was added to 2.3 ml of diluting reagent and 50 µl of tissue standard (A) or 50 µl of 1% UltraPur nitric acid (B).

Recovery was calculated for each trace element:

((concentration A – concentration B) / (concentration of standard * 0.25)) * 100%

Results can be seen in table 2.10 below.

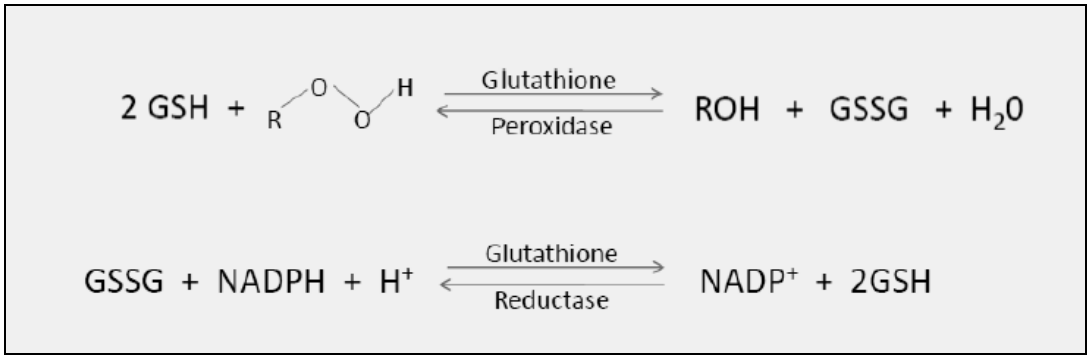
Almost all the recovery results achieved were acceptable, being between 90-110% and consistent across all the samples. The exception was selenium were recovery was lower in the third liver and muscle samples. Selenium concentration was far lower in these samples and the decision had already been made that due to high imprecision at low concentrations, muscle selenium was not going to be reported.

| Liver 1 | Standard (µg/L) | A (µg/L) | B (µg/L) | Recovery (%) |
|---|---|---|---|---|
| Mg | 35000 | 24870 | 16020 | 101.1 |
| Fe | 8000 | 5364.5 | 3166 | 109.9 |
| Cu | 10000 | 8427.5 | 5788 | 105.6 |
| Zn | 7000 | 4701.5 | 2959 | 99.6 |
| Se | 200 | 73.48 | 27.64 | 91.7 |
| Liver 2 | Standard (µg/L) | A (µg/L) | B (µg/L) | Recovery (%) |
| Mg | 35000 | 30470 | 21370 | 104.0 |
| Fe | 8000 | 6697 | 4571.5 | 106.3 |
| Cu | 10000 | 11265 | 8671 | 103.8 |
| Zn | 7000 | 5596.5 | 3869 | 98.7 |
| Se | 200 | 85.71 | 37.81 | 95.8 |
| Liver 3 | Standard (µg/L) | A (µg/L) | B (µg/L) | Recovery (%) |
| Mg | 35000 | 22875 | 13885 | 102.7 |
| Fe | 8000 | 6983.5 | 4841 | 107.1 |
| Cu | 10000 | 6903.5 | 4252 | 106.1 |
| Zn | 7000 | 4598.5 | 2886 | 97.9 |
| Se | 200 | 50.87 | 8.12 | 85.5 |
| Muscle 1 | Standard (µg/L) | A (µg/L) | B (µg/L) | Recovery (%) |
| Mg | 35000 | 35020 | 26020 | 102.9 |
| Fe | 8000 | 3908 | 1721.5 | 109.3 |
| Cu | 10000 | 2742 | 77.345 | 106.6 |
| Zn | 7000 | 6430 | 4738.5 | 96.7 |
| Se | 200 | 49.81 | 5.67 | 88.3 |
| Muscle 2 | Standard (µg/L) | A (µg/L) | B (µg/L) | Recovery (%) |
| Mg | 35000 | 26245 | 17180 | 103.6 |
| Fe | 8000 | 3380.5 | 1228 | 107.6 |
| Cu | 10000 | 2718.5 | 52.895 | 106.6 |
| Zn | 7000 | 4655 | 3001 | 94.5 |
| Se | 200 | 45.61 | 3.30 | 84.6 |
| Muscle 3 | Standard (µg/L) | A (µg/L) | B (µg/L) | Recovery (%) |
| Mg | 35000 | 25845 | 16875 | 102.5 |
| Fe | 8000 | 3034.5 | 953.1 | 104.1 |
| Cu | 10000 | 2723 | 60.33 | 106.5 |
| Zn | 7000 | 2965 | 1278.5 | 96.4 |
| Se | 200 | 43.83 | 3.06 | 81.5 |

**Table 2.10** Results of recovery experiment using 3 liver and 3 muscle samples

## 3.1 Principle of the method

Glutathione peroxidase was measured as described by Paglia and Valentine[90]. GPX catalyses the oxidation of Glutathione (GSH) by Cumene Hydroperoxide. In the presence of Glutathione Reductase (GR) and NADPH, the oxidised Glutathione is immediately converted to the reduced form with a concomitant oxidation of NADPH to $NADP^+$. The oxidation of NADPH is used to monitor the progress of the reaction via a decrease in absorbance measured at 340nm.




Drabkin's reagent ($K_3Fe[CN_6]$) is added prior to the assay to minimise the positive interference caused by the pseudoperoxidative activity of haemoglobin.
Red blood cell GPX activity is reported per gram of haemoglobin. Haemoglobin is initially reduced to methaemoglobin using Drabkin's reagent and then reacts with the cyanide in the Drabkin's reagent to form cyanmethaemoglobin. The absorbance of cyanmethaemoglobin is this measured at the main wavelength of 546nm and a bichromatic wavelength of 700nm. The absorbance of this derivative at 546nm is directly proportional to the haemoglobin content in the blood.

## 3.2 Specimen Requirements

### *3.2.1 Red blood cells*

50µl of red blood cells were required. Red blood cells are obtained from blood collected in specialist sodium heparin trace element-free blood tubes. The samples were separated by centrifugation within 4 hours of collection. The plasma was removed along with careful removal of the buffy coat by pipette. Red blood cells were stored at -50˚C and defrosted before use.

### *3.2.2 Whole blood*

100µl of whole blood was required, collected in specialist sodium heparin trace element-free blood tubes. Samples were stored at -50˚C and defrosted before use.

## 3.3 Reagents

Ransel Glutathione Peroxidase kit (Randox, Co. Antrim, UK)
Drabkin's reagent (Gainland Chemical Company, Sandycroft, Deeside, UK)
Bovine haemoglobin (Sigma Aldrich, St Louis, Missouri, USA)

## *3.4 Preparation of reagents*

### *3.4.1 Ransel Glutathione Peroxidase kit*

Diluting Reagent (reagent 3) – the contents of one vial were reconstituted with 200ml of deionised water. It was stored between 2-8˚C and was stable for 4 weeks.

Buffer (reagent 1b) – this was ready to use and stable at 4˚C until expiry date.

Working Reagent (reagent 1a) – the contents of one vial were reconstituted with 10ml of buffer (reagent 1b). This was then stable at 4˚C for 48 hours. This was heated to 37˚C in a water bath before the assay was performed.

Cumene Hydroperoxide (reagent 2) – 20µl was added by reverse pipetting technique to 10ml of deionised water. This was then mixed by vortex mixer for 2 minutes. This was required to be prepared fresh for every assay. This was heated to 37˚C in a water bath before the assay was performed.

*3.4.2 Drabkin's reagent*

25ml of concentrate was diluted to 1l with deionised water. Reconstitued reagent was stable for 4 months when stored in a dark bottle at room temperature.

*3.4.3 Double strength Drabkin's reagent*

25ml of concentrate was diluted to 500mL with deionised water. Reconstitued reagent was stable for 4 months when stored in a dark bottle at room temperature.

*3.4.4 Haemoglobin standard*

2g of bovine haemoglobin was dissolved in 6mL of deionised water. This was then transferred to a 10mL volumetric flask and diluted to 10mL with deionised water.

The haemoglobin standard value was determined by spectrophotometry: the prepared standard was diluted 1:50 in Drabkin's reagent (50µL Hb standard + 2.5mL Drabkin's reagent) and the absorbance measured at 546nm and 700nm. The haemoglobin standard value was then calculated:

$$\text{Hb g/dL} = \frac{(\text{Abs 546nm} - \text{Abs 700nm}) \times 64458 \times 50}{44000 \times 1 \times 10}$$

Where 64458 is the molecular weight of haemoglobin and 44000 is the molar extinction coefficient.
The assigned value was ~13.1g/dL. The standard was stored in 150µL aliquots at -50˚C.

## 3.5 Pre-analytical specimen preparation

### *3.5.1 Red blood cell Glutathione Peroxidase*

50µL of red cells were added to 450µL of diluting reagent (reagent 3). The samples were then mixed by inversion then centrifuged at 10000rpm for 10 minutes.

### *3.5.2 Whole blood Glutathione Peroxidase*

100µL of whole blood was added to 400µL of diluting reagent (reagent 3). The samples were then mixed by inversion then centrifuged at 10000rpm for 10 minutes.

## 3.6 Glutathione Peroxidase analysis

200µL of sample supernatant and 200µL of double strength Drabkin's reagent were added to auto-analyser cups. Deionised water was run as a sample blank. Samples were analysed within 20 minutes of adding double strength Drabkin's reagent.

Samples were run on a Sapphire 350 Spectrophotometer (Audit Diagnostics, Carrigtwohill, Ireland) autoanalyser using a kinetic method measuring the decrease in absorbance at 360nm over 4 minutes. 5µL of sample, 10µL of working reagent and 250µL of cumene hydroperoxide were auto-pipetted by the autoanalyser. The molar absorptivity coefficent of NADPH used was 8412.

Glutathione peroxidase concentration was calculated by the Sapphire 350 Spectrophotometer using the following formula:

$$\text{Gluthione peroxidase (U/L)} = 8412 \times \Delta A \text{ 340nm/ minute}$$

### 3.7 Haemoglobin analysis

200µL of sample supernatant were added to auto-analyser cups.
Samples were run on a Sapphire 350 Spectrophotometer autoanalyser (Audit Diagnostics, Carrigtwohill, Ireland) measuring absorbance at a wavelength of 546nm with a bichromatic correction at 700nm. 10µL of sample, and 240µL of Drabkin's reagent were auto-pipetted by the autoanalyser.

#### *3.7.1 Calibration*

Haemoglobin standard was used for calibration of the haemoglobin assay and diluted 1:5 as per whole blood samples. The Sapphire software calculated a factor from the absorbency value of haemoglobin standard and used this to calculate haemoglobin concentrations in patient samples:

$$\text{Factor} = \frac{[\text{calibrator}]}{\text{Abs 546nm} - \text{Abs 700nm}}$$

$$\text{Hb in patient sample} = \text{factor} \times (\text{Abs 546nm} - \text{Abs 700nm})$$

### 3.8 Calculation of results

$$\text{Red blood cell GPX (U GPX/g Hb)} = \frac{\cancel{20}(\text{GPX-water blank})}{\cancel{20} \times \text{Hb}}$$

GPX is multiplied by 20 to correct for the effect of dilution (diluting reagent and double strength Drabkin's reagent). Hb is multiplied by 20 to correct for different dilution of samples and standard (samples are diluted 1:10 while calibrator is diluted1:5) and to convert haemoglobin to g/L.

$$\text{Whole blood GPX (U GPX/g Hb)} = \frac{\cancel{10}(\text{GPX-water blank})}{\cancel{10} \times \text{Hb}}$$

GPX is multiplied by 10 to correct for the effect of dilution (diluting reagent and double strength Drabkin's reagent). Hb is multiplied by 10 to convert haemoglobin to g/L.

## 3.9 Method development

A method for measuring whole blood Glutathione Peroxidase (GPX) was already in use within the laboratory. However, many changes had been made to the method including the use of a new spectrophotometer since the method had last been validated. A full method evaluation was therefore undertaken.

The existing method had a 1:21 dilution of whole blood with diluting reagent (reagent 3) for sample preparation and a 1:20 dilution of sample supernatant with Drabkin's reagent for haemoglobin measurement.

### *3.9.1 Precision*

Precision was assessed for the overall assays (i.e. Units GPX/ g Hb) with an aim of achieving a coefficient of variation (CV) <10%. From 75 patient samples initially assayed high, medium and low range GPx activity pools were selected. These were analysed to establish intra and inter assay coefficients of variation. Intra-assay precision was assessed by the measurement of the same sample 20 times. CV was 4% for the high activity pool, 9.8% for the medium activity pool and 10% for the low activity pool.

Inter-assay precision was measured by assaying individual samples of low, mid and high activity pools on separate days, using freshly prepared reagents and calibrator each time. It was planned to investigate inter assay CV over 20 separate assay runs. However the CV for the low, medium, and high activity pools was unacceptable at 11.3%, 11.4% and 13.5% respectively. Investigation of this indicated that the problem lay with precision of the haemoglobin assay. Another assay in the laboratory that also relied on the Sapphire spectrophotometer for Hb measurement reported an increased CV in its internal quality control material. The problem was traced to contaminated cuvettes and these were replaced. The CV for the internal QC for the other assay returned to its previous level.

*3.9.1.1 Haemoglobin Concentration*

Haemoglobin was being measured at very low concentrations. An existing method in the laboratory also used the Sapphire for the measurement of Haemoglobin but used a concentration that was higher and had better precision. A standard curve was created by measuring absorbance over a range of haemoglobin concentrations (fig 3.1).

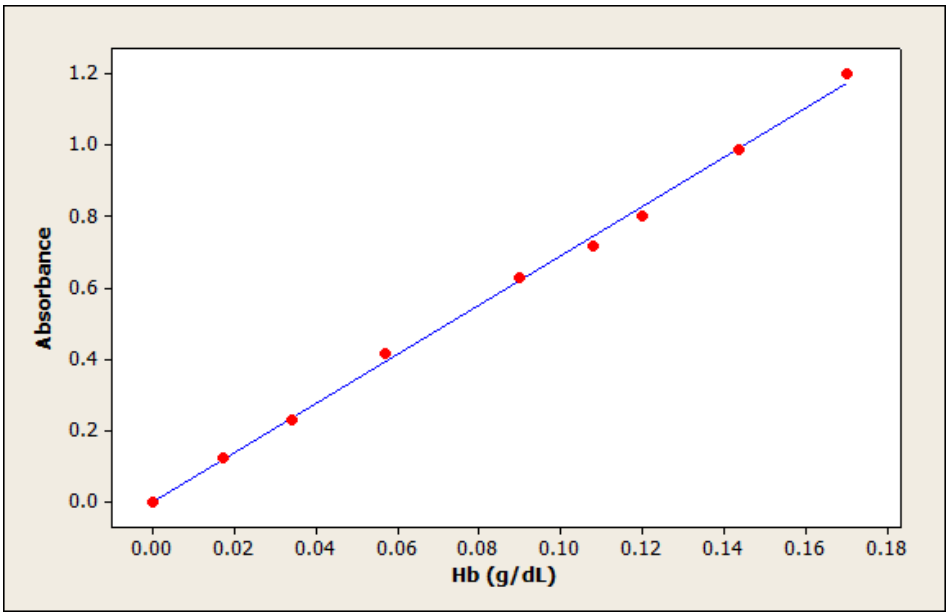



**Fig 3.1** Standard curve of Hb (g/dL) against Absorbance

The volume of Drabkin's Reagent added to the haemolysate was changed to a 1:5 dilution. Consequently a 7g/dL sample would be diluted to 0.066g/dL and an 18g/dL sample to 0.17g/dL. The standard curve shows linearity over this range.

*3.9.1.2 Concentration of Sample*

Precision was then checked for a given sample intra-assay. This sample had a very low GPX activity (approx 16U/g Hb) and the CV was 24.2%. It was felt that at such low concentration (1:21) precision may be poorer and small variations in the results would be amplified. The concentration was changed to a 1:5 dilution (100µl of sample to 400µl of diluting agent) and linearity was confirmed (fig 3.2).

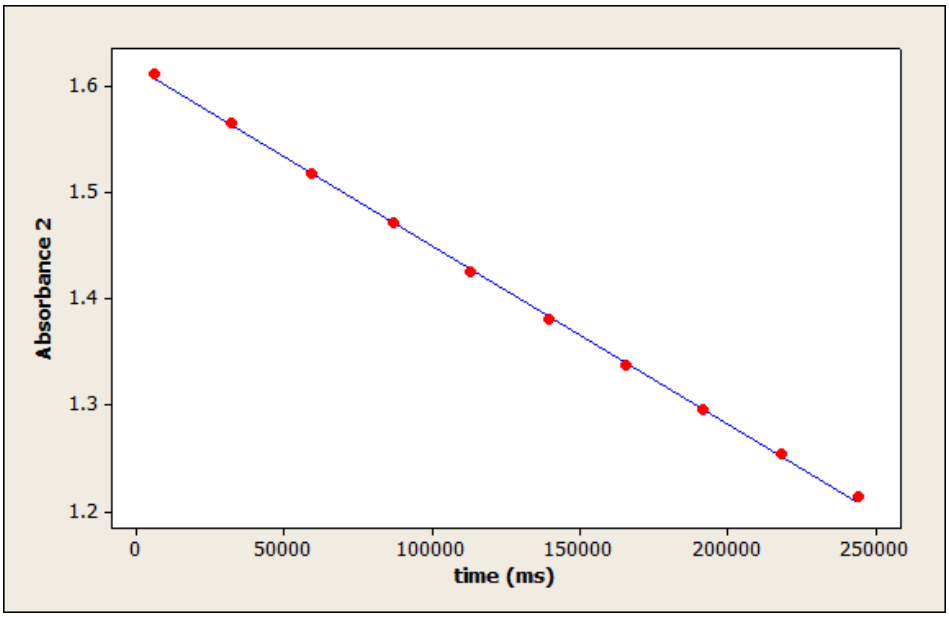



**Fig 3.2** Absorbance at 340nm versus time in milliseconds for a sample at a 1:5 dilution

To compensate for the increase in concentration of the haemoslyate, haemoglobin concentration was altered to give a concentration of 0.12g/dL (absorbance approx 0.6) for a sample with a haemoglobin of 14g/ dL. This was achieved by altering the dilution of the haemosylate with Drabkin's Solution to 1:24.

*3.9.1.3 Precision Studies*

With the above modification to the assays, the precision studies were restarted. Intra-assay precision was assessed using a single patient sample repeated 20 times in one batch. Results can be seen in table 3.1a. The overall intra-assay CV for the combination of the GPX and Hb assays was 3.6%.

| Sample | U GPX | Hb | U GPX/g Hb |
|---|---|---|---|
| 1 | 540.5 | 13.1 | 41.26 |
| 2 | 546.1 | 12.9 | 42.33 |
| 3 | 551.3 | 13.2 | 41.77 |
| 4 | 530.7 | 13 | 40.82 |
| 5 | 564.8 | 12.9 | 43.78 |
| 6 | 548.4 | 12.5 | 43.87 |
| 7 | 541.7 | 12.9 | 41.99 |
| 8 | 520.9 | 12.9 | 40.38 |
| 9 | 543.4 | 12.7 | 42.79 |
| 10 | 565.8 | 12.9 | 43.86 |
| 11 | 534.2 | 12.9 | 41.41 |
| 12 | 547.9 | 12.5 | 43.83 |
| 13 | 563.2 | 12.5 | 45.06 |
| 14 | 511 | 12.8 | 39.92 |
| 15 | 571.6 | 12.7 | 45.01 |
| 16 | 551.2 | 12.9 | 42.73 |
| 17 | 523.5 | 12.1 | 43.26 |
| 18 | 563.5 | 12.6 | 44.72 |
| 19 | 552.8 | 12.7 | 43.53 |
| 20 | 555.1 | 12.7 | 43.71 |
| mean | 546.38 | 12.77 | 42.80 |
| SD | 16.20 | 0.25 | 1.52 |
| CV(%) | 3.0 | 2.0 | 3.6 |

**Table 3.1a** GPX activity (U GPX), Hb, and GPX activity/g Hb for 20 samples (1-20) performed within one batch. Mean, standard deviation, and CV values are given for each.

Inter-assay precision was assessed by measuring a patient sample 20 times on separate days, using new reagents and calibrator each time. Results are shown in table 3.1b. The overall CV for the combination of GPX and Hb assays was 7.7%.

| Sample | U GPX | Hb (g/dL) | U GPX/g Hb |
|---|---|---|---|
| 1 | 563.7 | 12.7 | 44.39 |
| 2 | 590.4 | 12.5 | 47.23 |
| 3 | 595.4 | 12.5 | 47.63 |
| 4 | 562.4 | 12.1 | 46.48 |
| 5 | 544.4 | 13.3 | 40.93 |
| 6 | 580.1 | 13.4 | 43.29 |
| 7 | 556.3 | 13.2 | 42.14 |
| 8 | 570.7 | 13.4 | 42.59 |
| 9 | 536.5 | 13.2 | 40.64 |
| 10 | 526.3 | 13.3 | 39.57 |
| 11 | 554.1 | 13.6 | 40.74 |
| 12 | 574.6 | 11.5 | 49.97 |
| 13 | 585.3 | 13.7 | 42.72 |
| 14 | 592.8 | 13.5 | 43.91 |
| 15 | 599.5 | 12.5 | 47.96 |
| 16 | 624.3 | 12 | 52.03 |
| 17 | 627.4 | 12.6 | 49.79 |
| 18 | 597.5 | 13.2 | 45.27 |
| 19 | 594.5 | 13.1 | 45.38 |
| 20 | 580.2 | 13.3 | 43.62 |
| mean | 577.82 | 12.93 | 44.81 |
| SD | 26.67 | 0.60 | 3.46 |
| CV(%) | 4.6 | 4.6 | 7.7 |

**Table 3.1b** GPX activity (U GPX), Hb, and GPX activity/g Hb for 20 alloquots of one sample (1-20) each performed on separate days using new reagents and calibrator. Mean, standard deviation, and CV values are given for each.

*3.9.2 Accuracy*

Accuracy of the assays (GPX and haemoglobin) could not be assessed as no reference materials were available.

*3.9.3 Red blood cell versus whole blood values*

Glutathione Peroxidase can be measured on red bloods cells as well as whole blood. In order to determine concentration differences in GPX and Hb measurements, three samples were analysed. Fresh samples were collected in Lithium heparin and 500µL of whole blood was alloquoted. The remaining sample was spun at X x g for 10 mins to separate plasma and red blood cells, plasma and the buffy coat was carefully pipetted and discarded, and 500µL of red cells pipetted and stored.

GPX activity and Haemoglobin concentration were calculated as above for red blood cells and whole blood. All samples were analysed in duplicate and mean results are given in table 3.2.

| | | U GPX | Hb (g/dL) | U GPX/ g Hb |
|---|---|---|---|---|
| 1 | Red cells | 392.9 | 27.4 | 35.4 |
| | Whole blood | 566.6 | 13.4 | 27.9 |
| 2 | Red cells | 588.9 | 26.6 | 49.9 |
| | Whole blood | 1000.6 | 13.9 | 44.9 |
| 3 | Red cells | 654.8 | 26.3 | 55.0 |
| | Whole blood | 1040.7 | 13.6 | 45.1 |

**Table 3.2** GPX activity (U), Haemoglobin concentration (g/dL) and GPX activity/ g Hb (U/ g Hb) for 3 samples (1-3), measured in whole blood and red blood cells.

The concentration of haemoglobin was approximately double for red blood cells versus whole blood. Therefore, for red blood cells, the original dilution of sample with Diluting Agent was changed from 1:5 to 1:10 (50µL sample with 450µL diluting agent). This would bring the concentration of Hb measured by the spectrophotometer into the same range as was used for calculating precision of the assay. The red cell values for GPX activity/ g Hb are slightly lower than whole blood values, likely due to the small contribution from plasma GPX but not significantly as to affect assay precision.

*3.9.3.1 Haemoglobin calibrator for RBC Glutathione Peroxidase Assay*

In order to dilute the red cells to get concentrations of haemoglobin comparable to that in the precision studies, the bovine haemoglobin used for calibration would need to be diluted (calibrator has a concentration of ~13g/dL). This would then be at a lower concentration than the concentration levels which were validated. It was felt that the calibrator could continue to be diluted 1:5 and the Hb values obtained for samples doubled. To see if this would be accurate, GPX activity and Hb was measured in red blood cells using three patient samples. For each sample, the calibrator was either diluted 1:10, or diluted 1:5 with the values obtained being doubled. All samples were analysed in duplicate and mean results are given in table 3.3. Using a paired T-test, no difference was found in the values obtained when the calibrator was diluted 1:5 and the result doubled (p=0.723).

| | | U GPX | Hb (g/dL) | Hb x2 | U GPX/g Hb |
|---|---|---|---|---|---|
| 1 | Calibrator 1:10 | 383.7 | 27.4 | | 28.01 |
| | Calibrator 1:5 | | 13.4 | 26.8 | 28.63 |
| 2 | Calibrator 1:10 | 570.8 | 26.6 | | 42.92 |
| | Calibrator 1:5 | | 13.9 | 27.8 | 41.06 |
| 3 | Calibrator 1:10 | 529.5 | 26.3 | | 40.27 |
| | Calibrator 1:5 | | 13.6 | 27.2 | 38.93 |

**Table 3.3** GPX activity (U), Haemoglobin concentration (g/dL) and GPX activity/ g Hb (U/ g Hb) for 3 samples (1-3), measured in red blood cells with the calibrator (bovine haemoglobin) either diluted 1:5 or 1:10 with diluting agent.

*3.9.4 Anticoagulant*

In order to ascertain if there is any difference in GPX activity measured in red blood cells or whole blood collected in EDTA or Lithium heparin, fresh samples were collected from 5 healthy volunteers, and 500µL of whole blood was aliquoted. Red blood cells were separated as before and 500µL of red cells pipetted and stored. GPX was measured in duplicate as above and the mean results can be seen in table 3.4. A paired t-test was performed, and no difference in GPX activity measure was found between the two anticoagulants; whole blood (p=0.643), red blood cells (p=0.505).

| Whole Blood | EDTA GPX Activity (U/g Hb) | Li Hep GPX Activity (U/g Hb) |
|---|---|---|
| 1 | 31.9 | 32.0 |
| 2 | 31.9 | 32.1 |
| 3 | 33.1 | 32.5 |
| 4 | 32.1 | 32.6 |
| 5 | 33.7 | 34.5 |
| **Red Blood Cell** | **EDTA GPX Activity (U/g Hb)** | **Li Hep GPX Activity (U/g Hb)** |
| 1 | 31.9 | 29.9 |
| 2 | 33.5 | 32.0 |
| 3 | 30.1 | 30.3 |
| 4 | 31.3 | 27.5 |
| 5 | 30.4 | 33.5 |

**Table 3.4** GPX activity (U/g Hb) on 5 samples with the anticoagulant EDTA or Lithium heparin and separated into whole blood or red blood cells.

*3.9.5 Stability*

As the assay is for use in a specialist trace element laboratory, samples will be sent from labs throughout the country for analysis. The stability of samples in storage conditions of room temperature (20˚C) for two days, to simulate delivery by first class post, was compared to storage at -50˚C for two days. 5 patient samples taken in both lithium heparin and EDTA sample tubes were used. All samples were analysed in duplicate and mean results are given in table 3.5.

| | *GPX (U/g Hb)* | | | |
|---|---|---|---|---|
| Sample | Li Hep -50˚C | EDTA -50˚C | Li Hep 2/7 20˚C | EDTA 2/7 20˚C |
| 1 | 30.7 | 30.6 | 33.1 | 32.7 |
| 2 | 35.1 | 34.7 | 35.0 | 32.8 |
| 3 | 25.8 | 27.2 | 25.9 | 25.3 |
| 4 | 25.3 | 24.2 | 26.3 | 25.9 |
| 5 | 26.8 | 26.8 | 24.3 | 25.6 |

**Table 3.5** GPX activity/g Hb for 5 samples taken in Li heparin and EDTA and stored at -50˚C or 20˚C for 2/7

Friedman nonparametric analysis was used to examine differences between samples stored at 20˚C or -50˚C, by anticoagulant used. There was no difference found in measured GPX activity between storage methods; Li heparin p=0.951, EDTA p=0.931.

*3.9.6 Reference intervals*

120 samples were obtained from male and female healthy volunteers, not hospitalised or living in an institution, aged 25-75 years old. The samples were collected by the MRC for a social health study but were available for use for this reference range. Whole blood and red blood cells were obtained in the anticoagulant EDTA. All were analysed in duplicate and the mean values calculated.

*3.9.6.1 Whole blood reference interval*

Mean whole blood GPX results from the 120 samples can be seen in appendix 1.

A probability plot (fig 3.3a) and a histogram (GPX values have been rounded to the nearest 5 U/g Hb) (fig 3.3b) of whole blood GPX reference interval data were plotted and had a normal distribution (p=0.147).

The reference range was calculated as a 95% interpercentile interval by calculating two standard deviations from the mean. The mean result was 33.56 U/g Hb and 1 SD was 9.39, therefore the **reference interval is 15-52 U/g Hb**.

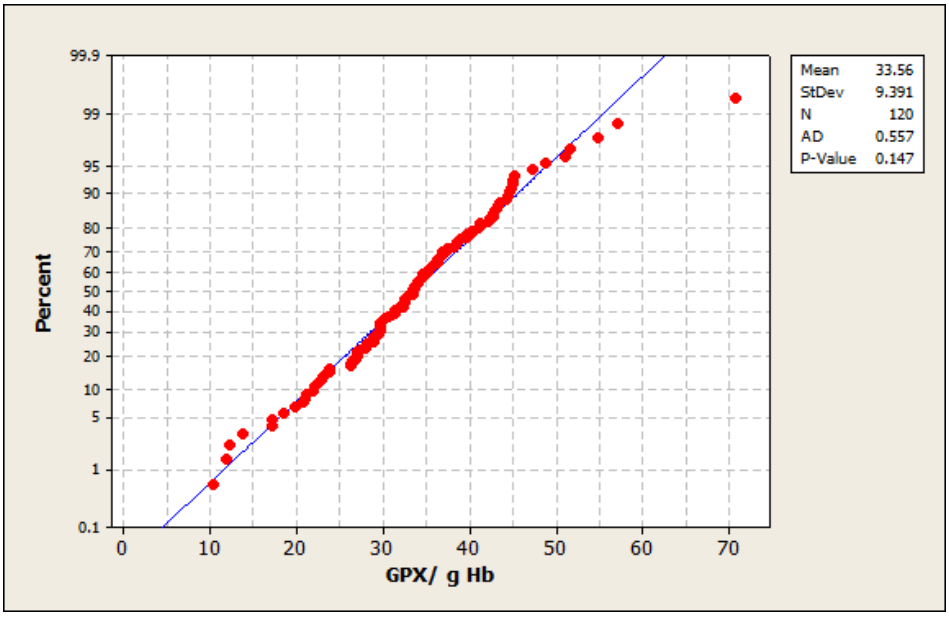



**Fig 3.3a** Probability plot of whole blood GPX reference interval

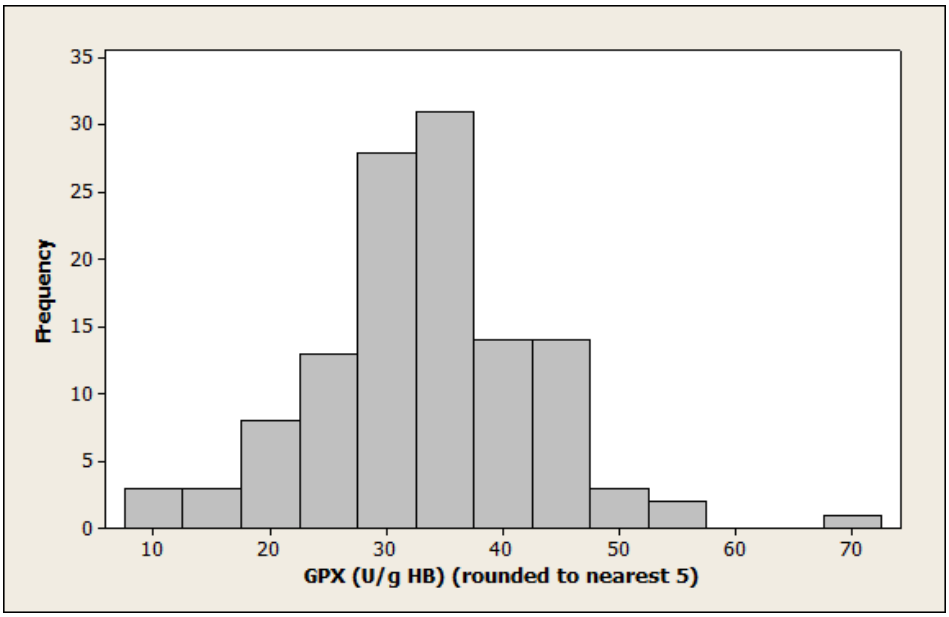



**Fig 3.3b** Histogram of whole blood GPX reference interval samples (U/g Hb)

*3.9.6.2 Red blood cell reference interval*

Mean red blood cell GPX results from the 120 samples can be seen in appendix 1.

A probability plot (fig 3.4a) and a histogram (GPX values have been rounded to the nearest 5 U/g Hb) (fig 3.4b) of whole blood GPX reference interval data were plotted and a normal distribution was seen (p=0.505).

The reference range was calculated as a 95% inter-percentile interval by calculating two standard deviations from the mean. The mean result was 35.85 U/g Hb and 1 SD was 9.03, therefore the **reference interval is 18-54 U/g Hb**.

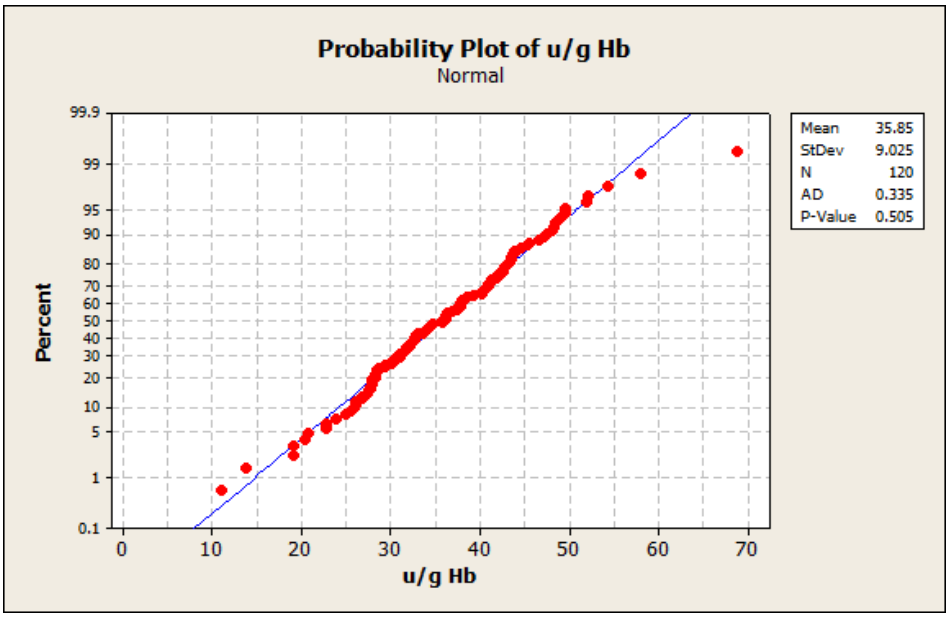



**Fig 3.4a**. Probability plot of red blood cell GPX reference interval

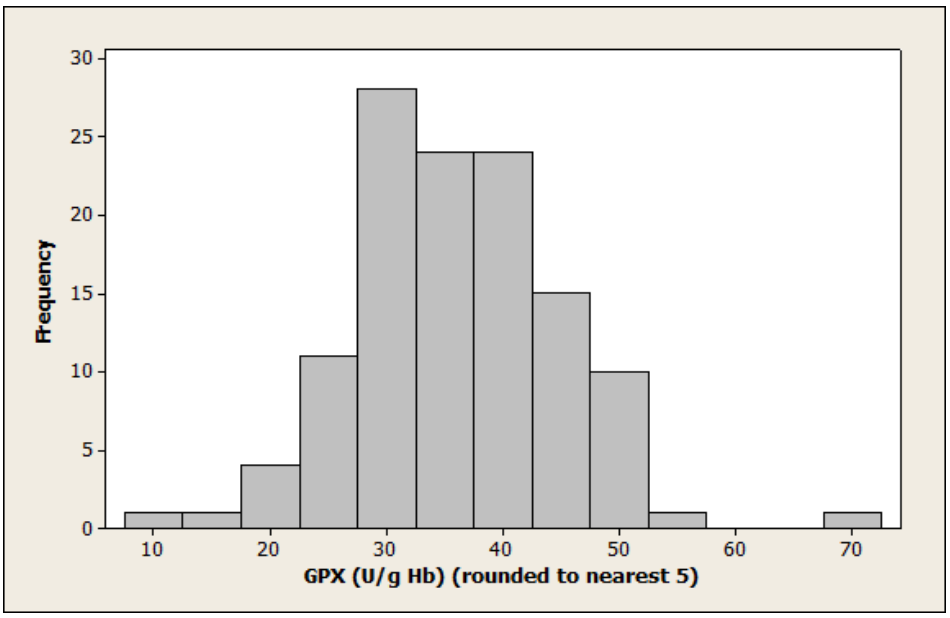



**Fig 3.4b**. Histogram of red blood cell GPX reference interval samples (U/g Hb)

*3.10 Summary*

- The existing Glutathione Peroxidase method in use previously was imprecise, likely due to high sample dilution resulting in low concentrations.
- Increasing sample concentrations achieved coefficients of variation of 3.6% intra-assay at 42.8 U/g Hb (546 U/L GPX, 12.7 g/dL Hb) and 7.7% 44.8 U/g Hb (578 U/L GPX, 12.9 g/dL Hb) inter-assay.
- Both EDTA and lithium heparin are suitable anticoagulants.
- Glutathione Peroxidase is stable at room temperature for 2 days, allowing sample delivery by first class postage.
- The reference interval for whole blood is **15-52** U/g Hb; red blood cells **18-54** u/g Hb.

# Chapter 4
# Other Methods

## 4.1 Serum Albumin

Serum Albumin was performed as part of the routine patient care using the routine biochemistry laboratory at Glasgow Royal Infirmary. This was a bromcresol purple albumin method by Abbot Laboratories on an Abbot Architect Analyser (Abbot Park, Ilinois).

The principle of the method is that bromocresol purple bind specifically with human albumin to form a coloured complex. The absorbance of the complex at 604 nm is directly proportional to the albumin concentration in the sample.

The intra-assay precision was 0.5% at 24 g/L, 0.5% at 33 g/L and 0.4% at 42 g/L. Inter-assay the precision was 0.8% at 25 g/L, 0.6% at 32 g/L and 0.5% at 38 g/L.

## 4.2 Serum C-reactive protein

Serum C-reactive protein was performed as part of the routine patient care using the routine biochemistry laboratory at Glasgow Royal Infirmary. This was a latex immunoassay method by Abbot Laboratories (CRP Vario) on an Abbot Architect Analyser (Abbot Park, Illinois). The assay is a high-sensitivity CRP method, with a limit of detection of 0.01 mg/L.

The principle of the method is that an antigen-antibody reaction occurs between CRP in a sample and anti-CRP antibody, which has been absorbed to latex particles and agglutination results. This agglutination is detected as an absorbance change (572 nm) with the change being proportional to the quantity of CRP in the sample.

The intra-assay precision was 0.7% at 17.3 mg/L and 0.6% at 50 mg/L. Inter-assay the precision was 2.7% at 7 mg/L, 0.5 % at 41 mg/L and 2.1% at 173 mg/L.

## 4.3 Ceruloplasmin

Plasma ceruloplasmin concentration was measured for use in the study using the routine method in the biochemistry laboratory at Stobhill Hospital, Glasgow. This was an immunoturbidimetric method by Abbot Laboratories on an Abbot Architect Analyser (Abbot Park, Illinois).

The principle of the method is that an immunoturbidimetric reaction occurs between an anti-ceruloplasmin polyclonal antiserum and its corresponding antigen in the presence of polyethylene glycol. The turbidity of the immune complex is proportional to the concentration of the analyte in the sample.

The intra-assay precision was 1.1% at 0.2 g/L and 2.7% at 0.49 g/L. Inter-assay the precision was 1.8% at 0.16 g/L and 2.3% at 0.42 g/L.

## 4.4 Ferroxidase activity

Plasma ferroxidase activity measurement was performed by Dr David Preiss (Specialist Registrar, Chemical Pathology, Glasgow Royal Infirmary).

### *4.4.1 Principle of the method*

The principle of the method is that the serum sample is incubated with a known amount of ferrous ion ($Fe^{2+}$) in buffer solution and then during the catalytic oxidation by ceruloplasmin, ferrous ions are oxidized to ferric ions ($Fe^{3+}$). The chromogen forms a highly coloured blue complex in combination with ferrous ions but it does not form a coloured complex with ferric ions. The chromogen is added at the end of the incubation period and a coloured complex is quickly formed between the chromogen and the remaining non-oxidized ferrous ions, and absorbance is measured spectrophotometrically at 600 nm.

*4.4.2 Reagents*

*Buffer solution 0.45 mol/L acetate buffer solution, pH 5.8:*

36.91 g of sodium acetate (BDH, Poole, UK) was dissolved in 1000 mL of deionised water. 25.9 mL reagent grade glacial acetic acid (BDH, Poole, UK) was diluted to 1000 mL with deionised water. 940 mL of the sodium acetate solution was added to 60 mL acetic acid solution, giving 0.45 mol/L acetate buffer with pH 5.8 checked by pH meter

*Substrate Solution 367 µmol/L:*

9.9 g thiourea (Sigma Aldrich, St Louis, Missouri, USA) was dissolved in 1000 mL deionised water, before the addition of 0.144 g iron (II) ammonium sulphate [$Fe(NH_4)_2(SO_4)_2.6H_2O$] (Sigma Aldrich, St Louis, Missouri, USA). The order in which reagents is added is crucial. A drop of Brij 35 solution (Sigma Aldrich, St Louis, Missouri, USA) was added thereafter. The substrate solution is stable at $4^o$ Celsius for at least six months.

Volumes of the acetate buffer and substrate solution were mixed before each run in a ratio of 14:3. The mixing of the reagents prior to the addition of sample had no measurable detrimental effect. The mixture is referred to as Reagent A hereafter. The concentration of $Fe^{2+}$ in the sample mixture after addition of Reagent A, sample and chromogen solution was chosen as 60 µmol/. Reagent A is also stable for at least 6 months at $4^o$ Celsius but is only stable for 24 hours if left unrefrigerated.

*Chromogen Solution:*

0.8899g of 3-(2-pyridyl)-5,6-bis(2-[5-furylsulfonic acid])-1,2,4-triazine (Sigma Aldrich, St Louis, Missouri, USA) was dissolved in 100 ml of the acetate buffer solution. This chromogen is light yellow in solution but forms a deep blue colour in contact with $Fe^{2+}$ with peak absorption at 590-600 nm. The chromogen was present in excess.

*4.4.3 Procedure*

Samples were run on a Sapphire 350 Spectrophotometer (Audit Diagnostics, Carrigtwohill, Ireland).

2 µL of sample was added to 284 µL of Reagent A at 0 seconds thus commencing the reaction at 37°C. After a further 225 seconds, 20 µL of chromogen solution was added as a stop reagent and chromogen. The change in absorption (which reflects the fall in $Fe^{2+}$ concentration) was then measured at a wavelength of 600 nm after a further 25 seconds. Zero absorption is taken before the addition of the chromogen due to the instantaneous development of blue coloration.

*4.4.4 Calibration*

There are no available sources of human enzymatic ceruloplasmin calibrators. A two-point negative calibration curve was therefore used:

a. Zero calibrator: The first calibrator, reagent grade water, gave maximal absorbance in the region of ≈1.5 – 1.7A. This is equivalent to a sample with no ferroxidase activity, all $Fe^{2+}$ ions remaining in the $Fe^{2+}$ form. Water was ascribed a ferroxidase value of 0.0 U/L.

b. Maximum calibrator: 25 mmol/L potassium EDTA solution gave effectively zero absorbance due to chelation of all available $Fe^{2+}$. This is equivalent to a sample with theoretically maximal ferroxidase activity where all 60 µmol/L $Fe^{2+}$ has been converted to $Fe^{3+}$. The EDTA calibrator was ascribed the maximal ferroxidase value of 2448 units/L, as described below.

### *4.4.5 Calculation of maximum ferroxidase value and derivation of units*

The difference in the ferrous ion concentration before (known) and after (unknown) the enzymatic reaction indicates the amount of oxidized ferrous ion. The amount of enzyme that converts 1 µmol of substrate into product per minute is defined as one unit. Using Fe concentration of 60 µmol/L gives a possible maximum ferroxidase activity of 2448 U/L with an undiluted sample.

$$\text{Enzyme activity} = \frac{[\Delta\text{substrate concentration}] \text{ X dilution factor}}{\text{Reaction time}}$$

Maximum Ferroxidase Activity

= [$\Delta Fe^{2+}$ concentration x dilution factor] / reaction time

= [60 µmol/L x 306µL/ 2 µL] / 3.75 min

### *4.4.6 Precision*

The intra-assay precision was 1.8% at 1253 U/L 3.1% at 808.3/L and 4.6% at 381 U/L. Inter-assay the precision was 6.5% at 783 U/L 6.8% at 665 U/L and 12.1% at 241 U/L.

# Chapter 5

## Experimental design

The aim of this study was to compare the concentrations of trace metals and magnesium in plasma and red blood cells with liver and muscle levels in the non-inflamed patient, and also to be able to investigate the changes in the concentrations of trace metals and magnesium in plasma and red blood cells during the acute phase response. Therefore this study was designed to allow tissue and blood sampling prior to the development of an acute phase response, and subsequent blood sampling during the acute phase response.

### 5.1 Approvals

Favourable ethical opinion was received from the Glasgow Royal Infirmary Research Ethics Committee; management approval was granted by NHS Greater Glasgow and Clyde (appendix 2).

### 5.2 Inclusion Criteria

- Patient in Glasgow Royal Infirmary under the care of Consultant Surgeon Mr Paul Horgan and due to have a liver resection for metastatic cancer, primary cancer or benign cyst.

### 5.3 Exclusion Criteria

- Blood transfusion in previous 3 months or blood transfusion given intra- or post-operatively.

Suitable participants were identified and consented by a member of Mr Paul Horgan's team on the day prior to the operation (invite letter, patient information sheet, consent form can be found in appendix a).

The aim was to recruit 30 participants. No previous studies were available on which to base a power calculation and as such this study should be considered as a preliminary investigation.

### 5.4 Samples

The following samples were obtained by a member of Mr Paul Horgan's team and then transferred to the biochemistry laboratory:

Day 0 (in theatre during operation):

- Approximately 300g of healthy liver tissue from the resected lobe avoiding any areas of tumour – placed in plain universal container
- Approximately 150g of rectus muscle – placed in plain universal container
- 20mL of blood collected in sodium heparin trace element – free blood tubes
- Routine serum sample for liver function tests including albumin and C-reactive protein
- Routine EDTA sample for haematology including haematocrit

Day 1-3 (collected daily while patient is an inpatient):

- 20mL of blood collected in sodium heparin, trace element free blood tubes
- Routine serum sample for liver function tests including albumin and C-reactive protein
- Routine EDTA sample for haematology including haematocrit

The protocol is summarised in figure 5.1.

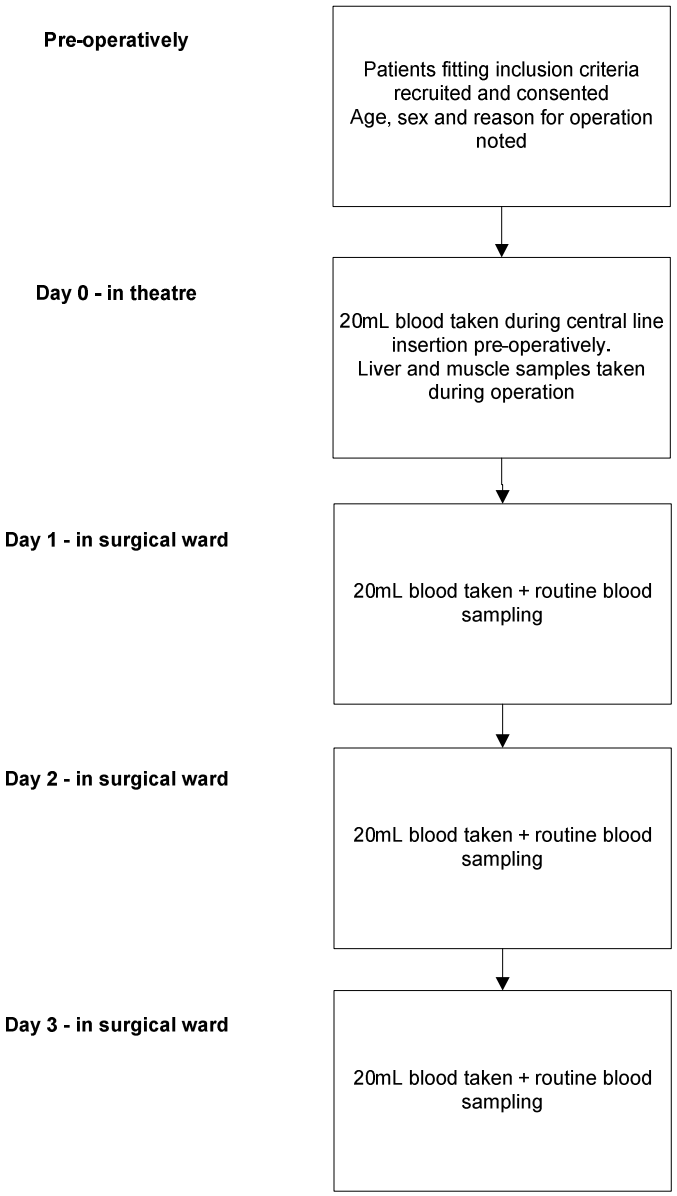



**Figure 5.1** Summary of protocol

## 5.5 Processing and storage of samples

Tissue samples were stored at -50˚C in the universal container they were collected in.

The sodium heparin blood samples were separated by centrifugation within 4 hours of collection. The plasma was removed and separated into four aliquots. The buffy coat was carefully removed by pipette leaving the red blood cells in the original blood tube. Red blood cell and plasma samples were stored at -50˚C.

Serum samples for biochemistry and EDTA whole blood for haematology were processed as part of the routine patient care.

## 5.6 Statistical Methods

All statistical analyses were performed using Minitab Version 15 (Microsoft).

Statistical methods are described throughout the results section and briefly here.

Given the small sample size and differences in numbers of samples each day, nonparametric methods of comparison were used when possible.

### *5.6.1 Normality of data*

The Anderson-Darling test of normality was used to assess the normality of results data with $p<0.05$ taken to indicate non-normal distribution.

### *5.6.2 Comparisons between groups*

Kruskal-Wallis non-parametric one-way analysis of variance was used to make comparisons between groups with $p<0.05$ taken to indicate statistical significance.

Where a difference between groups was found, Mann-Whitney comparisons were performed it identify which group or groups were different. The Bonferroni correction was used to correct the p-value for multiple comparisons (n) and $p=(0.05/n)$ was used to indicate statistical significance.

True (non-transformed) data was used for these analyses.

### *5.6.3 Relationship between two variables*

To assess possible linear relationships between two variables, simple linear regression was used with $p<0.05$ taken to indicate statistical significance.

Transformed data was used to fulfil the underlying assumption of the data being normally distributed.

# Chapter 6

## Participant Demographics and baseline results

Throughout the results sections, “day 0” will refer to the sample taken immediately pre-operatively, and “day 1”, “day 2” and “day 3” refer to 24, 48 and 72 hours post operatively respectively.

## 6.1 Participants

### *6.1.1 Participant Demographics*

31 participants were recruited who were having liver resections between April 2008 and July 2009. There were 14 males and 17 females. Mean age was 64 (range 35-79) years. 26 patients had liver resections due to metastatic colorectal cancer, 1 because of metastatic breast cancer, 2 because of hydatid cysts and 2 had liver resections due to radiological liver lesions and previous cancers but infact had normal histopathology.

None of the participants had a blood transfusion in the 3 months prior to the operation or in the 3 day post-operatively.

All of the participants, with the exception of the two with hydatid cysts, had bowel preparation prior to surgery. This consisted of 4 sachets of Klean-Prep (polyethylene gycol) reconstituted with 4 litres of water taken the day prior to surgery.

Age, sex and reason for operation for each participant can be found in appendix 3, alongside baseline biochemistry results.

### *6.1.2 Numbers of samples available at each time point*

CRP and Albumin were available for all participants on days 0-3. Liver and Muscle samples were available for all participants on days 0. Plasma and red cell trace metal samples were available for all participants on day 0, 22 participants on day 1, 15 participants on day 2 and 10 participants on day 3. Appendix 3 contains details of which samples were available for which participants.

## 6.2 C-Reactive Protein

### *6.2.1 Normality of data*

To ensure the normality of the data and exclude any outlying samples, the Anderson-Darling test for normality was performed. The results are summarised below and further details and probability plots can be found in appendix 4.

Serum CRP Day 0 (pre-operative sample) was found to not be normally distributed ($p<0.005$) and was therefore logarithmically transformed to achieve normality ($p=0.132$). Post-operatively, CRP on day 1, CRP on day 2, and CRP on day 3 were all found to be normally distributed ($p=0.849$, 0.635, 0.907 respectively).

In order to allow pooling of results, it would be ideal to log transform all CRP data; unfortunately logarithmic transformation of day 2 and 3 rendered them non-normal (day 1 $p=0.356$, day 2 $p<0.005$, day 3 $p=0.015$) so this was not done.

Transformed data was used for linear regression analysis only.

### *6.2.2 Change in CRP with the acute phase response*

The CRP increases during the acute phase response with the peak CRP seen on day 2. The median CRP and the interquartile range from Day 0-3 can be seen in table 6.1 and also graphically in figure 6.1.

Kruskal-Wallis (non-parametric) one way analysis of variance was performed to see if CRP concentration varied during the acute phase response; it was found to vary significantly ($p<0.001$).

Mann-Whitney comparisons were performed to assess the significance of change each day (Bonferroni correction $p=0.017$ is significant): day 0-1 $p<0.001$, day 1-2 $p<0.001$, day 2-3 $p=0.430$. Therefore, a significant increase in CRP occurs between day 0 and day 1, and day 1 and day 2.

| | Median CRP (mg/L) (IQR) |
|---|---|
| Day 0 | 5.0 (1.3-7.4) |
| Day 1 | 95 (69-122) |
| Day 2 | 164 (137-217) |
| Day 3 | 154 (114-207) |

**Table 6.1** Median (IQR) values of CRP (mg/L) on days 0-3

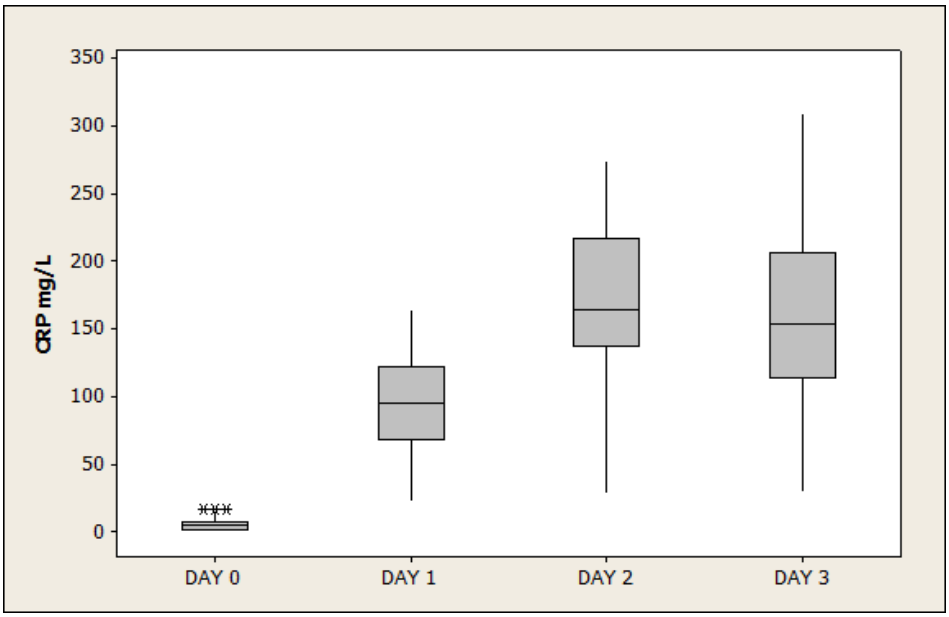



**Fig 6.1** Boxplot of median and interquartile range of CRP (mg/L) on Day 0-3

## 6.3 Albumin

### *6.3.1 Normality of data*

Using the Anderson-Darling test for normality, Albumin Day 0, Day 1, Day 2 and Day 3 were found to all be all normally distributed (p=0.306, 0.177, 0.267, 0.135 respectively). Further details and probability plots can be found in appendix 4.

*6.3.2 Change in Albumin with the acute phase response*

Serum Albumin falls with the acute phase response and has not started to rise by day 3. The change in median Albumin and the interquartile range from Day 0-3 can be seen in table 6.2 and also graphically in figure 6.2.

Kruskal-Wallis (non-parametric) one way analysis of variance was performed to see if albumin concentration varied during the acute phase response; it was found to vary significantly ($p<0.001$).

Mann-whitney comparisons were performed to assess the significance of change each day (Bonferroni correction $p=0.017$ is significant): day 0-1 $p<0.001$, day 1-2 $p=0.136$, day 2-3 $p=0.075$. Therefore, the significant decrease in serum albumin occurs between day 0 and day 1.

| | Median Albumin (g/L) (IQR) |
|---|---|
| **Day 0** | 38 (36-41) |
| **Day 1** | 28 (26-32) |
| **Day 2** | 27 (26-30) |
| **Day 3** | 26 (23-29) |

**Table 6.2** Median (IQR) values of albumin (g/L) on days 0-3

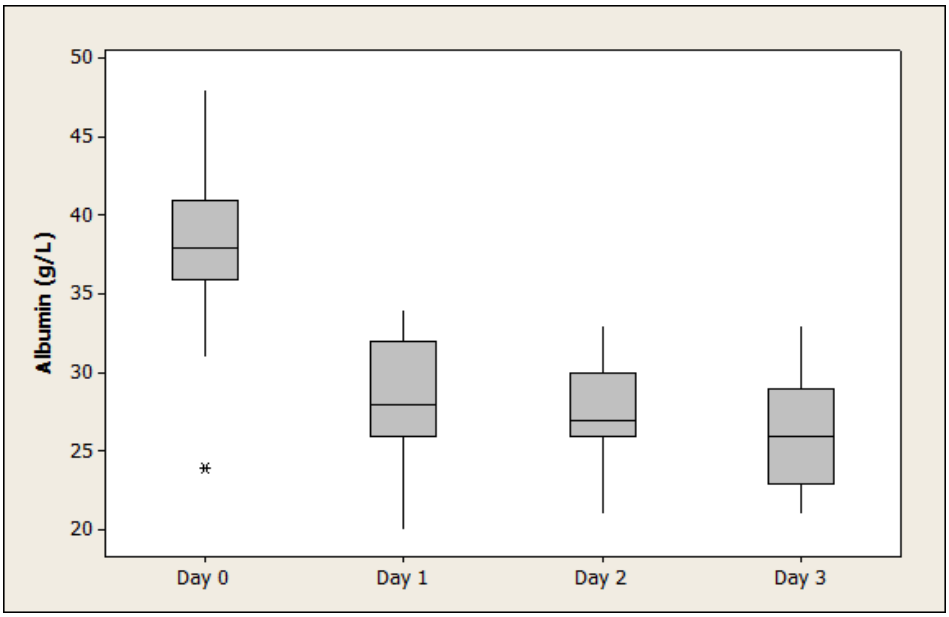



**Fig 6.2** Boxplot of median and interquartile range of Albumin (g/L) on Day 0-3.

## 6.4 Haematocrit

### *6.4.1 Normality of data*

Using the Anderson-Darling test for normality, haematocrit on day 0 was normally distributed (p=0.760). Examination of probability plots for haematocrit on day 1, 2 and 3 revealed outlying samples: day 1 samples 8, 10 and 21; day 2 samples 10, 18 and 22; day 3 sample 10. With these removed all samples were normally distributed: day 0 p=0.760, day 1 p=0.208, day 2 p=0.322 and day 3 p=0.410. Further details and probability plots can be found in appendix 4.

### *6.4.2 Change in Haematocrit with the acute phase response*

Haematocrit falls from day 0 to day 1-3 – this may be due to the acute phase response or intravenous fluid therapy. The change in median haematocrit and the interquartile range from Day 0-3 can be seen in table 6.3 and also graphically in figure 6.3.

Kruskal-Wallis (non-parametric) one way analysis of variance was performed to see if haematocrit concentration varied during the acute phase response; it was found to vary significantly ($p<0.001$).

Mann-Whitney comparisons were performed to assess the significance of change each day (Bonferroni correction p=0.017 is significant): day 0-1 $p<0.001$, day 1-2 p=0.108, day 2-3 p=0.301. Therefore, the significant decrease in haematocrit occurs between day 0 and day 1.

| | Median haematocrit (i/L) (IQR) |
|---|---|
| **Day 0** | 0.391 (0.368-0.419) |
| **Day 1** | 0.332 (0.309-0.356) |
| **Day 2** | 0.322 (0.298-0.338) |
| **Day 3** | 0.312 (0.293-0.330) |

**Table 6.3** Median (IQR) values of haematocrit (i/L) on days 0-3

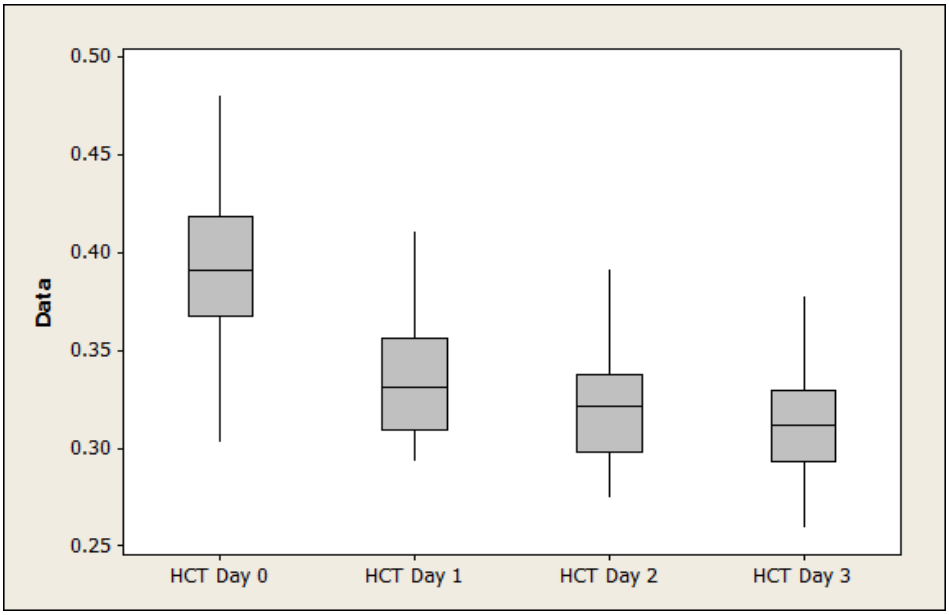



**Fig 6.3** Boxplot of median and interquartile range of haematocrit (i/L) on Day 0-3.

## 6.5 The relationship between changes in albumin and haematocrit

Changes in haematocrit are due to dilution from fluid retention by the body as a physiological part of the acute phase response, and possibly the effect of high volumes of intravenous fluids given post-operatively. Changes in albumin may also be a dilution effect, or may be due to the albumin moving from the intravascular to the extracellular compartments.

To investigate this the percentage change in the haematocrit from day 0 to day 1-3 was compared to the percentage change in albumin concentration from day 0 to day 1-3 using simple linear regression (table 6.4 and fig 6.4).

There is a linear relationship between the percentage change in haematocrit from day 0 and the percentage change in albumin for day 0, on day 1-3. However, between 55-65% of the variance in serum albumin is not accounted for by change in haematocrit, suggesting that the change in serum albumin concentration may be due to more than simple dilution, perhaps including redistribution to the extracellular compartment.

As dilution appears to play a role in the change in serum albumin concentration, metal: albumin ratios shall be examined where appropriate to correct for these changes.

| | **% change in haematocrit Vs % change in albumin** | |
|---|---|---|
| | $r^2$(%) | p value |
| Day 1 | 44.4 | <0.001 |
| Day 2 | 35.0 | 0.001 |
| Day 3 | 34.9 | 0.001 |

**Table 6.4** Relationship between % change in haematocrit and % change serum albumin concentration from day 0 on days 1-3, using simple linear regression

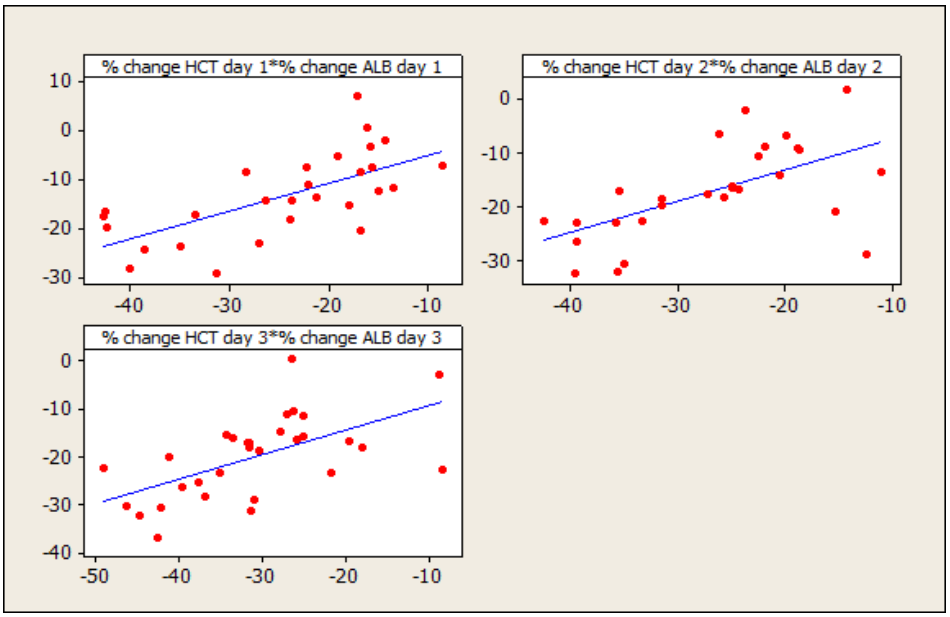



**Fig 6.4** Scatterplot of % change in haematocrit versus % change serum albumin concentration from day 0 on days 1-3

## 6.6 Copper

### *6.6.1 Normality of data and outliers*

The Anderson-Darling test for normality was performed to ensure the normality of the data and exclude any outlying samples. The results are summarised below and further details and probability plots can be found in appendix 4.

Liver copper concentrations were found to be normally distributed (p=0.100).

Plasma copper concentrations on days 0, 2 and 3 were normally distributed (p=0.661, 0.132 and 0.782 respectively). Plasma copper concentration on day 1 was not normally distributed (p=0.015). After logarithmic transformation, days 0-3 were all normally distributed (p=0.595, 0.097, 0.390 and 0.576 respectively).

Plasma ferroxidase activity was non-normal on day 0 (p=0.018). An outlier (study number 18) was identified and removed from future analyses. After this removal, plasma ferroxidase activities on days 0-3 were all normally distributed (p=0.656, 0.411, 0.874 and 0.828 respectively).

Initial examination of probability plots for plasma ceruloplasmin concentration showed outlying samples on day 0 (study number 21) and day 1 (study number 1); these were removed from further analyses. Day 2 was seen to be non-normally distributed (p=0.045). Logarithmic transformation and removal of outliers resulted in days 0-3 all being normally distributed (p=0.6, 0.599, 0.118 and 0.804 respectively).

Day 0 and day 1 red blood cell copper concentrations were non-normally distributed (both $p<0.005$). As the data was right-skewed it was logarithmically transformed, but day 0 and day 1 were still non-normal ($p<0.005$). Three high concentration outlying samples from day 0 (study numbers 11, 20, and 26) and one high concentration outlying sample (study number 26) from day 1 were therefore removed from further analyses and the distribution was normalised ($p=0.280$ and $p=0.933$ respectively).

Transformed data was used for linear regression analysis only.

### *6.6.2 Copper status of the study population at baseline*

As plasma copper is routinely measured in our laboratory, a reference interval of 10-22µmol/L is in place. The plasma copper status of the study population at baseline was investigated. Median plasma copper concentration was 15.1 µmol/L (inter-quartile range 12.6-16.5). The range of plasma copper concentrations, and the relationship with the reference interval, can be seen in figure 6.5.

Within the study population, the majority of samples fall within our laboratory reference interval with only one sample below (9 µmol/L) and one above (24 µmol/L).

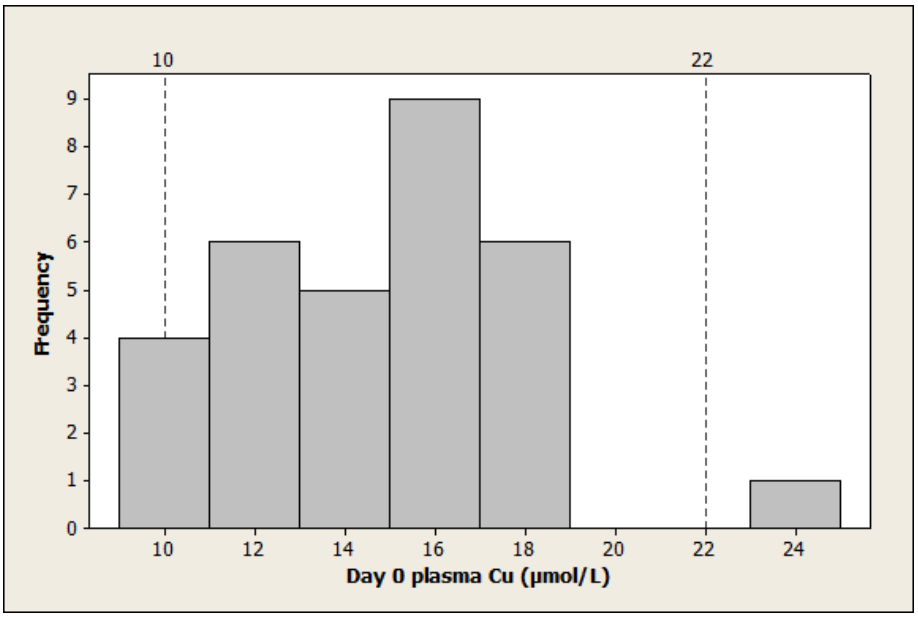



**Fig 6.5** Histogram of plasma copper concentration (µmol/L) on day 0. Upper (22 µmol/L) and lower (10 µmol/L) limits of the reference interval are marked by dotted lines.

*6.6.3 The contribution of baseline CRP to plasma copper concentration*

To investigate if the participants with a higher plasma copper concentration had a higher CRP, the data was split into tertiles of day 0 CRP: tertile 1 0.5-2.2 g/L, tertile 2 3.0-6.8 g/L, tertile 3 7.0-17.0 g/L.

Median (IQR) of plasma copper by tertile of CRP was: tertile 1 12.9 µmol/L (11.5-15.5), tertile 2 13.4 µmol/L (11.7-15.6), tertile 3 16.9 µmol/L (15.7-17.8); this is shown in fig 6.6. Kruskal-Wallis (non-parametric) one way analysis of variance was performed and a difference in plasma copper concentration was found between the three tertiles (p=0.019). Mann-Whitney comparisons were performed (Bonferroni correction p=0.017 is significant): there was no difference in copper concentration between tertile 1 and 2 (p=0.699) but there was a difference between tertile 1 and tertile 3 (p= 0.011). The difference between tertile 2 and tertile 3 was close to statistical significance (p=0.027). Therefore

the higher plasma copper concentrations seen at baseline in the study participants are related to differences in CRP concentration. The relationship between plasma copper concentration and CRP are discussed in section 8.3.

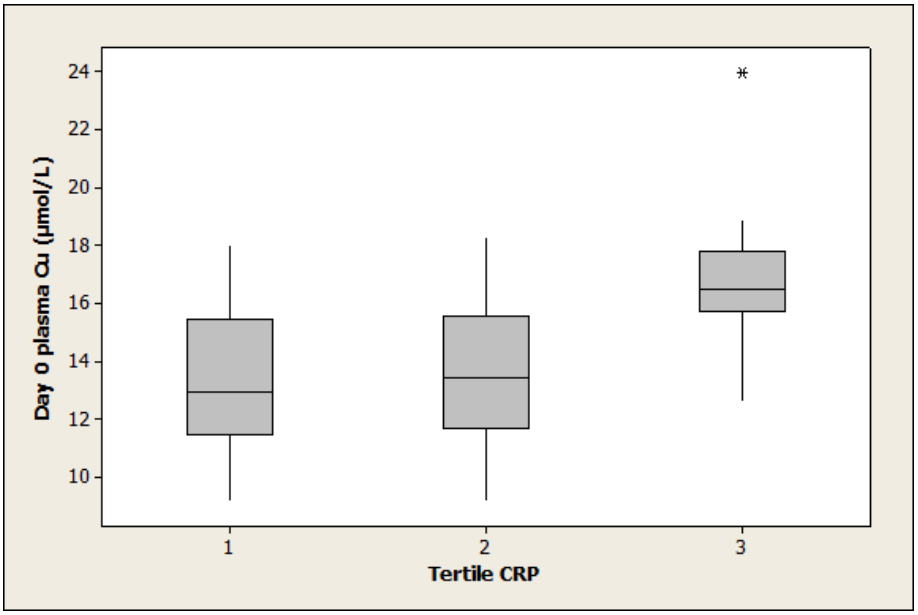



**Fig 6.6** Boxplot of plasma copper concentration (µmol/L) by tertile of CRP

## 6.7 Zinc

### *6.7.1 Normality of data and outliers*

To ensure the data followed a normal distribution to allow statistical analyses to take place, the Anderson-Darling normality test was performed. The results are summarised below and further details and probability plots can be found in appendix 4.

Muscle zinc concentrations (µg/g dry weight) were found to be non-normally distributed ($p<0.005$) therefore the two outlying data points (samples 13 and 29) were removed for the zinc statistical analyses. With outliers removed the muscle zinc was normally distributed ($p=0.14$).

Analysis of a probability plot of plasma zinc concentration (µg/L) on Day 0 revealed an outlying sample (sample 10) and this was also removed from further statistical analysis of zinc results. After this removal the plasma zinc concentration on Day 0 was normally distributed (p=0.727). Plasma zinc concentration (µg/L) on day 1, 2 and 3 all had a normal distribution (p=0.642, 0.778, 0.965 respectively).

Liver zinc concentrations (µg/g dry weight) were normally distributed (p=0.915).

RBC zinc concentrations (µg/ µg Fe) on day 0-3 were all normally distributed (p=0.715, 0.532, 0.123, 0.433 respectively).

*6.7.2 Zinc status of the study population at baseline*

As plasma zinc is routinely measured in our laboratory, a reference interval of 12-18 µmol/L is in place. The plasma zinc status of the study population at baseline was investigated. Median plasma zinc concentration was 11.6 µmol/L (inter-quartile range 10.6-12.8). The range of plasma zinc concentrations, and the relationship with the reference interval, can be seen in figure 6.7.

Within the study population, 16 samples fall below our laboratory reference interval with the rest of the samples all being at the lower end of the reference interval.

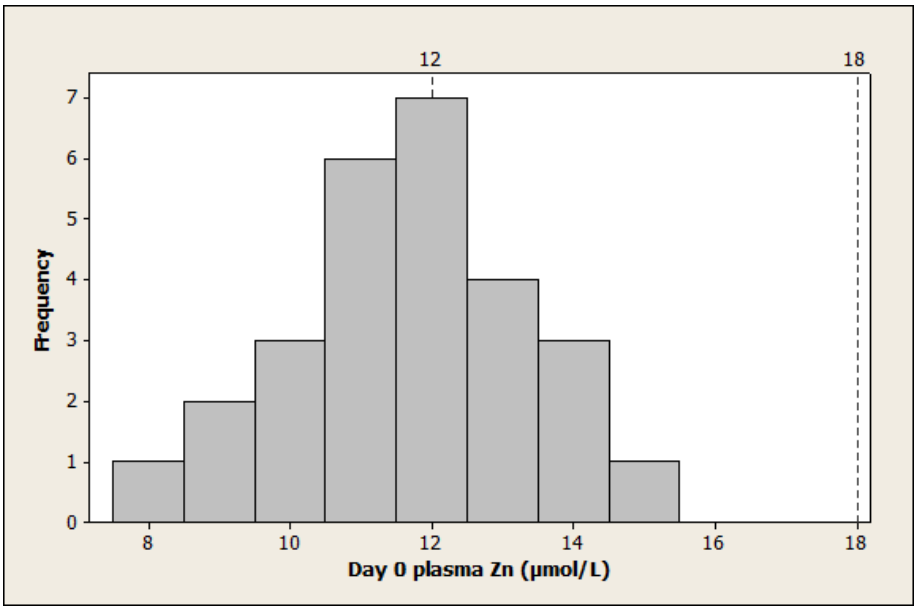



**Fig 6.7** Histogram of plasma zinc concentration (µmol/L) on day 0. Upper (18 µmol/L) and lower (12 µmol/L) limits of the reference interval are marked by dotted lines.

*6.7.3 The contribution of baseline CRP to plasma zinc concentration*

To investigate if the participants with a lower plasma zinc concentration had a higher CRP, the data was split into tertiles of day 0 CRP: tertile 1 0.5-2.2 g/L, tertile 2 3.0-6.8 g/L, tertile 3 7.0-17.0 g/L.

Median (IQR) of plasma zinc by tertile of CRP was: tertile 1 11.4 µmol/L (10.6-12.4), tertile 2 11.3 µmol/L (9.8-13.0), tertile 3 12.0 µmol/L (10.9-13.8); this is shown in fig 6.8. Kruskal-Wallis (non-parametric) one way analysis of variance was performed and no difference in plasma zinc concentration was found between the three tertiles (p=0.436). Therefore the low plasma zinc concentrations seen at baseline in the study participants are not due to differences in CRP concentration.

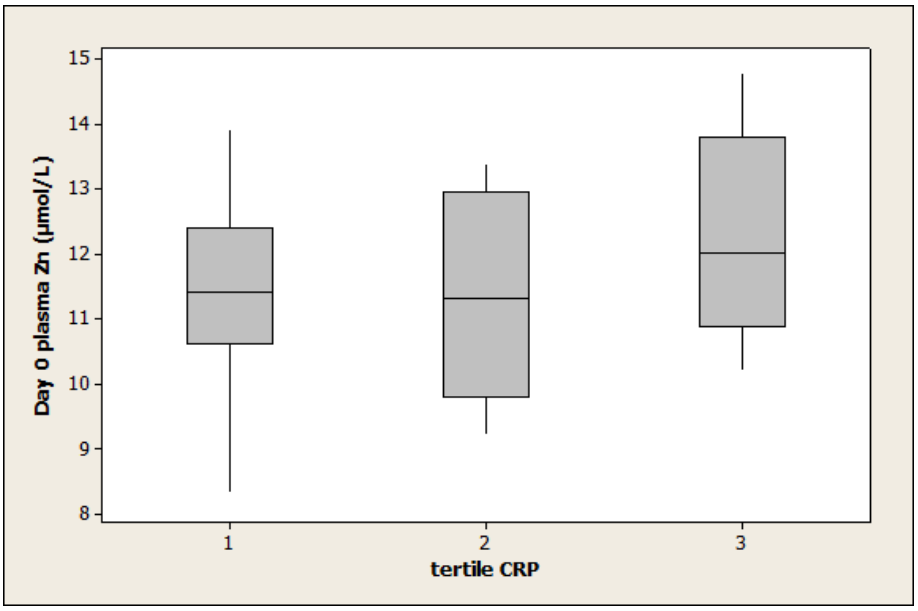



**Fig 6.8** Boxplot of plasma zinc concentration (µmol/L) by tertile of CRP

## 6.8 Selenium

### *6.8.1 Normality of data and outliers*

The Anderson-Darling test for normality was performed to ensure the normality of the data and exclude any outlying samples. The results are summarised below and further details and probability plots can be found in appendix 4.

Liver selenium concentrations were found to be normally distributed (p=0.232).

The Anderson-Darling normality test was performed on plasma selenium concentrations on day 0-3 and outlying samples were identified. Outlying samples (day 0 sample 10, day 1 samples 20 and 29) were removed and all plasma selenium concentrations on day 0-3 were normally distributed; day 0 p=0.726, day 1 p=0.522, day 2 p=0.891, day 3 p=0.559.

Examination of probability plots of red blood cell selenium concentration on days 0-3 revealed outlying samples. Sample 20 on day 0, 1 and 2 was removed from analysis and red blood cell selenium concentration was then normally distributed; day 0 p=0.479, day 1 p=0.625, day 2 p=0.233, day 3 p=0.257.

Red blood cell glutathione peroxidase was normally distributed on day 0-3; day 0 p=0.353, day 1 p=0.702, day 2 p=0.748, day 3 p=0.391.

### *6.8.2 Selenium status of the study population at baseline*

As plasma selenium is routinely measured in our laboratory, a reference interval of 0.8-2.0 μmol/L is in place. The plasma selenium status of the study population at baseline was investigated. Median plasma selenium concentration was 0.96 μmol/L (inter-quartile range 0.80-1.09). The range of plasma copper concentrations, and the relationship with the reference interval, can be seen in figure 6.9.

Within the study population, 6 samples fall below our laboratory reference interval with the rest of the samples all being at the lower end of the reference interval.

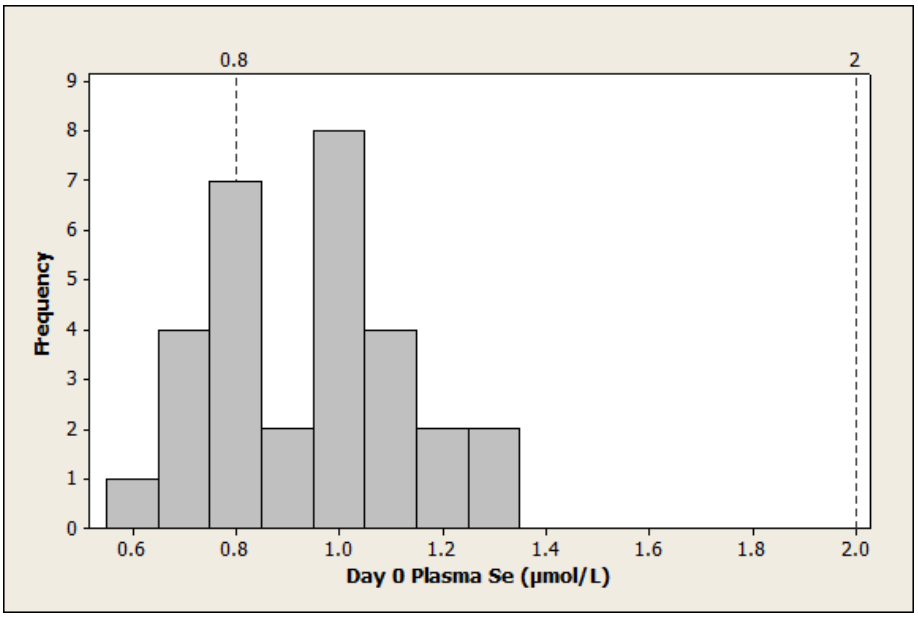



**Fig 6.9** Histogram of plasma selenium concentration (µmol/L) on day 0. Upper (2.0 µmol/L) and lower (0.8 µmol/L) limits of the reference interval are marked by dotted lines.

*6.8.3 The contribution of baseline CRP to plasma selenium concentration*

To investigate if the participants with a lower plasma selenium concentration had a higher CRP, the data was split into tertiles of day 0 CRP: tertile 1 0.5-1.9 g/L, tertile 2 2.2-6.8 g/L, tertile 3 7.0-17.0 g/L.

Median (IQR) of plasma selenium by tertile of CRP was: tertile 1 0.96 µmol/L (0.71-1.13), tertile 2 0.80 µmol/L (0.84-1.12), tertile 3 0.98 µmol/L (0.83-1.04); this is shown in fig 6.10. Kruskal-Wallis (non-parametric) one way analysis of variance was performed and no difference in plasma selenium concentration was found between the three tertiles (p=0.991). Therefore the low plasma selenium concentrations seen at baseline in the study participants are not due to differences in CRP concentration.

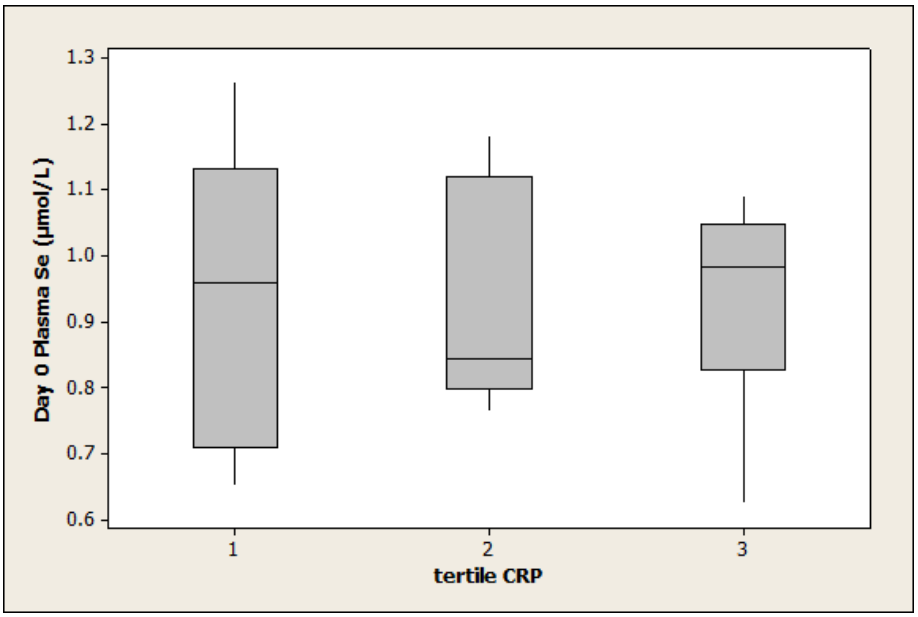



**Fig 6.10** Boxplot of plasma selenium concentration (µmol/L) by tertile of CRP

## Chapter 7

## The Relationship between blood and tissue concentrations at baseline

### 7.1 Copper

The relationship between blood concentrations of copper, ceruloplasmin concentration, ferroxidase activity and tissue concentrations of copper at baseline, when the subject has a low C-reactive peptide and is non-inflamed, allows recommendations to be made as to whether plasma or red blood cell copper concentrations are a reliable measure of tissue status in the non-inflamed patient. Therefore, simple linear regression was performed to examine the relationship between red blood cell, plasma, and liver copper concentrations, along with ceruloplasmin concentration and ferroxidase activity. The use of copper: ceruloplasmin ratios allows the relationship between copper concentration, ceruloplasmin concentration and the concentrations of other potential markers of copper status to be examined while correcting for any effect of plasma dilution due to intravenous fluid replacement given to the patient post-operatively.

Table 7.1 shows the strength of linear association between each of the copper measures.

| | $r^2$ (p value) | | | | | |
|---|---|---|---|---|---|---|
| | Liver Cu | Log red blood cell Cu | Log plasma Cu | Log Cp | Log Plasma Cu:Log Cp | Ferroxidase Activity |
| Liver Cu | | **15.9% (0.036)** | 4.3% (0.264) | 7.3% (0.142) | 2.9% (0.357) | 0.6% (0.691) |
| Log red blood cell Cu | **15.9% (0.036)** | | 1.7% (0.515) | 8.7% (0.128) | 3.2% (0.341) | 0.0% (0.926) |
| Log Plasma Cu | 4.3% (0.264) | 1.7% (0.515) | | **33.5% (0.001)** | **56.5% (<0.001)** | 3.3% (0.334) |
| Log Cp | 7.3% (0.142) | 8.7% (0.128) | **33.5% (0.001)** | | **94.4% (<0.001)** | 1.6% (0.501) |
| Log Plasma Cu:Log Cp | 2.9% (0.357) | 3.2% (0.341) | **56.5% (<0.001)** | **94.4% (<0.001)** | | 10% (0.102) |
| Ferroxidase Activity | 0.6% (0.691) | 0.0% (0.926) | 3.3% (0.334) | 1.6% (0.501) | 10% (0.102) | |

**Table 7.1** Results of linear regression analysis of each copper measure and its association with other copper measures. Values shown are $r^2$ (p value for significance). Significant results are highlighted in bold type. (Cp= ceruloplasmin).

Scatterplots can be seen at the end of this chapter, showing the relationship between each of the other copper measures and liver copper concentration (fig 7.1a-e), red blood cell copper concentration (fig 7.2a-d), plasma copper concentration (fig 7.3a-c), plasma ceruloplasmin concentration (figure 7.4a-b), plasma copper: ceruloplasmin ratio (figure 7.5).

The only linear relationship that exists between tissue and blood measures of copper is between liver copper concentration and red blood cell copper concentration ($r^2$=15.9%, p=0.036). Plasma copper concentration does not have a linear relationship with liver copper concentration ($r^2$=4.3%, p=0.264).

There is a linear relationship between plasma copper and plasma ceruloplasmin concentration ($r^2$=33.5%, p=0.001), which is unsurprising as ceruloplasmin is the main copper carrying protein in blood.

## 7.2 Zinc

Examination of the relationship between blood concentrations of zinc and tissue concentrations of zinc at baseline (day 0), when the subject has a low C-reactive peptide and is not inflamed, allows recommendations to be made as to whether plasma or red blood cell zinc concentrations are a reliable measure of tissue status in a patient without inflammation. Therefore, simple linear regression was performed to examine the relationship between red blood cell, plasma, liver and muscle zinc concentrations. Table 7.2 shows the strength of linear association between each of the zinc measures.

| | $r^2$ (p value) | | | |
|---|---|---|---|---|
| | Liver Zn | Red blood cell Zn | Plasma Zn | Muscle Zn |
| Liver Zn | | 5.3% (0.240) | **14.4% (0.046)** | 3.6% (0.339) |
| Red blood cell Zn | 5.3% (0.240) | | 0.1% (0.896) | 7.4% (0.161) |
| Plasma Zn | **14.4% (0.046)** | 0.1% (0.896) | | 0% (0.924) |
| Muscle Zn | 3.6% (0.339) | 7.4% (0.161) | 0% (0.924) | |

**Table 7.2** Results of linear regression analysis of each selenium measure and its association with other selenium measures. Values shown are $r^2$ (p value for significance). Statistically significant results are highlighted in bold type.

Scatterplots can be seen at the end of this chapter, showing the relationship between each of the other zinc measures and liver selenium concentration (fig 7.6a-c), red blood cell zinc concentration (fig 7.7a-b) and plasma zinc concentration (fig 7.8).

On day 0 when there is not a significant acute phase response, there is a weak linear relationship between plasma zinc concentration and liver zinc concentration ($r^2$=14.4%, p=0.046); this relationship does not exist between other blood and tissue measures of zinc.

## 7.3 Selenium

The relationship between blood concentrations of selenium and glutathione peroxidase activity and tissue concentrations of selenium at baseline (day 0), when the subject has a low C-reactive peptide and is not inflamed, allows recommendations to be made as to whether plasma or red blood cell selenium concentrations are a reliable measure of tissue status in a patient without inflammation. Therefore, simple linear regression was performed to examine the relationship between red blood cell, plasma, and liver selenium concentrations along with glutathione peroxidase activity. Table 7.3 shows the strength of linear association between each of the selenium measures.

| | $r^2$ (p value) | | | |
|---|---|---|---|---|
| | Liver Se | Red blood cell Se | Plasma Se | RBC Glutathione Peroxidase activity |
| Liver Se | | **17.1% (0.023)** | **43.1% (<0.001)** | **22.6% (0.008)** |
| Red blood cell Se | **17.1% (0.023)** | | **29.7% (0.002)** | **19.1% (0.016)** |
| Plasma Se | **43.1% (<0.001)** | **29.7% (0.002)** | | **33.6% (0.001)** |
| RBC Glutathione Peroxidase activity | **22.6% (0.008)** | **19.1% (0.016)** | **33.6% (0.001)** | |

**Table 7.3** Results of linear regression analysis of each selenium measure and its association with other selenium measures. Values shown are $r^2$ (p value for significance). Statistically significant results are highlighted in bold type.

Scatterplots can be seen at the end of this chapter, showing the relationship between each of the other selenium measures and liver selenium concentration (fig 7.9a-c), red blood cell selenium concentration (fig 7.10a-b), plasma selenium concentration (fig 7.11).

There are linear relationships between liver selenium concentration and red blood cell selenium concentration ($r^2$=17.1%, p=0.023), plasma selenium concentration ($r^2$=43.1%, p<0.001) and red blood cell glutathione peroxidase activity ($r^2$=22.6%, p=0.008). A linear relationship also exists between red blood cell selenium concentration and plasma selenium concentration ($r^2$=29.7%, p=0.002). Both red blood cell selenium concentration and plasma selenium concentration have a linear relationship with red blood cell glutathione peroxidase activity; $r^2$=19.1% (p=0.016) and $r^2$=33.6% (p=0.001) respectively.

*Liver copper*

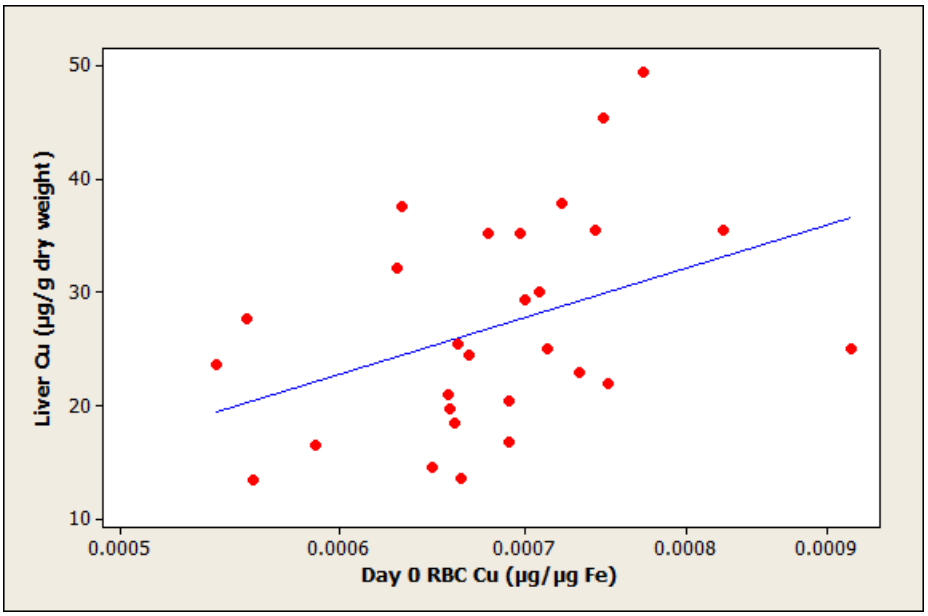



**Fig 7.1a** Scatterplot of liver Cu (µg/g dry weight) versus log red blood cell Cu (µg/µg Fe)

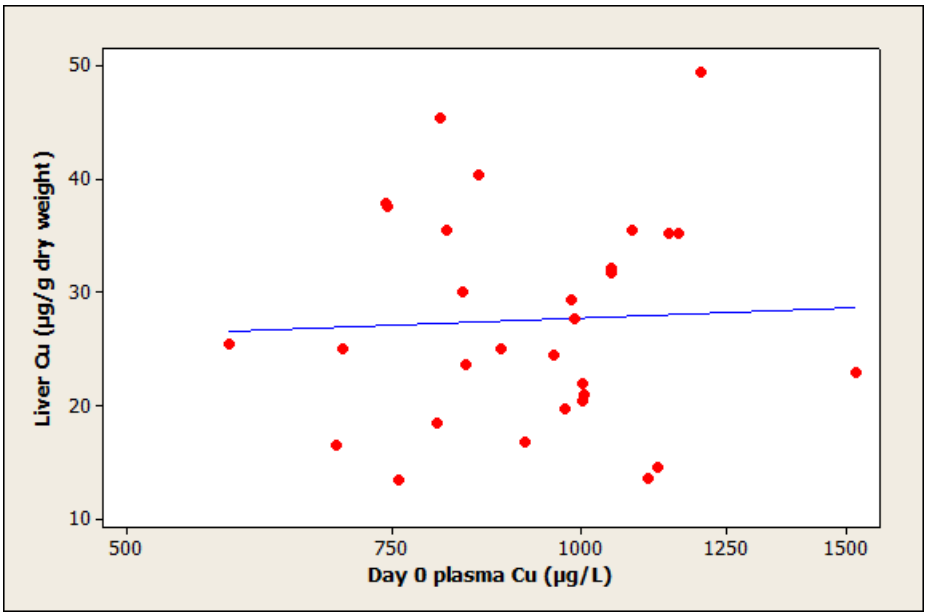



**Fig 7.1b** Scatterplot of liver Cu (µg/g dry weight) versus log plasma Cu (µg/L)

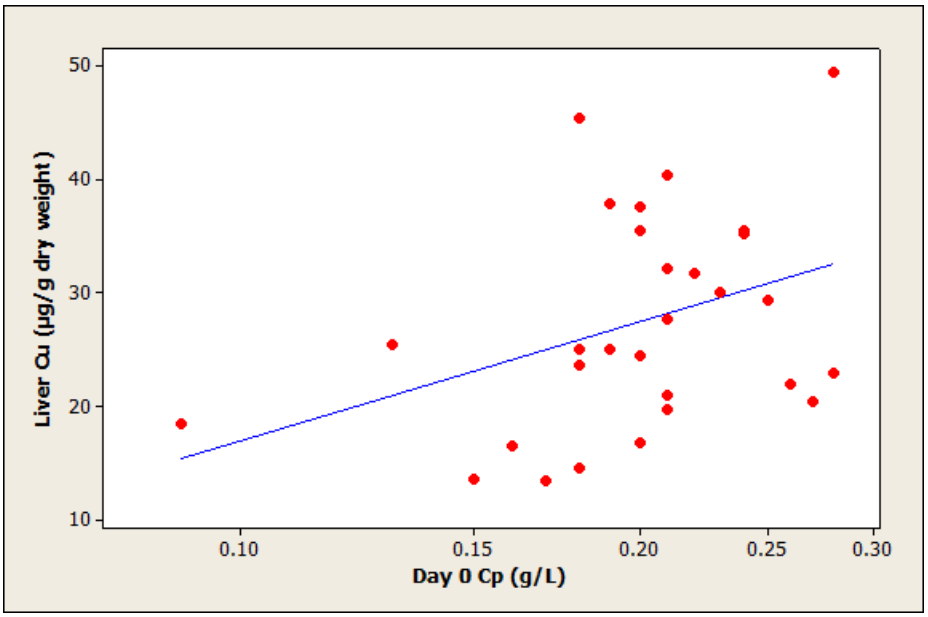



**Fig 7.1c** Scatterplot of liver Cu (µg/g dry weight) versus log plasma Ceruloplasmin (g/L)

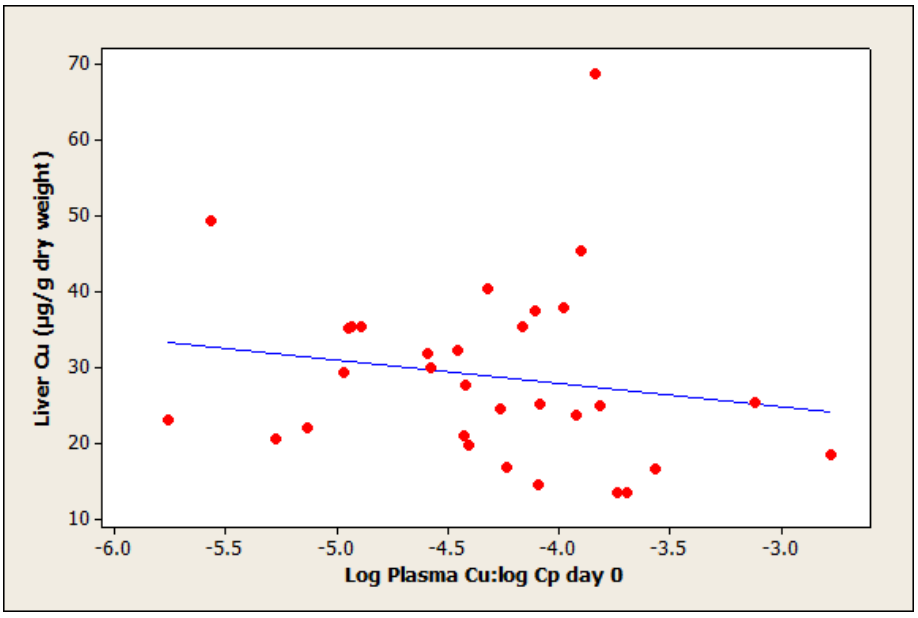



**Fig 7.1d** Scatterplot of liver Cu (µg/g dry weight) versus log plasma copper: log plasma Ceruloplasmin ratio

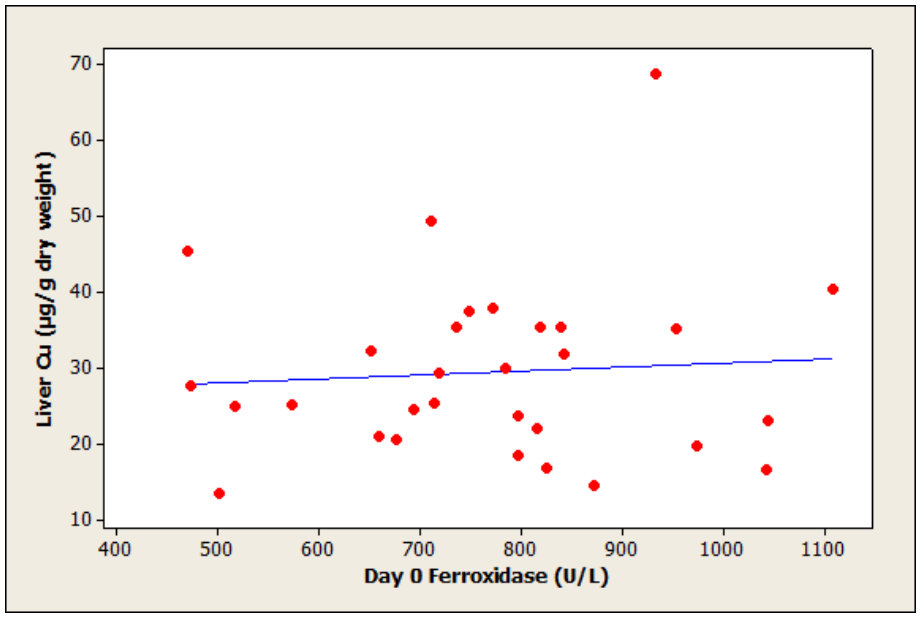



**Fig 7.1e** Scatterplot of liver Cu (µg/g dry weight) versus plasma ferroxidase activity (U/L)

*Red blood cell copper*

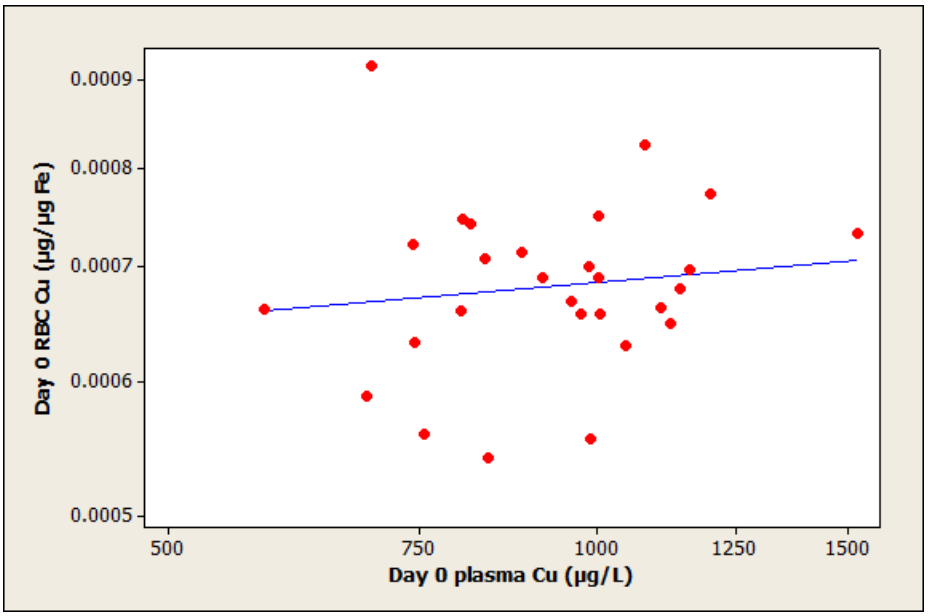



**Fig 7.2a** Scatterplot of log red blood cell Cu (µg/µg Fe) versus log plasma Cu (µg/L)

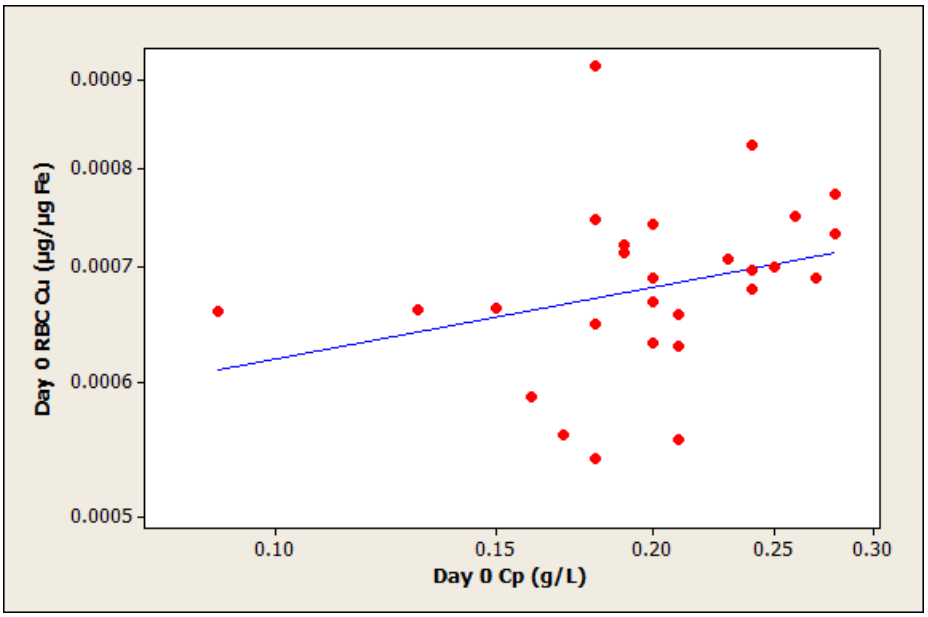



**Fig 7.2b** Scatterplot of log red blood cell Cu (µg/µg Fe) versus log plasma ceruloplasmin (g/L)

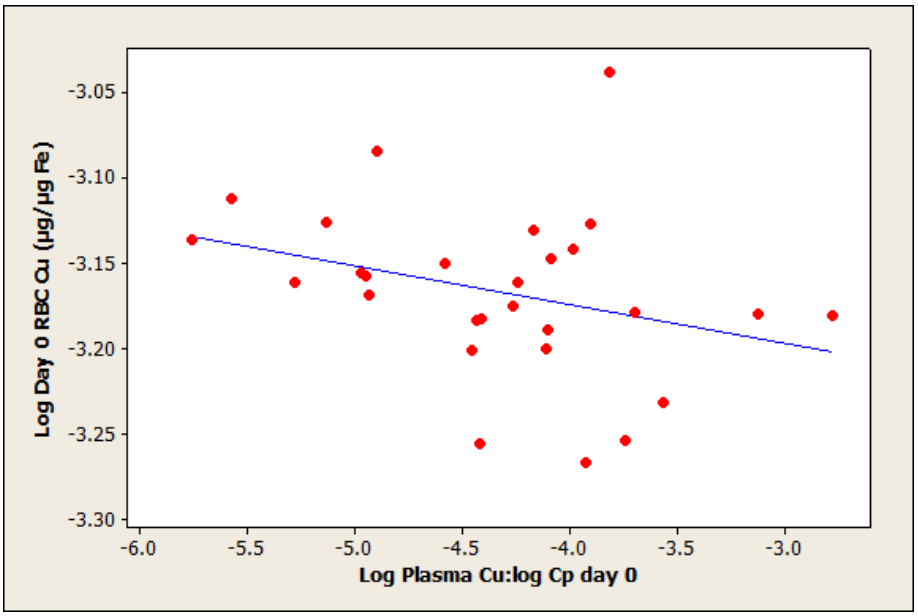



**Fig 7.2c** Scatterplot of log red blood cell Cu (µg/µg Fe) versus log plasma copper: log plasma Ceruloplasmin ratio

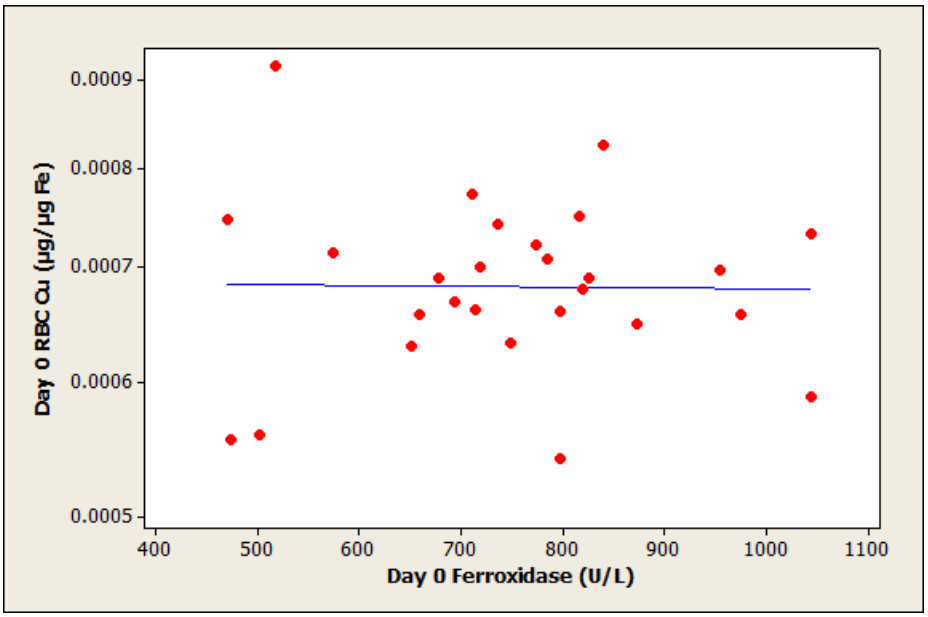



**Fig 7.2d** Scatterplot of log red blood cell Cu (µg/µg Fe) versus plasma ferroxidase activity (U/L)

*Plasma copper*

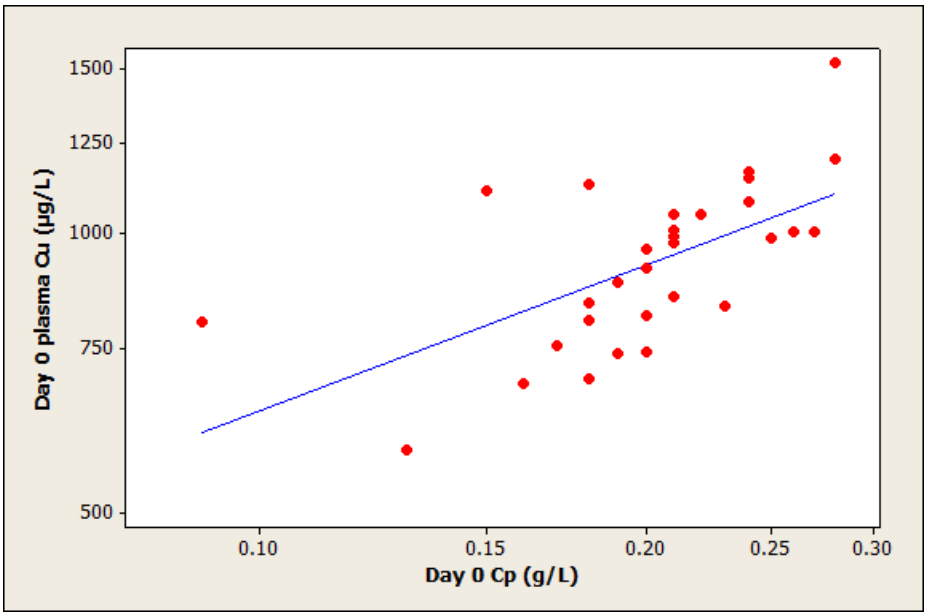



**Fig 7.3a** Scatterplot of log plasma Cu (µg/L) versus log plasma ceruloplasmin (g/L)

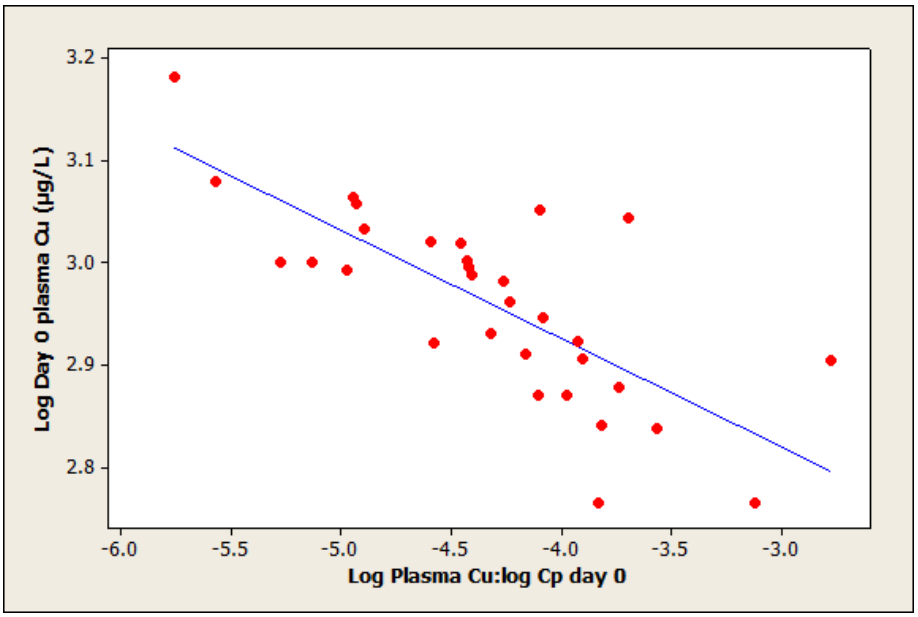



**Fig 7.3b** Scatterplot of log plasma Cu (µg/L) versus log plasma copper: log plasma ceruloplasmin ratio

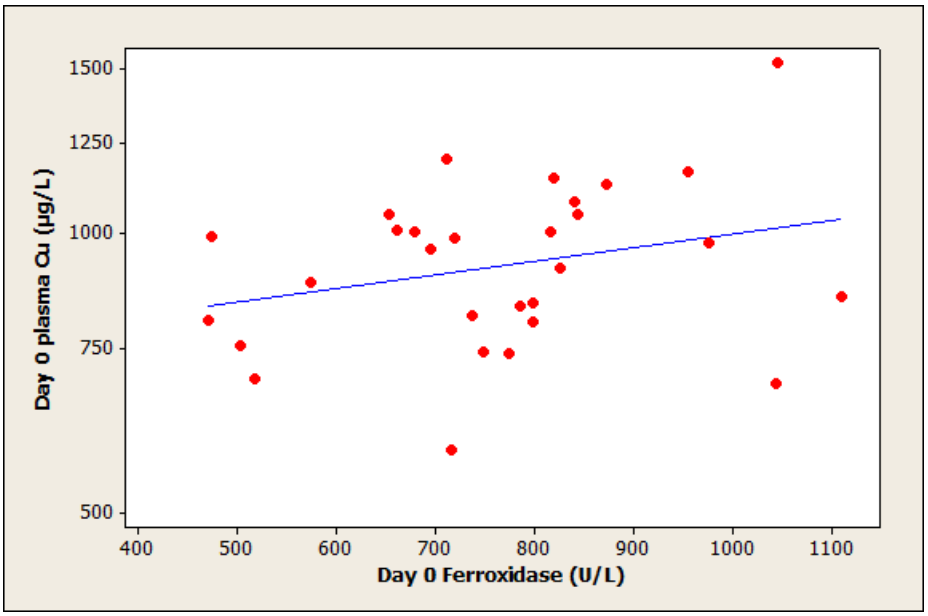



**Fig 7.3c** Scatterplot of log plasma Cu (µg/L) versus plasma ferroxidase activity (U/L)

*Ceruloplasmin*

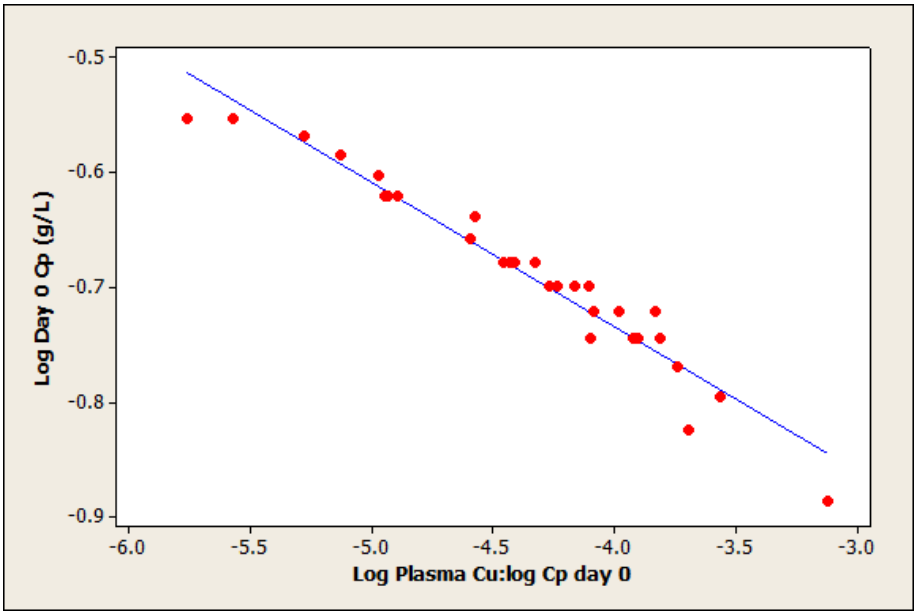



**Fig 7.4a** Scatterplot of log plasma ceruloplasmin (g/L) versus log plasma copper: log plasma ceruloplasmin ratio

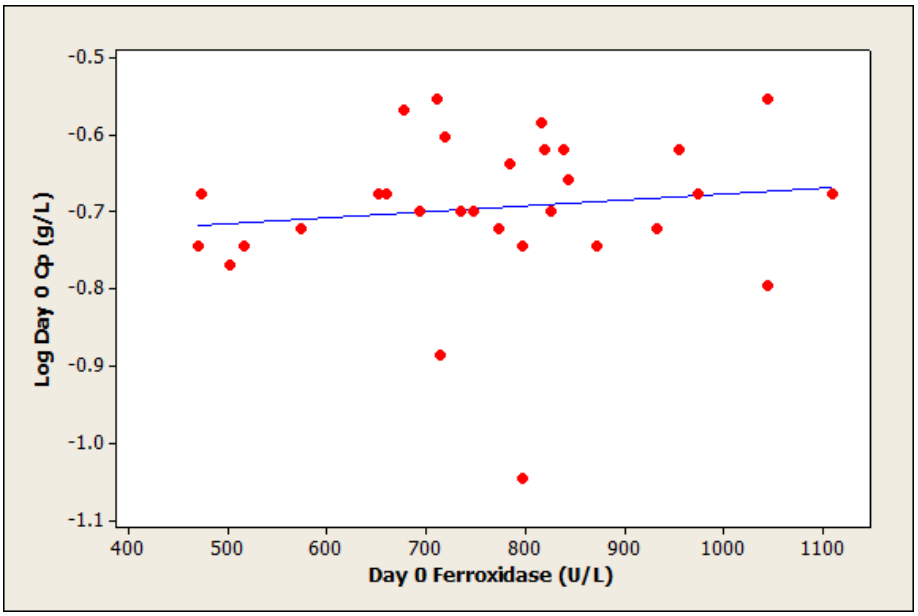



**Fig 7.4b** Scatterplot of log plasma ceruloplasmin (g/L) versus plasma ferroxidase activity (U/L)

*Plasma copper: plasma ceruloplasmin ratio*

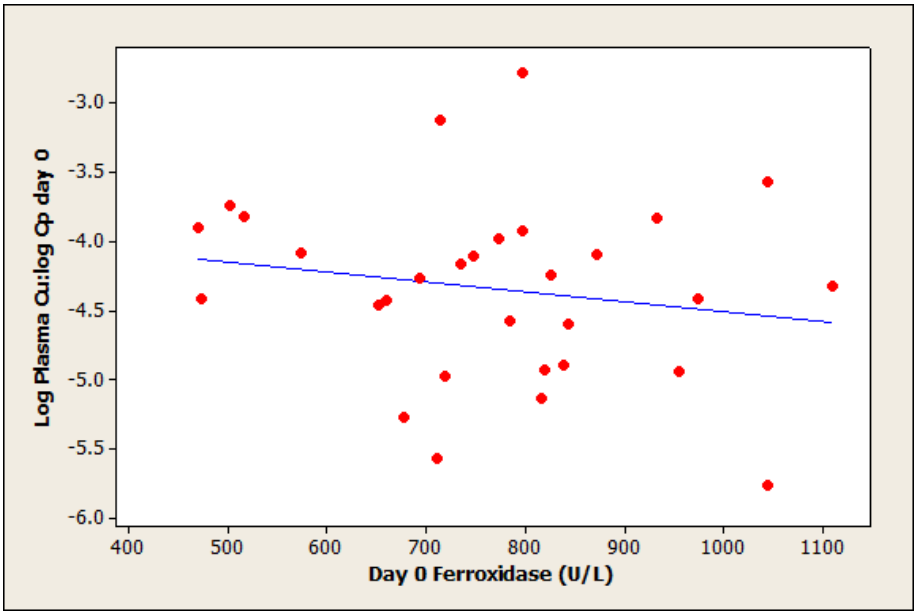



**Fig 7.5** Scatterplot of log plasma ceruloplasmin (g/L) versus plasma ferroxidase activity (U/L)

*Liver zinc*

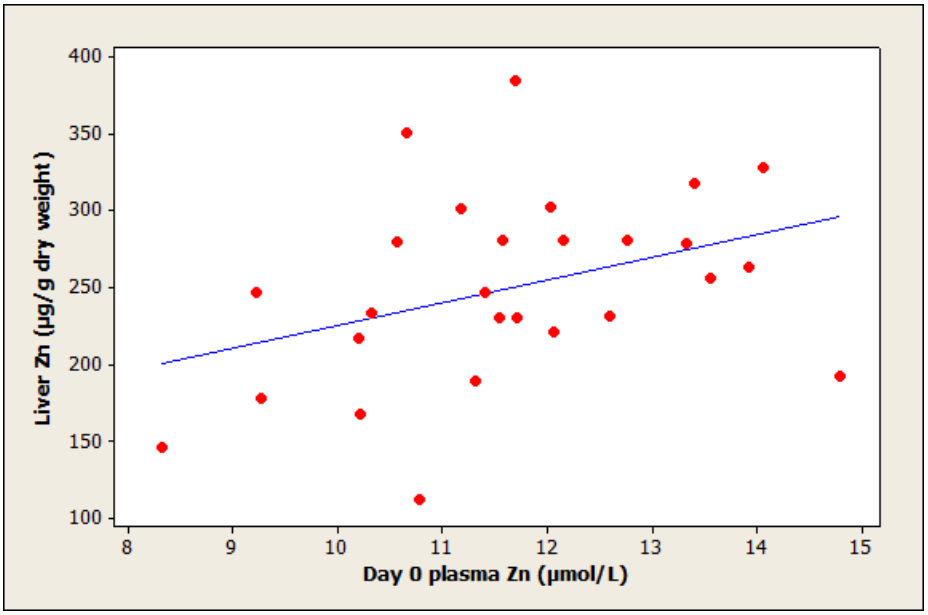



**Fig 7.6a** Scatterplot of Liver Zn (µg/g dry weight) versus Plasma Zn on Day 0 (µmol/L)

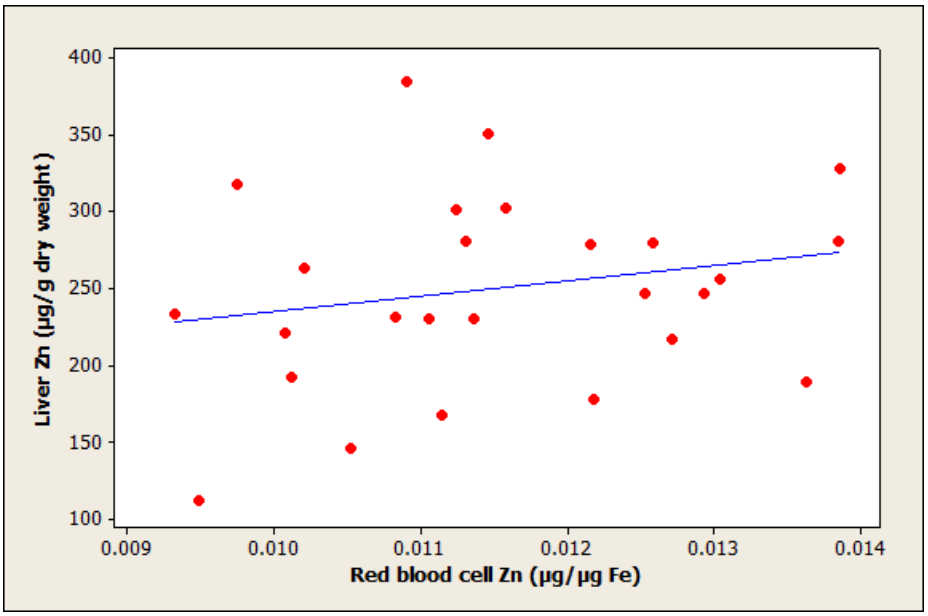



**Fig 7.6b** Scatterplot of Liver Zn (µg/g dry weight) versus RBC Zn on Day 0 (µg/µg Fe)

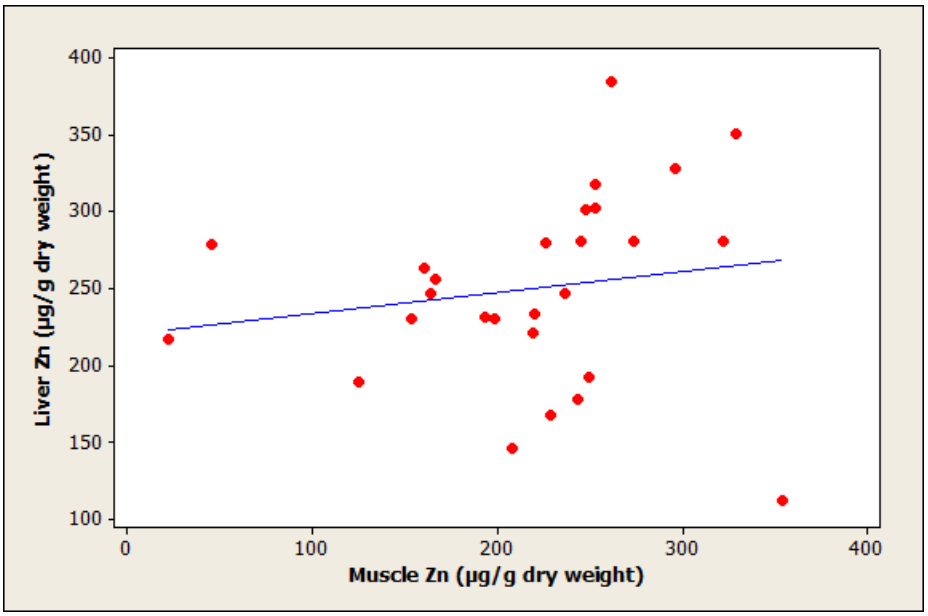



**Fig 7.6c** Scatterplot of Liver Zn (µg/g dry weight) versus Muscle Zn (µg/g dry weight)

*Red blood cell zinc*

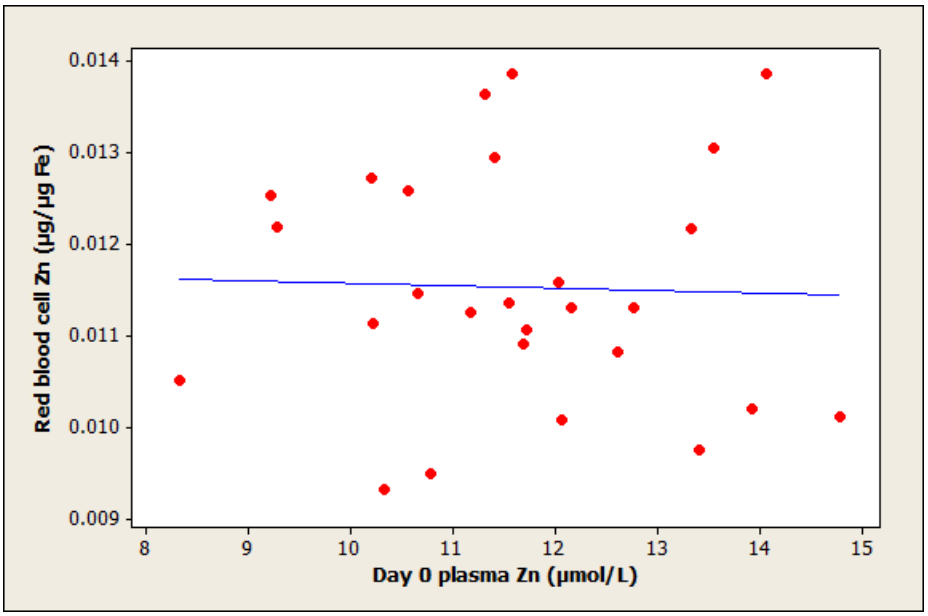



**Fig 7.7a** Scatterplot of RBC Zn (µg/ µg Fe) versus Plasma Zn on Day 0 (µmol/L)

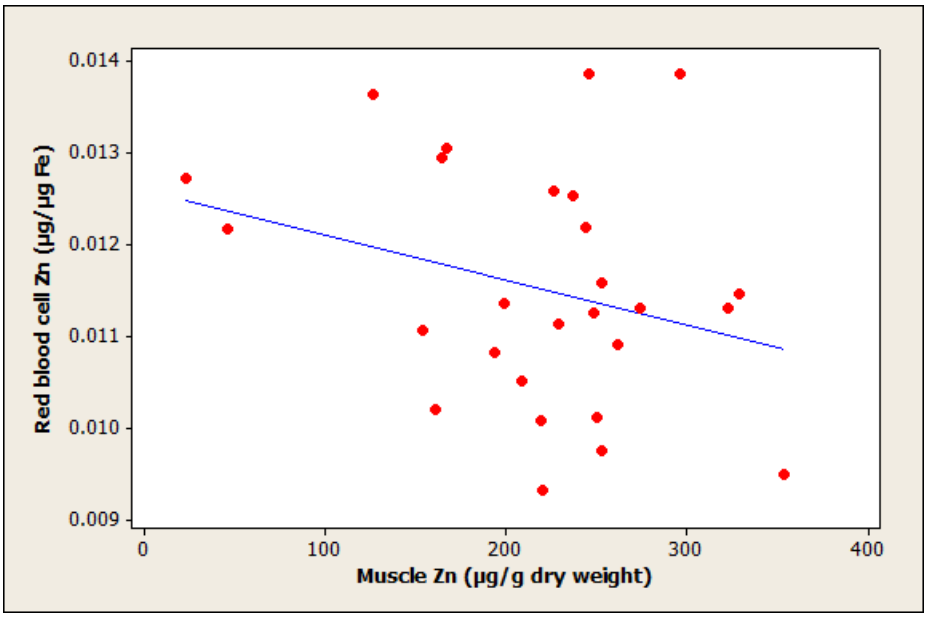



**Fig 7.7b** Scatterplot of RBC Zn (µg/ µg Fe) versus Muscle Zn (µg/g dry weight)

*Plasma zinc*

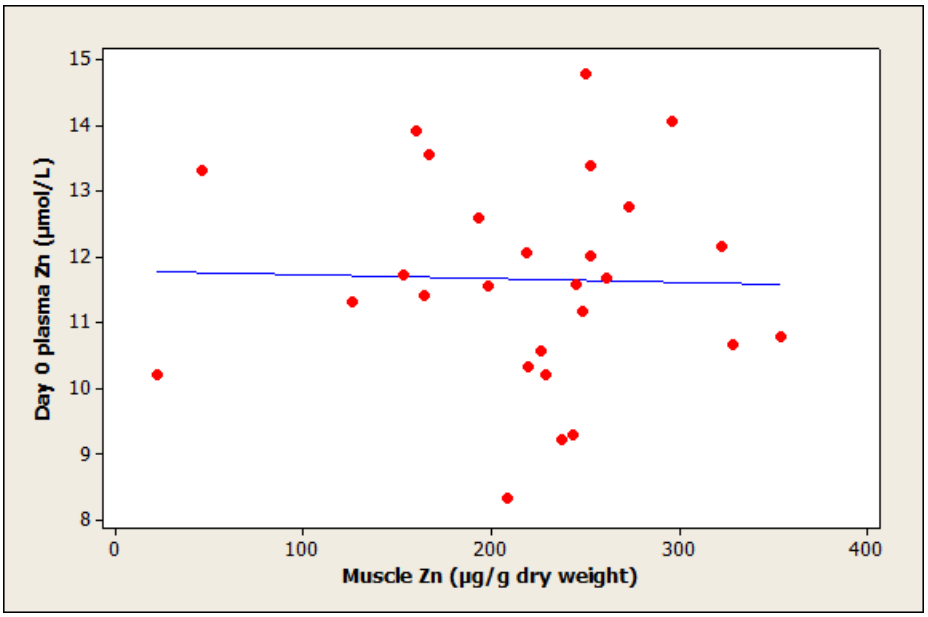



**Fig 7.8** Scatterplot of Plasma Zn (µmol/L) versus Muscle Zn (µg/g dry weight)

*Liver Selenium*

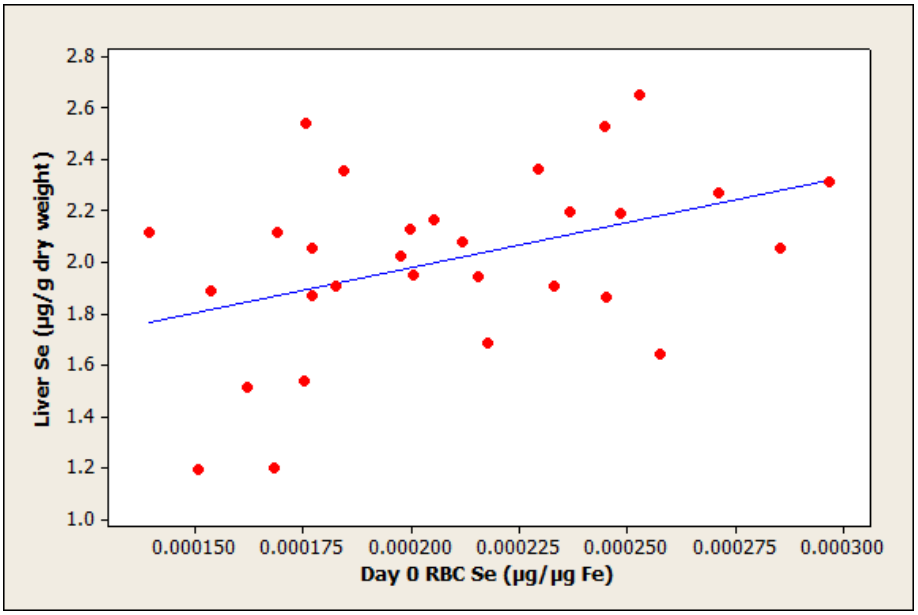



**Fig 7.9a**. Scatterplot of Liver Se (µg/g dry weight) versus RBC Se (µg/µg Fe) on day 0

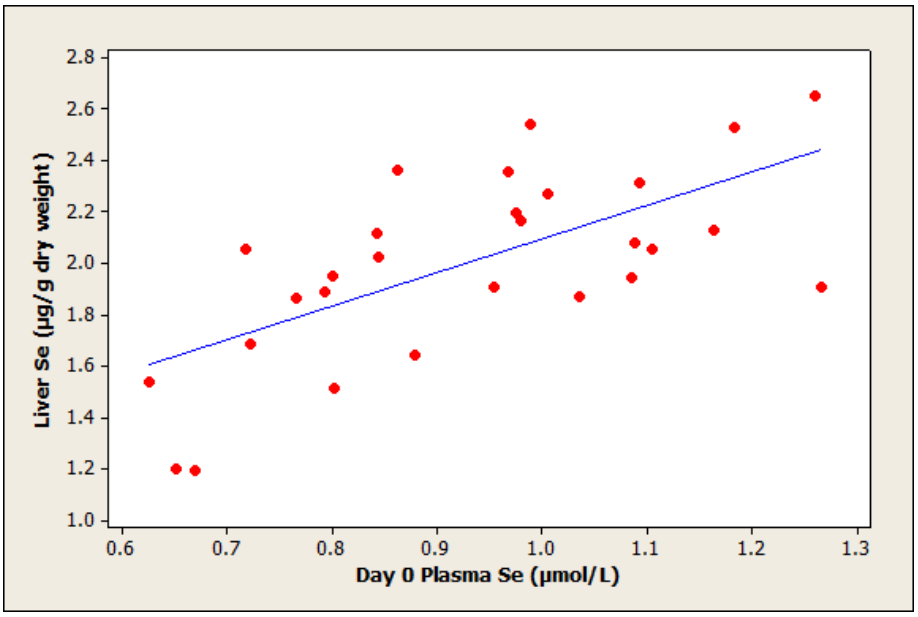



**Fig 7.9b**. Scatterplot of Liver Se (µg/g dry weight) versus plasma Se (µmol/L) on day 0

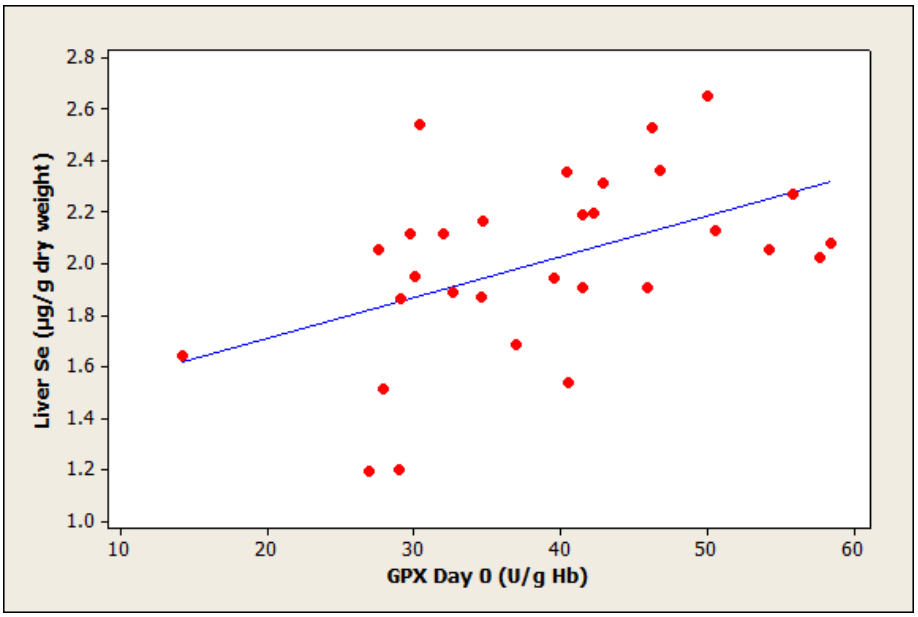



**Fig 7.9c**. Scatterplot of Liver Se (µg/g dry weight) versus RBC glutathione peroxidase activity (U/g Hb) on day 0

*RBC Selenium*

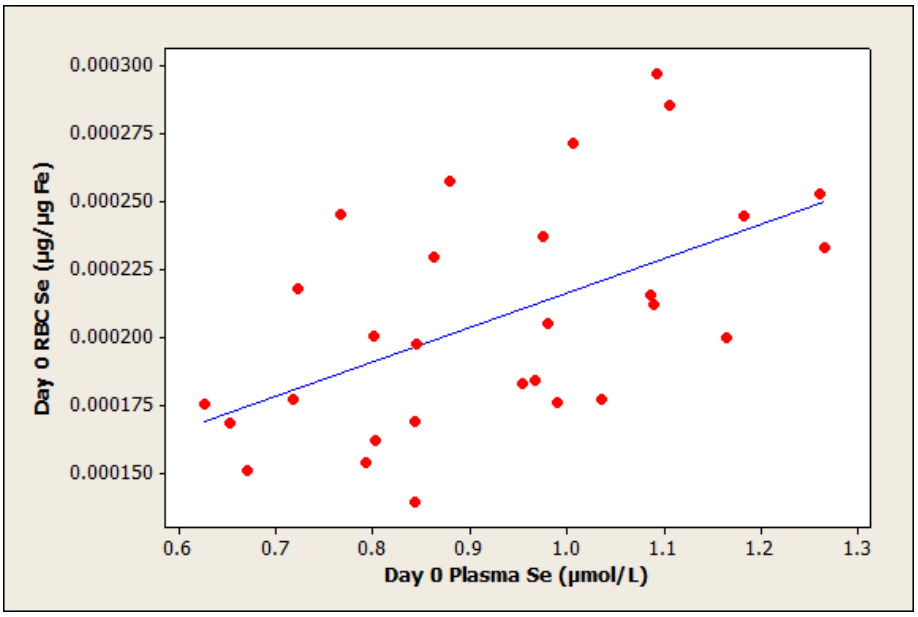



**Fig 7.10a** Scatterplot of RBC Se (µg/µg Fe) versus plasma Se (µmol/L) on day 0

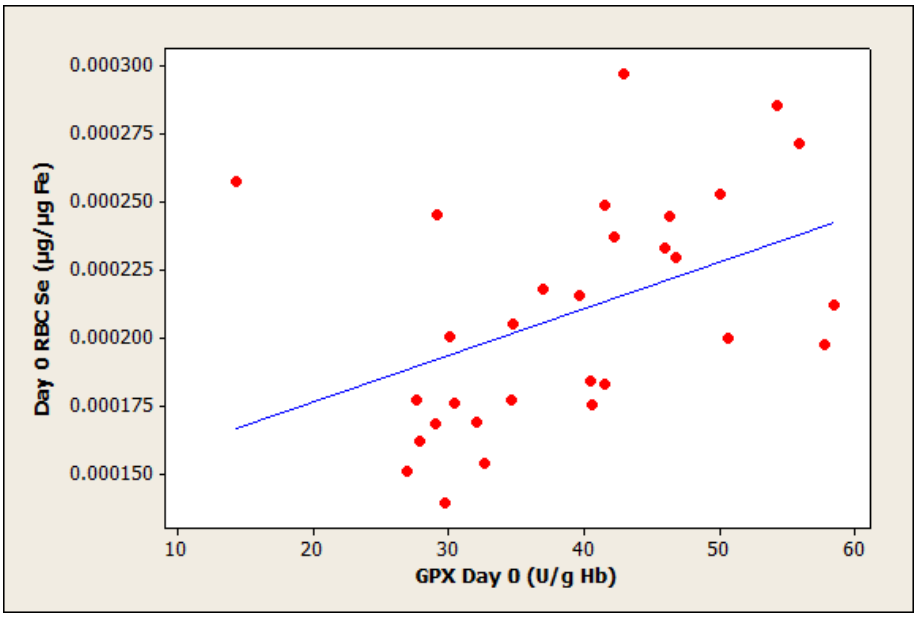



**Fig 7.10b** Scatterplot of RBC Se (µg/µg Fe) versus RBC glutathione peroxidase activity (U/g Hb) on day 0

*Plasma Selenium*

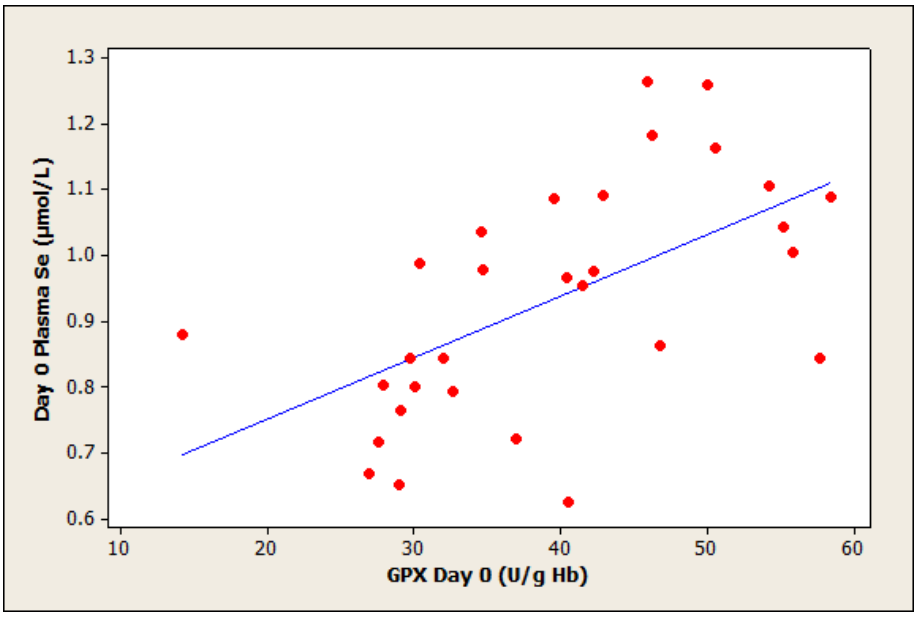



**Fig 7.11** Scatterplot of plasma Se (µmol/L) versus RBC glutathione peroxidase activity (U/g Hb) on day 0

# Chapter 8

## Copper and the acute phase response

### 8.1 Change in plasma copper, red blood cell copper and ceruloplasmin concentration, plus plasma ferroxidase activity, with the acute phase response

On days 1-3, while the patients are post-operative, a systemic acute phase response develops, marked by a rise in CRP and a fall in serum albumin. Kruskal-Wallis (non-parametric) one way analysis of variance was performed to see if red blood cell copper concentration, plasma copper concentration, plasma ceruloplasmin concentration or plasma ferroxidase activity varies during the acute phase response.

The change in the concentration of RBC copper can be seen in fig 8.1a, plasma copper in fig 8.1b, plasma ceruloplasmin in fig 8.1c and plasma ferroxidase activity in fig 8.1d. The change in the plasma copper: ceruloplasmin ratio can be seen in fig 8.1e. Median values of red blood cell copper concentration, plasma copper concentration, plasma ceruloplasmin concentration and plasma ferroxidase activity on days 0-3, are shown in table 8.1.

While there is a suggestion that plasma copper decreases on day 1, there is not any significant difference between the median plasma copper concentration on days 0-3 ($p=0.097$). There is no significant change in red blood cell copper concentration ($p=0.519$), plasma ceruloplasmin concentration ($p=0.152$), plasma ferroxidase activity ($p=0.510$) or plasma copper: ceruloplasmin ratio ($p=0.221$) on days 0-3.

| | Median (IQR) | | | | |
|---|---|---|---|---|---|
| | Day 0 | Day 1 | Day 2 | Day 3 | P value for variance |
| RBC Cu (µg/ µg Fe) | 0.000685 (0.000650-0.000729) | 0.000655 (0.000617-0.000718) | 0.000696 (0.000609-0.000734) | 0.000739 (0.000601-0.000862) | 0.519 |
| Plasma Cu (µmol/L) | 15.1 (12.6-16.5) | 11.4 (10.3-15.5) | 13.5 (10.0-14.2) | 14.6 (13.5-17.6) | 0.097 |
| Cp (g/L) | 0.20 (0.18-0.24) | 0.19 (0.17-0.20) | 0.20 (0.18-0.24) | 0.21 (0.19-0.26) | 0.152 |
| Ferroxidase (U/L) | 779 (673 - 850) | 710 (652 - 787) | 739 (562 - 837) | 691 (576 – 810) | 0.510 |
| plasma Cu:log Cp ratio | 4502 (3943-4783) | 4273 (3719-4752) | 4239 (3493-4423) | 4372 (4155-4372) | 0.221 |

**Table 8.1** Median (IQR) values of red blood cell copper concentration, plasma copper concentration, plasma ceruloplasmin (Cp) concentration and plasma ferroxidase activity on days 0-3. The significance of variance (p value) is from a Kruskal-Wallis (non-parametric) one way analysis of variance.

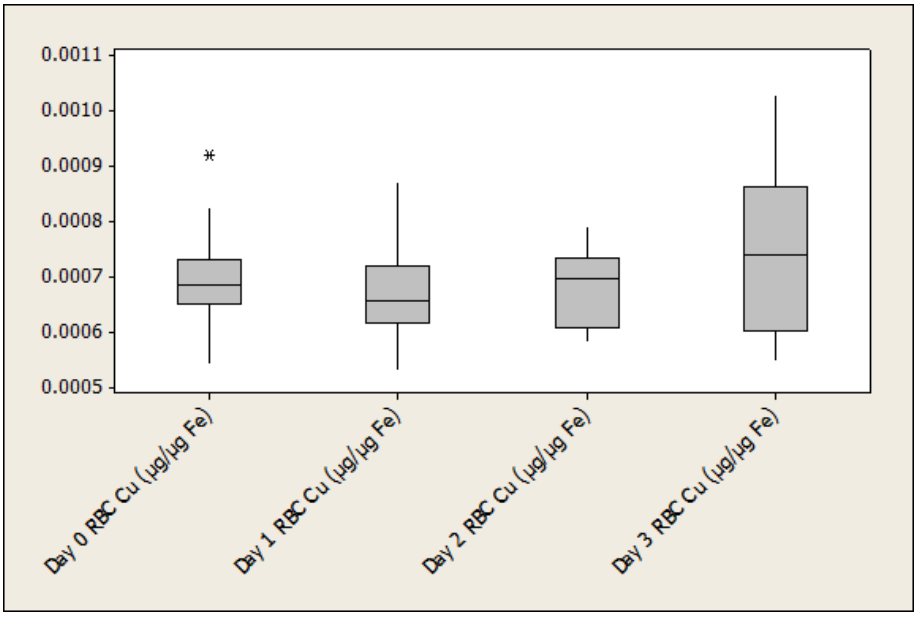



**Fig 8.1a** Boxplot of median red blood cell copper concentration (µg/ µg Fe) on day 0-3

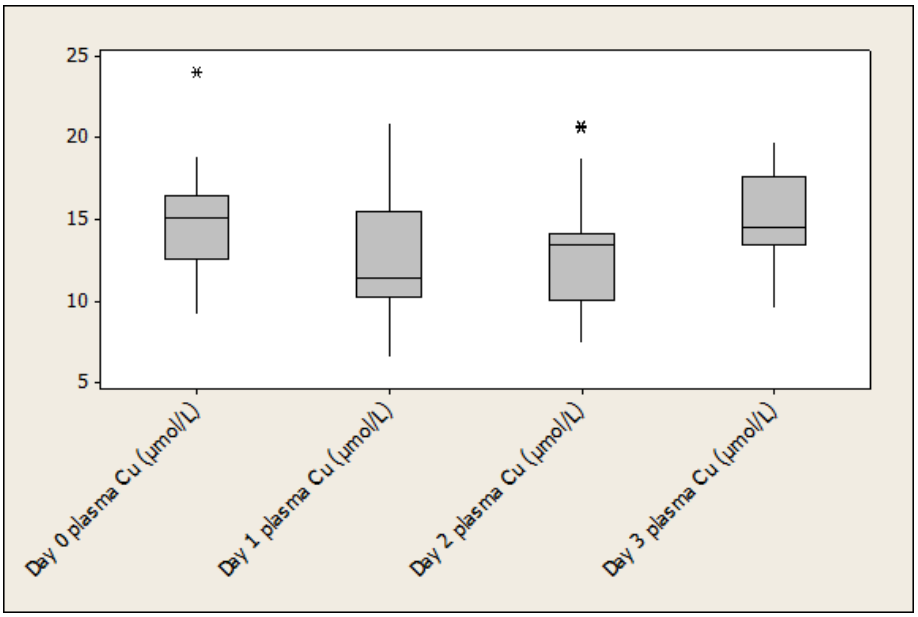



**Fig 8.1b** Boxplot of median plasma copper concentration (µmol/L) on day 0-3

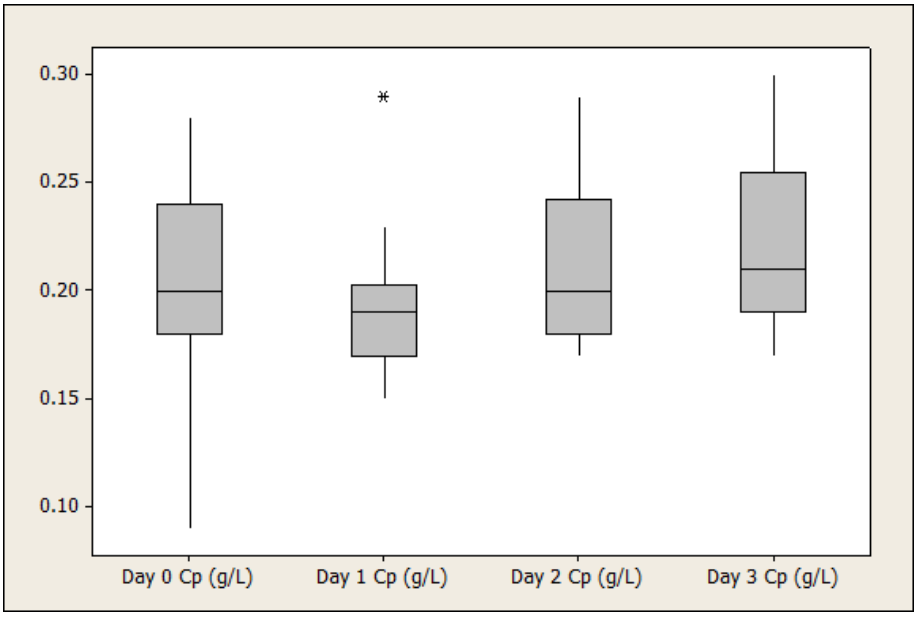



**Fig 8.1c** Boxplot of median plasma ceruloplasmin concentration (g/L) on day 0-3

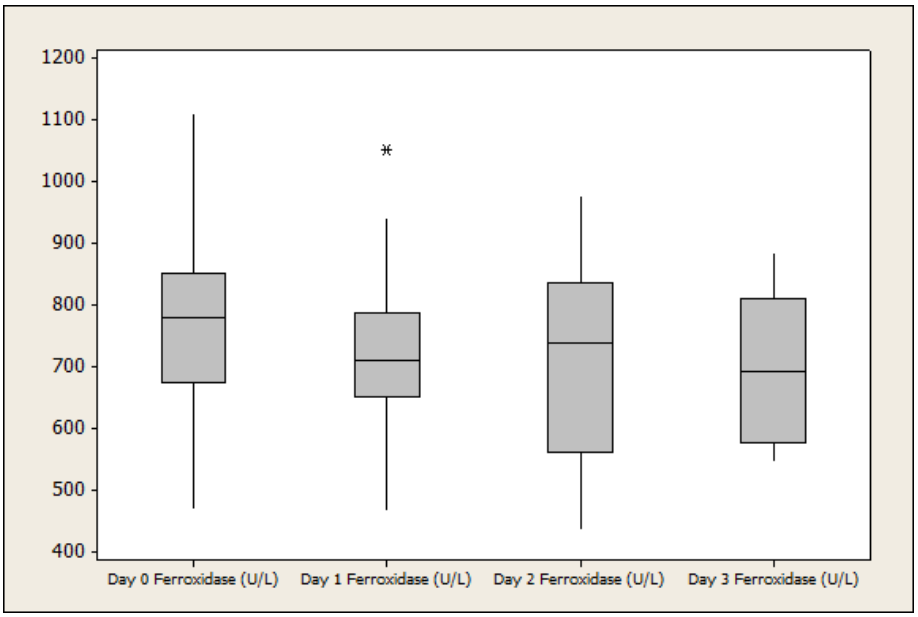



**Fig 8.1d** Boxplot of median plasma ferroxidase activity (U/L) on day 0-3

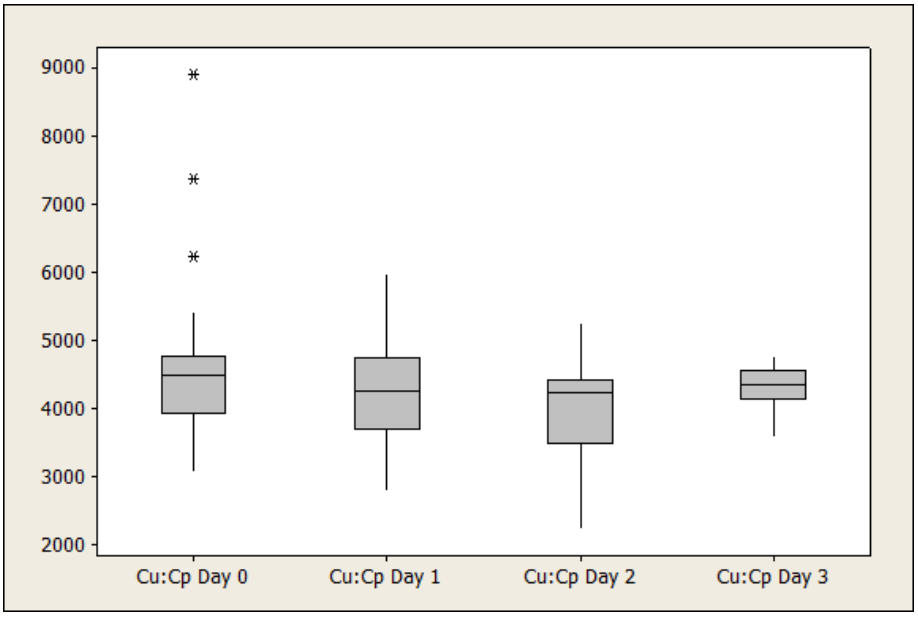



**Fig 8.1e** Boxplot of median plasma copper: ceruloplasmin ratio on day 0-3

## 8.2 Changes in linear associations during the acute phase response

### *8.2.1 Red blood cell copper and liver copper concentration*

Although none of the blood measures were found to change significantly during days 0-3, there may be differences in their associations. Red blood cell copper concentration has been found have a linear relationship with liver copper concentration on day 0. Simple linear regression was performed to examine the relationship on days 1-3; results are in table 8.2 and can be seen graphically in fig 8.2.

Despite not changing significantly during days 0-3, red blood cell copper concentration, which has a weak linear relationship with liver copper concentration on day 0 (p=0.04), does not have a linear relationship with liver copper on day 1 (p=0.480) or day 2 (p=0.782). A stronger linear relationship forms again on day 3 (p=0.019).

| | **Liver Cu(µg/g dry weight) Vs RBC copper (µg/µg Fe)** | |
|---|---|---|
| | $r^2$(%) | p value |
| Day 0 | 15.1 | 0.041 |
| Day 1 | 2.8 | 0.480 |
| Day 2 | 0.7 | 0.782 |
| Day 3 | 51.9 | 0.019 |

**Table 8.2** Relationship between RBC copper (µg/ µg Fe) on day 0-3 with liver copper concentration (µg/g dry weight), using simple linear regression.

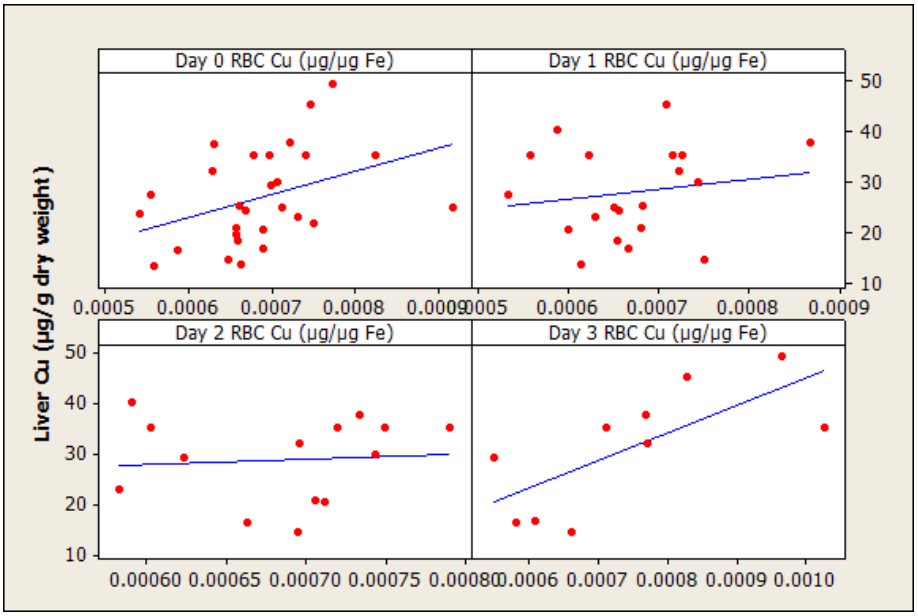



**Fig 8.2** Scatterplot of liver Cu (µg/g dry weight) versus log red blood cell Cu (µg/µg Fe) on day 0-3

*8.2.2 Plasma copper, ceruloplasmin and liver copper concentration*

None of plasma copper concentration, plasma ceruloplasmin or plasma copper: ceruloplasmin were found have a linear relationship with liver copper concentration on day 0. Simple linear regression was performed to examine the relationship on days 1-3; results are in table 8.3 and can be seen graphically in fig 8.3a-c.

While there is no linear relationship between plasma copper concentration, plasma ceruloplasmin or plasma copper: ceruloplasmin and liver copper on days 0-2, a trend does develop by day 3. Although not reaching statistical significance, all three measures form a stronger, more linear relationship with liver copper by day 3. Copper and Cp are all closely related as ceruloplasmin is the main copper carrying protein in blood, so all three measures showing a similar pattern strengthens this not simply being a chance finding.

| | Liver Cu (µg/g dry weight) Vs plasma Cu (µg/L) | |
|---|---|---|
| | $r^2$(%) | p value |
| Day 0 | 1.7 | 0.517 |
| Day 1 | 1.1 | 0.645 |
| Day 2 | 2.0 | 0.615 |
| Day 3 | 32.9 | 0.083 |
| | **Liver Cu (µg/g dry weight) Vs plasma Cp (g/L)** | |
| | $r^2$(%) | p value |
| Day 0 | 7.3 | 0.142 |
| Day 1 | 2.8 | 0.460 |
| Day 2 | 0.5 | 0.807 |
| Day 3 | 34.9 | 0.097 |
| | **Liver Cu (µg/g dry weight) Vs Plasma Cu:Cp** | |
| | $r^2$(%) | p value |
| Day 0 | 2.9 | 0.357 |
| Day 1 | 1.5 | 0.601 |
| Day 2 | 0.1 | 0.903 |
| Day 3 | 37.4 | 0.080 |

**Table 8.3** Relationship between log plasma copper (µg/L), log plasma ceruloplasmin (g/L) and log plasma copper: log plasma ceruloplasmin ratio with liver copper concentration (µg/g dry weight) on days 0-3, using simple linear regression.

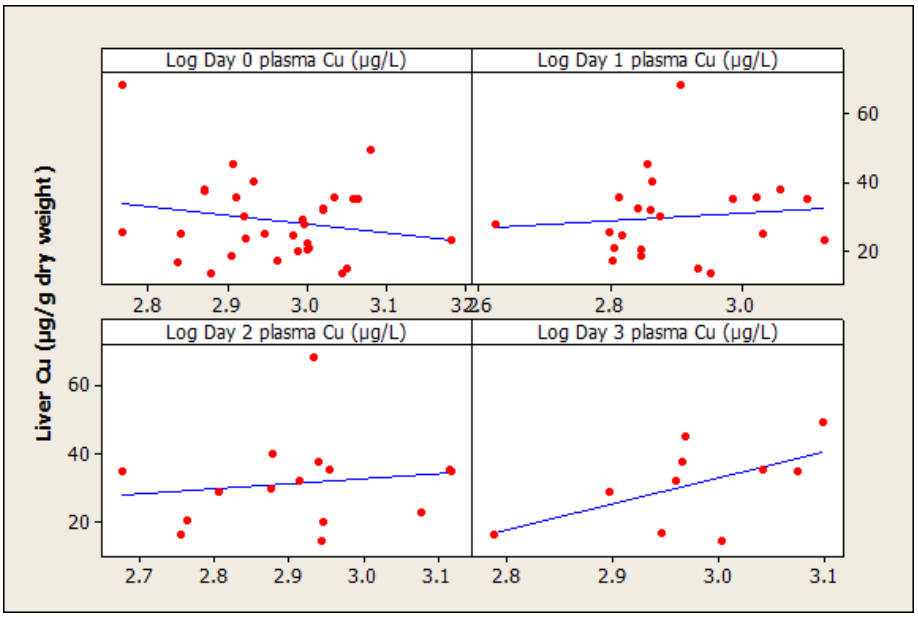



**Fig 8.3a** Scatterplot of liver Cu (µg/g dry weight) versus log plasma Cu (µg/L) on day 0-3

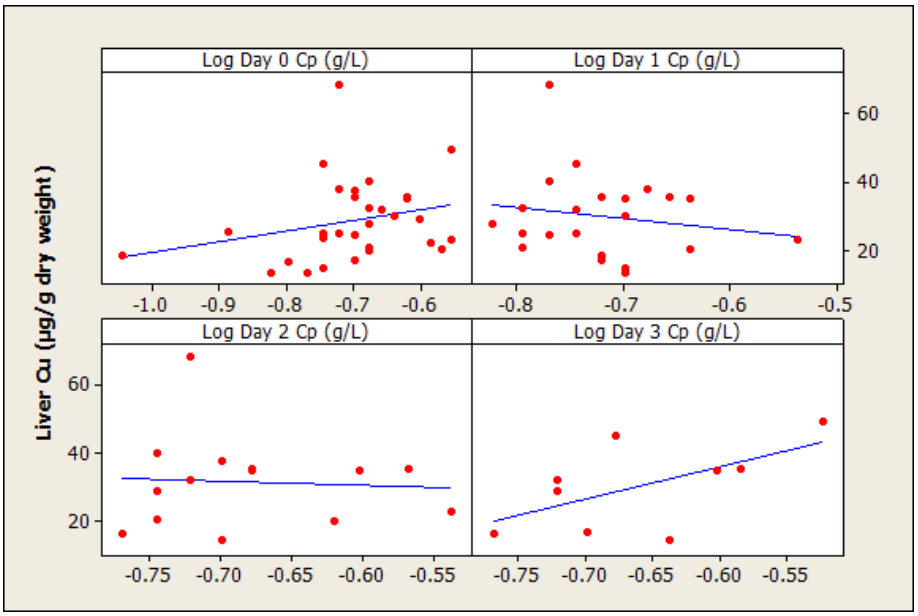



**Fig 8.3b** Scatterplot of liver Cu (µg/g dry weight) versus log plasma ceruloplasmin (g/L) on day 0-3

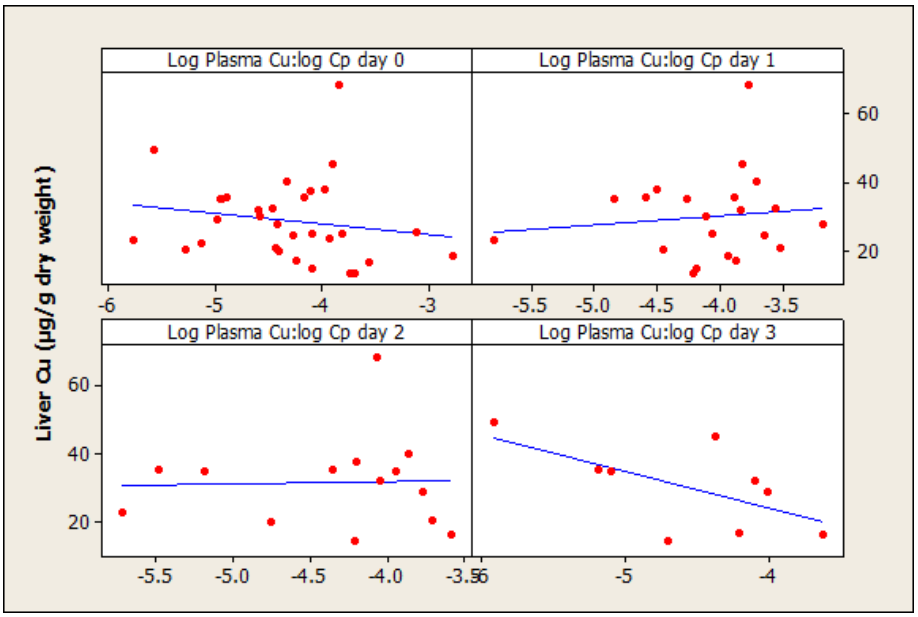



**Fig 8.3c** Scatterplot of liver Cu (µg/g dry weight) versus log plasma Cu: log plasma ceruloplasmin ratio on day 0-3

*8.2.3 Plasma copper and ceruloplasmin concentration*

As mentioned, ceruloplasmin is the main copper carrying protein in plasma. A linear relationship between plasma copper and plasma ceruloplasmin concentration was found on day 0 ($r^2$=33.5%; p=0.001). Simple linear regression was performed to investigate this relationship on days 1-3; results are in table 8.4 and can be seen graphically in fig 8.4.

The linear relationship between plasma copper and plasma ceruloplasmin concentration which was found on day 0 continues on days 1-3 and appears to strengthen with 86% of the variation in plasma copper concentration being explained by plasma ceruloplasmin concentration on day 3 (p<0.001).

| | **Plasma Cu (µg/L) Vs Ceruloplasmin (g/L)** | |
|---|---|---|
| | $r^2$(%) | p value |
| Day 0 | 33.5 | 0.001 |
| Day 1 | 54.8 | 0.000 |
| Day 2 | 57.7 | 0.002 |
| Day 3 | 86.3 | 0.000 |

**Table 8.4** Relationship between log plasma copper (µg/L) versus log plasma ceruloplasmin (g/L) on day 0-3, using simple linear regression.

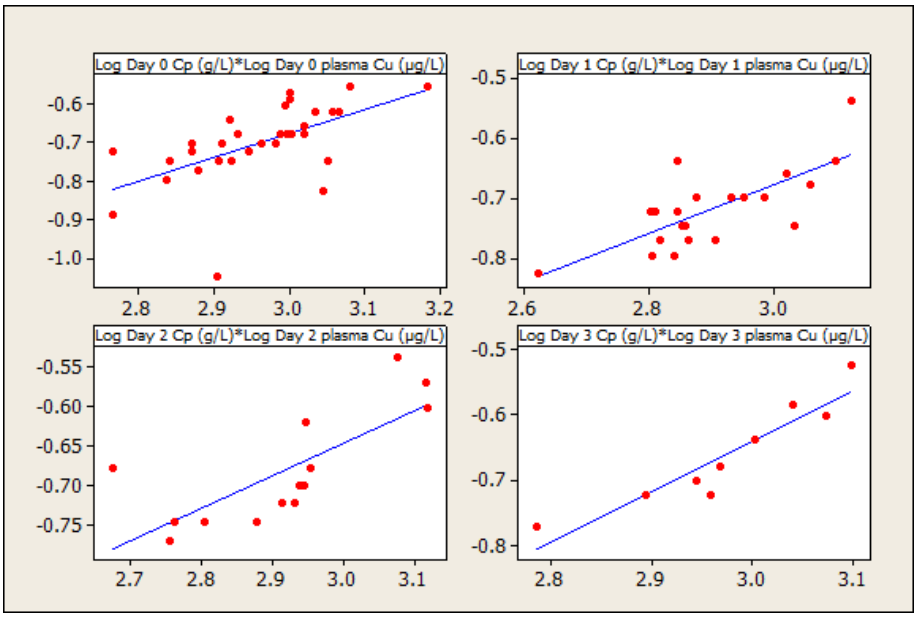



**Fig 8.4** Scatterplots of log plasma Cu (µg/L) versus log plasma ceruloplasmin (g/L) on day 0-3

## 8.3 Relationship between plasma copper, plasma ceruloplasmin and CRP concentration

C Reactive protein is one of the main markers of the acute phase response. While none of plasma copper concentration, plasma ceruloplasmin or plasma copper: ceruloplasmin ratio changed significantly during the acute phase response, there may be a relationship between their concentration and the concentration of CRP. Simple linear regression was performed to investigate this relationship on days 0-3; results are in table 8.5 and can be seen graphically in fig 8.5a-c.

On day 0, there is a linear relationship between plasma copper concentration ($r^2$=23.1%; p=0.006), plasma ceruloplasmin concentration ($r^2$=13.1%; p=0.045) and therefore plasma copper: ceruloplasmin ratio ($r^2$=24.2%; p=0.005) with CRP concentration. This relationship is not present on days 1-3.

| | CRP (g/L) Vs plasma Cu (µg/L) | |
|---|---|---|
| | $r^2$(%) | p value |
| Day 0 | 23.1 | 0.006 |
| Day 1 | 0.7 | 0.721 |
| Day 2 | 7.3 | 0.331 |
| Day 3 | 3.5 | 0.606 |
| | **CRP (g/L) Vs plasma Cp (g/L)** | |
| | $r^2$(%) | p value |
| Day 0 | 13.1 | 0.045 |
| Day 1 | 1.1 | 0.648 |
| Day 2 | 14.1 | 0.185 |
| Day 3 | 6.8 | 0.498 |
| | **CRP (g/L) Vs Plasma Cu:Cp** | |
| | $r^2$(%) | p value |
| Day 0 | 24.2 | 0.005 |
| Day 1 | 0.5 | 0.773 |
| Day 2 | 15.0 | 0.171 |
| Day 3 | 7.4 | 0.478 |

**Table 8.5** Relationship between log plasma copper (µg/L), log plasma ceruloplasmin (g/L), log plasma copper: log plasma ceruloplasmin ratio and CRP concentration (g/L) (log CRP day 0) on days 0-3, using simple linear regression.

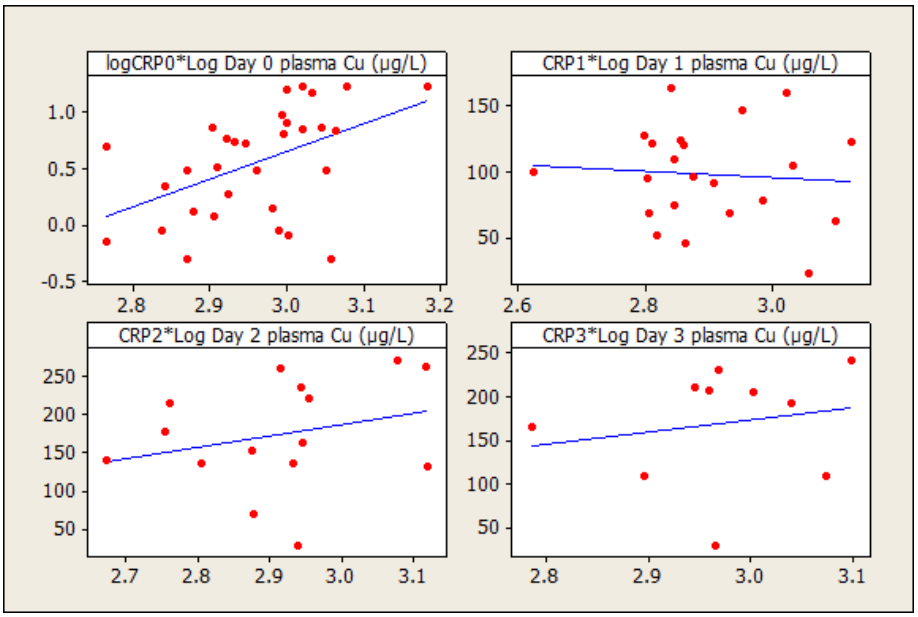



**Fig 8.5a** Scatterplots of CRP (g/L) versus log plasma copper (µg /L) on day 0-3

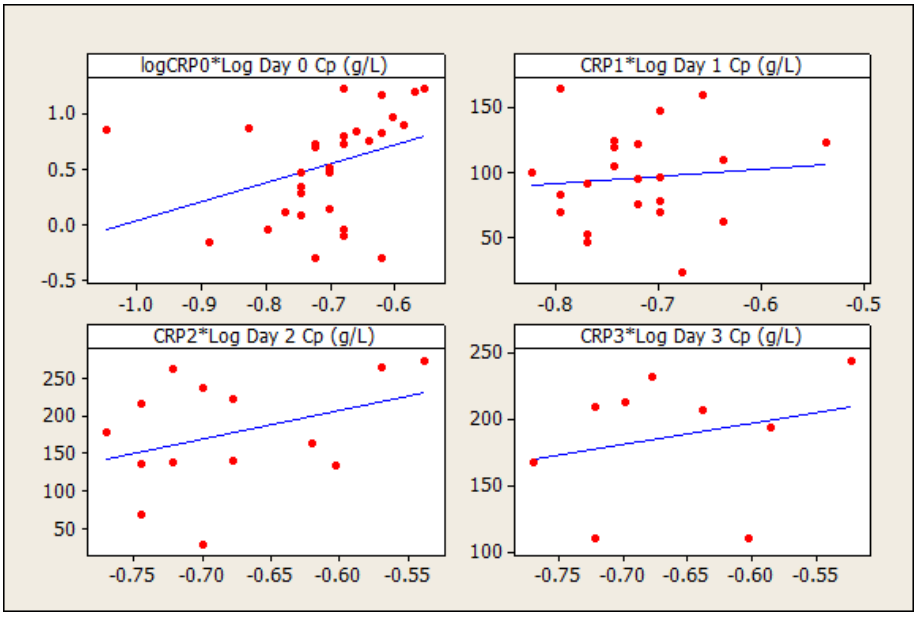



**Fig 8.5b** Scatterplots of CRP (g/L) versus log plasma ceruloplasmin (g /L) on day 0-3

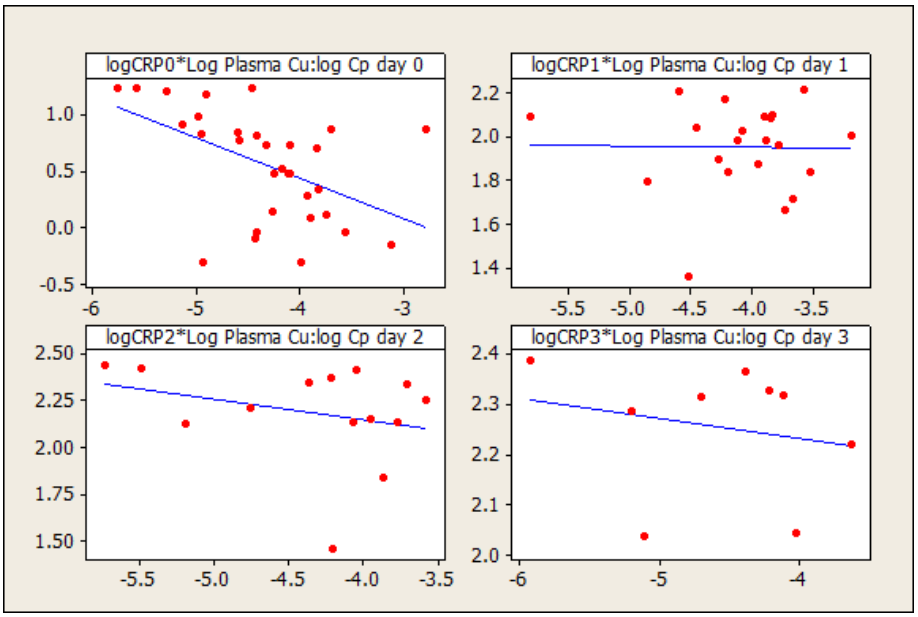



**Fig 8.5c** Scatterplots of CRP (g/L) versus log plasma Cu: log plasma ceruloplasmin ratio on day 0-3

To further examine the relationship between CRP and plasma copper, ceruloplasmin and plasma copper: ceruloplasmin ratio, the data was split into tertiles of day 0 CRP: tertile 1 0.5-2.2 g/L, tertile 2 3.0-6.8 g/L, tertile 3 7.0-17.0 g/L. Simple linear regression analysis was performed (fig 8.6a-c, table 8.6)

When day 0 data is split into tertiles of CRP concentration, the relationship between plasma copper concentration and CRP disappears, possibly as a result of small numbers. However the relationships between plasma ceruloplasmin concentration and CRP concentration, and ceruloplasmin: copper ratio and CRP concentration are present in tertile 2 and 3 (CRP 3.0-17.0 g/L). Of note, there is no linear relationship between plasma and liver copper concentration when only those samples with a CRP concentration in tertile 1 were included ($r^2$ 1.1%, p=0.771).

| | CRP (g/L) Vs plasma Cu (µg/L) | |
|---|---|---|
| | $r^2$(%) | p value |
| Tertile 1 | 0.1 | 0.921 |
| Tertile 2 | 8.0 | 0.398 |
| Tertile 3 | 29.6 | 0.104 |
| | **CRP (g/L) Vs plasma Cp (g/L)** | |
| | $r^2$(%) | p value |
| Tertile 1 | 3.2 | 0.623 |
| Tertile 2 | 49.8 | 0.015 |
| Tertile 3 | 40.1 | 0.049 |
| | **CRP (g/L) Vs Plasma Cu:Cp** | |
| | $r^2$(%) | p value |
| Tertile 1 | 4.7 | 0.546 |
| Tertile 2 | 43.4 | 0.027 |
| Tertile 3 | 40.1 | 0.049 |

**Table 8.6** Relationship between log plasma copper (µg/L), log plasma ceruloplasmin (g/L), log plasma copper: log plasma ceruloplasmin ratio and tertile 1-3 CRP concentration (g/L) days 0, using simple linear regression.

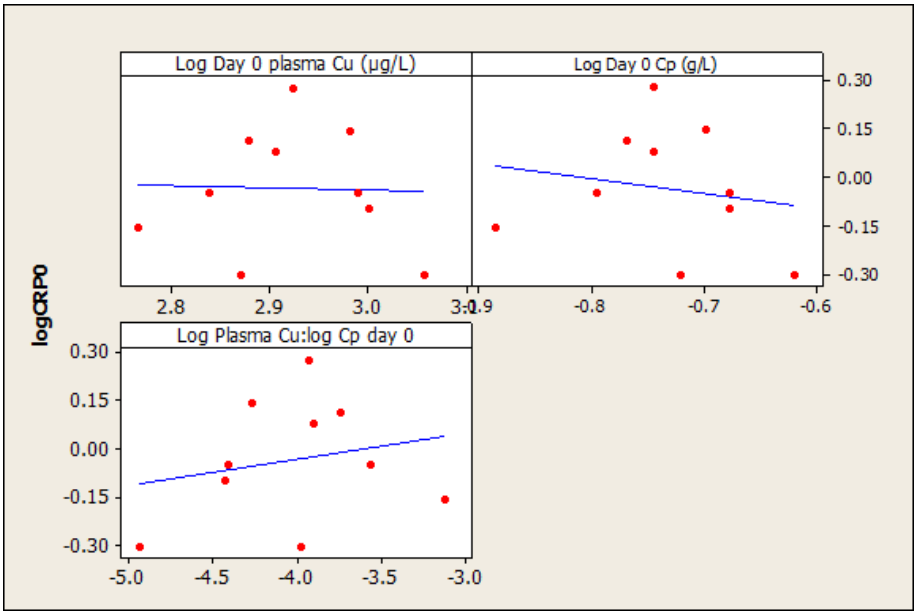



**Fig 8.6a** Scatterplots of tertile 1 CRP (g/L) versus log plasma Cu, log plasma ceruloplasmin and log plasma copper: ceruloplasmin ratio on day 0

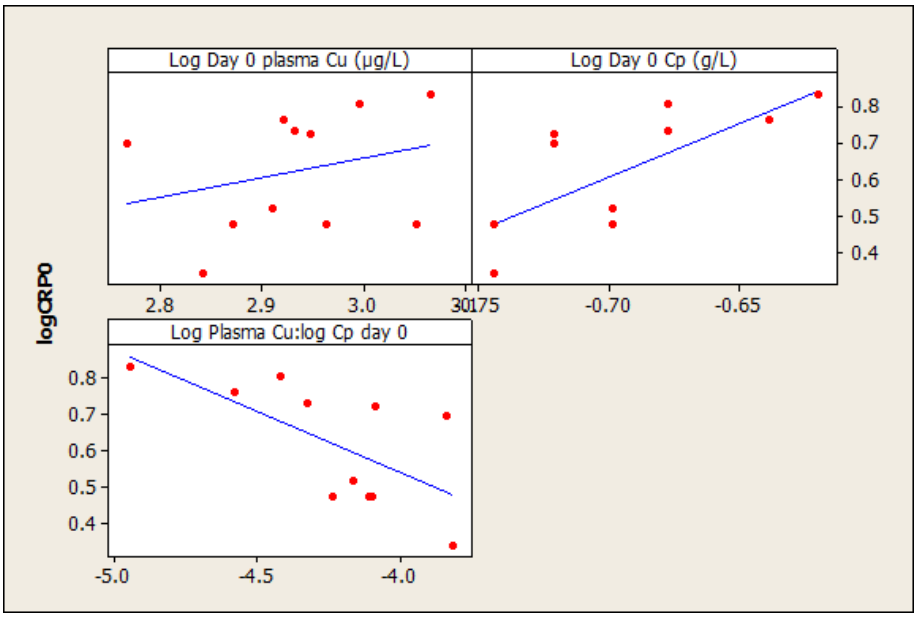



**Fig 8.6b** Scatterplots of tertile 2 CRP (g/L) versus log plasma Cu, log plasma ceruloplasmin and log plasma copper: ceruloplasmin ratio on day 0

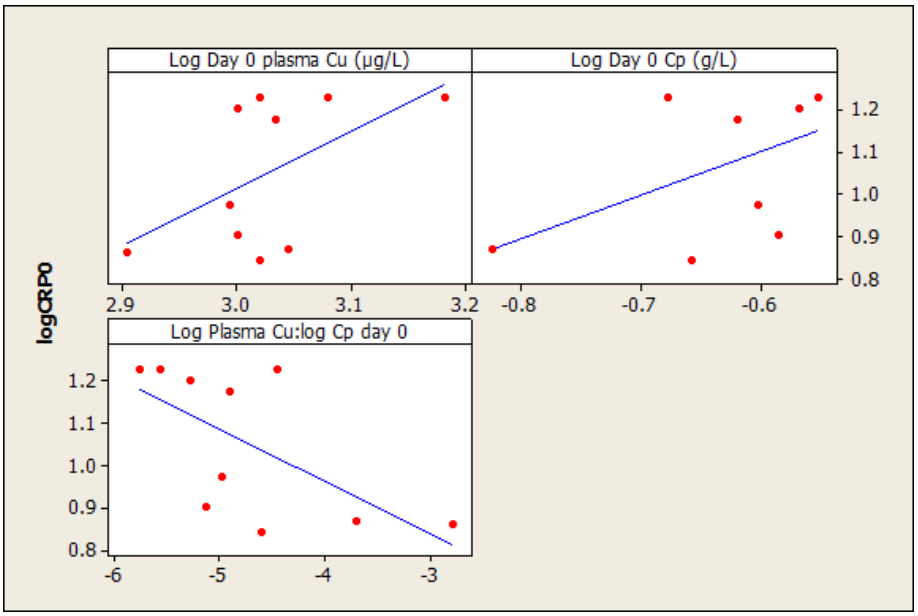



**Fig 8.6c** Scatterplots of tertile 3 CRP (g/L) versus log plasma Cu, log plasma ceruloplasmin and log plasma copper: ceruloplasmin ratio on day 0

## 8.4 Relationship between red blood cell copper concentration, plasma ferroxidase activity and CRP concentration

Simple linear regression was performed to investigate the relationship between red blood cell copper concentration, plasma ferroxidase activity and CRP concentration on days 0-3. Results are in table 8.7 and can be seen graphically in fig 8.7a-b.

There is no linear relationship between either red blood cell copper concentration or plasma ferroxidase activity, and C reactive protein concentration.

| | **CRP (g/L) Vs RBC Cu (µg/µg Fe)** | |
|---|---|---|
| | $r^2$(%) | p value |
| Day 0 | 5.8 | 0.219 |
| Day 1 | 9.9 | 0.164 |
| Day 2 | 2.6 | 0.562 |
| Day 3 | 0.4 | 0.861 |
| | **CRP (g/L) Vs ferroxidase (U/L)** | |
| | $r^2$(%) | p value |
| Day 0 | 0.8 | 0.634 |
| Day 1 | 2.1 | 0.516 |
| Day 2 | 13.6 | 0.194 |
| Day 3 | 0.1 | 0.928 |

**Table 8.7** Relationship between log red blood cell copper (µg/µg Fe), plasma ferroxidase activity (U/L) and CRP concentration (mg/L) (log CRP day 0) on days 0-3, using simple linear regression.

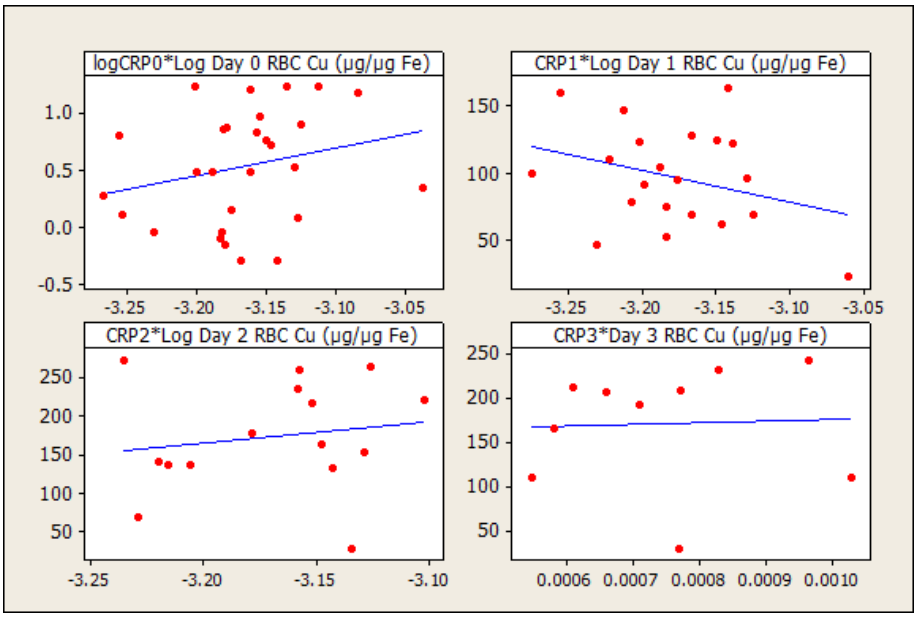



**Fig 8.7a** Scatterplots of CRP (mg/L) versus log red blood cell copper (µg/ µg Fe) on day 0-3

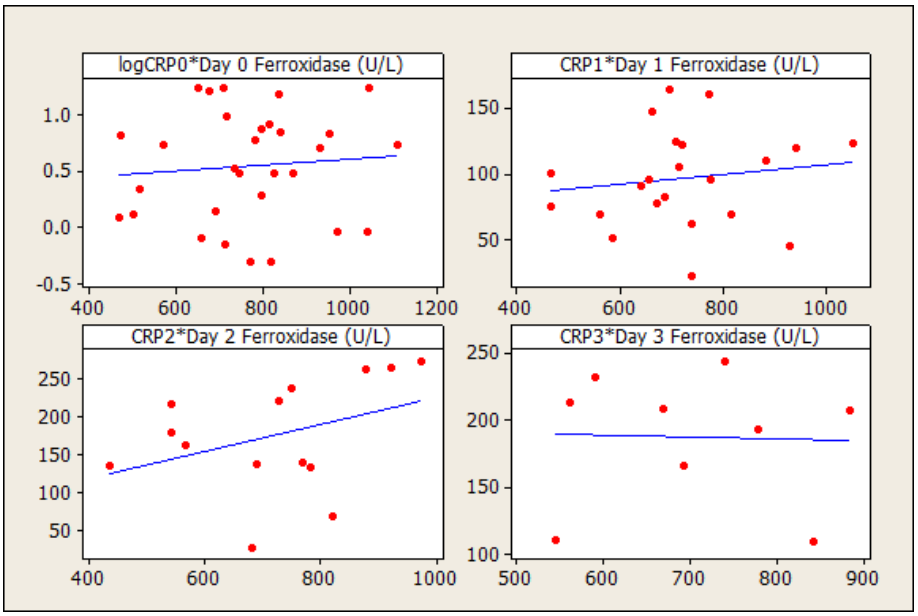



**Fig 8.7b** Scatterplots of CRP (mg/L) versus plasma ferroxidase (U/L) on day 0-3

## 8.5 Relationship between liver copper concentration and red blood cell copper concentration during the acute phase response

As red blood cell copper was the only blood measure of copper which was found to have a linear relationship with liver copper, and as red blood cell copper concentration does not change during the acute phase response, it may be a suitable marker of copper status regardless of the inflammatory status of the patient. However, as seen in table 8.2, the linear relationship between red blood cell copper and liver copper was not seen on days 1 and 2.

All red blood cell copper concentrations were combined and figure 8.8 shows the red blood cell copper concentrations for day 0-3 plotted against their respective liver copper concentration.

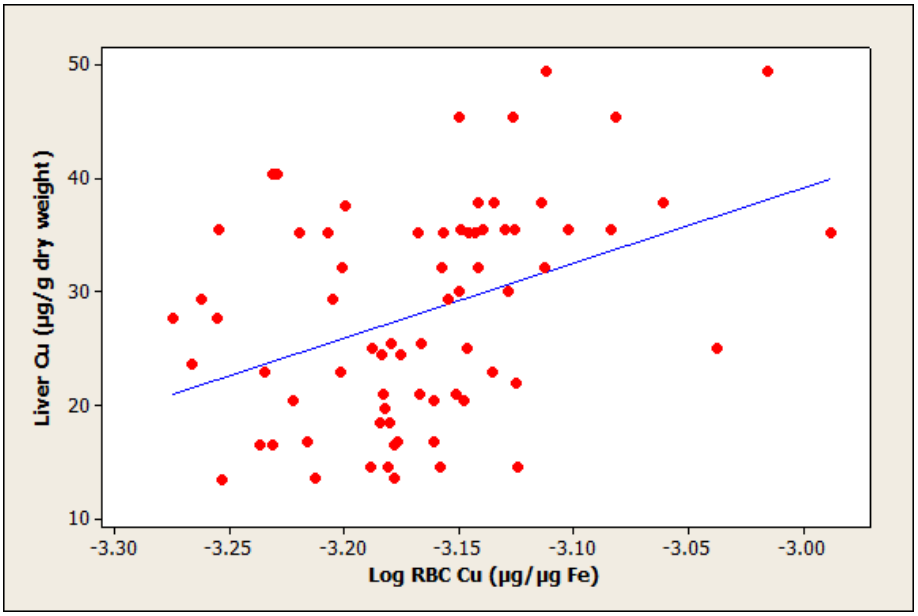



**Fig 8.8** Scatterplots of liver copper (µg/g dry weight) versus log red blood cell copper (µg/ µg Fe) on day 0-3

Simple linear regression analysis was performed: $r^2$ 15.6% (p=0.001). However, the sample size of each day is not equal, with day 0 having the most complete sample, this means that day 0, where there is known to be a linear relationship with liver copper concentration, will have dominance over the overall result.

To see if the relationship existed if day 0 was excluded, the analysis was repeated using red blood cell concentrations from day 1-3 only (fig 8.9).

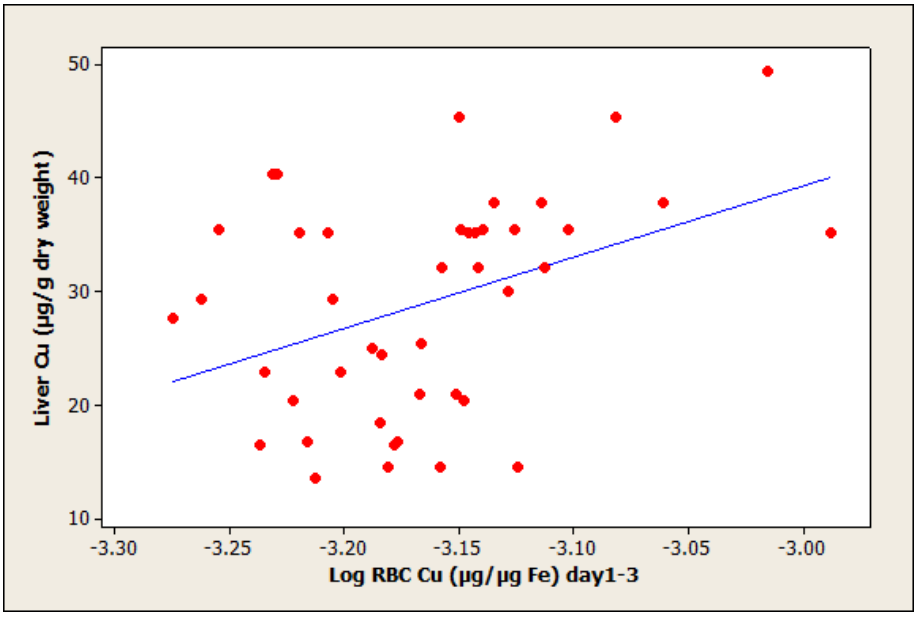



**Fig 8.9** Scatterplots of liver copper (µg/g dry weight) versus log red blood cell copper (µg/ µg Fe) on day 1-3

The linear relationship was found to still exist despite the exclusion of day 0 from analyses; $r^2$ 15.7%, p=0.008.

## 8.6 Summary of findings - Copper

None of the blood copper measures (plasma copper concentration, red blood cell copper concentration, ceruloplasmin concentration, ceruloplasmin: copper ratio, plasma ferroxidase activity) changed significantly during the evolution of the acute phase response on the 3 days post-operatively.

In the pre-operative samples, when the patients were not inflamed, red blood cell copper concentration has a linear relationship with liver copper concentration. This relationship changes during the evolution of the acute phase response and is no longer linear on days 1 and 2 but becomes linear again on day 3.

When all red blood cell concentrations from day 0-3 were pooled, inflamed and non-inflamed samples, there was still a linear relationship with liver copper; however, due to different numbers of samples for each day, the non-inflamed samples will be dominant and this result may not be truly representative of the relationship during inflammation. However the linear relationship persisted when the non-inflamed samples were excluded.

There is also a linear relationship between plasma copper concentration and ceruloplasmin concentration which is present both when the patients are not inflamed and through the evolution of the acute phase response on the 3 days immediately post-operatively.

Copper, ceruloplasmin and the copper: ceruloplasmin ratio do not have a linear relationship with liver copper concentration when non-inflamed or in the first two days of the acute phase response. However, by day 3 of the acute phase response all three measure have a strengthened linear relationship with liver

copper concentration, but, possibly due to small sample size, these did not reach full significance.

Copper, ceruloplasmin and the copper: Ceruloplasmin ratio all have a linear relationship with C-reactive protein on day 0, when the C-reactive protein concentration is low. Further examination of this relationship by splitting the data into tertiles of CRP shows that the linear relationships between ceruloplasmin concentration and CRP, and copper: ceruloplasmin ratio and CRP are present in tertile 2 and 3 of CRP. These tertiles correspond to a CRP of 3.0-17.0g/L.

## Chapter 9

## Zinc and the acute phase response

### 9.1 Change in plasma and red blood cell zinc with the acute phase response

Over days 1-3 an acute phase response develops as shown by an increase in serum CRP and a fall in serum albumin; in order to see if red blood cell or plasma concentrations of zinc change during this acute phase, Kruskal-Wallis (non-parametric) one way analysis of variance was performed.

The acute phase response evolved over day 1-3 as shown by an increase in CRP and a fall in serum albumin. The change in the concentration of RBC Zinc can be seen in fig 9.1a and plasma Zinc in fig 9.1b.

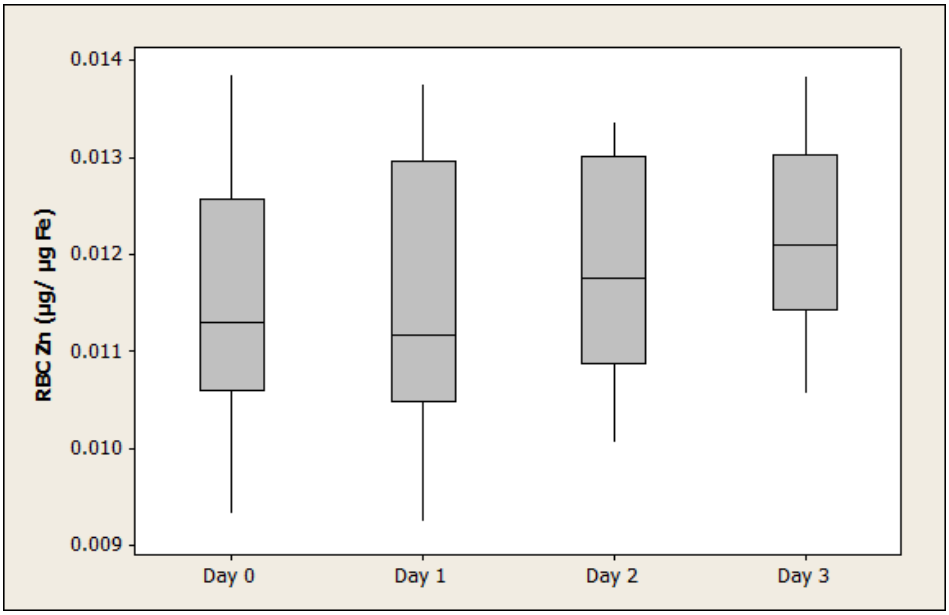



**Fig 9.1a** Boxplot of median red blood cell zinc concentration (µg/ µg Fe) on day 0-3.

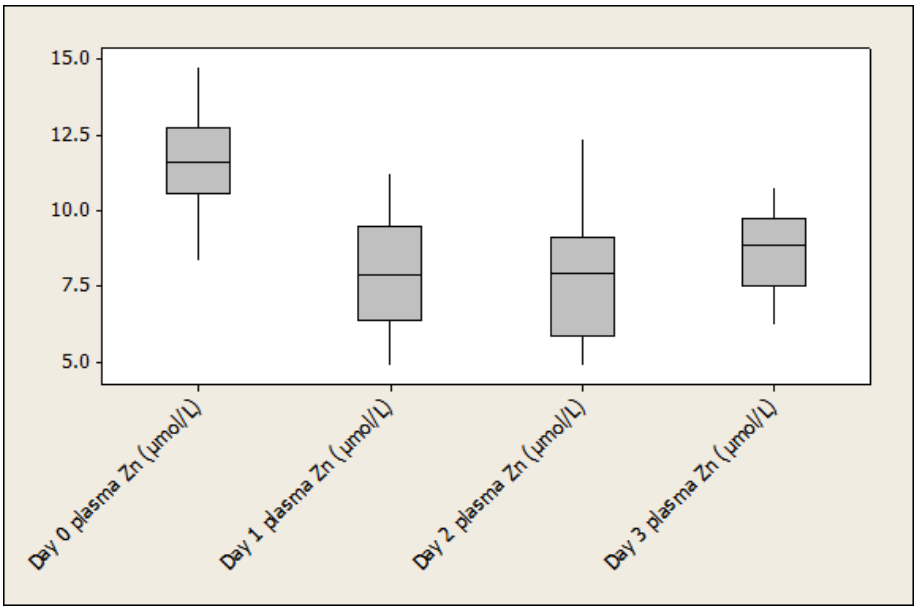



**Fig 9.1b** Boxplot of median plasma zinc concentration (µmol/L) on day 0-3

*RBC Zinc*

RBC zinc does not change significantly over day 0-3 (p=0.510); median values (IQR) were day 0 0.011 (0.011 – 0.013) µg/ µg Fe, day 1 0.011 (0.011 – 0.013) µg/ µg Fe, day 2 0.012 (0.012 – 0.013) µg/ µg Fe and day 3 0.012 (0.011 – 0.013) µg/ µg Fe. Therefore, red blood cell zinc concentration does not change during the acute phase response.

*Plasma Zinc*

Kruskal-Wallis (non-parametric) one way analysis of variance shows that plasma zinc concentration changes significantly from day 0 to day 1-3 ($p<0.001$). However there is no significant change in concentration between day 1, day 2 and day 3 (p=0.533). Median (IQR) values of plasma were 11.6 (10.6 – 12.8) µmol/L on day 0, 7.9 (6.4 – 9.5) µmol/L on day 1, 7.9 (5.9- 9.1) µmol/L on day 2 and 8.9 (7.5 – 9.7) µmol/L on day 3.

The median (IQR) percentage change in plasma zinc from day 0 to day 1 was -31.8% (-47.28 - -15.22), day 0 to day 2 -28.89% (-42.52 - -16.78) and day 0 to day 3 -25.4% (-30.9 - -15.06); there was no significant difference between these percentage changes (p=0.357).

Plasma zinc concentration decreases with the evolution of the acute phase response.

## 9.2 Tertiles of liver zinc

In order to examine the effect of liver zinc concentration on the changes in plasma zinc concentration seen during the evolution of acute phase response, data was split into tertiles by liver zinc concentration; there were 9, 10 and 9 patient samples in each of the low, medium and high tertiles respectively. Analysis of variance of CRP and Albumin was performed to ensure there were no differences in CRP or Albumin between tertiles on any of day 0-3; as it was not normally distributed, the logarithmic transformation of CRP day 0 was used. Mean values (SD) for CRP and Albumin by liver tertile can be seen in table 9.1. No differences in CRP or Albumin concentrations were seen between tertiles of liver zinc.

| | Tertile liver zinc (range liver zinc (µg/g dry weight)) | | | |
|---|---|---|---|---|
| | 1 (111.9-220.9) | 2 (230.7-280.1) | 3 (280.9-384.5) | p value for variance |
| CRP day 0 (mg/L) | 5.6(2.8) | 6.6(3.2) | 6.6(3.2) | 0.974 |
| CRP day 1 (mg/L) | 92.11(25.0) | 102.4(34.3) | 102.0(38.1) | 0.753 |
| CRP day 2 (mg/L) | 163.2(32.5) | 176.0(73.0) | 177.4(61.6) | 0.852 |
| CRP day 3 (mg/L) | 160.7(39.3) | 175.6(76.4) | 153.6(59.9) | 0.726 |
| Alb day 0 (g/L) | 37.3(7.57) | 37.9(3.81) | 39.0(2.0) | 0.772 |
| Alb day 1 (g/L) | 27.8(4.63) | 28.3(3.68) | 28.6(2.6) | 0.903 |
| Alb day 2 (g/L) | 26.1(3.26) | 28.1(3.04) | 27.6(2.51) | 0.339 |
| Alb day 3 (g/L) | 26.0(3.75) | 26.4(3.53) | 25.3(2.35) | 0.778 |

**Table 9.1** Mean values (SD) of CRP (mg/L) and Albumin (g/L) on day 0-3 for tertiles of liver zinc concentration (µg/g dry weight).

### *9.2.1 Plasma zinc concentrations between tertiles of liver zinc*

To see if the relationship found between plasma zinc concentration and liver zinc concentration is maintained during the acute phase response, Kruskal-Wallis non-parametric analysis of variance was performed between the medians of plasma zinc concentration for each of the three tertiles of liver zinc concentration on days 0-3; this was also performed for the percentage change in plasma zinc to ascertain if the decrease in plasma zinc concentration with the acute phase response is related to the liver zinc concentration.

Median concentration of plasma zinc (μmol/L) on day 0-3 and median percentage change in plasma zinc, from day 0 to day 1-3, divided by tertile of liver zinc, are shown in table 9.2. The median concentration of plasma zinc (μmol/L) for each liver tertile, at each time point, can be seen graphically in figure 9.2a and the median percentage change in plasma zinc concentration between day 0 and day 1-3, for tertiles of liver zinc concentration, can be seen in figure 9.2b.

While none of the plasma zinc concentrations vary statistically significantly across the tertiles of liver zinc concentration, a trend can be seen for plasma zinc concentration to increase through the tertiles. A trend also exists for the percentage change in plasma zinc: while none of the percentage changes in plasma zinc levels vary with statistical significance, on day 1 and day 2 the trend is for the lowest tertile to have the largest percentage drop in plasma zinc concentration, and the highest tertile to drop the least.

| | Tertile liver zinc (range liver zinc (µg/g dry weight)) | | | |
|---|---|---|---|---|
| | 1 (111.9-220.9) | 2 (230.7-280.1) | 3 (280.9-384.5) | p value for variance |
| Plasma Zn day 0 (µmol/L) | 10.5 (9.5-11.9) | 11.6 (10.5-13.4) | 12.0 (11.4-13.1) | 0.122 |
| Plasma Zn day 1 (µmol/L) | 6.4 (4.8-7.9) | 7.4 (6.0-10.1) | 9.1 (7.4-9.4) | 0.296 |
| Plasma Zn day 2 (µmol/L) | 5.7 (5.5-6.0) | 8.0 (6.8-9.6) | 9.1 (8.2-9.9) | 0.063 |
| Plasma Zn day 3 (µmol/L) | 7.3 (6.2-9.5) | 9.8* | 9.0 (8.3-9.8) | 0.368 |
| Change plasma Zn day 1 (%) | -45.26 (-51.09 - -35.05) | -31.67 (-49.93 - -11.77) | -22.36 (-47.46 - -13.12) | 0.305 |
| Change plasma Zn day 2 (%) | -40.77 (-44.5 - -34.10) | -32.59 (-47.6 - -11.9) | -22.61 (-28.39 - -15.57) | 0.211 |
| Change plasma Zn day 3 (%) | -25.57 (-28.79 - -16.42) | -24.97* | -25.28 (-31.61 - -7.7) | 0.895 |

**Table 9.2** Median (IQR) values of plasma zinc concentration (µmol/L) on day 0-3 and % change in plasma zinc concentration between day 0 and day 1-3, for tertiles of liver zinc concentration. *only 1 sample available in this category.

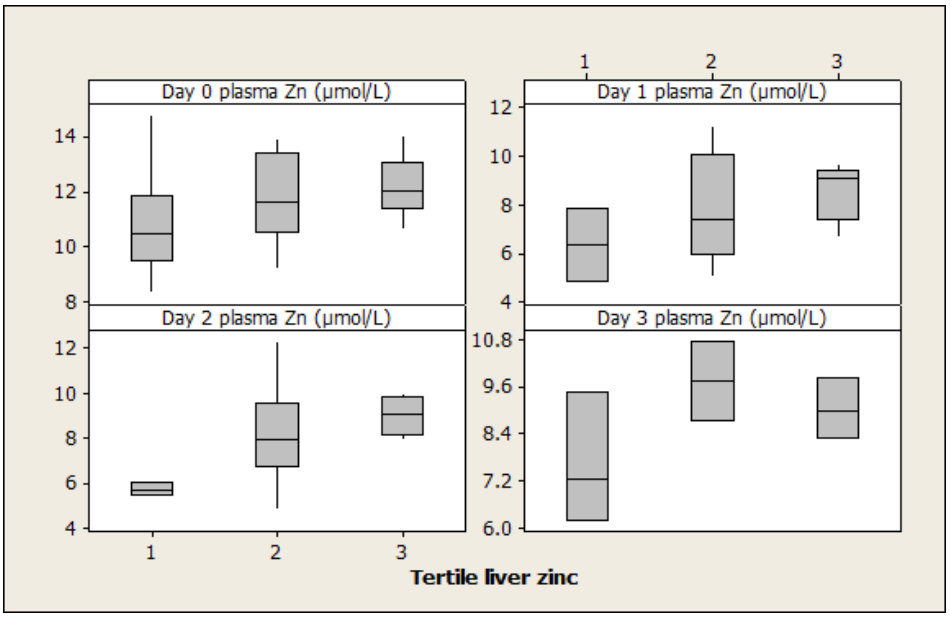



**Fig 9.2a**. Boxplot of median plasma zinc concentration (µmol/L) on day 0-3 for tertiles of liver zinc concentration

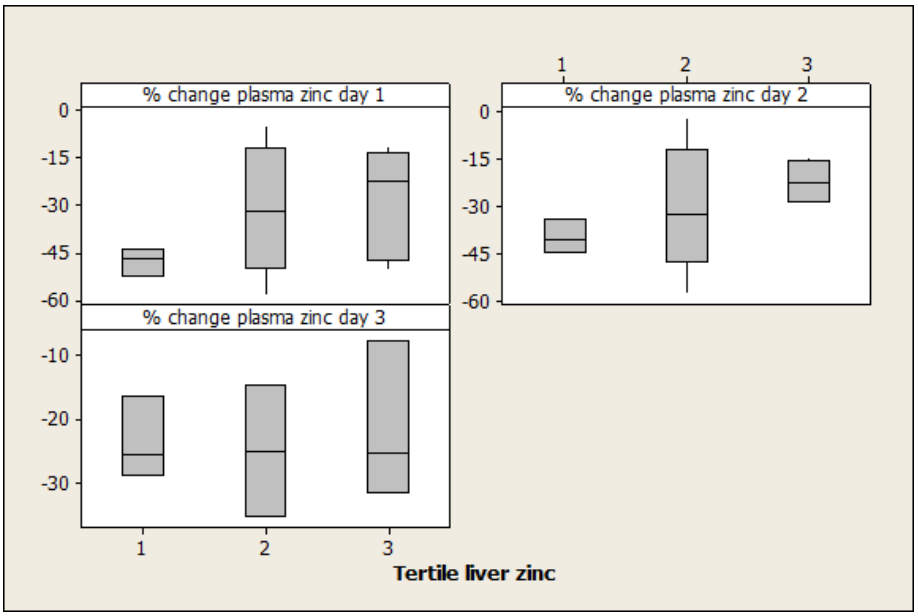



**Fig 9.2b** Boxplot of median % change in plasma zinc concentration between day 0 and day 1-3, for tertiles of liver zinc concentration

*9.2.2 Zinc: Albumin ratios*

The use of zinc:albumin ratios allows the relationship between albumin concentration, zinc concentration and tertile of liver zinc concentration to be examined while correcting for any effect of plasma dilution due to intravenous fluid replacement given to the patient post-operatively. Figure 9.3a shows the median zinc:albumin ratio on days 0-3. Using Mann-Whitney comparisons (Bonferroni correction p=0.017 is significant), the zinc:albumin ratio decreases from day 0 to day 1 (p=0.064), is similar or day 1 and 2 (p=0.645) and increases again on day 3 (p=0.022).

Figure 9.3b shows the median zinc:albumin ratio on days 0-3, divided by tertile of liver zinc concentration. Values for the median zinc:albumin ratio for each liver zinc tertile on days 0-3 and an analysis of variance can be seen in table 9.3.

On day 0 there is no difference between the zinc:albumin ratios for the three tertiles of liver zinc. However, by day one there is an decrease in the zinc:albumin ratio in the lowest tertile, and to a smaller effect in the middle tertile, relative to the highest tertile of liver zinc (p=0.063); this difference appears to have resolved by day 3 (p=0.249). As we know there is no difference in albumin concentrations between the tertiles (table 9.1), the decrease in zinc:albumin ratio must reflect a greater drop in plasma zinc concentration on day 1 in the lowest tertile of liver zinc, relative to the drop in albumin concentration, than the highest tertile of liver zinc.

| | Tertile liver zinc (range liver zinc (µg/g dry weight)) | | | |
|---|---|---|---|---|
| | 1 (111.9-220.9) | 2 (230.7-280.1) | 3 (280.9-384.5) | p value for variance |
| Zn:Alb day 0 | 19.58 (14.84-24.99) | 21.25 (18.30-22.29) | 20.16 (19.05-21.44) | 0.783 |
| Zn:Alb day 1 | 15.28 (12.93-17.34) | 18.26 (14.79-21.83) | 20.13 (18.55-20.59) | 0.063 |
| Zn:Alb day 2 | 16.48 (15.61-17.63) | 18.55 (14.48-22.08) | 20.60 (19.02-23.02) | 0.148 |
| Zn:Alb day 3 | 21.60 (18.43-25.77) | 22.50* | 22.60 (19.41-23.84) | 0.249 |

**Table 9.3** Median (IQR) values of zinc:albumin ratio on day 0-3 for tertiles of liver zinc concentration. *only 1 sample available in this category.

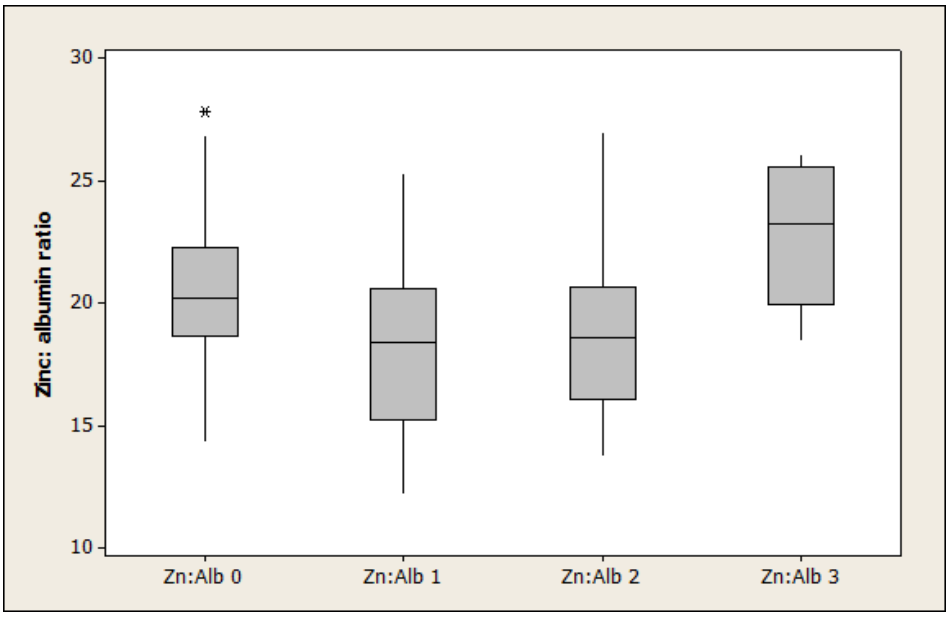



**Fig 9.3a** Boxplot of median zinc:albumin ratio on day 0-3

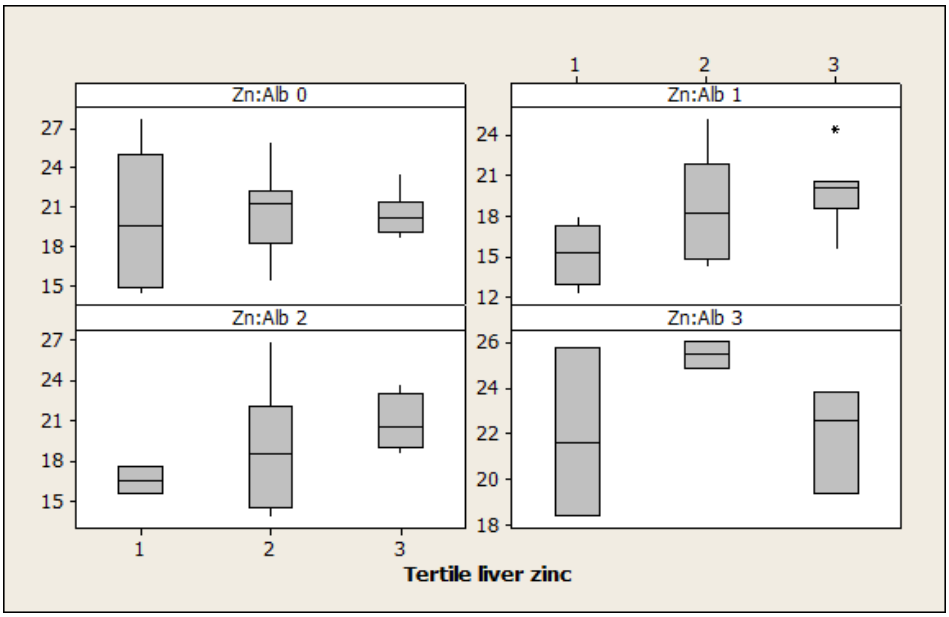



**Fig 9.3b** Boxplot of median zinc:albumin ratio on day 0-3 for tertiles of liver zinc concentration

## 9.3 Regression analysis

### *9.3.1 Plasma versus liver zinc concentrations*

In order to further examine the relationship between plasma zinc concentration, percentage decrease in plasma zinc and liver zinc concentration during the acute phase response, simple linear regression was performed; the results can be seen in table 9.4. The relationship between plasma zinc concentration (µg/L) and liver zinc concentration (µg/g dry weight) on day 0-3 can be seen in figure 9.4a and the relationship between the percentage change in plasma zinc concentration and liver zinc concentration (µg/g dry weight) on day 1-3 can be seen in figure 9.4b.

The weak linear relationship found on day 0 between plasma and liver zinc concentration is no longer statistically significant on days 1-3. There is no relationship between the percentage change in plasma zinc concentration on days 1-3 and liver zinc concentration.

| | **Liver Zn (µg/g dry weight) Vs plasma zinc (µg/L)** | |
|---|---|---|
| | $r^2$(%) | p value |
| Day 0 | 14.4 | 0.046 |
| Day 1 | 11.4 | 0.145 |
| Day 2 | 24.7 | 0.084 |
| Day 3 | 26.6 | 0.191 |
| | **Liver Zn (µg/g dry weight) Vs % change plasma zinc** | |
| | $r^2$(%) | p value |
| Day 1 | 7.2 | 0.253 |
| Day 2 | 9.2 | 0.313 |
| Day 3 | 5.2 | 0.588 |

**Table 9.4** Relationship between both plasma zinc (µg/L) on day 0-3, and percentage change in plasma zinc from day 0 (%) on day 1-3, with liver zinc concentration (µg/g dry weight) using simple linear regression.

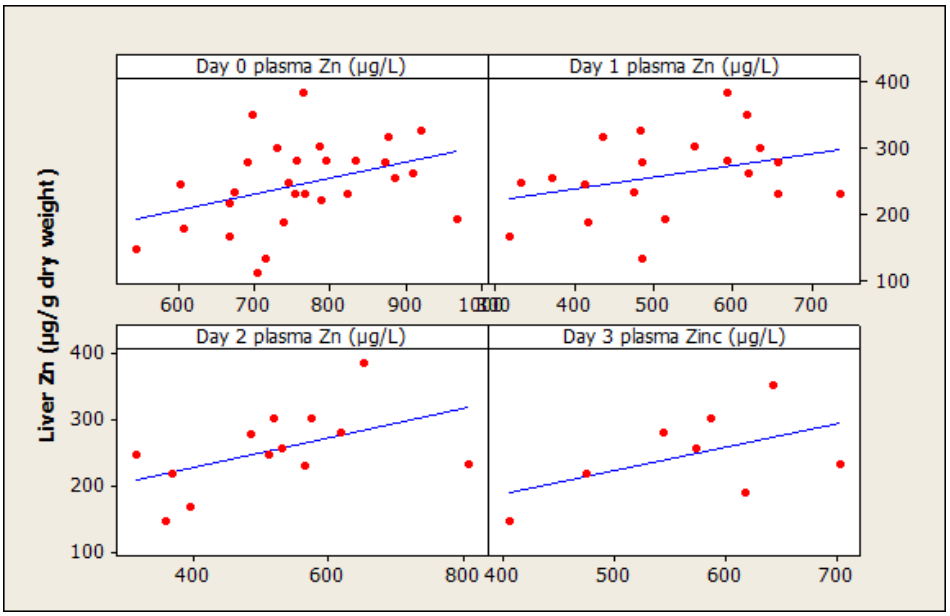



**Fig 9.4a** Scatterplots of plasma zinc concentration (µg/L) versus liver zinc concentration (µg/g dry weight) on day 0-3.

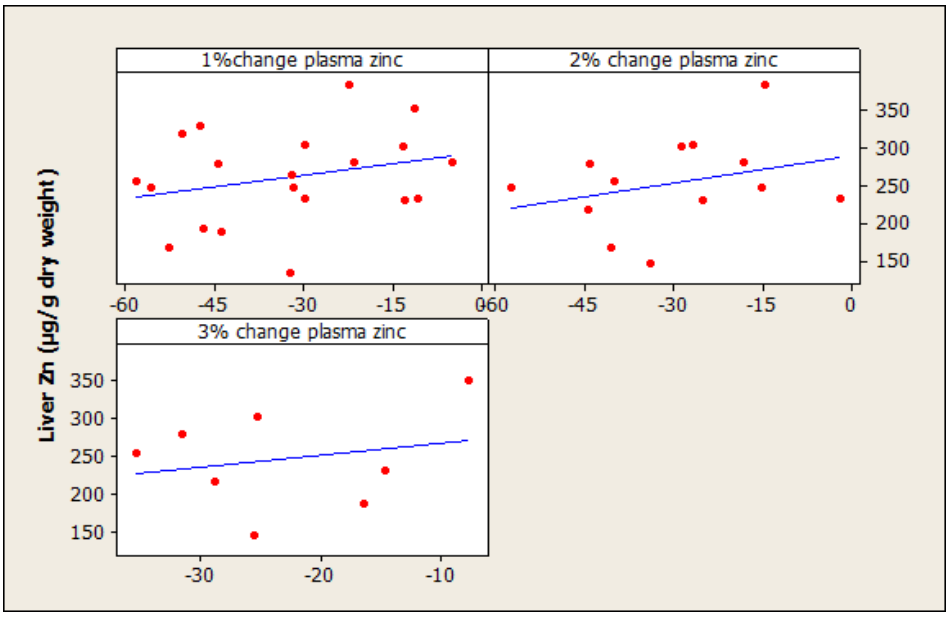



**Fig 9.4b** Scatterplots of % change plasma zinc concentration from day 0 versus liver zinc concentration (µg/g dry weight) on day 1-3

*9.3.2 Plasma zinc concentrations versus albumin*

Linear regression was performed to examine the relationship between serum albumin concentration and plasma zinc concentration on day 0-3, and % change in plasma zinc concentration on day 1-3; the results can be seen in table 9.5. The relationship between plasma zinc concentration (µg/L) and serum albumin concentration (g/L) on day 0-3 can be seen in figure 9.5a. The relationship between % change of plasma zinc concentration and serum albumin concentration (g/L) on day 1-3 can be seen in figure 9.5b.

While there is no linear relationship between serum albumin and plasma zinc concentrations on day 0, during days 1-3 a stronger and statistically significant linear relationship between serum albumin concentration and plasma zinc concentration develops. There is a suggestion of linear relationship between the % change in plasma zinc and the serum albumin on days 1 and 2, though this does not quite reach statistical significance; the relationship has lessened by day 3.

| | **Albumin (g/L) Vs plasma zinc (µg/L)** | |
|---|---|---|
| | $r^2$(%) | p value |
| Day 0 | 2.5 | 0.420 |
| Day 1 | 30.1 | 0.012 |
| Day 2 | 46.5 | 0.010 |
| Day 3 | 45.1 | 0.068 |
| | **Albumin (g/L) Vs % change plasma zinc** | |
| | $r^2$(%) | p value |
| Day 1 | 14.8 | 0.094 |
| Day 2 | 25.7 | 0.077 |
| Day 3 | 15.3 | 0.338 |

**Table 9.5** Relationship between serum albumin (g/L) and plasma zinc (µg/L) on day 0-3 and % change plasma zinc on day 1-3, using simple linear regression.

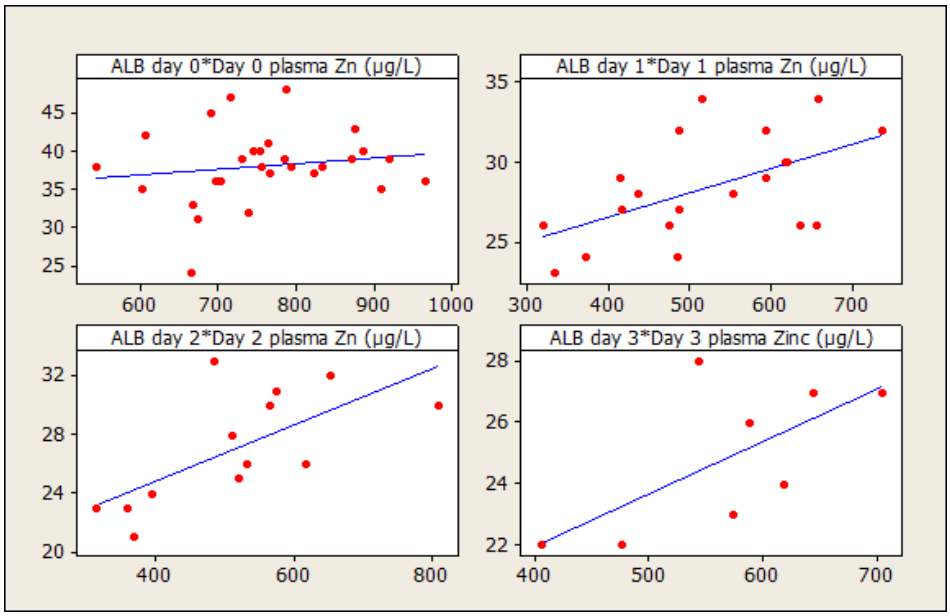



**Fig 9.5a** Scatterplots of plasma zinc concentration (µg/L) versus serum albumin concentration (g/L) on day 0-3

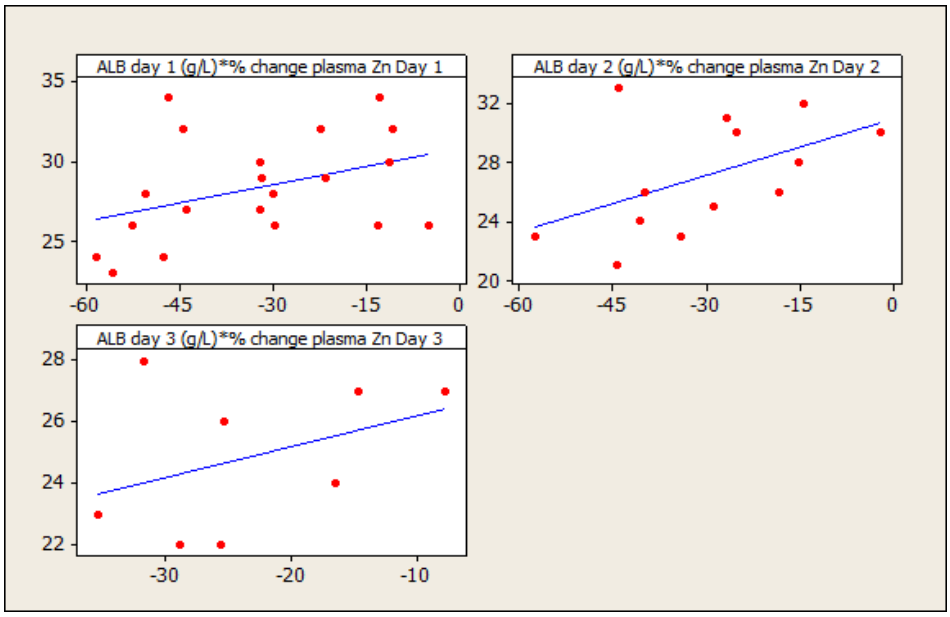



**Fig 9.5b** Scatterplots of % change plasma zinc concentration versus serum albumin concentration (g/L) on day 1-3

*9.3.3 Plasma zinc concentrations versus Zinc: Albumin ratio*

To further examine the relationship between the zinc: albumin ratio and plasma zinc concentration on days 0-3, and % change in plasma zinc concentration on day 1-3 , linear regression was carried out; the results can be seen in table 9.6. The relationship between plasma zinc concentration (µg/L) and the zinc: albumin ratio on day 0-3 can be seen in figure 9.6a. The relationship between % change of plasma zinc concentration and the zinc: albumin ratio on day 1-3 can be seen in figure 9.6b.

There is a statistically significant linear relationship between plasma zinc concentration and the zinc: albumin ratio on days 0-3 ($p \leq 0.01$). The relationship between the percentage change in plasma zinc concentration and the zinc: albumin ratio is statically significant on days 1 and 2 ($p \leq 0.001$), but absent by day 3 ($p=0.264$).

| | **Zinc: albumin Vs plasma zinc (µg/L)** | |
|---|---|---|
| | $r^2$(%) | p value |
| Day 0 | 38.5 | 0.000 |
| Day 1 | 75.3 | 0.000 |
| Day 2 | 74.6 | 0.000 |
| Day 3 | 70.0 | 0.010 |
| | **Zinc: albumin Vs % change plasma zinc** | |
| | $r^2$(%) | p value |
| Day 1 | 67.6 | 0.000 |
| Day 2 | 65.1 | 0.001 |
| Day 3 | 20.2 | 0.264 |

**Table 9.6** Relationship between zinc: albumin ratio and plasma zinc (µg/L) on day 0-3 and % change plasma zinc on day 1-3, using simple linear regression.

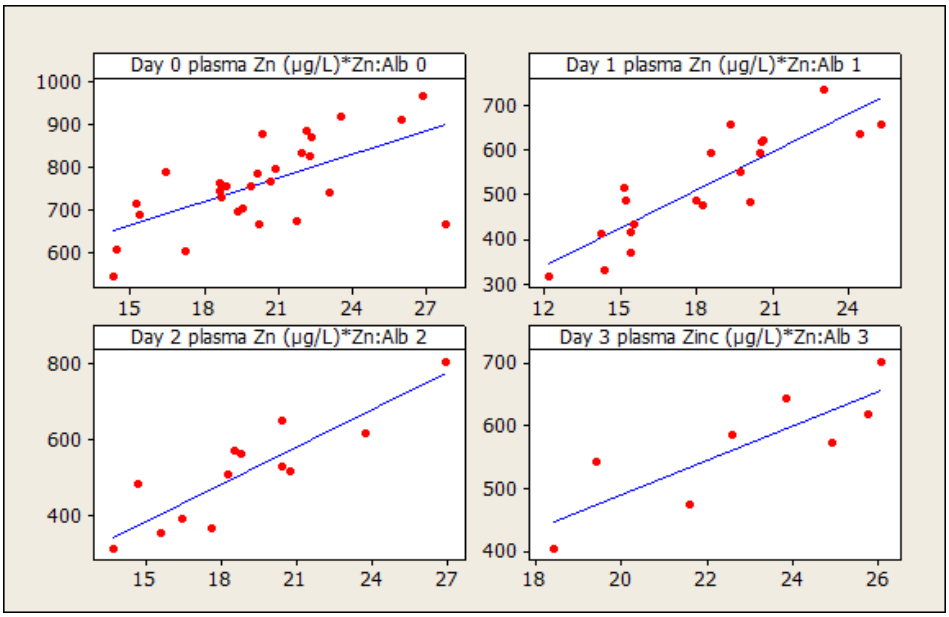



**Fig 9.6a** Scatterplots of plasma zinc concentration (µg/L) versus zinc: albumin ratio on day 0-3.

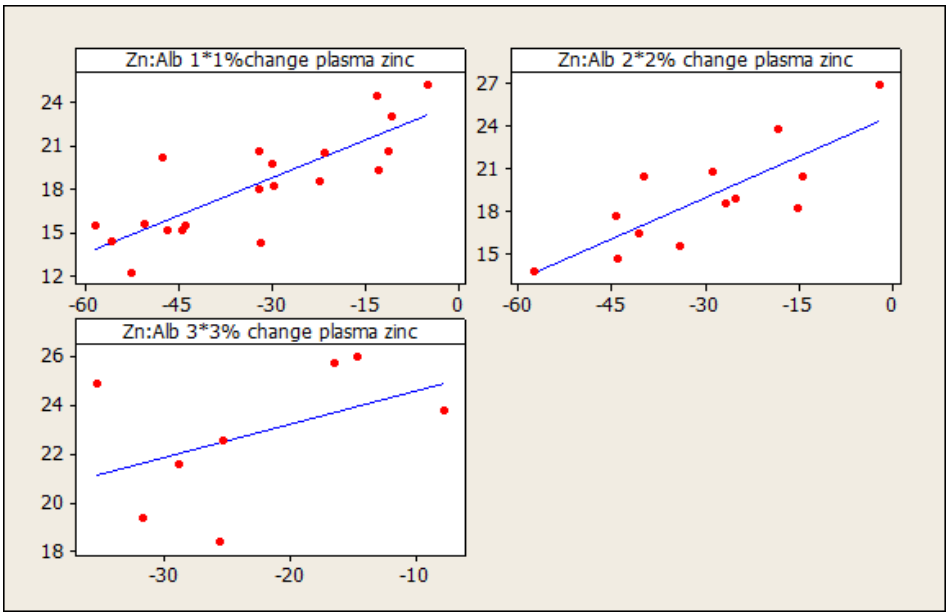



**Fig 9.6b** Scatterplots of % change plasma zinc versus zinc: albumin ratio on day 1-3

*9.3.4 Plasma zinc concentrations versus C-reactive protein*

In order to examine the relationship between C-reactive protein concentration and plasma zinc concentration on days 0-3, and % change in plasma zinc concentration on day 1-3 , linear regression was carried out; the results can be seen in table 9.7. The logarithmic transformation of CRP day 0 was used. The relationship between plasma zinc concentration (µg/L) and serum CRP concentration (mg/L) on day 0-3 can be seen in figure 9.7a. The relationship between % change of plasma zinc concentration and serum albumin concentration (g/L) on day 1-3 can be seen in figure 9.7b.

No linear relationship is found between plasma zinc concentration and serum C-reactive protein concentration over days 0-3 or between the % change in plasma zinc concentration and serum C-reactive protein concentration over days 1-3.

| | **CRP (mg/L) Vs plasma zinc (µg/L)** | |
|---|---|---|
| | $r^2$(%) | p value |
| Day 0 | 6.4 | 0.193 |
| Day 1 | 7.2 | 0.254 |
| Day 2 | 1.2 | 0.721 |
| Day 3 | 1.0 | 0.812 |
| | **CRP (mg/L) Vs % change plasma zinc** | |
| | $r^2$(%) | p value |
| Day 1 | 1.4 | 0.618 |
| Day 2 | 2.6 | 0.596 |
| Day 3 | 0.1 | 0.958 |

**Table 9.7** Relationship between CRP (mg/L) and plasma zinc (µg/L) on day 0-3, and % change plasma zinc on day 1-3, using simple linear regression.

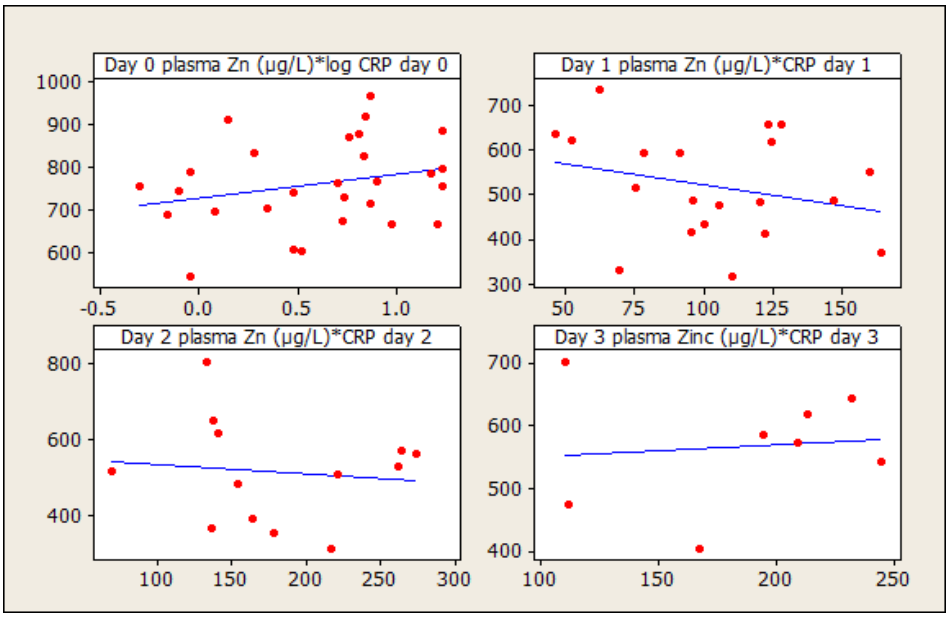



**Fig 9.7a** Scatterplots of plasma zinc concentration (µg/L) versus CRP concentration (mg/L) on day 0-3

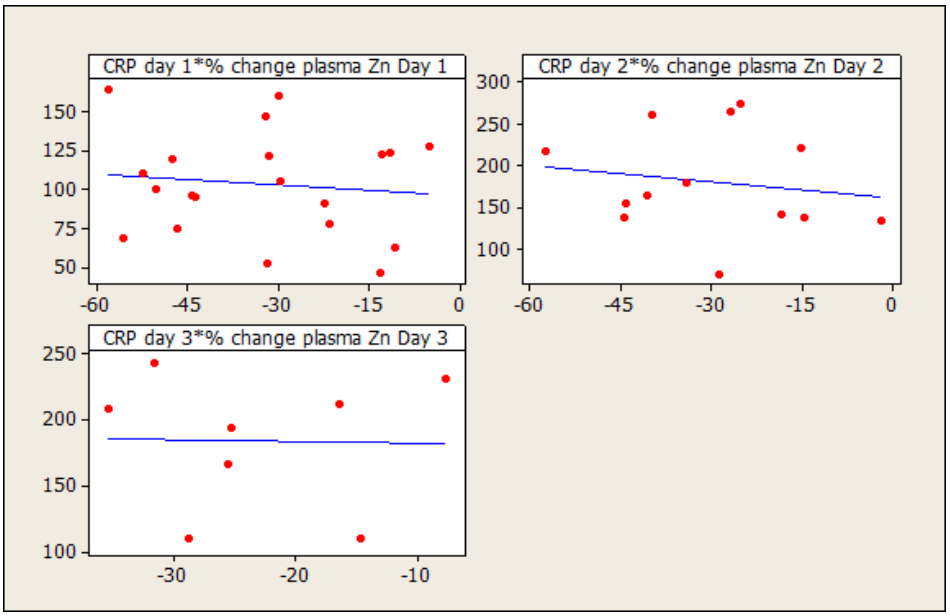



**Fig 9.7b** Scatterplots of % change in plasma zinc concentration versus CRP concentration (mg/L) on day 1-3

## 9.4 Prediction of liver zinc and day 0 plasma zinc

Given that plasma zinc is the only marker related to liver zinc status, yet it changes with the acute phase response, it would be of value to find a model to predict the "non-inflamed" plasma zinc, or the liver concentration of zinc, during the acute phase response. Regression analysis was used to find predictors of baseline plasma or liver zinc concentrations during the acute phase response seen on days 1-3.

### *9.4.1 Plasma zinc*

The relationship between plasma zinc concentration and liver zinc concentration on day 0 can be seen in fig 7.6a. The relationship between plasma zinc concentration on days 1-3 and plasma zinc concentration on day 0 can be seen in fig 9.8. Table 9.8 shows the results of linear regression analysis of the relationship between both plasma zinc day 0 (μg/L) and liver zinc concentration (μg/g dry weight), with plasma zinc concentration on day 1-3, using simple linear regression.

A linear relationship exists between plasma zinc and liver zinc concentrations beyond day 0, but the relationship is no longer statistically significant. There is no relationship between plasma zinc on day 0 and day 1, but by day 2 and day 3 there is a suggestion of a linear relationship between the plasma zinc concentration and the plasma zinc concentration from day 0, however it does not reach statistical significance.

| | Plasma zinc (µg/L) Vs Liver Zn (µg/g dry weight) | |
|---|---|---|
| | $r^2$(%) | p value |
| Day 0 | 14.4 | 0.046 |
| Day 1 | 11.4 | 0.145 |
| Day 2 | 24.7 | 0.084 |
| Day 3 | 26.6 | 0.191 |
| | **Plasma zinc (µg/L) Vs Plasma zinc Day 0 (µg/L)** | |
| | $r^2$(%) | p value |
| Day 1 | 0.6 | 0.752 |
| Day 2 | 25.3 | 0.080 |
| Day 3 | 44.6 | 0.070 |

**Table 9.8** Relationship between both plasma zinc day 0 (µg/L) and liver zinc concentration (µg/g dry weight), with plasma zinc concentration on day 0-3 using simple linear regression.

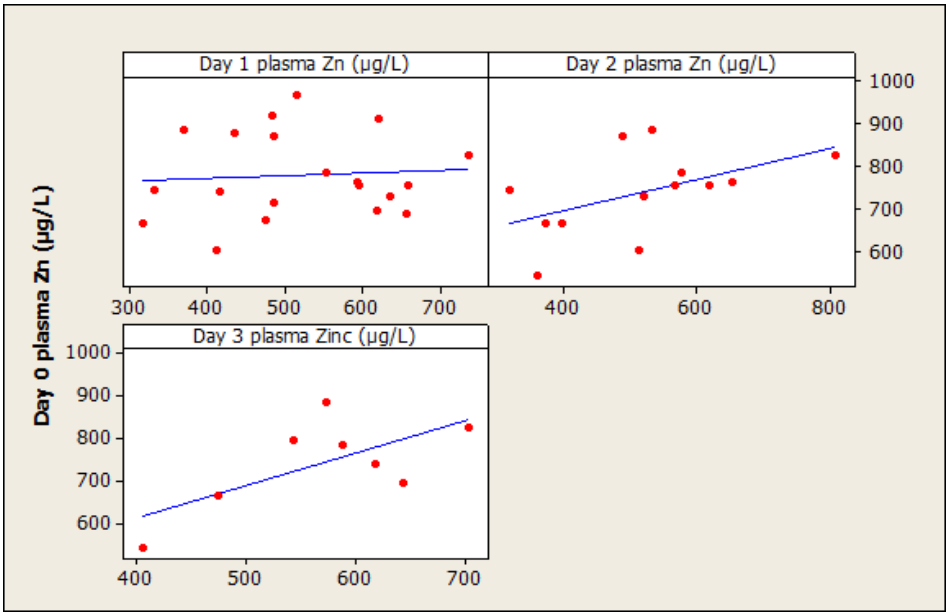



**Fig 9.8** Scatterplots of plasma zinc concentration (µg/L) on day 1-3 versus plasma zinc concentration (µg/L) on day 0.

*9.4.2 Albumin*

Figure 9.9a shows the relationship between serum albumin concentration on day 0-3 and plasma zinc concentration on day 0. Figure 9.9b shows the relationship between serum albumin concentration on day 0-3 and liver zinc concentration on day 0. The results of linear regression analysis of the relationship between both plasma zinc day 0 (µg/L) and liver zinc concentration (µg/g dry weight), with serum albumin concentration on day 1-3, using simple linear regression, can be seen in table 9.9.

There is no relationship between serum albumin concentration on day 0-3 and liver zinc concentration. A linear relationship between serum albumin concentration and plasma zinc concentration, that is not present on day 0, develops over day 1-3. The linearity is statistically significant on day 2 (p=0.009) when serum albumin explains 23.5% of the variability of the plasma zinc concentration on day 0.

| | **Serum Albumin (g/L) Vs Plasma Zn day 0 (µg/L)** | |
|---|---|---|
| | $r^2$(%) | p value |
| Day 0 | 2.5 | 0.420 |
| Day 1 | 9.0 | 0.120 |
| Day 2 | 23.5 | 0.009 |
| Day 3 | 12.9 | 0.061 |
| | **Serum Albumin (g/L) Vs Liver Zn (µg/g dry weight)** | |
| | $r^2$(%) | p value |
| Day 0 | 1.6 | 0.525 |
| Day 1 | 1.5 | 0.535 |
| Day 2 | 8.8 | 0.125 |
| Day 3 | 0.2 | 0.833 |

**Table 9.9** Relationship between both plasma zinc day 0 (µg/L) and liver zinc concentration (µg/g dry weight), with serum albumin concentration on day 0-3, using simple linear regression.

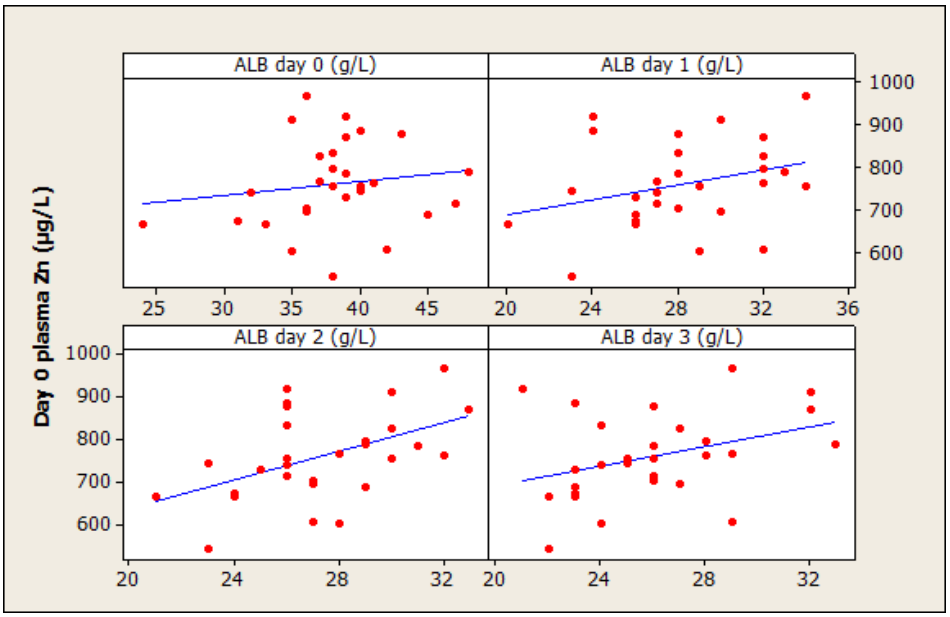



**Fig 9.9a** Scatterplots of serum albumin concentration (g/L) on day 0-3 versus plasma zinc concentration (µg/L) on day 0.

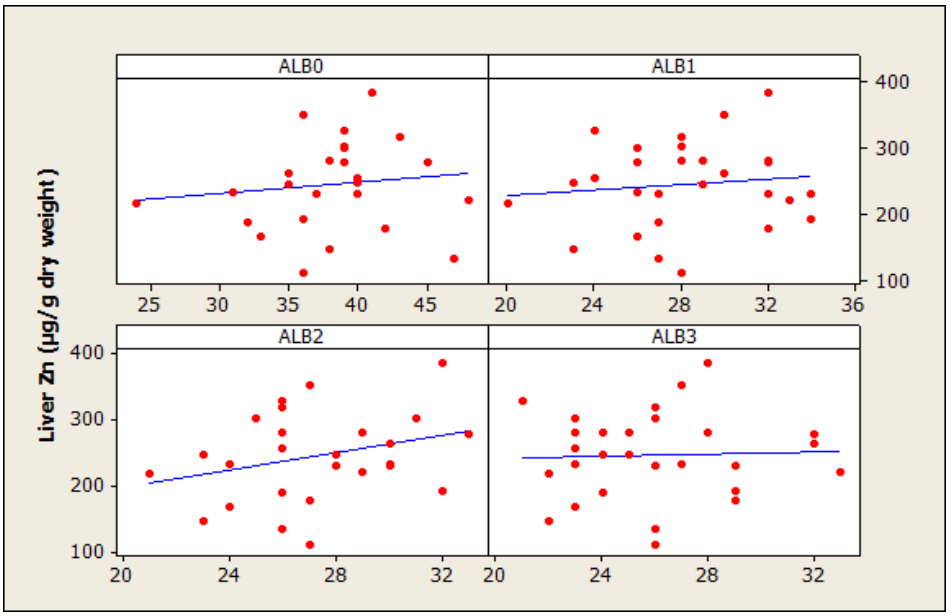



**Fig 9.9b** Scatterplots of serum albumin concentration (g/L) on day 0-3 versus liver zinc concentration (µg/g dry weight)

*9.4.3 Zinc: Albumin ratio*

The relationship between the zinc: albumin ratio on day 0-3 and plasma zinc concentration on day 0 can be seen in Figure 9.10a. Figure 9.10b shows the relationship between the zinc: albumin ratio on day 0-3 and liver zinc concentration on day 0. The results of linear regression analysis of the relationship between both plasma zinc day 0 (µg/L) and liver zinc concentration (µg/g dry weight), with the zinc: albumin ratio on day 1-3, using simple linear regression, can be seen in table 9.10.

There is a linear relationship between the zinc: albumin ratio and the plasma zinc concentration on day 0, but this then disappears on day 1 and day 2. There is an $r^2$ of 36.7% by day 3 but this is not statistically significant (p=0.111). There is no linear relationship between the zinc: albumin ratio and the liver zinc concentration on days 0-3.

| | **Zinc: Albumin ratio Vs Plasma Zn day 0 (µg/L)** | |
|---|---|---|
| | $r^2$(%) | p value |
| Day 0 | 38.5 | 0.000 |
| Day 1 | 0.1 | 0.910 |
| Day 2 | 10.6 | 0.277 |
| Day 3 | 36.7 | 0.111 |
| | **Zinc: Albumin ratio Vs Liver Zn (µg/g dry weight)** | |
| | $r^2$(%) | p value |
| Day 0 | 2.4 | 0.435 |
| Day 1 | 4.8 | 0.093 |
| Day 2 | 8.4 | 0.335 |
| Day 3 | 3.8 | 0.642 |

**Table 9.10** Relationship between both plasma zinc day 0 (µg/L) and liver zinc concentration (µg/g dry weight), with albumin: zinc ratio on day 0-3, using simple linear regression.

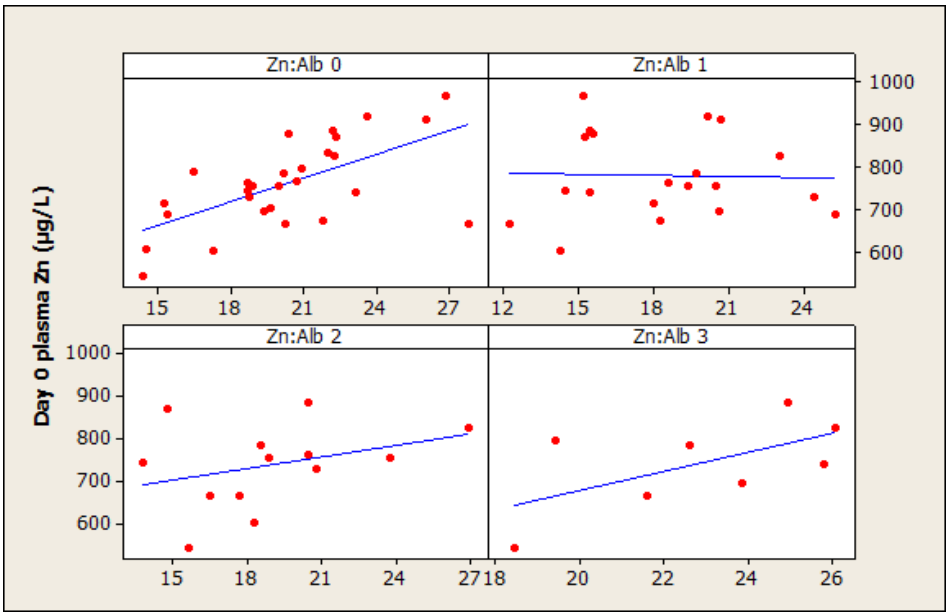



**Fig 9.10a** Scatterplots of zinc: albumin ratios on day 0-3 versus plasma zinc concentration (µg/L) on day 0

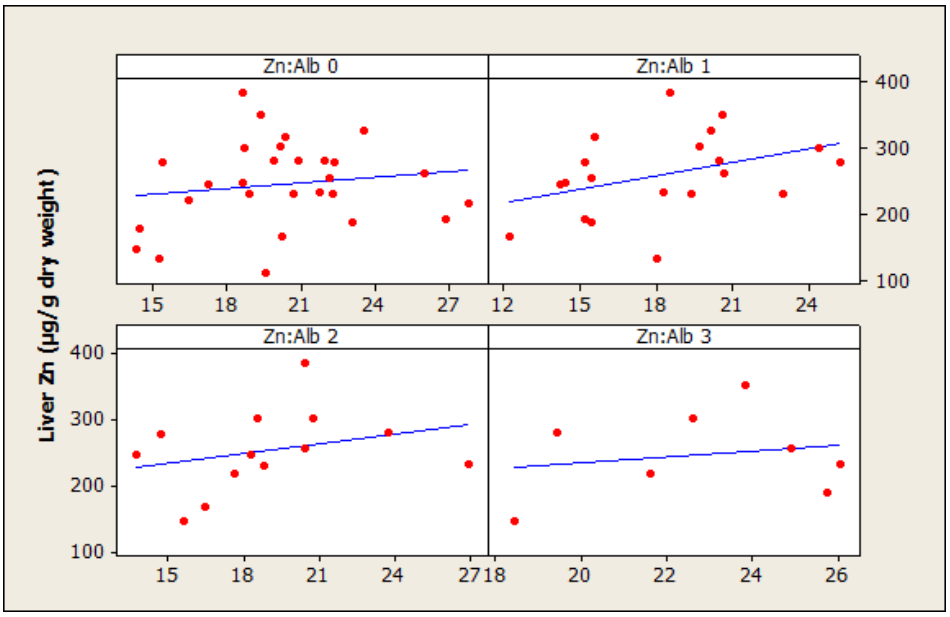



**Fig 9.10b** Scatterplots of zinc: albumin ratios on day 0-3 versus liver zinc concentration (µg/g dry weight)

*9.4.4 C - reactive protein*

Figure 9.11a shows the relationship between CRP concentration on day 0-3 and plasma zinc concentration on day 0. Figure 9.11b shows the relationship between CRP concentration on day 0-3 and liver zinc concentration on day 0. The logarithmic transformation of day 0 CRP was used. The results of linear regression analysis of the relationship between both plasma zinc day 0 (µg/L) and liver zinc concentration (µg/g dry weight), with CRP concentration on day 0-3, using simple linear regression, can be seen in table 9.11.

There is no relationship between either plasma zinc day on day 0 or liver zinc, and CRP on day 0-3.

| | **CRP (mg/L) Vs Plasma Zn day 0 (µg/L)** | |
|---|---|---|
| | $r^2$(%) | p value |
| Day 0 | 6.4 | 0.193 |
| Day 1 | 0.1 | 0.904 |
| Day 2 | 0.0 | 0.987 |
| Day 3 | 2.2 | 0.456 |
| | **CRP (mg/L) Vs Liver Zn (µg/g dry weight)** | |
| | $r^2$(%) | p value |
| Day 0 | 0.0 | 0.999 |
| Day 1 | 1.1 | 0.596 |
| Day 2 | 0.0 | 0.920 |
| Day 3 | 0.3 | 0.777 |

**Table 9.11** Relationship between both plasma zinc day 0 (µg/L) and liver zinc concentration (µg/g dry weight), with CRP (mg/L) on day 0-3, using simple linear regression.

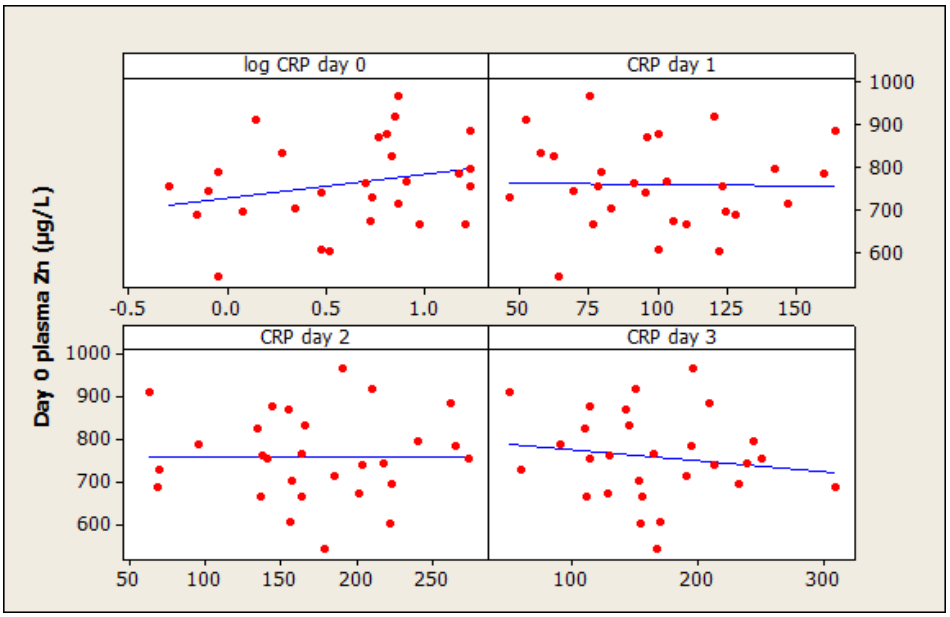



**Fig 9.11a** Scatterplots of CRP (mg/L) on day 0-3 versus plasma zinc concentration (µg/L) on day 0

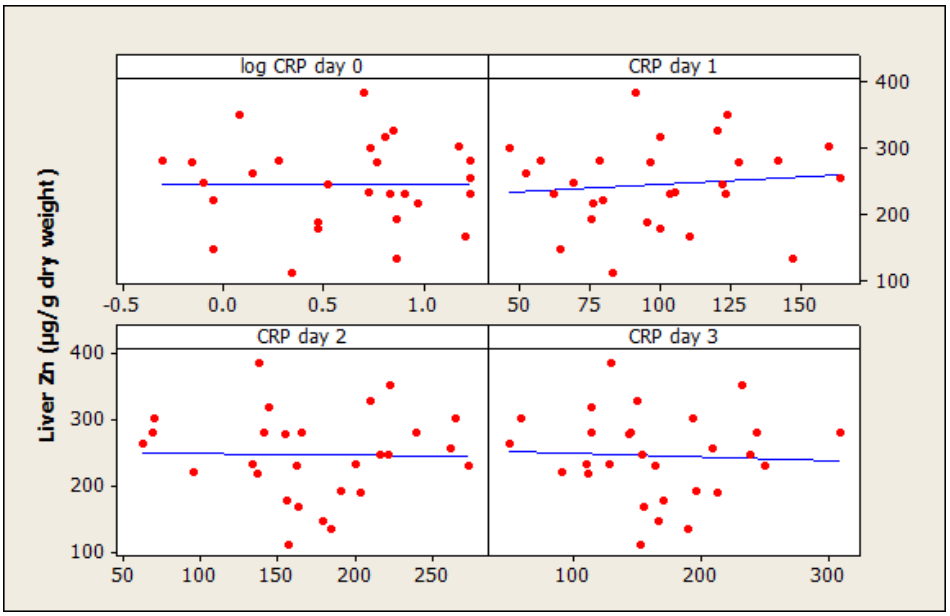



**Fig 9.11b** Scatterplots of CRP (mg/L) on day 0-3 versus liver zinc concentration (µg/g dry weight)

*9.4.5 Multiple linear regression*

Ideally, a model would exist to predict zinc status during the acute phase response. Plasma zinc status on day 0 and liver zinc concentration were used as markers of zinc status. As they are likely to be available in clinical practice, a model using plasma zinc concentration (μg/L), serum albumin concentration (g/L), zinc: albumin ratio and serum C-reactive protein (mg/L) was built for each of day 1-3. Results can be seen in table 9.12.

| | | $R^2$(%) | p value |
|---|---|---|---|
| **Day 1** | | | |
| Plasma Zinc Day 0 | | | |
| | Plasma Zinc Day 1 | 0.6 | 0.752 |
| | + Albumin Day 1 | 5.3 | 0.630 |
| | +Zn: Alb Day 1 | 7.1 | 0.751 |
| | + CRP Day 1 | 9.7 | 0.802 |
| Liver Zinc | | | |
| | Plasma Zinc Day 1 | 11.4 | 0.145 |
| | + Albumin Day 1 | 14.1 | 0.275 |
| | +Zn: Alb Day 1 | 15.6 | 0.423 |
| | + CRP Day 1 | 15.6 | 0.607 |
| **Day 2** | | | |
| Plasma Zinc Day 0 | | | |
| | Plasma Zinc Day 2 | 25.3 | 0.080 |
| | + Albumin Day 2 | 33.5 | 0.130 |
| | +Zn: Alb Day 2 | 37.5 | 0.217 |
| | + CRP Day 2 | 39.1 | 0.353 |
| Liver Zinc | | | |
| | Plasma Zinc Day 2 | 24.7 | 0.084 |
| | + Albumin Day 2 | 36.5 | 0.103 |
| | +Zn: Alb Day 2 | 39.1 | 0.197 |
| | + CRP Day 2 | 42.8 | 0.290 |
| **Day 3** | | | |
| Plasma Zinc Day 0 | | | |
| | Plasma Zinc Day 3 | 44.6 | 0.070 |
| | + Albumin Day 3 | 44.6 | 0.228 |
| | +Zn: Alb Day 3 | 73.0 | 0.123 |
| | + CRP Day 3 | 77.8 | 0.226 |
| Liver Zinc | | | |
| | Plasma Zinc Day 3 | 26.6 | 0.191 |
| | + Albumin Day 3 | 48.5 | 0.190 |
| | +Zn: Alb Day 3 | 52.3 | 0.351 |
| | + CRP Day 3 | 55.1 | 0.550 |

**Table 9.12a** Multiple linear regression to predict plasma zinc concentration on day 0 and liver zinc concentration on each of day 1-3. Each step involves the addition of a factor to the model.

None of the regression models reached statistical significance, probably due to insufficient numbers. However, a combination of plasma zinc concentration, albumin concentration, zinc: albumin ratio and CRP was the strongest model for predicting both plasma zinc concentration on day 0 and liver zinc concentration.

In clinical practice it is unlikely there would be a clean rise and fall of the acute phase response, so the stage of the acute phase response would not be known. Therefore, any equation to predict zinc status would have to be able to be used at any stage of the acute phase response. The results of day 1-3 were combined and the prediction model involving plasma zinc concentration (µg/L), serum albumin concentration (g/L), zinc: albumin ratio and serum C-reactive protein (mg/L) was repeated; the results can be seen in table 9.12b.

| | | $R^2$ (%) | p value |
|---|---|---|---|
| **Day 1-3** | | | |
| Plasma Zinc Day 0 | | | |
| | Plasma Zinc | 8.6 | 0.062 |
| | + Albumin | 16.8 | 0.031 |
| | +Zn: Alb | 21.0 | 0.032 |
| | + CRP | 21.5 | 0.063 |
| Liver Zinc | | | |
| | Plasma Zinc | 15.8 | 0.010 |
| | + Albumin | 18.4 | 0.021 |
| | +Zn: Alb | 18.5 | 0.054 |
| | + CRP | 18.5 | 0.110 |

**Table 9.12b** Multiple linear regression to predict plasma zinc concentration on day 0 and liver zinc concentration. Each step involves the addition of a factor to the model. Factors are a combination of the results from day 1-3.

16.8% of the variance of plasma zinc from day 0 can be explained by the plasma zinc concentration and the albumin (p=0.031). 18.5% of the liver zinc concentration can be explained by plasma zinc concentration, serum albumin concentration and the zinc: albumin ratio (p=0.054). The addition of CRP to the model did not strengthen it in either case. Neither of these models is strong enough to be used for the prediction of zinc status during the acute phase response in clinical practice.

## 9.5 Summary of findings - Zinc

Plasma zinc and the zinc: albumin ratio both decrease with the evolution of the acute phase response on days 1-3.

In the pre-operative samples, when the patients were not inflamed, plasma zinc concentration has a weak linear relationship with liver zinc concentration.

Exploring this relationship further by splitting the data into tertiles of liver zinc shows that the trend was for plasma zinc to be highest in the highest liver zinc tertile and lowest in the lowest liver zinc tertile; however, this result does not reach statistical significance. No significant linear relationship existed between plasma zinc and liver zinc concentration during the acute phase response.

A linear relationship between plasma zinc concentration and albumin concentration, which is not present on day 0 when the patients are non-inflamed, developed during days 1-3 of the acute phase response.

Albumin, plasma zinc and the plasma zinc: albumin ratio cannot be used to create a model for predicting the non-inflamed plasma zinc or liver zinc as a means of assessing zinc status during the acute phase response.

# Chapter 10

## Selenium and the acute phase response

### 10.1 Change in plasma selenium, red blood cell selenium and red blood cell glutathione peroxidase activity, with the acute phase response

On days 1-3, in the post-operative period, a systemic acute phase response develops marked by a rise in CRP and a fall in serum albumin. Kruskal-Wallis (non-parametric) one way analysis of variance was performed to see if red blood cell or plasma selenium concentration, or red blood cell glutathione peroxidase activity varies during the acute phase response. The use of the selenium: albumin ratio allows the relationship between selenium concentration and albumin concentration to be examined while correcting for any effect of plasma dilution due to intravenous fluid replacement given to the patient post-operatively.

The change in the concentration of red blood cell selenium can be seen in fig 10.1a, plasma selenium in fig 10.1b, the plasma selenium: albumin ratio in fig 10.1c and red blood cell glutathione peroxidase in fig 10.1d. Median values, inter-quartile ranges and the results of Kruskal-Wallis (non-parametric) one way analysis of variance can be seen in table 10.1.

There is no significant change in red blood cell selenium concentration ($p=0.647$), plasma selenium: albumin ratio ($p=0.959$), or red blood cell glutathione peroxidase activity ($p=0.526$) over days 0-3.

Plasma selenium concentration changes significantly over days 0-3 ($p<0.001$). Mann-Whitney comparisons were performed (Bonferroni correction $p=0.017$ is significant): day 0 to day 1 $p<0.001$, day 1 to day 2 $p=0.867$, day 2 to day 3 $p=0.890$. Therefore the plasma selenium does change significantly during the acute phase response, with the significant change being between day 0 and day 1.

| | Median (IQR) | | | | |
|---|---|---|---|---|---|
| | Day 0 | Day 1 | Day 2 | Day 3 | P value for variance |
| RBC Se (µg/µg Fe) | 0.000203 (0.000176-0.000245) | 0.000207 (0.000188-0.000234) | 0.00020 (0.000165-0.000231 | 0.000202 (0.000160-0.000214) | 0.647 |
| Plasma Se (µmol/L) | 0.96 (0.80-1.09) | 0.61 (0.57-0.78) | 0.67 (0.52-0.92) | 0.69 (0.54-0.82) | <0.001 |
| Se:Alb ratio | 2.02 (1.72-2.14) | 1.94 (1.67-2.19) | 1.95 (1.69-2.37) | 2.04 (1.84-2.25) | 0.959 |
| RBC GPX (U/g Hb) | 40.39 (30.38-46.78) | 42.03 (31.97-45.12) | 39.56 (30.68-51.11) | 33.61 (30.28-40.7) | 0.526 |

**Table 10.1** Median (IQR) values of red blood cell selenium concentration, plasma selenium concentration, plasma selenium: albumin ratio and red blood cell glutathione peroxidase activity on days 0-3. The significance of variance (p value) is from Kruskal-Wallis (non-parametric) one way analysis of variance.

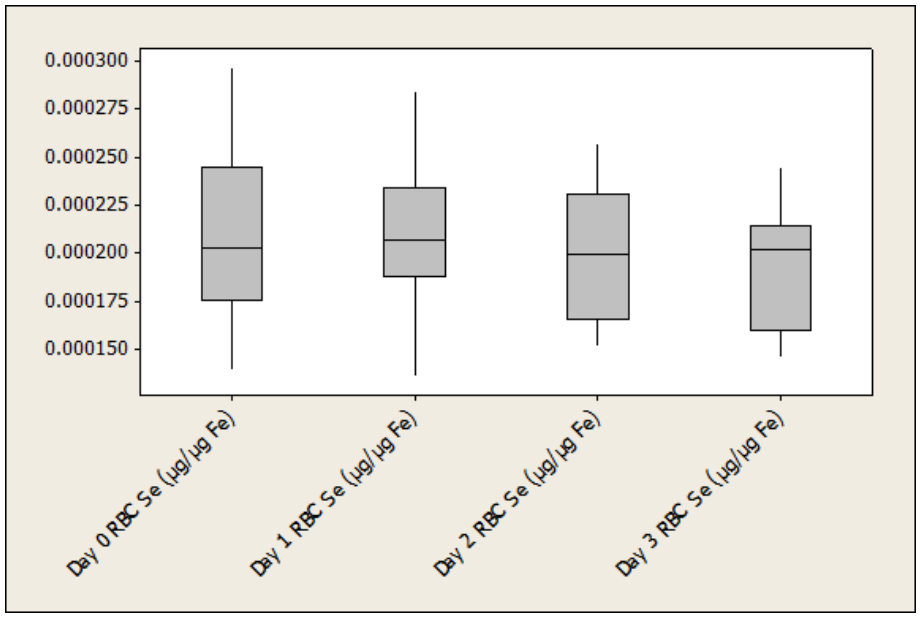



**Fig 10.1a** Boxplot of red blood cell selenium concentration (µg/ µg Fe) on day 0-3

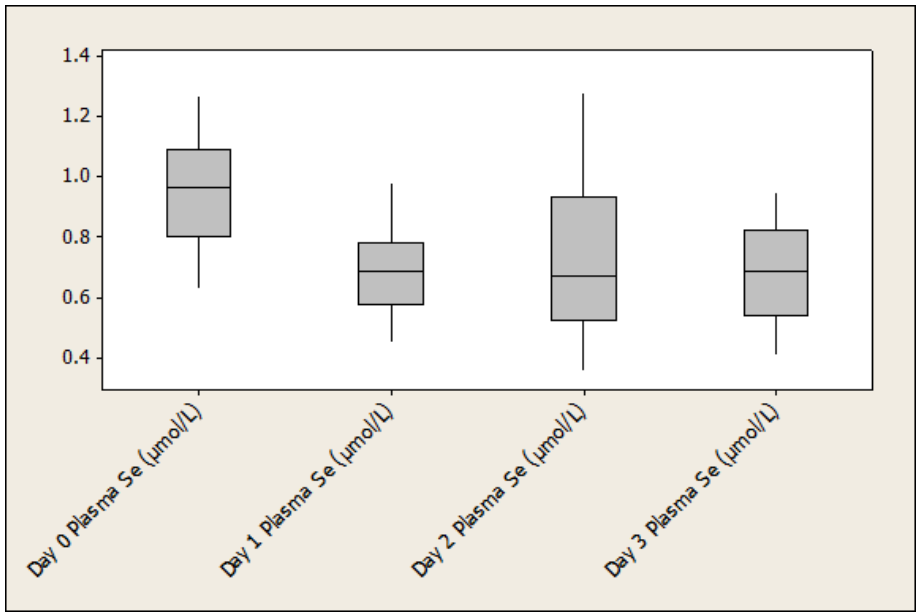



**Fig 10.1b** Boxplot of plasma selenium concentration (µmol/L) on day 0-3

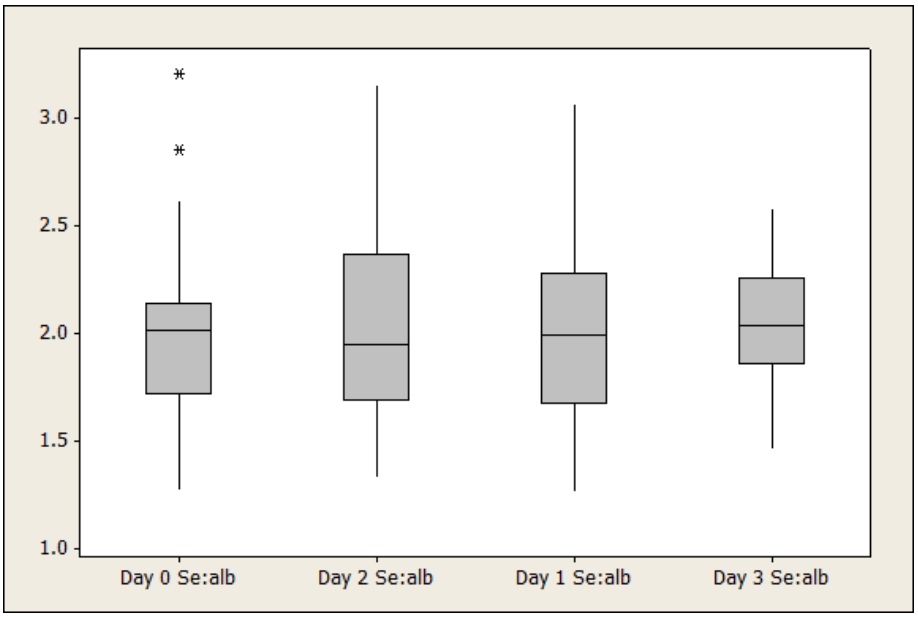



**Fig 10.1c** Boxplot of plasma selenium: albumin ratio on day 0-3

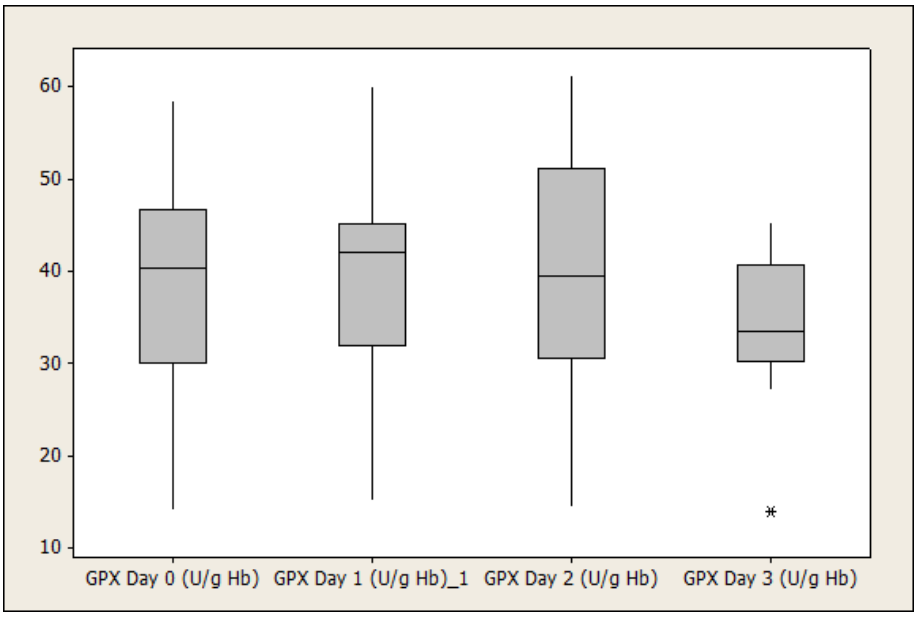



**Fig 10.1d** Boxplot of red blood cell glutathione peroxidase activity (U/g Hb) on day 0-3

## 10.2 Factors affecting changes in plasma selenium concentration

### *10.2.1 Tertiles of liver selenium*

In order to examine the effect of liver selenium concentration on the changes in plasma selenium concentration seen during the evolution of acute phase response, data was split into tertiles by liver selenium concentration; there were 10, 11 and 10 patient samples in each of the low, medium and high tertiles respectively. One way analysis of variance of CRP and Albumin was performed to ensure there were no differences in CRP or Albumin between tertiles on any of day 0-3; as it was not normally distributed, the logarithmic transformation of CRP day 0 was used. Mean values (SD) for CRP and Albumin by liver tertile can be seen in table 10.2. No differences in CRP or Albumin concentrations were seen between tertiles of liver selenium.

| | Tertile liver selenium (range liver selenium (µg/g dry weight)) | | | |
|---|---|---|---|---|
| | 1 (1.19-1.91) | 2 (1.91-2.12) | 3 (2.19-2.57) | p value for variance |
| CRP day 0 (mg/L) | 6.0(6.1) | 4.5(4.4) | 7.1(5.8) | 0.772 |
| CRP day 1 (mg/L) | 89.9(35.5) | 91.7(24.9) | 103.3(42.0) | 0.644 |
| CRP day 2 (mg/L) | 151.0(71.7) | 177.4(47.0) | 176.0(67.8) | 0.570 |
| CRP day 3 (mg/L) | 155.3(77.3) | 163.3(55.7) | 158.1(60.4) | 0.960 |
| Alb day 0 (g/L) | 38.1(6.4) | 36.5(3.9) | 40.3(2.9) | 0.180 |
| Alb day 1 (g/L) | 28.2(4.6) | 28.5(3.0) | 29.5(3.8) | 0.727 |
| Alb day 2 (g/L) | 27.1(3.3) | 26.6(2.7) | 28.8(2.8) | 0.277 |
| Alb day 3 (g/L) | 25.2(3.3) | 26.0(2.9) | 27.2(3.5) | 0.390 |

**Table 10.2** Mean values (SD) of CRP (mg/L) and Albumin (g/L) on day 0-3 for tertiles of liver selenium concentration (µg/g dry weight).

### *10.2.2 Plasma selenium concentrations between tertiles of liver Selenium*

To see if the relationship found between plasma selenium concentration and liver selenium concentration is maintained during the acute phase response, Kruskal-Wallis non-parametric analysis of variance was performed between the medians of plasma selenium concentration for each of the three tertiles of liver selenium concentration on days 0-3; this was also performed for the percentage change in plasma selenium to ascertain if the decrease in plasma selenium concentration with the acute phase response is related to the liver selenium concentration.

Median concentration of plasma selenium (μmol/L) on day 0-3 and median percentage change in plasma selenium, from day 0 to day 1-3, divided by tertile of liver selenium, are shown in table 10.3. The median concentration of plasma selenium (μmol/L) for each liver tertile, at each time point can be seen graphically in figure 10.2a and the median percentage change in plasma selenium concentration between day 0 and day 1-3, for tertiles of liver selenium concentration, can be seen in figure 10.2b.

The plasma selenium concentration across liver selenium tertile 1-3 is statistically significantly different on day 2; this is due to tertile 3 being higher than tertile 1 and 2 and fits with the relationship seen between liver and plasma selenium on day 0. The percentage change in plasma selenium on day 1 is less in tertile 1 than tertiles 2 and 3 ($p= 0.012$), but this does not persist on day 2.

| | Tertile liver selenium (range liver selenium (µg/g dry weight)) | | | |
|---|---|---|---|---|
| | 1 (111.9-220.9) | 2 (230.7-280.1) | 3 (280.9-384.5) | p value for variance |
| Plasma Se day 0 (µmol/L) | 0.78 (0.66-0.90) | 0.98 (0.84-1.11) | 1.01 (0.97-1.14) | 0.005 |
| Plasma Se day 1 (µmol/L) | 0.73 (0.65-0.78) | 0.62 (0.52-0.71) | 0.87 (0.60-0.95) | 0.064 |
| Plasma Se day 2 (µmol/L) | 0.63 (0.39-0.77) | 0.52 (0.47-0.67) | 0.98 (0.82-1.11) | 0.034 |
| Plasma Se day 3 (µmol/L) | 0.55 (0.41-0.69) | 0.65* | 0.86 (0.63-0.94) | 0.229 |
| Change plasma Se day 1 (%) | -17.0 (-20.1 - -5.1) | -33.8 (-42.6 - -24.8) | -28.0 (-38.3 - -22.5) | 0.012 |
| Change plasma Se day 2 (%) | -28.7 (-39.3 - -7.6) | -26.8 (-56.5 - -20.5) | -20.0 (-31.6 - 9.2) | 0.434 |
| Change plasma Se day 3 (%) | -28.0 (-35.7 - -15.0) | -20.5* | -20.4 (-20.4 - -4.6) | 0.607 |

**Table 10.3** Median (IQR) values of plasma selenium concentration (µmol/L) on day 0-3 and % change in plasma selenium concentration between day 0 and day 1-3, for tertiles of liver selenium concentration. *only 1 sample available in this category.

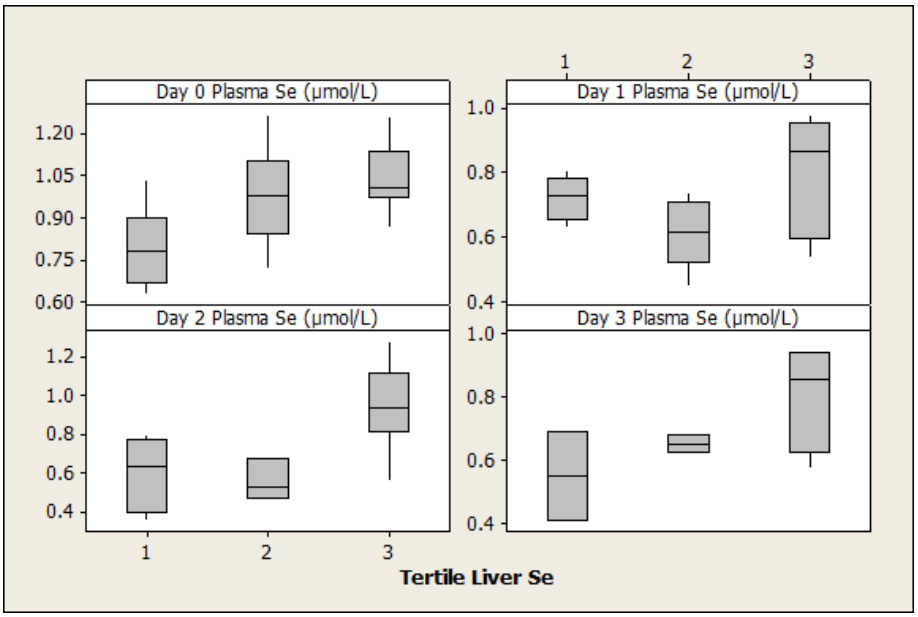



**Fig 10.2a** Boxplot of median plasma selenium concentration (µmol/L) on day 0-3 for tertiles of liver selenium concentration

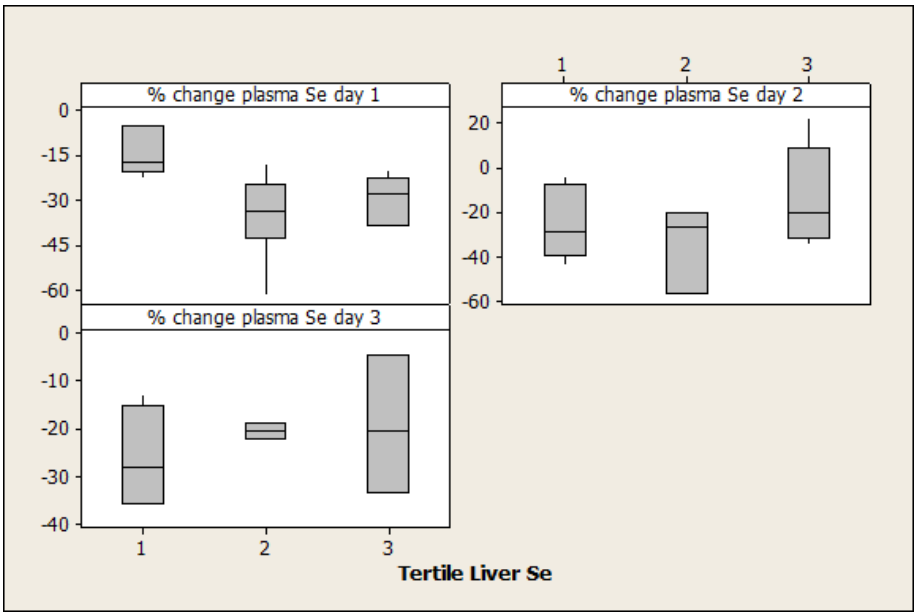



**Fig 10.2b** Boxplot of median % change in plasma selenium concentration between day 0 and day 1-3, for tertiles of liver selenium concentration

*10.2.3 Relationship between plasma selenium on day 0 and days 1-3*

To investigate whether the change in plasma selenium concentration on days 1-3 is related to the plasma selenium concentration on day 0 simple linear regression was performed. The relationship between the plasma selenium concentration on day 0 and the plasma selenium concentration on day 1-3 can be seen in figure 10.3a and the relationship between the plasma selenium concentration on day 0 and the percentage change in plasma selenium concentration from day 0, on day 1-3, can be seen in figure 10.3b. Result of linear regression analysis can be seen in table 10.4.

There is a linear relationship with the percentage fall in plasma selenium from day 0 and the plasma selenium concentration on day 0 on days 1 and 3, but not on day 2. However, looking at the Scatterplot (fig 10.3b) it can be seen that this relationship is reversed on day 3 from day 1. The lower the plasma selenium on day 0, the lower the percentage drop in plasma selenium from day 0; the higher the plasma selenium on day 0, the lower the percentage drop from day 0 is by day 3. In other words, if you have higher plasma selenium it has a greater percentage reduction on day 1 but also a smaller percentage reduction by day 3, therefore although it drops by a greater percentage it recovers more quickly. This may of course simply be regression to the mean and would require further investigation.

Despite differences in percentage change in plasma selenium from day 0 that are related to plasma selenium concentration on day 0, a linear relationship remains with the initial plasma selenium concentration on day 0 throughout day 1-3; those who had the highest plasma selenium on day 0 maintained their rank despite increased percentage drops in selenium concentration.

| | Plasma Se day 0 (µmol/L) Vs Plasma Se day1-3 (µmol/L) | |
|---|---|---|
| | $r^2$(%) | p value |
| Day 1 | 23.0 | 0.038 |
| Day 2 | 39.5 | 0.016 |
| Day 3 | 83.4 | 0.001 |
| | **Plasma Se day 0 (µmol/L) Vs % change plasma Se day1-3 (µmol/L)** | |
| | $r^2$(%) | p value |
| Day 1 | 25.0 | 0.029 |
| Day 2 | 1.3 | 0.694 |
| Day 3 | 38.3 | 0.045 |

**Table 10.4** Relationship between plasma selenium on day 1-3 (µmol/L) and the percentage change in plasma selenium form day 0, on day 1-3 with plasma selenium on day 0 (µmol/L), using simple linear regression.

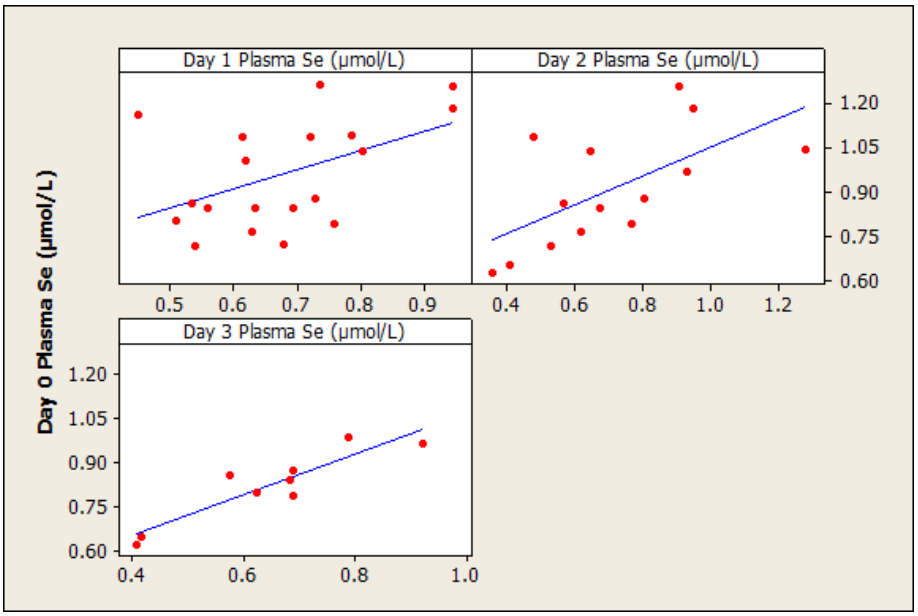



**Fig 10.3a** Scatterplot of plasma selenium on day 0 (µmol/L) versus plasma selenium (µmol/L) on day 1-3

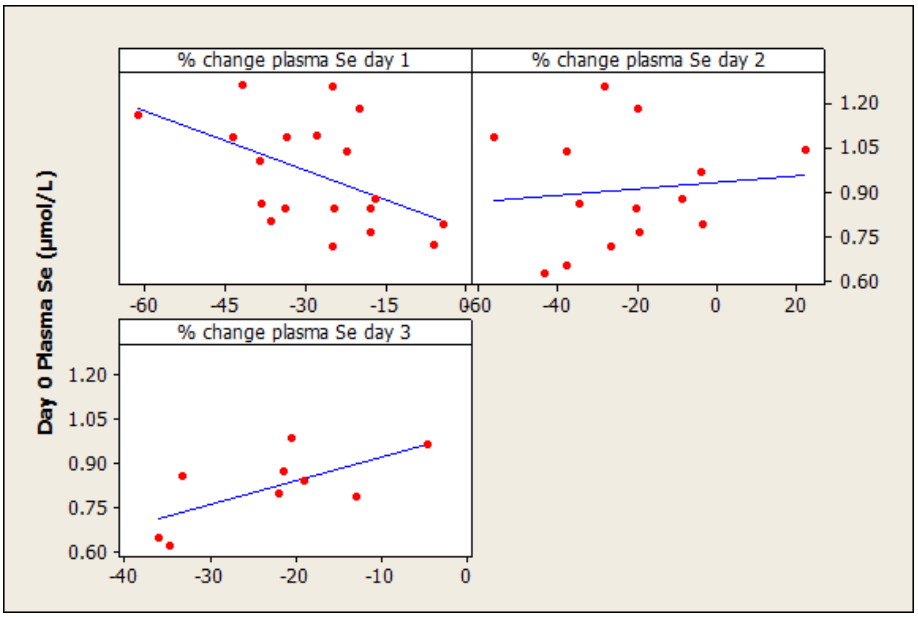



**Fig 10.3b** Scatterplot of plasma selenium on day 0 (µmol/L) versus % change in plasma selenium concentration from day 0, on day 1-3

*10.2.4 Relationship between plasma selenium and C-reactive protein*

Simple linear regression was performed to investigate the relationship between plasma selenium concentration and CRP concentration on days 0-3. Results are in table 10.5 and can be seen graphically in fig 10.4.

There is no linear relationship between plasma selenium concentration and C reactive protein concentration.

| | CRP (g/L) Vs plasma Se (µmol/L) | |
|---|---|---|
| | $r^2$(%) | p value |
| Day 0 | 0.3 | 0.780 |
| Day 1 | 5.5 | 0.322 |
| Day 2 | 2.8 | 0.548 |
| Day 3 | 0.3 | 0.885 |

**Table 10.5** Relationship between plasma selenium concentration (µmol/L) and CRP concentration (mg/L) (log CRP day 0) on days 0-3, using simple linear regression.

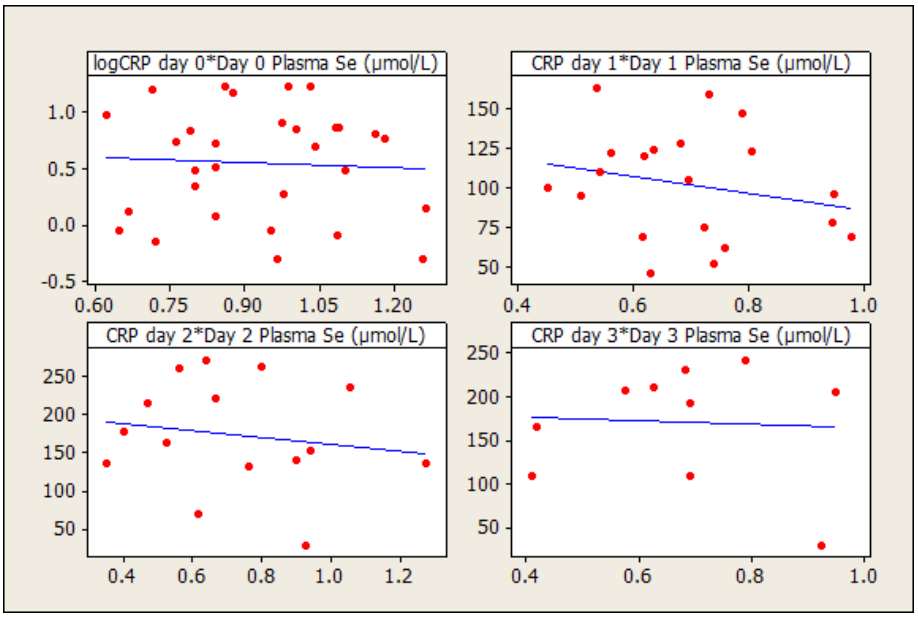



**Fig 10.4** Scatterplot of plasma selenium concentration (µmol/L) versus CRP concentration (mg/L) on day 0-3

### 10.3 Relationship between plasma selenium and serum albumin

Simple linear regression was performed to investigate the relationship between plasma selenium concentration and serum albumin concentration on days 0-3. Results are in table 10.6 and can be seen graphically in fig 10.5.

There is an increasingly strong linear relationship between plasma selenium concentration and serum albumin concentration on day 0-3. The relationship strengthens over days 0-3, and by day 3 the serum albumin concentration is responsible for 90.8% of the variation in plasma selenium concentration.

| | **Albumin (g/L) Vs plasma Se (µmol/L)** | |
|---|---|---|
| | $r^2$(%) | p value |
| Day 0 | 16.7 | 0.025 |
| Day 1 | 36.2 | 0.005 |
| Day 2 | 70.4 | <0.001 |
| Day 3 | 90.8 | <0.001 |

**Table 10.6** Relationship between plasma selenium concentraion (µmol/L) and serum albumin concentration (g/L) on days 0-3, using simple linear regression.

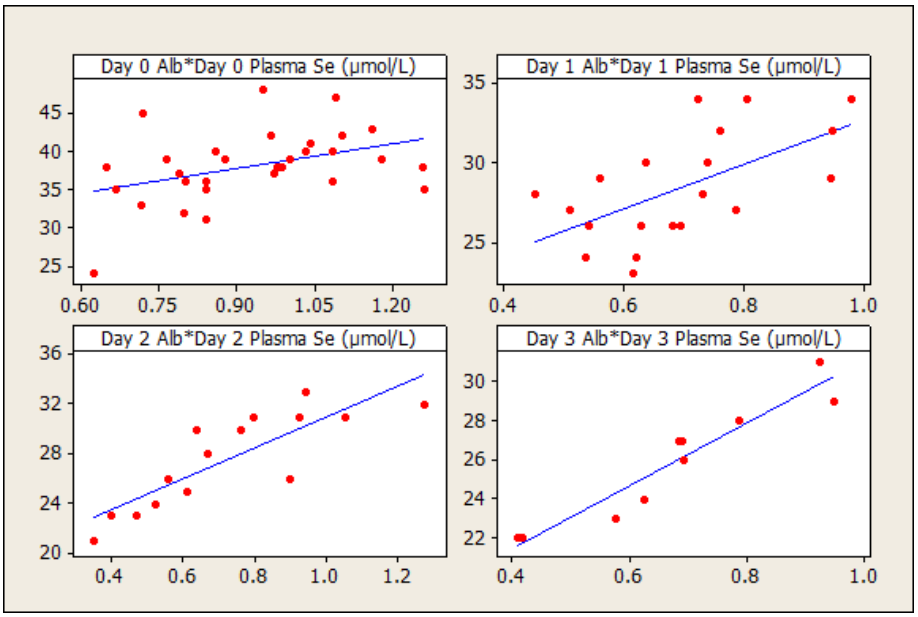



**Fig 10.5** Scatterplot of plasma selenium concentration (µmol/L) versus serum albumin concentration (g/L) on day 0-3

## 10.4 Relationship between liver and plasma selenium and albumin on days 0-3

Liver selenium is of interest as it acts as a surrogate for whole body tissue status. Simple linear regression was performed to investigate the relationship between plasma selenium concentration, plasma selenium:albumin ratio, serum albumin concentration and liver selenium concentration on days 0-3. Results are in table 10.7 and can be seen graphically in fig 10.6a-c.

The linear relationship between plasma selenium and liver selenium concentration seen on day 0 disappears on day 1 but returns on day 2 and 3 despite changes in plasma selenium concentration. When corrected for serum albumin by use of the selenium: albumin ratio, the linear relationship with liver selenium is present throughout days 0-3. There is not a significant relationship between liver selenium concentration and serum albumin concentration, though a pattern does exist most likely reflecting the strong relationship that both liver selenium and serum albumin have with plasma selenium.

| | Liver Se (µg/g dry weight) Vs plasma Se (µmol/L) | |
|---|---|---|
| | $r^2$(%) | p value |
| Day 0 | 37.1 | <0.001 |
| Day 1 | 9.9 | 0.177 |
| Day 2 | 59.4 | 0.001 |
| Day 3 | 50.4 | 0.022 |
| | **Liver Se (µg/g dry weight) Vs Se:alb ratio** | |
| | $r^2$(%) | p value |
| Day 0 | 16.7 | 0.022 |
| Day 1 | 39.5 | 0.002 |
| Day 2 | 67.5 | <0.001 |
| Day 3 | 54.8 | 0.014 |
| | **Liver Se (µg/g dry weight) Vs Serum albumin (g/L)** | |
| | $r^2$(%) | p value |
| Day 0 | 7.5 | 0.135 |
| Day 1 | 6.7 | 0.161 |
| Day 2 | 12.4 | 0.052 |
| Day 3 | 9.5 | 0.092 |

**Table 10.7** Relationship between plasma selenium (µmol/L), plasma selenium: albumin ratio and serum albumin concentration (g/L) with liver selenium concentration (µg/g dry weight) on days 0-3, using simple linear regression.

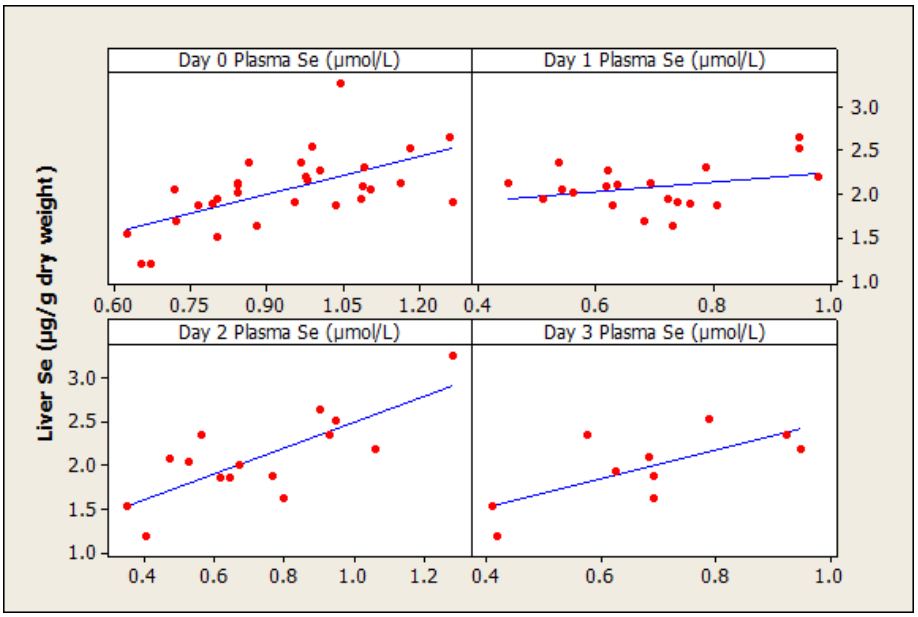



**Fig 10.6a** Scatterplot of plasma selenium concentration (µmol/L) versus liver selenium concentration (µg/g dry weight) on day 0-3

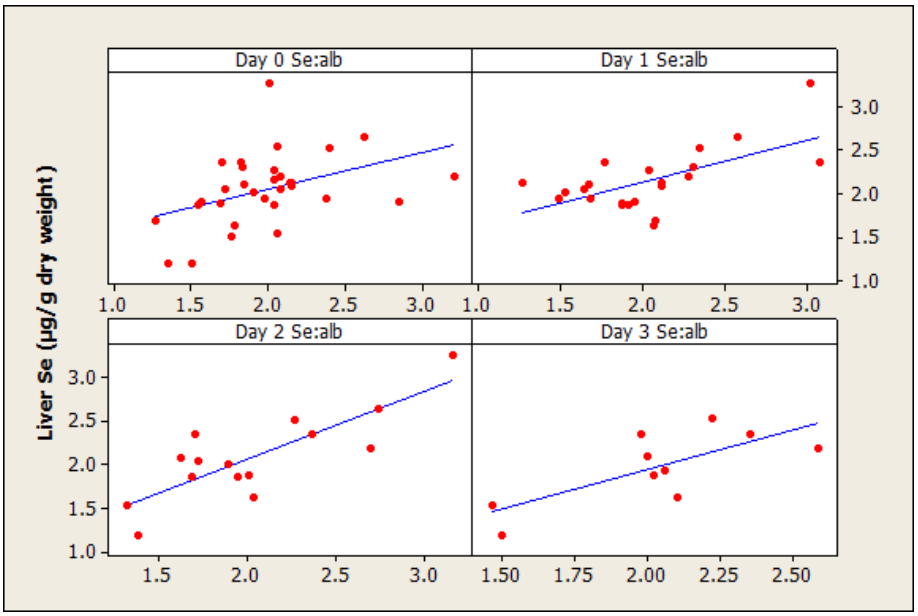



**Fig 10.6b** Scatterplot of plasma selenium: albumin ratio versus liver selenium concentration (µg/g dry weight) on day 0-3

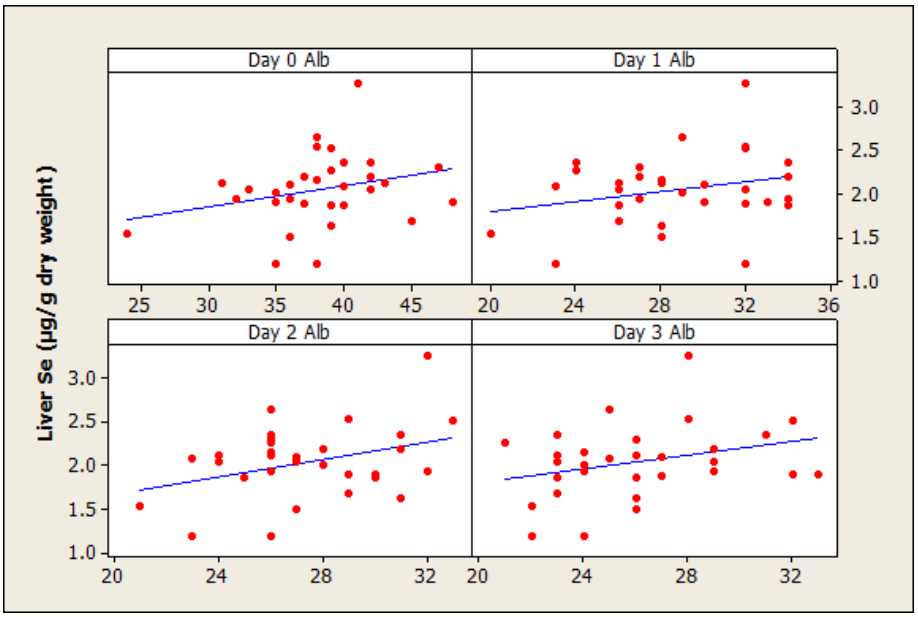



**Fig 10.6c** Scatterplot of serum albumin (g/L) versus liver selenium concentration (µg/g dry weight) on day 0-3

## 10.5 Correcting plasma selenium for albumin during the acute phase response

The changes seen in plasma selenium during the acute phase response appear to be strongly related to changes in serum albumin and correcting for serum albumin by use of the selenium: albumin ratio is strongly correlated with liver selenium concentration. To replicate clinical practice, data from day 0-3 were collated and the relationship with liver selenium concentration examined by use of simple linear regression (fig 10.7a).

Using all results from day 0-3, the selenium: albumin ratio accounted for 38.5% of the variance in liver selenium concentration ($p<0.001$).

As plasma selenium was related to liver selenium anyway on day 0, when the patient were not inflamed, day 0 was excluded and the analysis repeated (fig 10.7b).

Using only results from day 1-3 when plasma selenium alone was unreliable, the selenium: albumin ratio accounted for 50.5% of the variance in liver selenium concentration ($p<0.001$).

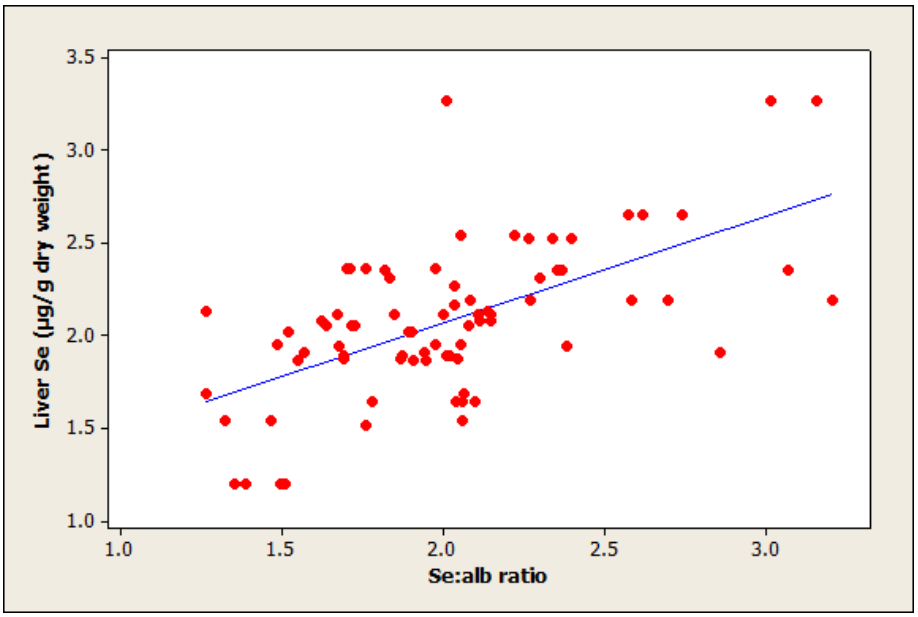



**Fig 10.7a** Scatterplot of plasma selenium: albumin ratio versus liver selenium concentration (µg/g dry weight) using all results from day 0-3

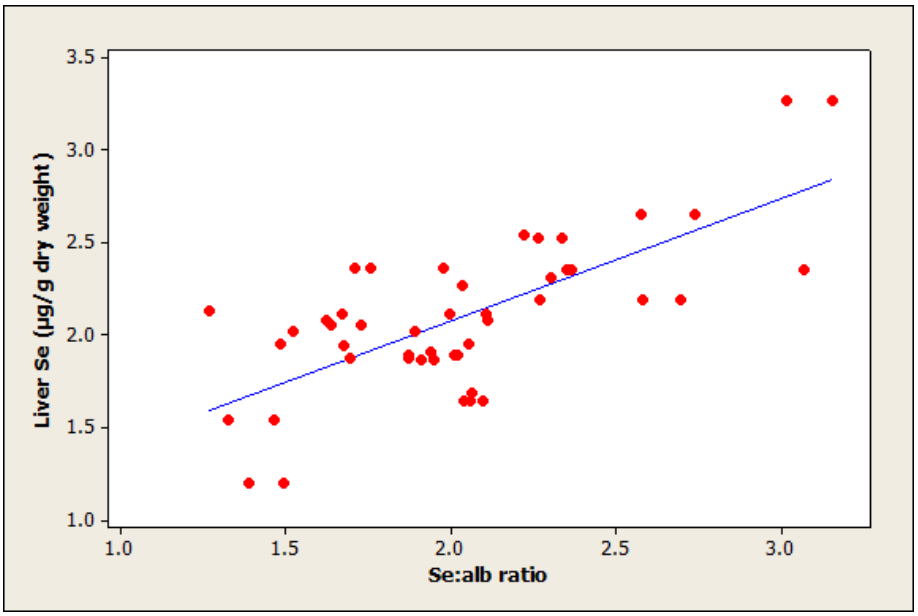



**Fig 10.7b** Scatterplot of plasma selenium: albumin ratio versus liver selenium concentration (µg/g dry weight) using all results from day 1-3

## 10.6 Summary of findings - Selenium

In this study population plasma selenium concentrations are low compared to the local laboratory population reference interval of 0.8-2.0 μmol/L, with a range of 0.6-1.3 μmol/L found on day 0.

On day 0, when the patients were not inflamed, linear relationships existed between all the selenium measures: red blood cell selenium, red blood cell glutathione peroxidase activity, plasma selenium concentration and liver selenium concentration.

Red blood cell selenium concentration and red blood cell glutathione peroxidase activity do not change during the acute phase response. Plasma selenium falls during the acute phase response with the decrease being in the first 24 hours.

There is a linear relationship with the percentage fall in plasma selenium from day 0 and the plasma selenium concentration on day 0 on days 1 and 3, but not on day 2. This relationship is reversed on day 3 from day 1 as the lower the plasma selenium on day 0, the lower the percentage drop in plasma selenium from day 0; the higher the plasma selenium on day 0, the lower the percentage drop from day 0 is by day 3. Therefore the higher plasma selenium has a greater percentage reduction on day 1 but also a smaller percentage reduction by day 3: although it drops by a greater percentage it recovers more quickly.

Plasma selenium concentration has a strong linear relationship with serum albumin concentration and this strengthens during the acute phase response.

There is a linear relationship between the selenium: albumin ratio and liver selenium concentration throughout the acute phase response.

During the acute phase response, when pooling data from days 1-3, there is a linear relationship between the selenium: albumin ration and liver selenium concentration. The selenium: albumin ratio accounts for 50.5% of the variance in liver selenium concentration making it a potential surrogate marker for selenium status during the acute phase response.

## Chapter 11

## Magnesium

## The relationship between tissue and blood concentrations of Magnesium and the effect of the acute phase response

### 11.1 Normality of data and outliers

To ensure the normality of the data and exclude any outlying samples, the Anderson-Darling test for normality was performed. The results are summarised below and further details and probability plots can be found in appendix 4.

Liver magnesium concentrations were found to be normally distributed ($p=0.423$).

An outlying sample (sample 13) was seen when examining a probability plot of muscle magnesium and this sample was excluded from all further magnesium analysis. With this sample removed muscle magnesium was normally distributed ($p=0.2$).

Red blood cell magnesium levels on day 0 and day 1 were not normally distributed. Outlying samples from day 0 (samples 14, 20 and 26) and day 1 (samples 20 and 31) were removed from further analyses. After these were removed days 0-3 were normally distributed; day 0 $p=0.119$, day 1 $p=0.434$, day 2 $p=0.836$, day 3 $p=0.518$.

The Anderson-Darling normality test was performed on plasma magnesium concentrations on day 0-3 and all were normally distributed; day 0 $p=0.528$, day 1 $p=0.734$, day 2 $p=0.992$, day 3 $p=0.507$.

## 11.2 Magnesium status of the study population at baseline

As plasma magnesium is routinely measured in our laboratory, a reference interval of 0.7-1.0 mmol/L is in place. The plasma magnesium status of the study population at baseline was investigated. Median plasma magnesium concentration was 0.72 mmol/L (inter-quartile range 0.64-0.84). The range of plasma magnesium concentrations, and the relationship with the reference interval, can be seen in figure 11.1.

Within the study population, a number of samples fall below our laboratory reference interval with the median being at the lower limit of the reference interval.

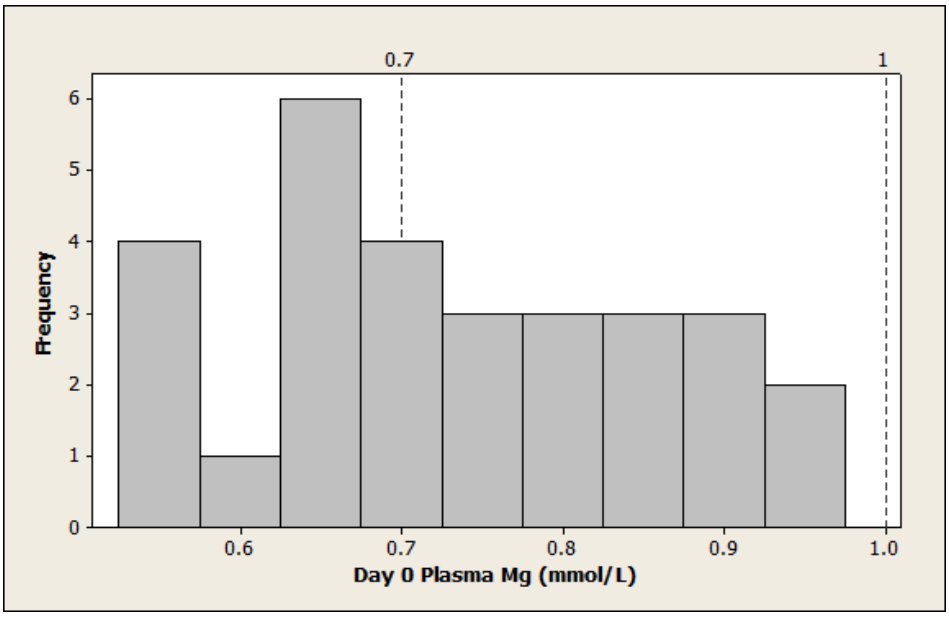



**Fig 11.1** Histogram of plasma magnesium concentration (mmol/L) on day 0. Upper (1.0mmol/L) and lower (0.7mmol/L) limits of the reference interval are marked by dotted lines

### 11.3 Relationship between blood and tissue magnesium concentrations at baseline

The relationship between tissue and blood concentrations of magnesium at baseline, without the contribution of a systematic inflammatory response (characterised by a "normal" C-reactive protein), allows recommendations to be made as to whether plasma or red blood cell magnesium concentrations are a reliable measure of tissue status in the non-inflamed patient. Simple linear regression was used to examine the relationships between liver, muscle, red blood cell and plasma magnesium.

There is no linear relationship between either RBC magnesium concentration on day 0 ($r^2$=2.5%, p=0.417) (fig 11.2a) or plasma magnesium concentration on day 0 ($r^2$=1.2%, p=0.517) (fig 11.2b) and muscle magnesium concentration.

There is also no linear relationship between RBC magnesium concentration on day 0 ($r^2$=0.7%, p=0.662) (fig 11.3a) or plasma magnesium concentration on day 0 ($r^2$=0.5%, p=0.712) (fig 11.3b) and liver magnesium concentration.

No significant linear relationship was found between liver and muscle magnesium concentrations ($r^2$=2.1%, p=0.394) (fig 11.4) or between red blood cell concentrations on day 0 and plasma magnesium concentrations on day 0 ($r^2$= 1.2, p=0.578) (fig 11.5).

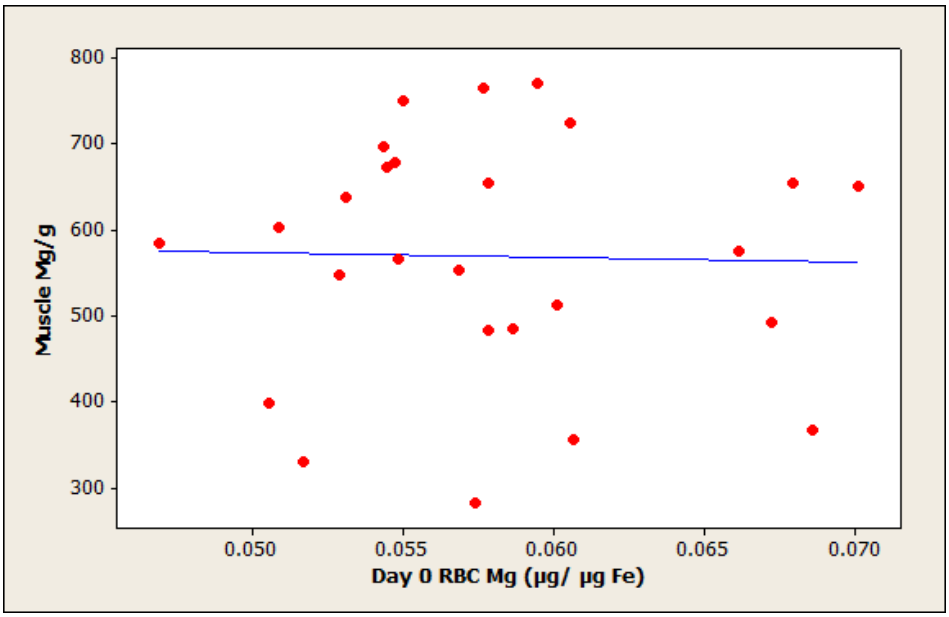



**Fig 11.2a** Scatterplot of Muscle Mg (µg/g dry weight) versus RBC Mg on Day 0 (µg/ µg Fe)

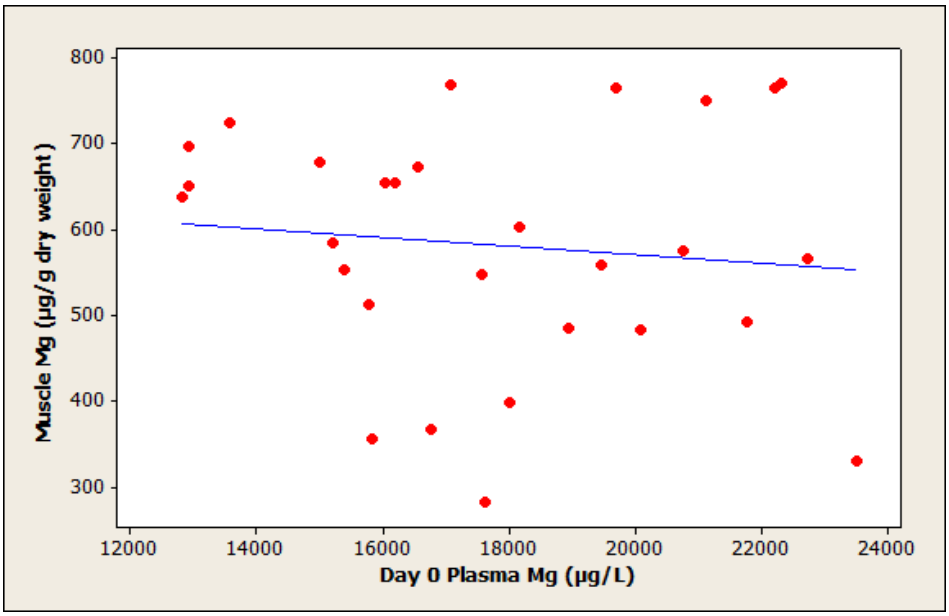



**Fig 11.2b** Scatterplot of Muscle Mg (µg/g dry weight) versus Plasma Mg on Day 0 (µg/L)

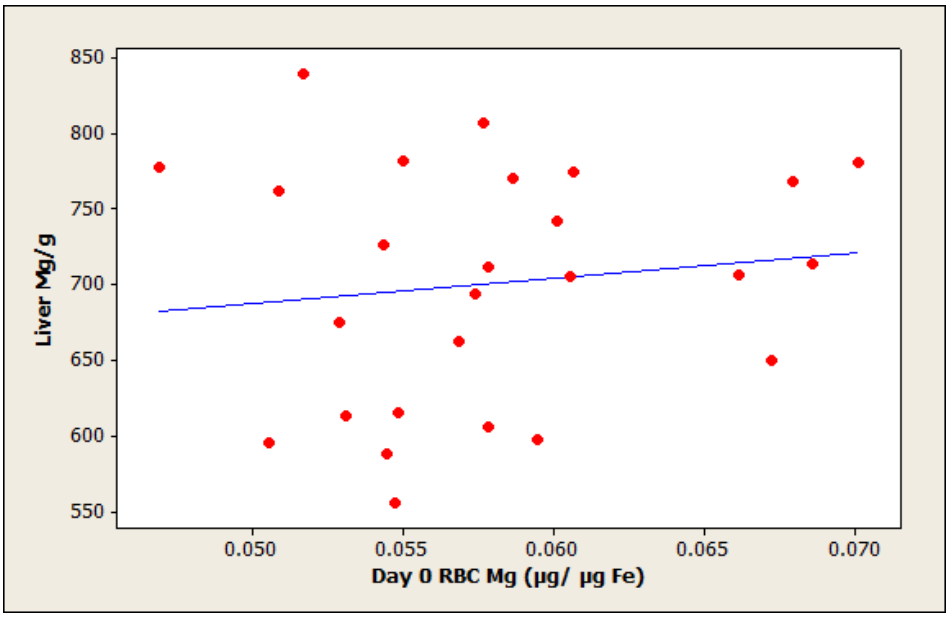



**Fig 11.3a** Scatterplot of Liver Mg (µg/g dry weight) versus RBC Mg on Day 0 (µg/ µg Fe)

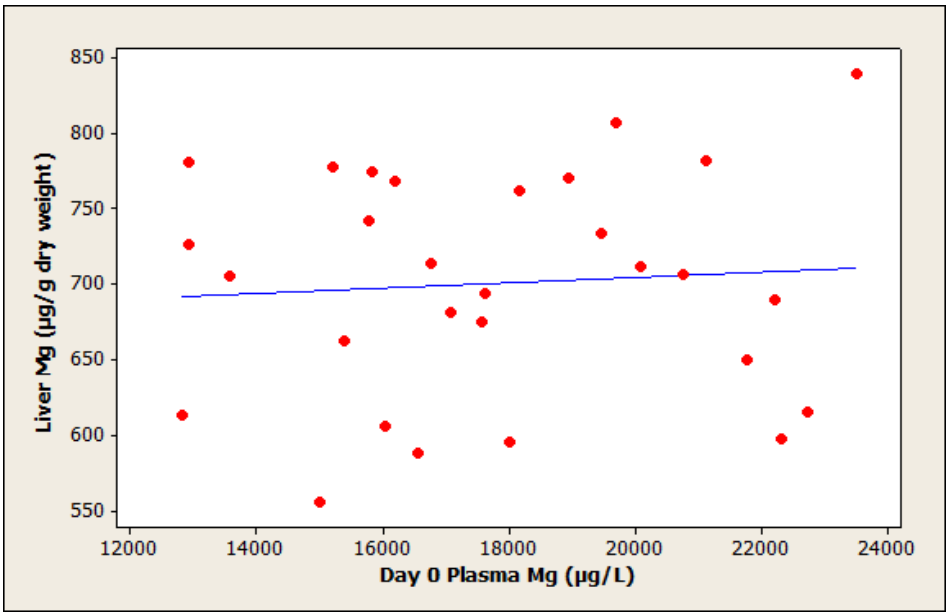



**Fig 11.3b** Scatterplot of Liver Mg (µg/g dry weight) versus Plasma Mg on Day 0 (µg/L)

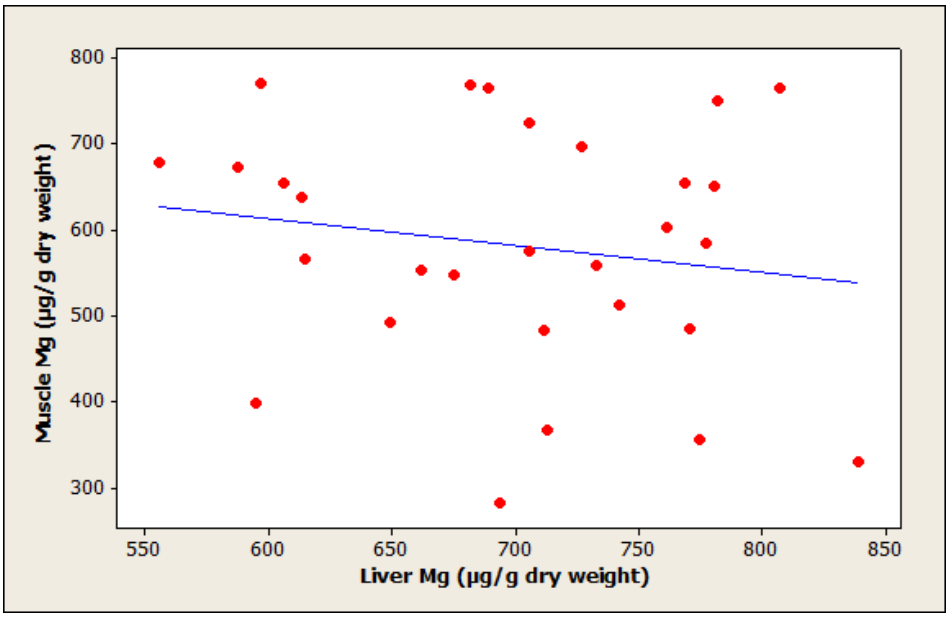



**Fig 11.4** Scatterplot of Muscle Mg (µg/g dry weight) versus Liver Mg (µg/g dry weight)

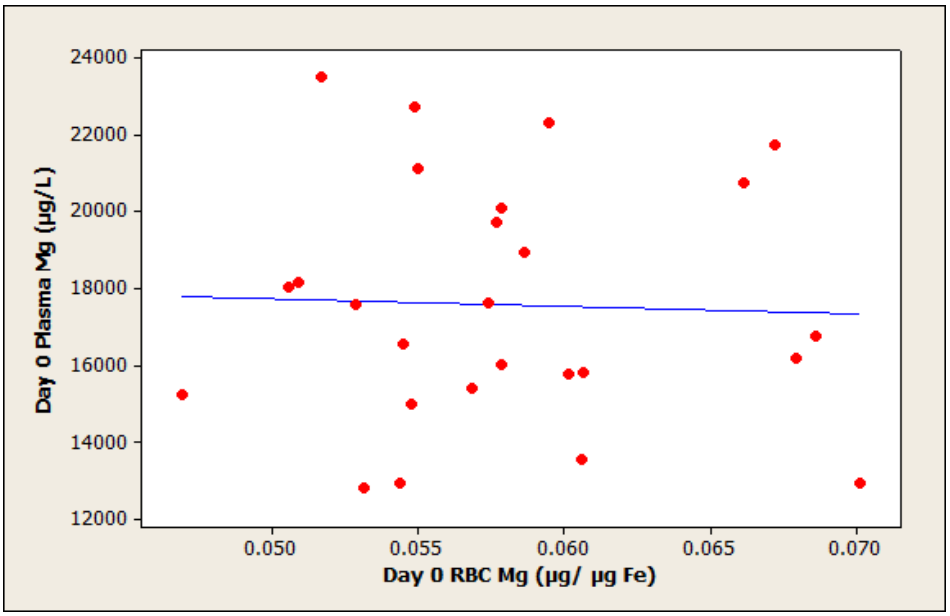



**Fig 11.5** Scatterplot of Plasma Mg (µg/L) versus RBC Mg (µg/ µg Fe) on Day 0

## 11.4 Change in plasma and red blood cell magnesium with the acute phase response

On days 1-3, in the post-operative period, a systemic acute phase response develops marked by a rise in CRP and a fall in serum albumin. Kruskal-Wallis (non-parametric) one way analysis of variance was performed to see if red blood cell or plasma magnesium concentration varied during the acute phase response. The change in the concentration of RBC magnesium can be seen in fig 11.6a and plasma magnesium in fig 11.6b.

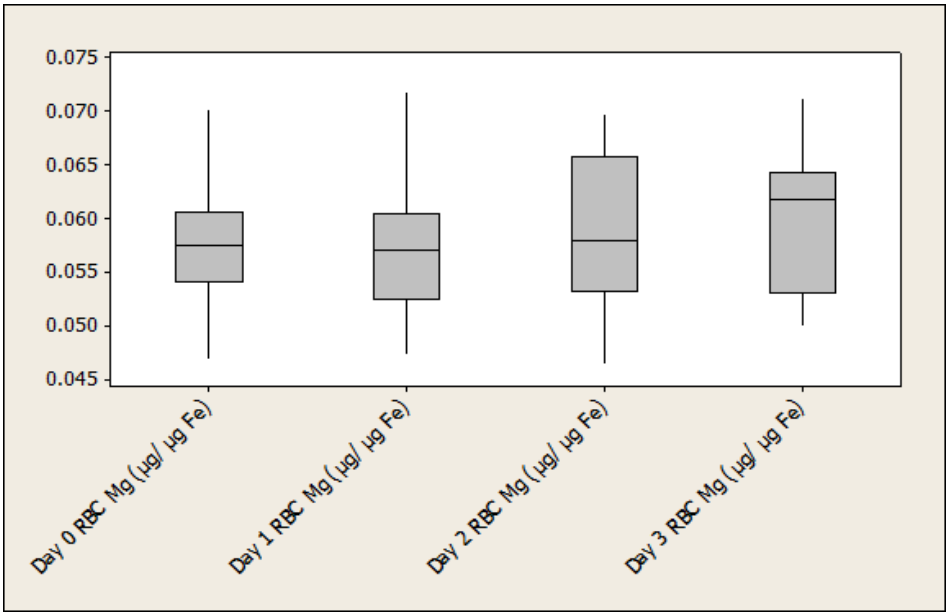



**Fig 11.6a** Boxplot of median red blood cell magnesium concentration (µg/ µg Fe) on day 0-3

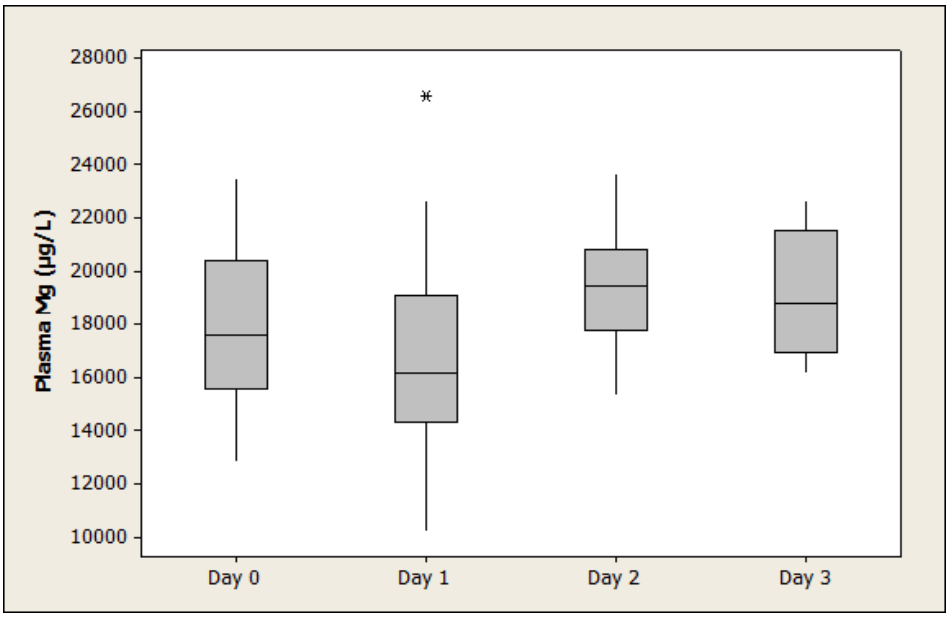



**Fig 11.6b** Boxplot of median plasma magnesium concentration (µg/L) on day 0-3

*11.4.1 Red blood cell Magnesium*

Median values of red blood cell Magnesium (µg/ µg Fe) on days 0-3 are shown in table 11.1. There is no significant change in RBC magnesium over day 0-3 (p=0.743) (fig 11.6a).

| | Median RBC Magnesium (µg/ µg Fe) (IQR) |
|---|---|
| Day 0 | 0.058 (0.054-0.061) |
| Day 1 | 0.057 (0.052-0.060) |
| Day 2 | 0.058 (0.053-0.066) |
| Day 3 | 0.062 (0.053-0.064) |

**Table 11.1** Median (IQR) values of red blood cell Magnesium (µg/ µg Fe) on days 0-3

*11.4.2 Plasma Magnesium*

Median values of plasma magnesium (µg/g L) on days 0-3, and the percentage change in plasma magnesium from day 0 on days 1-3 are shown in table 11.2. Kruskal-Wallis (non-parametric) one way analysis of variance showed that plasma magnesium concentration varies significantly over days 0-3 (p=0.049) (fig 11.6b).

Mann Whitney comparisons were performed to examine the differences between each day (Bonferroni correction p=0.017 is significant): there was no significant change in concentration between day 0 and day 1 (p=0.298) but the plasma magnesium concentration changes significantly from day 1 to day 2 (p=0.014). From day 2 to day 3 the difference in plasma magnesium concentration was not significant (p=0.777). Differences between the median percentage change in plasma magnesium from day 0 to day 1-3 did not reach significance (p=0.059)(fig 11.6c).

Plasma magnesium concentration decreases with the evolution of the acute phase response on day 1 and returns to baseline level on day 2 and 3. Although not reaching a statistically significant difference (p=0.059), it can be seen that there is a negative percentage change of plasma magnesium on day 1 (approx 11%) which normalises on day 2.

| | Median Plasma Magnesium (µg/ L) (IQR) |
|---|---|
| Day 0 | 17580 (15595 - 20420) |
| Day 1 | 16175 (14328 - 19115) |
| Day 2 | 19445 (17775 - 20835) |
| Day 3 | 18810 (16955 - 21550) |
| | Median % change in Plasma Magnesium (IQR) |
| Day 1 | -11.79% (-23.59 - 8.25) |
| Day 2 | 3.41% (-9.36 – 34.37) |
| Day 3 | 1.3% (-5.71 - 18.21) |

**Table 11.2** Median (IQR) values of Plasma Magnesium (µg/L) on days 0-3, and the percentage change in Plasma Magnesium from day 0 on days 1-3

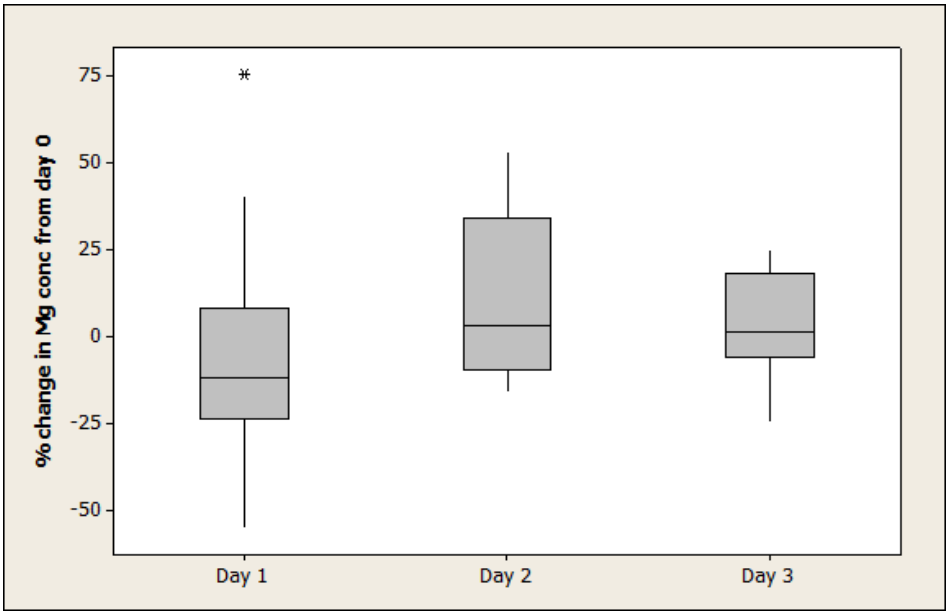



**Fig 11.6c** Boxplot of percentage change in plasma magnesium concentration from baseline (day 0) on day 1-3

## 11.5 Factors affecting changes in plasma magnesium concentration

### *11.5.1 Tertiles of plasma magnesium*

Magnesium is utilised intra-cellularly and it may be that the decrease in plasma magnesium seen on day 1 is due to the shifting of magnesium into cells. In order to examine what factors affect the changes in plasma magnesium, firstly plasma magnesium was split into tertiles to allow visualisation of any patterns. There were 10, 9 and 9 samples in each of the low medium and high (1-3) tertiles respectively.

Kruskal-Wallis (non-parametric) one way analysis of variance of CRP and Albumin was performed to ensure there were no differences in CRP or Albumin between tertiles on any of day 0-3; as it was not normally distributed, the logarithmic transformation of CRP day 0 was used. Median values (IQR) for CRP and Albumin by plasma tertile can be seen in table 11.3a. No differences in CRP or Albumin concentrations were seen between tertiles of plasma magnesium.

Figure 11.7a shows plasma magnesium concentrations for each of the three tertiles over days 0 to 3. Table 11.3b shows the median values for plasma magnesium concentration for each of the three tertiles over days 0 to 3, and the p values for the variance between tertiles on each day (Kruskal-Wallis one way analysis of variance).

Obviously, median values of plasma magnesium on day 0 are significantly different by tertile of day 0 plasma magnesium ($p<0.001$), but by day 1 there is no difference between the concentrations of plasma magnesium for the 3 tertiles of plasma magnesium concentration. Visually, in fig 11.7a, it appears that the lowest concentration tertile (tertile 1) increases, while the highest concentration tertile decreases.

To examine this further, the relationship between the percentage change in plasma magnesium on days 1 to 3, and the initial plasma concentration on day 0 was examined; fig 11.7b shows this relationship. Table 11.3c shows median values for the percentage change in plasma magnesium concentration from day 0 and the p values for the variance between tertiles on each day (Kruskal-Wallis one way analysis of variance).

Therefore, the percentage change in the plasma magnesium concentration from day 0 to day 1 and day 2 differs by tertile; the lowest tertile (tertile 1) increases and the highest tertile (tertile 3) decreases. There is not a statistically significant difference at day 3, but it is not known if this is due to changes pertaining to the resolution of the acute phase response, or simply a result of a small sample size.

| | Tertile plasma magnesium (range plasma magnesium (mmol/L)) | | | |
|---|---|---|---|---|
| | 1 (0.53-0.66) | 2 (0.67-0.78) | 3 (0.80-0.97) | p value for variance |
| CRP day 0(mg/L) | 2.6 (0.9-5.5) | 5.8 (3.3-7.7) | 4.7 (1.8-17) | 0.193 |
| CRP day 1(mg/L) | 95.5 (66.0-123.5) | 96.0 (65.5-107.5) | 86.5 (66.0-127.8) | 0.823 |
| CRP day 2(mg/L) | 156.5 (89.3-191.3) | 164 (144.5-209) | 185 (140-245.5) | 0.392 |
| CRP day 3(mg/L) | 153.5 (120.3-175) | 155 (119-214) | 176 (113.3-220.8) | 0.911 |
| Alb day 0 (g/L) | 41.5 (37.5-45.5) | 37 (34.5-39) | 38 (34.3-40.5) | 0.063 |
| Alb day 1 (g/L) | 28.5 (25.5-32.25) | 27 (26-32) | 28 (26.3-32.5) | 0.909 |
| Alb day 2 (g/L) | 27.5 (26-29.5) | 27 (24-31) | 26 (26-30) | 0.837 |
| Alb day 3 (g/L) | 26 (22.8-29.5) | 27 (23-29) | 25.5 (23.8-28.3) | 0.935 |

**Table 11.3a** Median values (IQR) of CRP (mg/L) and Albumin (g/L) on day 0-3 for tertiles of plasma magnesium concentration

| | Tertile plasma magnesium | | | |
|---|---|---|---|---|
| | 1 | 2 | 3 | p value for variance |
| Plasma Mg day 0 (mmol/L) | 0.62 (0.53-0.65) | 0.72 (0.69-0.74) | 0.88 (0.82-0.92) | <0.001 |
| Plasma Mg day 1 (mmol/L) | 0.77 (0.62-0.87) | 0.64 (0.57-0.72) | 0.66 (0.59-0.79) | 0.411 |
| Plasma Mg day 2 (mmol/L) | 0.73 (0.69-0.83) | 0.74 (0.69-0.95) | 0.83 (0.80-0.85) | 0.418 |
| Plasma Mg day 3 (mmol/L) | 0.72* | 0.70* | 0.84 (0.76-0.93) | 0.087 |

**Table 11.3b** Median values (IQR) of plasma magnesium concentration (mmol/L) on day 0-3 for tertiles of plasma magnesium concentration at day 0. *only one value available.

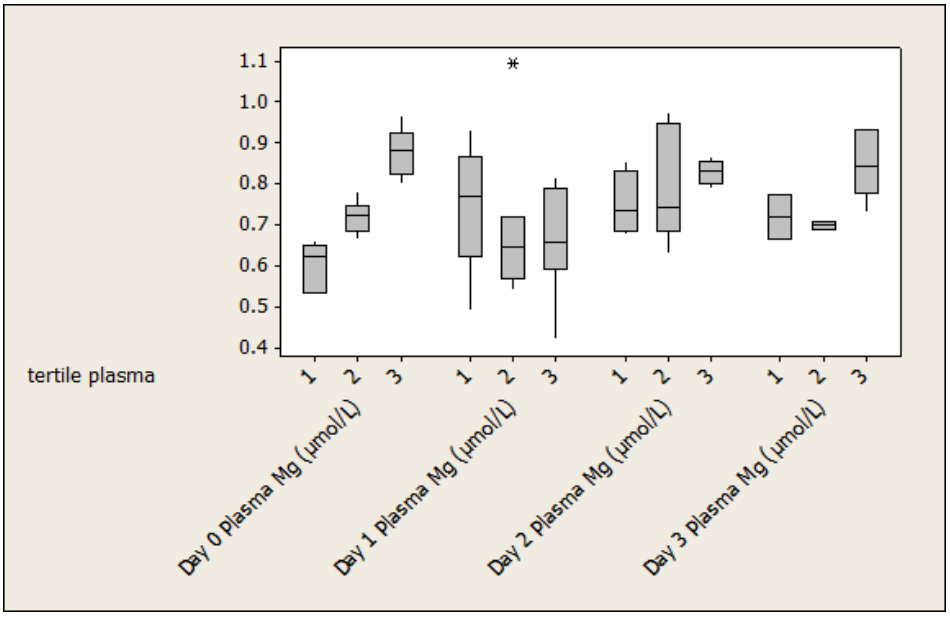



**Fig 11.7a** Boxplot of plasma magnesium concentration (mmol/L) for tertiles of plasma magnesium concentration (1-3), over days 0-3.

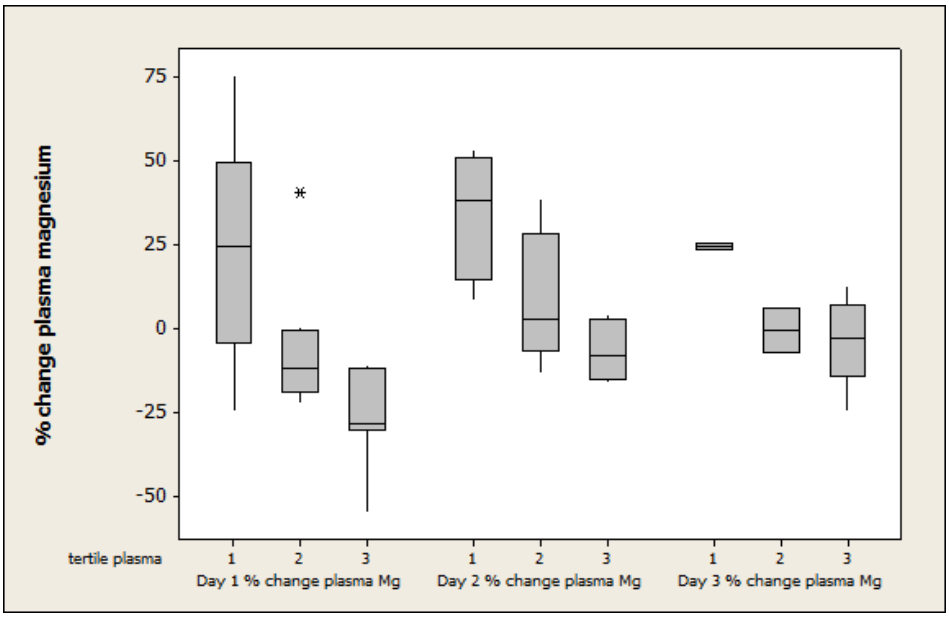



**Fig 11.7b** Boxplot of % change in plasma magnesium concentration from day 0, for tertiles of plasma magnesium concentration (1-3), over days 1-3

| | Tertile plasma magnesium | | | |
|---|---|---|---|---|
| | 1 | 2 | 3 | p value for variance |
| % change plasma Mg day 1 | 24.4 (-4.4-75.5) | -11.78 (-18.9-0.3) | -28.49 (-30.2--11.8) | 0.013 |
| % change plasma Mg day 2 | 38.2 (14.4-50.7) | 2.8 (-6.7-28.2) | -8.13 (-14.92-2.68) | 0.027 |
| % change plasma Mg day 3 | 24.4* | -0.53* | -14.25 (-3.01-7.07) | 0.087 |

**Table 11.3c** Median values (IQR) of plasma magnesium concentration (µg/L) on day 0-3 for tertiles of plasma magnesium concentration at day 0.*only one value available.

*11.5.2 Albumin concentration*

In the circulation, magnesium is partly bound to protein, the most abundant of which is albumin. Albumin changes during the acute phase response on days 0-3 (fig 6.2) and this change may be why magnesium changes on day 1. Figure 11.8 shows that the change in magnesium does not follow the change in albumin.

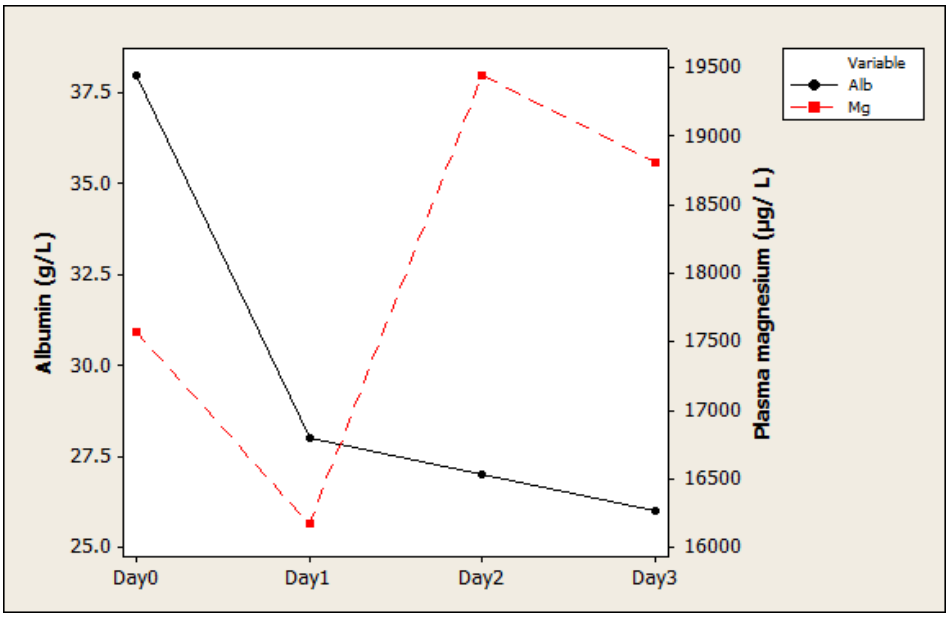



**Fig 11.8** Change in serum albumin concentration (g/L) and plasma magnesium concentration (µg/L), over days 0-3

In order to further examine the relationship between plasma magnesium and albumin concentration during the acute phase response, simple linear regression was performed on the percentage change from day 0 of both plasma magnesium and serum albumin; the results can be seen in table 11.4. Scatterplots of the percentage change in plasma magnesium from day 0 versus the percentage change in albumin from day 0, on days 1-3 can be seen in fig 11.9.

There is no relationship between the percentage change in plasma magnesium concentration and the percentage change in serum albumin concentration.

| | % change albumin Vs % change plasma magnesium | |
|---|---|---|
| | $r^2$(%) | p value |
| Day 1 | 1.1 | 0.610 |
| Day 2 | 6.5 | 0.379 |
| Day 3 | 0.0 | 0.989 |

**Table 11.4** Relationship between percentage changes in plasma magnesium from day 0 on day 1-3, with percentage changes in serum albumin from day 0 on day 1-3 using simple linear regression.

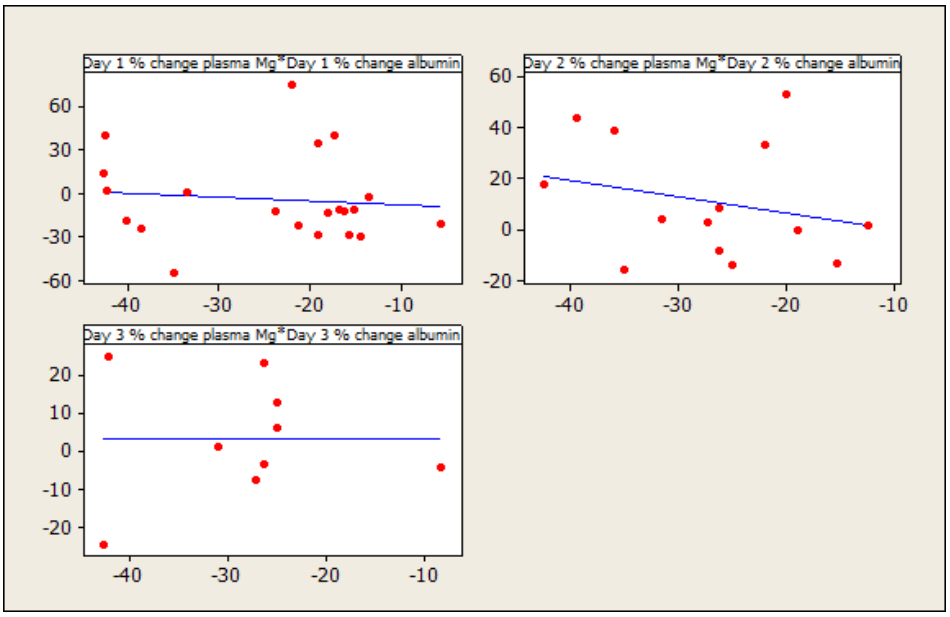



**Fig 11.9** Scatterplots of % change plasma magnesium concentration versus the percentage change in serum albumin concentration from day 0, on days 1-3

As it appears that the change in plasma magnesium is possibly related to the initial plasma magnesium (fig 11.7), the magnesium: albumin ratio was examined for the three tertiles of plasma magnesium. The use of the magnesium: albumin ratio allows the relationship between albumin concentration and magnesium concentration and tertile of plasma magnesium concentration to be examined while correcting for any effect of plasma dilution due to intravenous fluid replacement given to the patient post-operatively. Fig 11.10a shows the median magnesium: albumin ratio for each of the three tertiles over days 0-3, and table 11.5a gives the median values and result of Kruskal Wallis nonparametric analysis of variance for the differences between tertiles.

The percentage change in the magnesium: albumin ratio from day 0 to day 1-3, for all three tertiles is shown in figure 11.10b and table 11.5b. Kruskal Wallis nonparametric analysis of variance was performed to examine variance in results across the three tertiles.

These results show that while there is a difference in magnesium: albumin ratio on day 0 as expected, there is no difference in the magnesium: albumin ratio beyond day 1. There is a similar pattern of percentage change in the magnesium: albumin ratio as there is in the change in plasma magnesium concentration, with the lowest tertile increasing and the highest tertile decreasing. As table 11.3a shows, there is no difference in the albumin concentration on day 1-3 between tertiles.

The combination of these results confirm that the change in magnesium on day 1, when all three tertiles become similar, is not simply a function of change in the serum albumin concentration and therefore there must be other independent determinants of the change in plasma magnesium concentration seen during the acute phase response.

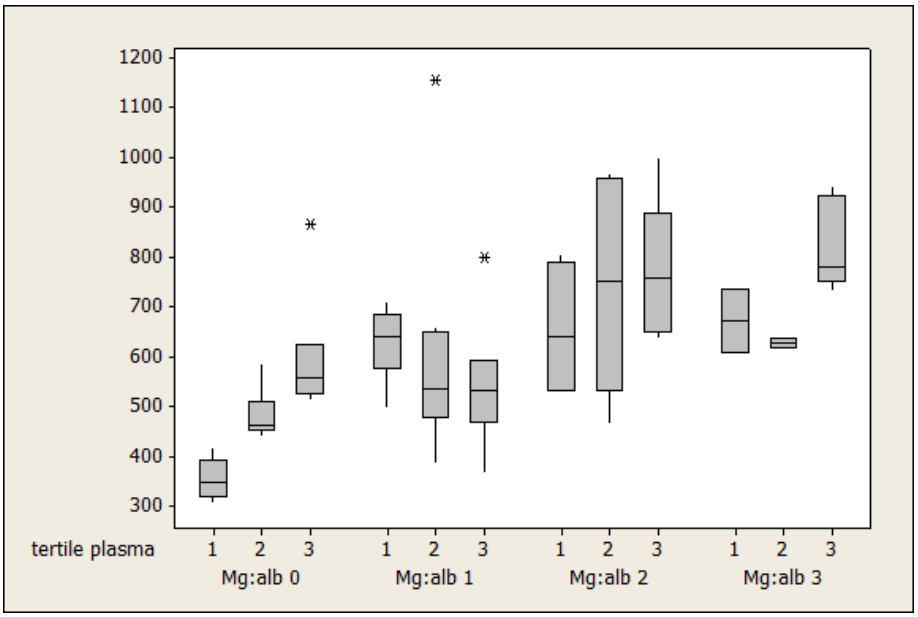



**Fig 11.10a** Boxplot of plasma magnesium: albumin ratio on day 0-3, for tertiles of plasma magnesium concentration (1-3)

| | Tertile plasma magnesium | | | |
|---|---|---|---|---|
| | 1 | 2 | 3 | p value for variance |
| Mg:Alb day 0 | 346.1 (319.4-392.1) | 459.9 (450.8-509.9) | 555.7 (526.1-623.3) | <0.001 |
| Mg:Alb day 1 | 639.7 (576.8-685.4) | 536.1 (477.1-648.8) | 531.9 (469.1-591.9) | 0.247 |
| Mg:Alb day 2 | 639.3 (532.8-790.2) | 752.7 (531.7-958.2) | 759.2 (649.9-890.1) | 0.553 |
| Mg:Alb day 3 | 671.2* | 628.0* | 779.7 (752.1-924.7) | 0.087 |

**Table 11.5a** Median values (IQR) of plasma magnesium: albumin ratios on day 0-3 for tertiles of plasma magnesium concentration at day 0 (1-3). *only one value available.

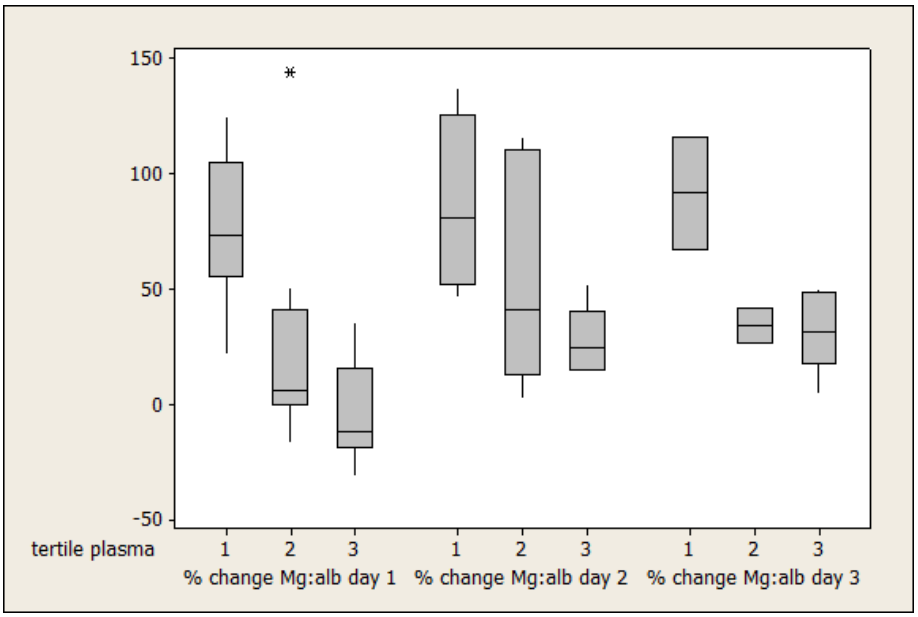



**Fig 11.10b** Boxplot of percentage change in plasma magnesium: albumin ratio from baseline (day 0) on day 1-3, for tertiles of plasma magnesium concentration (1-3)

| | Tertile plasma magnesium | | | |
|---|---|---|---|---|
| | 1 | 2 | 3 | p value for variance |
| % change Mg:Alb day 1 | 73.4 (55.5-105.1) | 6.0 (-16.1-41.2) | -11.7 (-18.6-15.6) | 0.007 |
| % change Mg:Alb day 2 | 80.9 (12.9-125.7) | 41.3 (12.9-110.6) | 24.47 (15.3-41.7) | 0.122 |
| % change Mg:Alb day 3 | 91.9* | 35.4* | 31.6 (18.1-48.6) | 0.117 |

**Table 11.5b** Median values (IQR) of plasma magnesium: albumin ratio on day 0-3 for tertiles of plasma magnesium concentration at day 0.*only one value available.

*11.5.3 Plasma magnesium on day 0*

In order to further examine the relationship between plasma magnesium concentration on day 0 when the patients were not inflamed, the percentage decrease in plasma magnesium and plasma magnesium concentration on days 1-3 during the acute phase response, simple linear regression was performed; the results can be seen in table 11.6. The relationship between plasma magnesium concentration (µmol/L) on day 0 and the plasma magnesium concentrations (µmol/L) on days 1-3, can be seen in figure 11.11a. The relationship between the percentage change in plasma magnesium concentration from day 0 to day 1-3 can be seen in figure 11.11b (day 1), figure 11.11c (day 2), and figure 11.11d (day 3); reference lines are added at 0.7mmol/L which is the lower limit of our laboratory reference interval.

There is no relationship between the plasma magnesium concentration on day 0 and the plasma magnesium concentration on day 1-3 ($p=0.435$, 0.317, 0.096 respectively). There is however a linear relationship between the percentage change in plasma magnesium from day 0 to day 1 ($p=0.001$), day 0 to day 2 ($p<0.001$) and day 0 and day 3 ($p=0.01$). This is depicted well in figure 11.11b: the reference line at 0.7mmol/L, the lower limit of the reference interval, shows that the majority of subjects with a plasma magnesium on day 0 above this concentration have a decrease in plasma magnesium concentration during the acute phase response, while the majority below this concentration have an increase in plasma magnesium concentration.

The strength of the linear relationship between plasma magnesium on day 0 and the change during the acute phase response is seen by the results of the regression analysis: 46.4% of the variance of plasma magnesium concentration from day 0 on day 1 can be explained by the plasma magnesium concentration on day 0, with this figure being 65.6% on day 2 and 64.0% on day 3. Therefore, the changes in plasma magnesium seen during the acute phase are predominantly a factor of the baseline plasma magnesium concentration.

| | Plasma Mg day 0 Vs plasma Mg day 1-3 (µmol/L) | |
|---|---|---|
| | $r^2$(%) | p value |
| Day 1 | 3.2 | 0.435 |
| Day 2 | 8.3 | 0.317 |
| Day 3 | 34.5 | 0.096 |
| | **Plasma Mg day 0 (µmol/L) Vs %change plasma Mg day 1-3** | |
| | $r^2$(%) | p value |
| Day 1 | 46.4 | 0.001 |
| Day 2 | 65.6 | <0.001 |
| Day 3 | 64.0 | 0.010 |

**Table 11.6** Relationship between plasma magnesium on day 0 and days 1-3 (µmol/L) and plasma magnesium on day 0 (µmol/L) and the percentage change in plasma magnesium on days 1-3, using simple linear regression.

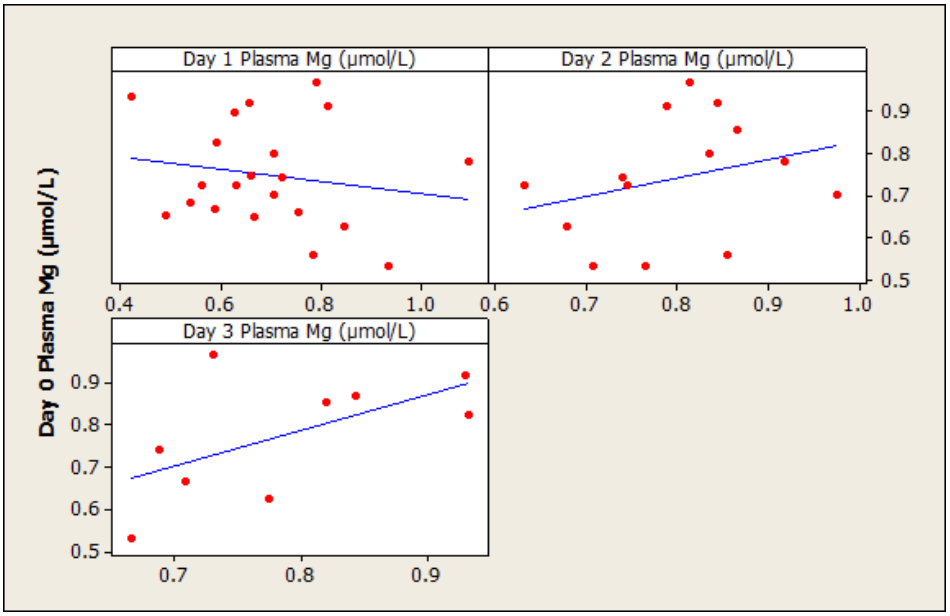



**Fig 11.11a** Scatterplot of plasma magnesium concentration on day 0 (mmol/L) versus plasma magnesium concentration on day 1-3 (mmol/L)

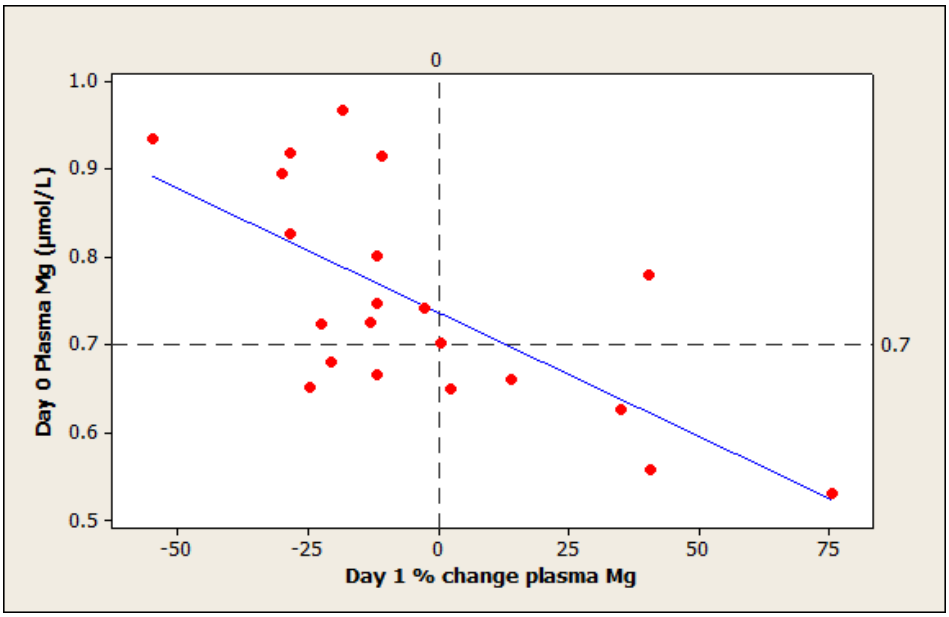



**Fig 11.11b** Scatterplot of plasma magnesium concentration on day 0 (mmol/L) versus % change in plasma magnesium concentration on day 1

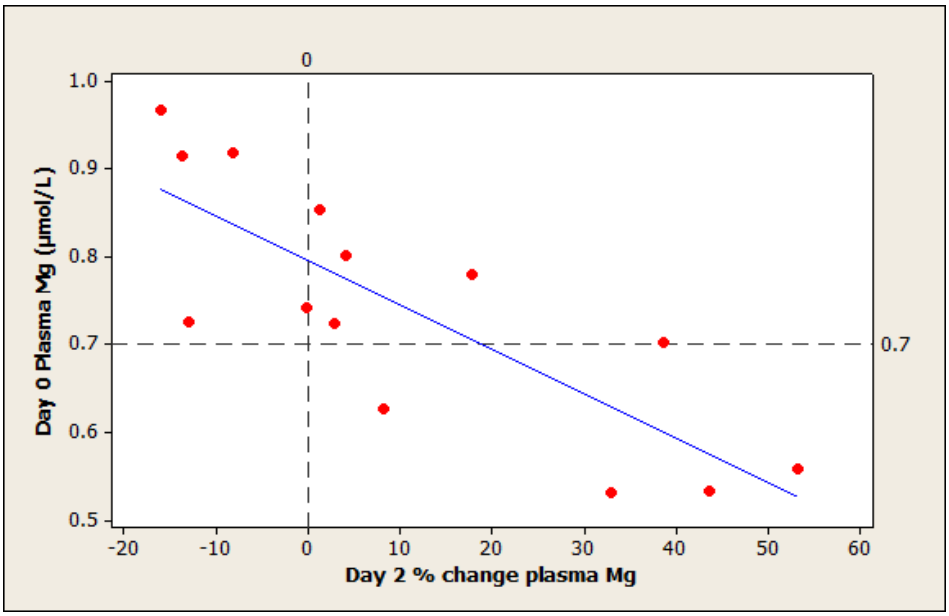



**Fig 11.11c** Scatterplot of plasma magnesium concentration on day 0 (mmol/L) versus % change in plasma magnesium concentration on day 2

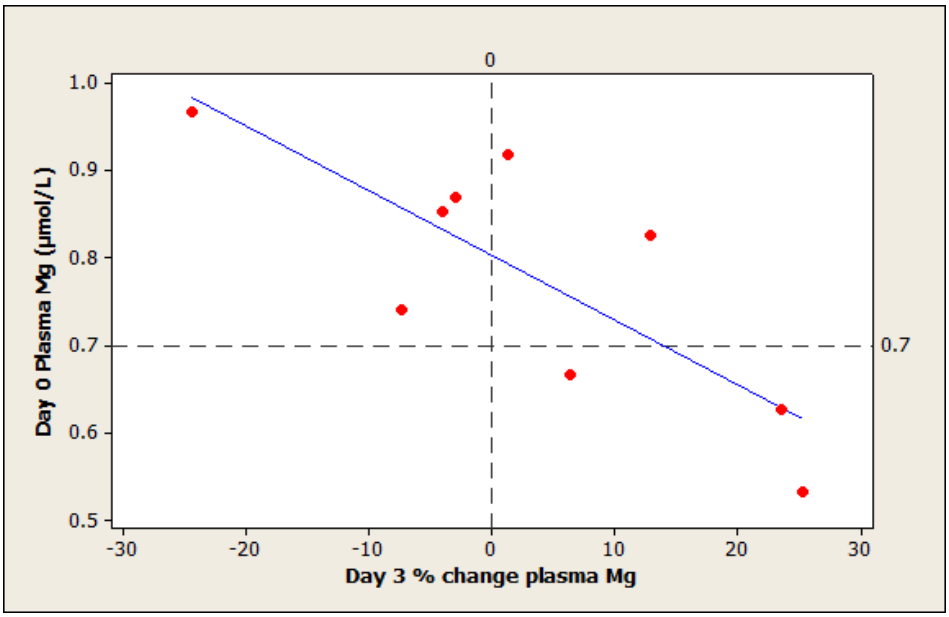



**Fig 11.11d** Scatterplot of plasma magnesium concentration on day 0 (µmol/L) versus % change in plasma magnesium concentration on day 3

*11.5.4 Muscle Magnesium concentration*

Linear regression was performed to examine the relationship between muscle magnesium concentration on day 0 and plasma magnesium concentration on day 0-3, and the percentage change in plasma magnesium concentration on day 1-3; the results can be seen in table 11.7. The relationship between plasma magnesium concentration (mmol/L) and muscle magnesium concentration (µg/g dry weight) on day 0-3 can be seen in figure 11.12a. The relationship between the percentage change in plasma magnesium concentration and muscle magnesium concentration (µg/g dry weight) on day 1-3 can be seen in figure 11.12b.

No linear relationship was found between plasma magnesium concentration and muscle magnesium concentration over days 0-3 or between the percentage change in plasma magnesium concentration and muscle magnesium concentration over days 1-3.

| | **Muscle Magnesium (µg/g dry weight) Vs plasma magnesium(mmol/L)** | |
|---|---|---|
| | $r^2$(%) | p value |
| Day 0 | 1.2 | 0.571 |
| Day 1 | 1.5 | 0.602 |
| Day 2 | 17.7 | 0.134 |
| Day 3 | 7.8 | 0.466 |
| | **Muscle Magnesium (µg/g dry weight) Vs % change plasma magnesium** | |
| | $r^2$(%) | p value |
| Day 1 | 4.5 | 0.356 |
| Day 2 | 25.4 | 0.066 |
| Day 3 | 23.8 | 0.183 |

**Table 11.7** Relationship between muscle magnesium concentration (µg/g dry weight) and plasma magnesium (mmol/L) on day 0-3, and % change plasma magnesium on day 1-3, using simple linear regression.

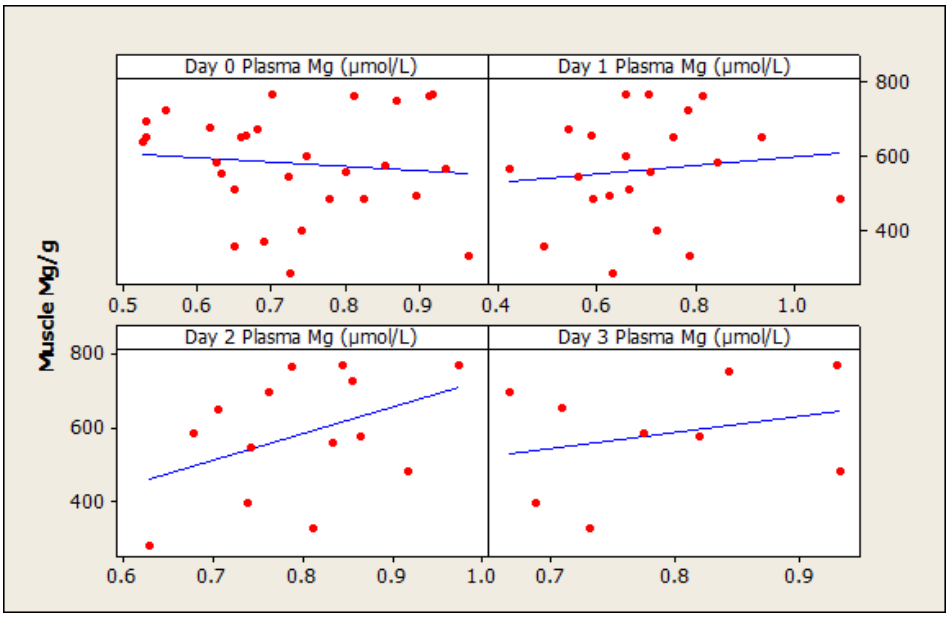



**Fig 11.12a** Scatterplots of muscle magnesium concentration (µg/g dry weight) Vs plasma magnesium concentration (mmol/L) on day 0-3

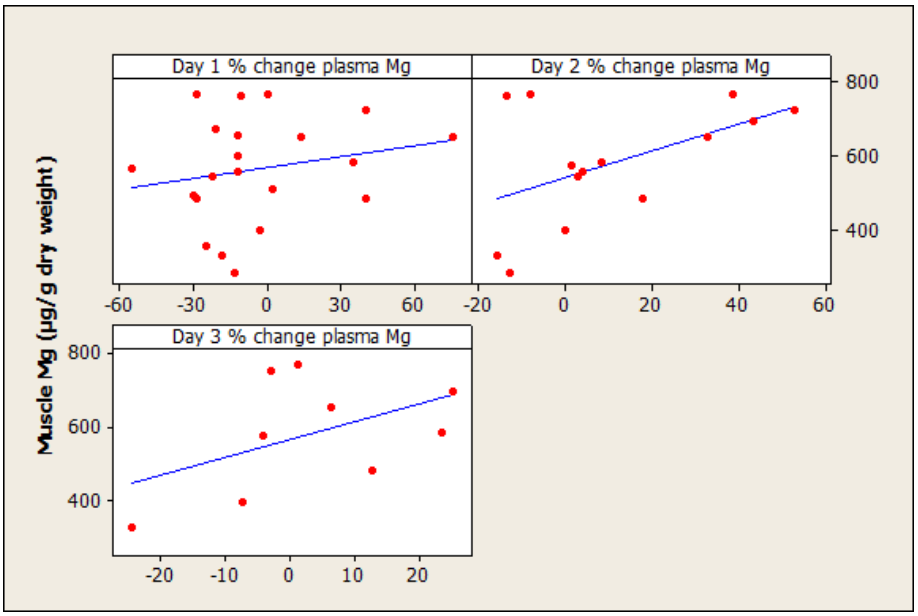



**Fig 11.12b** Scatterplots of muscle magnesium concentration (µg/g dry weight) Vs % change in plasma magnesium concentration on day 1-3

*11.5.5 Liver Magnesium Concentration*

In order to further examine the relationship between plasma magnesium concentration, the percentage decrease in plasma magnesium and liver magnesium concentration during the acute phase response, simple linear regression was performed; the results can be seen in table 11.8. The relationship between plasma magnesium concentration (mmol/L) and liver magnesium concentration (µg/g dry weight) on day 0-3 can be seen in figure 11.13a and the relationship between the percentage change in plasma magnesium concentration and liver magnesium concentration (µg/g dry weight) on day 1-3 can be seen in figure 11.13b.

There was no relationship found between plasma magnesium concentration and muscle magnesium concentration over days 0-3 or between the percentage change in plasma magnesium concentration and liver magnesium concentration over days 1-3.

| | **Liver Magnesium (µg/g dry weight) Vs plasma magnesium (mmol/L)** | |
|---|---|---|
| | $r^2$(%) | p value |
| Day 0 | 0.5 | 0.712 |
| Day 1 | 15.3 | 0.079 |
| Day 2 | 0.3 | 0.863 |
| Day 3 | 5.4 | 0.546 |
| | **Liver Magnesium (µg/g dry weight) Vs % change plasma magnesium** | |
| | $r^2$(%) | p value |
| Day 1 | 14.2 | 0.093 |
| Day 2 | 1.4 | 0.685 |
| Day 3 | 0.4 | 0.874 |

**Table 11.8** Relationship between liver magnesium concentration (µg/g dry weight) and plasma magnesium (mmol/L) on day 0-3, and % change plasma magnesium on day 1-3, using simple linear regression.

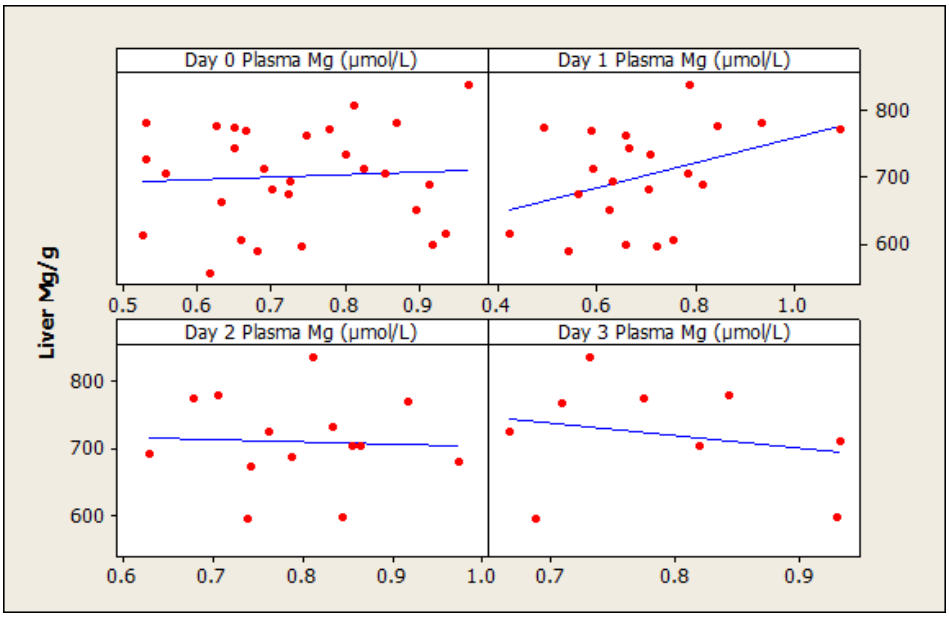



**Fig 11.13a** Scatterplots of liver magnesium concentration (µg/g dry weight) Vs plasma magnesium concentration (mmol/L) on day 0-3

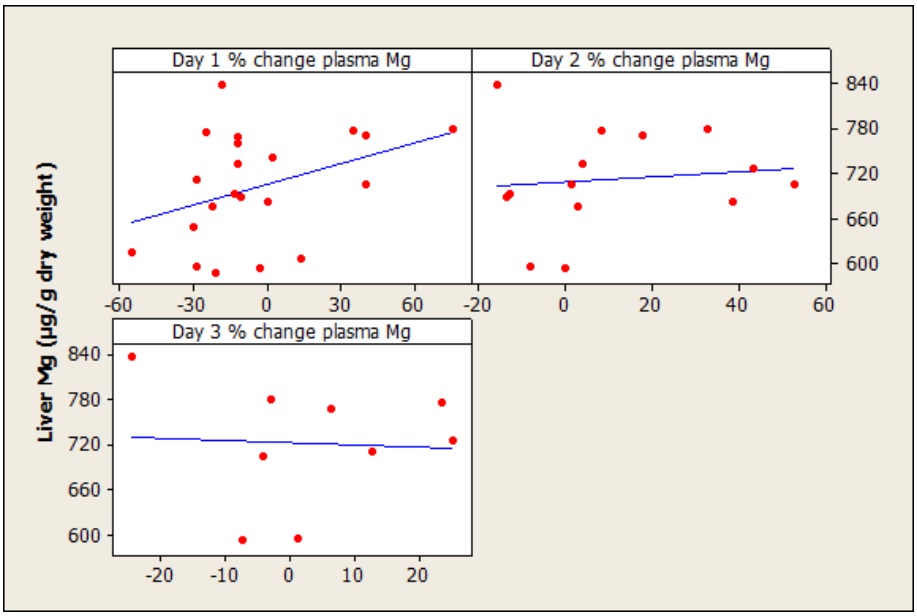



**Fig 11.13b** Scatterplots of liver magnesium concentration (µg/g dry weight) Vs % change in plasma magnesium concentration on day 1-3

*11.5.6 C Reactive Protein Concentration*

The relationship between C-reactive protein concentration and plasma magnesium concentration on days 0-3, and % change in plasma magnesium concentration on day 1-3 was examined by linear regression analysis; the results can be seen in table 11.9. The logarithmic transformation of CRP day 0 was used. The relationship between plasma magnesium concentration (mmol/L) and serum CRP concentration (mg/L) on day 0-3 can be seen in figure 11.14a. The relationship between % change of plasma magnesium concentration and serum CRP concentration (mg/L) on day 1-3 can be seen in figure 11.14b.

No linear relationship is found between plasma magnesium concentration and serum C-reactive protein concentration over days 0-3 or between the % change in plasma magnesium concentration and serum C-reactive protein concentration over days 1-3.

| | **CRP (mg/L) Vs plasma magnesium(mmol/L)** | |
|---|---|---|
| | $r^2$(%) | p value |
| Day 0 | 11.2 | 0.075 |
| Day 1 | 1.8 | 0.563 |
| Day 2 | 3.3 | 0.534 |
| Day 3 | 6.8 | 0.498 |
| | **logCRP (mg/L) Vs % change plasma magnesium** | |
| | $r^2$(%) | p value |
| Day 1 | 1.4 | 0.610 |
| Day 2 | 6.6 | 0.377 |
| Day 3 | 10.1 | 0.404 |

**Table 11.9** Relationship between CRP concentration (mg/L) and plasma magnesium (mmol/L) on day 0-3, and % change plasma magnesium on day 1-3, using simple linear regression.

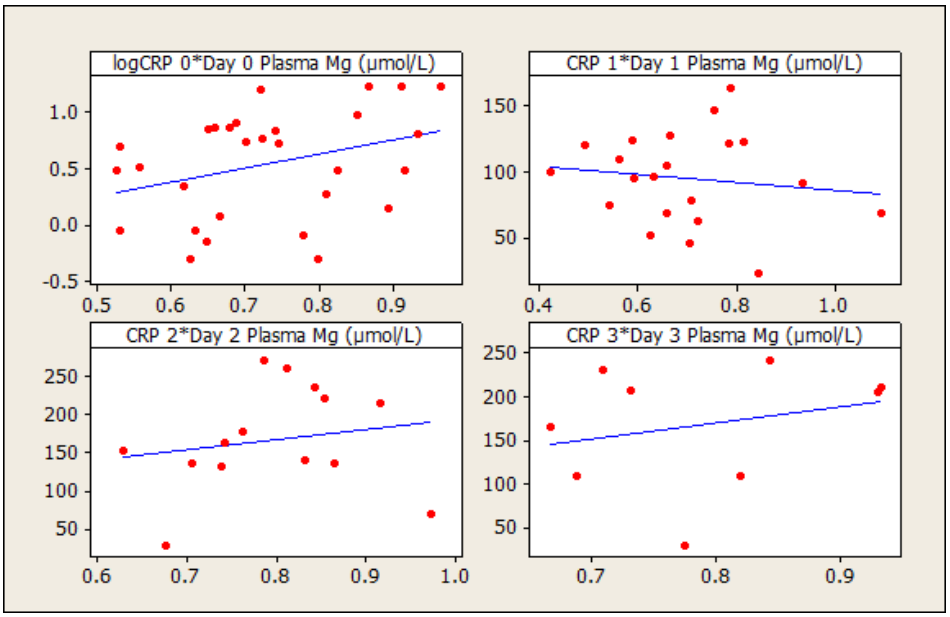



**Fig11.14a** Scatterplots of CRP concentration (mg/L) Vs plasma magnesium concentration (mmol/L) on day 0-3

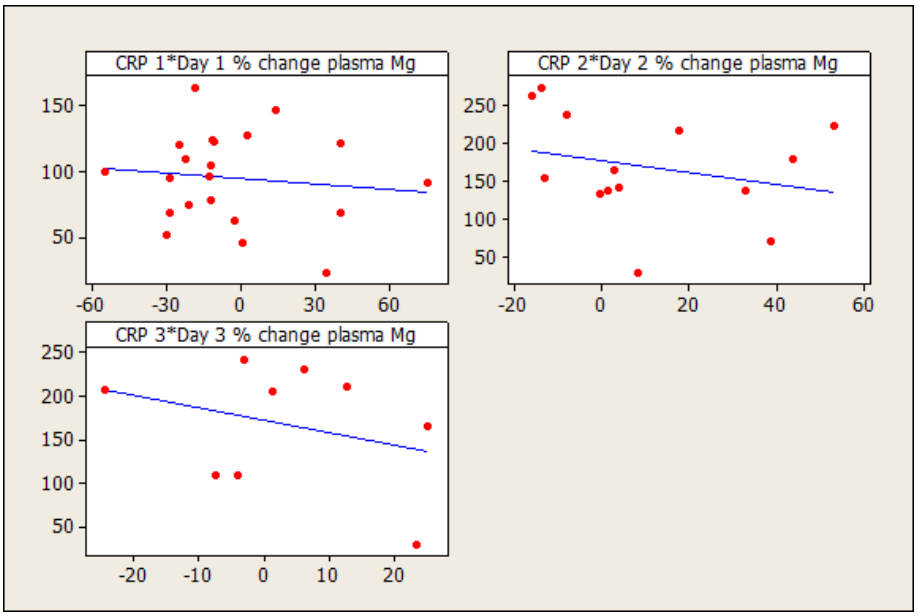



**Fig 11.14b** Scatterplots of CRP concentration (mg/L) Vs % change in plasma magnesium concentration on day 1-3

*11.5.7 Red Blood Cell Magnesium Concentration*

In order to examine the relationship between red blood cell magnesium concentration on day 0 and plasma magnesium concentration on day 0-3, and the relationship between the percentage change in red blood cell magnesium concentration and the percentage change in plasma magnesium concentration on day 1-3, linear regression analysis was carried out; the results can be seen in table 11.10. The relationship between plasma magnesium concentration (mmol/L) and red blood cell magnesium concentration (µg/ µg Fe) on day 0-3 can be seen in figure 11.15a. The relationship between the percentage change in plasma magnesium concentration and the percentage change in red blood cell magnesium concentration on day 1-3 can be seen in figure 11.15b.

No linear relationship was found between plasma magnesium and red blood cell magnesium concentration on days 0-3. Also, there was no relationship between the percentage change in plasma magnesium and the percentage change in the red blood cell concentration.

| | **RBC magnesium (µg/ µg Fe) Vs plasma magnesium(mmol/L)** | |
|---|---|---|
| | $r^2$(%) | p value |
| Day 0 | 1.2 | 0.578 |
| Day 1 | 0.1 | 0.905 |
| Day 2 | 23.3 | 0.083 |
| Day 3 | 17.7 | 0.259 |
| | **% change RBC magnesium Vs % change plasma magnesium** | |
| | $r^2$(%) | p value |
| Day 1 | 8.5 | 0.200 |
| Day 2 | 5.8 | 0.408 |
| Day 3 | 10.5 | 0.394 |

**Table 11.10** Relationship between RBC magnesium concentration (µg/ µg Fe) and plasma magnesium concentration (mmol/L) on day 0-3, and between the % change in RBC and plasma magnesium on day 1-3, using simple linear regression.

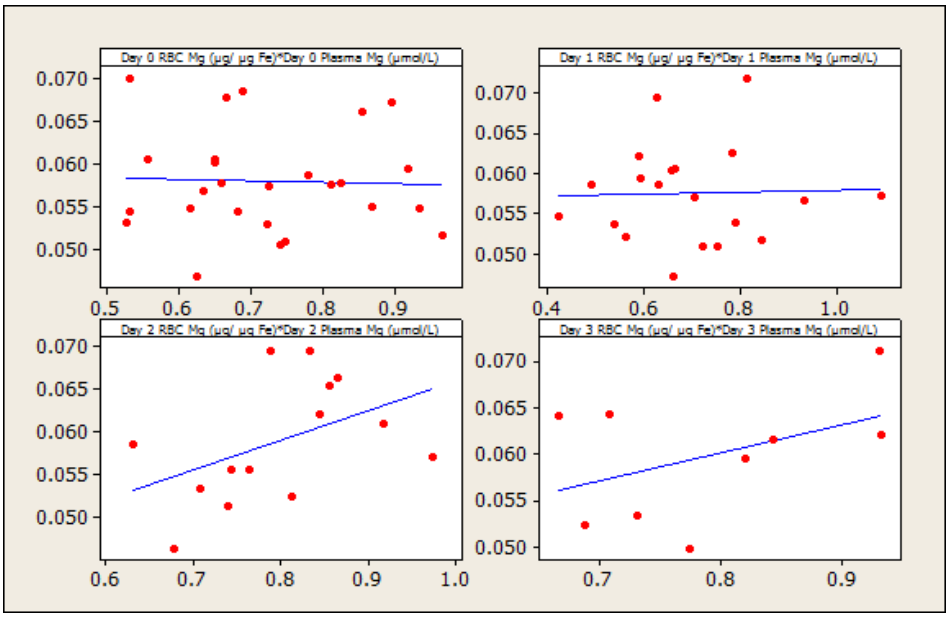



**Fig 11.15a** Scatterplots of RBC magnesium concentration (µg/ µg Fe) Vs plasma magnesium concentration (mmol/L) on day 0-3

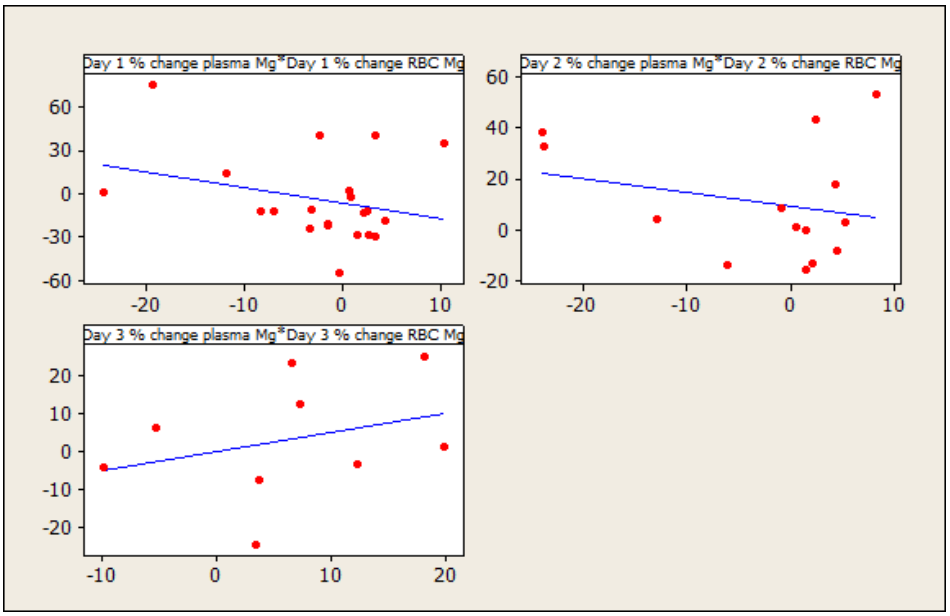



**Fig 11.15b** Scatterplots of % change in RBC magnesium concentration Vs % change in plasma magnesium concentration on day 1-3

### 11.6 Summary of findings – Magnesium

No relationship was found between plasma, red blood cell, liver or muscle magnesium concentration in the non-inflamed patient.

Plasma magnesium changed significantly during the acute phase response, through an approximate 11 % decrease on day 1. However, when this was examined more closely the change in plasma magnesium ranged from a positive to a negative change. Examination of tertiles of plasma magnesium concentration on day 0, when the patients were not inflamed, showed that the change during the acute phase was related to the baseline concentration of magnesium (median of 24% increase for the lowest tertile, 28% decrease for the highest tertile on day 1). The change in plasma magnesium concentration resulted in there being no difference in plasma magnesium concentration between the tertiles by day 1 of the acute phase response.

The relationship between the plasma magnesium concentration and the possible factors that may be related to the change in plasma magnesium concentration were explored. No relationship was found with albumin, C-reactive protein, liver magnesium concentration, muscle magnesium concentration or red blood cell magnesium concentration and either the plasma magnesium concentration or the change in plasma magnesium concentration from baseline.

There was a relationship between the change in plasma magnesium concentration during the acute phase response and the initial plasma magnesium when the patient was non-inflamed on day 0. The baseline plasma magnesium concentration accounted for 46.4% of the change in plasma magnesium concentration from day 0 on day 1, 65.6% on day 2 and 64.0% on day 3.

Therefore, it can be concluded that the plasma magnesium concentration changes during the acute phase response but the direction and magnitude of change is related to the baseline, non-inflamed, plasma magnesium concentration. This resulted in a similar range of magnesium concentration in all individuals regardless of their baseline plasma magnesium concentration.

# Chapter 12 - Discussion

## 12.1 Suitability of methods for the routine clinical laboratory

The requirements for an assay for use in a research setting and that which is to be used in a clinical setting can be different. The number of samples received may be far greater than in research, the instruments may be used for a number of assays meaning that a short sampling time is vital, the staff processing the samples will be performing a number of different tasks simultaneously so processes should be as simple as possible and be feasible in the routine reception area, and staff performing the analysis will have to be trained in this along with many other techniques within the laboratory, so the assay must be easy to learn and simple to perform. As the results will be used for clinical decision making they must be precise and ideally accurate. As reference materials are difficult to find for trace elements, local reference intervals would need to be developed to set decision levels for treatment.

The methods developed for this study are both practical and robust enough for use in a routine clinical laboratory setting. Pre-analytical steps were simplified to ensure they could be followed with minimal error in a busy laboratory reception, with erythrocyte preparation simply involving centrifugation and separation, avoiding any laborious cell washing stage; this had no detrimental effect on the reproducibility of results.

ICP-MS was used for the plasma and erythrocyte analysis as it allows multi-element analysis over a wide range of concentrations and a low sample volume allowing all the elements to measured simultaneously, making this a very efficient method for use in a routine setting. To eliminate any possible matrix effect, acid –digestion was performed. Acid-digestion can be laborious and add a further source for error and contamination; however, in the method developed the digestion took place in the tube that would be used for analysis on the

instrument, meaning that diluting reagent simply had to be added to the digested sample before analysis.

Iron was used as a mean of expressing the erythrocyte results as a surrogate for haemoglobin, based on the previous results from our laboratory showing that the haemoglobin concentration of red blood cells obtained via a standard haematology analyser and the iron concentration measured by ICP-MS correlate over a wide range of concentrations[2]. Iron was measured using a less abundant isotope as it was at far higher concentration than the other elements, allowing it to also be analysed on the same sample simultaneously with the other elements.

The trace element and magnesium methods developed had precision that made them suitable for use in a clinical setting. The plasma methods all had an inter-assay coefficient of variation <6% and the highest coefficient of variation for an erythrocyte method was magnesium at 11.7%, which are all suitable for clinical use. A recovery experiment, which is used to estimate proportional systematic error, particularly from a sample matrix effect, indicated no significant problems.

The glutathione peroxidase method was a simple kit method; however, possibly because it was designed for use on farm animals with far higher GPX activity as well as humans, the manufacturer's recommended dilutions resulted in poor precision which would render it unsuitable for clinical use. Changing the concentrations used resulted in an inter-assay CV of <8% for the combined GPX and haemoglobin assay, which is very acceptable for routine use. A local laboratory reference interval was developed allowing a decision limit to be set to guide clinicians when to supplement.

## 12.2 Choice of patient cohort

Patients undergoing liver lobe resection for metastatic lesions were chosen for this study as they were having their liver resected as part of routine care, allowing the study sample to be taken from a healthy area of the resected tissue, with rectus muscle sampled from the incision site. The operation itself acted as the stimulus for the acute-phase response allowing changes in plasma and erythrocyte trace element and magnesium concentrations to be studied post-operatively. As liver biopsy can have significant risks of haemorrhage, it would not have been possible to perform this study on healthy volunteers. Patients undergoing liver biopsy for diagnostic purposes would also have been unsuitable as the liver tissue may have been diseased.

However, the suitability of the patient cohort used in this study could be questioned. Patients with metastatic cancer may well have had recent loss of appetite and weight making then at higher risk of micronutrient deficiencies and therefore not representative of the population. They may also have had systemic inflammation at baseline that affected circulating micronutrient levels. These patients are selected for surgery on the basis of the surgeon's opinion of their likelihood of a successful outcome, based on clinical and radiological findings including nutritional state; these clinical decisions result in the selection of a cohort who have a low baseline C-reactive protein concentration and albumin concentration within the normal range. Also, as the study is focusing on the relationship between tissue and blood concentrations, the overall status of the cohort is unlikely to be a source of bias.

It should however be noted that due to the nature of the operation the postoperative period is not only a time of an acute-phase reaction, but also when a large volume of intravenous fluids may be given and the patient will be fasted; these may affect the micronutrient concentrations and should be accounted for, through the use of metal: protein ratios for example. Blood transfusion would also be a major source of error but this operation results in

less than 100mL of blood loss on average, so this combined with good pre-operative health avoids the need for pre or post-operative blood transfusion interfering with results.

The other issue with all but two of the participants is that they had bowel preparation prior to surgery. This consisted of a large volume of osmotic laxative which may have left them mildly dehydrated and resulted in a loss of magnesium. As only 2 participants did not have bowel prep it is impossible to analyse the two groups separately to examine for any affects of the bowel prep; however the 2 unaffected (number 1 and 29) had no difference in their haematocrit compared to the rest of the study participants. Certainly the bowel preparation should not have affected the main results: the majority of participants were affected and the use of metal: protein ratios would correct for any differences in fluid shifts as a result, and the tissue and blood correlations should have remained static even if there was loss of magnesium or trace elements as a result.

### 12.3 Utility of erythrocyte trace element concentrations

Erythrocytes have a life span of around 12 weeks, meaning that measurements of their concentration reflect the status when the erythrocytes were made. It could therefore be argued that erythrocyte trace element concentration does not reflect current body status, but the status several months previously. However, that is making the presumption that erythrocyte trace element concentration is stable from the time of erythrogenesis, rather than a dynamic store that varies in response to whole body status. This is unknown currently and would require futures work, although using the example of selenium where the majority of the element in incorporated into seleno-proteins at the time of erythrogenesis, a constant concentration for the lifespan of the cell would seem likely.

Would a constant concentration and therefore reflection of status of a longer time period be a major disadvantage? Certainly in patients on long term parenteral nutrition, one of the most established reasons for measuring trace element status, knowledge of the status over the previous 2-3 months would be advantageous. In patients with severe acute illness it is difficult to know as there are so many more unknowns. If a patient developed an acute severe illness on the background of chronic deficiency then this would be reflected in their erythrocyte concentration. However, if they started off with sufficient stores, over what time period would deficiency develop due to increased requirements and decreased intake during acute illness? This is unknown, as are the benefits of treating such deficiencies. It could certainly be argued that knowledge of their baseline status would give an indication as to the risk of developing deficiency during acute illness.

Blood transfusion in the previous 3 months would render the erythrocyte measure uninterpretable; this is a big concern in severe acute illness where blood transfusions are often required and also in patients on medium-term parenteral nutrition where ongoing inflammatory states leave them anaemic.

This is probably the major flaw of this measurement and if used in routine practice referring clinicians would have to be made fully aware of this.

### 12.4 What is a significant result?

The results of the regression analysis in this study that were found to be "statistically significant", did not always show very strong associations; for example red blood cell copper reflects only 16% of the variance in liver copper concentration. That weak association would not normally get a blood measure accepted as a marker of tissue status; however, these results should be taken in the context of the current clinical situation.

To use copper as an example, 5073 thousand plasma samples were received by the Scottish Trace Element and Micronutrient Laboratory last year, showing that there is a demand for knowledge of copper status, yet plasma copper concentration, which is measured in laboratories all over the world, has no relationship to liver copper concentration. It could be argued that given the lack of a suitable blood marker of status, and the apparent lack of knowledge of the limitations of these tests by referring clinicians, that these tests should not be offered at all. It is however difficult to go back from providing a test to providing no test at all, and a suitable alternative is required; while the relationships seen in this work are not strong, they are at least present and will represent a vast improvement on the current tests used, allowing studies to be carried out to attempt to elucidate the clinical relevance of trace element status.

It may also be the case that the relationship between erythrocyte/plasma and liver status would strengthen in patients who were deficient; as these are the main group that are of clinical interest this should be investigated further.

## 12.5 Copper

All but one of the study participants had a baseline plasma copper concentration which was within the laboratory reference interval, giving reassurance that they are representative of the general population.

Despite the evolution of acute phase response on the 3 days post-operatively, there was no significant change in the any of the blood copper measures (plasma copper concentration, red blood cell copper concentration, ceruloplasmin concentration, ceruloplasmin: copper ratio and plasma ferroxidase activity). This was unfortunate as it did not allow for full study of the factors that contribute the changes in plasma copper and ceruloplasmin concentrations during the acute phase. However, this was not unexpected as previous studies have shown that the rise in plasma ceruloplasmin concentration, and consequently the rise in plasma copper concentration, is not maximal until 7 days post-operatively[46;47]. It would not have been possible to have continued this study for seven days as the patients tended to be discharged from hospital on the third or fourth day after the operation but would not have been well enough to have returned to the hospital or research facility for blood sampling on day 7.

Erythrocyte copper was the only blood measure to reflect liver status at baseline; neither plasma copper nor ceruloplasmin concentration was related to liver copper concentration. This is possibly due to the study participants being copper replete, as supplementation studies have shown both plasma copper and ceruloplasmin to only increase in response to copper supplements if the participants have a low copper or ceruloplasmin concentration at baseline[22;38]. The linear relationship between red blood cell copper and liver copper, although statistically significant, is not strong, with liver copper only explaining 15.9% of the variation in red blood cell copper. The relationship was no longer statistically significant on days 1 and 2, but was again on day 3; however this may be a reflection of the small sample size as when days 1-3 were pooled the linear relationship was seen again.

The linear relationship between plasma copper and plasma ceruloplasmin was not surprising as ceruloplasmin is the main carrier protein for copper[20]. Although no relationship was seen between liver copper concentration and either copper or ceruloplasmin, when non-inflamed or in the first two days of the acute phase response, by day 3 both measures appeared to have a strengthening linear relationship with liver copper, though these did not quite reach statistical significance, possibly due to small numbers. It may be that this is due to initial changes of ceruloplasmin concentration, and consequently copper concentration, in response to the acute phase and that the magnitude of these changes is related to liver copper concentration, but further work with a larger sample size that followed the changes over several more days of the acute phase response would be required to prove this.

A surprising finding was the positive linear relationship between both plasma copper and plasma ceruloplasmin and C-reactive protein on day 0, when the patients were not inflamed. This relationship did not exist on days 1-3 when the CRP was far higher, though it is unknown whether this reflected a lag-time in the up-regulation of ceruloplasmin in response to the acute phase and whether this relationship would have been seen again by day 7. The relationship was not seen across the full spectrum of CRP concentration, but instead only when the CRP was greater than 3 g/L; this may reflect an increase in ceruloplasmin concentration in response to chronic low-grade inflammation. This is almost certainly the explanation for recent findings that plasma copper concentration is positively correlated with body mass index and low-density lipoprotein concentrations. Both obesity and atherosclerotic disease are thought to be inflammatory diseases[91;92] (or at least causally related in the case of atherosclerosis) but a large epidemiological study would be required to show that the association of plasma copper concentrations with these conditions was attenuated by the addition of CRP to the statistical model before this was conclusive. It is a factor which would interfere with plasma copper being a useful marker for plasma status even when patients have a CRP within the reference interval, though when the relationship between plasma copper in patients with a CRP <3 g/L and liver copper was examined there was no relationship.

It would appear that red blood cell copper may be a better biomarker of tissue copper status than plasma copper or ceruloplasmin, but further work would be required to examine the relationship between erythrocyte and liver copper during a longer period of an acute phase response, and also to examine the change in erythrocyte copper concentration with copper supplementation.

## 12.6 Zinc

The majority of study participants had a plasma zinc concentration below the reference interval. It is difficult to know the full significance of this. While no reference materials were available to check the accuracy of the method, the average value samples measured produced results that were slightly lower than the average value set, but this was not of sufficient magnitude to explain the degree of deficiency seen in the study population. It does not appear to be related to chronic low-grade inflammation as there is no relationship with baseline CRP. It should therefore be presumed that these results are genuine and that any associations seen are in a depleted population and cannot be assumed to automatically apply to a zinc replete population.

Plasma zinc concentration at baseline had a linear relationship with liver zinc concentration; erythrocyte zinc was not related to liver zinc concentration. This fits with the findings from supplementation studies that show that while plasma zinc concentration responds to zinc supplementation, erythrocyte zinc does not[49]. This would suggest that plasma zinc concentration is the better biomarker for zinc status at baseline; however plasma zinc concentration falls by over 25% in the first 24 hours of the acute phase response.

Plasma zinc concentration is associated with albumin concentration during the acute phase response, but not at baseline. This may be due to different mechanisms for zinc homeostasis during acute inflammation when there may be increased tissue requirements for zinc. This is certainly suggested in the trends of zinc: albumin ratios for each tertile of liver zinc: the plasma zinc concentration has a far greater decrease in relation to albumin concentration in patients who had low liver zinc at baseline. This may be due to low tissue levels requiring the uptake of zinc from the plasma into tissue; however a larger study, possibly with serial tissue measurements and/or the use of isotope tracers would be required to prove this.

If the decrease in plasma zinc concentration seen during the acute phase response was solely due to a decrease in albumin concentration, then it should be possible to adjust the results to gain an idea of true zinc status. However, the decrease in albumin concentration is not the only factor that affects plasma zinc concentration, with underlying tissue status possibly also having an effect. So, while plasma zinc concentration may be a useful biomarker for tissue status out-with the acute phase response, neither plasma or erythrocyte zinc appear to be reliable biomarkers of tissue status during the acute phase response. This is very unfortunate, as the possibility that underlying tissue status determines the change in plasma zinc during the acute phase response suggests that it is a vital micronutrient during that time.

## 12.7 Selenium

Plasma selenium concentrations in the study cohort are low compared to the laboratory reference interval. 6 participants have a plasma selenium concentration below the reference interval with the median value only being 0.96μmol/L while the reference interval is 0.8-2.0μmol/L. It is actually very likely that this is a true result as it has been noted that local plasma selenium concentrations have fallen in recent years, possibly due to European food policy, and Scotland is now felt to have low selenium status[12]. It should therefore be noted that the results of this study are from a selenium deficient population and that they may not hold true for a selenium replete population.

At baseline there were linear associations between all selenium measures including liver, erythrocyte and plasma concentrations and red blood cell glutathione peroxidase; this reflects the relative selenium deficiency of the population. Similar results have been found in New Zealand, a low selenium status population, but not in selenium replete populations; this is felt to be because glutathione peroxidase activity plateaus, despite increasing selenium availably, once it has reached its maximum activity[93]

Plasma selenium concentration, but not erythrocyte selenium concentration or glutathione peroxidase activity, falls in the first 24 hours of the acute phase response. A linear relationship with the percentage fall in plasma selenium from day 0 and the plasma selenium concentration on day 0 on days 1 and 3 was found but on inspection this relationship was opposite on days 1 and 3: the lower the plasma selenium on day 0, the lower the percentage drop in plasma selenium was from day 1, yet the higher the plasma selenium on day 0, the lower the percentage drop from day 0 is by day 3. In other words, the higher the plasma selenium at baseline, the greater the percentage reduction on day 1, but quicker it recovers to baseline levels by day 3. This may be due to mechanisms that move all available selenium into tissues (or excretion) leaving a required amount circulating in the plasma, but if the tissues are already selenium replete then

this selenium is not utilised and is redistributed to the plasma once the acute phase response is over. It may however, simply be regression to the mean with random changes around a fixed mean leading to weak linear relationships seen on days 1 and 3. It is hard to avoid this phenomena in a study such as this, although a larger sample size may help reduces the chance of finding a relationship at random.

There is a strong linear relationship between plasma selenium concentration and serum albumin concentration on day 0-3 but the relationship actually strengthens over the evolution of the acute phase response, and by day 3 the serum albumin concentration is responsible for 90.8% of the variation in plasma selenium concentration. As the albumin concentration falls, which it did equally in all participants regardless of baseline plasma selenium status, the selenium which is normally bound to albumin would have to either be excreted or move into tissues. So if you have higher plasma selenium but a similar drop in albumin then there is no alternative but to have a larger drop in plasma selenium concentration during the acute phase response.

When the plasma selenium is corrected for serum albumin concentration change by use of the selenium: albumin ratio, the linear relationship with liver selenium is present throughout days 0-3. This raises the question as to whether plasma selenium: albumin ratio could be used to assess selenium status during the acute phase response. When only results from during the acute phase response were used the plasma selenium: albumin ratio accounted for 50.5% of the variance in liver selenium concentration, making this appear to be like a reliable measure. Further work would be required to study the reliability of the selenium: albumin ratio over a range of baseline selenium statuses and also over a longer period of the acute phase response.

Erythrocyte selenium would appear to be a superior biomarker for selenium status than plasma selenium in that it does not change during the acute phase response. Glutathione peroxidase activity does not change during the acute

phase response either, though is a more labour intensive assay than erythrocyte selenium. The effects of chronic inflammation would need to be studied along with the association between erythrocyte and liver selenium concentrations in a replete population.

## 12.8 Magnesium

Magnesium is not a trace element but instead is an abundant cation with very important roles in physiology. It is therefore not unexpected that the mechanisms controlling plasma concentration during the acute phase response are very different from that of the trace elements. There was no relationship between any of the tissue or blood measures, suggesting that control mechanisms for plasma magnesium are independent of tissue status, which is to be expected given the vital importance of maintaining plasma magnesium concentration.

Erythrocyte magnesium remained constant during the acute phase response. Plasma magnesium concentration changed dependent on the initial plasma concentration with a decrease in plasma concentration for those in the highest tertile at baseline and an increase for those in the lowest baseline tertile. When this was examined further there was a linear relationship between the percentage change in plasma magnesium concentration during the acute phase response and the plasma magnesium concentration at baseline; this appeared to be independent to the change in albumin concentration. Therefore, during the acute phase response the plasma magnesium concentration alters to a set level (between 0.65 and 0.8 mmol/L) in all the patients, regardless of the initial plasma magnesium concentration; it is likely that this is due to a homeostatic mechanism and may have a protective effect. Whether this is in response to the acute phase or to starvation is unknown, but it is independent of liver, muscle and erythrocyte magnesium concentrations and also CRP concentration. There is also a suggestion of a return to the baseline pattern of magnesium concentration by day 3, but whether this is due to resolution of the acute phase response, a return to normal eating by the patient or simply an aberrant result due to small numbers of samples on day 3, is unknown. There is certainly some evidence that plasma magnesium levels during health are genetically determined[94] and therefore a return to the baseline results would be expected.

It is unlikely that these results are caused by regression to the mean but this should be kept in mind. The mean plasma magnesium level does appear to change for day 0 to day 1, though this difference does not quite reach statistical significance (p=0.059_ probably due to the small sample. The other factor against regression to the mean being the explanation for these results is that the relationship between plasma magnesium on day 1 and the change in plasma magnesium on days 1,2 and 3 are consistent and strong with baseline plasma magnesium explaining 65% of the variance in the percentage change.

Overall the patient group have a low plasma magnesium concentration compared to the laboratory reference interval, with mean plasma magnesium of 0.72mmol/L. The assay achieved values very similar to the assigned values of the quality control material used to assess accuracy, so this is unlikely to be an analytical problem. Given that homeostatic mechanisms appear to regulate the plasma magnesium to a concentration that is below the population reference interval, without evidence of harm, it may be that this reference interval is inappropriate and needs to be reviewed.

Again, the use of bowel preparation should be considered as a potential interfering source in these results. All but 2 patients had bowel preparation; interestingly the 2 who did not had initial plasma magnesium of 0.6 and 0.9 mmol/L which is in keeping with the rest of the cohort. If the participants who had bowel preparation lost a significant amount of magnesium during it, would that have affected the results? It would certainly not affect the tissue and blood correlations; it the patient became magnesium depleted during this time, then if the plasma or erythrocyte concentrations were a reflection of tissue concentration you would expect them to fall too and the relationship remain constant. It also seems unlikely to be an explanation of the changes in magnesium concentration seen post operatively, which are seen even when using magnesium:albumin rations to account for any differences in fluid status. While you may expect a patient who is magnesium depleted to correct that over the next few days, this would not explain the decreases in plasma magnesium in those whi had a higher plasma magnesium at teh time of operation.

Unfortunately no insight could be gained into which biomarker was the best surrogate for tissue magnesium status. It may be that a population with a low plasma magnesium concentration would be required to study this; homeostatic mechanisms will tightly control plasma magnesium concentrations, maintaining a tight plasma concentration range throughout a range of tissue concentrations. However, this study has discovered new factors in the regulation of plasma magnesium concentration and placed doubt on the accuracy of currently accepted reference intervals.

### 12.9 Strengths and Weaknesses

The major strength of this work is the generation of a unique data set of paired tissue and blood samples in patients who are then followed through the evolution of the acute phase response. This has allowed conclusions to be drawn about which blood measures best reflect tissue status. It has also allowed insight into the physiological changes in trace metal and magnesium concentration during the acute phase response, in the context of tissue status. As this work used methods easily performed in the routine clinical laboratory, the findings are directly transferable to clinical practice.

The main weakness of this work goes hand in hand with the unique nature of this dataset, in that the sample size is limited resulting in limited power; where correlations and significant changes were seen, the limited power means the size of these may be underestimated.

The other major weakness include the incomplete sample collection: this was due to a combination of early discharge home and ward changes and is not unexpected in a study which relied fully on NHS staff to collect samples. In hindsight using dedicated research staff for sample collection would have resulted in a more complete data set, but at additional financial cost. Also, the length of hospital stay post-operatively meant that the acute phase increase in copper concentration was not able to be studied and future work will be required in this area. However, what would have been possible was more complete baseline data collection including body mass index and weight change prior to surgery; though again the small sample size would have made sub-group analyses challenging.

Potential bias in the study participants has been discussed in section 12.2. These include the potential for preoperative malnutrition due to cancer cachexia, which could have been better understood by having collected details of

pre=operative weight loss. Also the effects of bowel preparation, intravenous fluids and fasting pre and post operatively had potential to influence results to a degree; though these are unlikely to affect tissue and blood correlations by their very nature.

The suitability of the patient group and generalisability of the results could therefore be questioned; however these are a very “well” patient group despite their diagnosis and have low baseline inflammatory status. It is difficult to find another patient group with whom this study would be ethically possible as liver biopsies would not be permitted on volunteers.

Another problem with this study was the use of skeletal tissue measurements. Selenium and copper concentrations in skeletal tissue were too low to measure accurately and were excluded from analyses. It may be possible to alter the methods to make this possible, including using a lower volume of acid for digestion and increasing the integration time when analysing the samples. Zinc and magnesium concentrations could be measured but no correlations were found. This may be due to the status of the participants. It may be that homeostatic mechanisms exist to maintain plasma zinc and magnesium at the expenses of the metals stored in muscle, or vice versa. Again, examination of cohorts with low magnesium and zinc status may show positive correlations between blood and muscle concentrations that are not otherwise seen.

**12.10 How are we to assess whether trace element deficiency is present and supplementation helpful?**

To assess whether trace element deficiency is present you need a suitable marker of deficiency and a definition of deficiency. The marker should reflect the status at a tissue level. In this study a number of potential markers were identified but all the correlations with tissue status were weak. This does not necessarily make them useless for identifying deficiency, but further work would be required to ensure a particular marker was sensitive enough to changes in status to pick up a patient who developed deficiency. It may be the case that one single marker is not going to be identified which can accurately identify deficiency, and a range of plasma, cellular and functional measures would be required in combination with corrections for carrier protein status.

How you define deficiency is a much harder question. Deficiency would be a state whereby low levels of a trace element result in an adverse clinical outcome. For many analytes population reference intervals are used to define deficiency; however this definition is at the mercy of the status of the population. If an entire population were relatively deficient in an element then deficiency would not be correctly identified. Conversely, a population with high levels of an element would result in a reference interval which incorrectly labelled patients with deficiency when they may still have a concentration far above the level at which they would be adversely affected. However, in the absence of a better definition, population reference intervals may help identify severe deficiency. Large scale epidemiological studies would be required to identify concentrations of trace elements which appeared to be related to adverse clinical outcomes; but even then that does not prove a causal link.

A causal link between a deficiency state and an adverse clinical outcome could only truly be established if a supplementation study showed a reversal or prevention of the adverse clinical outcome. This would require an accurate measure of deficiency to ensure the correct population were being targeted.

Lack of a way to define deficiency, or not selecting the study cohort on the basis of status, may be why supplementation studies have so far had little success in improving outcomes. It would also be useful if a marker of supplementation which increased in proportion to the amount of supplement given was found; erythrocyte selenium is known to increase with supplementation and the same should be established for copper.

## 12.11 Future work

There are still many unknowns in measuring trace element status and a lot of work is required before the full validity of measurements and their usefulness in clinical practice are known.

*The relationship between blood and tissue concentrations over a range of trace element status*

In the current work, the cohort had low plasma selenium and zinc status, and normal plasma copper status; this may have an effect on the relationships seen with potential for stronger relationships in deficiency. Examining these relationships over a range of baseline status would be beneficial as the relationship in deficiency is the clinically relevant model. However, finding a cohort with low plasma status that liver samples could be obtained from would be very difficult.

*The change in erythrocyte trace element concentration during prolonged inflammation*

While we know from previous work that erythrocyte concentration does not change over 7 days in a simple model of the acute phase response (post knee arthroplasty), this is not the typical patient model in which trace element concentration is of clinical interest. Investigating the change in erythrocyte concentration in a patient with a prolonged severe inflammatory illness would be advantageous as confounding factors of decreased erythrogenesis; iron deficiency, poor oral intake and increased utilisation would become important and may affect the concentration and its relationship with tissue status. This would help answer the question as to whether erythrocyte concentrations of trace elements where determined at the time of erythrogenesis or where

dynamic in response to tissue status. Such a study could be performed in patients in intensive care, or with severe inflammatory conditions such as rheumatoid arthritis or inflammatory bowel disease.

*The change in erythrocyte copper concentration with supplementation*

Erythrocyte selenium is known to increase with supplementation and erythrocyte zinc is known to not increase with supplementation but no such study has been performed for copper. Given that erythrocyte copper appears to be a potentially useful marker of copper status, such a study would be useful. This study would have to use a palatable and bioavailable form of copper for supplementation and supplementation would have to continue for over 12 weeks to allow incorporation into new erythrocytes.

*The development of population reference intervals*

Given the lack of suitable reference materials, local laboratory reference intervals for erythrocyte trace elements and magnesium would have to be developed. It should be noted that population reference intervals and optimal concentrations required for health are not synonymous; however, until more information is known about the potential risks from subclinical deficiency and an optimal concentration for health is ascertained, population reference intervals provide useful guidance for clinicians.

*The relationship between erythrocyte and plasma concentrations and clinical outcomes*

There is no point having a blood test if you are not able to make clinical recommendations on the basis of the results which will have a positive effect on

patient outcomes. This is the major missing area in trace element research. However, until you have a valid marker of deficiency, it is difficult to select a patient cohort who may benefit from supplementation. For example, there have been many studies of selenium supplementation in intensive care which have had inconclusive results[15;95]; it may be that selecting a cohort of patients who are truly selenium deficient would result in a positive effect on outcome.

## 12.12 Conclusions

- Erythrocyte copper concentration, not plasma copper, is weakly related to liver copper concentration and does not change during the acute phase response. This suggests erythrocyte copper is a potential suitable biomarker for tissue copper status during the acute phase response, however further studies looking at the relationship over a longer period of inflammation are required.

- Plasma copper concentration is related to C-reactive protein concentration when the patient does not have an inflammatory response. This may be the explanation for epidemiological studies linking higher copper status and cardiovascular disease and should be considered when interpreting plasma copper results.

- Plasma zinc concentration is weakly related to liver zinc concentration, but the concentration decreases during the acute phase response. The decrease in concentration may be related to liver concentration; however it is not possible to accurately 'correct' for the decrease by adjusting for the serum albumin concentration. Erythrocyte zinc concentration is not related to liver zinc concentration. Neither plasma nor erythrocyte zinc appear to be a reliable biomarker for zinc status during the acute phase response.

- Plasma and erythrocyte selenium concentration, and erythrocyte glutathione peroxidase concentration, all have a linear relationship with liver selenium concentration. However plasma selenium concentration decreases during the acute phase response; adjustment for serum albumin concentration may be possible but this requires further research. Erythrocyte selenium concentration or erythrocyte glutathione peroxidase activity both appear to be potential biomarkers of tissue selenium status,

but these studied would have to be repeated in a selenium replete population.

- Plasma magnesium concentration changes during the acute phase response to what appears to be a set concentration, regardless of the original plasma magnesium. This homeostatic set-point concentration is lower than the standard laboratory reference interval and the validity of this reference interval during acute illness needs to be re-visited.

**Appendix 1 – Reference range values**

*Whole blood glutathione peroxidase (U/ g Hb) for reference interval*

| Sample | Hb (g/L) | GPX U/L | GPX U/ g Hb |
|---|---|---|---|
| 1 | 18.8 | 542.5 | 28.86 |
| 2 | 15.7 | 517.5 | 32.96 |
| 3 | 16.9 | 871.5 | 51.57 |
| 4 | 19.7 | 811.3 | 41.18 |
| 5 | 19.1 | 653.3 | 34.20 |
| 6 | 18.1 | 727.8 | 40.21 |
| 7 | 17.6 | 629.1 | 35.74 |
| 8 | 18.8 | 495 | 26.33 |
| 9 | 15.8 | 545.2 | 34.51 |
| 10 | 17.7 | 580.4 | 32.79 |
| 11 | 17.7 | 421.7 | 23.82 |
| 12 | 15.4 | 576.7 | 37.45 |
| 13 | 17.4 | 505.9 | 29.07 |
| 14 | 17.5 | 789.3 | 45.10 |
| 15 | 17.4 | 546 | 31.38 |
| 16 | 12.3 | 363.7 | 29.57 |
| 17 | 19.2 | 537.1 | 27.97 |
| 18 | 15.7 | 579.5 | 36.91 |
| 19 | 15 | 446.3 | 29.75 |
| 20 | 17.1 | 551.5 | 32.25 |
| 21 | 11.1 | 133.1 | 11.99 |
| 22 | 17.6 | 369.6 | 21.00 |
| 23 | 13.1 | 378.4 | 28.89 |
| 24 | 17.6 | 465.8 | 26.47 |
| 25 | 18 | 310.1 | 17.23 |
| 26 | 12.8 | 305.8 | 23.89 |
| 27 | 19.6 | 458.9 | 23.41 |
| 28 | 19.7 | 590.6 | 29.98 |
| 29 | 14.6 | 535.4 | 36.67 |
| 30 | 15.1 | 676.1 | 44.77 |

| Sample | Hb (g/L) | GPX U/L | GPX U/ g Hb |
|---|---|---|---|
| 31 | 17.6 | 475.5 | 27.02 |
| 32 | 13 | 742.1 | 57.08 |
| 33 | 17.4 | 563.9 | 32.41 |
| 34 | 16.7 | 704.8 | 42.20 |
| 35 | 17.4 | 592.2 | 34.03 |
| 36 | 15.8 | 669.2 | 42.35 |
| 37 | 14.1 | 771.9 | 54.74 |
| 38 | 13.6 | 422.6 | 31.07 |
| 39 | 11.6 | 451.7 | 38.94 |
| 40 | 15 | 653.5 | 43.57 |
| 41 | 16.2 | 560.6 | 34.60 |
| 42 | 13.8 | 622 | 45.07 |
| 43 | 15.5 | 566.3 | 36.54 |
| 44 | 14.6 | 561.6 | 38.47 |
| 45 | 14.7 | 636.3 | 43.29 |
| 46 | 17.6 | 724.9 | 41.19 |
| 47 | 10.5 | 356.3 | 33.93 |
| 48 | 15.7 | 453.1 | 28.86 |
| 49 | 10.3 | 728.4 | 70.72 |
| 50 | 17.1 | 523.9 | 30.64 |
| 51 | 17 | 568.9 | 33.46 |
| 52 | 15.8 | 572.8 | 36.25 |
| 53 | 14.3 | 382.8 | 26.77 |
| 54 | 20.4 | 465.8 | 22.83 |
| 55 | 15.6 | 596.2 | 38.22 |
| 56 | 14 | 573.5 | 40.96 |
| 57 | 15.5 | 790.8 | 51.02 |
| 58 | 17.1 | 555.5 | 32.49 |
| 59 | 14.1 | 543.9 | 38.57 |
| 60 | 14.7 | 655.2 | 44.57 |
| 61 | 13.1 | 618.1 | 47.18 |
| 62 | 15 | 446.6 | 29.77 |
| 63 | 14.2 | 492.1 | 34.65 |
| 64 | 13 | 384.9 | 29.61 |

| Sample | Hb (g/L) | GPX U/L | GPX U/ g Hb |
|---|---|---|---|
| 65 | 12.8 | 624.4 | 48.78 |
| 66 | 18.2 | 644.8 | 35.43 |
| 67 | 14.8 | 497 | 33.58 |
| 68 | 13.7 | 497.5 | 36.31 |
| 69 | 14.7 | 631.3 | 42.95 |
| 70 | 18.2 | 612.1 | 33.63 |
| 71 | 15.4 | 324.7 | 21.08 |
| 72 | 16 | 553.1 | 34.57 |
| 73 | 15 | 604.6 | 40.31 |
| 74 | 14.3 | 503.9 | 35.24 |
| 75 | 14 | 599.4 | 42.81 |
| 76 | 14 | 454.4 | 32.46 |
| 77 | 15.7 | 525.5 | 33.47 |
| 78 | 13.9 | 436.3 | 31.39 |
| 79 | 13.3 | 567.3 | 42.65 |
| 80 | 13.4 | 368.4 | 27.49 |
| 81 | 15.8 | 350.7 | 22.20 |
| 82 | 15.6 | 308.7 | 19.79 |
| 83 | 13 | 285.8 | 21.98 |
| 84 | 12.9 | 382.4 | 29.64 |
| 85 | 13.6 | 587.2 | 43.18 |
| 86 | 16.3 | 339 | 20.80 |
| 87 | 13.7 | 486.9 | 35.54 |
| 88 | 12.3 | 151.7 | 12.33 |
| 89 | 14.3 | 387.7 | 27.11 |
| 90 | 13.9 | 364.5 | 26.22 |
| 91 | 16.1 | 474.7 | 29.48 |
| 92 | 14 | 314.1 | 22.44 |
| 93 | 13.6 | 368.7 | 27.11 |
| 94 | 14.6 | 335.7 | 22.99 |
| 95 | 16.2 | 300 | 18.52 |
| 96 | 13.1 | 446.1 | 34.05 |
| 97 | 14 | 144.3 | 10.31 |
| 98 | 13.9 | 238.9 | 17.19 |

| Sample | Hb (g/L) | GPX U/L | GPX U/ g Hb |
|---|---|---|---|
| 99 | 12.5 | 439 | 35.12 |
| 100 | 13.6 | 406.1 | 29.86 |
| 101 | 10.1 | 139.2 | 13.78 |
| 102 | 13 | 409 | 31.46 |
| 103 | 16.5 | 464.4 | 28.15 |
| 104 | 13.7 | 446 | 32.55 |
| 105 | 14.6 | 547.4 | 37.49 |
| 106 | 15.1 | 504.2 | 33.39 |
| 107 | 15.9 | 513.4 | 32.29 |
| 108 | 14.4 | 637.1 | 44.24 |
| 109 | 15.2 | 428.9 | 28.22 |
| 110 | 16.3 | 518.4 | 31.80 |
| 111 | 12.6 | 501.2 | 39.78 |
| 112 | 12.8 | 569.5 | 44.49 |
| 113 | 12.9 | 475.5 | 36.86 |
| 114 | 15 | 446.3 | 29.75 |
| 115 | 15.9 | 572 | 35.97 |
| 116 | 15.5 | 600.6 | 38.75 |
| 117 | 13.2 | 524.5 | 39.73 |
| 118 | 13.4 | 603.7 | 45.05 |
| 119 | 13.7 | 497.6 | 36.32 |
| 120 | 12.3 | 372.9 | 30.32 |

*Red blood cell glutathione peroxidase (U/ g Hb) for reference interval*

| Sample | Hb (g/L) | GPX U/L | GPX/ g Hb |
|---|---|---|---|
| 1 | 31 | 492 | 31.74 |
| 2 | 29 | 534.8 | 36.88 |
| 3 | 23.6 | 811.8 | 68.80 |
| 4 | 37.4 | 882.4 | 47.19 |
| 5 | 30.4 | 635.2 | 41.79 |
| 6 | 39.2 | 745.5 | 38.04 |
| 7 | 30.2 | 632.1 | 41.86 |
| 8 | 30.8 | 464.7 | 30.18 |
| 9 | 29.4 | 595.2 | 40.49 |
| 10 | 34.2 | 613.6 | 35.88 |
| 11 | 35 | 416.5 | 23.80 |
| 12 | 33 | 677.4 | 41.05 |
| 13 | 37.8 | 536.9 | 28.41 |
| 14 | 31.8 | 788.1 | 49.57 |
| 15 | 27.8 | 192 | 13.81 |
| 16 | 28.4 | 354.3 | 24.95 |
| 17 | 31.2 | 626.3 | 40.15 |
| 18 | 28.4 | 456.3 | 32.13 |
| 19 | 31.4 | 486.9 | 31.01 |
| 20 | 27.2 | 659.7 | 48.51 |
| 21 | 31 | 525.8 | 33.92 |
| 22 | 29.4 | 567.5 | 38.61 |
| 23 | 26.8 | 423.7 | 31.62 |
| 24 | 28.4 | 479.7 | 33.78 |
| 25 | 20 | 190.7 | 19.07 |
| 26 | 28.6 | 394.5 | 27.59 |
| 27 | 29.6 | 419.6 | 28.35 |
| 28 | 33 | 540.7 | 32.77 |
| 29 | 28.6 | 621.7 | 43.48 |
| 30 | 30.4 | 464.7 | 30.57 |
| 31 | 28 | 684.7 | 48.91 |
| 32 | 29 | 840.4 | 57.96 |

| Sample | Hb (g/L) | GPX U/L | GPX/ g Hb |
|---|---|---|---|
| 33 | 28.6 | 480.1 | 33.57 |
| 34 | 31.8 | 673.6 | 42.36 |
| 35 | 28.4 | 574.1 | 40.43 |
| 36 | 25.2 | 538.1 | 42.71 |
| 37 | 27.6 | 750 | 54.35 |
| 38 | 27.6 | 389.8 | 28.25 |
| 39 | 29.6 | 337.6 | 22.81 |
| 40 | 27.6 | 581.2 | 42.12 |
| 41 | 31.2 | 489.6 | 31.38 |
| 42 | 31.2 | 457.5 | 29.33 |
| 43 | 26.6 | 548.1 | 41.21 |
| 44 | 30.4 | 548.4 | 36.08 |
| 45 | 29.8 | 676.4 | 45.40 |
| 46 | 30.4 | 691.8 | 45.51 |
| 47 | 28.6 | 462.5 | 32.34 |
| 48 | 31 | 437.2 | 28.21 |
| 49 | 29.4 | 601.7 | 40.93 |
| 50 | 29.4 | 551.2 | 37.50 |
| 51 | 28.8 | 515.5 | 35.80 |
| 52 | 28 | 528.5 | 37.75 |
| 53 | 27.2 | 398.7 | 29.32 |
| 54 | 28.2 | 364.8 | 25.87 |
| 55 | 27.8 | 609.7 | 43.86 |
| 56 | 30.2 | 413 | 27.35 |
| 57 | 28.6 | 741.8 | 51.87 |
| 58 | 30.4 | 482.5 | 31.74 |
| 59 | 30.2 | 564.3 | 37.37 |
| 60 | 30.2 | 649.1 | 42.99 |
| 61 | 28.2 | 582.4 | 41.30 |
| 62 | 27.8 | 423.3 | 30.45 |
| 63 | 29 | 502.6 | 34.66 |
| 64 | 29 | 404.4 | 27.89 |
| 65 | 29.4 | 698.9 | 47.54 |
| 66 | 26.4 | 476.8 | 36.12 |

| Sample | Hb (g/L) | GPX U/L | GPX/ g Hb |
|---|---|---|---|
| 67 | 28 | 460.5 | 32.89 |
| 68 | 28 | 483 | 34.50 |
| 69 | 28.8 | 621.6 | 43.17 |
| 70 | 28 | 480.8 | 34.34 |
| 71 | 27.4 | 261.9 | 19.12 |
| 72 | 27.2 | 442.7 | 32.55 |
| 73 | 30.2 | 569.8 | 37.74 |
| 74 | 32.8 | 441 | 26.89 |
| 75 | 29 | 551.9 | 38.06 |
| 76 | 28 | 391.6 | 27.97 |
| 77 | 35.6 | 464.1 | 26.07 |
| 78 | 30.4 | 420.7 | 27.68 |
| 79 | 28.8 | 541.8 | 37.63 |
| 80 | 27.4 | 151.1 | 11.03 |
| 81 | 28.6 | 373.5 | 26.12 |
| 82 | 28.2 | 392.4 | 27.83 |
| 83 | 26.6 | 369.3 | 27.77 |
| 84 | 22.4 | 320.6 | 28.63 |
| 85 | 23.4 | 443.4 | 37.90 |
| 86 | 26.4 | 353.3 | 26.77 |
| 87 | 27.4 | 582.2 | 42.50 |
| 88 | 27.6 | 286.6 | 20.77 |
| 89 | 25.6 | 417.3 | 32.60 |
| 90 | 20.2 | 334.4 | 33.11 |
| 91 | 28.2 | 508.9 | 36.09 |
| 92 | 29 | 371.6 | 25.63 |
| 93 | 28.2 | 462.3 | 32.79 |
| 94 | 27.6 | 428.9 | 31.08 |
| 95 | 30.4 | 346.9 | 22.82 |
| 96 | 25.4 | 613.2 | 48.28 |
| 97 | 27 | 276.3 | 20.47 |
| 98 | 24.4 | 379.4 | 31.10 |
| 99 | 27.2 | 653.5 | 48.05 |
| 100 | 24.4 | 569 | 46.64 |

| Sample | Hb (g/L) | GPX U/L | GPX/ g Hb |
|---|---|---|---|
| 101 | 27.8 | 395.3 | 28.44 |
| 102 | 26 | 531.3 | 40.87 |
| 103 | 21.4 | 420.9 | 39.34 |
| 104 | 26.8 | 585 | 43.66 |
| 105 | 27.2 | 595.3 | 43.77 |
| 106 | 27 | 491.4 | 36.40 |
| 107 | 25.2 | 430.9 | 34.20 |
| 108 | 26.4 | 687.5 | 52.08 |
| 109 | 24.8 | 373.6 | 30.13 |
| 110 | 25 | 502.2 | 40.18 |
| 111 | 25.2 | 537.6 | 42.67 |
| 112 | 26.6 | 659.7 | 49.60 |
| 113 | 25.6 | 490.1 | 38.29 |
| 114 | 26.2 | 419.9 | 32.05 |
| 115 | 25.6 | 520.5 | 40.66 |
| 116 | 25.2 | 562.4 | 44.63 |
| 117 | 23.2 | 502.3 | 43.30 |
| 118 | 25.8 | 560.3 | 43.43 |
| 119 | 24.2 | 435.2 | 35.97 |
| 120 | 24.6 | 446.3 | 36.28 |

**Appendix 2**
Patient Information Sheet V2 02/02/2008

**A study of the relationship between tissue and blood concentrations of trace metals and micronutrients, and the effects of the acute inflammatory response on these concentrations.**

**A comparison of blood, liver, and muscle levels of metals and vitamins in patients who have had an operation.**

You are being invited to take part in a research study. Before you decide whether to take part, it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with others if you wish. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

Thank you for reading this.

What is the purpose of this study?

For normal body metabolism, skin to heal and to help fight infections, we need to take the correct amount of micronutrients (metals, such as copper and zinc and vitamins) in our food.

In our laboratory we routinely measure the concentrations of metals and vitamins in patients' bloods. This is particularly important in patients who receive their food directly to their blood (total parenteral nutrition) and also patients in intensive care.

You may have noticed that when you are sick for any reason, such as a common cold or the flu, you tend to not feel hungry and may have a high temperature. These symptoms are part of what is called the inflammatory response. Your body has an inflammatory response to a very wide variety of things such as infection, trauma (such as breaking a leg), surgery etc.

Other features of the inflammatory response include changes to the concentration of many components of the blood. These include a decrease in the concentration of the metal zinc in the blood even though the amount of zinc in the body is normal. The concentration of the metal copper can increase in the blood during inflammation, even when the body is lacking in copper. We are now able to measure these metals and vitamins in blood cells, which we not able to do in the past.

We are looking for a better way to measure the amounts of metals and vitamins in the body. We would like to compare the concentrations of metals and vitamins in blood cells with the levels in body tissues, to see if the blood cells contain similar amounts to other parts of the body, such as the liver and muscle. We would like to obtain liver and abdominal muscle samples from patients undergoing operations on their livers and compare their metal and vitamin concentrations to those measured in the blood. We would also like to measure the blood after the operation, whilst there is an inflammatory response, to see which type of blood cells keep a constant level of metals and vitamins.

Why have I been chosen?

You have been asked to participate in this study because you are undergoing a liver resection (operation where part of your liver is removed). This will allow us, if you agree to participate, to use a small amount of the spare liver tissue removed at the time of your operation.

We hope to recruit 50 patients.

Do I have to take part?

No, it is up to you to decide whether or not to take part. Whether or not you decide to take part will not in any way affect your treatment.

If you do decide to take part you will be given this information sheet to keep and be asked to sign a consent form. If you decide to take part you are still free to withdraw at any time and without giving a reason. A decision to withdraw at any time, or a decision not to take part, will not affect the standard of care you receive.

What will happen to me if I take part?

The majority of the study will take place during your hospital admission. It will not affect the treatment you receive in any way.

During your surgery, when part of the liver is removed, there will be left over liver that is normally destroyed. We wish to take a liver sample from the part of the liver that has been removed. We will take 2 x 1$cm^3$ of this liver tissue (about the size of 2 garden peas).

We will also take a small slither of muscle from your abdominal wall. Mr Horgan and his team will have to cut through your abdominal wall (your tummy) to enable them to see and operate on your liver. When they are finishing up their surgery and closing your wound, they will take a very thin strip from the abdominal wall under the skin, measuring 5mm by 10cm (about the size of the lead inside a pencil).

We will measure metal and vitamin levels on these samples.

We will take blood samples to measure the level of inflammation in your blood and also the concentrations of vitamins and metals in your blood. We will do this on the day before your surgery and one, two, three and six days after surgery. We will take 4 tubes of blood (around 20 ml or 4 teaspoons of blood) each time.

All efforts will be made to try and take the blood samples at the same time as routine bloods are being taken during your hospital stay.

If you have had a blood transfusion in the last three months this will have an effect on the concentrations on vitamins and metals in your blood cells (as they are will not be all your own), therefore you would not be able to participate in the study.

We appreciate that this can seem confusing so we have summarised what will happen to you below:

| | |
|---|---|
| Day of admission to hospital | A doctor will come and speak to you about taking part in the study. You will have routine bloods taken and at this time we will take 4 further tubes (20ml or 4 teaspoons) as part of the study. |
| Day of the operation | When Mr Horgan is starting the operation and is cutting through your abdominal wall (tummy) to get to your liver, a very small slither of muscle will be taken (5mm by 10cm - about the size of the lead inside a pencil).<br><br>During the operation, when Mr Horgan has removed part of your liver, a small part of the liver that is removed is used for the study (2 x 1cm$^3$ - about the size of 2 garden peas). |
| Each morning after the operation whilst you are in hospital | Mr Horgan's team will be taking bloods from you each day to monitor your recovery from the operation. At this time an additional 4 blood tubes will be taken for the study. |

It may be the case that in the future new tests are invented and it would be helpful if we could look at blood and tissues levels of it. Therefore, so we can get the maximum benefit from your donated samples, we would ask you to consider letting us keep your samples for use in future research projects. Your samples will be kept anonymised (without your name on them) so that they cannot be traced to you.

<u>What do I have to do?</u>

There are no restrictions placed on you if you take part in this study, for example there are no changes in diet, lifestyle or medication required.

What are the side effects of taking part?

Collecting the liver and muscle samples will not cause any significant discomfort to you over and above that of your operation. The muscle sample will have no effect on your wound, how it will look or how it will heal.

As with every blood test, there is a risk of pain or bruising on the arm from the needle. We will try to reduce this risk by having experienced persons taking your blood sample and trying to coordinate sampling so that the additional samples are collected with the routine bloods tests that your doctor requests. 4 tubes (20ml or 4 teaspoons) can seem like a lot of blood, but we can reassure you that this is very small compared to how much blood is in your body and you will have no ill-effects from this.

What are the possible disadvantages and risks of taking part?

There will be no alteration in your treatment if you participate in this study. We, therefore, do not you feel you will be disadvantaged by taking part in this study.

What are the possible benefits of taking part?

You will not benefit from taking part in this study as your treatment is unchanged.

We hope that the results of this test will help us treat future patients with nutritional problems better.

What if something goes wrong?

If you are harmed by taking part in this research project, there are no special compensation arrangements. If you are harmed due to someone's negligence, then you may have grounds for a legal action but you may have to pay for it. Regardless of this, if you wish to complain, or have any concerns about any aspect of the way you have been approached or treated during the course of this study, the normal National Health Service complaints mechanisms may be available to you.

Will my taking part in this study be kept confidential?

All information which is collected about you during the course of the research will be kept strictly confidential. Any information about you which leaves the hospital will have your name and address removed so that you cannot be recognised from it.
As a matter of courtesy, we will write to your GP to inform them that you have participated.

What will happen to the results of the research study?

You will not be identified in any report or publication of results from this study. We hope the study results will be published in a journal aimed specifically at specialist doctors and scientists. Results may be presented at meetings of specialist doctors and scientists.

Your samples will be analysed up to one year after you have left hospital. This, plus the fact that this is such an unknown area, means that there would be no results which we feel would be significant to your health, that we would want your doctor to act on.

Who is organising and funding the research?

The research is organised by the Scottish Trace Element and Micronutrient Reference Laboratory in the Department of Biochemistry at Glasgow Royal Infirmary. Financial support for the study may be sought from NHS research funds in Scotland. None of the researchers involved are being paid over and above their normal salary for undertaking this research.

Who has reviewed the study?

This study has been reviewed by the Glasgow Royal Infirmary Research Ethics Committee.

Contact for further information

If you require further information please contact:

Dr Jennifer Logue Tel: 0141 211 4390
Department of Biochemistry
McEwan Building
Glasgow Royal Infirmary
84 Castle Street
Glasgow
G4 0SF

Or ask a member of Mr Paul Horgan's team during your hospital admission.

Thank you for taking the time to read this information sheet.

**Scottish Trace Element and Micronutrient Reference Laboratory**
Department of Biochemistry
McEwan Building
Glasgow Royal Infirmary

**Department of Surgery**
Glasgow Royal Infirmary
84 Castle Street
Glasgow
G4 0SF

Date:

Dear

We are currently doing research involving patients who are having your type of surgery. We would like to invite you to take part. Enclosed is an information leaflet explaining why we are doing the research and what it will involve if you agreed to take part. Please take time to read the information and discuss it with others if you wish. When you come into hospital for your operation you will be able to discuss it further and ask any questions you may have, before deciding whether to take part.

Thank you for taking the time to read this.

Yours Sincerely

Dr Jennifer Logue
SpR in Chemical Pathology
Scottish Trace Element and Micronutrient Reference Laboratory
Glasgow Royal Infirmary

Mr Paul Horgan
Senior Lecturer and Honorary Consultant
Department of Surgery
Glasgow Royal Infirmary

Consent form V2 02/02/2008

Centre Number:
Study Number:
Patient Identification Number for this trial:

**CONSENT FORM**

**Title of Project**: **A study of the relationship between tissue and blood concentrations of trace elements and micronutrients, and the effects of the acute inflammatory response on these concentrations.**

Name of Researcher: Dr Jennifer Logue

Please initial box

1. I confirm I have read and understand the information sheet dated 02/02/2008 (Version 2) for the above study. I have had the opportunity to consider the information, ask questions, and have had these answered satisfactorily. ☐
2. I understand that my participation is voluntary and that I am free to withdraw at a time without giving any reason, without my medical care or legal rights being affected. ☐
3. I understand that relevant sections of my medical notes may be looked at by individuals from regulatory authorities the NHS trust, where it is relevant to my taking part in this research. I give permission for these individuals to have access to to my records. ☐
4. I agree to my GP being informed of my participation in the study. ☐
5. I agree to my tissue samples being stored for use in future studies. ☐
6. I agree to take part in the above study.

| _____ | _____ | _____ |
|---|---|---|
| Name of Patient | Date | Signature |
| _____ | _____ | _____ |
| Name of Person taking consent | Date | Signature |

When completed, 1 for patient, 1 for researcher site file, 1 (original) to be kept in medical notes.

Management approval 02/07/2008

North Glasgow University Hospitals Division

NHS
Greater Glasgow and Clyde

Research & Development Office
4th Floor Walton Building
Glasgow Royal Infirmary
84 Castle St
Glasgow
G4OSF
Telephone: 0141 211 0475
Fax: 0141 232 0752
Email: Fiona.Graham.gri@northglasgow scot.nhs.uk

Wednesday, 02 July 2008

Dr J. Logue
SpR in Chemical Pathology and Metabolic Medicine
Dept Biochemistry
McEwan Building
Glasgow Royal Infirmary
G4 0SF

Dear Dr Logue,

**Project Title.** A study of the relationship between tissue and blood concentrations of trace elements and micronutrients and the effects of the acute inflammatory response on these concentrations.
**Investigator:** Dr J. Logue
**R&D Reference:** RN08PA001
**COREC:** 07/S0704/109

We are pleased to inform you that, based on the information provided, the above project has been granted overall Management Approval and you may now proceed. This approval includes Finance and favourable Research Ethics Committee opinions.

Further management approval will be required for amendments that increase patient numbers, increase or change the test procedures or bring about a change in pharmacy requirements. Please contact the R&D office if you wish to discuss any future amendments.

Thank you for your current and future collaboration

Yours Sincerely,

Dr Fiona Graham
Academic Research Co-ordinator
Glasgow Royal Infirmary

**Glasgow Royal Infirmary REC**

**LIST OF SITES WITH A FAVOURABLE ETHICAL OPINION**

*For all studies requiring site-specific assessment, this form is issued by the main REC to the Chief Investigator and sponsor with the favourable opinion letter and following subsequent notifications from site assessors. For issue 2 onwards, all sites with a favourable opinion are listed, adding the new sites approved.*

| | | | | | |
|---|---|---|---|---|---|
| **REC reference number:** | 07/S0704/109 | **Issue number:** | 1 | **Date of issue:** | 31 January 2008 |
| **Chief Investigator:** | Dr Jennifer Logue | | | | |
| **Full title of study:** | A study of the relationship between tissue and blood concentrations of trace elements and micronutrients, and the effects of the acute inflammatory response on these concentrations. | | | | |

*This study was given a favourable ethical opinion by Glasgow Royal Infirmary REC on 31 January 2008. The favourable opinion is extended to each of the sites listed below. The research may commence at each NHS site when management approval from the relevant NHS care organisation has been confirmed.*

| *Principal Investigator* | *Post* | *Research site* | *Site assessor* | *Date of favourable opinion for this site* | *Notes (1)* |
|---|---|---|---|---|---|
| Dr Jennifer Logue | Specialist Registrar in Chemical Pathology and Metabolic Medicine | GLASGOW ROYAL INFIRMARY | Glasgow Royal Infirmary REC | 31/01/2008 | |

Approved by the Chair on behalf of the REC:

S Macgregor ..... (Signature of ~~Chair~~/Co-ordinator)

(delete as applicable)

S MACGREGOR ..... (Name)

**Appendix 3 Demographics and baseline results of participants and available samples:**

| Study number | Sex | Age | Reason for liver resection | Albumin (g/L) | CRP (mg/L) | Haematocrit (i/L) | Plasma Mg (mmol/L) | Plasma Cu (µmol/L) | Plasma Zn (µmol/L) | Plasma Se (µmol/L) | Samples available |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | F | 76 | Hydatid cyst | 40 | 17 | 0.402 | 0.9 | 24.0 | 11.6 | 1.0 | 0,1,2 |
| 2 | M | 76 | Liver metastases (Colorectal) | 39 | 5.8 | 0.36 | 0.7 | 13.1 | 13.3 | 1.2 | 0,1,2 |
| 3 | M | 61 | Liver metastases (Colorectal) | 42 | 3 | 0.437 | 0.5 | 11.7 | 9.3 | 1.1 | 0 |
| 4 | F | 62 | Liver metastases (Colorectal) | 37 | 8 | 0.392 | 0.7 | 15.8 | 11.7 | 1.0 | 0 |
| 5 | F | 76 | Liver metastases (Colorectal) | 40 | 0.8 | 0.368 | 0.8 | 15.8 | 11.4 | 1.1 | 0,1,2 |
| 6 | M | 73 | Liver metastases (Colorectal) | 32 | 3 | 0.357 | 0.8 | 14.4 | 11.3 | 0.8 | 0,1,3 |
| 7 | F | 35 | Liver metastases (Breast Ca) | 35 | 1.4 | 0.322 | 0.9 | 15.1 | 13.9 | 1.3 | 0,1 |
| 8 | M | 72 | Liver metastases (Colorectal) | 38 | 0.9 | 0.375 | 0.5 | 10.8 | 8.3 | 0.7 | 0,1,3 |
| 9 | F | 79 | Liver metastases (Colorectal) | 38 | 0.5 | 0.391 | 0.8 | 18.0 | 11.6 | 1.3 | 0,1,2 |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 10 | M | 66 | Liver metastases (Colorectal) | 42 | 3 | 0.481 | 0.9 | 17.7 | 18.5 | 1.7 | 0,1,2,3 |
| 11 | F | 71 | Liver metastases (Colorectal) | 39 | 5.4 | 0.363 | 0.7 | 13.4 | 11.2 | 0.8 | 0,1,2 |
| 12 | M | 48 | Liver metastases (Colorectal) | 40 | 17 | 0.409 | 1.0 | 16.5 | 13.6 | 0.9 | 0,1,2,3 |
| 13 | F | 69 | Liver metastases (Colorectal) | 35 | 1.3 | 0.391 | 0.9 | 11.9 | 14.8 | 0.7 | 0 |
| 14 | M | 68 | Liver metastases (Colorectal) | 33 | 16 | 0.408 | 0.7 | 15.8 | 10.2 | 0.7 | 0,1,2,3 |
| 15 | M | 73 | Liver metastases (Colorectal) | 24 | 9.4 | 0.431 | 0.9 | 15.5 | 10.2 | 0.6 | 0,2,3 |
| 16 | M | 54 | Liver metastases (Colorectal) | 38 | 17 | 0.303 | 0.9 | 18.9 | 12.2 | 1.0 | 0 |
| 17 | F | 51 | Liver metastases (Colorectal) | 43 | 6.4 | 0.398 | 0.9 | 15.6 | 13.4 | 1.2 | 0,1 |
| 18 | F | 60 | Liver metastases (Colorectal) | 47 | 7.4 | 0.384 | 0.7 | 17.5 | 10.9 | 1.1 | 0,1 |
| 19 | M | 57 | Liver metastases (Colorectal) | 36 | 1.2 | 0.386 | 0.7 | 6.4 | 10.7 | 0.8 | 0,1,3 |
| 20 | M | 64 | Liver metastases (Colorectal) | 41 | 5 | 0.429 | 0.5 | 9.2 | 11.7 | 1.0 | 0,1,2 |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 21 | M | 62 | Liver metastases (Colorectal) | 36 | 7.3 | 0.454 | 0.7 | 12.6 | 14.8 | 1.1 | 0,1 |
| 22 | M | 53 | Hepatocellcular carcinoma | 36 | 2.2 | 0.432 | 0.6 | 10.9 | 10.8 | 0.8 | 0 |
| 23 | F | 47 | Liver metastases (Colorectal) | 31 | 5.3 | 0.388 | 0.7 | 13.9 | 10.3 | 0.8 | 0,1 |
| 24 | F | 64 | Resection – no cancer on histopathology | 45 | 0.7 | 0.408 | 0.6 | 9.2 | 10.6 | 0.7 | 0,1 |
| 25 | F | 73 | Liver metastases (Colorectal) | 35 | 3.3 | 0.384 | 0.6 | 12.8 | 9.2 | 0.8 | 0,1,2 |
| 26 | F | 50 | Liver metastases (Colorectal) | 39 | 7 | 0.419 | 0.7 | 16.5 | 14.1 | 1.0 | 0,1 |
| 27 | F | 53 | Resection – no cancer on histopathology | 48 | 0.9 | 0.447 | 0.6 | 15.3 | 12.1 | 1.0 | 0 |
| 28 | F | 68 | Liver metastases (Colorectal) | 37 | 6.8 | 0.413 | 0.7 | 18.3 | 12.6 | 0.8 | 0,1,2,3 |
| 29 | F | 52 | Hydatid cyst | 42 | 0.5 | 0.367 | 0.6 | 11.7 | 11.0 | 1.0 | 0,1,2,3 |
| 30 | M | 43 | Liver metastases (Colorectal) | 38 | 1.9 | 0.377 | 0.8 | 13.2 | 12.8 | 1.0 | 0 |
| 31 | F | 71 | Liver metastases (Colorectal) | 39 | 15 | 0.364 | 0.8 | 17.0 | 12.0 | 0.9 | 0,1,2,3 |

## Appendix 4

### Normality of data - expanded details

*C-Reactive Protein*

To ensure the normality of the data and exclude any outlying samples, the Anderson-Darling test for normality was performed.

Serum CRP Day 0 (pre-operative sample) was found to not be normally distributed ($p<0.005$) (fig A4.1a) and was therefore logarithmically transformed to achieve normality ($p=0.132$) (fig A4.1b). Post-operatively, CRP on day 1, CRP on day 2, and CRP on day 3 were all found to be normally distributed ($p=0.849$, 0.635, 0.907 respectively)(fig A4.1c).

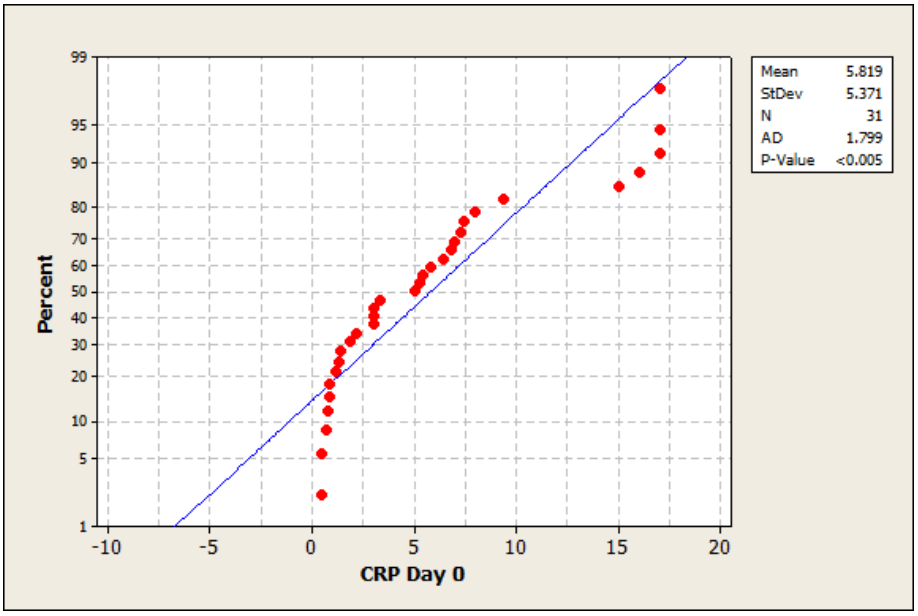



**Fig A4.1a** Probability plot of CRP concentration on day 0 (mg/L)

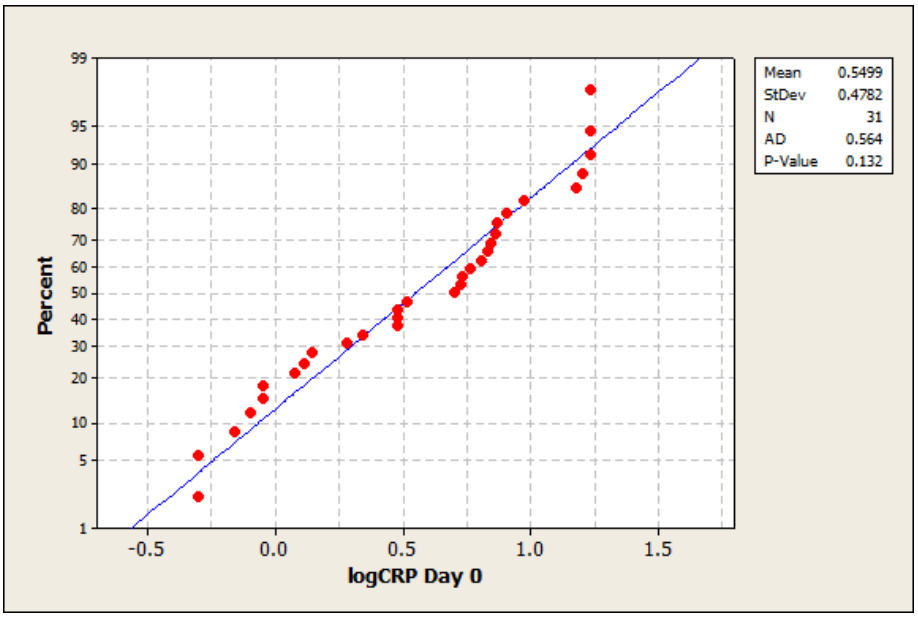



**Fig A4.1b** Probability plot of the logarithmic transformation of CRP concentration on day 0 (mg/L)

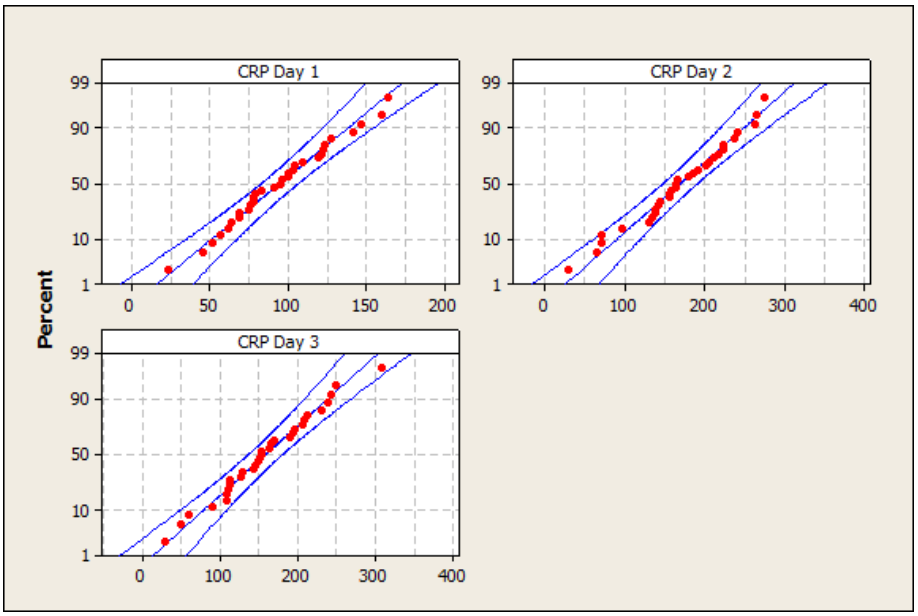



**Fig A4.1c** Probability plots of CRP concentration on day 1-3 (mg/L)

In order to allow pooling of results, it would be ideal to log transform all CRP data; unfortunately logarithmic transformation of day 2 and 3 rendered them non-normal (day 1 p=0356, day 2 p<0005, day 3 p=0.015; fig A4.2) so this was not done.

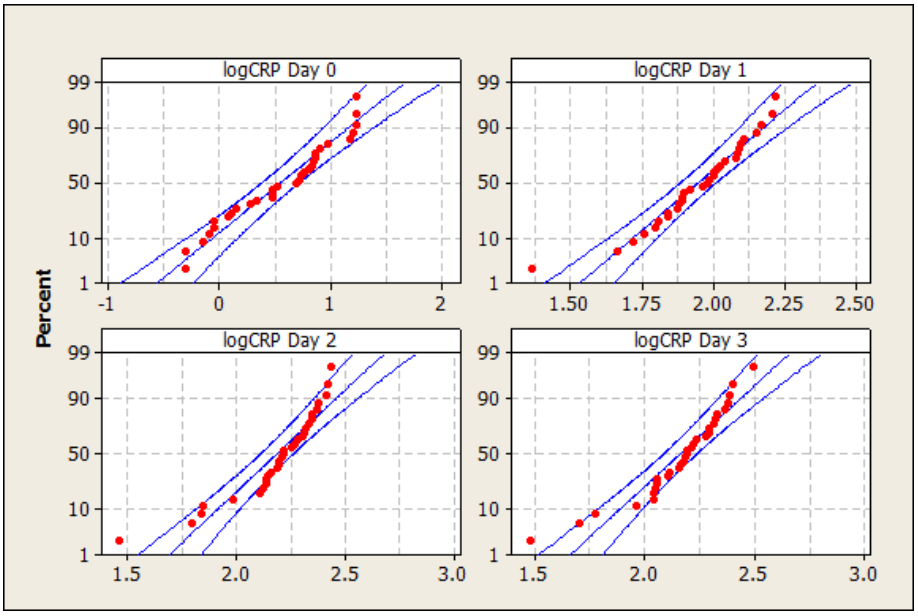



**Fig A4.2** Probability plots of logCRP concentration on day 0-3 (mg/L)

*Albumin*

Using the Anderson-Darling test for normality, Albumin Day 0, Day 1, Day 2 and Day 3 were found to all be all normally distributed (p=0.306, 0.177, 0.267, 0.135 respectively) (fig A4.3).

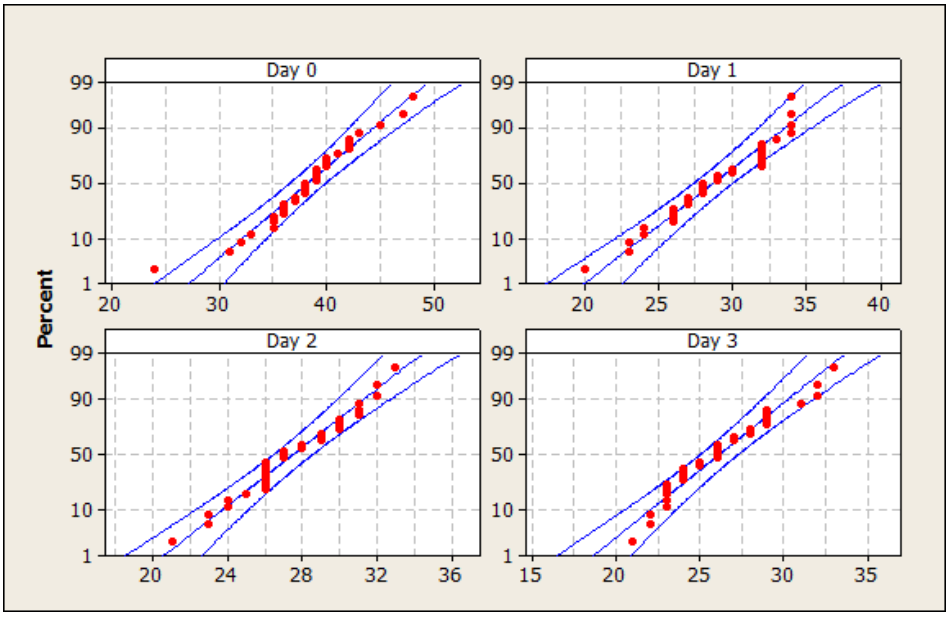



**Fig A4.3** Probability plots of albumin concentration on day 0-3 (g/L)

*Haematocrit*

*Normality of data*

Using the Anderson-Darling test for normality, haematocrit Day 0 was normally distributed (p=0.760). Examination of probability plots for haematocrit on day 1, 2 and 3 revealed outlying samples (fig A4.4a): day 1 samples 8, 10 and 21; day 2 samples 10, 18 and 22; day 3 sample 10. With these removed all samples were normally distributed: day 0 p=0.760, day 1 p=0.208, day 2 p=0.322 and day 3 p=0.410 (fig A4.4b).

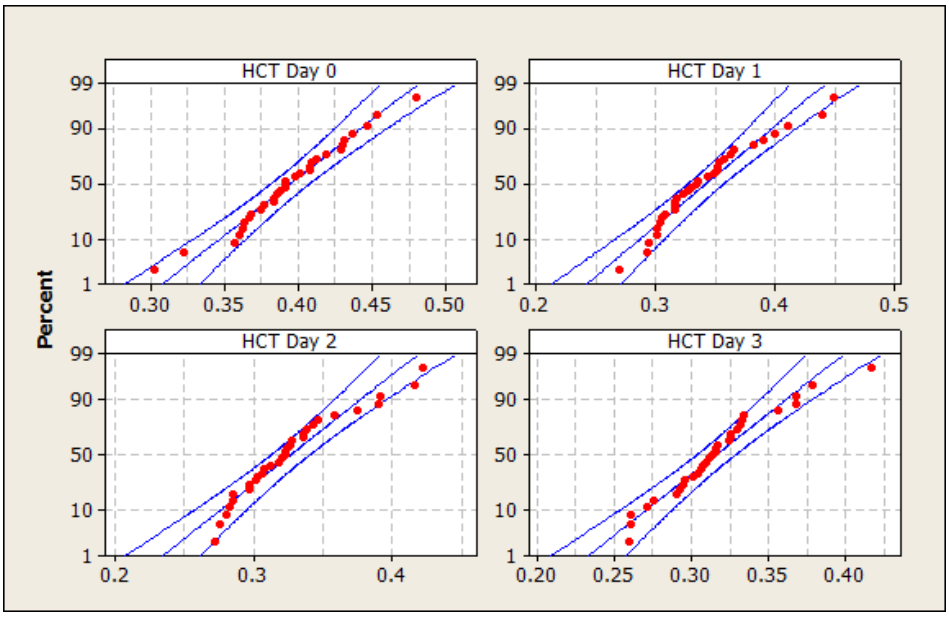



**Fig A4.4a** Probability plots of haematocrit concentration (i/L) on day 0-3

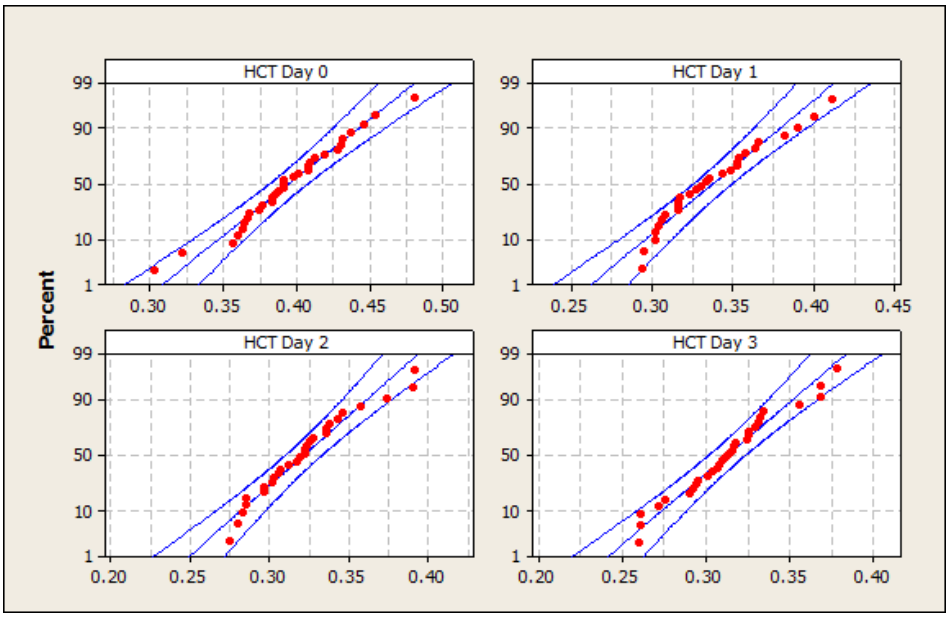



**Fig A4.4b** Probability plots of haematocrit concentration (i/L) on day 0-3 with outlying samples removed

*Copper*

*Liver copper concentration*

The Anderson-Darling test for normality was performed to ensure the normality of the data and exclude any outlying samples. Liver copper concentrations were found to be normally distributed (p=0.100) (fig A4.5).

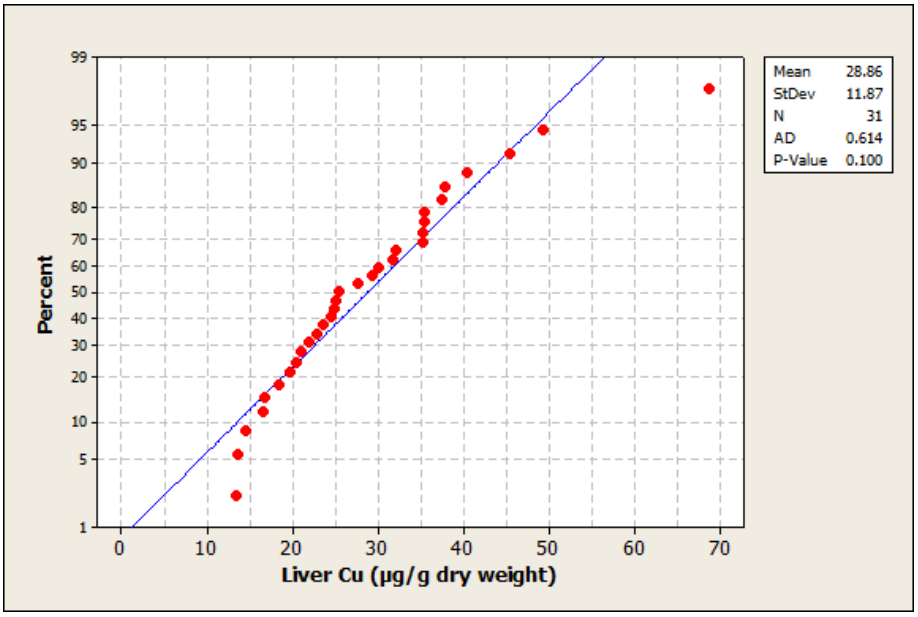



**Fig A4.5** Probability plot of liver copper concentration (µg/g dry weight)

*Plasma copper concentration*

Plasma copper concentrations on days 0, 2 and 3 were normally distributed (p=0.661, 0.132 and 0.782 respectively; fig A4.6a). Plasma copper concentration on day 1 was not normally distributed (p=0.015) and a histogram was draw to examine the pattern of distribution (fig A4.6b). After logarithmic transformation, days 0-3 were all normally distributed (p=0.595, 0.097, 0.390 and 0.576 respectively; fig A4.6c).

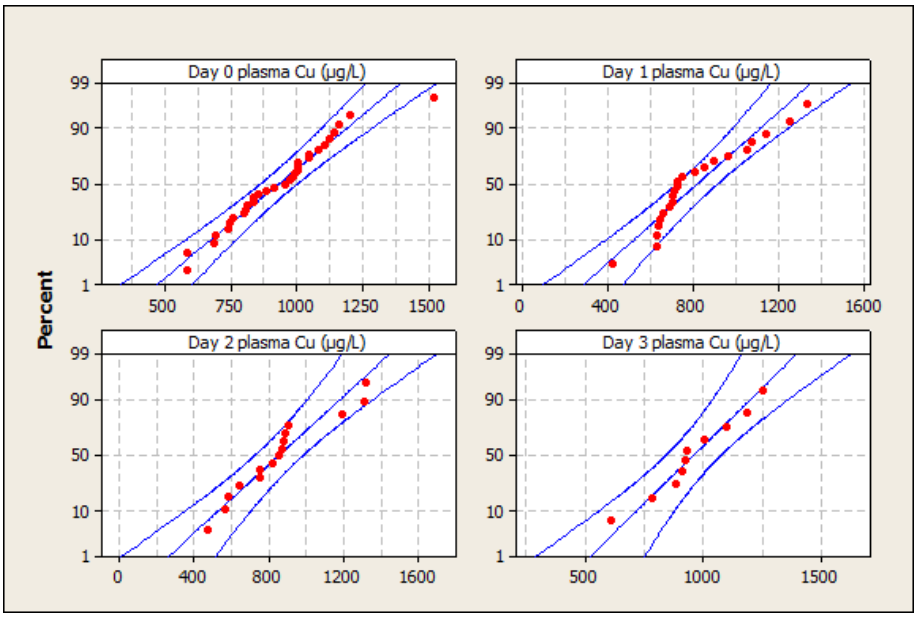



**Fig A4.6a** Probability plot of plasma copper concentration (µg/L) on day 0-3

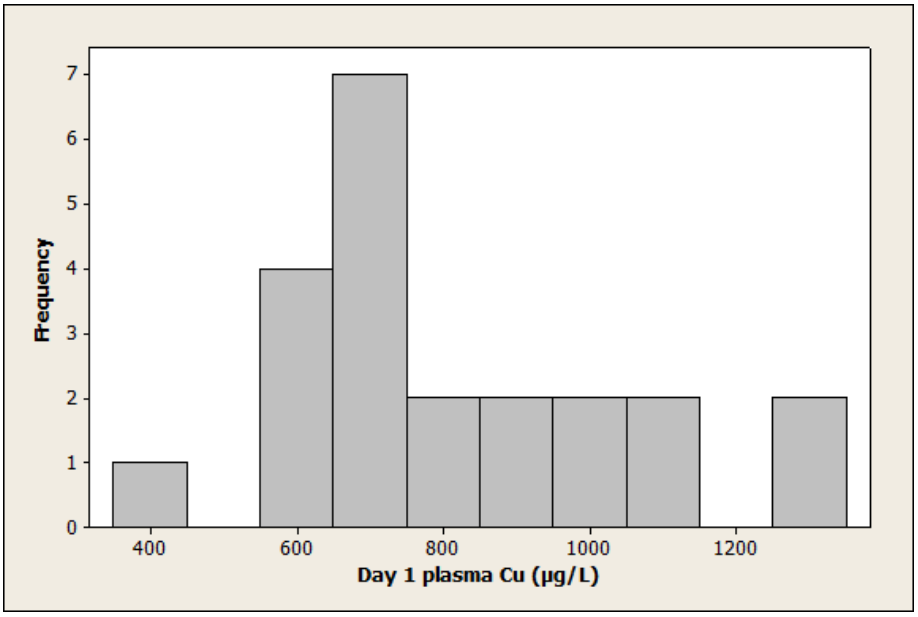



**Fig A4.6b** Histogram of plasma copper concentration (µg/L) on day 1

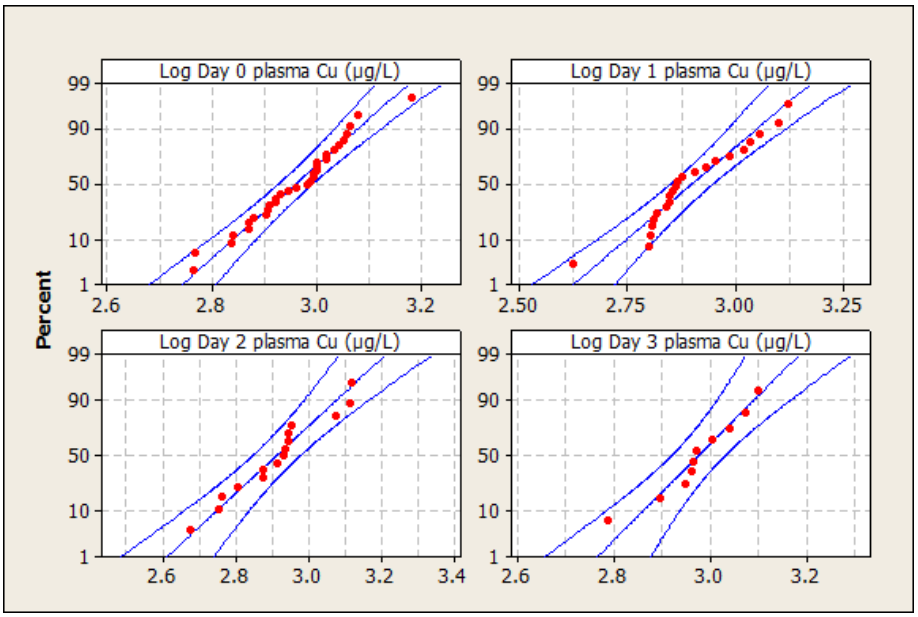



**Fig A4.6c** Probability plot of log plasma copper concentration (µg/L) on day 0-3

*Plasma ferroxidase* activity

Plasma ferroxidase activity was non-normal on day 0 (p=0.018; fig A4.7a). An outlier (study number 18) was identified and removed from future analyses. After this removal, plasma ferroxidase activities on days 0-3 were all normally distributed (p=0.656, 0.411, 0.874 and 0.828 respectively; fig A4.7b).

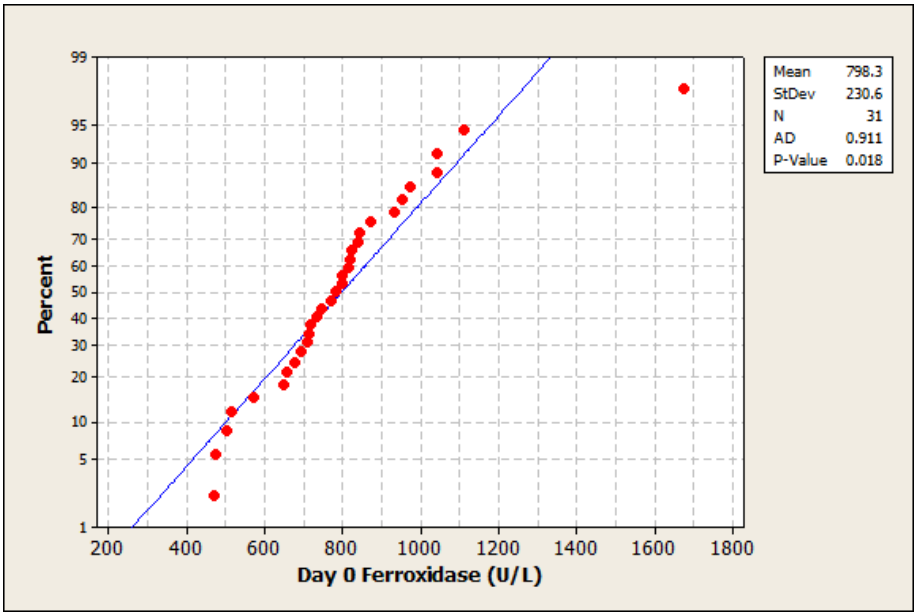



**Fig 4.7a** Probability plot of plasma ferroxidase activity (U/L) on day 0

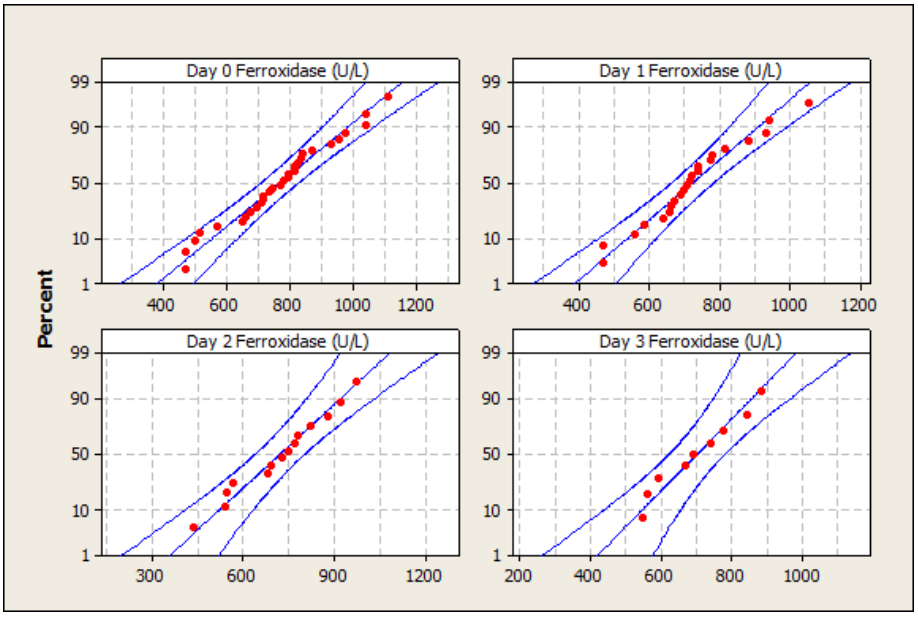



**Fig A4.7b** Probability plot of plasma ferroxidase activity (U/L) on day 0-3 with study number 18 removed from day 0

*Plasma ceruloplasmin concentration*

Initial examination of probability plots for plasma ceruloplasmin concentration showed outlying samples on day 0 (study number 21) and day 1(study number 1) (fig A4.8a); these were removed from further analyses. Day 2 was seen to be non-normally distributed (p=0.045) (fig A4.7b). Logarithmic transformation and removal of outliers resulted in days 0-3 all being normally distributed (p=0.6, 0.599, 0.118 and 0.804 respectively) (fig A4.7c).

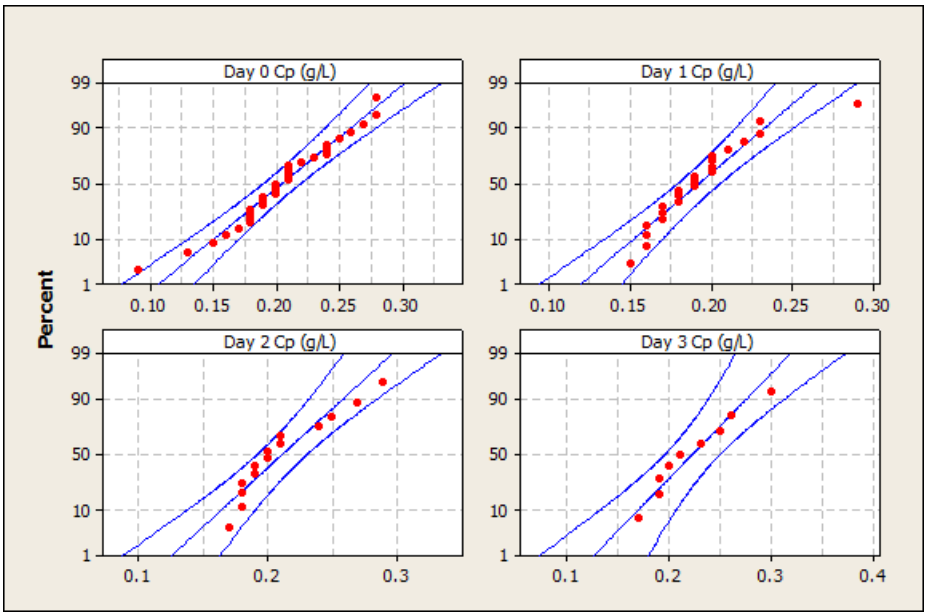



**Fig A4.7a** Probability plots of plasma ceruloplasmin concentration (g/L) on day 0-3

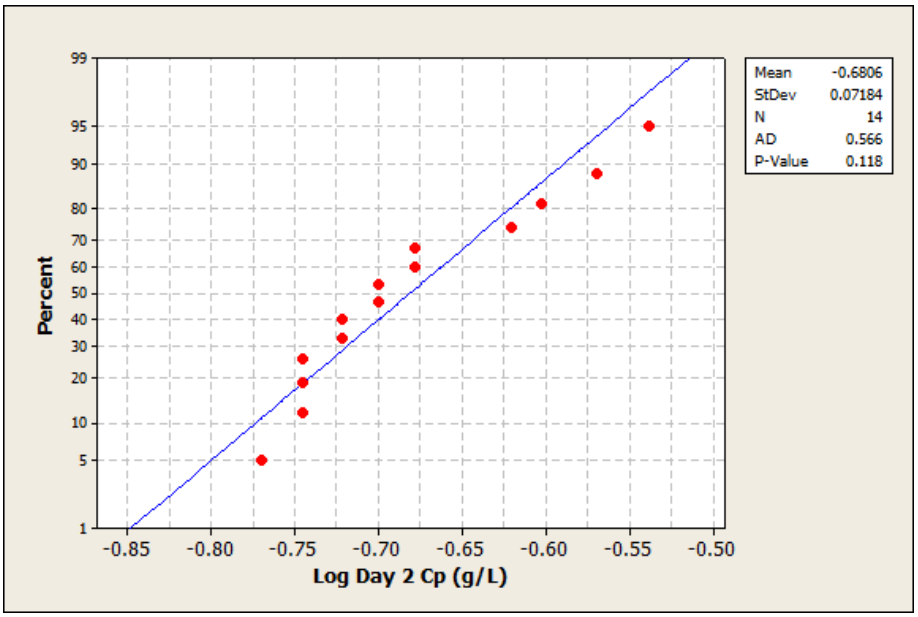



**Fig A4.7b** Probability plot of log plasma ceruloplasmin concentration (g/L) on day 2

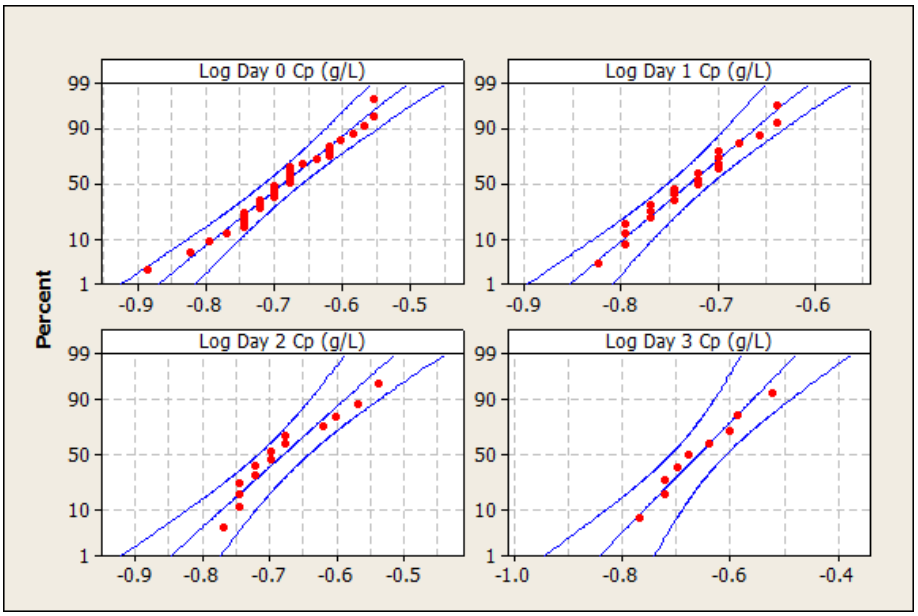



**Fig A4.7c** Probability plots of log plasma ceruloplasmin concentration (g/L) on day 0-3 (study number 21 removed from day 0, study number 1 removed from day 1).

*Red blood cell copper concentration*

Day 0 and day 1 red blood cell copper concentrations were non-normally distributed (both $p<0.005$). Dot plots were generated of red blood cell copper concentrations on days 0-3 (fig A4.8a) and these showed day 0 and day 1 red blood cell copper concentrations to be right-skewed. The data was therefore logarithmically transformed, but day 0 was still non-normal ($p<0.005$, fig A4.8b). Three high concentration outlying samples (study numbers 11, 20, and 26) were therefore removed from further analyses and the distribution was normalised ($p=0.280$; fig A4.8c).

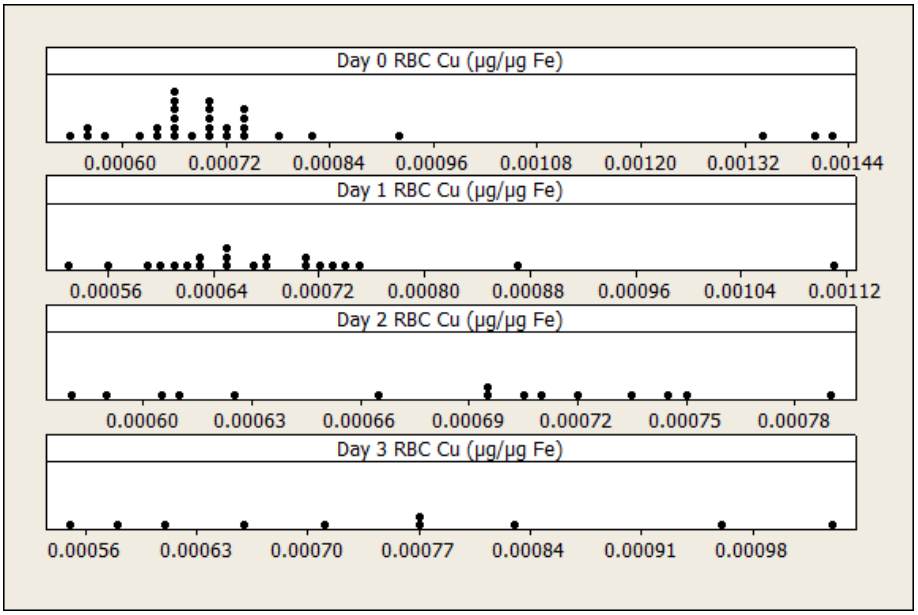



**Fig A4.8a** Dot plots of red blood cell copper concentration (µg/µg Fe) on day 0-3

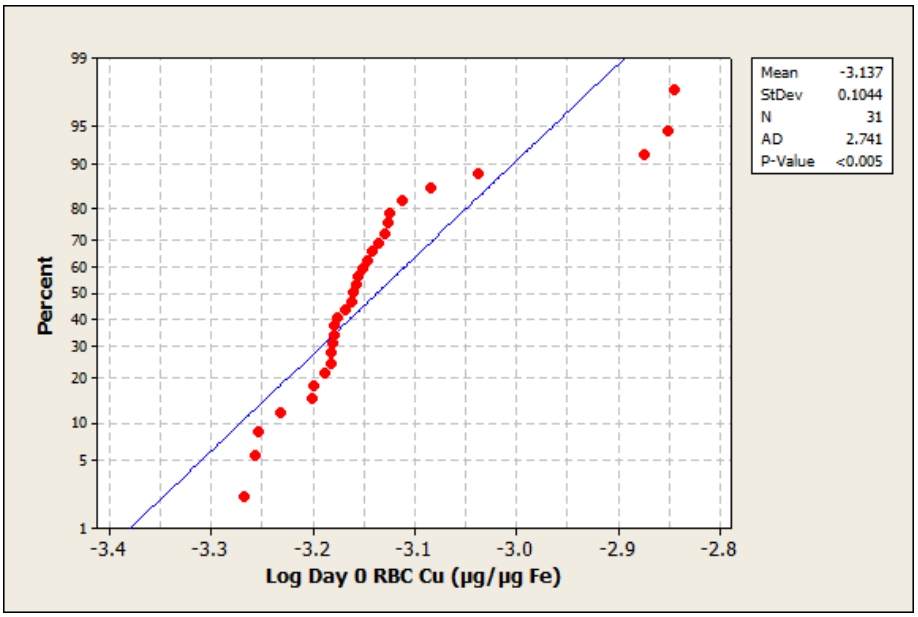



**Fig A4.8b** Probability plot of log red blood cell copper concentration (µg/µg Fe) on day 0

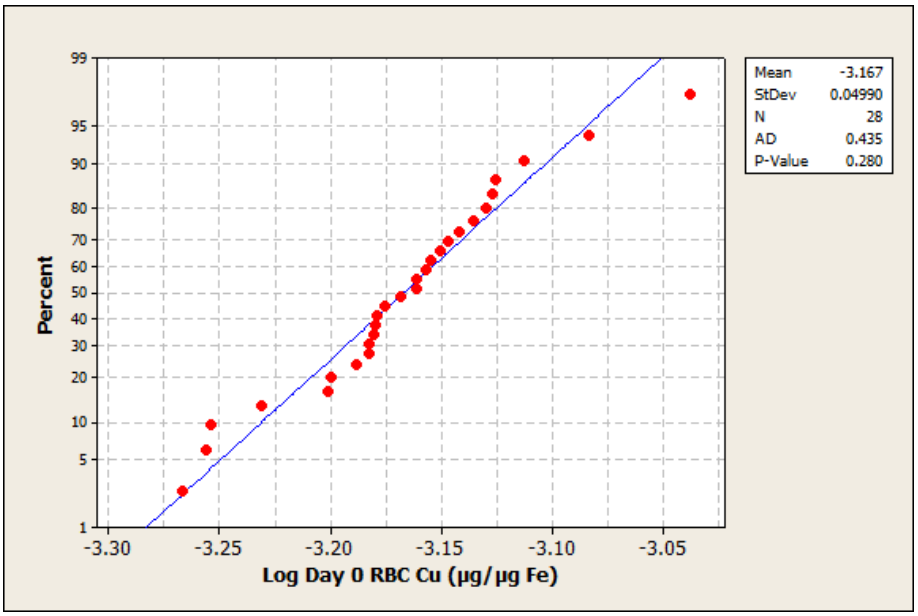



**Fig A4.8c** Probability plot of log red blood cell copper concentration (µg/µg Fe) on day 0 (study number 11, 20 and 26 removed)

The concentrations of red blood cell copper day 1 were also still non-normal ($p<0.053$, fig A4.8d) after logarithmic transformation. One high concentration outlying sample (study number 26) was therefore removed from further analyses and the distribution was normalised ($p=0.933$, fig A4.8e).

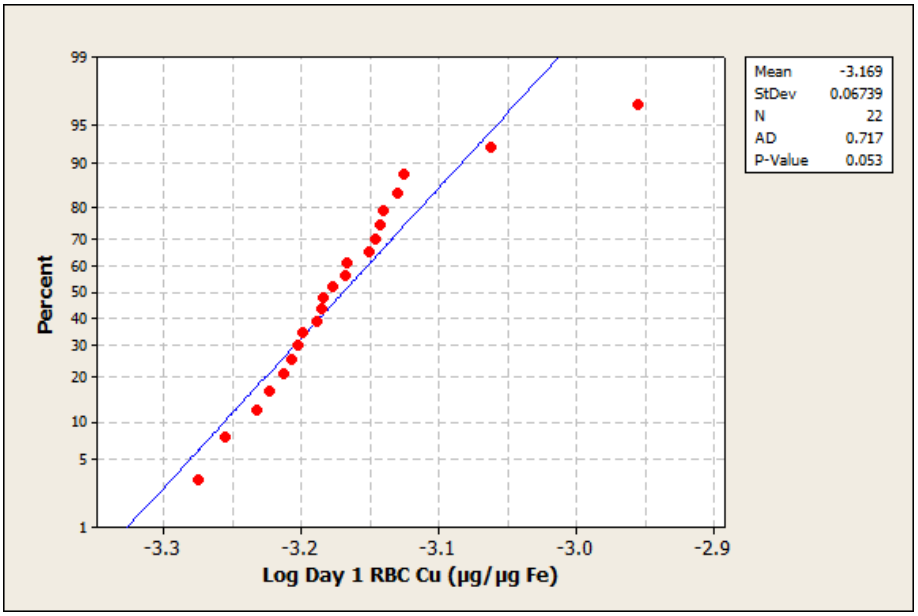



**Fig A4.8d** Probability plot of log red blood cell copper concentration (µg/µg Fe) on day 1

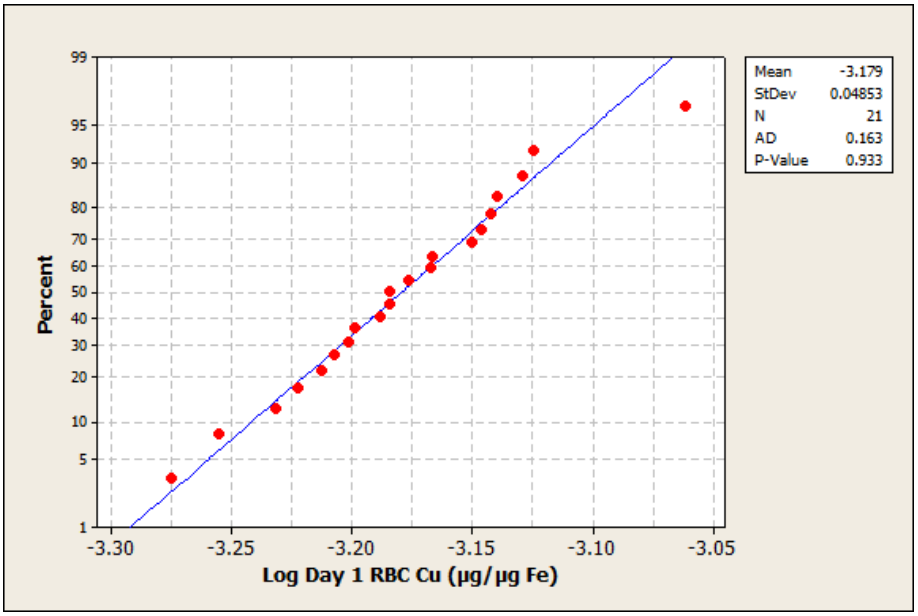



**Fig A4.8e** Probability plot of log red blood cell copper concentration (µg/µg Fe) on day (study number 26 removed)

After logarithmic transformation and the removal of outlying samples the red blood cell copper concentration on day 0-3 were all normally distributed (p=0.28, 0.933, 0.173 and 0.889 respectively; fig A4.8f).

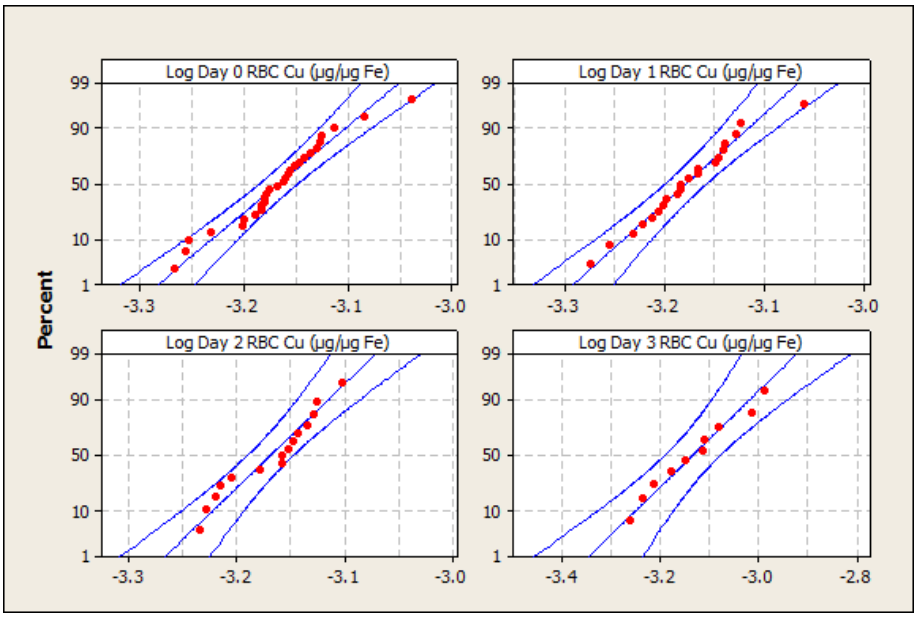



**Fig A4.8f** Probability plots of log red blood cell copper concentration (µg/µg Fe) on day 0-3 (study numbers 11, 20 and 26 removed from day 0, study number 26 removed from day 1).

*Zinc*

To ensure the data followed a normal distribution to allow statistical analyses to take place, the Anderson-Darling normality test was performed.

Muscle zinc concentrations (µg/g dry weight) were found to be non-normally distributed ($p<0.005$, fig A4.9a). Logarithmic transformation of the data did not normalise it therefore the two outlying data points (samples 13 and 29) were removed for the zinc statistical analyses. With outliers removed the muscle zinc was normally distributed ($p=0.14$, fig A4.9b).

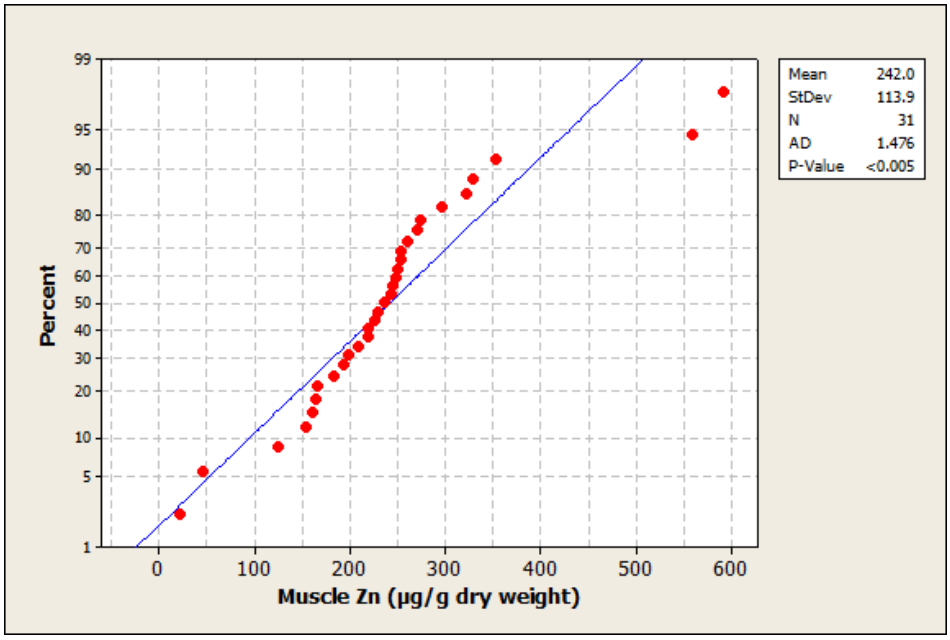



**Fig A4.9a** Probability plot of muscle zinc concentration (µg/g dry weight) with all 31 samples

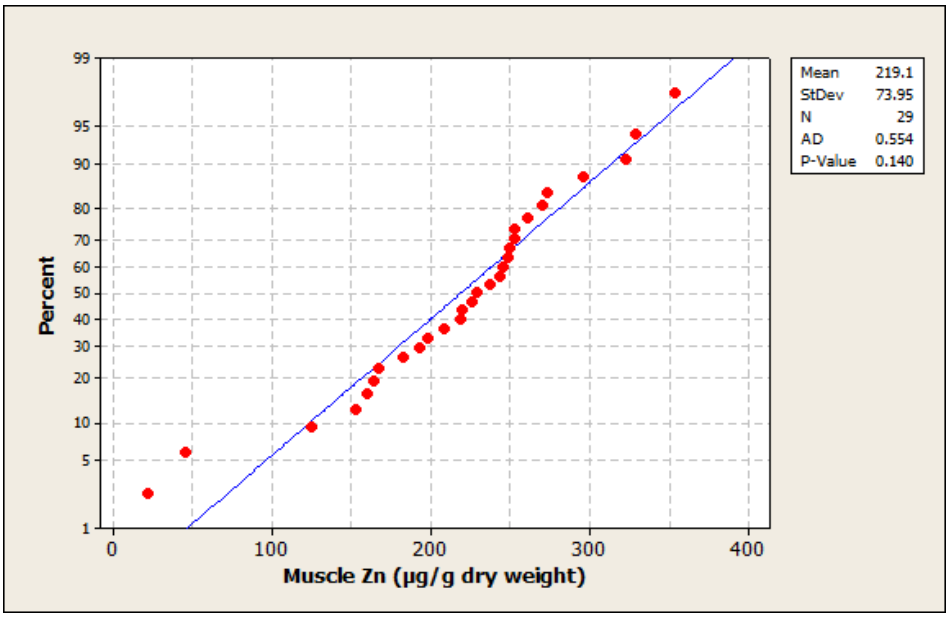



**Fig A4.9b** Probability plot of muscle zinc concentration (µg/g dry weight) excluding samples 13 and 29

Analysis of a probability plot of plasma zinc concentration (µg/L) on Day 0 (fig A4.10) revealed an outlying sample (sample 10) and this was also removed from further statistical analysis of zinc results. After this removal the plasma zinc concentration on Day 0 was normally distributed (p=0.727).

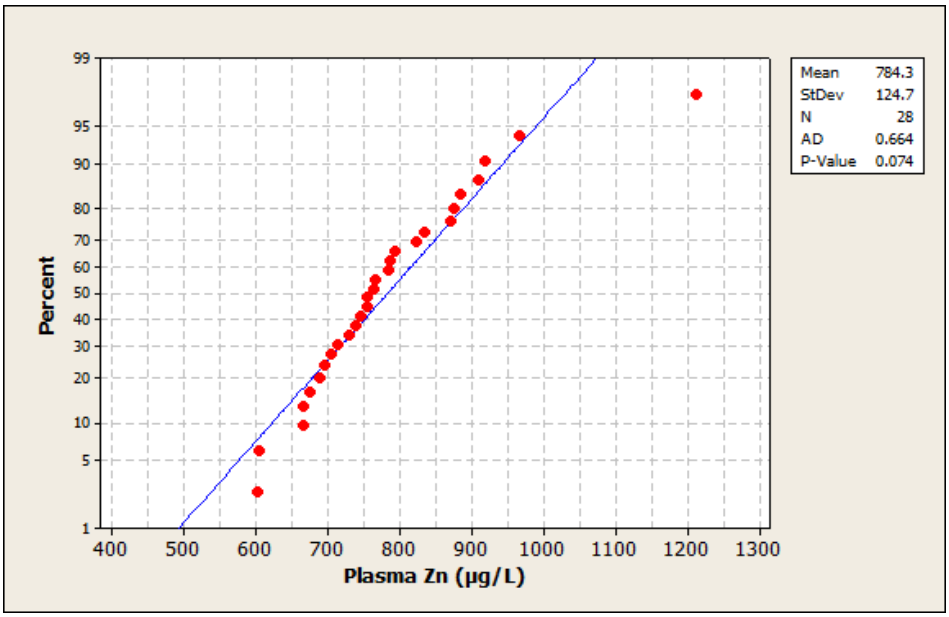



**Fig A4.10** Probability plot of plasma zinc concentration (µg/L) on day 0, excluding samples 13 and 29

Plasma zinc concentration (µg/L) on day 1, 2 and 3 all had a normal distribution (p=0.642, 0.778, 0.965 respectively).

Liver zinc concentrations (µg/g dry weight) were normally distributed (p=0.915).

RBC zinc concentrations (µg/ µg Fe) on day 0-3 were all normally distributed (p=0.715, 0.532, 0.123, 0.433 respectively).

The Anderson-Darling test for normality was performed to ensure the normality of the data and exclude any outlying samples.

Liver selenium concentrations were found to be normally distributed (p=0.232) (fig A4.11).

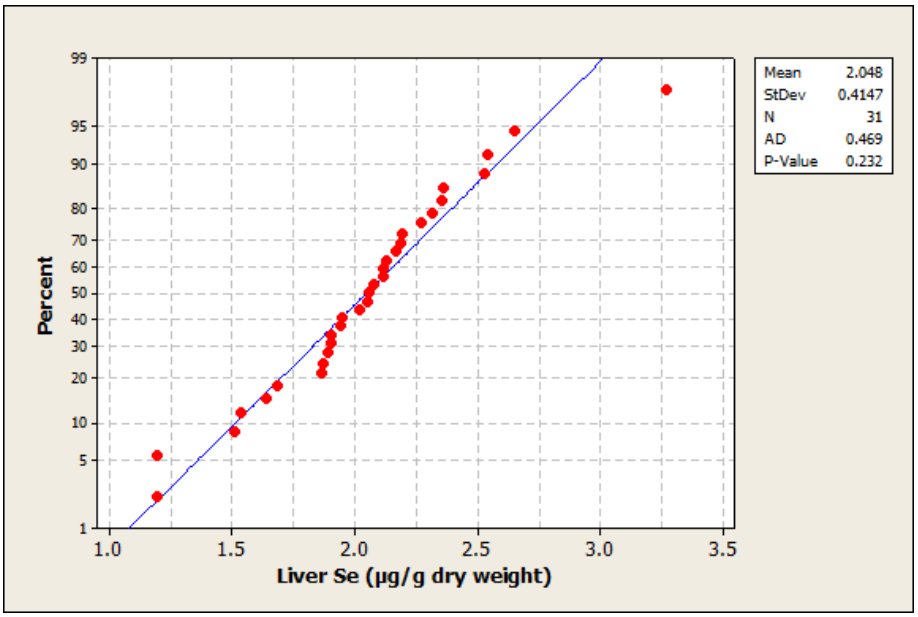



**Fig A4.11** Probability plot of liver selenium concentration (µg/g dry weight)

The Anderson-Darling normality test was performed on plasma selenium concentrations on day 0-3 and outlying samples were identified; day 0 p=0.284, day 1 p=0.035, day 2 p=0.891, day 3 p=0.559. Probability plots of plasma selenium concentrations on day 0-3 can be seen in fig A4.12a. Outlying samples (day 0 sample 10, day 1 samples 20 and 29) were removed and all plasma selenium concentrations on day 0-3 were normally distributed; day 0 p=0.726, day 1 p=0.522, day 2 p=0.891, day 3 p=0.559. Probability plots of plasma selenium concentrations on day 0-3 with outlying samples removed can be seen in fig A4.12b.

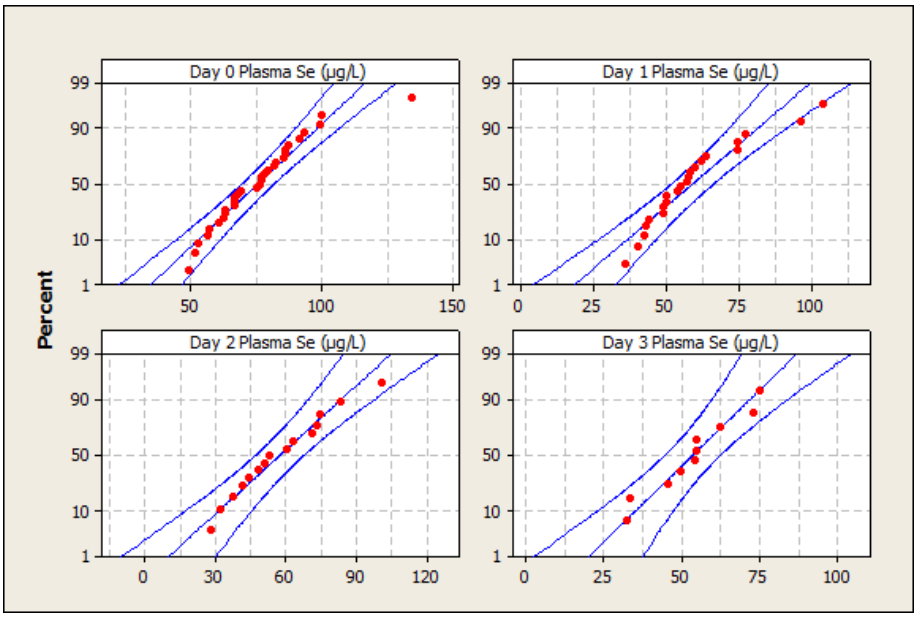



**Fig A4.12a** Probability plot of plasma selenium concentration (µg/L)

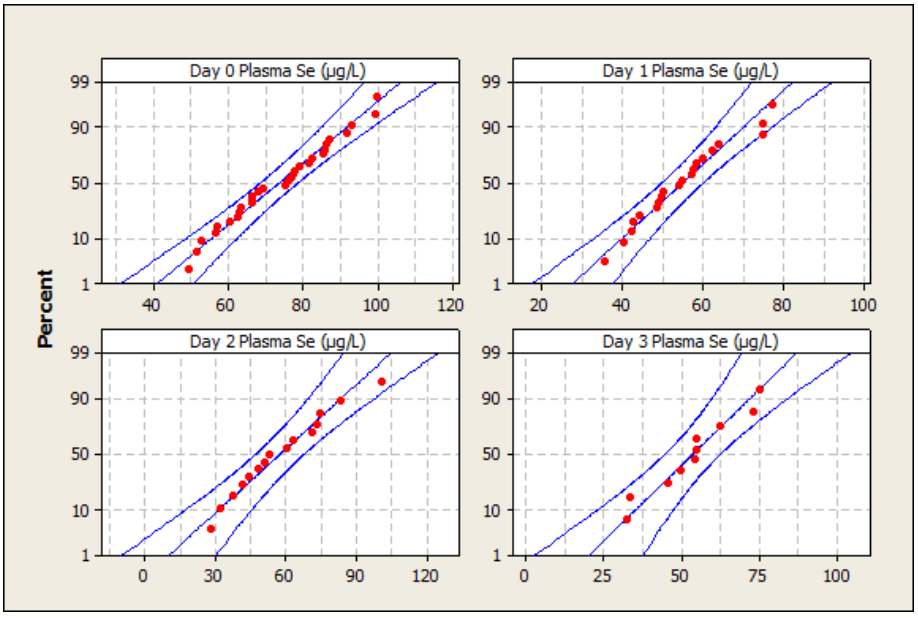



**Fig A4.12b** Probability plot of plasma selenium concentration (µg/L) day 0 sample 10 and day 1 samples 20 and 29 removed

Examination of probability plots of red blood cell selenium concentration on days 0-3 revealed outlying samples (fig A4.13a); day 0 p=0.026, day 1 p=0.372, day 2 p=0.226, day 3 p=0.257. Sample 20 on day 0, 1 and 2 was removed from analysis and red blood cell selenium concentration was then normally distributed (fig A4.13b); day 0 p=0.479, day 1 p=0.625, day 2 p=0.233, day 3 p=0.257.

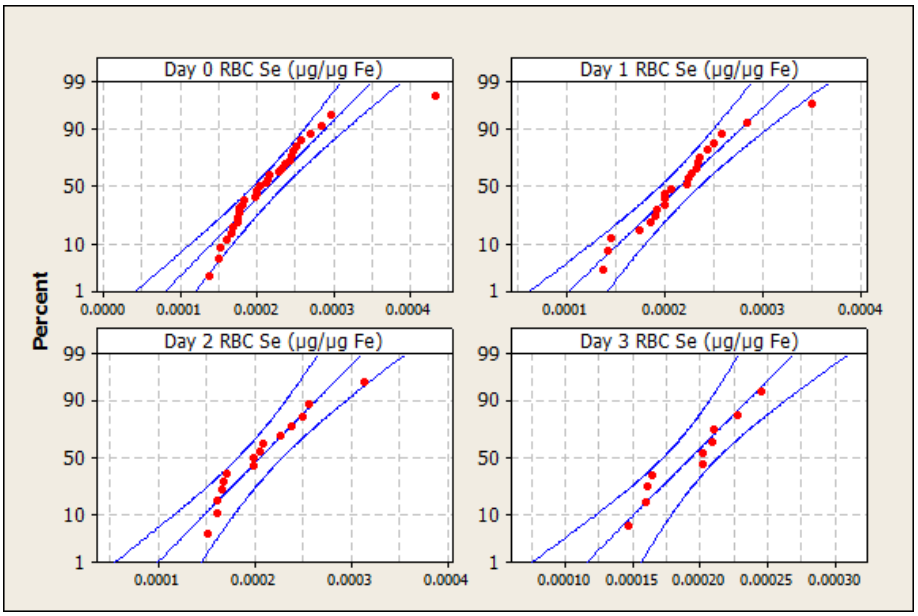



**Fig A4.13a** Probability plots of RBC selenium concentration on day 0-3 (µg/µg Fe)

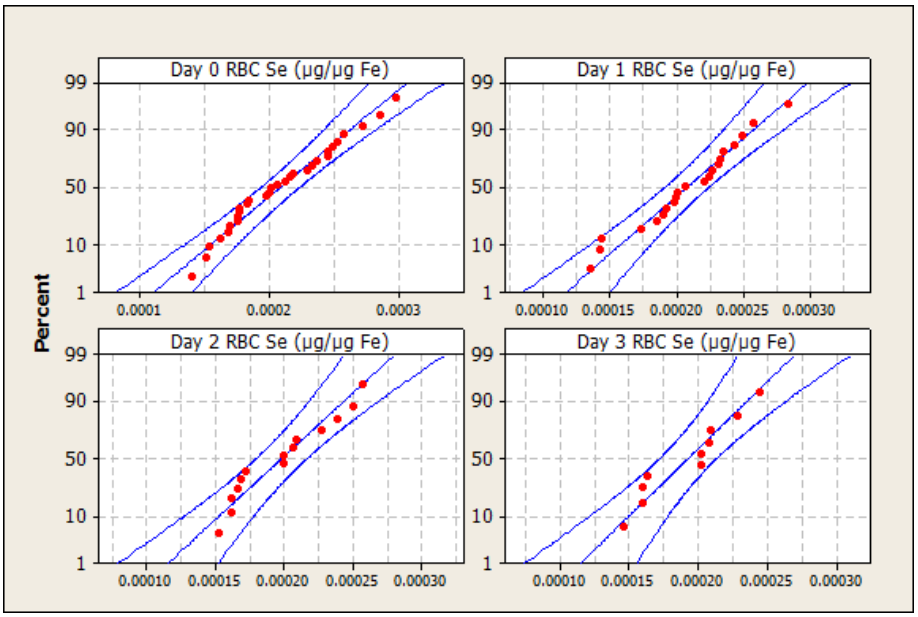



**Fig A4.13b** Probability plots of RBC selenium concentration on day 0-3 (µg/µg Fe) with sample 20 removed on days 0-2

Red blood cell glutathione peroxidase was normally distributed on day 0-3; day 0 p=0.353, day 1 p=0.702, day 2 p=0.748, day 3 p=0.391 (fig x).

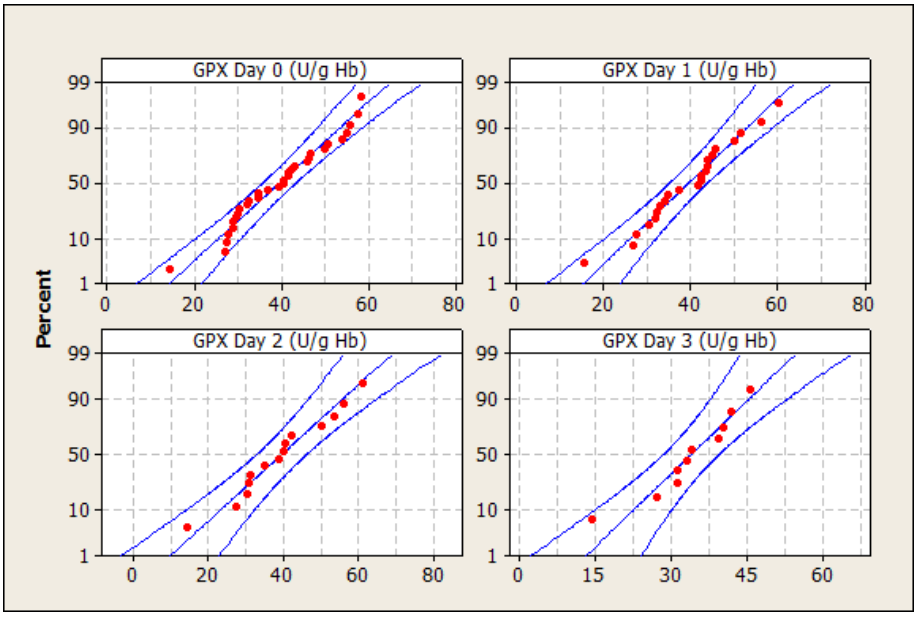



**Fig** A4.14 Probability plots of RBC glutathione peroxidase activity on day 0-3 (U/g Hb)

*Magnesium*

To ensure the normality of the data and exclude any outlying samples, the Anderson-Darling test for normality was performed.

Liver magnesium concentrations were found to be normally distributed (p=0.423).

An outlying sample (sample 13) was seen when examining a probability plot of muscle magnesium (fig A4.15) and this sample was excluded from all further magnesium analysis. With this sample removed muscle magnesium was normally distributed (p=0.2).

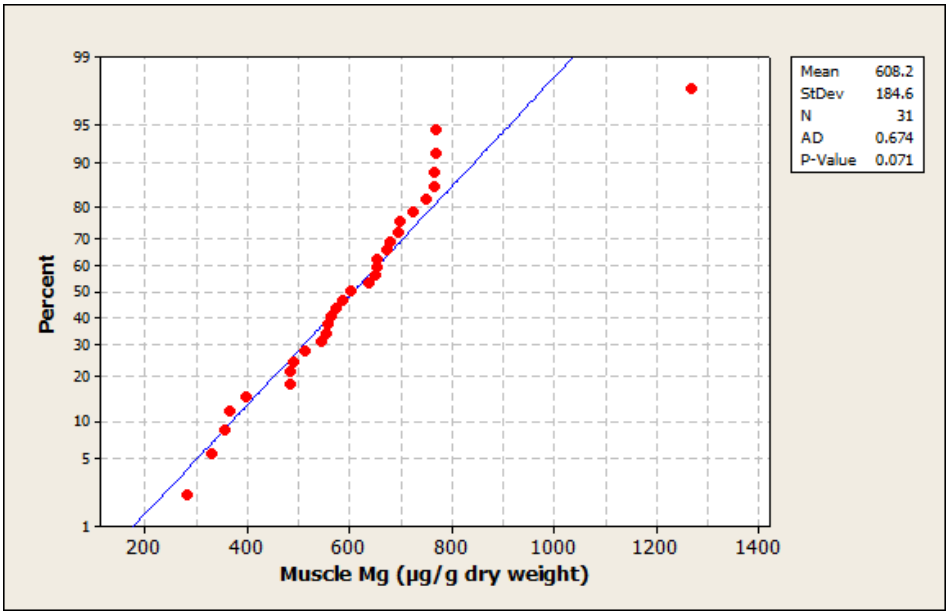



**Fig 4.14** Probability plot of muscle magnesium concentration (µg/g dry weight)

Red blood cell magnesium levels on day 0 and day 1 were not normally distributed; p=0.048 and p=0.030 respectively. Examination of a probability plot

(fig A4.15a) and histogram (fig A4.15b) of day 0 RBC magnesium showed outlying samples (Samples 14, 20 and 26) and these was removed from further analyses. Samples 20 and 31 were outliers on day 1 and were also removed (fig A4.15c). After these were removed days 0-3 were normally distributed and probability plots can be seen in fig A4.15d; day 0 p=0.119, day 1 p=0.434, day 2 p=0.836, day 3 p=0.518.

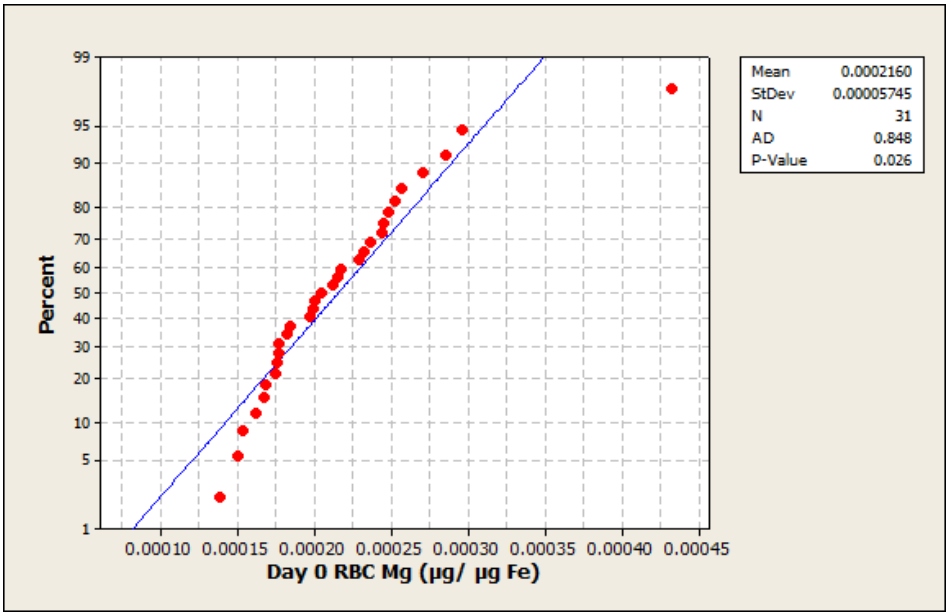



**Fig A4.15a** Probability plot of day 0 RBC magnesium concentration (µg/µg Fe)

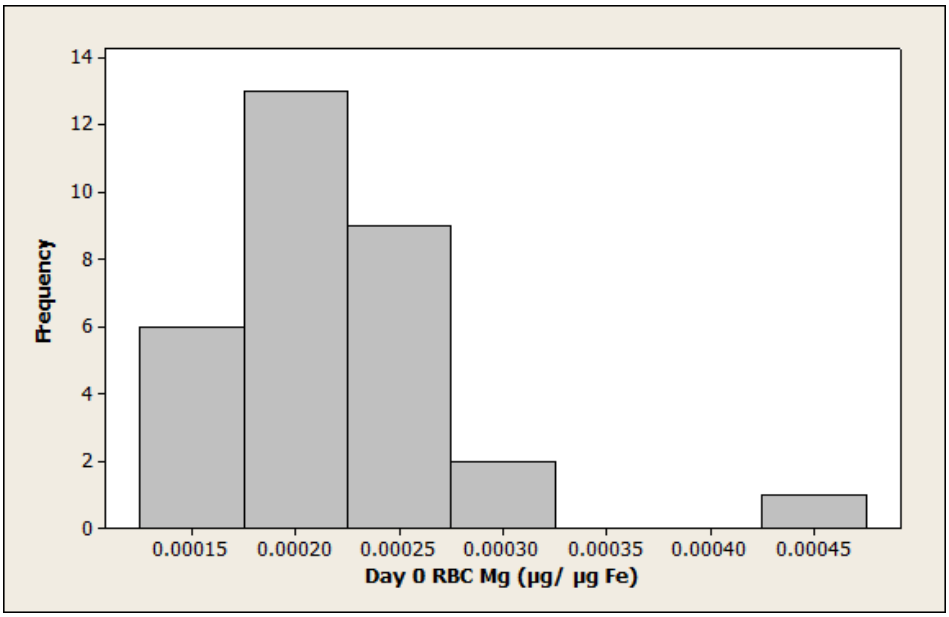



**Fig A4.15b** Histogram of day 0 RBC magnesium concentration (µg/µg Fe)

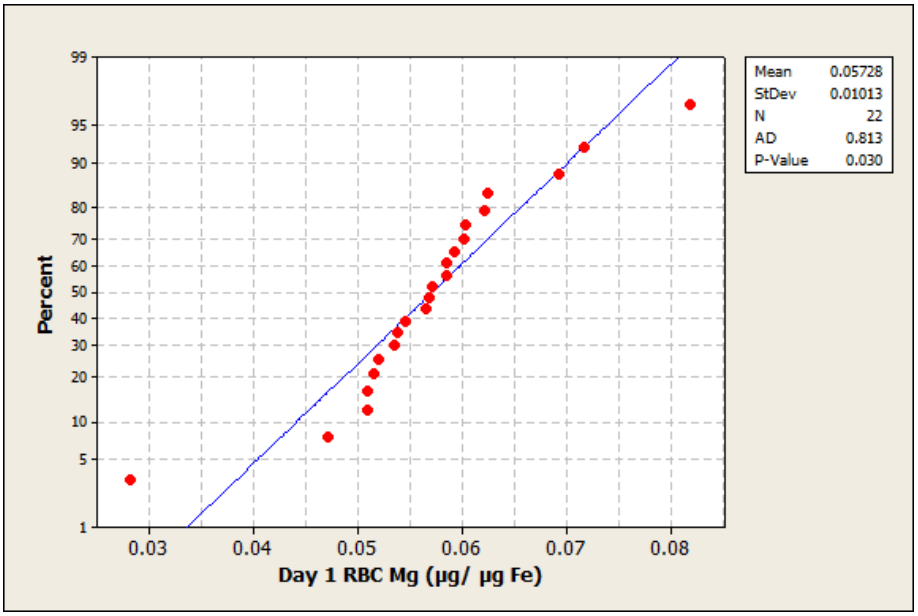



**Fig A4.15c** Probability plot of day 1 RBC magnesium concentration (µg/µg Fe)

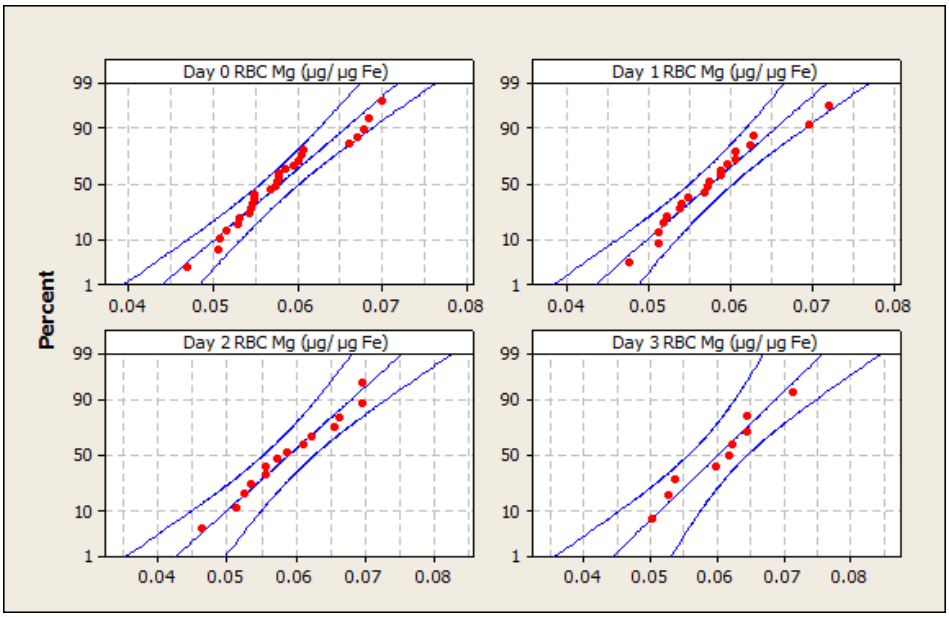



**Fig A4.15d** Probability plots of RBC magnesium concentration on day 0-3 (µg/µg Fe)

The Anderson-Darling normality test was performed on plasma magnesium concentrations on day 0-3 and all were normally distributed; day 0 p=0.528, day 1 p=0.734, day 2 p=0.992, day 3 p=0.507. Probability plots of plasma magnesium concentrations on day 0-3 can be seen in fig A4.16.

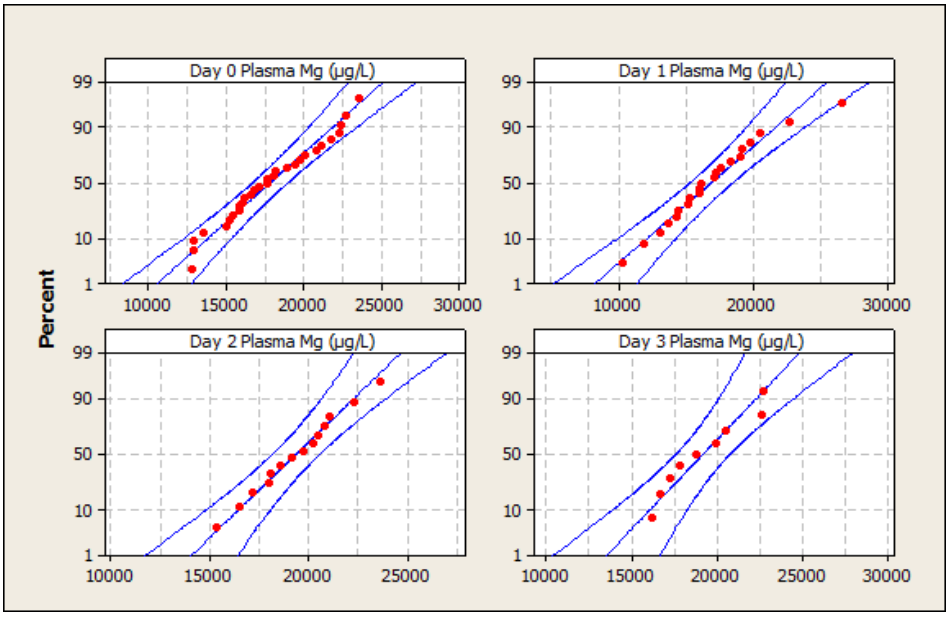



**Fig 4.16**. Probability plots of Plasma magnesium concentration on day 0-3 (µg/L)

(1) Galloway P, McMillan DC, Sattar N. Effect of the inflammatory response on trace element and vitamin status. *Ann Clin Biochem* 2000; 37 ( Pt 3):289-297.

(2) Oakes EJ, Lyon TD, Duncan A, Gray A, Talwar D, O'Reilly DS. Acute inflammatory response does not affect erythrocyte concentrations of copper, zinc and selenium. *Clin Nutr* 2008; 27(1):115-120.

(3) Tietz Textbook of Clinical Chemistry and Molecular Diagnostics. 4th ed. Elsevier; 2006.

(4) Tietz Fundamentals of Clinical Chemistry. Saunders; 2001.

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