AN INVESTIGATION OF THE DETERMINANTS OF PLASMA GLUCOSE AND MICRONUTRIENT CONCENTRATIONS IN PATIENTS WITH CRITICAL ILLNESS

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Abstract

This thesis describes a series of observational studies that examine the relationship between the systemic inflammatory response, glucose, micronutrients concentrations and outcomes in patients with critical illness with reference to a large nutrition screen cohort. Data and stored samples were available from cohorts which had been defined previously. The critical illness cohort comprised of medical or surgical patients ≥ 18 years admitted to the Intensive Care Unit (ICU) of the Glasgow Royal Infirmary (GRI) between September 2006 and December 2008. The nutrition screen cohort comprised samples and associated information which had been processed by the Pathological Biochemistry Department of the GRI. The samples had been received for vitamin and / or trace element assessment of patients from both GRI and other Scottish hospitals between January 2006 and March 2013. Samples had been referred to the lab for analysis of a number of variables related to nutrition, including glucose, albumin, C-reactive protein (CRP), lutein, lycopene, vitamins A, B1, B2, B6, C, D and E, and zinc, selenium, copper, and manganese. Data was not available for all variables for all samples, hence studies varied in the number of observations. plasma glucose was measured in sodium floride blood sample whole-blood samples underwent for routine analysis of concentrations of lutein, lycopene, α -carotene and β -carotene, 25-hydroxyvitamin D (25 (OH) D), ascorbic acid (vitamin C), α-tocopherol (vitamin E) in plasma, zinc, selenium, copper, B1, B2, B6 in plasma and red cells.

In Chapter 2 the relationship between glucose and markers of the systemic inflammatory response was examined in detail in patients from the critical illness cohort (n=100). The results of this study showed that plasma glucose concentration - even within the context of tight insulin protocol - was influenced by many factors. Surgical and medical patients differed in their requirements for insulin; medical patients have higher plasma glucose and insulin

administration accordingly compared with surgical patients. However, catecholamine and steroid administration were also associated with higher insulin requirements.

In Chapter 3 the relationship between plasma asymmetric dimethylarginine (ADMA) and related arginine metabolism (homoarginine, arginine, symmetric dimethylarginine (SDMA)) and outcome was examined in patients from critical illness cohort (n=104). Patients with critical illness experience metabolic disorders including catabolism and hyperglycaemia and these were associated with poor outcome. Plasma ADMA and SDMA concentrations were higher in patients with critical illness and were also associated with disease severity and mortality. In contrast, plasma homoarginine concentrations were lower in patients with critical illness and were also associated with disease severity and mortality. These results suggest that ADMA metabolism is perturbed with likely knock on effects on nitric oxide synthase (NOS) and endothelial function. There is a need for further work on in vivo dimethylaminohydrolase (DDAH) activity in critical illness and the effect of critical illness on the cationic amino acid transporters (CAT)-mediated exchange of ADMA between intra and extra-cellular compartments. It was proposed that ADMA and SDMA may not only be indicators of the severity of illness and may even contribute to adverse events in patients with critical illness.

In Chapter 4 the relationship between markers of the systemic inflammatory response, as evidenced by CRP and albumin, and mortality, in patients from critical illness cohort (n=261) was examined. In this cohort, the combination of CRP and albumin predicted ICU and hospital mortality as effectively as APACHE II.

In Chapter 5 the relationship between markers of the systemic inflammatory response and plasma glucose was examined in a nutrition screen cohort (n=5248). The results of this study

showed that plasma concentrations of glucose were independently positive and negative associated with both CRP and albumin respectively, and were consistent with the systemic inflammatory response as having an impact on glucose concentrations. However, such relationships were not apparent in patients with critical illness (n=116). It was concluded that plasma concentrations of glucose were independently associated with both CRP and albumin, and were consistent with the systemic inflammatory response as a determinant of its concentrations.

In Chapter 6 the relationship between markers of the systemic inflammatory response and plasma concentrations of carotenoids was examined in a nutrition screen cohort (n=1074). The results of this study showed that the clinical interpretation of plasma carotenoids requires knowledge of the magnitude of the systemic inflammatory response, even after adjustment for cholesterol. It was concluded that a reliable clinical interpretation can be made only for plasma lutein, lycopene and β-carotene if the CRP is less than 20 mg/L.

In Chapter 7 the relationship between markers of the systemic inflammatory response and plasma 25-hydroxyvitamin D (25(OH) D) was examined in a nutrition screen cohort (n=5327, and in patients from critical illness cohort n=117). The results of this study showed that plasma concentrations of 25(OH) D were independently associated with both CRP and albumin. It was concluded that the systemic inflammatory response was a major confounding factor in determining vitamin D status.

In Chapter 8 the relationship between markers of the systemic inflammatory response and plasma vitamin E (α-tocopherol) and vitamin C (ascorbic acid) was examined in a nutrition screen cohort (n=359, n=494 respectively and in patients from critical illness cohort n= 82). The results of this study showed that α-tocopherol/ cholesterol ratio and ascorbic acid were

independently associated with both CRP and albumin. It was concluded that the systemic inflammatory response was a major confounding factor in determining vitamin E and C status.

In Chapter 9 the relationship between markers of the systemic inflammatory response and plasma zinc, selenium was examined in a nutrition screen cohort (n=743, n=833, respectively and in patients from the critical illness cohort n= 114). The results of this study showed that plasma concentrations of zinc were associated with both CRP and albumin. However, the impact of the systemic inflammatory response (as evidenced by elevation of CRP concentrations) on plasma zinc concentrations could be largely adjusted by albumin concentrations. Plasma concentrations of selenium were associated with both CRP and albumin. However, the impact of the systemic inflammatory response on plasma selenium concentrations could not be reasonably adjusted by albumin concentrations. It was concluded that plasma zinc and selenium concentrations were independently associations with CRP and albumin as markers of systemic inflammatory response.

In Chapter 10 the relationship between markers of the systemic inflammatory response and red cell zinc, selenium and copper was examined in patients from critical illness cohort (n= 125). The results of this study showed that altered red cell concentrations of zinc, selenium and copper were likely to be more reliable measures of status in the presence of a systemic inflammatory response.

In Chapter 11 the relationship between markers of the systemic inflammatory response and red cell vitamins B1, B2 and B6 was examined in nutrition screen cohort (n= 553, n=251, n= 313, respectively and in patients from critical illness cohort n=94). The results of this study showed that in contrast to plasma concentrations of B1, B2 and B6, red cell concentrations do not fall as a part of the systemic inflammatory response. It was concluded that red cell

concentrations of B1, B2 and B6 were likely to be more reliable measures of status in the presence of a systemic inflammatory response.

In summary, studies in the present thesis showed that, during the systemic inflammatory response, plasma concentration of glucose had multiple determinants other than insulin. Furthermore, in the presence of systemic inflammatory response, plasma lutein, lycopene, α-carotene and β-carotene, 25 (OH) D, C, E, zinc, selenium, and copper were unreliable, and that intracellular micronutrients concentrations such as red cell zinc, selenium, copper, B1, B2 and B6, were more reliable as indicators of vitamin and trace element status in patients subject to nutrition screen and in patients with critical illness. These results have implications for the assessment of glucose and micronutrient status in the general population and for treatment in patients with critical illness.

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Author's declaration

The work presented in this thesis was performed entirely by myself except as indicated below:

1. I was assisted in the study design by Prof John Kinsella, Donald C. McMillan and Dr. Dinesh Talwar.
2. I was assisted in the collection of clinical data by Prof John Kinsella for critical illness cohort.
3. I was assisted in creating databases and performing statistical analysis by Prof. Donald C. McMillan.
4. Blood sampling was performed by the medical and nursing staff of the Intensive Care Unit, Glasgow Royal Infirmary. Routine laboratory analysis of plasma glucose and vitamins 25 (OH) D, B1, B2, B6, C, α-tocopherol, zinc, selenium, copper, lutein, lycopene, α- and β-carotene were performed by the laboratory staff of the Scottish Trace Element and Micronutrient Reference Laboratory, Glasgow Royal Infirmary.

This thesis has not been previously submitted for a degree or diploma at this or any other institution.

Rawia Ghashut

Publications

The work presented in this thesis has resulted in the following publications;

- Ghashut RA, McMillan DC, Kinsela J, Duncan A, Talwar D (2013). 'Quantitative data on the magnitude of the systemic inflammatory response and its effect on carotenoids status based on plasma measurements'. Clinical Nutrition ESPEN. 8 (5): e193-e199.
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Dedication

Dedicated to my daughter Arwa Naili, my father Abdosalam Ghashut and my mother Saida Shwekha for their love and support

Abbreviations

APACHE II	Acute physiology and Chronic Health Evaluation II score.
ADMA	Asymmetrical Dimethylarginine.
CAT	cationic amino acid transporters.
CRP	C-reactive protein.
DDAH	dimethylarginine dimethylaminohydrolase.
HPLC	High Performance Liquid Chromatography.
ICU	Intensive Care Unit
Lab-Glucose	Laboratory Glucose.
NOS	nitric oxide synthase.
SDMA	Symmetrical Dimethylarginine.
SOFA	Sequential Organ Failure Assessment.

1. Introduction

Hyperglycaemia, glucose intolerance and insulin resistance have significant effects on outcomes in patients with critical illness. A reduction of body antioxidant concentrations has been reported to have a similar effect. There is evidence that these metabolic derangements are associated with the systemic inflammatory response as evidenced by systemic inflammatory response syndrome (SIRS) criteria, the elevation of CRP and hypoalbuminemia. Hyperglycaemia and micronutrient deficiencies are also been associated with increased morbidity and mortality in this group. Researchers have therefore focused on the treatment of hyperglycaemia and on the provision of optimal nutrition and micronutrient supplements, in order to reduce organ dysfunction and mortality. The assessment of micronutrient status has been monitored either by measuring micronutrient concentrations in plasma or by using functional tests (enzymatic activity). However, since most micronutrients behave as acute phase reactants, the value of such plasma measurements has been questioned (Galloway et al. 2000). Recent advances in methods of micronutrient assessment allow the direct measurement of their concentrations within plasma and red cells (Talwar et al. 2003a). For example, based on these direct methods it has been shown that redistribution of B-vitamins between plasma and blood cells takes place during elective surgery (Gray et al. 2004) and critical illness (Talwar et al. 2003b; Quasim et al. 2005). This questions the resultant frequency and severity of micronutrient deficiency in these patients.

1.1. Defining critical illness

An intensive care unit describes the specialised area in a hospital for patients with potential or established organ failure, which offer comprehensive care and support in relation to the diagnosis, treatment and prevention of multi-organ failure (Department of health 1999;

Capuzzo et al. 2010). Critically ill patients have been defined as those "*with potential recoverable diseases who can benefit from more detailed observation and treatment than is generally available in the standard ward and departments*" (Department of health 1996).

1.2. Epidemiology.

In 2008 Wunsch and colleagues demonstrated that there was international variation in critical care services. They presented data from eight countries across North America and Western Europe (Table 1-1). The data shows that Germany had the highest number of acute care hospital beds, while the US had the fewest. Germany and Belgium had the highest number of ICU beds per 100,000 of population, with seven times as many beds compared with the UK, which had the lowest proportion (Wunsch et al. 2008)**.**

There was a wide variation in the ICU admission rates, e.g. Germany had ten times as many admissions to ICU compared with the UK, and six times as many compared with Canada (Wunsch et al. 2008). Although the US had the largest number of acute care hospitals and beds, it has the smallest portion of acute care hospital beds per 100,000 of population.

According to the Scottish Intensive Care Audit Group (SICSAG, 2008) data, since 1995 there has been a gradual and significant elevation in the number of patients admitted to ICU in Scotland. For example, in 2007 there were 18,157 patients admitted to high dependency units, while 10,453 were admitted to ICU, with an occupancy rate of 75% for each (SICSAG 2008; SICSAG 2014).

Table 1-1: Acute care hospital services population, and numbers of ICU and ICU beds, per head of the population in 2005 in eight different countries across North America and Western Europe (Adapted from Wunsch *et al*, 2008).

Countries	Acute care hospitals	Acute care hospitals beds	Acute care hospitals beds (per 100.000 population)	Total adult ICU	Total adult ICU Beds	% of total adult ICU Beds	adult ICU Beds (per 100,000 population)	Annual ICU admissions	Annual ICU admissions (per 100,000 population)
Germany	1,695	489,433	593	NA	20,259	4.1	24.6	1,941,347	2,353
Belgium	121	52,548	500	135	2,304	4.4	21.9	110,475	1,051
France	2,134	231,900	380	550	5,707	2.5	9.3	260,000	426
Spain	669	146,598	332	258	3,628	2.5	8.2	NA	NA
Netherlands	97	49,304	302	115	1,367	2.8	8.4	76,000	466
Canada	622	98,262	300	319	3,388	3.4	13.5	97,813	389
UK	1,815	179,512	298	268	2,131	1.2	3.5	130,000	216
US	7,914	655,664	221	5,980	59,162	9.0	20.0	5,700,000	1,923

1.3. The cost of ICU care.

It has been reported that the US is by far the highest spender on healthcare (currently 15% of gross domestic product), followed by Canada, Germany and France ranged between 9-11% , and the UK and Spain spending the least at 7- 8 % (Wunsch et al. 2008).

It has been estimated that annual spending in the UK intensive care was approximately £675 million, which was estimated to be 2% of the UK's hospital budget (Cronin et al. 2007). It has been estimated that the daily cost per intensive care bed was £1,000-£1,800 (The intensive care society 2006).

1.4. ICU admission criteria.

The Society of Critical Care Medicine (SCCM, 1999) provided criteria for patients with critical illness who are entitled to receive treatment in ICU according to their disease. There are three different models, which are: the diagnosis model, the objective variables model and the prioritisation model (SCCM 1999).

The diagnosis model specified medical conditions or diseases that were severe enough for treatment in the ICU, and classified these medical disorders under the following categories; cardiovascular diseases, pulmonary or respiratory system diseases, gastrointestinal disorders, neurological disorders, endocrine disorders, drug overdose and surgical causes. In contrast, the objective variables model specified the medical examination and investigation findings such as vital signs, laboratory values, radiographic findings, electrocardiographic findings and physical findings at the acute onset (SCCM 1999).

Other criteria, Other criteria, known as levels of care, that specified the patient's medical condition and their need to admission to the ICU accordingly; this criteria is used principally in the UK, which includes levels 0 to 3, in Table 1-2:

Table 1-2: Levels of Care (Department of Health. Comprehensive critical care—a review of adult critical care services. London: Department of Health, 2000 cited in Hudson & Boyd, 2007, p 117).

Level 0	*Patients whose needs can be met through normal ward care in an acute hospital.*
Level 1	*Patients at risk of their condition deteriorating, or those recently relocated from higher levels of care, whose needs can be met on an acute ward with additional advice and support from the critical care team.*
Level 2	*Patients requiring more detailed observation or intervention, including support for a single failing organ system or postoperative care and those 'stepping down' from higher levels of care.*
Level 3	*Patients requiring advanced respiratory support alone or basic respiratory support together with support of at least two organ systems. This level includes complex patients requiring support for multiorgan failure'.*

1.5. ICU length of stay and patient outcomes.

Although the median length of stay in ICU as well as in high dependency units in the UK is around two days, the mean length of stay is 4.3 and 2.7 days in ICU and high dependency units respectively. This was dependent on the cause of admission. For example, patients with primary respiratory diagnosis or in septic shock stay much longer than other groups of patients (SICSAG 2014). It has been reported that following admission to ICU in Scotland in 2014 the hospital mortality rate was 19% and reflects the severity of illness and co-morbidity among these patients (SICSAG 2014).

1.6. Scoring systems for illness severity.

APACHE score

Some specific scoring systems such as the Acute Physiology and Chronic Health Evaluation (APACHE) scoring system were adapted specifically to assess patients with critical illness. In 1985 APACHE II was introduced, it is a simpler form of the APACHE and contains 12 physiological variables that include age and chronic health (Figure 1-1) (Knaus et al. 1985; Knaus et al. 1991).

APACHE III was launched in 1991 and is similar to APACHE II, apart from containing more information about the patient's previous location and the medical issue necessitating their ICU admission. Although APACHE III is the newest form of this scoring system, APACHE II is the most commonly used form in the UK (Knaus et al. 1991).

The APACHE II severity of disease classification system

Physiologic Variable	High Abnormal Range					Low Abnormal Range				
	+4	+3	+2	+1	0	+1	+2	+3	+4	Points
Temperature - rectal (°C)	≥41°	39 to 40.9°		38.5 to 38.9°	36 to 38.4°	34 to 35.9°	32 to 33.9°	30 to 31.9°	≤29.9°	
Mean Arterial Pressure - mm Hg	≥160	130 to 159	110 to 129		70 to 109		50 to 69		≤49	
Heart Rate (ventricular response)	≥180	140 to 179	110 to 139		70 to 109		55 to 69	40 to 54	≤39	
Respiratory Rate (non-ventilated or ventilated)	≥50	35 to 49		25 to 34	12 to 24	10 to 11	6 to 9		≤5	
Oxygenation: A-aDO2 **or** PaO2 (mm Hg) a. FIO2 ≥0.5 record A-aDO2 b. FIO2 <0.5 record PaO2	≥500	350 to 499	200 to 349		<200 PO2>70	 PO2 61 to 70		 PO2 55 to 60	 PO2<55	
Arterial pH (preferred) Serum HCO3 (venous mEq/l) (not preferred, but may use if no ABGs)	≥7.7 ≥52	7.6 to 7.69 41 to 51.9		7.5 to 7.59 32 to 40.9	7.33 to 7.49 22 to 31.9		7.25 to 7.32 18 to 21.9	7.15 to 7.24 15 to 17.9	<7.15 <15	
Serum Sodium (mEq/l)	≥180	160 to 179	155 to 159	150 to 154	130 to 149		120 to 129	111 to 119	≤110	
Serum Potassium (mEq/l)	≥7	6 to 6.9		5.5 to 5.9	3.5 to 5.4	3 to 3.4	2.5 to 2.9		<2.5	
Serum Creatinine (mg/dl) Double point score for acute renal failure	≥3.5	2 to 3.4	1.5 to 1.9		0.6 to 1.4		<0.6			
Hematocrit (%)	≥60		50 to 59.9	46 to 49.9	30 to 45.9		20 to 29.9		<20	
White Blood Count (total/mm3) (in 1000s)	≥40		20 to 39.9	15 to 19.9	3 to 14.9		1 to 2.9		<1	
Glasgow Coma Score (GCS) Score = 15 minus actual GCS										
A. Total Acute Physiology Score (sum of 12 above points)										
B. Age points (years) ≤44=0; 45 to 54=2; 55 to 64=3; 65 to 74=5; ≥75=6										
C. Chronic Health Points (see below)										
Total APACHE II Score (add together the points from A+B+C)										

Figure 1-1: The Acute Physiology and Chronic Health Evaluation (APACHE) II score system.

(Knaus et al. 1985)

SOFA score

The Sequential Organ Failure Assessment (SOFA) is a combined score that grades from one to four and includes six organ systems: cardiovascular, respiratory, renal, neurological, hepatic and coagulation (Figure 1-2). This is carried out on a daily basis while the patient is in ICU; it is a dynamic score that estimates the severity of the illness and the progression of organ dysfunction over time. It has been proposed that using this scoring system in a repetitive manner is a useful predictor of ICU mortality rates (Vincent et al. 1996; Capuzzo et al. 2010).

The use of scoring systems remains controversial. Their main use is in comparative audit, such as comparing patient outcomes with other hospital unit. Scoring systems that include mortality rates are used to assess ICU unit performance and is known as Standardised Mortality Ratio. This ratio is calculated by dividing the actual mortality rate by the predicted rates, if the score < 1, this means that the ICU performance was better than predicted (Hudson and Boyd 2007).

SOFA score	1	2	3	4
Respiration **PaO_2/FiO_2 mmHg**	<400	<300	<200 With respiratory support	<100 With respiratory support
Coagulation **Platelets x $10^3/mm^3$**	<150	<100	<50	<20
Liver **Bilirubin. mg/dl (µmol/L)**	1.2-1.9 (20-32)	2.0-5.9 (33-101)	6.0-11.9 (102-204)	>12.0 (>204)
Cardiovascular **Hypotension** [a]	MAP <70 mmHg	Dopamine ≤ 5 or Dobutamine (any dose)	Dopamine < 5 or epinephrine ≤ 0.1 or norepinephrine ≤ 0.1	Dopamine > 1.5 or epinephrine > 0.1 or norepinephrine > 0.1
Central Nervous System **Glasgow coma score**	13-14	10-12	6-9	< 6
Renal **Creatinine. mg/dL (µmol/L) or urine output**	1.2-1.9 (110-170)	2.0-3.4 (171-299)	3.5-4.9 (300-440) or <500 mL/day	>5.0 (>440) or <200 mL/day

[a] adrenergic agents administered for at least one hour (doses given are in µg/Kg. min)

Table 1-3: Sequential Organ Failure Assessment (SOFA) score system.

(Vincent et al. 1996)

1.7. Specific causes of ICU admissions.

It has been reported that in the UK the most common source of ICU admission was from operating theatres (60-70%), and those from emergency departments (Hudson and Boyd 2007). The ICU post-operative admissions were mainly from emergency surgery. In contrast, the high dependency unit's admissions were mainly from elective planned surgery (SICSAG 2011)

The most common medical issues or causes of ICU admissions are:

- *'Previous severe cardio-respiratory illness such as acute myocardial infarction, chronic obstructive pulmonary disease, and stroke.*
- *Late-stage vascular disease involving the aorta.*
- *Age more than 70 with limited physiological reserve in one or more vital organs*
- *Extensive surgery for carcinoma such as oesophagectomy, gastrectomy, and cystectomy.*
- *Acute abdominal catastrophe with haemodynamic instability such as peritonitis, perforated viscus, pancreatitis.*
- *Acute massive blood loss more than 8 units.*
- *Septicaemia.*
- *Positive blood culture or septic focus.*
- *Respiratory failure: PaO2 <8.0 kPa on FiO2 >0.4 or mechanical ventilation >48 hours.*
- *Acute renal failure: urea >20 mmol/l or creatinine >260 μmol/l'* (Hudson and Boyd 2007).

1.8. The systemic inflammatory response

The systemic inflammatory response is a complex response to acute and chronic injury and is associated with a number of clinical conditions, such as tissue injury, infection, sepsis, ischemia, burns, multiple trauma, haemorrhagic shock, and immunologically mediated organ injury (Bone et al. 1992; Levy et al. 2003). Systemic inflammation may generally be beneficial to the host until its clinical manifestations become unmanageable by the body (Bone 1996).

Almost all patients with critical illness display systemic inflammatory response syndrome (SIRS) (Pittet et al. 1995). This generalised inflammatory response may lead to progressive organ failure, which has a high mortality rate (Lobo et al. 2003). The systemic inflammatory response includes an elevation of CRP and a reduction in plasma albumin concentrations. In contrast, urine and tissue albumin concentration increase (Gabay and Kushner 1999) (Figure 1-3).

The SIRS is manifested by two or more of the following clinical conditions:

Temperature	> 38º C or < 36º C;
heart rate	> 90 beats per minute
respiratory rate	> 20 breaths per minute or PaCO2 <32 mm Hg
white blood cell count	>12,000/ cu mm, <4,000/ cu mm, or >10% immature (band) forms

Table 1-4: The systemic inflammatory response syndrome (Bone et al. 1992; Levy et al. 2003)

1.8.1. Local reaction at the site of injury or infection.

At this stage the source of infection such as traumatic injury, infection or burn injury may enhance the release of a wide range of mediators. These include cytokines such as

interleukin-1, interleukin-6 and Tumour Necrosis Factor (TNF-α). These induce a proinflammatory state and there may be a release of mediators that have multiple overlapping effects and which are designed to limit any new damage, as well as to ameliorate any damage that has already occurred.

These mediators may destroy the damaged tissue and pathogenic organisms, neoplastic cells and any foreign antigens stimulate the growth of new tissue. This response is followed directly by a compensatory anti-inflammatory response through the release of interleukins such as interleukin -4, 10, 11 and 13, soluble Tumour Necrosis Factor (TNF-α) receptors and transforming growth factor-β to ensure that the proinflammatory mediator effects do not become destructive and which in turn may restore homeostasis.

At this stage if the original site of injury or infection is severe, proinflammatory and anti-inflammatory mediators will appear in the systemic circulation in large amounts. Proinflammatory mediators usually present in the systemic circulation and work as a part of the normal response to infection, and as a warning signal that the local microenvironment is not able to control the initiating insult.

The proinflammatory mediators enhance the migration of neutrophils, T cells, B cells, platelets, and coagulation factors to the site of injury or infections; this process enhances the compensatory systemic anti-inflammatory response, which in turn may quickly down-regulate the initial proinflammatory response. At this stage a few signs and symptoms may appear, however marked organ dysfunction is quite rare.

1.8.2. Excessive systemic inflammation.

At this stage there may be a loss of regulation of the proinflammatory response, which may lead to a massive systemic reaction that manifests itself as the systemic inflammatory response syndrome. The pathological changes include: a) endothelial dysfunction and

increased microvascular permeability, b) sludging of platelets, c) stimulation of the coagulation system, and d) vasodilatation, redistribution of blood flow, and fluid transudation all of which may lead to profound shock. Ultimately organ dysfunction and failure may occur unless homeostasis is restored.

Where there is multiple organ dysfunction this can result in immunologic dissonance and characterised by inappropriate excessive responses of the immunomodulatory system. In this stage there is an overwhelming systemic inflammation that may sustain and multiple organ dysfunction increasing risk of death.

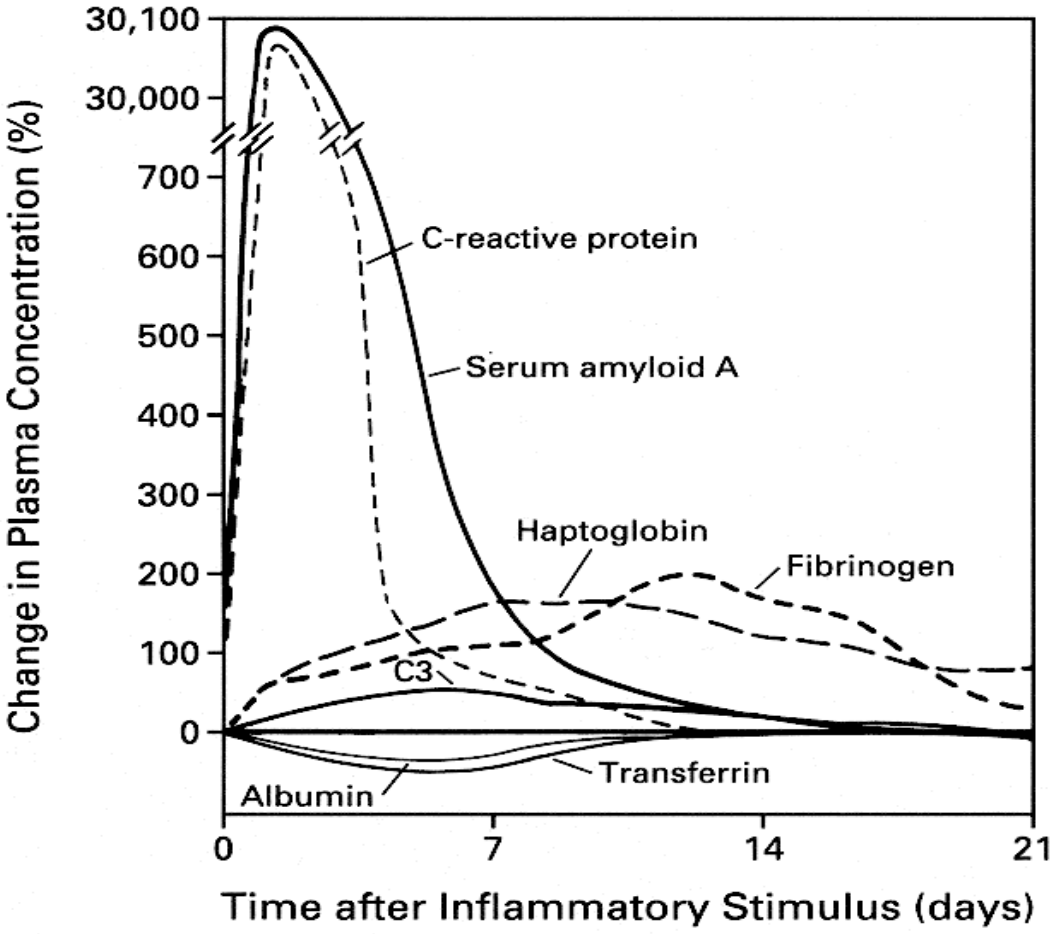


Figure 1-2: Acute phase proteins and the systemic inflammatory response.

(From Gitlin & Cotlen 1987 as modified in Gabay & Kushner 1999)

1.9. Management of the critically-ill patients.

Sepsis.

"Sepsis encompasses a spectrum of illness that ranges from minor signs and symptoms through to organ dysfunction. Sepsis ranks in the top 10 causes of death and it is a life-threatening condition, especially amongst patients with a chronic disease" (Bone et al. 1997; Russell 2007; Lever 2007). The most common source of the bacteria infection includes:

- The lining of the brain (meningitis).
- The lungs (bacterial pneumonia).
- The bowel (usually seen with peritonitis).
- The kidneys (upper urinary tract infection or pyelonephritis).
- The liver or the gall bladder.
- The skin (cellulitis).
- In hospitalized patients, common sites of infection include intravenous lines, surgical wounds, surgical drains, and sites of skin breakdown such as bedsores (Russell 2007).

The symptoms of sepsis include a fall in blood pressure, which may lead to shock, and may be associated with multi organ dysfunction. A change in mental status and an increase in the respiratory rate, could indicate early signs, as well as other symptoms such as: chills, shaking, confusion or delirium, decreased urine output, fever or hypothermia, warm skin, hyperventilation, headache, tachycardia, and skin rash (Russell 2007).

Pathophysiology of sepsis.

It has been reported that sepsis may have five stages in the development of multiple organ dysfunction, which are: (1) local reaction at the site of injury or infection; (2) initial systemic response; (3) massive systemic inflammation; (4) excessive immunosuppression; and (5) immunologic dissonance (Bone et al. 1997).

Patients with sepsis on admission to ICU often receive intravenous antibiotics, oxygen, parenteral fluids. Also vasopressor and inotropic may be required, as well as dialysis in case of renal failure and mechanical ventilation in case of respiratory failure. Treatment with powerful anti-inflammatory drugs, such as corticosteroids or recombinant human activated protein C is no longer routinely used (Dellinger et al. 2008).

Initial resuscitation.

Resuscitation should be started immediately in patients with severe sepsis or sepsis induced hypoperfusion shock which is characterised as severe hypotension or elevation in serum lactate > 4 mmol/l. During the first six hours the following resuscitation goals are have been recommended.

- '*Central venous pressure (CVP) 8–12 mm Hg.*
- *Mean arterial pressure ≥65 mm Hg.*
- *Urine output ≥0.5 mL.kg-1.hr-1.*
- *Central venous (superior vena cava) oxygen saturation ≥70%, or mixed venous ≥65%'* (Dellinger et al. 2008).
- If venous oxygen saturation target is not achieved the following steps of treatment may be considered
- *Consider further fluid.*

- *Transfuse packed red blood cells if required to hematocrit of ≥30% and/or*
- *Dobutamine infusion max 20 μg.kg−1.min−1'* (Dellinger et al. 2008).

Diagnosis of sepsis.

Diagnosis of sepsis needs to be confirmed by body fluid cultures, such as blood, urine, cerebrospinal fluid, wound swab, and respiratory secretions. These should be collected before antibiotic treatment begins in order to identify the causative organisms (Dellinger et al. 2008). Other tests could be carried out, such as blood gases, renal function tests, blood differential red cell and white blood cell counts (Dellinger et al. 2008). Furthermore, imaging studies such as an ultrasound scan must be considered to identify the source of infection to remove a foreign body or drain an abscess (Dellinger et al. 2008).

Antibiotic therapy.

Administration of antibiotics is recommended to start as early as possible and at the initial recognition of septic shock; a broad spectrum antibiotic is highly recommended in order to cover different suspected organisms.

Antibiotic therapy regimen may take seven-to-ten days and should be reassessed daily to '*optimise efficacy, prevent resistance, avoid toxicity & minimise costs*' (Dellinger et al. 2008). The treatment may take longer than ten days if patients have a slow response, undrainable foci of infection, or in patients with immunologic deficiencies. Finally, antibiotic therapy may be stopped if the cause of inflammation is found to be non-infectious (Dellinger et al. 2008).

Fluid therapy.

Crystalloids and colloids fluids are commonly used during resuscitation to maintain central venous pressure ≥ 8 mmHg and ≥ 12 mmHg if the patient is on mechanical ventilation.

Fluid challenge with either 1000 ml of crystalloid or 300 to 500 ml of colloids that are given over 30 minutes may be considered with simultaneously assessment of the improvement of haemodynamic status. In cases with sepsis-induced tissue hypoperfusion, large fluid volumes with rapid administration may be required. Recently it has been established that colloids are not associated with better outcomes compared to crystalloids (Dellinger et al. 2008).

Drugs.

- Vasopressor agents such as nor-epinephrine or dopamine may by required to maintain the main arterial blood pressure ≥ 65 mmHg.
- Inotropic therapy such as dobutamine or epinephrine may be required in patients with myocardial dysfunction.
- Steroids such as dexamethasone may be required if a patient with septic shock has hypotension that poorly responds to fluid resuscitation measures and vasopressor agents (Dellinger et al. 2008).

Sepsis complications may include disseminated intravascular coagulation, problems with blood flow to vital organs such as brain, heart and kidneys, septic shock and death (Russell 2007).

1.10. Hyperglycaemia in patients with critical illness (pathophysiology).

Patients with critical illness may suffer from hyperglycaemia, glucose intolerance and insulin resistance even without pre-existing diabetes (Falciglia et al. 2009). This elevation of plasma glucose concentrations due to the presence of acute illness is known as stress diabetes or diabetes of injury.

Patients with or without diabetes who suffer from hyperglycaemia during critical illness may have increased morbidity and mortality rates (Van den Berghe et al. 2006; Treggiari et al. 2008). Hyperglycaemia may influence the outcome of patients with critical illness through the suppression of immune function; inducing oxidative stress and endothelial damage, hepatocyte mitochondrial damage, and may potentiate tissue ischemia due to inflammation and acidosis (Treggiari et al. 2008).

1.11. Diabetes of injury.

Schricker (2001) has demonstrated that there may be a marked insulin resistance post-operatively that may lead to hyperglycaemia, even without complications or sepsis, and this may persist up to 20 days following surgery; this situation has been called ‘ diabetes of injury’(Schricker 2001).

Acute illness may enhance the catabolic response by the release of inflammatory cytokines and counter regulatory hormones such as catecholamines, cortisol, glucagon, and growth hormone, as well as stimulating the sympathetic nervous system that may lead to elevation in cortisol, noradrenaline and adrenaline concentrations (Vanhorebeek and Van den Berghe 2006). Drugs, such as exogenous catecholamines and glucocorticoids, may suppress the secretion and the action of insulin, may inhibit glycogen synthesis, stimulate gluconeogenesis and alter insulin-mediated glucose uptake by tissues. This may result in insulin resistance, nitrogen loss and hyperglycaemia. Intravenous fluid such as dextrose and parenteral nutrition, may also contribute to hyperglycaemia (Saberi et al. 2008; Ead 2009; Kavanagh and McCowen 2010). Figure 1-4 below shows the pathophysiology of hyperglycaemia in critical illness.

It has been reported that hyperglycaemia may extend to the post-operative care period depending on the magnitude of surgery. For example, with major surgical operations, such

as cardiovascular surgery, plasma glucose levels increase up to 10- 12 mmol/l and stay at this level for more than 24 hours following surgery in non-diabetic patients, even in minor operations the hyperglycaemia may persist more than five days after surgery (Marks 2003; Rahman and Beattie 2004; Ead 2009).

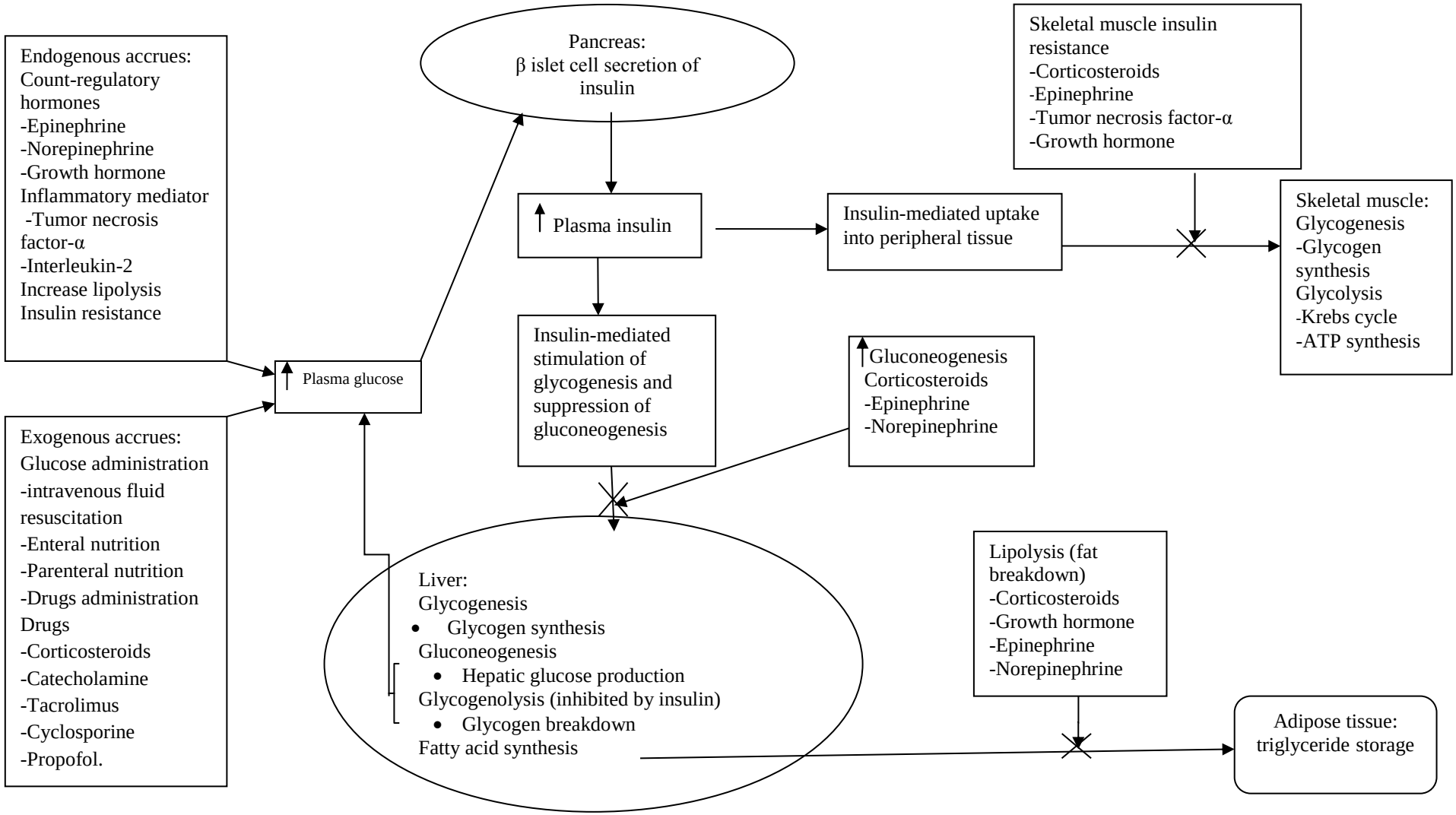


Figure 1-4: The pathophysiology of hyperglycaemia in critical illness Adapted from (Anger and Szumita 2006)

1.12. Hyperglycaemia and cell damage

Oxidative stress.

Oxidative stress is an imbalance between reactive oxygen species (ROS) production and antioxidants. Free oxygen radicals are missing an electron which may make them highly active and unstable and they cause to oxidative damage to cell membranes, proteins and genes by reducing the bioavailability of endothelium-derived nitric oxide, which in turn may lead to vasoconstriction, smooth muscle cell proliferation and platelet activation (Papaharalambus and Griendling 2007; Hatzitolios et al. 2009). In addition, free radicals may enhance the deposition of low-density lipoproteins (LDLs) in the walls of blood vessels and, as a result, atherosclerotic changes may take place (Papaharalambus and Griendling 2007; Hatzitolios et al. 2009).

Advanced Glycosylated end- products (AGEs).

AGEs are complex compounds formed as a result of non-enzymatic glycosylation reactions of proteins and lipoproteins (Goh and Cooper 2008). AGEs are normally formed at a constant and slow rate throughout life. Chronic hyperglycaemia may accelerate glycosylation of proteins and lipoproteins, which may lead to production of AGEs in the walls of the blood vessels (Goh and Cooper 2008; Hatzitolios et al. 2009).

AGEs may form irreversible bonds with and between proteins to alter their structures and activity. AGEs may interact with cell surface AGE-binding receptors (known as RAGE). When AGEs bind to their corresponding receptors this can lead to LDL lipoprotein deposition, enhancement of the oxidative reaction, enhancement of the formation of foam cells and the atheromatous plaque, and alteration of the gene transcription. In addition,

AGEs may lead to endothelial dysfunction, macrophage activation and impaired vascular smooth muscle-cell function (Goh and Cooper 2008; Hatzitolios et al. 2009).

Specific protein kinase C isoforms activity and Aldose reductase activity (the polyol pathway).

Hyperglycaemia may activate protein kinase C (PKC). The activation of PKC has a significant influence in blood vessels and endothelial function. Normally, glucose uptake by cells is dependent on insulin. However, in some cells, such as nerves, it enters without insulin. The glucose is metabolized principally by the glycolytic pathway, with less than 5% of the glucose being metabolised by the polyol pathway (Hatzitolios et al. 2009).

The intracellular polyol pathway is the intracellular converts of glucose to sorbitol by the effect of aldose reductase. This polyol pathway may promote formation of diacylglycerol, which in turn may stimulate PKC. Protein kinases (PKS) are a group consisting of 12 isoforms; stimulation of specific PKC isoforms, such as PKC-β and PKC-δ, may lead to the proliferation of blood vessel cells, impairment in glucose and lipid metabolism, reduction of vasodilatation, expression of atherosclerosis-promoting genes, and enhanced vascular permeability (Hatzitolios et al. 2009).

When the polyol is trapped inside the cells, it can create an osmotic gradient and make sodium and water flow into the cell. Sorbitol is converted to fructose by the effect of sorbitol dehydrogenase. High fructose may lead to AGEs and more damage may occur (Hatzitolios et al. 2009).

1.13. Glycaemic control in patients with critical illness.

Rigorous hyperglycaemia management protocols using insulin therapy have been reported (Van den Berghe et al. 2001). Intensive insulin therapy reduced the morbidity and

mortality rate regardless of those patients who had a previous history of diabetes or not (Krinsley 2006). These studies are summarised in Table 1-5. However, some studies, such as the original Van den Berghe et al (2001) paper and Preiser et al (2009) have been criticised because the population were predominately post cardiac surgery. It was already well known that following myocardial injury hyperglycaemia had significant harmful effects and associated with poor outcomes (Malmberg 1997; Malmberg et al. 2005). These, studies may not be representative of all critically ill patients and in particular those with sepsis. Other studies have major limitations, Krinsley et al (2004) demonstrated a substantial mortality reduction (an absolute reduction of over 6% and a relative reduction of 29%), and this was based on historical controls which severely reduce the validity of any conclusions. The Specialised Relative Insulin Nutrition Tables (SPRINT) study also demonstrated significant improvements in outcome but was also based on historical controls (Chase et al. 2008).

More recent studies such as the Normoglycaemia in Intensive Care Evaluation-Survival Using Glucose Algorithm Regulation (NICE-SUGAR Study 2009) have reported that patients who had intensive glucose control with plasma glucose target between 4.4-6.0 mmol/l had a higher mortality rate than conventional glucose control patients with plasma glucose target <10 mmol/l within 90 days of ICU admission. Also, severe hypoglycaemic episodes, where plasma glucose < 2.2 mmol/l, were more common in the intensive glucose control group (Van den Berghe et al. 2003; Cheung et al. 2006). However, the mortality rate among intensive glucose control group of patients was not clear, whether due to the reduction of plasma glucose concentration, the increase of insulin administration, the occurrence of hypoglycaemia or due to the study methodology (The NICE-SUGAR Study Investigators 2009). Further analysis of the data from the NICE Sugar trial confirmed the association between the hypoglycaemia and outcome but could not establish as causal link (The NICE-SUGAR Study Investigators 2009; Finfer et al. 2012). Several recent studies,

have also studied factors influencing glycaemic control. These studies have significant limitations, for example Dossett et al (2008) did not report on the intake of calories from nutrition support and Egi et al (2009) did not include any analysis of the drugs likely to influence insulin sensitivity (Dossett et al. 2008; Egi et al. 2009). Therefore, the treatment of hyperglycaemia in critically ill patients remains a controversial issue (Mehta et al. 2005). Moreover, the frequency of hyperglycaemia and/or hypoglycaemia and the association with the other medical conditions that occur in patients with critical illness requiring insulin therapy is not clear.

In a much larger study Flaciglia reported that hyperglycaemia was associated with severity of illness and outcome. This study looked at over 250,000 patients in 153 critical care units. Their definition of hyperglycaemia was based upon the averages of all of the laboratory glucose measurements made for each patient. Their findings indicated that in some patients hyperglycaemia was more difficult to treat and that hyperglycaemia was more strongly associated with a poor outcome in some medical diagnoses compared with surgical patients. It was not established whether the resistance to insulin *per se* or administering high dose of insulin was the cause of poor outcomes. Indeed, in the NICE sugar study a unique concern was that excessive insulin administration in the context of a tight glucose control protocol was the cause of the excess mortality.

Indeed, counter-regulatory hormone administration and fluid balance are the essential aspect of the treatment of hypovolaemic shock in patients with critical illness in order to increase cardiac output and systemic vascular resistance. Khoury and McGill (2011) reported that norepinephrine led to a reduction in insulin sensitivity in non-critically ill patients and suggested that during the haemodynamic support in the treatment of acute illness the insulin administration rate will need to be increased or decreased according to the administration or discontinuation of catecholamines (Khoury and McGill 2011). Therefore, insulin protocols do not take into account the physiological changes that may

occur due to the drugs administration in the intensive care setting such as catecholamines, which in turn, may increase the risk of both hypoglycaemia and hyperglycaemia. This may be avoided with better understanding of the metabolic effect of these drugs (Khoury and McGill 2011). However, this had a small study sample (n=11) and therefore it is difficult to generalize their findings to all critically ill patients. Most of these studies were in surgical patients, it may be inappropriate to generalise their findings to critical illness due to medical conditions. This may be because the cause of illness, or its management. Surgical and medical patients should possibly be considered separately both in terms of the clinical protocol and research investigations of hyperglycaemia control in patients with critical illness.

In the SPRINT study that also studied tight glycaemic control in a general ICU there was a similar frequency of severe hypoglycaemia. However, the incidence of moderate hypoglycaemia between 2.2 mmol/l and 4.4 mmol/l was 12.8%. They could not identify an association between glycaemia and mortality when the tight glycaemic protocol was used. In the SPRINT study the analysis of glycaemic control was over the entire ICU stay rather than on a daily basis and the use of drugs that influence glycaemia apart from insulin was not examined.

It has been established that hyperglycaemia in patients with critical illness may directly or indirectly have harmful effects independent of diabetes. However, it is not clear or established the frequency of this medical issue and the association with other different medical conditions that may critically ill patients suffer from (Van den Berghe et al. 2001). Several observational studies have shown that patients with critical illness may suffer from hyperglycaemia; this elevation of plasma glucose level may increase the morbidity and mortality rates thereafter independent of pre-existing diabetes. Hyperglycaemia has been reported in many studies to be more common in patients with critical illness, who suffer from acute myocardial infarction, acute stroke, sepsis, and postoperative cardiac surgery,

whether those patients had previous history of diabetes or not. However, it is not clear or established the frequency of this medical issue and the association with other different medical conditions that may critically ill patients suffer from. Some studies have shown that management of the hyperglycaemia has been considered to have beneficial effects but the ideal target for plasma glucose, as well as the critical ill population that would benefit most from this treatment remains controversial (Krinsley 2006).

In contrast, other studies have presented that treatment of hyperglycaemia did not reduce morbidity and mortality rates among those patients (Malmberg et al. 2005; Chuang et al. 2006; Treggiari et al. 2008; The NICE-SUGAR Study Investigators 2009).

There are a few important questions from this work;

- Why hyperglycaemia occurs in ICU patients without a previous history of diabetes (Is hyperglycaemia a marker or a cause of adverse outcomes)?

- Why hyperglycaemia appears to be so deleterious?

- What is the mechanism underlying the development of hyperglycaemia in critically ill patients?

- Are there are variable effects of hyperglycaemia in different diseases?

- What are the most appropriate glycaemic targets for different populations of ICU patients?

- Which population of ICU patients would benefit most from glycaemic control intervention?

1.14. The inflammatory response and outcome in critical illness

1.14.1. The prognostic role of C-reactive protein in critical illness

C-reactive protein (CRP) is an acute-phase protein that has been evaluated extensively in the clinical setting (Gabay and Kushner 1999; Ho et al. 2008). Although CRP is not diagnostic of sepsis and in particular reflects a systemic inflammatory state, in the clinical context it is routinely used to raise suspicions of infection and further investigations such as imaging and blood cultures. Moreover, a change in CRP concentrations can however suggest a response to treatment (Povoa et al. 2006) and may a reliable marker of outcomes during the post-ICU period (Ho et al. 2008; Grander et al. 2010; Ranzani et al. 2013). Ho and co-workers (2008) who studied the association between discharge CRP concentration and prediction of in-hospital mortality in 603 unselected patients with critical illness and showed that a high CRP concentration at ICU discharge was an independent predictor of in-hospital mortality after ICU discharge (Ho et al. 2008). A subsequent prospective study by Grander and co-workers (2010) who studied the maximum and discharge CRP concentrations in 765 nonsurgical patients with critical illness, reported that CRP levels during critical illness independently associated with post-ICU survival (Grander et al. 2010). In contrast, an observational study by Al-Subaie and co-workers (2010) who studied the CRP and albumin concentration on the day of ICU discharge in 1487 medical and surgical patients with critical illness, reported that plasma CRP was not associated with ICU readmissions and in-hospital mortality (Al Subaie et al. 2010). However, it might be expected that patients will only leave ICU when they are fit enough and therefore exit CRP concentration may not be useful.

1.14.2. The prognostic role of albumin in critical illness

Serum albumin is a negative acute-phase protein; thus, the degree of hypoalbuminemia in critically ill patients correlates with the intensity of the inflammatory response triggered by infection (Ho et al. 2008; Al Subaie et al. 2010; Ranzani et al. 2013). Hypoalbuminaemia at admission may be associated with ICU and hospital mortality and poor outcomes in patients with critical illness (Al Subaie et al. 2010). Hypoalbuminaemia in critically ill patients is mainly related to the underlying systemic inflammatory processes, including increased breakdown secondary to inflammatory mediators, loss through capillary leak, and renal injury (Ballmer 2001). The association between low albumin concentrations and poor outcome may be directly related to loss of colloid osmotic pressure and reduction in antioxidant effects, or may be secondary to an ongoing inflammatory process (Vincent et al. 2003). However, Cochrane review reported that "no evidence that albumin reduced mortality and a strong suggestion that it might increase the risk of death in patients with hypovolaemia, burns, or hypoproteinaemia" (Cochrane Injuries Group Albumin Reviewers 1998)

1.15. Micronutrients

Micronutrients are substances that are needed in small amounts and have significant physiological functions such as antioxidant effects. Antioxidants either inhibit or delay the oxidation of substrates (Berger and Shenkin 2006). There are a number of antioxidants such as uric acid, glutathione, albumin, bilirubin, vitamins and phenols, and enzymatic antioxidants such as superoxide dismutases, catalase, and the glutathione peroxidase (Berger and Shenkin 2006).

Endogenous antioxidant activity balances the reactive oxygen species production but the normal person loses 1% of antioxidants daily. The most important source of antioxidants is from nutrition. Trace elements, including selenium and zinc, are also essential elements of the defence mechanism. Nutrition antioxidants have several mechanisms; the most important one is as free radical scavengers, which include the following; neutralisation of free radicals such as reactive oxygen species production by quenching iron and reduction of the peroxide concentrations and repair of oxidised membranes (Heyland et al. 2005).

1.15.1. Carotenoids

Carotenoids enhance immune function (Hughes 1999) and appear to have a protective effect against ageing and several common diseases such as cancer, cardiovascular disease and stroke in epidemiological, however, trials of supplement have shown no benefit (Mayne 1996; Krinsky and Johnson 2005). The most common carotenoids that were found in measurable concentration in human plasma and intracellular are lycopene, lutein, α-carotene and β-carotene (Voutilainen et al. 2006). The properties of carotenoids appear to be mediated by a reduction of oxygen derived radicals (Bast et al. 1998).

1.15.2. Vitamin D

Although the principle metabolic effect of vitamin D is on maintaining bone health it may also effect the glycaemic control both directly and indirectly (Pittas et al. 2007). Directly, through enhancing the expression of insulin receptor, which in turn enhances insulin function for glucose transport (Maestro et al. 2000).). Indeed, a review by Badawi and co-workers (2010) reported that the active biological metabolite of vitamin D, 1, 25-dihydroxy vitamin D (1, 25$(OH)_2$D), stimulates insulin synthesis and secretion through vitamin D receptors (VDR). Several studies have discussed the relationship between single nucleotide polymorphisms (SNPs) in the genes regulating vitamin D receptor (VDR) and vitamin D-binding protein (DBP) with glucose intolerance and insulin secretion (Fu et al. 2009; Badawi et al. 2010).

This relationship explains the reported link between Vitamin D and diabetes type 2, whereby the overall risk rate of type 2 diabetes was reduced in individuals who consume at least 800 IU/d of vitamin D daily.

There is an immunomodulatory function of vitamin D, as VDRs are found in many types of immune cells. In addition, (1, 25$(OH)_2$D), has a significant influence in the modulation and production of immunostimulatory and immunosuppressive cytokines, for example interleukin 12 and interleukin 10 respectively (Badawi et al. 2010).

Vitamin D and calcium homeostasis may reduce the risk of type 1 and 2 diabetes (Pittas et al. 2007). Calcium may have a role in insulin secretion and function, but it works together with vitamin D (Pittas et al. 2007). The indirect method by which vitamin D regulates glucose concentration in blood is mainly by regulating the extracellular calcium levels and the influx of the calcium via cellular membrane. Insulin release depends mainly on this indirect method (Pittas et al. 2007). As a result, insulin secretion is a calcium dependant process, any inadequate vitamin D or calcium intake may disturb the balance in the influx

of the β-cells extracellular and intracellular calcium pools, which in turn may alter the normal insulin secretion. This may explain that the deficiency in these micronutrients may adversely affect the glycaemic control (Pittas et al. 2007). Overall, the supplement combination of both vitamin D and calcium may improve and optimize the glucose metabolism and control (Pittas et al. 2007). However, this has not been examined with critical illness.

1.15.3. Vitamin E and C

Vitamin E is a fat-soluble vitamin, which has potent anti-inflammatory and anti-oxidant functions. Vitamin E consumption may inhibit the peroxidation of low density lipoprotein (LDL), neutralize free radical produced from normal cellular metabolism. This in turn prevents the oxidative stress that hyperglycaemia may cause, reduce the expression of cytokine gene. Vitamin E may have a role in treatment and prevention of long-term hyperglycaemia complications by reducing protein glycation, lipid oxidation, and by suppressing platelet aggregation and adhesion (O'Connell 2001). Vitamin E deficiency may be associated with increasing the incident rate of diabetes, and having it as a supplement improved the glycaemic control and reduced oxidative stress markers. Recently, the relationship between vitamin E and vitamin C in improving insulin sensitivity has been discussed. Taking a 1000mg/day vitamin C combined with 400 IU/day vitamin E may reduce the development of insulin sensitivity resistance (Ristow et al. 2009).

1.15.4. Zinc and selenium

Zinc is importance in human health, as a cofactor in many catalytic processes and zinc also has a structural role. Selenium is essential for the catalytic activity of glutathione peroxidase (GSH-Px) protecting against membrane lipid peroxidation (Thomas et al.

1990). Zinc also combats oxidative stress as a cofactor in zinc superoxide dismutase. Zinc and selenium both play important functions in immune regulation (Fraker et al. 2000). Adequate trace element status is considered to be of particular importance in critically ill patients who have significantly reduced circulating and body stores of antioxidants, such as zinc superoxide dismutase and GSH-Px, therefore increasing reactive oxygen species (Heyland et al. 2005).

Concentrations of zinc and selenium decrease significantly following severe trauma, surgery, sepsis and severe systemic inflammatory response and remain low for several days and weeks (Berger et al. 1998; Heyland et al. 2006). It has been proposed that this reduction in trace element concentrations may deplete circulating antioxidants leading to an elevation of reactive oxygen species and so exacerbating the severity of illness (Berger and Chiolero 2007). Other groups have also suggested that decreased plasma trace elements concentrations are associated with increased severity of critical illness (Goode et al. 1995; Metnitz et al. 1999; Rinaldi et al. 2009).

It has been reported that the changing of the concentration of insulin and zinc in human pancreas has a similar effect. Zinc is required in insulin synthesis, secretion and storage; it also has shown to improve glucose levels in patients with type 2 diabetes and may delay the effect of oxidative stress and the development of diabetes chronic microvascular and macrovascular complications (Akhuemokhan et al. 2013).

1.15.5. B vitamins

Vitamins B1, B2 and B6 serve as cofactors for several enzymes important in energy metabolism. They are important for the production of reducing substances used in oxidant stress defences. There is evidence that plasma concentrations of B-vitamins, fall as part of the systemic inflammatory response and therefore this may be a confounding factor in their

interpretation. In order to address this issue the use of red cell measurements of B1 (Thiamine diphosphate), B2 (Flavin adenine dinucleotide) and B6 (pyridoxal phosphate) has been proposed.

Vitamin B1 is an important cofactor for enzymes involved on carbohydrate metabolism, also it is a cofactor for several enzymes, such as pyruvate dehydrogenase, oxoglutarate dehydrogenase and transketolase (Gray et al. 2004; Fattal-Valevski A 2011). Vitamin B2 and its two cofactors flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD). FAD was mainly found in red blood cells, FMN and FAD act as cofactors in oxidation-reduction reaction and in the respiratory chain and are thus involved in energy production (e.g. cytochrome, glutathione reductase, glutathione peroxidase, xanthine oxidase and methylene tetrahydrofolate reductase) (Talwar et al. 2003b; Talwar et al. 2005). Vitamin B2 is required for normal immune function, by maintenance of glutathione status (Grimble 1997). It also has a suppressive effect on the production of tissue inflammatory mediators and decreases plasma elevated nitric oxide levels (Kodama K et al. 2005). Vitamin B6, also known as pyridoxal phosphate, acts as cofactor for a wide variety of enzymes of intermediary metabolism, particularly proteins, carbohydrates, fats and homocysteine and synthesis of several neurotransmitters and heam (Leklem 1991; Quasim et al. 2005). These vitamins act as cofactors for many enzymes that regulate glucose, lipid and amino acid metabolism and neurotransmitter synthesis (O'Connell 2001).

1.16. Assessment of vitamin and trace elements status in hospitalised patients

Vitamin and trace element status can be defined as the extent to which an individual's physiological need for vitamins and trace elements is being met. Optimal vitamin and trace element status is a balance between intake and requirements. Vitamin and trace element intakes depend on actual food consumption which can be influenced by various factors such as economic status, eating behaviour, emotional status, cultural influences,

disease, and the ability to absorb the vitamins and trace elements consumed. Vitamin and trace elements requirements are also influenced by many factors, including infection, disease, fever or trauma, growth and pregnancy. Adequate intake is necessary, but provision of excess supplements to patients who do not need them may be harmful. Single micronutrient deficiency states are comparatively easily recognised and treated. Subclinical deficiency, often of multiple micronutrients, is more difficult to recognise, and laboratory assessment is often complicated by the present of systemic inflammatory response. Shenkin concluded that "clinical benefit was most likely in those patients who are severely depleted and at risk of complications"(Shenkin 2006).

1.17. Aim of the thesis

The aim of the thesis is to examine the relationship between the systemic inflammatory response, as evidenced by both CRP and albumin, on plasma glucose, plasma and red cell concentrations of vitamins and trace elements in a nutrition screen cohort and a critical illness cohort.

Table 1-5: Summary of the hyperglycaemia studies.

Author (S)/ year	Subject	Population	Findings	Comments
Malmberg, 1997 DIGAMI 1 study. Stockholm/Sweden.	Hyperglycemia and patients with critical illness.	620 patients with diabetes and acute myocardial infarction. Medical ICU.	A significant reduction in haemoglobin A1c in both groups. Significantly more in infusion group. 30% reduction of one year mortality rate in intervention. 97% of all deaths in both groups occurred due to cardiovascular causes. There was a significant reduction in these events in the intervention group	-The subjects were patients with diabetes. -Cardiac patients (MI). -The findings may suggest that long term glycaemic control may be important in the prevention cardiovascular causes of death in patients with diabetes. -After DIGAMI study the intervention to control plasma glucose levels in patients with myocardial infarction become a standard practice. -Glycaemia focussed study.
Scott *et al*, 1999. England, the UK.	Hyperglycemia and patients with critical illness.	50 patients with stroke. Medical ICU.	-Glucose potassium insulin (GKI) infusion treatment to control mild to moderate hyperglycemia in patients with stoke is reported to be safe, practical, and has a significant improvement of lowering the high plasma glucose without any significant episodes of hypoglycaemia , cardiovascular events and excess mortality rate at first four week post stroke.	-Pilot study. -Non- diabetic patients. -Patients with acute stroke.

Van Den Berghe *et al*, 2001. Belgium	Hyperglycemia and patients with critical illness.	1,548 critically ill patients/ surgical ICU.	Significant reduction mortality rate. Decrease in overall hospital mortality rate, bloodstream infection, acute renal failure that required dialysis or heamofltration, red cell transfusion, clinical illness polyneuropathy, the need for mechanical ventilation.	- Prospective randomized controlled study. - Heterogeneous surgical ICU population, lead to difficulties to extrapolate the result to medical ICU patients. -63% cardiac patients. -Male: Female ratio 71:29. -Single-centre study.
Van Den Berghe *et al*, 2003 Belgium	Hyperglycemia and patients with critical illness.	Extension study to Van Den Berghe *et al*, 2001 study. Same number of patients.	Normoglycaemia was achieved safely within 24 hours of the ICU admission.	- Heterogeneous medical and surgical. ICU patients.
Krinsley, 2004 Stamford, Conn, the USA.	Hyperglycemia and patients with critical illness.	1,600 medical-surgical ICU critically ill patients.	-No significant changes in ICU infection acquired rate. -Significant improvement of patient's glucose levels. - Significant improvement in renal function. -Significant reduction in mortality rate between control and treatment group by 21% to 15% respectively (29% reduction in overall mortality rate of ICU patients after applying the protocol). - ICU length of stay was decreased from	- Heterogeneous medical and surgical ICU patients.

			3.6 days in control group to 3 days in treatment group.	
Malmberg, 2005 DIGAMI 2 study. Stockholm/Sweden	Hyperglycemia and patients with critical illness.	1253 patients with diabetes and acute myocardial infarction.	-There was no significant improvement in the morbidity and mortality rates in patients who have diabetes and presented with acute myocardial infarction after acutely induction of long term insulin treatment compared with conventional treatment. - The study epidemiological analysis demonstrates that the plasma glucose level is a strong, independent prognostic factor for long term mortality among those patients, which in turn glucose control is an essential part of their management.	-Multicentre prospective randomized blinded controlled study. -44 centres were in Sweden, Finland, Norway, Denmark, The Netherlands, and the UK. -Glycaemia focussed study.
The CREATE-ECLA Trial Group Investigation, 2005 The USA	Hyperglycemia and patients with critical illness.	20,210 patients with ST-segment elevation myocardial infarction (STEMI).	- No marked difference in the mortality rate within 7 and 30 days. -No significant differences between the both groups in the occurrence of ventricular fibrillation, tachycardia, advanced second or third degree heart block, electromechanical dissociation, and fluid volume overload. -A new episode of heart failure in both groups after 7 and 30 days of ICU	- Randomized controlled study - Cardiac patients. - High dose of GIK infusion has neutral effect on mortality.

			admission. -Hypoglycaemia episodes were more common in intervention group as well as hyperkalemia. - High dose of GIK infusion has no significant effect on the mortality rate on patients with ST- segment elevation myocardial Infarction.	
Van Den Berghe *et al*, 2006 Belgium	Hyperglycemia and patients with critical illness.	1,200 medical ICU patients.	- hypoglycemia 73% and 62% in conventional and intensive treatment group respectively. - A reduction of the ICU mortality rate by 10% among intensive insulin therapy group who stayed more than five days. - A significant improvement in the time of mechanical ventilation weaning, discharge from medical ICU and hospital among intensive insulin treatment group of patients.	- Prospective randomized controlled study. - Heterogeneous medical ICU patients. - Single-centre study.
Krinsley,2006 Stamford, Conn, the USA.	Hyperglycemia and patients with critical illness.	5,365 non-cardiac surgical ICU patients.	- Hospital mortality rate was low among patients who plasma glucose level was between 4.0 -5.5 mmol/l, and increased progressively with the increasing in the level of plasma glucose. - The mortality rate was 40% higher among patients with diabetes.	-Prospective cohort study. - Heterogeneous medical, surgical and trauma ICU patients.

			- Hypoglycemic episodes were 1% in control group and 2% in intervention group.	
Cheung and colleagues (2006) New South Wales, Australia.	Hyperglycemia and patients with critical illness.	240 patients with acute myocardial infarction.	- There was no significant difference in mortality rate between two groups. -There were low rate of cardiac failure and of reinfarction rate in the insulin infusion therapy group. -There was 13 hypoglycaemia events in insulin infusion therapy group compare with only 2 events in the conventional therapy group.	-Multicentre open-label randomized controlled clinical study. - 6 hospitals in the state of New South Wales, Australia.
Treggiari *et al*, 2008 Washington/ the USA	Hyperglycemia and patients with critical illness.	10,456 medical, surgical, trauma and neurosurgical ICU patients.	-Plasma glucose at ICU admission was lower after applying of intensive insulin treatment protocol. -The proportions of patients receiving insulin infusion increased dramatically over the study periods. -The average SAPS II and APS III scores were lower in period III than in periods I and II. -Reduction in the requirement of mechanical ventilation. -An increase in hypoglycaemia episodes three to four times from period I to III.	- Cohort study. - Heterogeneous ICU patients.

			-Hypoglycemic was the main cause of death. -The overall ICU mortality rate was significantly different between periods II and III compared with period I. -There was an increase in the mortality rate in patients that stayed ≤ 3 days in ICU. -A policy of intensive insulin therapy in a group of ICUs from a single institution was not associated with a decrease in hospital mortality**.**	
Egi *et al*, 2008 Australia and New Zealand.	Hyperglycemia and patients with critical illness.	4,946 ICU patients.	-There was a strong association between plasma glucose level and the mortality rate in non-diabetic critically ill patients compared with those with diabetes. -The mortality rate was four times greater in patients without diabetes. -This may support the hyperglycaemia may have poor outcome in critically ill patients who do not have pervious history of diabetes.	- Retrospective observational cohort study. - Heterogeneous ICU patients.

The NICE-SUGAR 2009 Australia and New Zealand.	Hyperglycemia and patients with critical illness.	6,104 medical and surgical ICU patients from 42 hospitals.	-Patients treated with intensive glucose control had a higher mortality rate than conventional glucose control patients in 90 days of ICU admission.	- Randomized controlled study. - Evaluation of the ICU mortality rate at 90 days of ICU admission. - Heterogeneous ICU patients.

2. The relationship between glucose concentrations and clinicopathological factors within the context of an insulin protocol in patients with critical illness.

2.1. Introduction

Acute illness leads to a significant metabolic response. An important mechanism is through the enhancement of the catabolic response by the release of pro-inflammatory cytokines such as interleukin-1 (IL-1), interleukin-6 (IL6), interleukin-8 (IL8) and tumour necrosis factor (TNF) (Hill 1999) and counter regulatory hormones such as: catecholamines, cortisol, glucagon, and growth hormone (Desborough 2000). This may in turn lead to hyperglycaemia, glucose intolerance and insulin resistance which are associated with multiple organ dysfunction and increased morbidity and mortality (Vanhorebeek and Van den Berghe 2006; Egi et al. 2008). The overall metabolic effect of the hormonal changes is increased catabolism which mobilises substrates to provide energy sources, and a mechanism to retain salt and water and maintain fluid volume and cardiovascular homeostasis (Desborough 2000).

The elevation in plasma glucose concentrations may occur due to the increase of glucose production in liver and increase insulin resistance in skeletal muscle (Cuthbertson 1979). Moreover, secretion of pituitary hormones and stimulation of sympathetic nervous system appear to have a secondary effect on both the release and action of cortisol, norepinephrine, and epinephrine (Van den Berghe et al. 2001; Vanhorebeek and Van den Berghe 2006), resulting in increase in plasma glucose concentration by increasing glucagon secretion and reducing the insulin secretion.

Finally, the use of some drugs in ICU such as exogenous catecholamines and glucocorticoids may suppress the secretion and the action of insulin, and in turn may inhibit glycogen synthesis, stimulate gluconeogenesis and alter insulin-mediated glucose uptake by tissues. Clearly these exogenous hormones may exacerbate insulin resistance, nitrogen loss and hyperglycaemia (Saberi et al. 2008; Ead 2009; Kavanagh and McCowen 2010; Thomas et al. 2010). The degree of insulin resistance induced by exogenous hormones such as glucocorticoids is greater in the critically ill compared to healthy individuals receiving these drugs (Pretty et al. 2011). Therefore, the clinical situation is complex and involves combinations of endogenous and exogenous hormones leading to alterations of glucose homeostasis and therefore a tendency to produce hyperglycaemia (Khoury and McGill 2011). It is now recognised that hyperglycaemia may directly or indirectly have harmful effects on organs (Van den Berghe et al. 2001). Several observational and interventional studies have shown that most patients with critical illness may suffer from hyperglycaemia and this has a significant effect on outcome (Krinsley 2006). However, it was not clear whether all patients with critical illness are equally susceptible to the adverse effects of hyperglycaemia and whether all would have similar benefit from treatment (Falciglia et al. 2009).

Therefore, the aim of the present study was to evaluate in detail glucose concentrations in patients with critical illness during their ICU admission and to examine the multiple determinants of hyperglycaemia and outcome, including severity of illness, administration and use of drugs that can influence glycaemic control.

2.2. Patients and methods

2.2.1. Patients and study design

Data and stored samples were available from cohorts which had been defined previously. The critical illness cohort comprised of medical or surgical patients ≥ 18 years admitted to the ICU of the Glasgow Royal Infirmary (GRI) between September 2006 and December 2008. At this time the ICU in Glasgow Royal Infirmary had nine beds with an annual admission with approximately 340 patients per year. The median length of stay was 6.9 days and the median predicted mortality rate was 37%. Only patients with an ICU stay of seven days were included in this retrospective observational study.

APACHE II score (Knaus et al. 1985) (predicted hospital mortality and ICU length of stay were recorded. Medical variables included prick (Point of Care (POC) capillary blood glucose concentration, laboratory plasma glucose concentration (Lab-Glucose), CRP, and white cell count, albumin and fluid balance were measured in daily basis in all patients. Drugs that may have influenced plasma glucose were also recorded such as insulin, epinephrine, norepinephrine, dobutamine and hydrocortisone, and the drug doses were recorded on a daily basis. The data were collected manually from the Royal Infirmary ICU computer using Carevue programme, such data was collected as part of routine clinical care.

Albumin was measured by a BCP dye-binding method and C-reactive protein was measured using an automated analyser (Architect, Abbott Diagnostics, USA). For C-reactive protein the limit of detection was 5 mg/l. The inter-assay coefficient of variation was less than 3% and 5% over the sample concentration range for albumin and C-reactive protein respectively. The limit of detection for albumin was 10 g/L.

There were several enteral feeding preparations used in the ICU such as Jevity (1 cal/ml), Jevity Plus (1.2 cal/ml), Jevity 1.5 (1.5 cal/ml) and Osmolite (1 cal/ml), Osmolite Plus (1.2

cal/ml), Peptisorb (1 cal/ml), Perative (1.31 cal/ml) and Nepro (2 cal/ml). Patients routinely received 2L of one of these preparations per day, amounting to an intake of 2000-2500 calories per day. The caloric intake was measured in each patient and presented on a daily basis.

The study was previously approved by the ethics committees of the North Glasgow NHS Trust and Multicentre Research Ethics Committee (MREC) Scotland. Where patients were unable to give signed informed consent, consent was obtained from the patients' next of kin or welfare guardian in accordance with the requirements of the Adults with Incapacity Scotland (2000) Act (Appendix 1, 2).

2.2.2. ICU insulin protocol

In the ICU, Glasgow Royal Infirmary, hyperglycaemia was treated according to Insulin Protocol Ward 25 GRI as of July the 5th 2006. This continued to be used over the period of the study. The target of plasma glucose concentration for critically ill patient in ICU was 4.4- 6.9 mmol/l. In order to maintain this concentration, patients who required insulin infusion were infused with a standard concentration of 50 units of insulin in 50 ml 0.9% saline (Blood glucose control protocol Figure 2-1 p. 91).

2.2.3. Statistical analysis

Data from patients with critical illness was presented as median and range. Comparisons between the medical and surgical critically groups were performed with the use of the Mann-Whitney *U* test. Correlations between variables were performed with the use of the Spearman's rank correlation. Data from different time points in the patient groups were tested for statistical significance with the use of the Wilcoxon signed rank test. All statistical tests were 2-tailed, and because of the number of statistical comparisons a *P*

value of <0.01 was considered to be significant. Analysis was performed with the use of SPSS software (version 19; SPSS Inc, Chicago, IL).

2.3. Results

2.3.1. Study population and baseline characteristics

In total, one hundred adult patients with critical illness (medical n=56, surgical n=44) were studied. With reference to glucose, there was a collection of the finger stick plasma glucose concentrations on each patient. The frequency of these measurements was dependent upon the stability of the patient's plasma glucose, clinical condition and nutrition intake in ICU.

Due to the number of observations only the first 50 patients were examined. In the first 50 patients over the first seven days of ICU stay, there was a total of 3,674 Point of Care (POC) capillary blood glucose measurements. In these patients, there was a median of 11 Point of Care (POC) capillary blood glucose measurements in a 24 hour period. On analysis of these 3,674 Point of Care (POC) capillary blood glucose measurements there were 5 (0.1%) were <2.2mmol/l, 242 (6.6 %) were between 2.2- 4.4 mmol/l, 1747 (47.6%) were between 4.4-6.9 mmol/l, 1,067 (29%) were between 6.9-9.0 mmol/l, and 613 (16.7%) were >9.0 mmol/l. The daily average of these individual POC capillary glucose measurements were consequently correlated with the daily Lab-Glucose measurements. These were significantly associated with each other over days 1-7 (r_s=0.802, P<0.001), (r_s = 0.564, P<0.001), (r_s = 0.428, P<0.01), (r_s = 0.606, P<0.001), (r_s = 0.519, P<0.001), (r_s = 0.441, P<0.01) respectively. Therefore, the daily Lab-Glucose measurements were subsequently correlated with other 24 hour measurements in all 100 patients.

The characteristics of the patients with critical illness on admission are shown in Table 2-1. The majority of patients were male (59%) and over the age of eighteen. The median ICU stay was 11.7 days; the median APACHE II score was greater than 22, and a predicted

hospital mortality rate of ≥ 40%. Sixty five patients survived and thirty five patients died in the ICU.

The admission clinicopathological characteristics of the patients with critical illness are shown in Table 2-1. The median of finger stick glucose measurements was similar to that of the Lab-Glucose measurements (6.4 mmol/l and 6.6 mmol/l respectively), which was above the normal values and close to the upper limit of the target glucose concentration in the insulin protocol that applied in the study period (Figure 2-1 p. 91). The median daily caloric intake was 30.8 Kcalories. The median CRP concentration was elevated at 134 mg/l, median white cell count was close to the upper normal level 11.7 x 10^9, and the median albumin concentration was low with median concentration of 16 g/l. The mean administration of insulin, norepinephrine, epinephrine, hydrocortisone and dobutamine were 0.8 U hour $^{-1}$, 0.5 mg hour^{-1}, 0.1 mg hour^{-1}, 1.5 mg hour^{-1}, 3.4 mg hour^{-1} respectively. The administration of insulin ranged from zero to 9.3 units per hour, norepinephrine from zero to 4.1 mg per hour, epinephrine from zero to 2.7 mg per hour, hydrocortisone from zero to 16 mg per hour and dobutamine from zero to 76.4 mg per hour.

The admission clinicopathological characteristics of surgical and medical patients with critical illness medical are shown in Table 2-2. Compared with medical patients, surgical patients more likely to be male (P <0.05) and had lower insulin administration on admission day (P <0.05). This was also the case in the subsequent six days of ICU stay, which was the mean of insulin administration in medical patients at the second day 2.6 u/hour and in surgical patients 1.2 u/hour (P <0.01), on the third day 2.8 u/hour for medical patients and 1.3 u/hour for surgical patients (P <0.01), 2.8 u/hour versus 1.2 u/hour in the fourth day (P <0.001), 3.0 u/hour versus 1.3 u/hour on the fifth day (P <0.001), 2.7 u/hour versus 1.6 u/hour on the sixth day (P <0.01), and 2.6 versus 1.3 u/hour in the seventh day (P <0.01).

The relationship between plasma glucose and other variables and ICU death is shown in Tables 2-3. Compared with survivors, on the admission day non-survivors were older (P <0.01), had higher albumin concentration (P < 0.01). On multivariate logistic regression analysis of the significant variable identified (Table 2-4), only albumin (OR 0.895, 95%CI 0.825-0.971, P =0.007) was independently associated with death in ICU.

2.3.2. Relationship between plasma glucose and other variables

The inter-relationships between plasma glucose , CRP, white cell count, albumin, fluid balance, insulin, norepinephrine, epinephrine, dobutamine and hydrocortisone were examined and presented in series of supplemental Tables (2-5, 2-6 and 2-7) for the first, fourth and seventh days after admission to ICU.

On the first, forth and seven days after admission to ICU the inter-relationships between plasma glucose , CRP, white cell count, albumin, fluid balance, insulin, norepinephrine, epinephrine, dobutamine and hydrocortisone were examined and presented in series of supplemental Tables (2-5, 2-6 and 2-7).

Plasma glucose was directly associated with insulin on admission day (r_s=0.321, P <0.01, Table 2-5). It was also had a direct associated with epinephrine on admission day (r_s=0.307, P <0.01; Table 2-5), with albumin on the fourth day (r_s =298, P <0.01; Table 2-6), and with fluid balance on the seventh day (r_s =285, P <0.01; Table 2-7). Caloric intake was inversely associated with norepinephrine on admission (r_s = -0.323, P =0.001; Table 2.5).

White cell count was directly associated with norepinephrine on admission day (r_s =0.262, P <0.01; Table 2-5). It was also associated with hydrocortisone on fourth day (r_s= 0.316, P <0.01; Table 2-6), and with fluid balance on the seventh day (r_s= 0.283, P <0.01; Table 2-7). Albumin was inversely associated with body fluid balance on admission day (rs= -

0.538, P <0.001; Table 2-5) and fourth day (r_s = -0.424, P <0.001; Table 2-6). It was also inversely associated with norepinephrine on fourth (r_s = -0.450, P <0.001; Table 2-6) and seventh day (r_s = - 290, P < 0.01; Table 2-7). Fluid balance was directly associated with epinephrine on the fourth day (r_s = 0.285, P <0.01; Table 2-6). It was also associated with norepinephrine on fourth (r_s = 0.595, P <0.001; Table 2-8), and with hydrocortisone on the fourth (r_s =258, P <0.01; Table 2-6) and seventh day (r_s = 0.285, P <0.01; Table 2-7).

Insulin administration was directly associated with hydrocortisone on the seventh day (r_s= 262, P < 0.01; Table 2-7). Norepinephrine was directly associated with dobutamine on admission day (r_s=259, P <0.01; Table 2-5), fourth (r_s= 0.304, P <0.01; Table 2-6) and seventh day (r_s= 0.277, P <0.01; Table 2-7), and with hydrocortisone on admission day (r_s= 0.499, P <0.001; Table 2-5), fourth (r_s= 0.359, P <0.001; Table 2-6) and seventh day (r_s= 308, P <0.01; Table 2-7). Epinephrine was directly associated hydrocortisone on admission day (r_s=391, P <0.01; Table 2-5) and seventh day (r_s= 0.272, P <0.01; Table 2-7).

Of the 100 patients who were admitted in the ICU, they were had two further follow up samples during their ICU stay first was on forth day and last was on seventh day (Tables 2-8 and 2-9 respectively). On the first follow up there was a significant increase in caloric intake (P <0.001), low albumin (P = 0.005), positive fluid balance (P =0.007) and high insulin administration (P < 0.001). On the last follow up there was a significant increase in caloric intake (P <0.001), low albumin (P = 0.011), high insulin administration (P < 0.001) and low norepinephrine and epinephrine administration (P <0.001, P = 0.003 respectively).

2.4. Discussion

The results of the present study show that, using a standard insulin protocol in patients with critical illness, few patients had glucose concentrations outwith the target results and in particular hypoglycaemia was a rare event. This was consisted with the target glucose concentration being in the range defined as tight control. Despite this tight insulin control, plasma glucose in patients with critical illness was also dependent on several variables including caloric intake and administration of corticosteroids.

These results are consistent with the work of Duska and Andel (2008) also reported that in a small study of 20 patients, that plasma insulin concentrations were determined more by endogenous secretion than insulin infusion, even during the systemic inflammatory response when exogenous insulin requirements are high (Duska and Andel 2008). For example, insulin administration may be interrupted and other drugs such as epinephrine and norepinephrine given that may destabilize the plasma glucose concentration (Braithwaite et al. 2006).

In the last two decades several studies have reported a relationship between the elevation of plasma glucose concentrations in patients with critical illness and their poor outcomes regarding a variety of settings and disorders including post-operative ICU surgical cardiac patients (Van den Berghe et al. 2001) post-operative ICU non cardiac patients (Krinsley 2006), mixed population from surgical and medical ICU (Scott et al. 1999; Krinsley 2004), myocardial infarction (Malmberg 1997; Cheung et al. 2006) , stroke (Malmberg 1997; Cheung et al. 2006) trauma (Scott et al. 1999), and neurosurgical ICU patients (Scott et al. 1999).

In view of this information more rigorous hyperglycaemia management protocols using insulin therapy have been undertaken in patients with critical illness (Van den Berghe et al. 2001). These studies reported that intensive insulin therapy reduced the morbidity and

mortality rate regardless of those patients had a previous history of diabetes or not (Van den Berghe et al. 2001; Krinsley 2004; Krinsley 2006; Van den Berghe et al. 2006; Treggiari et al. 2008). However, some of these studies, such as the original Van den Berghe et al (2001) paper and Preiser et al (2009) have been criticised because the population were predominately post cardiac surgery. Indeed, it was already well known that following myocardial injury hyperglycaemia had significant harmful effects and associated with poor outcomes (Diabetes Mellitus Insulin-Glucose Infusion in Acute Myocardial Infarction Study, (Malmberg 1997; Malmberg et al. 2005). Therefore, studies which have a population of patients with cardiac injury may not be representative of all critically ill patients and in particular those with sepsis. Other studies have major limitations, Krinsley et al (2004) demonstrated a substantial mortality reduction (an absolute reduction of over 6% and a relative reduction of 29.3%), and this was however based on historical controls which severely reduce the validity of any conclusions regarding cause and effect. The SPRINT study also demonstrated significant improvements in outcome but was also based on historical controls (Chase et al. 2008).

More recent studies such as NICE-SUGAR Study (2009) have reported that patients who had intensive glucose control with plasma glucose target between 4.4-6.0 mmol/l had a higher mortality rate than conventional glucose control patients with plasma glucose target <10 mmol/l within 90 days of ICU admission. Also, severe hypoglycaemic episodes, where plasma glucose < 2.2 mmol/l, were more common in the intensive glucose control group (Van den Berghe et al. 2003; Cheung et al. 2006). However, the cause behind the increasing of mortality rate among intensive glucose control group of patients was not clear, whether due to the reduction of plasma glucose concentration, the increase of insulin administration, the occurrence of hypoglycaemia or due to the study methodology (The NICE-SUGAR Study Investigators 2009). Further analysis of the data from the NICE Sugar trial confirmed the association between the hypoglycaemia and outcome but could

not establish as causal link (The NICE-SUGAR Study Investigators 2009; Finfer et al. 2012). Several recent studies, have also studied factors influencing glycaemic control. These studies have significant limitations, for example Dossett et al (2008) did not report on the intake of calories from nutrition support and Egi et al (2009) did not include any analysis of the drugs likely to influence insulin sensitivity (Dossett et al. 2008; Egi et al. 2009). Therefore, the treatment of hyperglycaemia in critically ill patients remains a controversial issue (Mehta et al. 2005). Moreover, the frequency of hyperglycaemia and/or hypoglycaemia and the association with the other medical conditions that occur in patients with critical illness requiring insulin therapy is not clear.

In a much larger study Flaciglia reported that hyperglycaemia was associated with severity of illness and outcome. This study looked at over 250,000 patients in 153 critical care units. Their definition of hyperglycaemia was based upon the averages of all of the laboratory glucose measurements made for each patient. Their findings indicated that in some patient's hyperglycaemia was more difficult to treat and that hyperglycaemia was more strongly associated with a poor outcome in some medical diagnoses compared with surgical patients. Neither this nor the present study established whether the resistance to insulin per se or administering high dose of insulin is a cause of poor outcomes. Indeed, in the NICE sugar study a unique concern was that excessive insulin administration in the context of a tight glucose control protocol was the cause of the excess mortality.

In the present study it was of interest that, on admission, surgical patients had less requirement for insulin to control glucose concentration on admission and over the subsequent 6 days, they also had less catecholamine administration. It was of also of interest that, at the daily level, when a counter-regulatory hormone such as norepinephrine was given there was a tendency for other counter-regulatory hormones to be given. Indeed, counter-regulatory hormone administration and fluid balance are the essential aspect of the treatment of critically ill patient with hypovolaemic shock in order to increase

cardiac output and systemic vascular resistance. Khoury and McGill (2011) reported that norepinephrine led to a reduction in insulin sensitivity in non-critically ill patients and suggested that during the haemodynamic support in the treatment of acute illness the insulin administration rate will need to be increased or decreased according to the administration or discontinuation of catecholamines (Khoury and McGill 2011). Therefore, insulin protocols may not take into account the physiological changes that may occur due to the drugs administration in the intensive care setting such as catecholamines, which in turn, it may increase the risk of both hypoglycaemia and hyperglycaemia. This may be avoided with better understanding of the metabolic effect of these drugs (Khoury and McGill 2011). However, this had a small study sample (n=11) and therefore it is difficult to generalize their findings to all critically ill patients. Nevertheless, as norepinephrine was used more frequently in medical patients there may be a relationship between this and hyperglycaemia treatment. Furthermore, as most of the studies that have investigated the benefits of glucose control using insulin in patients with critical illness have been in surgical patients this may suggest that medical patients behave differently. This may be related to the cause of illness, or its management. Taken together these findings would suggest that surgical and medical patients should be considered separately both in terms of the clinical protocol and research investigations of hyperglycaemia control in patients with critical illness.

In the present study, using detailed glucose analysis over 7 days, approximately 48% of plasma glucose concentrations were within the target range of 4.4-6.9 mmol/l, 7% were below and 45% were above the target range. More recent insulin protocols have widened the target range with the intention of reducing the frequency of hypoglycaemia. Of note in the present study was that the occurrence of clinical hyperglycaemia was very low. In the SPRINT study that also studied tight glycaemic control in a general ICU there was a similar frequency of severe hypoglycaemia. However, the incidence of moderate

hypoglycaemia between 2.2 mmol/l and 4.4 mmol/l was much higher in the SPRINT study 12.8% v 6.6%. Similar to the present study they could not identify an association between glycaemia and mortality when the tight glycaemic protocol was used. In the SPRINT study the analysis of glycaemic control was over the entire ICU stay rather than on a daily basis and the use of drugs that influence glycaemia apart from insulin was not examined.

It was also of interest that there was a significant relationship between albumin, fluid balance and catecholamines in the present study. This may have been expected to occur due to the development of systemic inflammatory response syndrome, capillary leak and vasodilatation, which was treated with fluids and counter-regulatory hormones administration.

Limitations of the present study were principally that it was a single centre study and a relatively small sample size. Furthermore, the cohort was mixed in term of surgical and medical patients and there was considerable variation in the severity of illness.

In summary, the present study differs from previous studies of glycaemic control in patients with critical illness in its detailed daily examination of glycaemic control. It has been shown that, despite concern over the accuracy of near patient testing of glycaemia, there was a direct relationship between the results of fingertip testing and laboratory measured glucose as measures of glycaemic control. Also, that insulin and counter regulatory hormone administration and caloric intake accounted for only a small part of the daily fluctuation of glucose concentrations in these patients with critical illness. Therefore, it would appear that a plasma glucose concentration, even within the context of an insulin protocol, is complex and multifactorial in patients with critical illness.

Table 2-1: Patient clinical characteristics on admission to ICU.

	Reference Intervals	Patients with critical illness (n=100)
Age (Year)	N/A	60 (18-86)
Gender (Male/Female)	N/A	59/41
ICU stay (Days)	N/A	11.7 (4.2-76.4)
Predicted mortality rate (%)	N/A	39.9 (2.0-95.2)
APACHE II	N/A	22 (6-40)
Admission cause (Surgical/Medical)	N/A	44/56
Alive/Dead	N/A	65/35
Finger stick glucose (mmol/l)	3.5-5.5	6.4 (2.9-16.7)
Lab-Glucose (mmol/l)	3.5-5.5	6.6 (2.8-30.6)
Intake (Kcalories/ml)	N/A	30.8 (10-83)
C-reactive protein (mg/l)	<10	134 (2-565)
White cell count (10^9/L)	4-11 x10^9	11.7 (0.5-45.4)
Albumin (g/l)	35-55	16 (10-39)
Fluid balance (ml)	N/A	856 (-176-5182)
Insulin (U hour $^{-1}$) [a]	N/A	0.8 (0.0-9.3)
Norepinephrine (mg hour^{-1}) [a]	N/A	0.5 (0.0-4.1)
Epinephrine (mg hour^{-1}) [a]	N/A	0.1 (0.0-2.7)
Hydrocortisone (mg hour^{-1}) [a]	N/A	1.5 (0.0-16.0)
Dobutamine (mg hour^{-1}) [a]	N/A	3.4 (0.0-76.4)

Median (range), [a] Mean (range).

Table 2-2: Patients clinical characteristics on admission to ICU according to specialities.

	Reference Intervals	Medical (n=56)	Surgical (n=44)	P-value
Age (Year)	N/A	57 (18-81)	62 (20-86)	0.249
Sex (Male/Female)	N/A	28/28	31/13	0.040
ICU stay (Days)	N/A	12 (4.6-65.1)	11.1 (4.2-76.4)	0.715
APACHE II	N/A	22 (9-38)	21 (6-40)	0.087
Predicted mortality rate (%)	N/A	41.9 (4.9-92.6)	37.8 (2.0-95.2)	0.181
Alive/Dead (%)	N/A	36/20 (36%)	29/15 (34%)	0.867
Finger stick glucose (mmol/l)	3.5-5.5	6.8 (3.4-16.7)	6.1 (2.9-9.9)	0.189
Lab-Glucose (mmol/l)	3.5-5.5	6.7 (2.8-30.6)	6.5 (3.1-13.2)	0.513
Intake (Kcalories/ml)	N/A	30.8 (10-80)	30.6 (10-83)	0.953
C-reactive protein (mg/l)	<10	134 (2-565)	132 (2-447)	0.865
White cell count (10^9/l)	4-11 x109	11.5 (0.5-45.5)	11.7 (2.4-31.3)	0.576
Albumin (g/l)	35-55	18 (10-39)	15 (10-32)	0.062
Fluid balance (ml)		781 (-176-4946)	941 (-25-5182)	0.650
Insulin (u/hour) [a]	N/A	1.2 (0.0-9.3)	0.4 (0.0-6.0)	0.036
Norepinephrine (mg $hour^{-1}$) [a]	N/A	0.5 (0.0-4.1)	0.6 (0.0-4.0)	0.967
Epinephrine (mg $hour^{-1}$) [a]	N/A	0.2 (0.0-2.7)	0.1 (0.0-1.6)	0.056
Hydrocortisone (mg $hour^{-1}$) [a]	N/A	2.0 (0.0-16.0)	0.9 (0.0-8.0)	0.122
Dobutamine (mg $hour^{-1}$) [a]	N/A	3.7 (0.0-76.4)	0.1 (0.0-50.0)	0.627

Median (range), [a] Mean (range).

Table 2-3: Patient characteristics and measurements between survivors and non-survivors on admission to ICU.

	Admission Survivors (n=65)	Admission Non-survivors (n=35)	P-value
Age (year)	57 (18-86)	67 (20-81)	0.008
Sex (Male/Female)	40/25	19/16	0.484
Patients (Medical/Surgical)	36/29	20/15	0.867
APACHE II	21 (6-40)	23 (10-34)	0.098
Predicted mortality rate (%)	34.8 (2.0-95.2)	42.5 (11.7-87.5)	0.113
ICU stay (Days)	11.0 (4.2-76.4)	12.8 (4.6-59.5)	0.515
Finger stick glucose (mmol/l)	6.6 (2.9-16.7)	6.1 (3.8-13.9)	0.285
Lab-Glucose (mmol/l)	6.6 (3.1-30.6)	6.6 (2.8-26.4)	0.591
Intake (Kcalories/ml)	30.0 (10-63)	33.7 (10-83)	0.211
C-reactive protein (mg/l)	122 (2-447)	141 (2-565)	0.908
White cell count (10^9/l)	10.8 (0.5-45.4)	12.2 (3.3-33.1)	0.435
Albumin (g/l)	19 (10-39)	14 (10-28)	0.005
Fluid balance (ml)	586 (-176-4663)	861 (-160-5182)	0.665
Insulin (U hour $^{-1}$) [a]	0.7 (0.0-9.3)	1.0 (0.0-8.0)	0.159
Norepinephrine (mg hour^{-1}) [a]	0.6 (0.0-4.1)	0.4 (0.0-4.0)	0.543
Epinephrine (mg hour^{-1}) [a]	0.1 (0.0-2.7)	0.1 (0.0-1.0)	0.701
Hydrocortisone (mg hour^{-1}) [a]	2.0 (0.0-16.0)	0.7 (0.0-8.0)	0.071
Dobutamine (mg hour^{-1}) [a]	4.2 (0.0-76.4)	2.0 (0.0-50.0)	0.532

Median (range), [a] Mean (range).

Table 2-4: The relationship between admission characteristics, the parameter, drugs and hospital mortality in patients with critical illness. Univariate and multivariate binary logistic regression analysis.

	Univariate analysis		Multivariate analysis	
	OR (95%CI)	P-value	OR (95%CI)	P-value
Age (years)	1.03 (1.004-1.06)	0.026		
Sex (male/female)	1.35 (0.586-3.09)	0.482		
Patients (medical/surgical)	1.07 (0.469-2.461)	0.866		
APACHE II score	1.05 (0.990-1.112)	0.103		
Predicted mortality rate (%)	1.013 (0.996-1.03)	0.135		
ICU length of stay (days)	1.008 (0.978-1.04)	0.596		
Lab-Glucose (mmol/L)	1.026 (0.927-1.136)	0.616		
Intake (K calories/ml)	1.023 (0.991-1.056)	0.154		
C-reactive protein (mg/l)	1.001 (0.997-1.004)	0.782		
White cell count (10^9/l)	1.012 (0.996-1.062)	0.608		
Albumin (g/l)	0.895 (0.830- 0.970)	0.007	0.895 (0.825-0.971)	0.007
Fluid balance (ml)	1.000 (1.000-1.001)	0.256		
Insulin (u/hour)	1.102 (0.877-1.384)	0.405		
Norepinephrine (mg $hour^{-1}$)	0.834 (0.505-1.376)	0.477		
Epinephrine (mg $hour^{-1)}$	0.905 (0.317-2.582)	0.851		
Hydrocortisone (mg $hour^{-1}$)	0.866 (0.738-1.017)	0.080		
Dobutamine (mg $hour^{-1}$)	0.982 (0.940-1.025)	0.400		

Table 2-5: The relationship between the parameter and drugs. Lab-Glucose, CRP, white cell count, Albumin, fluid balance, insulin, norepinephrine, epinephrine, hydrocortisone, and dobutamine in critically ill patients on admission day (n=100).

	Lab-Glucose	Caloric intake	CRP	White cell count	Albumin	Fluid balance	Insulin	Norepinephrine	Epinephrine	Hydrocortisone	Dobutamine
Lab-Glucose		0.101	-0.155	0.0101	-0.062	-0.109	0.321**	-0.023	0.307**	0.206*	0.176
Caloric intake			-0.093	-0.067	-0.019	0.160	0.136	-0.323**	-0.041	-0112	0.133
CRP				0.13	-0.17	0.112	-0.106	0.051	0.172	0.168	-0.115
White cell count					0.057	0.062	0.174	0.262**	-0.015	0.177*	0.093
Albumin						-0.538**	0.062	-0.250*	-0.025	-0.245*	0.128
Fluid balance							-0.116	0.119	0.035	0.067	-0.384*
Insulin								-0.002	0.249*	0.08	0.098
Norepinephrine									0.189	0.499***	0.259**
Epinephrine										0.391**	0.226*
Hydrocortisone											0.17
Dobutamine											

*p<0.05, **p<0.01, ***p<0.001, correlations between variables in the critically ill patients were carried out using the Spearman rank correlations (r_s).

Table 2-6: The relationship between the parameter and drugs Lab-Glucose, CRP, white cell count, albumin, fluid balance, insulin, norepinephrine, epinephrine, hydrocortisone, and dobutamine in critically ill patients on day 4 (n=100).

	Lab-Glucose	Caloric intake	CRP	White cell count	Albumin	Fluid balance	Insulin	Norepinephrine	Epinephrine	Hydrocortisone	Dobutamine
Lab-Glucose		0.014	0.004	0.027	0.298**	-0.162	0.033	-0.104	-0.022	0.101	0.109
Caloric intake			-0.079	-0.071	0.111	-0.031	0.184	-0.128	-0.066	-0.131	-0.058
CRP				0.038	-0.141	0.019	-0.013	0.07	0.072	-0.159	0.041
White cell count					-0.193	0.072	0.111	0.215*	-0.135	0.316**	0.061
Albumin						-0.424***	0.141	-0.382***	-0.036	-0.076	-0.088
Fluid balance							0.075	0.595***	0.285**	0.258*	0.185
Insulin								0.151	-0.169	0.039	0.037
Norepinephrine									0.246*	0.359***	0.304**
Epinephrine										0.026	0.087
Hydrocortisone											0.02
Dobutamine											

*p<0.05, **p<0.01, ***p<0.001, correlations between variables in the critically ill patients were carried out using the Spearman rank correlations (r_s)

Table 2-7: The relationship between the parameter and drugs Lab-Glucose, CRP, white cell count, albumin, fluid balance, insulin, norepinephrine, epinephrine, hydrocortisone, and dobutamine in critically ill patients on day 7 (n=100).

	Lab-Glucose	Caloric intake	CRP	White cell count	Albumin	Fluid balance	Insulin	Norepinephrine	Epinephrine	Hydrocortisone	Dobutamine
Lab-Glucose		0.125	0.081	0.137	0.069	0.285	0.148	0.09	-0.006	0.167	-0.034
Caloric intake			-0.127	-0.114	-0.029	-0.101	0.226	0.048	-0.006	-0.207	0.127
CRP				0.169	-0.174	-0.009	0.044	0.067	0.165	-0.023	0.141
White cell count					-0.194	0.283**	-0.038	0.116	-0.06	0.158	0.05
Albumin						-0.275*	0.034	-0.290**	-0.164	-0.041	0.013
Fluid balance							0.154	0.258*	0.087	0.285**	-0.02
Insulin								0.114	0.041	0.262**	0.014
Norepinephrine									-0.04	0.308**	0.277**
Epinephrine										0.272**	-0.01
Hydrocortisone											-0.037
Dobutamine											

*p<0.05, **p<0.01, ***p<0.001, correlations between variables in the critically ill patients were carried out using the Spearman rank correlations (r_s).

Table 2-8: The relationship between patient characteristics and plasma glucose concentrations in patients with critical illness on admission and day 4 after admission.

	Admission (n=100)	Follow up (n=100)	P-value[b]
Age (Year)	60 (18-86)		
Gender (Male/Female)	59/41		
ICU stay (Days)	11.7 (4.2-76.4)		
Predicted mortality rate (%)	39.9 (2.0-95.2)		
APACHE II	22 (6-40)		
Admission cause (Surgical/Medical)	44/56		
Finger stick glucose (mmol/l)	6.4 (2.9-16.7)	6.4 (3.9-20.0)	0.290
Lab-Glucose (mmol/l)	6.6 (2.8-30.6)	6.5 (3.7-19.5)	0.046
Intake (K calories/ml)	30.8 (10-83)	67.7 (20-104)	<0.001
C-reactive protein (mg/l)	134 (2-565)	116 (18-430)	0.440
White cell count (10^9/l)	11.7 (0.5-45.4)	11.7 (2.3-64.3)	0.363
Albumin (g/l)	16 (10-39)	14 (10-38)	0.005
Fluid balance (ml)	856 (-176-5182)	1881 (-10268-9629)	0.007
Insulin (U hour $^{-1}$) [a]	0.8 (0.0-9.3)	2.1 (0.0-17.0)	<0.001
Norepinephrine (mg hour^{-1}) [a]	0.5 (0.0-4.1)	0.4 (0.0-4.9)	0.032
Epinephrine (mg hour^{-1}) [a]	0.1 (0.0-2.7)	0.04 (0.0-1.0)	0.014
Hydrocortisone (mg hour^{-1}) [a]	1.5 (0.0-16.0)	2.3 (0.0-16.0)	0.071
Dobutamine (mg hour^{-1}) [a]	3.4 (0.0-76.4)	3.0 (0.0-117.0)	0.683

Median (range), [a] Mean (range), [b] Wilcoxon rank test.

Table 2-9: The relationship between patient characteristics and plasma glucose concentrations in patients with critical illness on admission and day 7 after admission.

	Admission (n=100)	Follow up (n=100)	P-value [b]
Age (Year)	60 (18-86)		
Gender (Male/Female)	59/41		
ICU stay (Days)	11.7 (4.2-76.4)		
Predicted mortality rate (%)	39.9 (2.0-95.2)		
APACHE II	22 (6-40)		
Admission cause (Surgical/Medical)	44/56		
Finger stick glucose (mmol/l)	6.4 (2.9-16.7)	6.5 (3.1-12.1)	0.109
Lab-Glucose (mmol/l)	6.6 (2.8-30.6)	6.2 (3.8-10.3)	0.024
Intake (K calories/ml)	30.8 (10-83)	96.1 (8.6-100)	<0.001
C-reactive protein (mg/l)	134 (2-565)	108 (10-397)	0.097
White cell count (10^9/l)	11.7 (0.5-45.4)	12.7 (4.6-53.1)	0.133
Albumin (g/l)	16 (10-39)	14 (10-35)	0.011
Fluid balance (ml)	856 (-176-5182)	806 (-2420-6303)	0.416
Insulin (U hour $^{-1}$) [a]	0.8 (0.0-9.3)	2.0 (0.0-9.6)	<0.001
Norepinephrine (mg hour^{-1}) [a]	0.5 (0.0-4.1)	0.1 (0.0-4.9)	<0.001
Epinephrine (mg hour^{-1}) [a]	0.1 (0.0-2.7)	0.01 (0.0-0.9)	0.003
Hydrocortisone (mg hour^{-1}) [a]	1.5 (0.0-16.0)	1.0 (0.0-8.0)	0.178
Dobutamine (mg hour^{-1}) [a]	3.4 (0.0-76.4)	0.5 (0.0-45.0)	0.015

Median (range), [a] Mean (range), [b] Wilcoxon rank test.

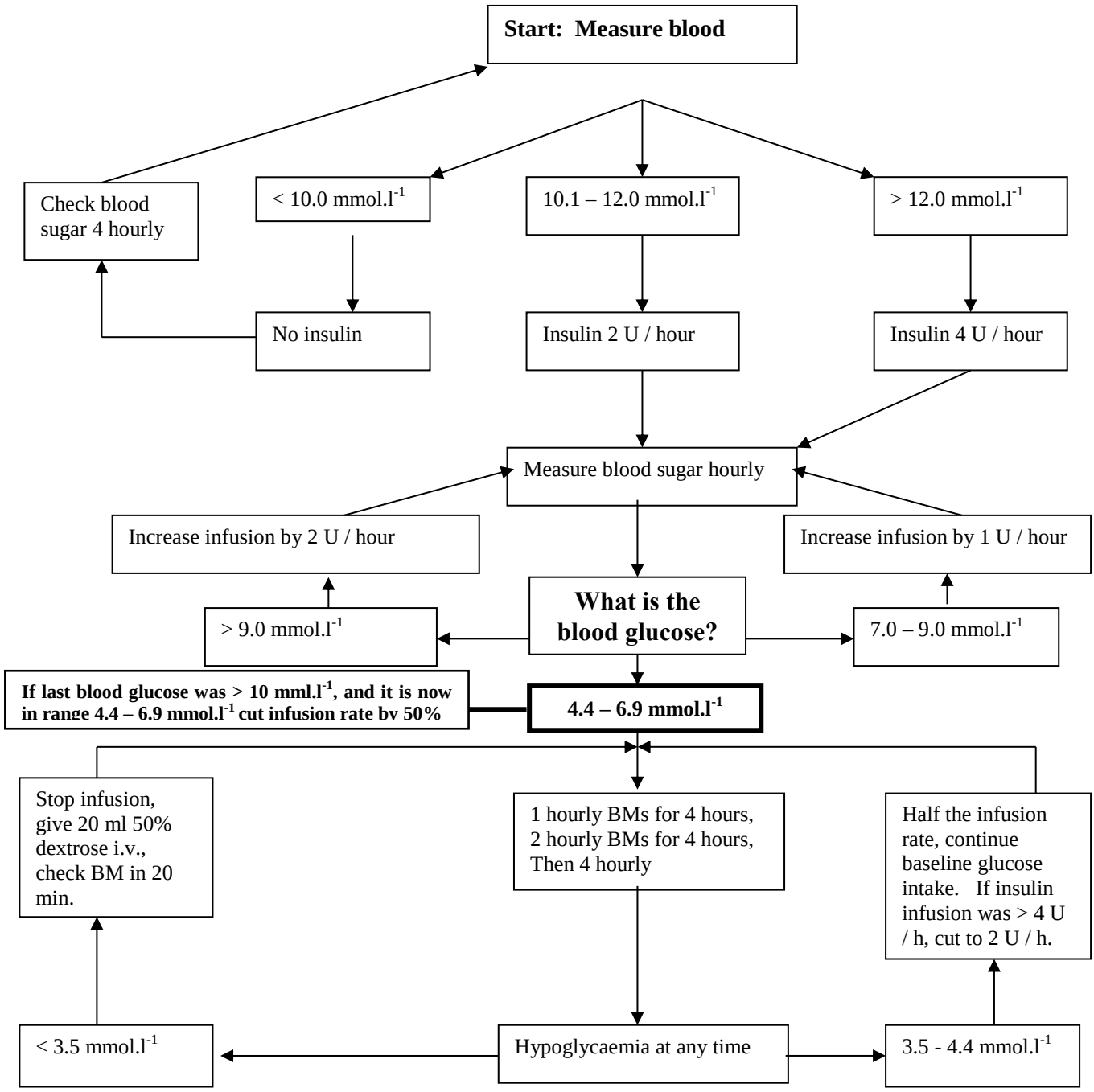
Figure. 2-1: Blood glucose control protocol, Insulin Protocol Ward 25 GRI (2006).

Patients admitted to ICU are frequently hyperglycaemic. This occurs for a number of reasons: reduced insulin production and sensitivity, severity of the acute illness, and the concurrent administration of other drugs affecting sensitivity to insulin.

We know that maintenance of normoglycaemia reduces both morbidity and mortality. The target for blood sugar of patients in ICU is 4.4 to 6.9 mmol.l^{-1}. This will require most patients admitted to ICU to receive insulin. The following guidelines aim to help us achieve this new target.

Insulin infusion, the standard concentration is 50 units insulin in 50 ml 0.9% saline. After ICU admission until normoglycaemia (4.4 to 6.9 mmol.l^{-1}) is reached 1-hourly blood sugar measurement is required.

All patients with blood sugar < 10 mmol.l^{-1} require either enteral feed (> 30 ml.h^{-1}) or 5% dextrose at 100 ml.h^{-1}.



3. The relationship between plasma asymmetrical dimethylarginine metabolism and patients with critical illness outcome.

3.1. Introduction

Patients with critical illness are often hypercatabolic and hyperglycaemic, and these metabolic disorders have been shown to have a significant influence on patient outcome (Siroen et al. 2005b). Furthermore, several studies have reported the beneficial effect of the treatment of metabolic response and controlling hyperglycaemia that consequently reduce the morbidity and mortality (Van den Berghe et al. 2001; Siroen et al. 2005b). However, the mechanisms by which these beneficial effects are brought about remain unclear. One possible mechanism is the amelioration of endothelial dysfunction related to endogenous nitric oxide synthase (NOS) inhibitors (Nijveldt et al. 2003; Siroen et al. 2005a; Ellger et al. 2008).

Endothelial dysfunction has been reported to be associated with an increase in insulin resistance and the circulating asymmetric dimethylarginine (ADMA). This may lead to a reduction in the bioavailability of nitric oxide (NO) (Figure 3-1) (Blackwell 2010; Siervo et al. 2011). An increase in ADMA concentrations and endothelial dysfunction have been associated with a broad range of several clinical disorders such as obesity, cardiovascular disease, chronic renal failure, diabetes and hypertension (Boeger 2006; Blackwell 2010; Siervo et al. 2011). Therefore, ADMA may play a role in developing of insulin resistance. This is consistent with the report that plasma ADMA concentration was increased in patients with impaired glucose tolerance (Nijveldt et al. 2003), diabetes (Can et al. 2011) and overweight individuals (Nijveldt et al. 2003). In addition, weight loss was associated with improved the insulin sensitivity and reduced plasma ADMA concentration (Nijveldt et al. 2003). It is of interest that, in patients with critical illness an elevated ADMA

concentration was associated with increased mortality (Nijveldt et al. 2003). Also, significant reduction in ADMA concentrations in critically ill patients who received intensive insulin treatments compared with patients who were treated conventionally. This reduction was associated with a significant improvement in mortality (Siroen et al. 2005b; Ellger et al. 2008). Therefore, there is evidence that tight regulation of glucose levels by intensive insulin treatment preserves dimethylaminohydrolase (DDAH) activity (Siroen et al. 2005b). Moreover, insulin may decrease the degradation of arginine and thereby reduce the production of ADMA (Siroen et al. 2005b). In contrast, Iapichino and co-workers reported that intensive insulin treatments, while achieving tight glucose control, did not reduce ADMA levels in patients with septic shock who were fed with no more than 25 kCal/kg per day (Iapichino et al. 2008).

It has been reported during acute inflammatory response of elective surgery plasma ADMA concentration decreases rapidly in the first 48 hours (Blackwell et al. 2011). It has also been shown that inflammatory markers, such as interleukin-6 (IL-6), CRP and tumour necrosis factor-α are inversely associated with ADMA concentrations and the ADMA:SDMA ratio, and this has been proposed to be the result of increased catabolism induced by an inflammatory response (Blackwell et al. 2009; Iapichino et al. 2010).

The aims of the present study were to examine the relationships between the concentrations of plasma ADMA and related compounds (homoarginine, arginine, SDMA), length of stay and mortality in patients with critical illness.

3.2. Patients and methods

Patients in the ICU of the Glasgow Royal Infirmary who were ≥ 18 years old, and had evidence of the systemic inflammatory response syndrome as per Bone's criteria (Bone et al. 1992; Levy et al. 2003), were studied. The study was approved by the ethics committees of the North Glasgow NHS Trust and Multicentre Research Ethics Committee (MREC) Scotland. Where patients were unable to give signed informed consent, consent was obtained from the patients' next of kin or welfare guardian in accordance with the requirements of the Adults with Incapacity Scotland (2000) Act (Appendix 1, 2).

Blood samples for population references values were obtained from laboratory staff, from local health centres and from people attending a cardiovascular risk clinic. None of the subjects were taking any vitamin supplements or evidence of a systemic inflammatory response (serum CRP <10 mg/ l) and previously reported (Blackwell et al. 2009)

3.2.1. Data collection.

Baseline demographics including age, ICU admission cause, and ICU length of stay were extracted from ICU computer using Carevue programme. Such data was collected as part of routine clinical care including laboratory plasma glucose, CRP, white cell count, albumin, creatinine, alanine aminotransferase (ALT) and liver function tests. Drugs and their dosages that may have influenced plasma glucose were also recorded such as insulin, epinephrine, norepinephrine, dobutamine and hydrocortisone. APACHE II score and predicted hospital mortality and SOFA scores were recorded.

3.2.2. ICU insulin protocol

Hyperglycaemia was treated according to a standardised insulin Protocol (Figure 2-1, p 91). The same protocol was used over the period of the study. The target of plasma glucose concentration for critically ill patient in ICU was 4.4- 6.9 mmol/l. In order to maintain this concentration, patients who required insulin infusion were infused with a standard concentration of 50 units of insulin in 50 ml 0.9% saline.

There were several parenteral feeding preparations used in the ICU such as Jevity (1 cal/ml), Jevity Plus (1.2 cal/ml), Jevity 1.5 (1.5 cal/ml) and Osmolite (1 cal/ml), Osmolite Plus (1.2 cal/ml), Peptisorb (1 cal/ml), Perative (1.31 cal/ml) and Nepro (2 cal/ml). Patients routinely receive 2L of one of these preparations per day, amounting to an intake of 2000-2500 calories per day.

3.2.3. Analytical methods

Collection and preparation of blood samples.

Venous blood samples (EDTA) were withdrawn on admission (day 1) and follow up sample which was the last day in ICU sample (died or discharge sample) (median 7, IQR 6-8, rang 5-15) for the analysis of plasma ADMA, arginine, homoarginine and SDMA. Blood samples were centrifuged (2500 g, 10 minutes), the plasma removed and stored at -70°C until analysis. Blood samples that formed part of patients' clinical care were handled in the usual way according to established standard operating procedures in the routine hospital laboratory.

Laboratory analysis

ADMA, arginine, homoarginine and SDMA were measured using isocratic high performance liquid chromatography (HPLC) with fluorescence detection as previously

described (Blackwell et al. 2009). The inter-assay coefficients of variation (CV) were approximately 3.0%. Reference values for the studied analytes were derived from 86 healthy people, and have already been published (Blackwell et al. 2009). These were used to construct reference intervals with which to compare the present results in patients with critical illness.

Plasma glucose level, white cell count, creatinine, alanine aminotransferase (ALT) were measured in accordance with the manufacturer's instructions on an automated analyzer (Architect; Abbott Diagnostics, Abbott Park, Chicago, IL) in the routine biochemistry laboratory which is accredited (Clinical Pathology Accreditation UK). For CRP, the limit of detection was 0.5 mg/L.

Albumin was measured by a BCP dye-binding method and C-reactive protein was measured using an automated analyser (Architect, Abbott Diagnostics, USA). For C-reactive protein the limit of detection was 5 mg/l. The inter-assay coefficient of variation was less than 3% and 5% over the sample concentration range for albumin and C-reactive protein respectively. The limit of detection for albumin was 10 g/L.

3.2.4. Statistical analysis

As the data was not normally distributed and to use a consistent statistical approach throughout the thesis the data was presented as median (median, IQR) and non-parametric testing used as appropriate. The relationship between patient characteristics and plasma arginine, homoarginine ADMA and SDMA concentrations were carried out with the use of the Mann-Whitney U test. Correlations between variables in the control and critically-ill groups were carried out using the Spearman rank correlation. Data from different time points in the patient groups were tested for statistical significance with the use of the Wilcoxon signed rank test. Outcome data were analysed by binary logistic regression

analysis. Analysis was performed with the use of SPSS software (version 19; SPSS Inc., Chicago, IL).

3.3. Results

In total, one hundred and four adult patients with critical illness (medical n=44, surgical n=60) admitted to Glasgow Royal Infirmary ICU in the period between September 2006 to March 2008 were studied. The characteristics of the patients with critical illness on admission were shown in Table 3-1. The majority of patients were male (69%) and had median age of 61 years. The median APACHE II score was 21, predicted hospital mortality rate was 39% and SOFA score was 7, 10 and 5 patients were on severe sepsis and septic shock respectively. Compared with controls (n=86), patients with critical illness had a lower median arginine and homoarginine concentrations and higher ADMA and SDMA concentrations (all $p< 0.001$). Also, critically ill patients had lower ADMA: SDMA and arginine: ADMA ratios and higher total dimethylarginine (sum of ADMA and SDMA) concentrations (both $p< 0.001$).

The median creatinine was close to the upper normal range, the median bilirubin was within the normal range and the median of alanine transaminase (ALT) was above the normal range. The median of plasma glucose measurements was above the normal range and close to the upper limit of the target glucose concentration in the insulin protocol that was applied during the study period. The median CRP concentration was elevated, median white cell count was close to the upper normal range, and the median albumin concentration was low. The median daily fluid balance was positive 1034 ml.

The mean infusion rate of insulin, norepinephrine, epinephrine, dobutamine and hydrocortisone were 0.9 Unit hour -1, 0.5 mg hour -1, 0.1 mg hour -1, 2.5 mg hour -1 and 1.9 mg hour -1 respectively. The administration of insulin ranged from zero to 9.3 units per hour, norepinephrine from zero to 6.0 mg per hour, epinephrine from zero to 2.7 mg per hour, dobutamine from zero to 76.4 mg per hour and hydrocortisone from zero to 16

mg per hour. The median ICU length of stay was 5.1 days. Seventy eight patients survived and twenty six patients died in the ICU.

The relationship between patient characteristics, plasma ADMA concentrations, other variables and ICU death was shown in Table 3-2. Non-survivors had significantly higher SOFA scores ($P < 0.001$), higher ADMA, SDMA and total dimethylarginine concentrations (all $P < 0.05$), lower homoarginine concentrations ($P < 0.028$) and lower albumin concentrations ($P < 0.017$). They also had a longer ICU length of stay ($P < 0.001$).

Univariate binary logistic regression analysis of clinical characteristics and dimethylarginine concentrations on admission to ICU and ICU death in critically ill patients was shown in Table 3-3. On univariate binary logistic regression analysis of the significant variables identified in comparisons of survivors and non-survivors, only SOFA scores ($P <0.011$), ICU length of stay ($P =0.007$) and ADMA ($P = 0.003$) were independently associated with ICU mortality.

Of the 104 patients who were admitted into the ICU, only 33 patients had last blood sample measurements of plasma ADMA concentrations (Table 3-4). The rest of the patients did not have a last sample measurement due to discharge (n = 61) or death (n = 10). The median time between admission and the last sample was 7 (range 5-15, IQR 6-8) days. There was a significant decrease in SOFA score ($P < 0.02$), arginine ($P <0.008$), homoarginine ($P <0.001$), ALT ($P < 0.012$), Lab-Glucose ($P <0.012$), and albumin ($P <0.012$). There was a significant increase in ADMA ($P <0.009$), ADMA: SDMA ratio ($P < 0.003$), and the administration of norepinephrine ($P < 0.017$).

3.4. Discussion

Plasma dimethylarginine, especially ADMA, have been previously reported to be associated with mortality in critically ill patients, and have been speculated to be a cause of endothelial dysfunction and multiple organ failure in this patient group (Blackwell et al. 2009; Iapichino et al. 2010). In the present study it was shown that plasma concentrations of ADMA and SDMA were significantly higher in critically ill patients compared with healthy controls and that both were significantly associated with disease severity and mortality. This association supports the hypothesis that ADMA metabolism may play a causative role in endothelial dysfunction through impairment of NOS activity. The strength of association between ADMA and mortality was striking (odds ratio of 31), and is consistent with a recent report in patients with severe sepsis (Blackwell et al. 2009; Iapichino et al. 2010).

In the present study SDMA was also associated with increased mortality. However, there was a strong association between SDMA and creatinine concentrations on admission (n= 104, r_s= 0.62, P<0.001) and on follow-up (n= 33, r_s= 0.76, P<0.001) and therefore plasma SDMA concentrations, in part, reflect renal function; most likely due to renal elimination being the predominant route of SDMA clearance. It has previously been speculated that by competing with arginine uptake at cationic amino acid transporters (CAT) SDMA can impair the delivery of the substrate arginine to NOS (Closs et al. 1997). However, it has not been established whether the concentrations encountered *in vivo* are sufficient to have a significant effect. Therefore, it may be that the association of SDMA with mortality is in part due to the effect of renal dysfunction on mortality.

ADMA and SDMA are generated by the methylation of arginine residues in proteins by type 1 protein arginine methyltransferases (type 1 PRMTs) and from type 2 protein arginine methyl transferases (type 2 PRMTs) respectively. Daily production of ADMA is

approximately 300 µmol with about 10% of this amount excreted unchanged into the urine. The majority is metabolized by DDAH to citrulline and dimethylamine. In contrast, SDMA is mainly excreted unchanged in urine and is increasingly being regarded as a sensitive marker of renal function (Kielstein et al. 2008; Can et al. 2011).

In the present study the ADMA: SDMA ratio was significantly lower in patients with critical illness compared with controls. The ADMA: SDMA ratio has previously been shown to be reduced in patients with acute infections, severe sepsis, post-operatively and in rats following lipopolysaccharide administration; it has been speculated that the reduction in ratio reflects an inflammation-induced increase in ADMA clearance through DDAH activity (Nijveldt et al. 2004; Zoccali et al. 2007; Iapichino et al. 2010; Blackwell et al. 2011). However, in no such study has DDAH activity *in vivo* been assessed, and therefore no conclusion can be reached about this at the present time; indeed in the present study there was no direct association between CRP and ADMA concentration. Inflammatory cytokines have been shown to both increase and decrease DDAH activity in different models (Cardounel et al. 2007;Ueda et al. 2003), but the net effect *in vivo*, taking into account the confounding effects of oxidative stress and NO itself, is unclear.

In the present study the ADMA concentration increased on subsequent sampling and is consistent with other studies. This pattern has also been shown in patients undergoing elective surgery who had a significant inflammatory response, although in those patients this was following a significant reduction in ADMA concentration from baseline (Blackwell et al. 2011). In these patients there was no change in SDMA concentrations, indicating differential handling of the dimethylarginines. However, there was no clear evidence of increased ADMA metabolism, and it may be have been that ADMA transport via CATs was the more important factor (Blackwell et al. 2011). This is an area which is worthy of further study in critical illness, as it is becoming clear that the plasma

concentrations may not reflect the pathophysiologically important tissue concentrations, which even in health are up to 10 – 20 times that found in plasma (Cardounel et al. 2007; Teerlink et al. 2009).

There are two distinct isoforms of DDAH. DDAH-1 is widely expressed, particularly in tissues such as liver, renal cortex, lung and immune tissues and cells. There are large arterio-venous gradients across organs such as the liver and kidney, and DDAH-1 has been described as the "guardian" of circulating ADMA concentration (Palm et al. 2007). In contrast, DDAH-2 is mainly found in tissues which express endothelial nitric oxide synthase (eNOS) and inducible nitric oxide synthase (iNOS) such as endothelium and smooth muscle cells of cardiovascular system (Blackwell 2010; Li et al. 2011). As such, DDAH-2 appear to be more involved in the regulation of local endothelial responses (Palm et al. 2007). Consequently, ADMA concentrations in plasma are regulated within fairly narrow limits in health, and it is clear that DDAH-mediated metabolism is the critical step in this regulation (Teerlink 2005; Blackwell 2010).

The activity of dimethylaminohydrolase (DDAH) is highly dependent on a cysteine residue at its catalytic site. This is susceptible to oxidation as a consequence of oxidative stress and may provide a mechanism that results in increased ADMA concentration and reduced NO formation (Blackwell 2010). This has been observed with hyperglycaemia and hyperhomocystinaemia (Blackwell 2010). Therefore, interaction between dimethylaminohydrolase (DDAH), ADMA and NOS may present a common pathway of several risk factors affecting the vascular endothelium.

Indeed, a recent study in critically ill rabbits showed that tissue DDAH activity was a stronger determinant of plasma than tissue ADMA concentration, suggesting that CAT-mediated exchange of ADMA between compartments might be even more important than changes in DDAH activity itself (Davids et al. 2012). Previous work has documented

increased fractional excretion of ADMA by the liver during endotoxaemia (Nijveldt et al. 2004), but, again, this does not differentiate between an increase in CAT-mediated uptake and a true increase in DDAH activity itself. In this regard, it has also been shown that methylarginine uptake into endothelial cells and macrophages increases with cytokine stimulation (Teerlink et al. 2009). With current limitations in knowledge, caution should be exercised when interpreting the ADMA: SDMA ratio that might not be useful as a simple surrogate marker of DDAH activity for these reasons.

A novel aspect of the present study was the measurement of homoarginine. Homoarginine is an amino acid largely generated from lysine and may promote endothelial function as a substrate for NO synthesis (Hecker et al. 1991), although it is probably present at too low a concentration for this to be important, given the relative concentration of arginine is about 30 times greater (Blackwell et al. 2009). A potentially more important role that has received some attention is in the inhibition of the enzyme arginase with consequent preservation of arginine for NOS (Yang and Ming 2006). Indeed, low homoarginine concentrations have been recently shown to be associated with mortality and cardiovascular events (Maerz et al. 2010). Furthermore, increased homoarginine concentrations during normal pregnancy are associated with increases in brachial artery diameter and flow-mediated dilatation (Valtonen et al. 2008). In the present study, homoarginine was positively associated with arginine (r_s = 0.641, P<0.001), that is consistent with the above concept. Indeed, homoarginine was significantly inversely associated with CRP (r_s= -0.349, P= 0.001) suggesting an effect of inflammation on homoarginine synthesis and/ or metabolism.

Limitations of the present study were principally that it was a single centre study and there was no validation cohort. Furthermore, the cohort was mixed in two of surgical and medical patients there was considerable variation in the severity of illness.

In summary, plasma ADMA and SDMA concentrations were higher in patients with critical illness and were associated with disease severity and mortality. In contrast, plasma homoarginine concentrations were lower in patients with critical illness and were also associated with disease severity and mortality. These results suggest that ADMA metabolism is perturbed with likely knock on effects on NOS and endothelial function. There is a need for further work on in vivo DDAH activity in critical illness and the effect of critical illness on the CAT-mediated exchange of ADMA between intra and extra-cellular compartments.

Table 3-1: Admission characteristics and arginine, homoarginine, ADMA and SDMA concentrations in normal subjects and patients with critical illness (n=104).

	Reference Intervals (Control) (n=86)	Patients with critical illness (n=104)	P-value*
Age (years)		61 (47-71)	
Gender (Male/Female) (%)		72 (69)/32 (31)	
Admission cause (Surgical/Medical) (%)		60 (58)/44 (42)	
APACHE II		21 (17-27)	
Predicted mortality rate (%)		38.9 (17.6-60.4)	
SOFA score		7.0 (4.0-9.0)	
Sever sepsis/septic shock (%)		10 (10)/5 (5)	
Arginine (µmol L^{-1})	65.7 (48.8-79.9)	18.4 (12.9-29.1)	<0.001
Homoarginine (µmol L^{-1})	1.9 (1.38-2.49)	0.58 (0.32-1.18)	<0.001
ADMA(µmol L^{-1})	0.45 (0.41-0.52)	0.54 (0.41-0.64)	<0.001
SDMA (µmol L^{-1})	0.38 (0.34-0.43)	0.75 (0.52-1.15)	<0.001
ADMA/SDMA ratio	1.20 (1.10-1.36)	0.73 (0.43-1.02)	<0.001
Arginine/ADMA ratio	125.7 (101.0-171.5)	36.7 (26.0-56.6)	<0.001
Total dimethylarginine	0.82 (0.76-0.88)	1.2 (1.0-1.83)	<0.001
Creatinine (umol/L)[§]	68-118	117 (78-171)	
Total Bilirubin (umol/l) [§]	5.1–17.0	10.5 (7.0-21.0)	
ALT (U/L) [§]	10-40	37 (18-115)	
Lab-Glucose (mmol/l) [§]	3.5-5.5	6.4 (5.5-8.1)	
C-reactive protein (mg/l) [§]	<10	119 (41-216)	
White cell count (10^9/L) [§]	4-11 x10^9	11.4 (7.9-18.5)	
Albumin (g/l) [§]	35-55	16 (12-21)	
Daily fluid balance (ml)		1034 (393-2343)	
Insulin (U hour $^{-1}$) [a]		0.9 (0.0-9.3)	
Norepinephrine (mg $hour^{-1}$) [a]		0.5 (0.0-6.0)	
Epinephrine (mg $hour^{-1}$) [a]		0.1 (0.0-2.7)	
Dobutamine (mg $hour^{-1}$) [a]		2.5 (0.0-76.4)	
Hydrocortisone (mg $hour^{-1}$) [a]		1.9 (0.0-16.0)	
ICU length of stay (Days)		5.1 (2.0-13.6)	
ICU death No/Yes		78 (75%)/26 (25%)	

Median (interquartile range, IQR), [§] reference interval, [a] Mean (range), * Mann-Whitney *U*.

Table 3-2: Admission patient characteristics and measurements between survivors and non-survivors (n=104).

	Admission Survivors (n=78)	Admission Non-survivors (n=26)	P-value*
Age (years)	60 (43-69)	67 (48-74)	0.175
Gender (Male/Female) (%)	55 (71)/23 (29)	17/9	0.625
Admission cause (Surgical/Medical) (%)	45 (58)/33 (42)	15 (58) /11 (42)	1.000
APACHE II	21 (16-26)	24 (18-31)	0.174
Predicted mortality rate (%)	35.3 (14.1-57.3)	45.6 (23.3-73.8)	0.061
SOFA score	5 (4-8)	8 (7-11)	0.001
Sever sepsis/septic shock (%)	7 (9)/5 (6)	3 (12)/0 (0)	
Arginine (µmol L^{-1})	17.5 (12.1-29.0)	19.6 (17.3-34.9)	0.281
Homoarginine (µmol L^{-1})	0.70 (0.35-1.30)	0.50 (0.26-0.77)	0.028
ADMA(µmol L^{-1})	0.49 (0.38-0.62)	0.59 (0.52-0.90)	0.003
SDMA (µmol L^{-1})	0.69 (0.50-1.10)	0.94 (0.57-1.72)	0.047
ADMA/SDMA ratio	0.72 (0.43-1.02)	0.79 (0.37-1.05)	0.866
Arginine/ADMA ratio	38.5 (25.7-59.2)	33.3 (26.0-49.2)	0.218
ADMA+SDMA	1.2 (0.92-1.68)	1.7 (1.2-2.5)	0.008
eGFR >30/ $\leq$30	10 (0-35)	11 (0-28)	0.747
Creatinine (umol/L)	115 (73-169)	118 (79-249)	0.605
Total Bilirubin (µmol/l)	10.5 (8-20)	10.5 (7-28)	0.980
ALT	38.5 (19-129)	37 (15-115)	0.556
Lab-Glucose (mmol/l)	6.4 (5.5-8.1)	6.8 (5.4-9.2)	0.642
C-reactive protein (mg/l)	108 (40-218)	125 (42-169)	0.870
White cell count (10^9/L)	10.8 (7.6-16.9)	16.3 (7.9-20.0)	0.223
Albumin (g/l)	17 (13-23)	13 (10-19)	0.017
Daily fluid balance (ml)	1006 (393-2166)	4793 (-7-5113)	0.167
Insulin (U hour $^{-1}$) [a]	0.9 (0.0-9.3)	0.7 (0.0-4.8)	0.874
Norepinephrine (mg hour-1) [a]	0.4 (0.0-2.5)	0.8 (0.0-6.0)	0.292
Epinephrine (mg $hour^{-1}$) [a]	0.1 (0.0-2.7)	0.1 (0.0-1.0)	0.894
Dobutamine (mg $hour^{-1}$) [a]	2.4 (0.0-76.4)	2.7 (0.0-50.0)	0.431
Hydrocortisone (mg hour-1) [a]	2.2 (0.0-16.0)	1.2 (0.0-8.0)	0.276
ICU length of stay (Days)	3 (1-10)	11.6 (6-19)	<0.001

Median (interquartile range, IQR), [a] Mean (range), * Mann-Whitney *U*.

Table 3-3: The relationship between clinical characteristics and ADMA on admission to ICU mortality in patients with critical illness. Univariate binary logistic regression analysis.

	Univariate analysis OR (95%CI)	P-value
Age (years)	1.02 (0.99-1.05)	0.292
Sex (male/female)	1.27 (0.49-3.25)	0.624
Patients (medical/surgical)	1.00 (0.41-2.46)	1.000
APACHE II score	1.05 (0.99-1.12)	0.138
Predicted mortality rate (%)	1.02 (0.99-1.03)	0.081
SOFA score	1.34 (1.12-1.61)	0.002
ICU length of stay (days)	1.05 (1.01-1.09)	0.007
Arginine (µmol L-1)	1.02 (0.99-1.05)	0.241
Homoarginine (µmol L-1)	0.39 (0.15-0.99)	0.048
ADMA(µmol L-1)	31.31 (3.34-293.18)	0.003
SDMA (µmol L-1)	1.54 (0.90-2.64)	0.118
ADMA/SDMA ratio	1.10 (0.30-4.06)	0.887
Arginine/ADMA ratio	0.99 (0.97-1.01)	0.243
ADMA+SDMA	1.66 (1.02-2.70)	0.041
eGFR >30/ <30	0.99 (0.97-1.02)	0.771
Creatinine (umol/L)	1.00 (0.99-1.00)	0.721
Bilirubin (umol/l)	1.00 (0.99-1.01)	0.858
ALT (u/l)	1.00 (1.00-1.00)	0.661
Lab-Glucose mmol/l	1.05 (0.94-1.16)	0.413
C-reactive protein (mg/l)	1.00 (0.99-1.01)	0.665
Albumin (g/l)	0.92 (0.85- 1.00)	0.063
Daily fluid balance (ml)	1.00 (1.00-1.00)	0.020
Insulin (U hour -1)	0.93 (0.70-1.24)	0.623
Norepinephrine (mg hour-1)	1.51 (0.95-2.40)	0.079
Epinephrine (mg hour-1)	0.89 (0.25-3.23)	0.864
Dobutamine (mg hour-1)	1.00 (0.96-1.05)	0.896
Hydrocortisone (mg hour-1)	0.92 (0.800-1.06)	0.263

OR, odd ratio

Table 3-4: The relationship between patient characteristics and plasma arginine, homoarginine, ADMA, and SDMA concentrations in patients with critical illness on admission and (follow-up) last sample median day 7 (range 5-15) (IQR 6-8)

	Patients with critical illness Admission (n=33)	Patients with critical illness Follow-up (n=33)	P-value*
Age (years)	64 (51-71)		
Sex (M/F) (%)	23 (70%) /10 (30%)		
Medical/ Surgical (%)	19 (58%) /14 (42%)		
APACHE II score	24 (18-29)		
Predicted mortality (%)	46.0 (21.8-71.9)		
SOFA score	8 (6-10)	6 (4-8)	0.002
Sever sepsis/septic shock	2/1		
Arginine (µmol L^{-1})	19.1 (12.8-31.8)	24.3 (21.5-35.6)	0.008
Homoarginine (µmol L^{-1})	0.56 (0.35-1.22)	0.33 (0.12-0.42)	<0.001
ADMA(µmol L^{-1})	0.60 (0.50-0.84)	0.71 (0.57-1.04)	0.009
SDMA (µmol L^{-1})	0.87 (0.53-1.44)	0.94 (0.51-1.49)	0.829
ADMA/SDMA ratio	0.75 (0.43-1.04)	0.89 (0.57-1.25)	0.003
Arginine/ADMA ratio	31.2 (16.3-44.2)	40.0 (27.0-46.3)	0.236
ADMA+SDMA	1.5 (1.04-2.44)	1.6 (1.16-2.52)	0.936
eGFR >30/ $\leq$30	20.0 (0.0-33.5)	17.0 (0.0-30.0)	0.604
Creatinine (umol/L)	126 (93-204)	167 (91-278)	0.626
Bilirubin (umol/l)	11 (7-20)	11 (6-27)	0.350
ALT (u/l)	57 (1-171)	42 (21-96)	0.012
Lab-Glucose mmol/l	7.4 (5.9-10.5)	6.2 (5.2-7.0)	0.012
C-reactive protein (mg/ l)	72 (13-156)	131 (56-173)	0.597
White cell count (10^9/L)	10.6 (7.4-18)	12.0 (9.2-20.8)	0.320
Albumin (g/ l)	15 (10-21)	13 (11-16)	0.012
Daily fluid balance (ml)	469 (-21-2000)	1491 (-275-2148)	0.778
Insulin (U $hour^{-1}$) [a]	1.0 (0.0-9.3)	2.1 (0.0-10.9)	0.148
Norepinephrine (mg $hour^{-1}$) [a]	0.4 (0.0-4.0)	0.5 (0.0-4.4)	0.017
Epinephrine (mg $hour^{-1}$) [a]	0.1 (0.0-2.7)	0.07 (0.0-1.6)	0.028
Dobutamine (mg $hour^{-1}$) [a]	3.9 (0.0-76.4)	4.5 (0.0-117)	0.028
Hydrocortisone (mg $hour^{-1}$) [a]	2.1 (0.0-16.0)	2.4 (0.0-16.0)	0.058
ICU length of stay (days)	16 (11-27)		
ICU death (no/ yes) (%)	17 (52)/16 (48)		

Median (interquartile range, IQR), [a] Mean (range), * Wilcoxon signed rank test.

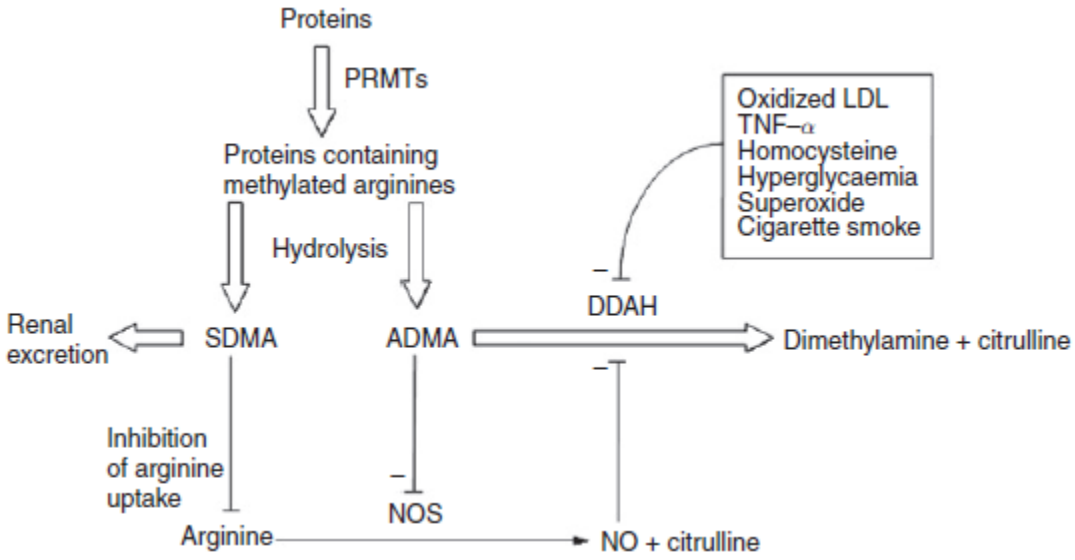


Figure 3-1: Interactions between oxidative stress, dimethylarginine dimethylaminohydrolase (DDAH), asymmetric dimethylarginine (ADMA) and nitric oxide synthase (NOS). SDMA, symmetric dimethylarginine; PRMTs, protein arginine methyltransferases; TNF-a, tumour necrosis factor-a (Blackwell, 2010)

4. The relationship between markers of the systemic inflammatory response and outcomes in patients with critical illness.

4.1. Introduction

Prediction of outcome in patients with critical illness by current scores such as APACHE II score and predicted mortality rate are complex and time consuming. The systemic inflammatory response has long been recognised to be associated with many forms of tissue injuries including surgery, trauma, burns and infections. It is composed of a number of stereotypical reactions that involve almost all organs in the human body such that the systemic inflammatory process is resolved and healing of tissue occurs (Gabay and Kushner 1999). It is becoming increasingly recognised that the systemic inflammatory response is not only an epiphenomena of the tissue injury but is also key to the resolution or exacerbation of the injury.

APACHE II score was applied to evaluate probabilities of hospital mortality which were compared with the actual mortality (Dieter et al, 1997). Expected or predicted mortality rate (EMR) is calculated by summing the predicted probability of death for all of the patients and dividing by the number of patients (Edward et al, 2005), however, predicted mortality rate has been developed for estimating the probability of mortality among ICU patients and it is calculated automatically in the Royal Infirmary ICU computers by Carevue programme, such data was collected as part of routine clinical care. It was calculated as described in Figure 1-1(see Chapter 1 p 27).

CRP is an acute-phase protein that has been evaluated extensively in the clinical setting (Gabay and Kushner 1999; Ho et al. 2008). Although CRP is not diagnostic of sepsis and in particular reflects a systemic inflammatory state, in the clinical context it is routinely used to raise suspicions of infection and further investigations such as imaging and blood

cultures. Moreover, a change in CRP concentrations can however suggest a response to treatment (Povoa et al. 2006) and may a reliable marker of outcomes during the post-ICU period (Ho et al. 2008; Grander et al. 2010; Ranzani et al. 2013). In addition to CRP, serum albumin may be an important short- and long-term marker for prognosis. Serum albumin is a negative acute-phase protein; thus, the degree of hypoalbuminemia in critically ill patients correlates with the intensity of the systemic inflammatory response.

The aim of the present study was to examine the relationship between the systemic inflammatory response, as evidenced by the combination of CRP and albumin, and mortality in patients with critical illness.

4.2. Patients and methods

Data and stored samples were available from cohorts which had been defined previously. The critical illness cohort comprised of medical or surgical patients ≥ 18 years admitted to the ICU of the Glasgow Royal Infirmary (GRI) between September 2006 and December 2008 and who had evidence of the systemic inflammatory response syndrome as per Bone's criteria (Bone et al. 1992; Levy et al. 2003), were studied. Briefly, APACHE II score (Knaus et al. 1985) and predicted hospital mortality, CRP and albumin were recorded. This cohort has been described previously (Vasilaki et al. 2008).

This investigation was conducted with the intent of developing local guideline to aid in the interpretation of patient with critical illness outcomes results. A selected prospective cohort arose from a prospective study of patients with critical illness. The patient data from the cohort was anonymised and de-identified prior to analysis (Andrew Duncan and Donald C McMillan respectively). In line with local ethical procedures written informed consent was obtained. The latter study was approved by the ethics committees of the North Glasgow NHS Trust and Multicentre Research Ethics Committee (MREC) Scotland. Where patients were unable to give signed informed consent, consent was obtained from the patients' next of kin or welfare guardian in accordance with the requirements of the Adults with Incapacity Scotland (2000) Act (Appendix 1, 2).

4.2.1. Analytical methods

Albumin was measured by a BCP dye-binding method and CRP was measured using an automated analyser (Architect, Abbott Diagnostics, USA). For CRP the limit of detection was 5 mg/l. The inter-assay coefficient of variation was less than 3% and 5% over the sample concentration range for albumin and CRP respectively. The limit of detection for albumin was 10 g/L.

4.2.2. Statistical analysis

Data was presented in median and range value. Correlations between variables in the convenience sample were carried out using the Spearman rank correlation. The cohorts were divided into three groups according to CRP concentrations ≤10, 11-80 and >80 mg/L as previously described (Marsik et al. 2008). The patient outcome either alive or dead were grouped according to 3 categories of albumin concentrations ≥ 35, 25-34,< 25 g/Las previously described (Goldwasser and Feldman 1997). Comparisons between the <50 and ≥ 50 of predicted mortality rate groups were performed with the use of the Mann-Whitney *U* test. Outcome data were analysed by binary logistic regression analysis. A P- value < 0.05 was considered significant and the analysis of the data was carried out using SPSS software (version 19; SPSS Inc, Chicago, Ill).

4.3. Result

The APACHE II severity of disease classification system was shown in Figure 1-1 (see Chapter 1 p 27). The characteristics of the patients with critical illness (n= 261) were shown in Table 4-1. The majority were older than 50 years (median 60 years), 78% of the patients were males, an APACHE II score of 23 and the associated median predicted mortality was 46% and had plasma CRP concentrations were above the normal range and plasma albumin concentrations were below the normal range. The median ICU stay was 7 days; 168 patients survived and 93 (36%) patients died in the ICU and hospital outcome was 150 survived and 111 (45%) died.

Sex was significant associated with APACHE II score (r_s= 0.151, P =0.015), predicted mortality rate (r_s= 0.205, P =0.001), ICU stay (r_s= 0.155, P =0.012) and hospital mortality (r_s= - 0.145, P =0.019). APACHE II score was significantly associated with predicted mortality rate (r_s= 0.906, P <0.001), ICU mortality (r_s= - 0.196, P =0.001) and hospital mortality (r_s= - 0.186, P =0.003). Predicted mortality rate was significant associated with CRP (r_s= 0.204, P =0.001), albumin (r_s= -0.194, P =0.002), ICU stay (r_s= 0.182, P =0.003), ICU mortality (r_s= - 0.250, P <0.001) and hospital mortality (r_s= - 0.266, P <0.001). Duration of ICU stay was significant associated with CRP (r_s= 0.152, P =0.014), albumin (r_s= - 0.178, P =0.004) and hospital mortality (r_s= - 0.143, P =0.021). ICU mortality was significantly associated with albumin (r_s= 0.169, P =0.006) and hospital mortality (r_s= 0.849, P <0.001). Hospital mortality was significant associated with albumin (r_s= 0.152, P =0.014).

A comparisons between the <50 and ≥ 50 of predicted mortality rate groups in the patients with critical illness cohort was shown in Table 4-2. Predicted mortality rate has been developed for estimating the probability of mortality among ICU patients and it is calculated automatically in the Royal Infirmary ICU computers by Carevue programme,

such data was collected as part of routine clinical care. Compared with patients with predicted mortality rate < 50%, patients with predicted mortality rate ≥50 % were older (P <0.001), more likely to be male (P =0.003), and had higher APACHE II score (P <0.001), higher CRP concentrations (P =0.009), lower albumin concentration (P =0.007) and higher CRP/albumin ratio (P =0.003). The ICU and hospital mortality rate was also higher in those patients (P =0.015, P =0.002 respectively).

A distribution of ICU survival rate according to CRP and albumin concentrations in the patients with critical illness cohort was shown in Table 4-3. The mortality rate was higher in patients with CRP concentration > 80 mg/l and albumin concentration < 25 g/l.

A distribution of hospital survival rate according to CRP and albumin concentrations in the patients with critical illness cohort was shown in Table 4-4. The mortality rate was higher in patients with CRP concentration > 80 mg/l and albumin concentration < 25 g/l.

4.4. Discussion

The results of the present study suggest that, the combination of CRP and albumin concentrations during critical illness were independently associated with outcome in patients with critical illness. Compared with the complex APACHE II score the simple combination of CRP and albumin effectively predicted ICU and hospital mortality. Given the widespread use of these blood protein markers it may be possible to rationalise the prediction of outcome in patients with critical illness.

The results of the present study were consistent with previous work. Ho and co-workers (2008) reported that in 603 unselected patients with critical illness a high CRP concentration at ICU discharge was an independent predictor of higher in-hospital mortality (Ho et al. 2008). Similarly, Grander and co-workers (2010) reported that in 765 nonsurgical patients with critical illness, CRP concentrations during critical illness were independently associated with poorer post-ICU survival (Grander et al. 2010). In contrast, an observational study by Al-Subaie and co-workers (2010) reported that in 1487 medical and surgical patients with critical illness a higher CRP concentration and low albumin concentrations at the day of discharge were not associated with ICU readmissions and in-hospital mortality (Al Subaie et al. 2010).

Recently Ranzani and co-workers (2014) reported that in 334 patients with critical illness, a higher CRP / albumin ratio at ICU discharge independently predicted of mortality within 90 days and that the combination had greater accuracy that CRP alone. In particular, CRP and albumin concentration at discharge predicted long-term outcome after an episode of sepsis. In the present study the CRP/ albumin ratio on admission was compared with that of the standard thresholds of CRP and albumin concentration in predicting ICU and hospital mortality. In the largest category (CRP >80 mg/l and albumin < 25 g/l, n=143) the

CRP/ albumin ratio afforded little additional prognostic value in predicting ICU mortality (P = 0.097) and no additional prognostic value in predicting hospital mortality (P = 0.287). Therefore, in the present study the CRP/ albumin ratio offers little additional prognostic value over the simple standard threshold measurement of and albumin in patients admitted to ICU.

An APACHE II score is based on 12 physiological variables that mainly assist the function of cardiovascular, respiratory and urinary tract systems, and is not validated for certain diseases states such as patients with burns (Vincent and Moreno 2010). Moreover, the APACHE II score does not assess liver function in patients with critical illness. The results of the present study suggest that synthetic liver function may play an important role in determining ICU outcome. The basis of such a relationship are unclear, however, an increased CRP is associated with a reduction of activity of a number of metabolically active detoxify enzymes such as cytochrome P450 (Morgan et al. 2008; Roberts and Hall 2013). Also a low albumin concentration is associated with a loss of colloid osmotic pressure and a reduction in the plasma availability of a number of micronutrients (Ghashut et al. 2013; Stefanowicz et al. 2013a; Ghashut et al. 2014).

In summary, the combination of CRP and albumin concentrations during critical illness was independently associated with mortality outcome. In this cohort, the combination of CRP and albumin predicted ICU and hospital mortality as effectively as APACHE II. Future research focusing on the association between the acute phase protein response post-ICU outcomes is warranted in order to understand their role long-term survival of patients with critical illness particularly in septic patient.

Table 4-1: Characteristics of patients with critical illness cohort (n=261).

	Reference interval	Patients with critical illness
Age (years)	N/A	60 (18-100)
Sex (Male/Female)	N/A	206 (78%) / 55 (22%)
APACHE II score	N/A	23 (3-45)
Predicted mortality rate (%)	N/A	46 (0.2 -97)
C-reactive protein (mg/l)	<10	134 (<10-565)
Albumin (g/l)	35-55	17 (<10-47)
ICU stay (days)	N/A	7 (<1 -76)
ICU outcome (alive/ dead)	N/A	168 (64%) / 93 (36%)
Hospital outcome (alive/ dead)	N/A	150 (55%) / 111 (45%)

(Median and range)

Table 4-2: Distribution of ICU and hospital mortality according to predicted mortality rate in the patients with critical illness cohort (n=261).

Predicted mortality rate

	<50% (n=147)	≥50% (n=114)	P- value
Age (years)	53 (18-86)	62 (10-100)	<0.001
Sex (Male/Female)	123 (84%) / 24 (16%)	83 (73%) / 31 (27)	0.033
APACHE II score	18 (3-30)	29 (21-45)	<0.001
C-reactive protein (mg/l)	120 (<1-434)	153 (7 – 565)	0.009
Albumin (g/l)	19 (5-47)	16 (<10-38)	0.007
ICU outcome alive/ dead (%)	104/ 43 (29%)	64 / 50 (44%)	0.015
Hospital outcome alive/dead (%)	97/50 (34%)	53 / 61 (54%)	0.002

Table 4-3: Distribution of ICU mortality according to CRP and albumin concentrations in the patients with critical illness cohort (n=261).

	CRP ≤10 (mg/l) n= (25)	CRP 11-80 (mg/l) n= (69)	CRP >80 (mg/l) n= (167)	Mortality (%) according to albumin
Albumin ≥35 (g/l) n= (14)	APACHE II= 16 PM= 2 % AM= 4/1 (20%)	APACHE II= 20 PM= 32% AM= 5/2 (40 %)	APACHE II= 23 PM= 42% AM= 2/0 (0 %)	APACHE II= 19 PM= 23% AM= 11/3 (21%)
Albumin 25-34 (g /l) n= (47)	APACHE II= 15 PM= 14% AM= 8/3 (27 %)	APACHE II= 21 PM= 21% AM=10/4 (29 %)	APACHE II= 24 PM= 55% AM= 15/7 (32 %)	APACHE II= 21 PM= 32% AM= 33/14 (30%)
Albumin <25 (g/l) n= (200)	APACHE II= 21 PM= 22% AM= 8/1 (11 %)	APACHE II= 25 PM= 50% AM=34/14 (29 %)	APACHE II= 23 PM= 53% AM= 82/61 (43 %)	APACHE II= 23 PM= 49% AM= 124/76 (38%)
Mortality (%) according to CRP	APACHE II= 17 PM= 15% AM= 20/5 (19%)	APACHE II= 24 PM= 46% AM= 49/20 (29%)	APACHE II= 23 PM= 53% AM= 99/68 (41%)	

PM predicted mortality rate (Median), AM actual mortality (alive/ dead).

Table 4-4: Distribution of hospital mortality according to CRP and albumin concentrations in the patients with critical illness cohort (n=261).

	CRP ≤10 (mg/l) n= (25)	CRP 11-80 (mg/l) n= (69)	CRP >80 (mg/l) n= (167)	Mortality (%) according to albumin
Albumin ≥35 (g/l) n= (14)	APACHE II= 16 PM= 2% AM= 4/1 (20 %)	APACHE II= 20 PM= 32% AM= 5/2 (40 %)	APACHE II= 23 PM= 42% AM= 2/0 (0 %)	APACHE II= 19 PM= 23% AM= 11/3 (21%)
Albumin 25-34 (g /l) n= (47)	APACHE II= 15 PM= 14% AM= 7/4 (36 %)	APACHE II= 21 PM= 21% AM= 8/6 (43 %)	APACHE II= 24 PM= 55% AM=14/8 (79 %)	APACHE II= 21 PM= 32% AM= 29/18 (39%)
Albumin <25 (g/l) n= (200)	APACHE II= 21 PM= 22% AM= 7/2 (22 %)	APACHE II= 25 PM= 50% AM= 30/18 (38 %)	APACHE II= 23 PM= 53% AM= 73/70 (49 %)	APACHE II= 23 PM= 49% AM= 110/90 (45%)
Mortality (%) according to CRP	APACHE II= 17 PM= 15% AM= 18/7 (28%)	APACHE II= 23 PM= 46% AM= 43/26 (38%)	APACHE II= 24 PM= 53% AM= 89/78 (47%)	

PM predicted mortality rate (Median), AM actual mortality (alive/ dead).

5. The relationship between markers of the systemic inflammatory response and plasma glucose concentrations.

5.1. Introduction

Acute illness has a profound effect on the metabolic response. As part of this metabolic response it has been reported that systemic inflammatory response is associated with the significant elevation of plasma glucose secondary to protein catabolism by the release of inflammatory cytokines and counter regulatory hormones (Hill 1999). This may lead to hyperglycaemia and insulin resistance resulting in organ dysfunction and increased morbidity and mortality (Krinsley 2006; Egi et al. 2008; Kavanagh and McCowen 2010).

However, it is not clear whether all patients are equally susceptible to the adverse effects of hyperglycaemia and whether all would have similar benefit from treatment (Falciglia et al. 2009) also optimal glucose concentration targets in the patients with systemic inflammatory response have not been well defined (Schuetz et al. 2014). In previous work there was evidence that insulin administration only accounted for a small part of the daily fluctuation of glucose concentrations in these patients with critical illness (Falciglia et al. 2009). Therefore, it would appear that regulation of plasma glucose concentrations, even within the context of an insulin protocol, is complex and multifactorial in patients with critical illness (Falciglia et al. 2009).

The aim of the present study was to examine the relationships between plasma glucose and systemic inflammatory response, as evidenced by the elevation of CRP and albumin concentrations in a large cohort of patients referred for assessment and also examine these relationships in patients with critical illness receiving an insulin protocol.

5.2. Patients and methods

5.2.1. Nutrition screen cohort

A total of 5248 consecutive heparin-treated whole-blood samples from 5248 patients were received from hospitals throughout Scotland between January 2006 and March 2013 for routine analysis of plasma measurements of glucose concentrations. If more than one set of plasma measurements of glucose results was available in a patient only the first sample was included into the analysis, leaving a total of 5248 plasma glucose results. As a regional centre blood samples were sent for analysis if the patient was considered at nutrition risk and was often secondary to a number of disease states. In addition, measurement of albumin and CRP was also recorded for these patients.

5.2.2. Critical illness cohort

Data and stored samples were available from cohorts which had been defined previously. The critical illness cohort comprised of medical or surgical patients ≥ 18 years admitted to the ICU of the Glasgow Royal Infirmary (GRI) between September 2006 and December 2008 and who had evidence of the systemic inflammatory response syndrome as per Bone's criteria (Bone et al. 1992; Levy et al. 2003), were studied. Briefly, APACHE II score (Knaus et al. 1985) and predicted hospital mortality and SOFA scores, CRP and albumin were recorded. This cohort has been described previously (Vasilaki et al. 2008).

This investigation was conducted with the intent of developing local guideline to aid in the interpretation of glucose results. There were two cohorts studied. The first (a large unselected retrospective cohort) arose from an audit of patients who had a sample sent to a regional laboratory for a nutrition screen. Approval for audit purpose was obtained from the local ethics committee of the North Glasgow NHS Trust. The second (a small selected

prospective cohort) arose from a prospective study of patients with critical illness. The patient data from the two cohorts was anonymised and de-identified prior to analysis (Andrew Duncan and Donald C McMillan respectively). In line with local ethical procedures written informed consent was obtained for the latter cohort only. The latter study was approved by the ethics committees of the North Glasgow NHS Trust and Multicentre Research Ethics Committee (MREC) Scotland. Where patients were unable to give signed informed consent, consent was obtained from the patients' next of kin or welfare guardian in accordance with the requirements of the Adults with Incapacity Scotland (2000) Act (appendix 1, 2).

5.2.3. Analytical methods

Plasma glucose samples were collected into blood tubes containing sodium fluoride as an anticoagulant. The sample was centrifuged at 3000 rpm for 10 minutes to separate the plasma from blood cells. Plasma samples were analysed on an automated platform (ARCHITECT; Abbott Diagnostics) using a commercial kit (Abbott Diagnostics) according to manufacturer's instructions. The Limit of detection for plasma glucose is 0.14 mmol/L with an inter assay Coefficient of Variation of $\leq 5\%$.

Albumin was measured by a BCP dye-binding method and CRP was measured using an automated analyser (Architect, Abbott Diagnostics, USA). For CRP the limit of detection was 5 mg/l. The inter-assay coefficient of variation was less than 3% and 5% over the sample concentration range for albumin and CRP respectively. The limit of detection for albumin was 10 g/L.

5.2.4. ICU insulin protocol

In the ICU, Glasgow Royal Infirmary, hyperglycaemia was treated according to Insulin Protocol Ward 25 GRI as of July the 5th 2006 (Blood glucose control protocol Figure 2-1, p 91). This continued to be used over the period of the study. The target of plasma glucose concentration for critically ill patient in ICU was 4.4- 6.9 mmol/l. In order to maintain this concentration, patients who required insulin infusion were infused with a standard concentration of 50 units of insulin in 50 ml 0.9% saline.

5.2.5. Statistical analysis

Data was presented in median and range value. Correlations between variables in the convenience sample were carried out using the Spearman rank correlation. The cohorts were divided into three groups according to CRP concentrations ≤10, 11-80 and >80 mg/L as previously described (Marsik et al. 2008). The concentration of individual glucose measurments were grouped according to 3 categories of albumin concentrations ≥ 35, 25-34,< 25 g/Las previously described (Goldwasser and Feldman 1997). A P- value < 0.05 was considered significant and the analysis of the data was carried out using SPSS software (version 19; SPSS Inc, Chicago, Ill).

5.3. Results

5.3.1. Screen cohort

The characteristics of the convenience sample for glucose (n= 5248) are shown in Table 5-1. The majority were younger than 50 years (median 35 years), female (57%) and had plasma albumin were below the normal range and plasma CRP and plasma glucose were above the normal range. Plasma glucose was significantly associated with age (r_s= 0.280, P <0.001) sex (r_s= -0.122, P <0.001), CRP (r_s= -0.217, P <0.001) and albumin (r_s= -0.164, P <0.001). CRP was significantly associated with age (r_s= 0.256, P <0.001), sex (r_s= -0.003, P <0.001) and albumin (r_s= -0.465, P <0.001). Albumin was significantly associated with age (r_s=-0.398, P <0.001) and sex (r_s= -0.151, P <0.001).

The effects of the magnitude of the systemic inflammatory response, as evidenced by CRP concentrations and albumin, on glucose are shown in Figure 5-1a, 5-1b and 5-1c**.**

The median plasma concentrations of glucose grouped according to CRP concentrations ≤10, 11-80 and >80 mg/L were 5.2, 5.5 and 6.0 mmol/l respectively (P <0.001) with an overall elevation of 13%. The median plasma concentrations of glucose grouped according to albumin concentrations ≥35, 25-34 and <25 g/l were 5.3, 5.8 and 6.2 mmol/l respectively (P <0.001) with an overall elevation of 15%.

A distribution of median plasma glucose concentration according to CRP and albumin concentrations in nutrition screen cohort was shown in Table 5-2. When albumin concentrations were ≥ 35 g/L, the median plasma concentrations of plasma glucose grouped according to CRP concentrations ≤10 and 11-80 mg/L were 5.2, 5.4 and 5.6 mmol/l (P <0.001) with an overall elevation of 7%. When albumin concentrations were 25-34 g/L, the median plasma concentrations of glucose grouped according to CRP

concentrations ≤10, 11-80 and >80 mg/L were 5.4, 5.9 and 6.0 mmol/l (P <0.001) with an overall elevation of 11%. When albumin concentrations were < 25 g/L, the median plasma concentrations of glucose grouped according to CRP concentrations ≤10, 11-80 and >80 mg/L were 5.6, 6.1 and 6.4 mmol/l (P =0.441) with an overall elevation of 14%. The deference between glucose concentration when albumin concentration were ≥ 35 and <25 g/l was 5.2 and 6.4 mmol/l with an elevation of 19% (P < 0.001).

5.3.2. Patients with critical illness

The characteristics of the critically ill cohort (n=116) are shown in Table 5-1. The majority were older than 50 years (median 60 years), male (66%), an APACHE II score of 21 and SOFA score of 7 and the associated median predicted mortality was 33.6%. The majority of patients were surgical (56%) and had a median length of ICU stay of 4 days and median hospital stay of 19 days. The median CRP and albumin concentrations were above and below the normal reference intervals respectively. Approximately half had some renal impairment as evidence by eGFR of <60ml/min.

The median plasma glucose was above the reference interval (6.4mmol/l), whereas patients with critical illness have low plasma glucose concentrations compared with general population of this nutrition screen cohort. CRP was significantly associated with albumin (r_s=-0.345, P <0.001).

The median plasma concentrations of glucose grouped according to CRP concentrations ≤10, 11-80 and >80 mg/L were 7.2, 6.3 and 6.3 mmol/l respectively (88%, P <0.486). The median plasma concentrations of glucose grouped according to albumin concentrations ≥35, 25-34 and <25 g/l were 7.6, 6.1 and 6.4 mmol/l respectively (84%, P <0.128*).*

A distribution of median plasma glucose concentration according to CRP and albumin concentrations in nutrition screen cohort was shown in Table 5-3. The deference between

glucose concentration when albumin concentration were <25 g/l was increased from 6.0 to 6.4 mmol/l with an elevation of 6% ($P < 0.001$).

Discussion

The results of the present study showed that, in a cohort of patients referred for a plasma glucose assessment, plasma concentrations of glucose were significantly higher with increasing magnitude of the systemic inflammatory response as evidenced by both changes in CRP and albumin concentrations. Furthermore, this effect was differential with increasing albumin concentration associated with a reduction in plasma glucose irrespective of the CRP concentrations. As a result, albumin appears to have independent effect than CRP on plasma concentrations of glucose. In contrast, in patients with critical illness the association of both CRP and albumin with glucose appeared weaker, this may related to the effect of the drugs supplementation such as insulin. Nevertheless, these results provide a basis for the better understanding of the impact of the systemic inflammatory response on plasma glucose concentrations and for its assessment and interpretation.

These results are consistent with the recent work of Schuetz and co-workers (2014) that reported elevation on plasma glucose in patients with non-critical-care setting was associated with a more pronounced inflammatory response (Schuetz et al. 2014). Taken together with the present work although it would appear that the magnitude of the systemic inflammatory response is an important determinant of plasma glucose concentration or it may be that it is the glucose concentrations determine the magnitude of the systemic inflammatory response. However, given that the administration of cortisol (an anti-inflammatory agent) is often associated with an increase in glucose concentrations it may be that glucose concentrations enhance the systemic inflammatory response.

The elevation in blood glucose concentrations may occur due to the increase of glucose production in liver and increase insulin resistance in skeletal muscle (Cuthbertson 1979).

Moreover, secretion of pituitary hormones and stimulation of sympathetic nervous system appears to have a secondary effect on both the release and action of cortisol, norepinephrine, and epinephrine (Van den Berghe et al. 2001; Vanhorebeek and Van den Berghe 2006), resulting in an increase in plasma glucose concentration by increasing glucagon secretion and reducing the insulin secretion. Moreover, the use of some drugs in ICU such as exogenous catecholamines and glucocorticoids may suppress the secretion and the action of insulin, and in turn may inhibit glycogen synthesis, stimulate gluconeogenesis and alter insulin-mediated glucose uptake by tissues. Clearly these exogenous hormones may exacerbate insulin resistance, nitrogen loss and hyperglycaemia (Saberi et al. 2008; Ead 2009; Kavanagh and McCowen 2010; Thomas et al. 2010) and complicate the relationship between plasma glucose concentration and the magnitude of the systemic inflammatory response.

In summary, plasma concentrations of glucose appear to be independently associated with both CRP and albumin and consistent with the systemic inflammatory response as a major confounding factor in determining its status. However, in patients with critical illness, within the context of an insulin protocol the relationship was more complex and multifactorial.

Table 5-1: Characteristics of nutrition screening, patient with critical illness cohorts and plasma glucose concentrations.

	Reference interval	Nutrition screen cohort (n= 5248)	Nutrition screen cohort (n= 116)	P- value
Age (years)	NA	35 (18-99)	60 (18-100)	0.043
Sex (Male/Female)	NA	2241 (43%)/ 2997 (57%)	76 (66%)/ 40 (34%)	<0.001
C-reactive protein (mg/l)	<10	5 (<10- 568)	109.0 (1.0- 565.0)	<0.001
Albumin (g/l)	35-55	40 (4-59)	17 (9-45)	<0.001
Plasma Glucose (mmol/l)	3.5-5.5	5.3 (1.0-31.0)	6.4 (2.8-30.6)	<0.001
APACHE II score	-	-	21 (3-38)	-

(Median and range)

Table 5-2: Distribution of median plasma glucose concentration according to CRP and albumin concentrations in the nutrition screen cohort (n=5248).

Plasma glucose	CRP ≤10 (mg/l)	11-80 (mg/l)	>80 (mg/l)	Plasma glucose according to albumin
Albumin ≥35 (g/l)	5.2 (1.2-54.3) (n=3450)	5.4 (3.3-25.3) (n=791)	5.6 (3.9-18.5) (n=144)	5.3 (1.2-54.3) (n=4385)
34-25 (g/l)	5.4 (1.8-21.6) (n=253)	5.9 (1.1-23.8) (n=281)	6.0 (2.8-30.5) (n=188)	5.8 (1.1-30.5) (n=722)
<25 (g/l)	5.6 (3.6-18.3) (n=14)	6.1 (0.6-18.8) (n=34)	6.4 (0.1-41.4) (n=93)	6.2 (0.1-41.4) (n=141)
Plasma glucose according to CRP	5.2 (1.2-54.3) (n=3717)	5.5 (0.6-25.3) (n=1106)	6.0 (0.1-41.4) (n=425)	

Median (range) (number of observation)

Table 5-3: Distribution of median plasma glucose concentration according to CRP and albumin concentrations in patients with critical illness cohort in admission (n=116).

Plasma glucose (mmol/l)	CRP ≤10 (mg/l)	11-80 (mg/l)	>80 (mg/l)
Albumin ≥35 (g/l)	- (n=3)	- (n=2)	- (n=1)
34-25 (g/l)	6.0 (n=6)	7.0 (n=8)	5.9 (n=5)
<25 (g/l)	7.5 (n=8)	6.1 (n=23)	6.4 (n=60)

When n<5 median value was not calculated

Figure 5-1a: The relationship between glucose (mmol/l) and CRP (log 10) (r_s= 0.217, *P* <0.001).

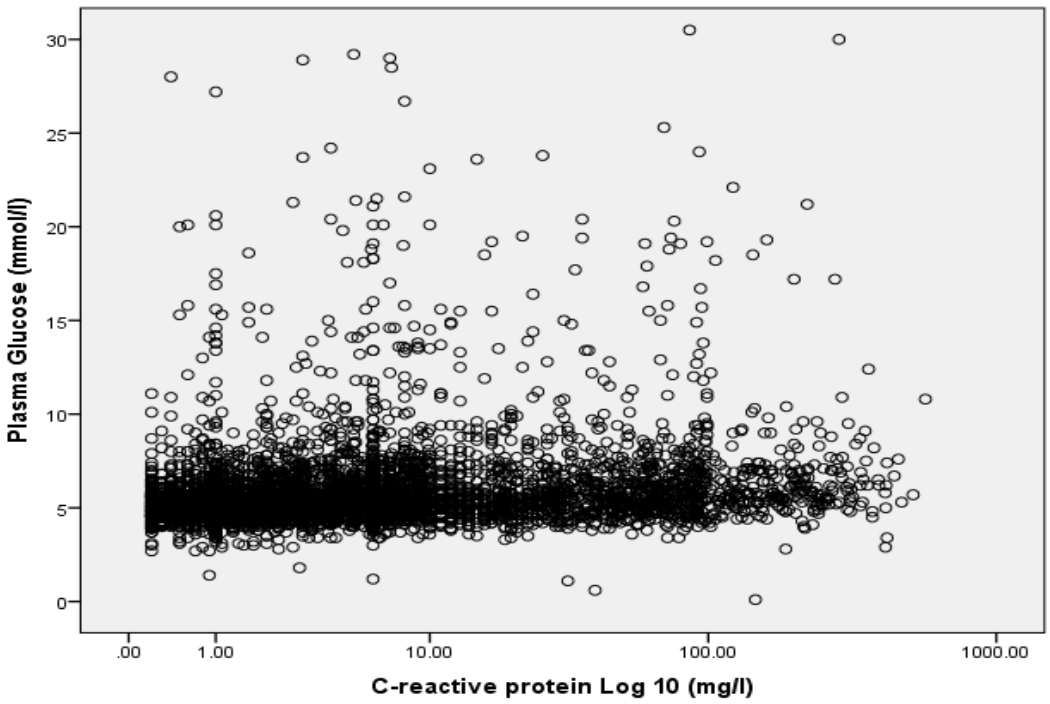


Figure 5-1b: The relationship between glucose (mmol/l) and albumin (r_s= - 0.164, *P* <0.001).

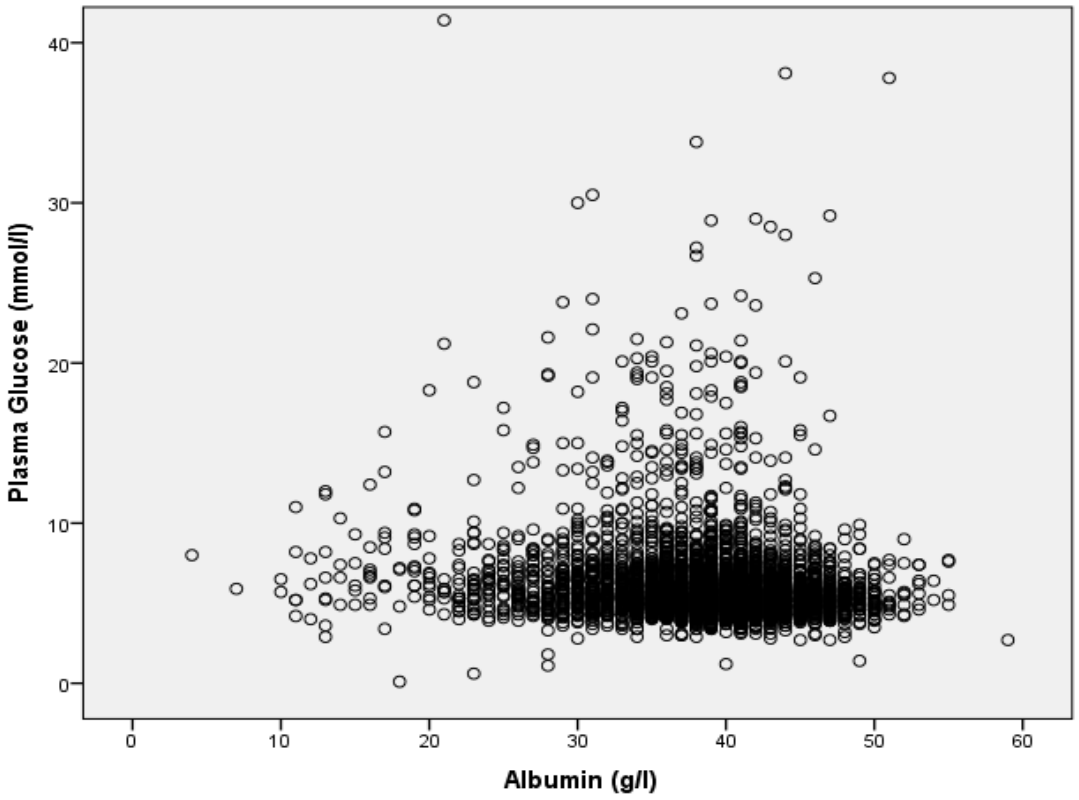
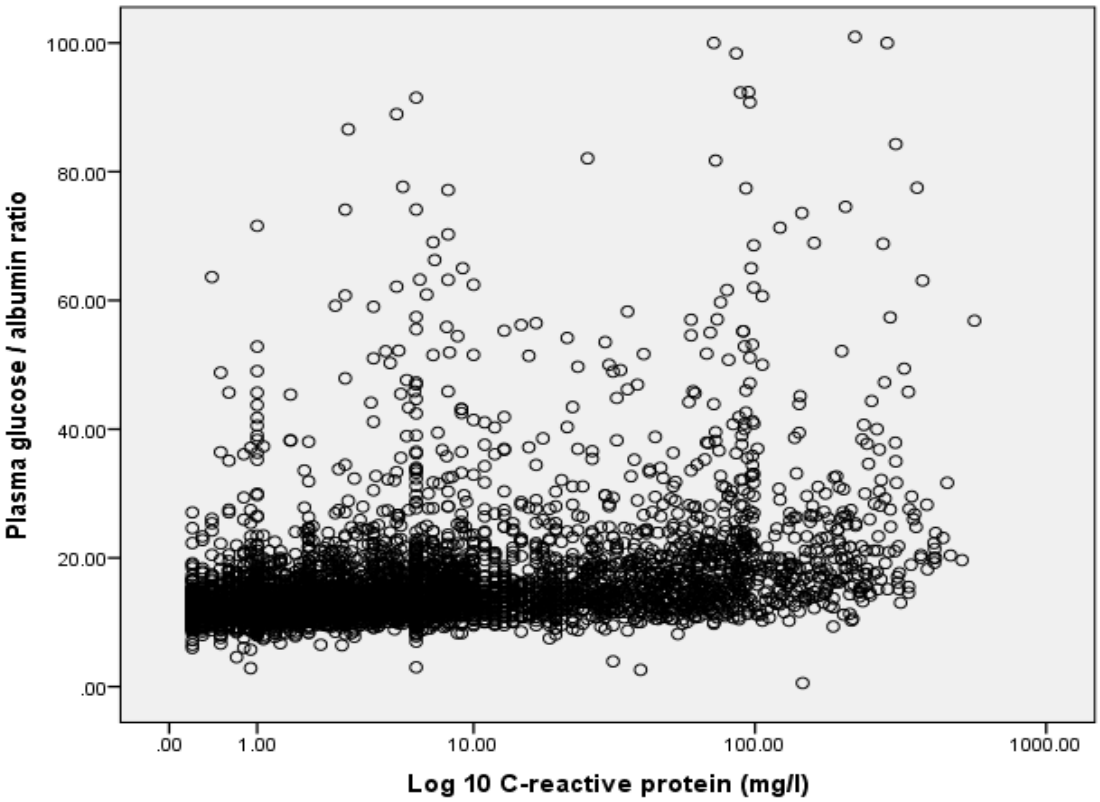


Figure 5-1c: The relationship between CRP (log 10) and plasma glucose adjusted to albumin (r_s= 0.407, *P* <0.001).



6. The relationship between markers of the systemic inflammatory response and plasma carotenoids concentrations.

6.1. Introduction

Carotenoids enhance immune function (Hughes 1999) and appear to have a protective effect against ageing and several common diseases such as cancer, cardiovascular disease and stroke (Mayne 1996; Krinsky and Johnson 2005). The properties of carotenoids appear to be mediated by a reduction of oxygen derived radicals (Bast et al. 1998). Currently, assessment of carotenoid status is based on their direct measurement in plasma. However, acute and chronic inflammation is associated with an alteration in concentrations of carotenoids in plasma independently of tissue stores (Quasim et al. 2003; Ford et al. 2003). For example, patients with non-small cell lung cancer and a systemic inflammatory response, as evidenced by elevated CRP concentrations, were found to have decreased plasma concentrations of carotenoids (Talwar et al. 1997; Gray et al. 2004).

Although the effect of the systemic inflammatory response on plasma carotenoids concentrations is known, it has not translated into widespread changes in clinical practice with regard to the routine interpretation and reporting of plasma micronutrient concentrations. Recently, it has been shown that a range of plasma micronutrient concentrations are inversely associated with CRP, commonly used as a marker of the systemic inflammatory response (Duncan et al. 2012b). In an unselected cohort of 1,303 patients, the systemic inflammatory response was associated with a major reduction in plasma zinc, selenium, vitamin A, C, D and B6 concentrations (Duncan et al. 2012b). As a result, it was concluded that the interpretation of plasma micronutrients should only be made when the CRP concentrations were known (Duncan et al. 2012b). Whether such a conclusion is valid for carotenoids is unclear. In particular, since carotenoids are carried in

cholesterol-rich lipoprotein, the effect of the systemic inflammatory response on plasma carotenoid concentrations may be corrected by calculating carotenoid / cholesterol ratios.

Therefore, the aim of the present study was to examine the effect of the magnitude of systemic inflammatory response on plasma carotenoids concentrations in a large cohort of patients whose blood samples were referred to the Scottish Trace Element and Micronutrient Reference Laboratory for assessment of their carotenoids status. This investigation was conducted with the aim of developing local guidelines to aid in the interpretation of results.

6.2. Patients and methods

A total of 1665 consecutive heparin-treated whole-blood samples from 1074 patients were received from hospitals throughout Scotland between December 2005 and December 2010 for nutrition analysis and routine carotenoid analysis. The individual analytes measured in a carotenoid screen are lutein, lycopene, α-carotene and β-carotene. If more than one set of carotenoid results was available for a patient, in order to reduce potential bias, only the most recent one was included into the analysis, leaving a total of 1074 carotenoid results. Of these patients 778 had cholesterol measured allowing carotenoids concentrations to be adjusted for cholesterol. Blood samples were sent to a regional centre for analysis if the patient was considered at a nutrition risk, and was often secondary to a number of disease states including kidney disease and critical illness.

Plasma lutein, lycopene, α-carotene and β-carotene concentrations were determined with an HPLC method (Talwar et al. 1998). Plasma was deproteinized with ethanol containing internal standards, and carotenoids were extracted into hexane. After a single hexane extraction the mean (±2 SD) linear recoveries of carotenoids added to plasma were as follows: lutein 94 (±5) %; lycopene 91 (±6) %; and α-carotene and β-carotene 89 (± 4) %. Analysis was carried out using reversed-phase HPLC (5-tam microbore; Phenomenex, Macclesfield, United Kingdom) and multiwavelength monitoring (Waters, Milford, MA). Carotenoids were detected at 450 mn. The precision (CV) of this method was < 9% intrabatch and < 16% interbatch for all analytes.

The laboratory participated in external quality assurance and proficiency testing programs (trace element quality assurance scheme; UK NEQAS for vitamin assays; UK NEQAS for CRP and cholesterol) throughout the time period of the study. Performance was acceptable

throughout, which indicated adequate accuracy and analytical precision. The limit of detection of the assays was ≤ 10ug/l for all carotenoids.

CRP and cholesterol were measured by using routine laboratory procedures with an automated analyzer (Architect; Abbott Diagnostics). The limit of detection or CRP varied from 0.2 mg/L (from 2007) to 1.0 mg/L (2006–2007) to 5.0 mg/L (2001–2006). Intraassay imprecision was 4%.

6.2.1. Statistics analysis

The concentrations of individual carotenoids and carotenoids after adjusted to cholesterol were separated according to 6 categories of CRP concentrations < 5, 6–10, 11–20, 21–40, 41–80, and >80 mg/L as previously described (Marsik et al. 2008). For each CRP category, data is presented as medians and 5th, 10th, 25th, 75th, and 95th percentiles. The concentration of each analyte was compared with the reference CRP category of ≤5 mg/L by using the Mann-Whitney *U* test. Correlations of plasma carotenoid concentrations compared with CRP and cholesterol were expressed as Spearman correlation coefficients. For each category of CRP concentration, the percentage of individual carotenoid concentrations below the lower limit of our laboratory reference range was calculated. The numerical proportions of low carotenoid concentrations for the categories were compared with that of ≤5 mg/L using a contingency table analysis, with the chi-square test (Kuskal-Wallis) for trend as appropriate. Due to the number of statistical comparisons carried out a *P* value <0.01 was considered to be significant. The statistical analysis was performed with the use of SPSS software (version 19; SPSS Inc, Chicago, Ill).

6.3. Results

The characteristics and the plasma concentrations of carotenoids of the cohort are shown in Table 6-1. The median age was 52 years (range 18-99), 58% of the patients were female and the median CRP concentration was < 6 mg/l. The median levels of plasma concentrations of lutein, lycopene and β-carotene carotenoids were above the limits of detection but below the laboratory reference interval. In contrast, median α-carotene concentrations were at the limit of detection of the assay. Circulating concentrations of lutein, lycopene, α-carotene and β-carotene were below the limit of detection in 165(15%), 238 (21%), 580 (52%) and 228 (20%) respectively. Therefore, α-carotene was not corrected for cholesterol.

The effect of the magnitude of the systemic inflammatory response, as evidenced by CRP concentrations, on median percentage changes in concentration of lutein, lycopene and β-carotene are shown in Figures 6-1a, 6-1b and 6-1c, respectively.

The median and the 5th, 10th, 25th, 75th and 95th percentiles of lutein, lycopene, α-carotene and β-carotene concentrations were grouped according to CRP categories and their statistical comparison are shown in Tables 6-2, 6-3, 6-4 and 6-5 respectively**.** With increasing CRP concentrations, plasma concentrations of lutein, lycopene and β-carotene significantly decreased. Median α-carotene results were at the limit of detection and the same trend was not evident; however, at the 75th percentile the same trend of decreasing results was found. For lutein this decrease was significant (P <0.01) when CRP concentrations were elevated between 10 and 20 mg/L (Table 6-2). For lycopene, α-carotene and β-carotene this decrease was significant (P <0.01) when CRP concentrations were elevated between 20 and 40 mg/L (Tables 6-3, 6-4 and 6-5, respectively). CRP

concentrations of >80 mg/L were associated with the largest median fall in lutein, lycopene and β-carotene in the range 60-70%.

Cholesterol concentration were directly correlated with lutein (r_s=0.330, P <0.001) lycopene (r_s= 0.297, P <0.001), α-carotene (r_s=0.233, P <0.001) and β-carotene (r_s=0.250, P <0.001) and so their ratio to cholesterol concentrations were determined. The same pattern of decreasing carotenoids / cholesterol concentration as CRP increased was observed in Figures 6-1d, e and f, respectively.

In order to compare the carotenoid values with and without adjustment for cholesterol the percentage change from the median reference value (<5mg/l) was calculated. For lutein there was a 69% reduction in median concentrations when CRP concentrations were > 80mg/L and when adjusted for cholesterol this was 42%. For lycopene there was a 68% reduction in lutein concentrations when CRP concentrations were >80mg/L and when adjusted for cholesterol this was 47%. For β -carotene there was a 64% reduction in the median concentrations when CRP concentrations were >80mg/L and when adjusted for cholesterol this was a 44%.

Levels of lutein, lycopene, beta-carotene, and alpha-carotene were strongly correlated with one another (r_s=0.60-0.76, P <0.001). Cholesterol was modestly correlated with the carotenoids (r_s =0.23-0.33, P <0.001). CRP was modestly correlated with the carotenoids (r_s =0.14-0.19, P <0.001).

The percentages of results below the lower end of the reference range at each CRP concentration category are shown in Table 6-6. For lutein, lycopene and β-carotene there were significantly more results below the lower laboratory reference range limit as CRP concentrations increased. For lutein these ranged from 60% to 90% (P <0.001).

6.4. Discussion

The results of the present study show that, as the magnitude of the systemic inflammatory response increased, as categorised by increasing increments of CRP concentrations, there was a significant fall in plasma concentrations of lutein, lycopene and β-carotene. The effect of the systemic inflammatory response in lowering plasma lutein, lycopene and β-carotene was apparent with both moderate and severe elevations of CRP concentrations. Indeed, it was shown that, as the severity of systemic inflammatory response increased, the proportion of results below the lower reference limit increased. Adjusting these carotenoid results for cholesterol reduced, but did not abrogate, this effect. Therefore, there is a risk, in the presence of a systemic inflammatory response, of misinterpreting plasma carotenoid concentrations as indicating deficiency.

In particular, the present data suggests that, in the presence of moderate or severe systemic inflammatory response (as indicated by a CRP concentration >20 mg/L), plasma concentrations of lutein, lycopene and β-carotene are not readily interpretable. Low plasma carotenoids, in the presence of such elevated CRP concentrations, could be an indication of either dietary insufficiency or chronic inflammation, and may suggest that increased supplementation of carotenoids may be warranted. In other words, low plasma values may be the first warning sign of carotenoid insufficiency, despite normal intracellular levels.

In the present study, the magnitude of the systemic inflammatory response was assessed using the circulating CRP concentration. CRP was then grouped into clinically meaningful thresholds in order to facilitate clinical recognition of such associations and to be consistent with the previous literature (Duncan et al. 2012b). It may be argued that some information about the nature of the relationship is lost by such analysis. However, the

present analysis does facilitate the recognition of the clinical impact of this important association.

The interpretation of the effect of the systemic inflammatory response on plasma α-carotene concentrations was problematical since the majority of observations were at the limit of detection of the assay. Nevertheless, at the 75th percentile there was a significant reduction in α-carotenoid concentration. Therefore, given the other carotenoid results it would not be surprising if a similar association was also true for α-carotene.

The carotenoids that were included in the present study are lutein, lycopene, α-carotene and β-carotene. Lycopene, α-carotene and β-carotene are hydrocarbons that are classified as very fat-soluble carotene. In contrast, lutein has at least one hydroxyl group and it is more polar than carotenes. As a result, in circulation, lycopene and β-carotene tend to be located mainly in the low-density lipoproteins (LDL), whereas lutein is more equally distributed between both low-density lipoproteins (LDL) and high-density lipoproteins (HDL) (Krinsky and Johnson 2005).

Since these carotenoids are carried in cholesterol-rich lipoproteins, it was hypothesised that the effect of the systemic inflammatory response on plasma carotenoid concentration may be corrected by calculating carotenoid/cholesterol ratios. However, this approach did not abrogate the effect of the systemic inflammatory response. This may be due to the complex effects of systemic inflammatory response on both carotenoids and cholesterol metabolism. The mechanisms are at present uncertain, and therefore in practice this means that correcting the carotenoid concentrations according to cholesterol in patients with systemic inflammatory response cannot be recommended.

It was also of interest that the correlation coefficients of plasma CRP and individual carotenoids were all relatively weak, which confirms that the association is highly variable

from patient to patient. This is perhaps not surprising because the data was accumulated from a wide cross-section of patients with differing types and severities of medical conditions, as well as varying degrees of carotenoids deficiencies. Furthermore, it might be expected that, in addition to redistribution to the extra-vascular compartments, increased utilization and turnover of carotenoids may occur in conditions associated with a systemic inflammatory response.

The results of the present study demonstrate the difficulty of interpreting plasma carotenoid concentrations in the presence of moderate or severe systemic inflammatory response; however, it does not help to find a better way to detect patients with real carotenoid deficiency. Alternative approaches of assessing micronutrient status in the presence of systemic inflammatory response are required and measurements in red blood cells, correcting with carrier proteins or the use functional measurements have been suggested (Talwar et al. 2003b; Duncan et al. 2012b). However, measurement of carotenoids in red cells is problematical and it remains to be determined what the optimal approach will be to detect carotenoid deficiency in patients. These results, however, do raise the issue of whether there are carotenoids concentrations below which deficiency is present irrespective of the systemic inflammatory response; such patients may truly be deficient but go untreated because of elevated CRP concentrations.

A potential effect of systemic inflammatory response on decreasing plasma concentrations of carotenoids has been previously reported (Ford et al. 2003; Gray et al. 2004; Galan et al. 2005). However, these studies did not clearly delineate such an association and there remains a general lack of awareness in routine clinical medicine. The basis of the consistent association between the magnitude of the systemic inflammatory response and low plasma concentrations of a wide variety of micronutrients including carotenoids is not clear (Duncan et al. 2012b). However, it is known that following an acute systemic

inflammatory response low plasma carotenoids concentrations return to normal levels (Talwar et al. 1997; Gray et al. 2004). This would suggest that redistribution of carotenoids from extracellular to intracellular compartments is an important component of this association. Alternative approaches of assessing micronutrient status in the presence of systemic inflammatory response are required. For example, measurements in red blood cells although useful for some vitamins (Talwar et al. 2003b; Galan et al. 2008) measurement of carotenoids in red cells are problematical and it remains to be determined what the optimal approach will be to detect carotenoid deficiency in patients.

The results of the present study, although clear because of their cross-sectional nature, cannot prove a causal effect of the systemic inflammatory response on carotenoids concentrations. There may be an underlying physiologic process that alters both the carotenoids concentrations and the systemic inflammatory response, so that the observed relations could at times be indirect.

In summary, the results of the present study indicate that the magnitude of the systemic inflammatory response is directly associated with a significant lowering effect on the plasma concentrations of carotenoids within the context of a routine clinical service. The basis of this association is not clear and may not be causal and so caution should exercise in clinical interpretation. Therefore, we recommend that clinical interpretation of plasma carotenoids only be made when the CRP concentration is known (an indicator of the inflammation) or the presence of systemic inflammatory response is excluded. Our data suggest that, in the presence of moderate or severe systemic inflammatory response (as indicated by a CRP concentration >20 mg/L), plasma concentrations of lutein, lycopene and β-carotene are not readily interpretable.

Table 6-1: Characteristics of nutrition screening cohort and plasma carotenoid concentrations (n=1074).

	Limits of detection	Reference intervals	Carotenoids screening cohort (*n*=1074)
Age (years)	N/A	N/A	52 (18-99)
Sex groups (Male / Female)	N/A	N/A	455 (42%) / 619 (58%)
C-reactive protein (mg/L)	<0.02	<10	6 (0.2-464)
Cholesterol (mg/L)	<0.161		4.3 (0.95-10.4)
Lutein (µg/L)	≤10	80-200	50.0 (<10-1088)
< 80 / 80-200 / >200			725 (68%) / 294 (27%) / 54 (5%)
Lutein/ cholesterol (µg/mmol)	N/A		12.1 (0.42-241.5)
Lycopene (µg/L)	≤10	100-300	50.0 (<10-694)
< 100 / 100-300 / >300			717 (67%) / 308 (29%) / 40 (4%)
Lycopene/ cholesterol (µg/mmol)	N/A		12.3 (0.73-135.3)
α-carotene (µg/L)	≤10	14-60	≤10 (≤10-1120)
< 14 / 14-60 / > 60			667 (62%) / 331 (31%) / 74 (7%)
β-carotene (µg/L)	≤10	90-310	55.0 (<10-2167)
< 90 / 90-310 / > 310			687 (64%) / 288 (28%) / 96 (9%)
β -carotene/ cholesterol (µg/mmol)	N/A		12.0 (1.1-386.3)

Median (range), N/A not applicable

Table 6-2: Distribution in percentiles of plasma lutein concentrations according to increments of CRP concentration (*n*=1074).

Plasma Lutein (µg/L)

CRP	Subjects	5th Percentile	10th Percentile	25th Percentile	Median (% change)	75th Percentile	90th Percentile	95th Percentile	*P*-value
	n (%)								
≤5 mg/L	483(45)	<10.0	<10.0	27.0	62.0 (100)	121.0	186.8	253.4	-
>5-10 mg/L	191(18)	<10.0	<10.0	28.0	70.0 (113)	117.0	159.6	206.6	0.680
>10-20 mg/L	124 (12)	<10.0	<10.0	17.0	49.0 (79)	87.5	136.5	169.0	0.004
>20-40 mg/L	92 (9)	<10.0	<10.0	14.0	42.0 (68)	76.5	109.1	131.8	<0.001
>40-80 mg/L	67 (6)	<10.0	<10.0	13.0	24.0 (39)	43.0	85.6	115.4	<0.001
>80 mg/L	113 (11)	<10.0	<10.0	<10.0	19.0 (31)	37.5	71.6	91.0	<0.001

Table 6-3: Distribution in percentiles of plasma lycopene concentrations according to increments of CRP concentration (*n*=1074).

Plasma lycopene (µg/L)

CRP	Subjects	5th Percentile	10th Percentile	25th Percentile	Median (% change)	75th Percentile	90th Percentile	95th Percentile	*P*-value
	n (%)								
≤5 mg/L	480 (45)	<10.0	<10.0	19.0	70.0 (100)	154.8	241.8	293.9	-
>5-10 mg/L	189 (18)	<10.0	<10.0	15.0	75.0 (107)	176.0	282.0	327.0	0.441
>10-20 mg/L	122 (11)	<10.0	<10.0	<10.0	49.5 (71)	145.5	247.0	274.4	0.200
>20-40 mg/L	92 (9)	<10.0	<10.0	<10.0	29.0 (41)	94.0	172.5	238.5	<0.001
>40-80 mg/L	67 (6)	<10.0	<10.0	13.0	34.0 (49)	60.0	124.4	152.0	<0.001
>80 mg/L	112(10)	<10.0	<10.0	<10.0	22.5 (32)	45.0	92.0	137.4	<0.001

Table 6-4: Distribution in percentiles of plasma α-Carotene concentrations according to increments of CRP concentration (*n*=1074).

Plasma α-Carotene (µg/L)

CRP	Subjects	5th Percentile	10th Percentile	25th Percentile	Median	75th Percentile	90th Percentile	95th Percentile	*P*-value
	n (%)								
≤5 mg/L	483 (45)	<10.0	<10.0	<10.0	<10.0	28.0	58.0	87.8	-
>5-10 mg/L	190 (18)	<10.0	<10.0	<10.0	<10.0	25.5	50.9	69.0	0.737
>10-20 mg/L	124(12)	<10.0	<10.0	<10.0	<10.0	18.8	30.0	65.8	0.118
>20-40 mg/L	92 (9)	<10.0	<10.0	<10.0	<10.0	15.0	28.4	35.4	0.007
>40-80 mg/L	67 (6)	<10.0	<10.0	<10.0	<10.0	16.0	32.0	79.6	0.070
>80 mg/L	113(11)	<10.0	<10.0	<10.0	<10.0	15.0	31.4	59.9	0.004

Table 6-5: Distribution in percentiles of plasma β-Carotene concentrations according to increments of CRP concentration (*n*=1074).

Plasma β-Carotene (µg/L)

CRP	Subjects	5th Percentile	10th Percentile	25th Percentile	Median (% change)	75th Percentile	90th Percentile	95th Percentile	*P*-value
	n (%)								
≤5 mg/L	481 (45)	<10.0	<10.0	18.0	72.0 (100)	173.5	335.8	4910	-
>5-10 mg/L	190 (18)	<10.0	<10.0	20.8	64.5 (90)	142.0	315.2	545.3	0.531
>10-20 mg/L	124 (12)	<10.0	<10.0	10.5	49.0 (68)	118.3	306.5	537.3	0.023
>20-40 mg/L	92 (9)	<10.0	<10.0	16.3	46.0 (64)	97.8	202.6	283.1	0.005
>40-80 mg/L	68 (6)	<10.0	<10.0	12.5	37.0 (51)	90.3	186.2	279.0	0.002
>80 mg/L	113 (11)	<10.0	<10.0	<10.0	26.0 (36)	61.5	142.8	229.6	<0.001

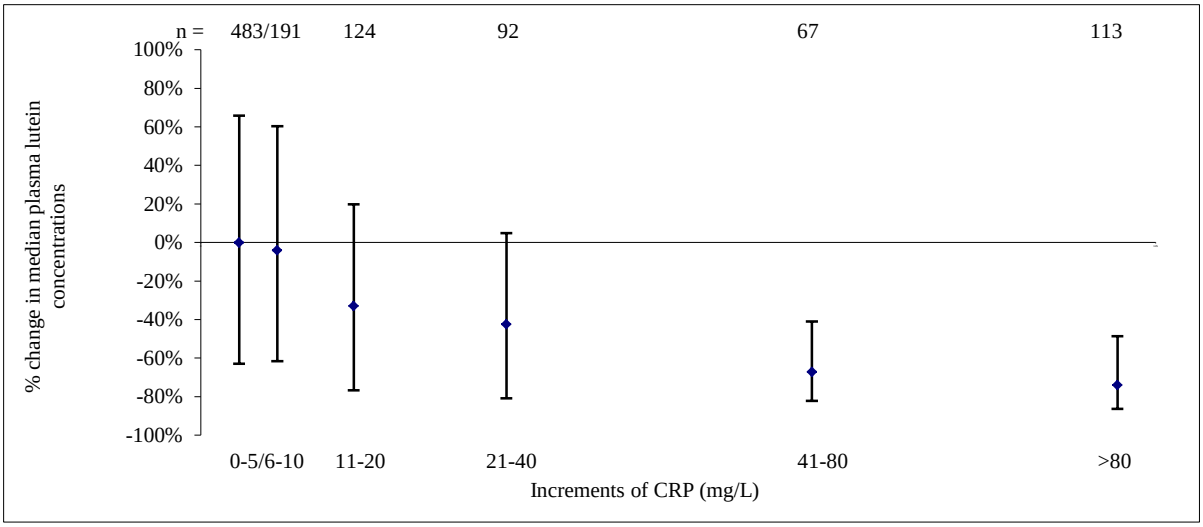
Table 6-6: Plasma carotenoids below the lower plasma reference range according to CRP concentrations.

CRP	Lutein (n=722)	Lutein/Cholesterol (n=527)	Lycopene (n=714)	Lycopene/Cholesterol (n=515)	β-Carotene (n=685)	B-carotene/Cholesterol (n=503)
	n (%)	*n* (%)	*n* (%)	*n* (%)	*n* (%)	*n* (%)
≤5 mg/L	288(60)	234(61)	295 (61)	236(62)	276 (57)	228(60)
>5-10 mg/L	106 (55)	73 (38)	107 (57)	73 (62)	115 (61)	75 (64)
>10-20 mg/L	92 (74)	64 (72)	80 (66)	56 (64)	83 (67)	58 (65)
>20-40 mg/L	72 (78)	51 (77)	73 (79)	49 (74)	68 (74)	48 (73)
>40-80mg/L	59 (88)	40 (91)	57 (85)	37 (84)	51 (75)	36 (80)
>80 mg/L	105 (93)	65 (89)	102 (91)	64 (89)	92 (81)	58 (79)
[a]*P* value	<0.001	0.427	0.141	0.636	0.113	0.854

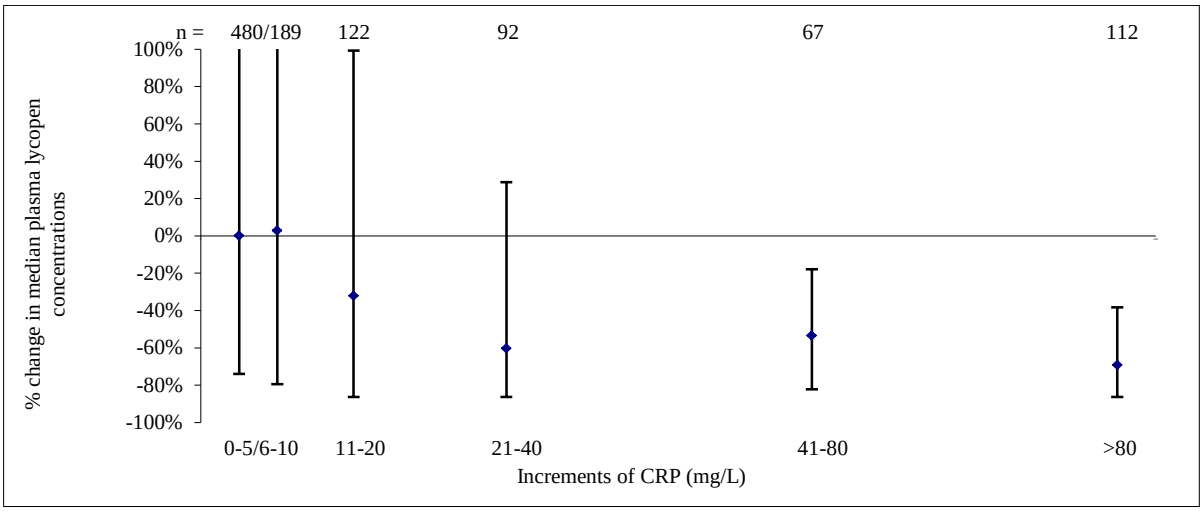
Lower limits: Lutein, 80 (µg/L), lycopene, 100 (µg/L), β-Carotene, 90 (µg/L), [a]Kuskal-Wallis

Figure 6-1a, 6-1b, 6-1c: Percentage changes in lutein (Figure 6-1a), lycopene (Figure 6-1c) and β-carotene (Figure 6-1e) with increasing increments of CRP Error bars represent the upper and the lower quartiles. CRP; C- reactive protein, n, number of patients.

1a



1b



1c

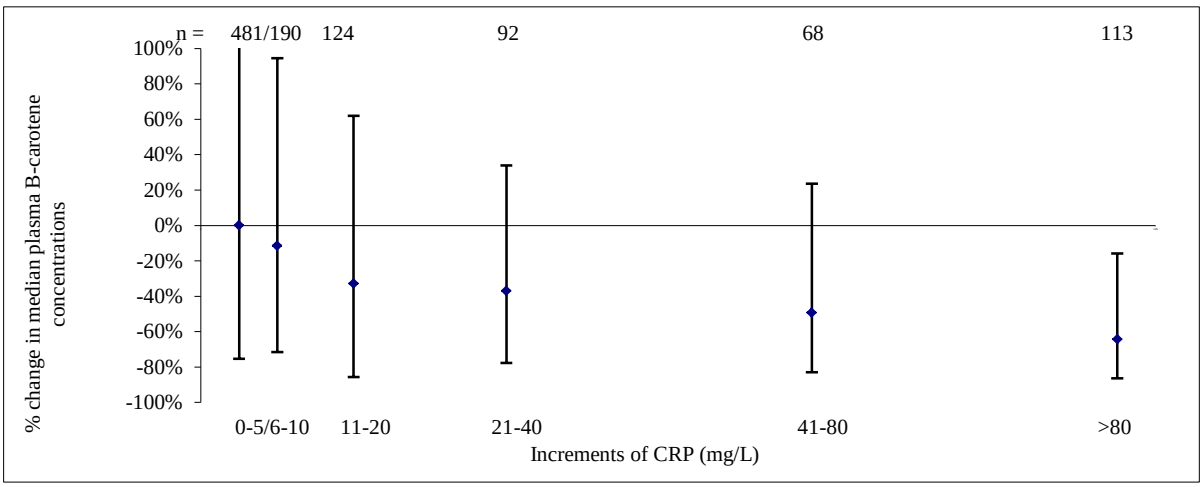
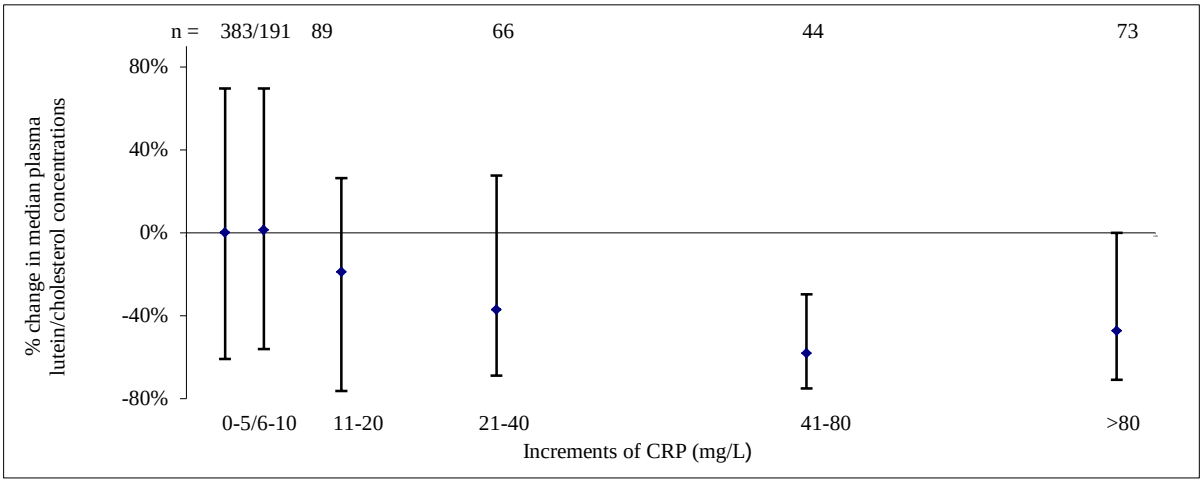
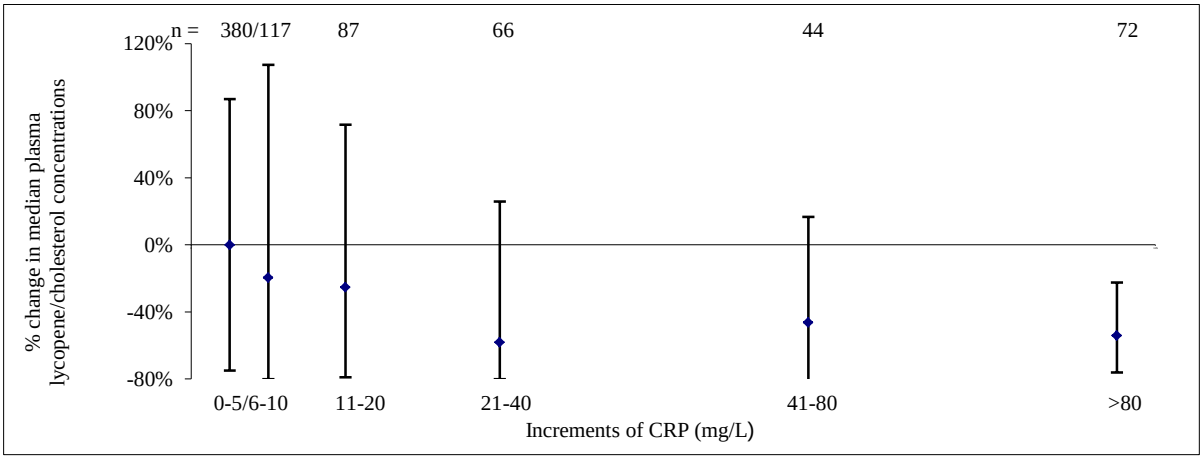


Figure 6-1d, 6-1e and 6-1f: Percentage changes in lutein/ cholesterol ratio (Figure 6-1b), lycopene/cholesterol ratio (Figure 6-1d) and β-carotene/cholesterol ratio (Figure 6-1f) with increasing increments of CRP Error bars represent the upper and the lower quartiles. CRP, C- reactive protein; n, number of patients.

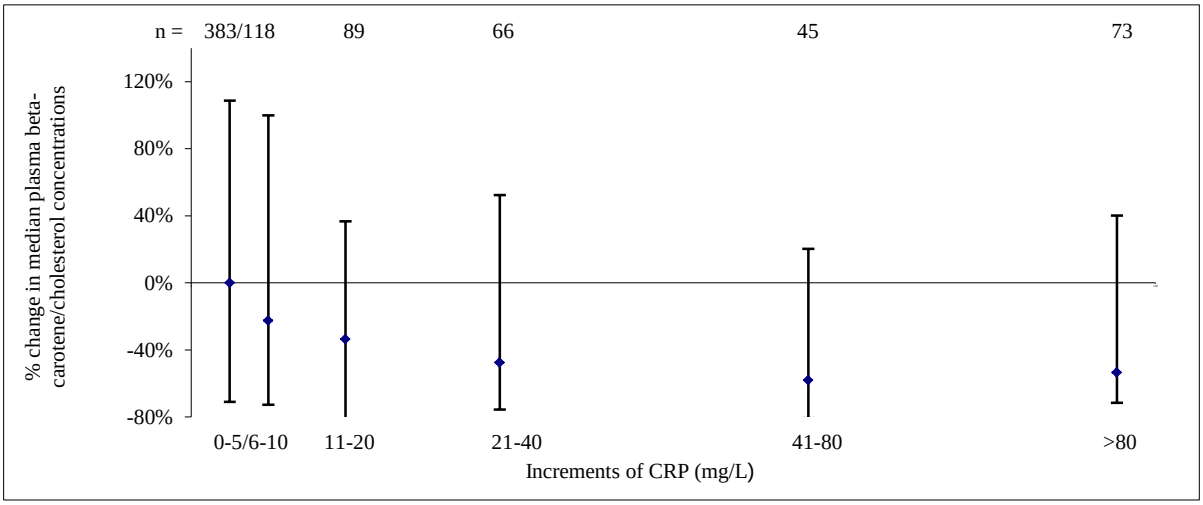
1d



1e



1f



7. The relationship between markers of the systemic inflammatory response and plasma vitamin 25 (OH) D concentrations.

7.1. Introduction

It has been recognised for some time that plasma 25-hydroxyvitamin D (25 (OH) D) deficiencies are associated with increased risks in relation to several diseases such as diabetes, hypertension, stroke, and heart disease (Danik and Manson 2012). However, there is the potential for confounding factors, since the presence of a systemic inflammatory response has been consistently reported to be associated with low plasma micronutrient concentrations (Galloway et al. 2000; Duncan et al. 2012b; Ghashut et al. 2013). In particular, it has recently been reported that the magnitude of systemic inflammatory response, as evidenced CRP, is a major factor associated with the lowering of circulating concentrations of the important lipid soluble vitamins, such as 25 (OH) D (Reid et al. 2011; Waldron et al. 2013).

Duncan and co-workers (Duncan et al. 2012b) reported the presence of an elevated systemic inflammatory response was associated with a significant reduction (41%) in plasma vitamin D concentration when CRP concentrations were > 80 mg/L compared to ≤5 mg/L. However, the variability of the association was such that plasma 25(OH) D concentrations were not readily adjusted for CRP concentrations (Duncan et al. 2012b).

In the blood 25 (OH)D is transported primarily bound to vitamin D binding protein (VDBP) but also to albumin and these fall as part of the systemic inflammatory response (Reid et al. 2011; Waldron et al. 2013). Quantitatively, albumin is the most important protein in the plasma and binds most plasma micronutrients, including 25 (OH) D. Moreover, albumin may be considered as a surrogate measure for other plasma binding

proteins, such as VDBP, and is routinely measured. Therefore, it may be useful in adjusting plasma 25 (OH) D concentrations for the effect of the systemic inflammatory response.

The aim of the present study was to examine the relationships between plasma 25 (OH) D, CRP and albumin concentrations in a large cohort of patients referred for vitamin D assessment, and also to examine these relationships in patients with critical illness.

7.2. Patients and methods

7.2.1. Nutrition screen cohort

A total of 7646 consecutive heparin-treated whole-blood samples from 5327 patients were received from hospitals throughout Scotland between January 2000 and March 2013 for routine analysis of plasma 25 (OH) D concentrations. If more than one set of plasma 25(OH) D results was available in a patient only the first sample was included in the analysis, leaving a total of 5327 plasma 25 (OH) D results. Blood samples were sent to a regional centre for analysis if the patient was considered at nutrition risk and was often secondary to a number of disease states. In addition, measurement of CRP and albumin was also recorded for these patients.

7.2.2. Critical illness cohort

Data and stored samples were available from cohorts which had been defined previously. The critical illness cohort comprised of medical or surgical patients ≥ 18 years admitted to the ICU of the Glasgow Royal Infirmary (GRI) between September 2006 and December 2008 and had evidence of the systemic inflammatory response syndrome as per Bone's criteria (Bone et al. 1992; Levy et al. 2003), and admitted in the period from September 2006 to December 2008 were studied. Briefly, APACHE II score (Knaus et al. 1985) and predicted hospital mortality and SOFA scores, CRP and albumin were recorded. This cohort have been described previously; their purpose was to study micronutrients concentrations in the critically ill (Stefanowicz et al. 2013a).

This investigation was conducted with the intent of developing local guidelines to aid in the interpretation of vitamin 25 (OH) D results. There were two cohorts studied. The first (a large convenience retrospective sample) arose from an audit of patients who had a

sample sent to a regional laboratory for a nutrition screen. The second (a small selected prospective cohort) arose from a prospective study of patients with critical illness. The patient data from the two cohorts was anonymised and de-identified prior to analysis (Andrew Duncan and Donald C McMillan respectively). In line with local ethical procedures written informed consent was obtained for the latter cohort only. The latter study was approved by the Ethics Committees of the North Glasgow NHS Trust and the Multicentre Research Ethics Committee (MREC) Scotland. Where patients were unable to provide signed informed consent, this was obtained from the patients' next of kin or welfare guardian in accordance with the requirements of the Adults with Incapacity Scotland (2000) Act.

7.2.3. Analytical methods

Plasma 25 (OH) D was measured by enzyme immunoassay kit (Immunodiagnostic Systems Ltd) until 2009, when tandem mass spectrometry was used (Waters Acuity; UPLC and TQD). Using routine standards and quality control, intra- and between assay imprecision was <10% in both methods over the concentration range of 25 to 120 nmol/L. Both methods had a similar lower limit of sensitivity of ~ 4 nmol/L.

Albumin was measured by a BCP dye-binding method and C-reactive protein was measured using an automated analyser (Architect, Abbott Diagnostics, USA). For CRP the limit of detection was 5 mg/l. The inter-assay coefficient of variation was less than 3% and 5% over the sample concentration range for albumin and CRP respectively. The limit of detection for albumin was 10 g/L.

7.2.4. Statistical analysis

Data was presented in median and range. Correlations between variables in the nutrition screen and critically-ill groups were carried out using the Spearman rank correlation. The large cohort was divided into three groups according to CRP concentrations ≤ 10, 11-80 and >80 mg/L as previously described (Marsik et al. 2008). The concentrations of individual 25(OH) D were grouped according to 3 categories of albumin concentrations (≤ 25, 25-34, ≥ 35 g/L) as previously described (Goldwasser and Feldman 1997). For each albumin category, data are presented as medians and 5th, 10th, 25th, 75th, and 95th percentiles. The concentration of each analyte was compared with the reference albumin category of ≥ 35 g/L by using the Mann-Whitney U test. With each CRP and albumin category the Kruskal-Wallis test was used for the comparison of more than two groups. A *P*- Value < 0.05 was considered significant and the analysis of the data was carried out using SPSS software (version 19; SPSS Inc, Chicago, Ill).

7.3. Results

7.3.1. Nutrition screen cohort

The characteristics of the convenience sample (n= 5327) are shown in Table 7-1. The majority were older than 50 years (median 58 years), female (67%) and had plasma CRP, albumin and 25 (OH) D in the normal range (median 31 µmol/l). Plasma 25 (OH) D was significantly associated with age (r_s=0.052, P <0.001), sex (males median 29nmol/l, females median 33nmol/l, P <0.001), CRP (r_s= -0.113, P <0.001) and albumin (r_s=0.192, P <0.001). Age was significantly associated with CRP (r_s= 0.275, P <0.001) and albumin (r_s=-0.292, P <0.001). CRP was significantly associated with albumin (r_s=-0.496, P <0.001).

The association between the magnitude of the systemic inflammatory response, as evidenced by CRP and albumin concentrations, on 25 (OH) D are shown in Figure 7-1a and 7-1b. The association of CRP and 25 (OH) D/ albumin ratio is shown in Figure 7-1c; the association of albumin and 25 (OH) D/ CRP ratio is shown in Figure 7-1d.

The median plasma concentrations of 25 (OH) D grouped according to CRP concentrations ≤10, 11-80 and >80 mg/L were 34, 27 and 22 nmol/l respectively (P <0.001) with an overall reduction of 35%. The median plasma concentrations of 25 (OH) D grouped according to albumin concentrations ≥35, 25-34 and <25 g/l were 34, 25 and 20 nmol/l respectively (P <0.001) with an overall reduction of 41%.

The median and the 5th, 10th, 25th, 75th, and 95th percentiles of plasma 25 (OH) D concentrations grouped according to albumin concentrations ≥35, 25-34 and <25 g/l for CRP concentrations ≤10, 11-80 and >80 mg/L are shown in Table 7-2. 3711 patients had CRP concentrations ≤ 10 mg/L; with decreasing albumin concentrations ≥35, 25-34 and

<25 g/l, median plasma concentrations of 25 (OH) D were significantly lower from 35 to 28 to 14 nmol/l (P <0.001). This decrease was significant when albumin concentrations were between 25-34 g/L (P <0.001) and when albumin concentrations were <25 g/L (P < 0.001). 1271 patients had CRP concentrations between 11-80 mg/L; with decreasing albumin concentrations ≥35, 25-34 and <25 g/l, median plasma concentrations of 25 (OH) D were significantly lower from 31 to 24 to 19 nmol/l (P <0.001). This decrease was significant when albumin concentration were 25-34 g/L (P <0.001) and when albumin concentrations were <25 g/L (P < 0.001). 345 patients had CRP concentrations >80 mg/L; with decreasing albumin concentrations ≥35, 25-34 and <25 g/l, median plasma concentrations of 25 (OH) D were not significantly altered varying from 19 to 23 to 23nmol/l.

When albumin concentrations were ≥35 g/L, the median plasma concentrations of 25 (OH) D grouped according to CRP concentrations ≤ 10, 11-80 and >80 mg/L were 35, 31 and 19.0 nmol/l (P <0.001) with an overall reduction of 46%. When albumin concentrations were 25-34 g/L, the median plasma concentrations of 25 (OH) D grouped according to CRP concentrations ≤ 10, 11-80 and >80 mg/L were 28, 24 and 23.0 nmol/l (P =0.013) with an overall reduction of 18%. When albumin concentrations were <25 g/L, the median plasma concentrations of 25 (OH) D grouped according to CRP concentrations ≤ 10, 11-80 and >80 mg/L were 14, 19.0 and 23.0 nmol/l (P =0.082). The median 25 (OH) D/ albumin ratio x100 for CRP concentrations ≤ 10, 11-80 and >80 mg/L were 85.0, 84.2 and 90.9 (P =0.002).

7.3.2. Patients with critical illness

The characteristics of the critically ill cohort (n=117) are shown in Table 7-3. The majority were older than 50 years (median 60 years), male (65%), an APACHE II score of

21 and SOFA score of 7 and the associated median predicted mortality was 34%. The majority of patients were surgical (55%) and had a median length ICU stay of 5 days and median hospital stay of 22 days. The median CRP and albumin concentrations were above and below the normal reference intervals respectively. Median adjusted calcium, and median urea and creatinine concentrations were within the normal reference interval. Approximately half had some renal impairment eGFR of <60ml/min. The median plasma 25(OH) D was 33 nmol/l. Plasma 25 (OH) D was significantly associated with age (r_s= 0.052, P <0.001), sex (r_s = 0.082, P <0.001), CRP (r_s = -0.113, P <0.001), albumin (r_s =0.192, P <0.001). Age was significantly associated with sex (r_s =0.030, P =0.029), CRP (r_s= -0.275, P <0.001) and albumin (r_s= -0.292, P <0.001). CRP was significantly associated with sex (r_s= -0.040, P =0.003) albumin (r_s=-0.496, P <0.001).

The association between the magnitude of the systemic inflammatory response, as evidenced by CRP and albumin concentrations, on 25 (OH) D in patients with critical illness were shown in Figure 7-2a and 7-2b. The association of CRP and 25 (OH) D/ albumin ratio was shown in Figure 7-2c; the association of albumin and 25 (OH) D/ CRP ratio is shown in Figure 7-2d.

7.4. Discussion

The results of the present study show that, in two different patient cohorts, plasma concentrations of 25 (OH) D were associated with the magnitude of the systemic inflammatory response as evidenced by both changes in CRP and albumin concentrations. This effect was differential with CRP most strongly associated with a reduction in plasma 25 (OH) D, where albumin concentrations were in the normal range (>35g/l) and with a much weaker association where albumin concentrations were very low (<25g/l). With normal CRP concentrations (≤ 10 mg/L), albumin had an independent effect on plasma concentrations of 25 (OH) D. These results provide a basis for the better understanding of the impact of the systemic inflammatory response on plasma vitamin D concentrations and for their assessment and interpretation.

There is now a wealth of data from observational studies that have shown associations between low concentrations of plasma 25 (OH) D and increased risks of cardiovascular disease, cancer, neurodegenerative diseases, disorders of glucose metabolism and death. Many factors, such as ageing, ethnicity, season, latitude, adiposity, physical activity, smoking and diet may impact on the link between low 25 (OH) D and the above health outcomes. In the present study of patients referred for a nutrition screen, some or all of the above factors may have impacted on plasma 25 (OH) D concentrations. However, all of the above potential confounding factors appear to be dependent on inflammatory processes for their effect on plasma 25 (OH) D concentrations. This is well illustrated in a recent study of the effect of elective knee arthroplasty on plasma 25 (OH) D concentrations (Reid et al. 2011). Indeed, inflammatory processes have been proposed as a major reason behind the discrepancy in effect of normal vs deficient plasma 25 (OH) D concentrations on health outcomes in observational studies (beneficial effect) and interventional (no effect) studies

of health outcomes (Autier et al. 2014). Similar associations seen in the critical illness cohort in the present study are also consistent with such a concept.

In the present study, the basis of the relatively low median plasma concentrations of 25(OH) D concentrations in those patients with normal CRP and albumin concentrations is not clear. However, at northerly latitudes, such as Scotland plasma concentrations of 25(OH) D concentrations are lower (Zgaga et al. 2011). Indeed, it is recognized that the majority of the Scottish population have plasma 25 (OH) D concentrations less than 50 nmol/l, considered to be borderline vitamin D status (Zgaga et al. 2011). In such patients with albumin concentrations in the normal range the basis of the inverse relationship between CRP and plasma 25 (OH) D might be due to consumption of vitamin D since the concentrations of other binding proteins such as VDBP are also likely to be in the normal range (Reid et al. 2011; Waldron et al. 2013). Further, there is some evidence that 25(OH)D can be actively taken up by inflammatory cells, particularly macrophages (Hewison 2010). In contrast, in those patients with low plasma albumin concentrations, the lack of such a relationship between CRP and plasma 25 (OH) D might be due to loss of vitamin D from the plasma compartment due to redistribution of its binding proteins vitamin D binding protein and albumin (Reid et al. 2011). More generally the associations of plasma concentrations of CRP, albumin and vitamin D may reflect the redistribution and /or the consumption of body stores by a chronic ongoing systemic inflammatory response. If this were the case it is likely that similar associations would apply to other micronutrients.

In order to confirm whether the combination of CRP and albumin can better account for the variability of plasma of 25 (OH) D, it would be important to have access to direct intracellular measurements. For example, red blood cells have been shown to be useful for vitamin B6, vitamin E, B2 zinc and selenium concentrations in subjects undergoing an

elective knee arthroplasty with a consequent acute systemic inflammatory response (Gray et al. 2004; Gray et al. 2005; Oakes et al. 2008) and in patients with critical illness (Vasilaki et al. 2008; Vasilaki et al. 2009; Vasilaki et al. 2010; Stefanowicz et al. 2013a). Whereas plasma concentrations of these vitamins deceased rapidly on activation of the systemic inflammatory response, red blood cell concentrations remained stable. Therefore, alongside the present measurements of plasma 25 (OH) D, CRP and albumin concentrations it would be important to examine intracellular concentrations of 25 (OH) D to better inform the effect of CRP and albumin on plasma 25 (OH) D. Also, this may provide insight into the nature of the effect, i.e. whether redistribution or consumption.

In summary, it is now recognized that plasma concentrations of 25 (OH) D were confounded in the presence of a systemic inflammatory response, as evidenced by CRP. The results of the present study show that albumin concentration also has an independent effect on plasma concentrations of 25 (OH) D. Therefore, plasma concentrations of 25 (OH) D may not be reliably interpreted in the presence of abnormal CRP (>10mg/l) and albumin concentrations (<35g/l). It may be that plasma concentrations of 25 (OH) D in the presence of normal CRP and albumin concentrations reliably indicate nutrition status of vitamin D and the need for supplementation.

Table 7-1: Characteristics of nutrition screen cohort and plasma 25 (OH) D concentrations.

	Reference interval	Nutrition screen cohort (n= 5327)
Age (years)	NA	58 (16-109)
Sex (Male/Female)	NA	1773 (33%)/ 3554 (67%)
CRP (mg/l)	<10	5.0 (0.19- 565.0)
Albumin (g/l)	35-55	38 (9-52)
25 (OH) D (nmol/l)	<25/25-50/>50	31 (6-1140)

(Median and range)

Table 7-2: Distribution in percentiles of plasma 25 (OH) D according to CRP and albumin concentrations (n=5327).

CRP concentrations ≤10 mg/L (n= 3711)									
Albumin	Subjects *n* (%)	5th Percentile	10th Percentile	25th Percentile	Median (% change)	75th Percentile	90th Percentile	95th Percentile	*P*-value
≥ 35 g/L	3294(89)	14.0	14.0	20.0	35.0 (100)	57.0	85.0	102.0	<0.001 a
25-34g/L	378 (10)	14.0	14.0	15.0	28.0 (80)	46.0	75.0	88.0	<0.001 b
<25g/L	39 (1)	14.0	14.0	14.0	14.0 (40)	34.0	64.0	67.0	<0.001 b
25 (OH) D/ albumin ratio x100		34.12	36.59	50.00	**85.0**	141.86	210.33	259.68	
CRP concentrations 11-80 mg/L (n= 1271)									
≥ 35 g/L	649 (51)	14.0	14.0	18.0	31.0 (100)	52.0	80.0	101.0	<0.001 a
25-34g/L	476 (37)	14.0	14.0	14.0	24.0 (77)	49.0	82.0	105.0	<0.001 b
<25g/L	146 (11)	14.0	14.0	14.0	19.0 (61)	37.8	76.7	138.5	<0.001 b
25 (OH) D/ albumin ratio x100		36.82	39.97	51.81	**84.2**	148.48	242.36	105.71	
CRP concentrations ≥ 80 mg/L (n= 345)									
≥ 35 g/L	42 (12)	14.0	14.0	14.0	19.0 (100)	26.5	52.6	72.5	0.284 a
25-34g/L	150 (43)	10.6	14.0	14.0	23.0 (121)	38.0	58.8	82.8	0.277 b
<25g/L	153 (44)	14.0	14.0	14.0	23.0 (121)	33.0	55.2	78.3	0.093 b
25 (OH) D/ albumin ratio x100		37.81	42.39	56.55	**90.9**	169.23	265.24	422.29	

a Kruskal Wallis, b Mann-Whitney *U* test. When n<5 median value was not calculated.

Table 7-3: Characteristics of ICU cohort and plasma vitamin D concentrations.

	Reference interval	Nutrition screen cohort (n= 117)
Age (years)	NA	60 (18-100)
Sex (Male/Female)	NA	76 (65%)/ 41 (35%)
C-reactive protein (mg/l)	<10	109.0 (1.0- 565.0)
Albumin (g/l)	35-55	17 (9-45)
25 (OH) D (nmol/l)	<25/25-50/>50	33 (13-1140)

(Median and range)

Figure 7-1a: The relationship between CRP (log 10) and 25 (OH) D in the nutrition screen cohort (r_s= -0.113, *P* <0.001).

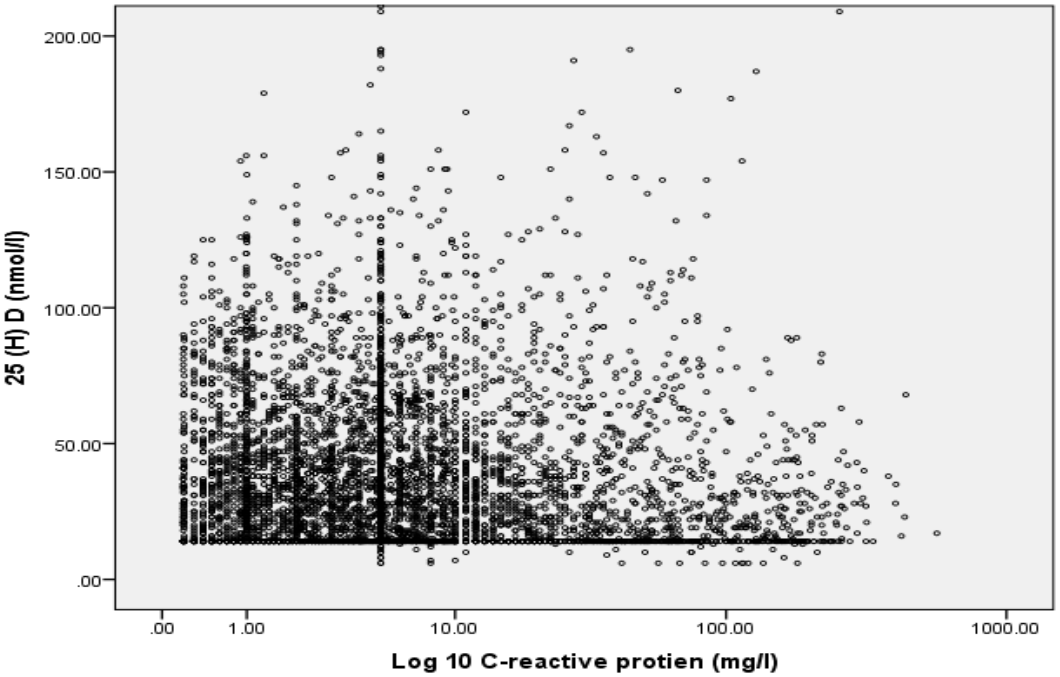


Figure 7-1b: The relationship between albumin and 25 (OH) D in the nutrition screen cohort (r_s= 0.192, *P* <0.001).

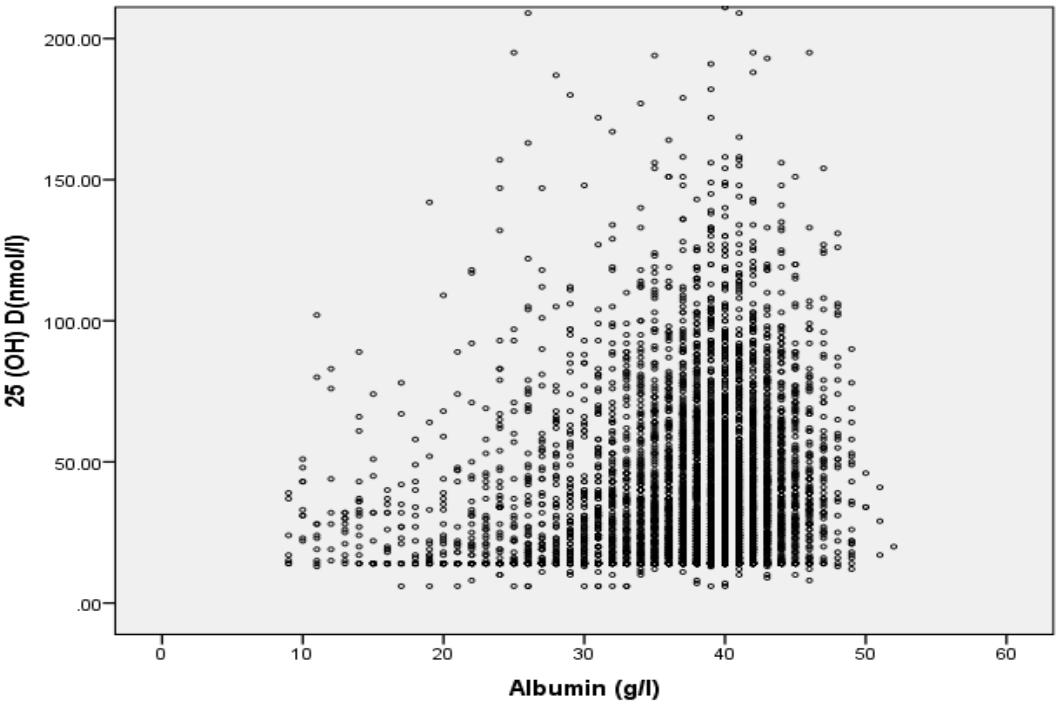


Figure 7-1c: The relationship between CRP (log 10) and 25 (OH) D adjusted to albumin in the nutrition screen cohort (r_s=- 0.053, *P* <0.001).

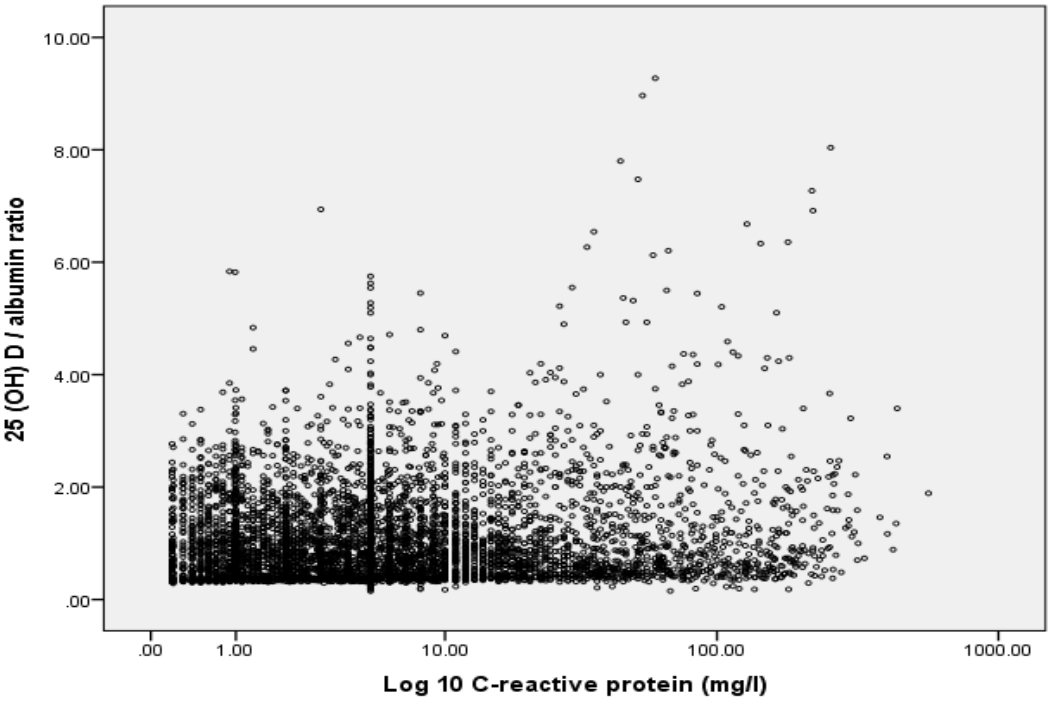


Figure 7-1d: The relationship between albumin and 25 (OH) D adjusted to CRP in the nutrition screen cohort (r_s=0.507, *P* <0.001).

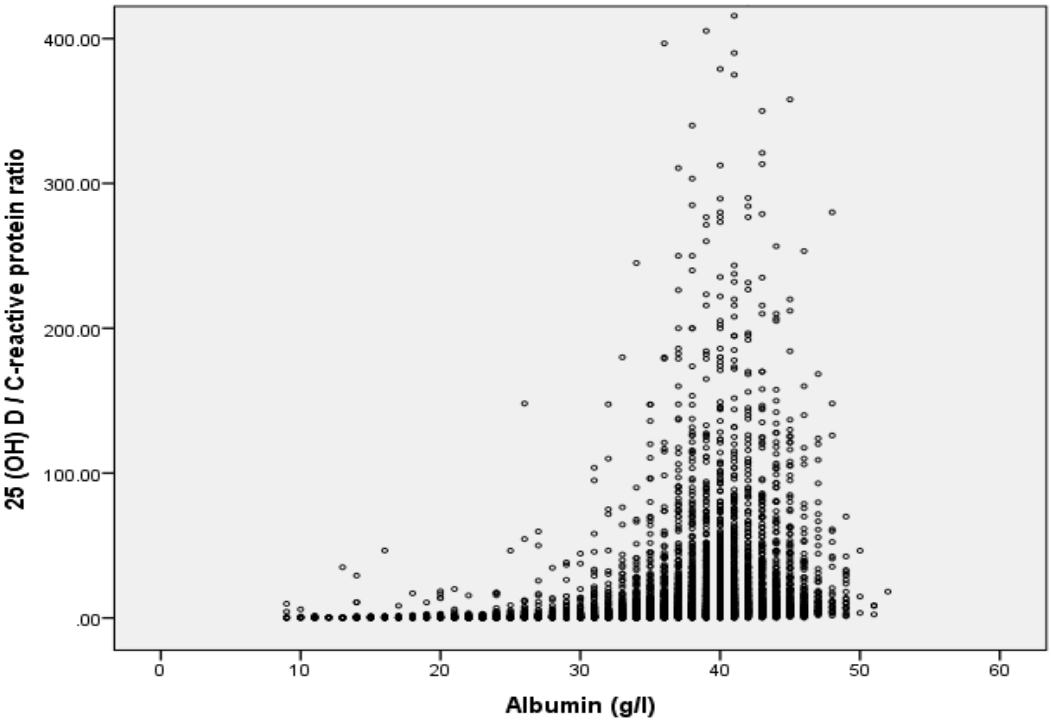


Figure 7-2a: The relationship between CRP (log 10) and 25 (OH) D in patients with critical illness (r_s= -0.221, *P* =0.017).

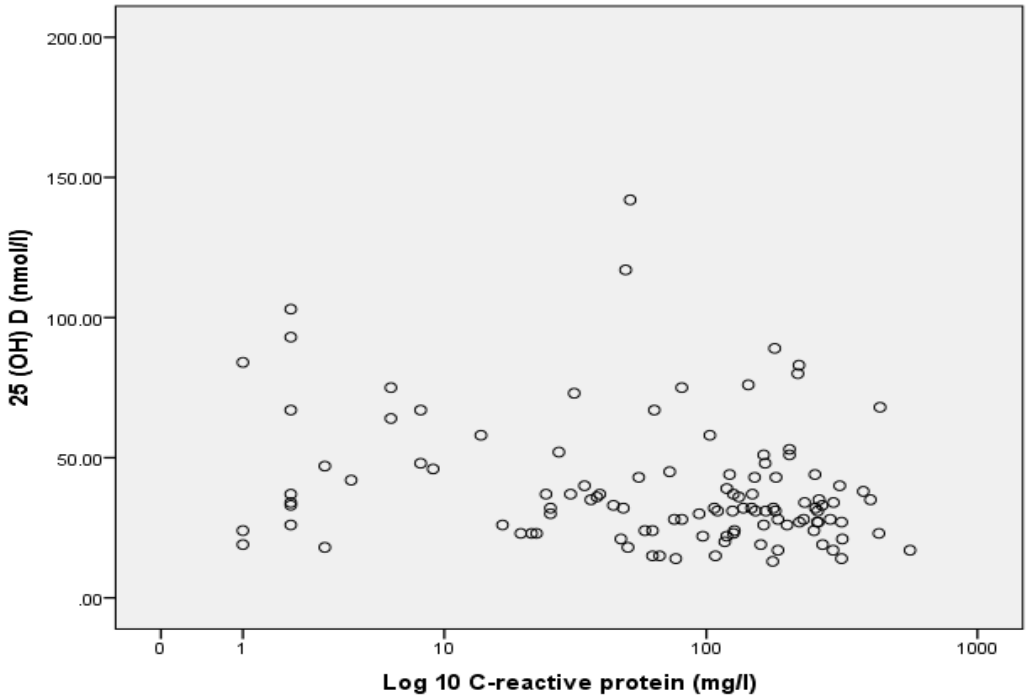


Figure 7-2b: The relationship between albumin and 25 (OH) D in patients with critical illness (r_s= 0.166, *P* =0.073).

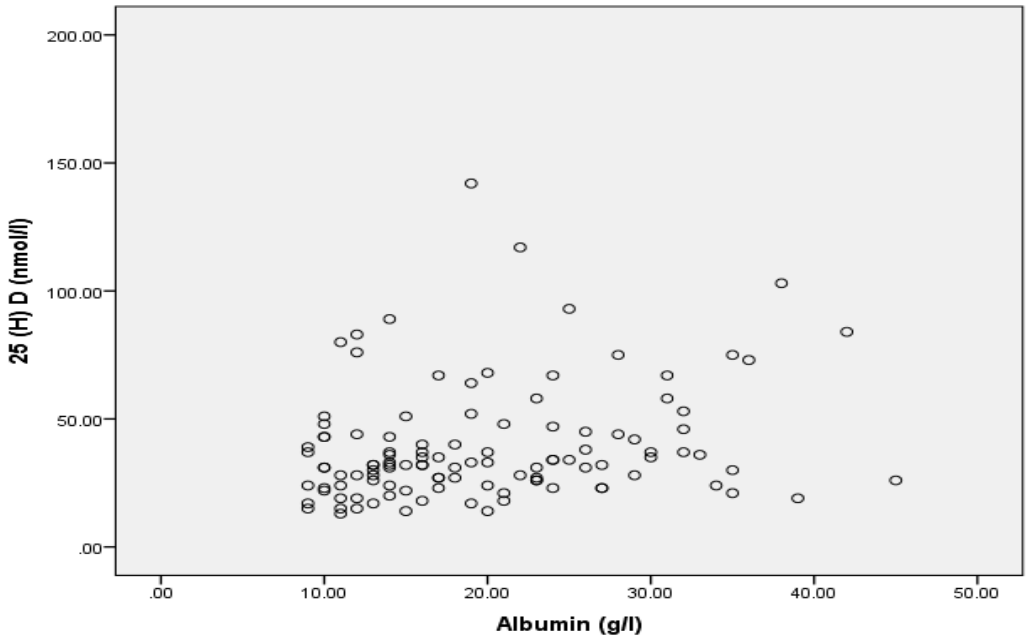


Figure 7-2c: The relationship between CRP (log 10) and 25 (OH) D adjusted to albumin in patients with critical illness (r_s= 0.016, *P* =0.863).

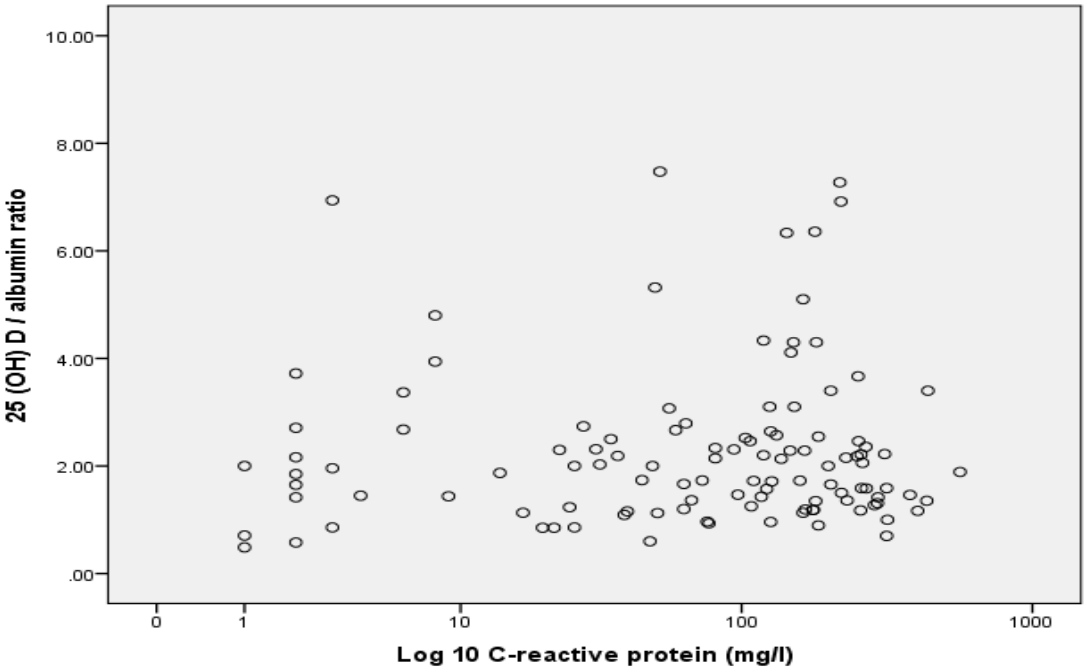
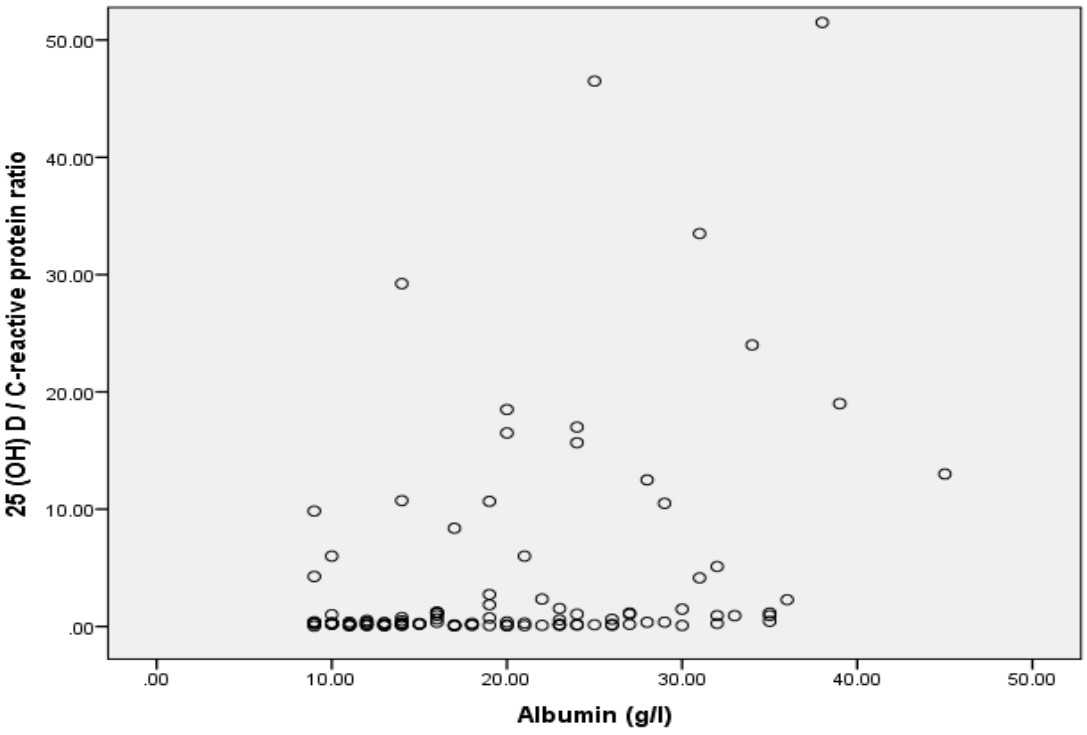


Figure 7-2d: The relationship between albumin and 25 (OH) D adjusted to CRP in patients with critical illness (r_s= 0.363, *P* <0.001).



8. The relationship between the markers of the systemic inflammatory response and plasma vitamin E and C concentrations.

8.1. Introduction

Vitamin E (α-tocopherol), a fat-soluble vitamin, and vitamin C (ascorbic acid), a water-soluble vitamin, both have complimentary and anti-oxidative properties (Padayatty et al. 2003). Vitamins E and C are chain-breaking antioxidants that prevent the peroxidation of lipids in cell membranes and lipoproteins (Nogueira et al. 2013). Vitamins E and C also act synergistically, as a peroxyl radical scavenger, preventing the propagation of free radicals in tissues, by reacting with them to form an α-tocopheroxyl radical that is then reduced back to α-tocopherol by a hydrogen donor such as vitamin C (Crimi et al. 2004; Nogueira et al. 2013). Vitamin E, because it is fat-soluble, is incorporated into cell membranes, which protects them from oxidative damage. It is also essential with cholesterol for the cell membranes structural stability (Chow 1975).

Vitamin E and C status is usually assessed by plasma measurement of α-tocopherol and ascorbic acid respectively, although their correlation with concentrations in tissues is not clearly established. Moreover, plasma concentrations of vitamin E are strongly associated with their carrier lipids, principally lipoprotein complexes, such as high density lipoprotein (HDL), very low density lipoprotein (VLDL) and chylomicrons. To overcome this limitation it has been proposed that the plasma α-tocopherol concentrations should be expressed in relation to plasma lipid concentrations, usually cholesterol (Thurnham et al. 1986; Doise et al. 2008; Vasilaki et al. 2009). Some workers have proposed either expressing plasma vitamin concentrations per lipid concentrations (Thumham et al. 2008) or measurement of intracellular vitamin concentrations (Talwar et al. 2003b). Indeed, in healthy subjects undergoing elective surgery, there is a significant transient decrease in

both α-tocopherol and cholesterol concentrations during the evolution of the systemic inflammatory response (Gray et al. 2005). Consistent with the above, when plasma α-tocopherol/ cholesterol, was applied there was no significant alteration in plasma α-tocopherol concentrations (Gray et al. 2005).

Duncan and co-workers (2012) reported that the impact of systemic inflammatory response on plasma α-tocopherol not adjusted for cholesterol and ascorbic acid concentrations when CRP concentrations were > 80 mg/L were associated with a 8% and 78% reduction (Louw et al. 1992; Galloway et al. 2000; Duncan et al. 2012b). However, the variability of the association was such that plasma α-tocopherol and ascorbic acid concentrations were not readily adjustable for CRP concentrations. Furthermore, Ghashut and co-workers (2014) have recently reported that plasma concentrations of fat soluble micronutrients such as 25 (OH) D may be confounded in the presence of a systemic inflammatory response with both CRP and albumin having an independent effect on plasma 25 (OH) D concentrations (Ghashut et al. 2014).

The aim of the present study was to examine the relationships between plasma α-tocopherol, ascorbic acid, CRP and albumin concentrations in a large cohort of patients referred for assessment and also examine these relationships in patients with critical illness.

8.2. Patients and methods

8.2.1. Nutrition screen cohort

A total of 359 consecutive heparin-treated whole-blood samples from 684 patients, and a total of 494 consecutive heparin-treated whole-blood samples from 916 patients, were received from hospitals throughout Scotland between January 2000 and March 2013 for routine analysis of plasma α-tocopherol and ascorbic acid concentrations. If more than one set of plasma α-tocopherol and ascorbic acid results was available in a patient, only the first sample was included in the analysis, leaving a total of 359 and 494 plasma α-tocopherol and ascorbic acid results, respectively. In the nutrition screen cohort < 20 % of patients had both vitamin E and C measurements. In order to have enough observations to examine the relationship between markers of the systemic inflammatory response and measurements of vitamin E and C we used the present approach. Blood samples were sent to a regional centre for analysis if the patient was considered at nutrition risk and was often secondary to a number of disease states. In addition, measurement of albumin and CRP was also recorded for these patients.

8.2.2. Critical illness cohort

Data and stored samples were available from cohorts which had been defined previously. The critical illness cohort comprised of medical or surgical patients ≥ 18 years admitted to the ICU of the Glasgow Royal Infirmary (GRI) between September 2006 and December 2008 and had evidence of the systemic inflammatory response syndrome as per Bone's criteria (Bone et al. 1992; Levy et al. 2003), were studied. Briefly, APACHE II score (Knaus et al. 1985) and predicted hospital mortality and SOFA scores, CRP and albumin were recorded. This cohort has been described previously (Vasilaki et al. 2008).

This investigation was conducted with the intent of developing local guidelines to aid in the interpretation of α-tocopherol and ascorbic acid results. Two cohorts were studied: the first (a large unselected retrospective cohort) arose from an audit of patients who had a sample sent to a regional laboratory for a nutrition screen. Approval for audit purpose was obtained from the local Ethics Committee of the North Glasgow NHS Trust. The second (a small selected prospective cohort) arose from a prospective study of patients with critical illness. The patient data from the two cohorts was anonymised and de-identified prior to analysis (Andrew Duncan and Donald C McMillan respectively). In line with local ethical procedures written informed consent was obtained for the latter cohort only. The latter study was approved by the ethics committees of the North Glasgow NHS Trust and Multicentre Research Ethics Committee (MREC) Scotland. Where patients were unable to give signed informed consent, consent was obtained from the patients' next of kin or welfare guardian in accordance with the requirements of the Adults with Incapacity Scotland (2000) Act (Appendix 1, 2).

8.2.3. Analytical methods

The within-batch imprecision was 3.7% at a concentration of 38 mol/L. α-tocopherol was measured by HPLC (Talwar et al. 1998). The intra-assay CV was, 9% for both analytes over the sample concentration range. The intra-assay imprecision was 4.9% at 59 nmol/L. Ascorbic acid was only measured if the plasma sample was prepared within 4 h and stabilized by diluting it with an equal volume of 6% metaphosphoric acid. Plasma ascorbic acid status was assessed by using a method based on that of Margolis and Davis (Margolis and Davis 1988). Briefly, plasma was stabilized and deproteinized with 60 g metaphosphoric acid/L (stored at 4–8°C and prepared fresh every 2 weeks) and centrifuged and an aliquot of supernatant fluid was injected on a C_{18} reversed-phase analytic column (Nucleosil). After separation, ascorbic acid was determined by coulometric

electrochemical detection (ESA 5100A). The within-batch imprecision was 3.7% at a concentration of 38 μmol/L.

8.2.4. Statistical analysis

Data was presented in median and range value. Correlations between variables in the convenience sample were carried out using the Spearman rank correlation. The cohorts were divided into three groups according to CRP concentrations ≤ 10, 11-80 and >80 mg/L as previously described (Marsik et al. 2008). The concentration of individual α-tocopherol/ cholesterol and ascorbic acid were grouped according to 3 categories of albumin concentrations ≥ 35, 25-34, < 25 g/L as previously described (Goldwasser and Feldman 1997). A P- value < 0.05 was considered significant and the analysis of the data was carried out using SPSS software (version 19; SPSS Inc, Chicago, Ill).

8.3. Results

The characteristics of the convenience sample for α-tocopherol (n= 359) are shown in Table 8-1. The majority were older than 50 years (median 52 years), male (52%) and had plasma albumin and cholesterol below the normal range, while plasma CRP and plasma α-tocopherol were in the normal range. Plasma α-tocopherol was significantly associated with sex (r_s=-0.342, P =0.001), cholesterol (r_s= -0.311, P =0.005) and albumin (r_s=0.491, P <0.001). CRP was significantly associated with cholesterol (r_s=-0.334, P =0.003). Albumin was significantly associated with cholesterol (r_s=-0.464, P <0.001).

The characteristics of the convenience sample for ascorbic acid (n= 494) are also shown in Table 8-1. The majority were older than 50 years (median 54 years), female (53%), and had plasma CRP and albumin and plasma ascorbic acid in the normal range. Plasma ascorbic acid was significantly associated with age (r_s=-0.268, P <0.001), CRP (r_s= -0.333, P <0.001) and albumin (r_s=0.588, P <0.001). Age was significantly associated with CRP (r_s= 0.259, P <0.001) and albumin (r_s=-0.251, P <0.001). CRP was significantly associated with albumin (r_s=-0.643, P <0.001).

8.3.1. Nutrition screen cohort for α-tocopherol / cholesterol ratio

The effects of the magnitude of the systemic inflammatory response, as evidenced by CRP concentrations and albumin, on α-tocopherol/ cholesterol ratio are shown in Figure 8-1a and 8-1b.

The median α-tocopherol/ cholesterol ratio grouped according to CRP concentrations ≤10, 11-80 and >80 mg/L were 5.9, 4.6 and 2.1 umol/l respectively (P <0.001) with an overall reduction of 64%. The median α-tocopherol/cholesterol ratio grouped according to

albumin concentrations ≥35, 25-34 and <25 g/l were 6.0, 5.5 and 2.1 umol/l respectively (*P* <0.001) with an overall reduction of 65%.

When albumin concentrations were ≥ 35 g/L, the median α-tocopherol/ cholesterol ratio grouped according to CRP concentrations ≤10 and 11-80 mg/L were 6.2 and 5.4 (*P* =0.134). When albumin concentrations were 25-34 g/L, the median α-tocopherol/ cholesterol ratio grouped according to CRP concentrations ≤10, 11-80 and >80 mg/L were 5.2, 6.0 and 4.6 (*P* =0.518) with an overall reduction of 12%. When albumin concentrations were < 25 g/L, the median α-tocopherol/ cholesterol ratio grouped according to CRP concentrations ≤10, 11-80 and >80 mg/L were 3.8, 2.4 and 1.7 (*P* =0.032) with an overall reduction of 55%.

8.3.2. Nutrition screen cohort for ascorbic acid.

The effects of the magnitude of the systemic inflammatory response, as evidenced by CRP and albumin concentrations, on ascorbic acid are shown in Figure 8-2a and 8-2b. The effect of the CRP on ascorbic acid/ albumin ratio is shown in Figure 8-2c.

The median plasma concentrations of ascorbic acid grouped according to CRP concentrations ≤10, 11-80 and >80 mg/L were 25.0, 15.0 and 6.0 µmol/l respectively (*P* <0.001) with an overall reduction of 78%. The median plasma concentrations of ascorbic acid grouped according to albumin concentrations ≥35, 25-34 and <25 g/l were 32.0, 13.0 and 5.0 µmol/l respectively (*P* <0.001) with an overall reduction of 84%.

When albumin concentrations were ≥ 35 g/L, the median plasma concentrations of ascorbic acid grouped according to CRP concentrations ≤10, 11-80 and >80 mg/L were 33.0, 30 and 31µmol/l (*P* =0.765). When albumin concentrations were 25-34 g/L, the median plasma concentrations of ascorbic acid grouped according to CRP concentrations ≤10, 11-80 and >80 mg/L were 12.0, 15 and 10.0 µmol/l (*P* =0.409) with an overall

reduction of 17%. When albumin concentrations were < 25 g/L, the median plasma concentrations of ascorbic acid grouped according to CRP concentrations ≤10, 11-80 and >80 mg/L were 3, 10 and 5.0 µmol/l (P =0.121). The median vitamin C/ albumin ration x 100 for CRP concentrations ≤10, 11-80 and >80 mg/L were 71.8, 53. 5 and 37.3 respectively (48%, p=0.050). The median vitamin C/ CRP ratio x 100 for albumin concentrations ≥35, 25-34 and <25 g/l were 711.5, 65.8 and 10.3 (99%, p<0.001).

8.3.3. Patients with critical illness

The effects of the magnitude of the systemic inflammatory response, as evidenced by CRP and albumin concentrations, on α-tocopherol/ cholesterol ratio are shown in Figure 8-1a and 8-1b**;** the effects on ascorbic acid are shown in Figure 8-2a and 8-2b, while the effect of the CRP on ascorbic acid/ albumin ratio is shown in Figure 8-2c.

The characteristics of the critically ill cohort (n=82) are shown in Table 8-1. The majority were older than 50 years (median 58 years), male (62%), with an APACHE II score of 21 and a SOFA score of 7; the associated median predicted mortality was 33.6%. The majority of patients were surgical (56%) and had a median length ICU stay of 4 days, and median hospital stay of 19 days. The median CRP and albumin concentrations were above and below the normal reference intervals respectively. Approximately half had some renal impairment as evidenced by eGFR of <60ml/min.

The median plasma α-tocopherol was within the normal reference interval (14.5umol/l), whereas patients with critical illness have extremely low α-tocopherol concentrations compared with the general population of this nutrition screen cohort, median plasma cholesterol concentration was slightly elevated. Plasma α-tocopherol was significantly associated with sex (r_s =0.211, P =0.022), CRP (r_s = -0.270, P =0.003) and with albumin (r_s =0.5847, P <0.001). Age was significant associated with albumin (r_s = -0.207, P

=0.024). CRP was significantly associated with albumin (r_s=-0.358, P <0.001) and cholesterol (r_s =0.366, P <0.001). Albumin was significantly associated with cholesterol (r_s =-0.416, P <0.001).

The median α-tocopherol/ cholesterol ratio grouped according to CRP concentrations ≤10, 11-80 and >80 mg/L were 4.0, 2.4 and 1.6 µmol/l respectively (85%, P <0.001). The median α-tocopherol / cholesterol ratio grouped according to albumin concentrations ≥35, 25-34 and <25 g/l were 4.8, 3.9 and 1.7 µmol/l respectively (65%, P <0.001*).*

The median plasma ascorbic acid was below the normal reference interval (4.0µmol/l), whereas patients with critical illness had extremely low vitamin C concentrations compared with the general population of this nutrition screen cohort. Plasma ascorbic acid was significantly associated with white cell ascorbic acid (r_s =0.327, P =0.035). CRP was significantly associated with albumin (r_s=-0.393, P <0.001).

The median plasma concentrations of ascorbic acid grouped according to CRP concentrations ≤10, 11-80 and >80 mg/L were 2.0, 5.0 and 5.0 µmol/l respectively (P =0.052). The median plasma concentrations of ascorbic acid grouped according to albumin concentrations ≥35, 25-34 and <25 g/l were 6.0, 8.0 and 4.0 µmol/l respectively (p=0.490).

8.4. Discussion

The results of this study show that, in two cohorts of patients referred for a nutrition screen, plasma concentrations of α-tocopherol/ cholesterol ratio and ascorbic acid were significantly altered by magnitude of the systemic inflammatory response as evidenced by both changes in CRP and albumin concentrations. Furthermore, this effect was differential, with albumin associated with a reduction in plasma α-tocopherol/ cholesterol ratio and ascorbic acid irrespective of the CRP concentrations. As a result, albumin appears to have a larger effect than CRP on α-tocopherol/ cholesterol ratio and ascorbic acid. Moreover, vitamin C concentrations were more profoundly affected by the presence of a systemic inflammatory response. These results provide a basis for the better understanding of the impact of the systemic inflammatory response on α-tocopherol/ cholesterol ratio and ascorbic acid concentrations, and for their assessment and interpretation.

In particular, the results of the present study question the use of the α-tocopherol/ cholesterol ratio, as proposed by Thurnham and co-workers (1986), to adjust for the effect of the systemic inflammatory response. Since, when expressed per mmol of cholesterol, α-tocopherol, concentrations were lower with decreasing concentrations of albumin.

The role of vitamin C in alleviating the oxidant stress and recycling the oxidized α-tocopherol is well recognised. Plasma α-tocopherol concentrations, in addition to being redistributed as part of the systemic inflammatory response, have been reported to be regenerated by vitamin C. Early work on fat autoxidation performed by Golumbic and Mattill (1941) reported the antioxygenic action of ascorbate in association with tocopherols (Golumbic and Mattill 1941). More recently, in vitro studies have shown that ascorbic acid reduces the tocopheroxyl radical (Packer et al. 1979) and thereby restores the radical-

scavenging activity of tocopherol (Niki et al. 1982; Doba et al. 1985; Lambelet et al. 1985; Niki 1987; Wayner et al. 1987). It would appear that the tocopheroxyl radical that forms in membranes reacts with ascorbic acid to yield tocopherol and the ascorbyl radical, the result of which is to maintain radical scavenging potential within the membrane by regenerating tocopherol and to transfer the oxidative challenge to the aqueous phase. This scheme is consistent with the observation in the present study that plasma vitamin C concentrations were lower compared with vitamin E concentrations for the same magnitude of the systemic inflammatory response, even in patients with critical illness in whom vitamin C concentrations were recognised to be profoundly low (Schorah et al. 1996; Nathens et al. 2002; Mishra et al. 2005).

The importance of the systemic inflammatory response in determining plasma vitamin C concentrations is highlighted by the relative inefficiency, whereby supplementation can prevent low concentrations following an inflammatory insult. For example, in healthy patients with normal pre–operative plasma concentrations of ascorbic acid, a single oral supplementation with 1,000 mg of ascorbic acid was unable to prevent the fall in postoperative plasma concentrations (Ruemelin et al. 2002). Therefore, it is reasonable to conclude that ascorbic acid deficiency and the need for supplementation should only be considered on the basis of low plasma ascorbic acid concentrations in the presence of plasma CRP and albumin concentrations in the normal range.

In summary, the results of the present study show that plasma concentrations of α-tocopherol/ cholesterol ratio and ascorbic acid are independently associated with both CRP and albumin and consistent with the systemic inflammatory response as a major confounding factor in determining their status. In particular, vitamin C concentrations were more profoundly affected by the presence of systemic inflammatory response. These

results provide a basis for the better understanding of the impact of the systemic inflammatory response on plasma vitamin E and C.

Table 8-1: Characteristics of nutrition screening, patient with critical illness cohorts and plasma vitamin E (α-tocopherol) and C (ascorbic acid) concentrations.

	Reference interval	Vitamin E nutrition screen (n=359)	Vitamin C nutrition screen (n=494)	Critical illness cohort (n= 82)	P-value
Age (years)	N/A	52 (15-100)	54 (16-90)	58 (18-86)	<0.001a
Sex (Male/Female)	N/A	185 (52%)/ 174 (48%)	234 (47%)/ 260 (53%)	51 (62%)/ 31 (38%)	<0.001a
C-reactive protein (mg/l)	<10	9.0 (0.20- 565.0)	10.0 (0.20- 565.0)	85.0 (1.0- 565.0)	<0.001a
Albumin (g/l)	35-55	31 (9-50)	33 (9-50)	18 (9-45)	<0.001a
Plasma α-tocopherol (umol/l)	12 - 46	21.0 (2.9-58.0)		15.1 (5.0 -36.0)	<0.001b
Cholesterol (mmol/l)	<5.2	4.5 (1.1-15.7)		2.2 (0.53-6.4)	<0.001b
α-tocopherol/ cholesterol ratio (µmol/ mmol)	5.2 (3.5-8.7)	5.2 (0.4-19.5)		6.6 (2.1-15.7)	<0.001b
Plasma ascorbic acid (µmol/l)	11-114		17.0 (0.9-262.0)	4.0 (0.9-136.0)	<0.001b

Median (Range) a Kruskal Wallis, b Mann-Whitney *U* test

Table 8-2: Distribution of plasma α-tocopherol adjusted to cholesterol according to CRP and albumin concentrations (n=359).

Plasma α-tocopherol/ cholesterol ratio	CRP ≤10 (mg/l)	11-80 (mg/l)	>80 (mg/l)	Plasma α-tocopherol/ cholesterol ratio according to albumin
Albumin ≥35 (g/l)	6.2 (3.2-10.7) (n=131)	5.4 (3.3-10.8) (n=18)	- (n=1)	6.0 (3.2-10.8) (n=150)
34-25 (g/l)	5.2 (0.97-10.4) (n=48)	6.0 (1.2-9.7) (n=38)	4.6 (1.3-8.3) (n=12)	5.5 (1.0-10.4) (n=98)
<25 (g/l)	3.8 (1.1-7.6) (n=15)	2.4 (0.9-17.0) (n=31)	1.7 (0.4-19.4) (n=65)	2.1 (0.4-19.5) (n=111)
Plasma α-tocopherol/ cholesterol ratio according to CRP	5.9 (1.0-10.7) (n=194)	4.6 (0.9-17.0) (n=87)	2.1 (0.4-19.5) (n=78)	

Median (range) (number of observation)

Table 8-3: Distribution of plasma vitamin C (ascorbic acid) (µmol/l) according to CRP and albumin concentrations (n=494).

Plasma vitamin C (ascorbic acid) (µmol/l)	CRP ≤10 (mg/l)	11-80 (mg/l)	>80 (mg/l)	Plasma C (ascorbic acid) according to albumin
Albumin ≥35 (g/l)	33 (0.99-235) (n=175)	30 (0.99-79.0) (n=44)	31 (17.0-66.0) (n=4)	32 (0.99-235) (n=223)
34-25 (g/l)	12 (0.90-126) (n=61)	15 (0.99-136) (n=66)	10 (0.99-61.0) (n=19)	13 (0.90-136) (n=146)
<25 (g/l)	3 (0.90-40.0) (n=12)	10 (0.90-262) (n=50)	5.0 (0.90-102.0) (n=63)	5 (0.90-262) (n=125)
Plasma C (ascorbic acid) according to CRP	25 (0.90-235) (n=248)	15 (0.90-262.0) (n=160)	6.0 (0.90-102.0) (n=86)	

Median (range) (number of observation)

Figure 8-1a: The relationship between α-tocopherol adjusted to cholesterol and CRP (log 10) (r_s= - 0.424, *P* <0.001) and) in patients with critical illness (r_s= - 0.391, *P* <0.001).

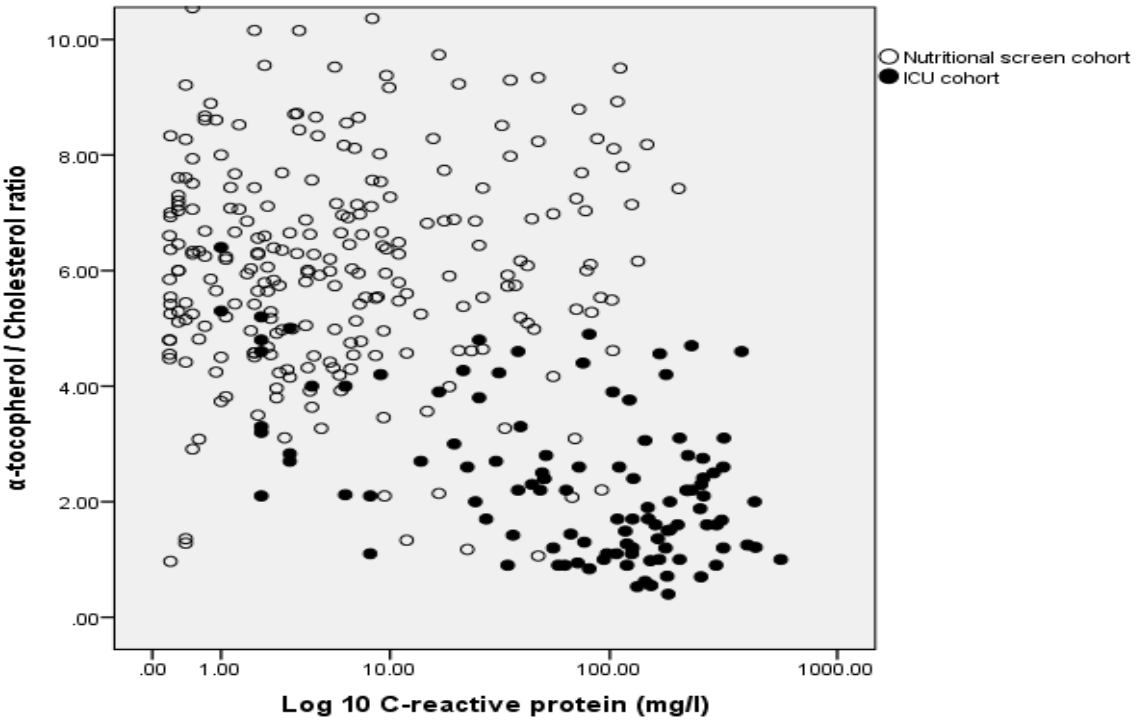


Figure 8-1b: The relationship between α-tocopherol adjusted to cholesterol and albumin (r_s= 0.531, *P* <0.001) and in patients with critical illness (r_s= 0.693, *P* <0.001).

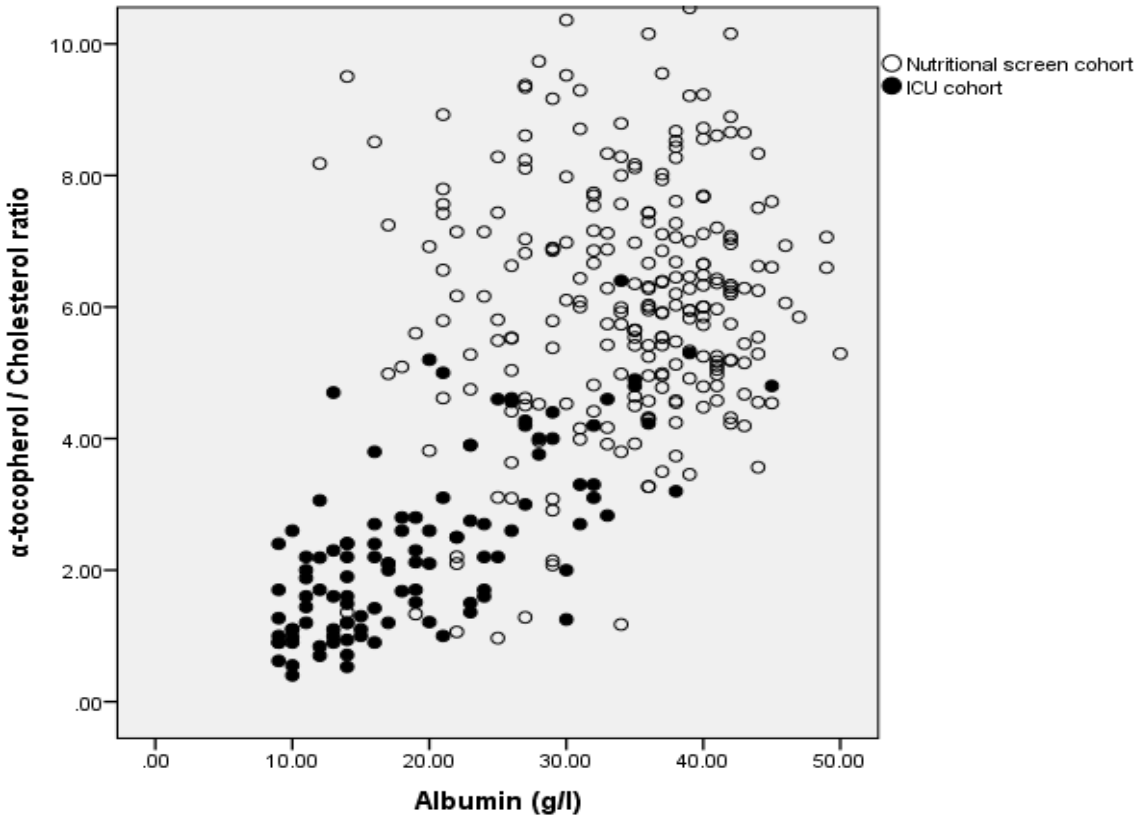


Figure 8-2a: The relationship between CRP (log 10) and vitamin C (µmol/l) (r_s= - 0.333, *P*<0.001) and in patients with critical illness (r_s= - 0.174, *P*=0.117).

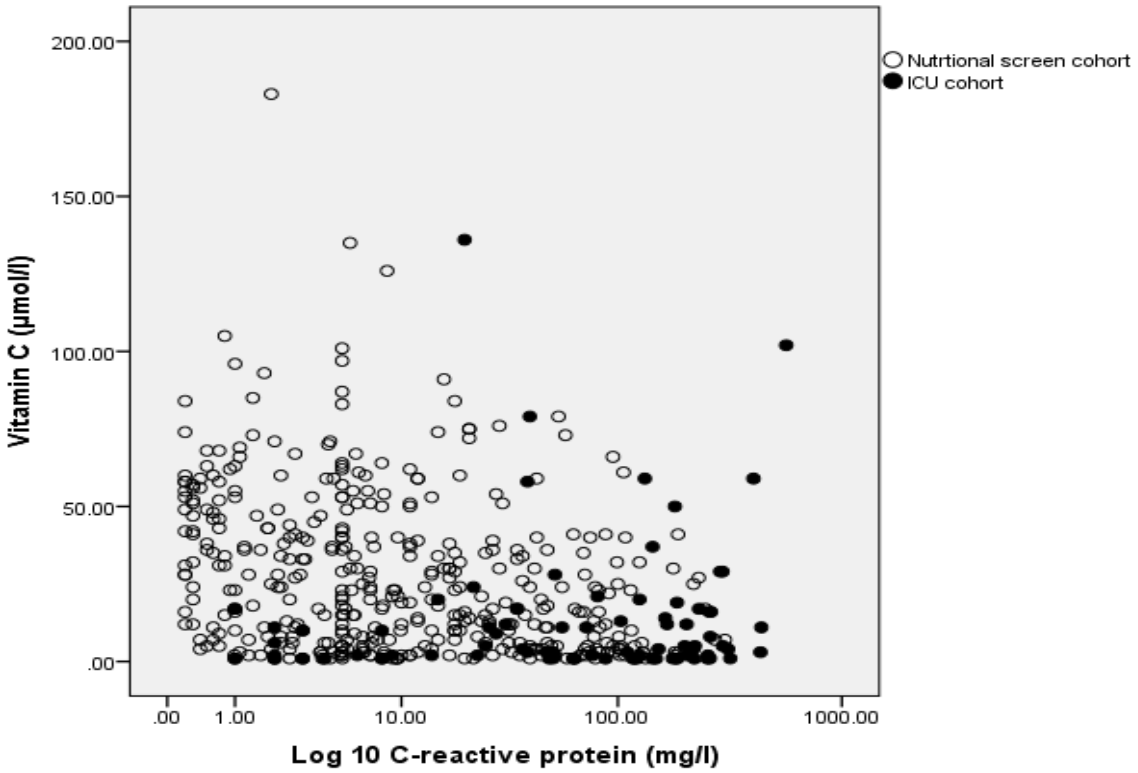


Figure 8-2b: The relationship between albumin and vitamin C (µmol/l) (r_s= 0.427, *P*<0.001) and in patients with critical illness (r_s= 0.205, *P*=0.064).

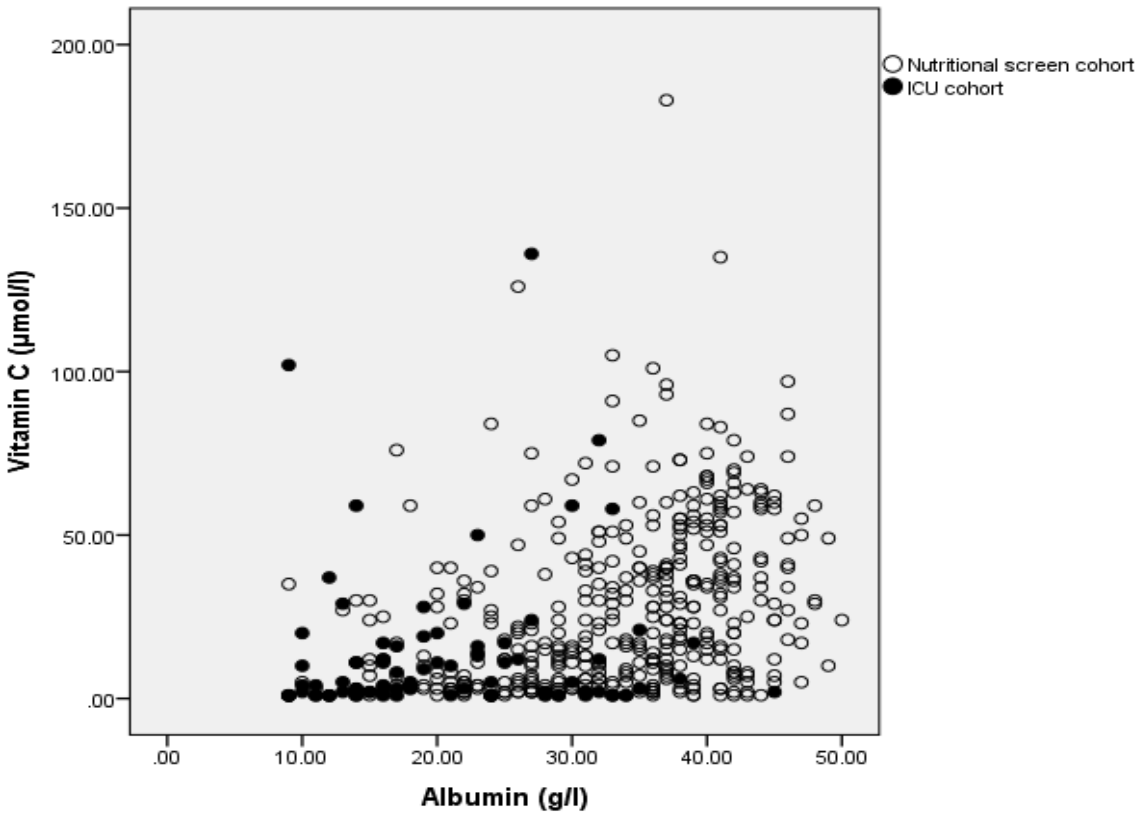
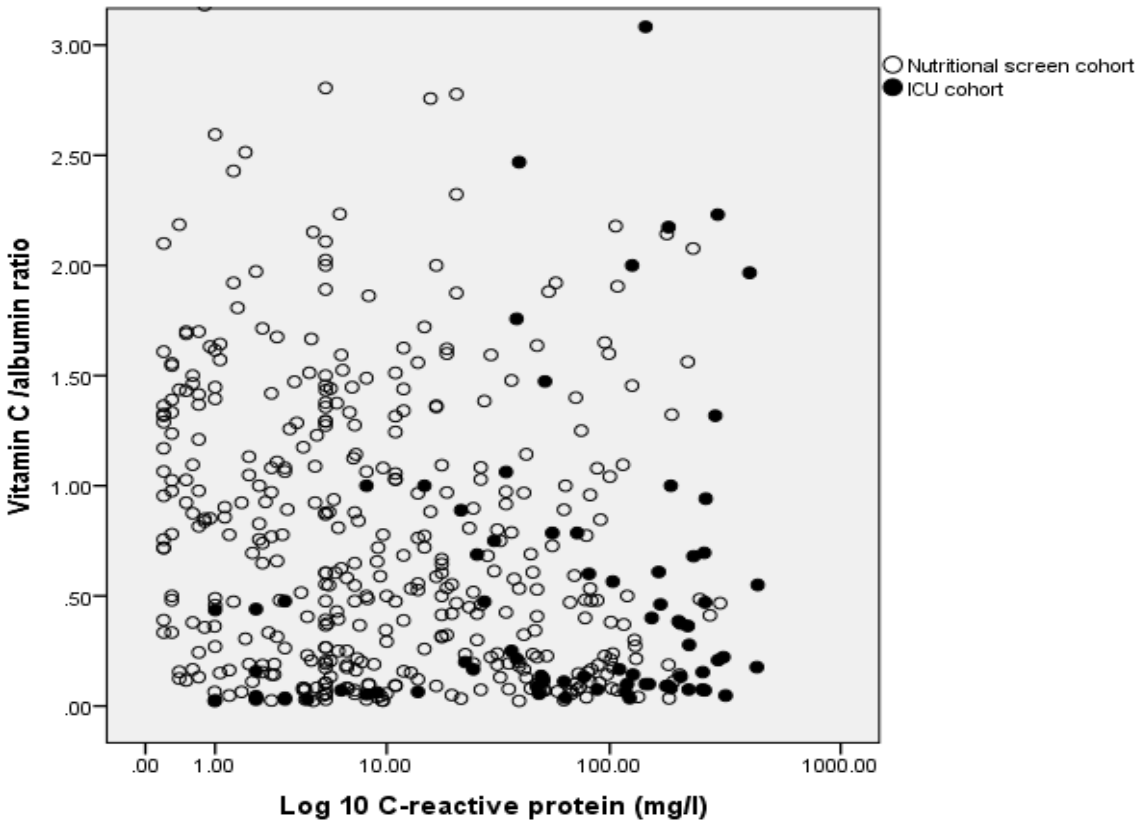


Figure 8-2c: The relationship between CRP (log 10) and vitamin C adjusted to albumin (r_s= - 0.179, P<0.001) and in patients with critical illness (r_s= - 0.266, P=0.016).



9. The relationship between markers of the systemic inflammatory response and plasma zinc and selenium concentrations.

9.1. Introduction

Trace elements are of vital importance in human health acting as cofactors in many catalytic processes and particularly, in the case of zinc, they have a structural role. Selenium is essential for the catalytic activity of glutathione peroxidase activity (GSH-Px) protecting against membrane lipid peroxidation (Thomas et al. 1990) while zinc also combats oxidative stress as cofactors in zinc superoxide dismutase. Zinc and selenium both play important functions in immune regulation (Fraker et al. 2000). With such roles, adequate trace element status is considered to be of particular importance in critically ill patients who have significantly reduced circulating and body stores of antioxidants, such as zinc superoxide dismutase and glutathione peroxidase activity, therefore compromise of reactive oxygen species (Heyland et al. 2005).

Concentrations of some circulating trace elements such as zinc and selenium are known to decrease significantly following severe trauma, surgery, sepsis and severe systemic inflammatory response and remain low for several days and weeks (Berger et al. 1998; Heyland et al. 2006). It has been proposed that this reduction in trace element concentrations may deplete circulating antioxidants leading to an elevation of reactive oxygen species and so exacerbating the severity of illness (Berger and Chiolero 2007). Other groups have also suggested that decreased plasma trace elements concentrations are associated with severity of critical illness (Goode et al. 1995; Metnitz et al. 1999; Rinaldi et al. 2009).

However, the presence of a systemic inflammatory response has been reported to be associated with low plasma antioxidant concentrations (Galloway et al. 2000; Duncan et al. 2012b). In particular, it has recently been reported that the magnitude of systemic inflammatory response, as evidenced by CRP, is associated with lower circulating concentrations of zinc and selenium (Duncan et al. 2012b).

Duncan and co-workers (2012) reported that the systemic inflammatory response was associated with a 20% and 50% reduction in plasma zinc and selenium concentration respectively when CRP concentrations were > 80 mg/l. However, the variability of the association was such that plasma zinc and selenium concentrations were not readily adjusted for CRP concentrations (Duncan et al. 2012b).

Oakes and co-workers (2008) reported that during the acute systemic inflammatory response plasma zinc fell by 36%, plasma selenium fell by 26% and albumin by 19% (Oakes et al. 2008). Therefore, it is of interest that albumin is quantitatively the most important protein in the plasma and binds several plasma micronutrients, including zinc and selenium (Blindauer et al. 2009). Albumin is routinely measured and may be considered as a surrogate measure for some plasma binding proteins of similar molecular weight. Therefore, albumin may be useful in adjusting plasma zinc and selenium concentrations for the effect of the systemic inflammatory response and it may be useful in assessing zinc and selenium status.

The aim of the present study was to examine the relationships between plasma zinc, selenium and the systemic inflammatory response in a large cohort of patients referred for zinc and selenium assessment and also examine these relationships in patients from critical illness cohort.

9.2. Patients and methods

9.2.1. Nutrition screen cohort

Plasma zinc measurements were obtained from an unselected cohort of 1305 consecutive heparin-treated whole- blood samples from 743 patients referred for nutrition assessment between January 2000 and March 2013. Similarly, plasma selenium measurements were obtained from an unselected cohort of 1244 samples from 833 patients. If more than one set of plasma zinc or selenium results was available in a patient only the first sample was included into the analysis, leaving a total of 743 and 833 plasma zinc and selenium results respectively. In the nutrition screen cohort < 20 % of patients had both zinc and selenium measurements. In order to have enough observations to examine the relationship between markers of the systemic inflammatory response and measurements of zinc and selenium we used the present approach. As a regional centre blood samples were sent for analysis if the patient was considered at nutrition risk and was often secondary to a number of disease states. In addition, measurement of CRP and albumin was also recorded for these patients.

9.2.2. Critical illness cohort

Data and stored samples were available from cohorts which had been defined previously. The critical illness cohort comprised of medical or surgical patients ≥ 18 years admitted to the ICU of the Glasgow Royal Infirmary (GRI) between September 2006 and December 2008 and who had evidence of the systemic inflammatory response syndrome as per Bone's criteria (Bone et al. 1992; Levy et al. 2003), and admitted in the period from September 2006 to December 2008 were studied. Briefly, APACHE II score (Knaus et al. 1985) and predicted hospital mortality and SOFA scores, CRP and albumin were recorded. This cohort has been described previously and the basic purpose of this cohort was to study micronutrients concentrations (Stefanowicz et al. 2013a).

This investigation was conducted with the intent of developing local guideline to aid in the interpretation of zinc and selenium results. There were two cohorts studied, the first (a large unselected retrospective cohort) arose from an audit of patients who had a sample sent to a regional laboratory for a nutrition screen. Approval for audit purpose was obtained from the local ethics committee of the North Glasgow NHS Trust. The second (a small selected prospective cohort) arose from a prospective study of patients with critical illness. The patient data from the two cohorts was anonymised and de-identified prior to analysis (Andrew D and Donald C McMillan respectively). In line with local ethical procedures written informed consent was obtained for the latter cohort only. The latter study was approved by the ethics committees of the North Glasgow NHS Trust and Multicentre Research Ethics Committee (MREC) Scotland. Where patients were unable to give signed informed consent, consent was obtained from the patients' next of kin or welfare guardian in accordance with the requirements of the Adults with Incapacity Scotland (2000) Act (Appendix 1, 2).

9.2.3. Analytical methods

Plasma zinc and selenium were measured by atomic absorption spectrometry (Perkin-Elmer) until 2006, and then it was measured by inductively coupled plasma mass spectrometry (Agilent Technologies). Intra-assay imprecision was 4% for these analyses.

Samples were centrifuged (500 g, 4˚C, 10 minutes) and the plasma removed for analysis. Samples were stored at -70 ˚C prior to analysis. Plasma was simultaneously analysed for zinc and selenium using a 7500 CE inductively-coupled plasma mass spectrometer (Agilent, USA) operated in reaction cell mode. Prior to analysis plasma was diluted 1 in 10 with a solution containing 2% butan-1-ol, 0.05% EDTA, 0.05% Triton-X-100, 1% ammonia and 25 ug/l of germanium which served as internal standard. The dried sample

was then digested in 500 µl of 60% ultrapure nitric acid at 70˚C for 4 hours and diluted with 4.5 mL of 1% nitric acid containing 25 ug/l of germanium. Plasma internal quality controls (Seronorm, Billingstad, Norway) and plasma and whole blood external quality assurance samples (TEQAS, Guildford, UK) were analysed.

Albumin was measured by a BCP dye-binding method and C-reactive protein was measured using an automated analyser (Architect, Abbott Diagnostics, USA). For C-reactive protein the limit of detection was 5 mg/l. The inter-assay coefficient of variation was less than 3% and 5% over the sample concentration range for albumin and C-reactive protein respectively. The limit of detection for albumin was 10 g/L.

9.2.4. Statistical analysis

Data was presented in median and range. Correlations between variables in the convenience sample were carried out using the Spearman rank correlation. The cohorts were divided into three groups according to CRP concentrations < 10, 11-80 and >80 mg/l as previously described (Marsik et al. 2008). Plasma CRP is recognised to be produced in an exponential manner and have a skewd distribution. Therefore taking the logarithm of CRP is a recognised approach to normalise the distribution prior to examining relationships The concentration of individual zinc and selenium were grouped according to 3 categories of albumin concentrations ≤ 25, 25-34, ≥ 35 g/l as previously described (Goldwasser and Feldman 1997). A P- value < 0.05 was considered significant and the analysis of the data was carried out using SPSS software (version 19; SPSS Inc, Chicago, Ill).

9.3. Results

9.3.1. Nutrition screen cohort for zinc and selenium

The characteristics of the zinc convenience sample (n= 743) are shown in Table 9-1. The majority were older than 50 years (median 55 years), female (52%) and had plasma CRP was elevated, albumin concentrations were low and zinc in the normal range. Plasma zinc was significantly associated with age (r_s=-0.118, P =0.001), sex (males median 9.7 µmol/l, females median 10.9 µmol/l, P <0.001), CRP (r_s= -0.404, P <0.001) and albumin (r_s=0.588, P <0.001). Age was significantly associated with CRP (r_s= 0.308, P <0.001) and albumin (r_s=-0.294, P <0.001). CRP was significantly associated with albumin (r_s=-0.606, P <0.001).

The association between the magnitude of the systemic inflammatory response, as evidenced by CRP and albumin concentrations and zinc are shown in Figure 9-1a and 9-1b. The association of CRP and zinc/ albumin ratio is shown in Figure 9-1c.

The median plasma concentrations of zinc grouped according to CRP concentrations ≤10, 11-80 and >80 mg/l were 11.4, 10.0 and 7.0µmol/l respectively (P <0.001) with an overall reduction of 39%. The median plasma concentrations of zinc grouped according to albumin concentrations ≥35, 25-34 and <25 g/l were 11.9, 10.0 and 6.3µmol/l respectively (P <0.001) with an overall reduction of 47%.

The characteristics of the selenium convenience sample (n= 833) are shown in Table 9-1. The majority were older than 50 years (median 56 years), female (54%) and had plasma CRP and albumin in the normal range, and selenium was slightly below the normal range (median 0.74 µmol/l). Plasma selenium was significantly associated with age (r_s=0.073, P =0.036), sex (males median 0.69 µmol/l, females median 0.78 µmol/l, P =0.008), CRP (r_s= -0.489, P <0.001) and albumin (r_s=0.600, P <0.001). Age was significantly associated

with CRP (r_s= 0.279, P <0.001) and albumin (r_s=-0.191, P <0.001). CRP was significantly associated with albumin (r_s=-0.599, P <0.001).

The association between the magnitude of the systemic inflammatory response, as evidenced by CRP and albumin concentrations and selenium are shown in Figure 9-2a and 9-2b. The association of CRP and selenium/ albumin ratio is shown in Figure 9-2c.

The median plasma concentrations of selenium grouped according to CRP concentrations <10, 11-80 and >80 mg/l were 0.86, 0.66 and 0.37µmol/l respectively (P <0.001) with an overall reduction of 57%. The median plasma concentrations of selenium grouped according to albumin concentrations ≥35, 25-34 and <25 g/l were 0.90, 0.63 and 0.34 µmol/l respectively (P <0.001) with an overall reduction of 62%.

The median distributions of plasma zinc according to CRP and albumin concentrations are shown in Table 9-2. The median plasma concentrations of zinc grouped according to CRP concentrations ≤10, 11-80 and >80 mg/l were 11.4, 10.0 and 7.0µmol/l respectively (P <0.001) with an overall reduction of 39%. The median plasma concentrations of zinc grouped according to albumin concentrations ≥35, 25-34 and <25 g/l were 11.9, 10.0 and 6.3µmol/l respectively (P <0.001) with an overall reduction of 47%.

When albumin concentrations were ≥ 35 g/l, the median plasma concentrations of zinc grouped according to CRP concentrations ≤10, 11-80 and >80 mg/l were 12.1, 11.5 and 9.0 µmol/l (P =0.006) with an overall reduction of 26%. When albumin concentrations were 25-34 g/l, the median plasma concentrations of zinc grouped according to CRP concentrations ≤10, 11-80 and >80 mg/l were 9.6, 10.5 and 9.7µmol/l (P =0.031) with an overall reduction of <1%. When albumin concentrations were < 25 g/l, the median plasma concentrations of zinc grouped according to CRP concentrations ≤10, 11-80 and >80 mg/l were 7.2, 7.0 and 5.5 µmol/l (P =0.016) with an overall reduction of 24%. The median

zinc/ albumin ratio x 100 for CRP concentrations ≤10, 11-80 and >80 mg/l were 31, 33 and 32 (*P* =0.029). The median Zinc / CRP ratio x 100 for albumin concentrations ≥35, 25-34 and <25 g/l were 25.1, 59.4 and 8.4 (*P* <0.001).

The median distributions of plasma selenium according to CRP and albumin concentrations are shown in Table 9-3. The median plasma concentrations of selenium grouped according to CRP concentrations <10, 11-80 and >80 mg/l were 0.89, 0.66 and 0.37µmol/l respectively (*P* <0.001) with an overall reduction of 57%. The median plasma concentrations of selenium grouped according to albumin concentrations ≥35, 25-34 and <25 g/l were 0.90, 0.63 and 0.34 µmol/l respectively (*P* <0.001) with an overall reduction of 62%.

When albumin concentrations were ≥ 35g/l, the median plasma concentrations of selenium grouped according to CRP concentrations ≤10, 11-80 and >80 mg/l were 0.93, 0.85 and 0.87µmol/l (*P* =0.013) with an overall reduction of 6%. When albumin concentrations were 25-34 g/l, the median plasma concentrations of selenium grouped according to CRP concentrations ≤10, 11-80 and >80 mg/l were 0.71, 0.66 and 0.48 µmol/l (*P* =0.001) with an overall reduction of 32%. When albumin concentrations were <25g/l, the median plasma concentrations of selenium grouped according to CRP concentrations ≤10, 11-80 and >80 mg/l were 0.43, 0.39 and 0.29 µmol/l (*P* =0.002) with an overall reduction of 33%. The median selenium /albumin ratio x 100 for CRP concentrations ≤10, 11-80 and >80 mg/l were 2.3, 2.1 and 1.8 (p<0.001). The median selenium / CRP ratio x 100 for albumin concentrations ≥35, 25-34 and <25 g/l were 0.54, 3.9 and 21.5 (*P* <0.001).

9.3.1. Patients with critical illness.

The characteristics of the critically ill cohort (n=114) are shown in Table 9-1. The majority were older than 50 years (median 60 years), male (65%), an APACHE II score of

21 and SOFA score of 7 and the associated median predicted mortality was 35.1%. The majority of patients were surgical (56%) and had a median length of ICU stay of 4 days and median hospital stay of 19 days. The median CRP and albumin concentrations were above and below the normal reference intervals respectively. Approximately half had some renal impairment as evidence by eGFR of <60ml/min. The median plasma zinc and selenium was 4.5 μmol/l and 0.29μmol/l respectively.

Plasma zinc was significantly associated with CRP (r_s= - 0.266, P =0.004) and albumin (r_s =0.348, P <0.001). Age was significantly associated with albumin (r_s = - 0.233, P =0.013). CRP was significantly associated with albumin (r_s =-0.377, P <0.001). Plasma selenium was significantly associated with CRP (r_s = -0.322, P <0.001) and Albumin (r_s =0.580, P <0.001). Age was significantly associated with albumin (r_s =-0.212, P =0.022). CRP was significantly associated with albumin (r_s =-0.362, P <0.001).

The association between the magnitude of the systemic inflammatory response, as evidenced by CRP and albumin concentrations, on zinc and selenium in patients with critical illness were shown in Figures 9-1a, 9-1b and 9-2a, 9-2b. The association of CRP and zinc/ albumin ratio and selenium/ albumin ratio were shown in Figure 9-1c and 9-2c respectively.

The median plasma concentrations of zinc grouped according to CRP concentrations ≤10, 11-80 and >80 mg/L were 6.8, 5.5 and 4.0 μmol/l respectively (P =0.007). The median plasma concentrations of zinc grouped according to albumin concentrations ≥35, 25-34 and <25 g/l were 8.0, 6.1 and 4.0 μmol/l respectively (P <0.001).

The median plasma concentrations of selenium grouped according to CRP concentrations ≤10, 11-80 and >80 mg/L were 0.51, 0.30 and 0.22 μmol/l respectively (P <0.001). The

median plasma concentrations of selenium grouped according to albumin concentrations ≥35, 25-34 and <25 g/l were 0.63, 0.52 and 0.22 µmol/l respectively (P <0.001).

9.4. Discussion

The results of the present study show that, in nutrition screen and critical illness patient cohorts, plasma zinc and selenium concentrations were independently associations with CRP and albumin as markers of systemic inflammatory response. In the case of zinc the effect was mainly associated with lower albumin concentrations and so the impact of the systemic inflammatory response (as evidenced by elevated CRP concentrations) could largely be adjusted to the albumin concentration. In the case of selenium the effect was associated with both CRP and albumin and so the impact of the systemic inflammatory response (as evidenced by elevation of CRP concentrations) could not be reasonably adjusted by either CRP or albumin concentrations alone.

One of the important implications of the present study is that when one encounters patients who have low plasma concentrations of zinc or selenium, it is difficult to differentiate real deficiencies of these trace elements from redistribution caused by systemic inflammation and therefore independent of nutrition deficiency. However, recognition of such a relationship would suggest that, in those patients who have a low plasma concentration of zinc or selenium and who have a CRP and albumin concentration in the normal range, such patients are likely to be deficient. In the case of those patients who have low plasma concentration of zinc or selenium and who have CRP and albumin concentrations out with the normal range it is difficult to determine whether deficiency is real or apparent. In these patients a number of approaches could be used to determine whether there was real deficiency. Firstly, serial measurements could be used to examine the changes in zinc or selenium and in CRP and albumin. Secondly, functional tests may be useful e.g. glutathione peroxidase activity (GSH-Px) in the case of selenium. Thirdly, intracellular measurements could be carried out e.g. red cell zinc or selenium measurements. Fourthly,

potential correction of zinc and selenium plasma levels for their carrier proteins (i.e. albumin for zinc and albumin/selenoproteins for selenium).

The reasons for the differential association of CRP and albumin with zinc and selenium are unclear. However, approximately 70 % of plasma zinc is bound to albumin while approximately 53% and 9% of plasma selenium is bound to selenoprotein P (SePP) or albumin respectively (Deagen et al. 1993; Harrison et al. 1996; Mostert et al. 1998; Nicholson et al. 2000; Stefanowicz et al. 2013b). Indeed, in the blood less than 1% of selenium is in free form. The concentrations of all these fall as part of the systemic inflammatory response (Oakes et al. 2008; Alhazzani et al. 2013) and therefore interpretation of plasma selenium remains problematical. Therefore, it is of interest that Stefanowicz and co-workers (2013) recently reported that erythrocyte GSH-Px activity was associated with plasma and erythrocyte selenium concentrations to a threshold point after which activity of the enzyme was found to reach a maximum and plateau. Further work examining the relationship between plasma selenium and erythrocyte GSH-Px activity in the presence of a systemic inflammatory response may shed some light on the importance of low plasma selenium concentrations.

The results of the present study examining the effect of the systemic inflammatory response on plasma zinc and selenium concentrations may also have implications for other trace elements and vitamins in plasma that fall as part of the systemic inflammatory response (Duncan et al. 2012b; Ghashut et al. 2013). Indeed, CRP is recognised to be the prototypical marker of the systemic inflammatory response (Gabay and Kushner 1999). In contrast, plasma albumin not only reflects the systemic inflammatory response but also reflects lean body mass and thus can be considered as a general marker of nutritional status (McMillan et al. 1998; Richards et al. 2012). Therefore, examining the different effects of these acute phase proteins gives an important insight into the basis of low plasma zinc and

selenium concentrations. Also albumin is a carrier protein for zinc and selenium in plasma and may reflect the extent of redistribution of these trace elements in the presence of systemic inflammatory response. Further work is required to determine whether both CRP and albumin have an independent impact on circulating concentrations of such micronutrients.

The present study has a number of limitations. In particular there was no information on the presence of chronic disease, co-morbidity or on BMI. Nevertheless, it is likely that the impact of these clinical factors on metabolic trace elements will have involved the systemic inflammatory response, at least in part (Hotamisligiil 2006; Jensen 2014).

In summary, the results of the present study show that plasma concentrations of zinc were associated with both CRP and albumin. Moreover, that the impact of the systemic inflammatory response (as evidenced by elevation of CRP) on plasma zinc concentration may be largely adjusted by the albumin concentrations. Plasma concentrations of selenium were associated with both CRP and albumin. However, the impact of the systemic inflammatory response (as evidenced by elevation in CRP) may not be readily adjusted by the albumin concentration. Similar relationships were also observed in the cohort of patients with critical illness.

Table 9-1: Characteristics of nutrition screening, patient with critical illness cohorts and plasma zinc and selenium.

	Reference interval	Nutrition screen cohort (Zinc) (n=743)	Nutrition screen cohort (Selenium) (n=833)	Critical illness patients (n= 114)	P- value [a]
Age (years)	N/A	55 (16-100)	56 (16-100)	60 (18-100)	0.068
Sex (Male/Female)	N/A	359 (48%)/ 384 (52%)	387 (46%)/ 446 (54%)	74 (65%)/ 40 (35%)	<0.001
C-reactive protein (mg/l)	<10	12.0 (0.19- 565.0)	10.0 (0.19- 565.0)	100.0 (1.0- 565.0)	<0.001
Albumin (g/l)	35-55	32 (<10-50)	35 (<10-50)	17 (9-45)	<0.001
Zinc (µmol/l)	12.0-18.0	10.5 (0.6-30.1)		4.5 (0.6-27.0)	<0.001
Selenium (µmol/l)	0.8-2.0		0.74 (0.1-5.68)	0.29 (0.01-5.68)	<0.001

Median (Range), [a] Mann-Whiney U Test.

Table 9-2: Distribution of plasma zinc (µmol/l) according to CRP (mg/l) and albumin (g/l) concentrations (n=743).

Plasma Zinc (µmol/l)	CRP ≤10 (mg/l)	11-80 (mg/l)	>80 (mg/l)	Plasma zinc according to albumin
Albumin ≥35 (g/l)	12.1 (6.5-30.1) (n=242)	11.5 (7.0-23.0) (n=70)	9.0 (5.5-15.5) (n=9)	11.9 (5.5-30.1) (n=321)
34-25 (g/l)	9.6 (3.5-25.5) (n=87)	10.5 (3.5-27.8) (n=100)	9.7 (3.0-15.5) (n=40)	10.0 (3.0-27.8) (n=227)
<25 (g/l)	7.2 (3.0-27.9) (n=26)	7.0 (0.6-27.0) (n=71)	5.5 (1.0-15.5) (n=98)	6.3 (0.6-27.9) (n=195)
Plasma zinc according to CRP	11.4 (3.0-30.1) (n=355)	10.0 (0.6-27.8) (n=241)	7.0 (1.0-15.5) (n=147)	

Median (range) (number of observation)

Table 9-3: Distribution in plasma selenium (µmol/l) according to CRP (mg/l) and albumin (g/l) concentrations (n=833).

Plasma Selenium (µmol/l)	CRP ≤10 (mg/l)	11-80 (mg/l)	>80 (mg/l)	Plasma selenium according to albumin
Albumin ≥35 (g/l)	0.93 (0.1-3.4) (n=315)	0.85 (0.3-2.6) (n=99)	0.87 (0.6-1.2) (n=8)	0.90 (0.1-0.3) (n=422)
34-25 (g/l)	0.71 (0.2-1.7) (n=86)	0.66 (0.1-1.4) (n=100)	0.48 (0.02-1.3) (n=41)	0.63 (0.02-1.7) (n=227)
<25 (g/l)	0.43 (0.1-1.1) (n=26)	0.39 (0.01-1.7) (n=70)	0.29 (0.04-5.7) (n=88)	0.34 (0.01-5.7) (n=184)
Plasma selenium according to CRP	0.89 (0.1-3.4) (n=427)	0.66 (0.01-2.6) (n=269)	0.37 (0.02-5.7) (n=137)	

Median (range) (number of observation)

Figure 9-1a: The relationship between CRP (log 10) and zinc (µmol/l) in the nutrition screen cohort (r_s= - 0.404, *P* <0.001) and in the critical illness cohort (r_s= -0.266, *P*=0.004).

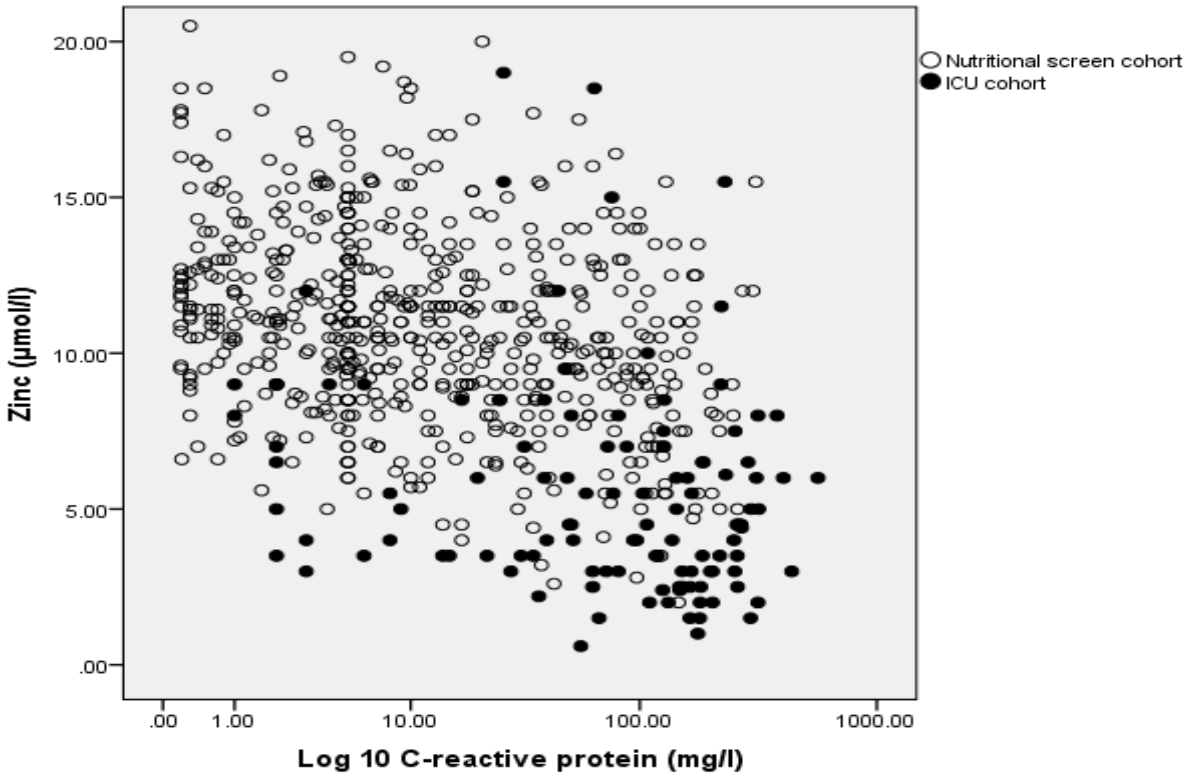


Figure 9-1b: The relationship between albumin and zinc (µmol/l) in the nutrition screen cohort (r_s= 0.588, *P*<0.001) and in the critical illness cohort (r_s= 0.348, *P*<0.001).

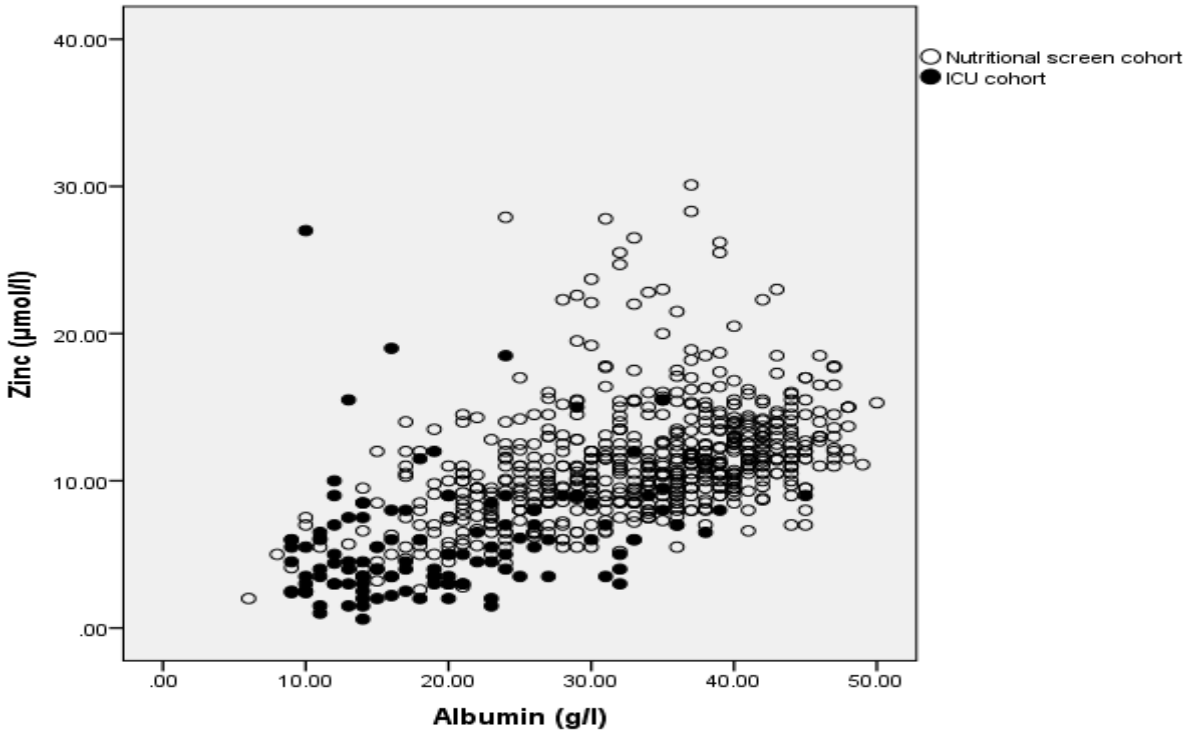


Figure 9-1c: The relationship between CRP (log 10) and zinc adjusted to albumin in the nutrition screen cohort (r_s= 0.074, *P*=0.045) and in the critical illness cohort (r_s= 0.038, *P*=0.114).

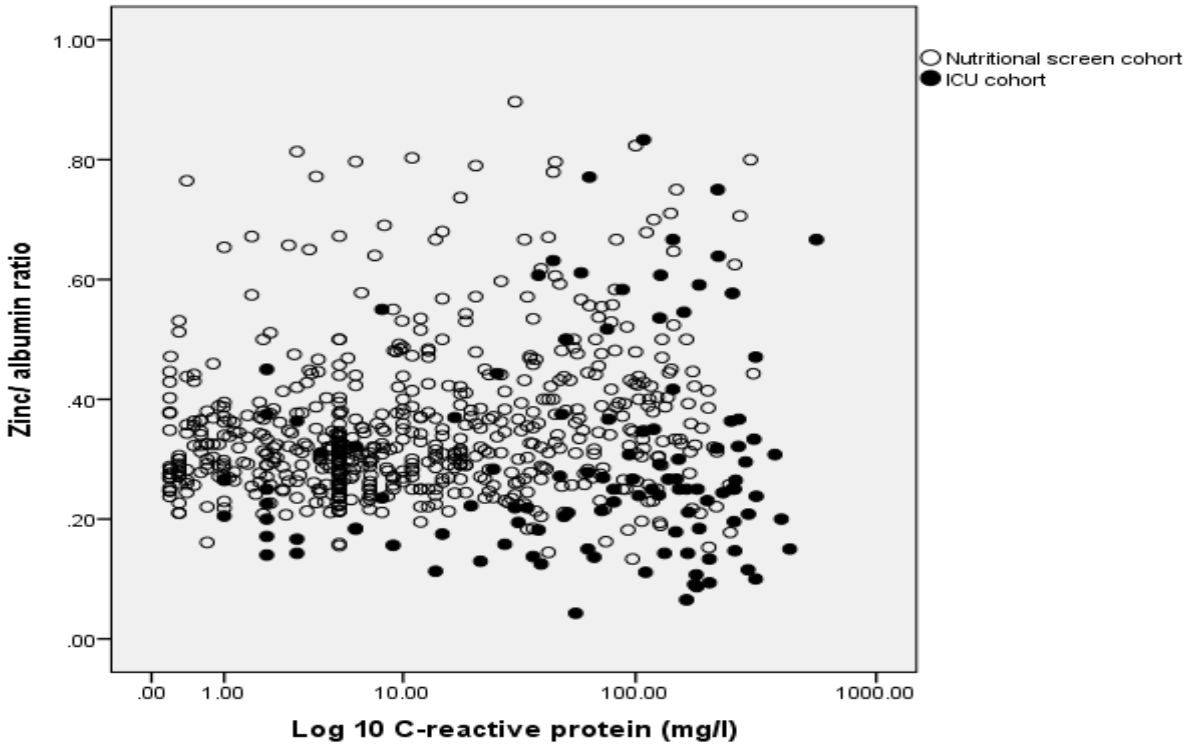


Figure 9-2a: The relationship between CRP (log 10) and selenium (µmol/l) in the nutrition screen cohort (r_s= -0.489, *P*<0.001) and in the critical illness cohort (r_s= -0.336, *P*<0.001).

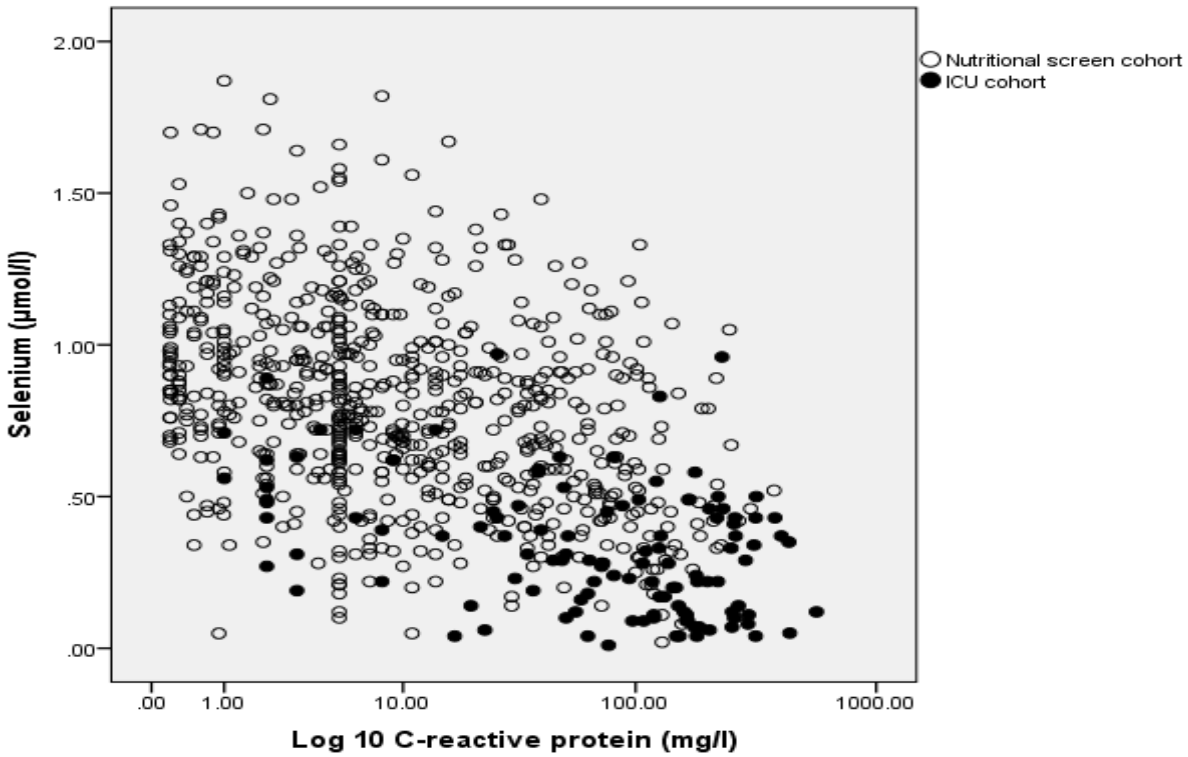


Figure 9-2b: The relationship between albumin and selenium (µmol/l) in the nutrition screen cohort (r_s= 0.600, *P*<0.001) and in the critical illness cohort (r_s= 0.588, *P*<0.001).

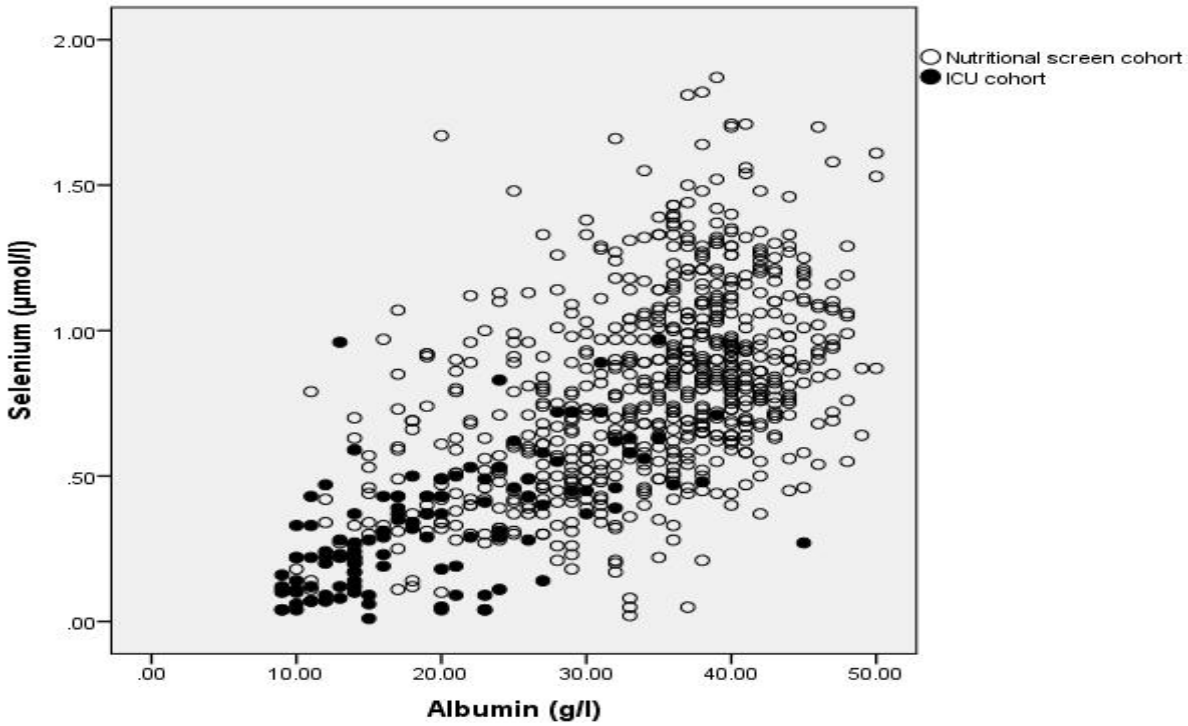
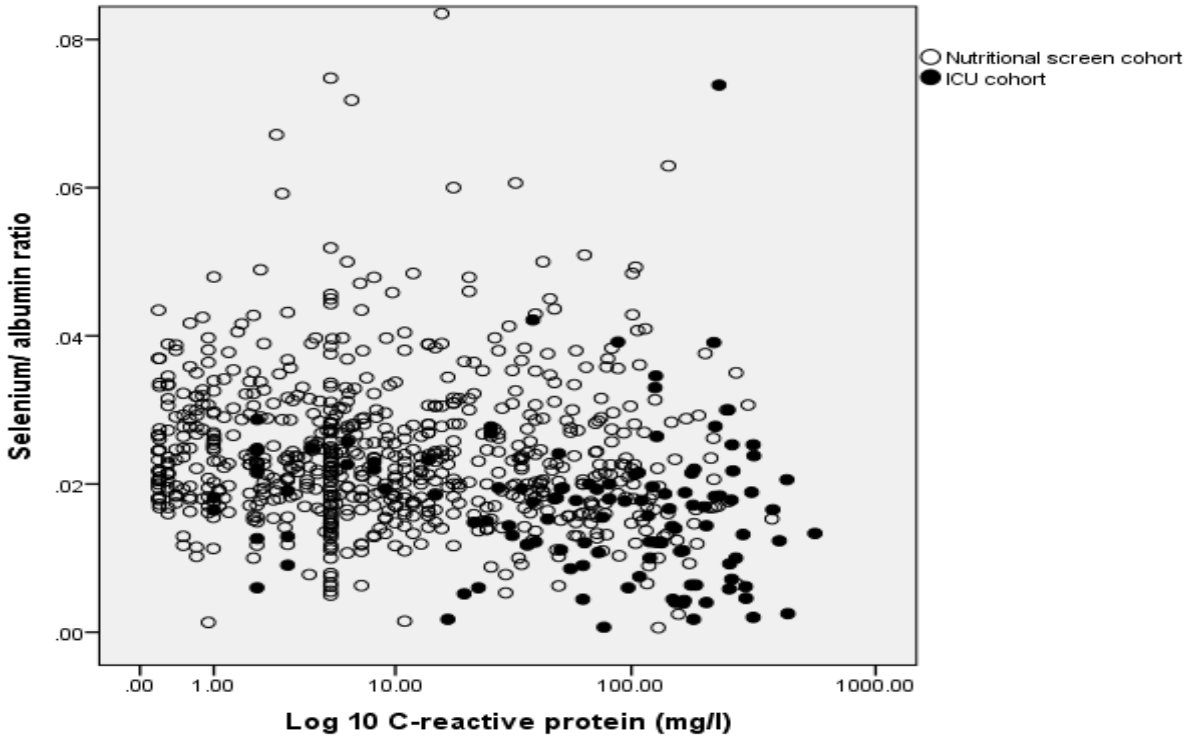


Figure 9-2c: The relationship between CRP (log 10) and selenium adjusted to albumin in the nutrition screen cohort (r_s= - 0.207, P<0.001) and in the critical illness cohort (r_s= - 0.163, P=0.081)



10. The relationship between markers of the systemic inflammatory response and plasma and red cell trace element concentrations, disease severity and outcome in patients with critical illness.

10.1. Introduction

Trace elements are of vital importance in human health acting as cofactors in many catalytic processes and particularly, in the case of zinc, they have a structural role. Selenium is essential for the catalytic activity of glutathione peroxidase (GSH-Px) protecting against membrane lipid peroxidation (Thomas et al. 1990) while copper and zinc also combat oxidative stress as cofactors in copper-zinc superoxide dismutase. Zinc and selenium both play important functions in immune regulation (Fraker et al. 2000). With such roles, adequate trace element status is considered to be of particular importance in critically ill patients who have significantly reduced circulating and body stores of antioxidants, such as copper-zinc superoxide dismutase and glutathione peroxidase, so impeding detoxification of reactive oxygen species (Heyland et al. 2005).

Concentrations of some circulating trace elements such as zinc and selenium, are known to decrease significantly following severe trauma, surgery, sepsis and severe systemic inflammatory response and remain low for several days and weeks (Berger et al. 1998; Heyland et al. 2006). It has been proposed that this reduction in trace element concentrations may deplete circulating antioxidants leading to an elevation of reactive oxygen species and so exacerbating the severity of illness (Berger and Chiolero 2007). Other groups have also suggested that decreased plasma trace elements concentrations are associated with severity of critical illness (Goode et al. 1995; Metnitz et al. 1999; Rinaldi et al. 2009).

A recent study of patients presenting to ICU reported that plasma zinc concentrations were inversely associated with Sequential Organ Failure Assessment scores (SOFA) (Cander et al. 2011). Furthermore, a study examining zinc metabolism in patients in ICU found plasma concentrations were lower in septic compared with non-septic adults after admission to ICU (Besecker et al. 2011). Similarly, a low plasma selenium concentration found after admission to ICU was associated with the systemic inflammatory response, multiorgan dysfunction (Manzanares et al. 2009) and an increase in mortality (Forceville et al. 1998).

Perhaps unwittingly such reports suggest a causative role of low circulating concentrations of plasma trace elements in these processes. In clinical practice a significant percentage of UK ICUs routinely request such measurements and low plasma zinc concentrations, purportedly indicating deficiency, has resulted in a significant minority of UK ICUs supplementing with high-dose zinc supplementation (Duncan et al. 2012a).

Although the direct measurement of essential trace elements in plasma is the most predominant method used to assess nutrition deficiency, interpretation of plasma concentrations of trace elements can be difficult in patients with a systemic inflammatory response. Rather than being indicative of nutrition deficiency, decreasing concentrations of plasma trace elements concentrations such as zinc, selenium, vitamin E, C and B6 are associated with the magnitude of inflammatory response as evidenced by increased CRP concentrations (Galloway et al. 2000; Duncan et al. 2012b). This relationship can be readily demonstrated by studying the evolution of the systemic inflammatory response following an elective knee arthroplasty (Oakes et al. 2008; Defi et al. 2011); plasma selenium and zinc concentrations initially decreased from baseline in association with a rise in CRP concentrations while the copper increased. Plasma concentration changes were transient and normalised without supplementation as CRP concentrations returned to

normal as the patient recovers. Erythrocyte trace element concentrations such as zinc and selenium, however, did not change significantly throughout the development of the systemic inflammatory response (Oakes et al. 2008).

This study was carried out to further investigate the value of plasma trace element measurement in ICU patients by investigating their association with the severity of illness and mortality. Glutathione peroxidase activity was also measured as an alternative marker of selenium status. Since erythrocyte concentrations of trace elements remained stable during elective surgery we also took the opportunity to similarly examine their relationship with severity of illness and outcome.

10.2. Patients and methods

10.2.1. Patients and study design

Patients admitted to ICU at the Glasgow Royal Infirmary, between September 2006 and April 2008 and who had respiratory failure requiring ventilatory support, were ≥18 years old, and who had evidence of the systemic inflammatory response syndrome as per Bone's criteria, (Bone et al. 1992) were studied. The systemic inflammatory response is a systemic response to a variety of severe clinical insults. The syndrome is manifested by 2 or more of the following conditions: temperature >38C or < 36C, heart rate > 90 beats/min, respiratory rate > 20 breath/min or $PaCO_2$< 4.3 kPa, and white cell count > 12000 or < 4000 cells/mm^3 or > 10% immature forms (Bone et al. 1992). Only two patients had been diagnosed with liver failure and the frequency and use of red cell transfusion was not recorded.

Venous blood was collected into heparinised trace element free tubes on admission to the ICU and, depending on the length of stay, on day four and day seven (± one day). APACHE II and SOFA scores were also recorded. Enteral feeding was usually started on day two using either Jevity (Abbott, US); 10mg/L zinc, 5.30µg/L selenium, 95 mg/L copper) or Osmolite (Abbott, US): 1.50mg/L zinc, 50µg/L selenium, 1.2 mg/L copper). Patients routinely receive 2L of one of these preparations per day, amounting to an intake of 2.2 to 2.60 mg zinc, 10.6 to 12.0 µg selenium, and 0.19 to 0.22 g copper. The reference nutrient intakes are: copper, 1.2 mg/day; zinc 9.5 mg/day; selenium 75 µg/day (males) and 60 µg/day (females) (Expert group on vitamins and minerals 2003).

The study was approved by the Multicentre Research Ethics Committee, Scotland. In cases where patients were unable to provide consent, it was obtained from their next of kin

or welfare guardian in accordance with the Adults with Incapacity Scotland (2000) Act (Appendix 1, 2).

10.2.2. Analytical Methods

Samples were centrifuged (500 g, 4˚C, 10 minutes) and the plasma removed for analysis. Packed erythrocytes were prepared by removing any residual plasma and the buffy layer. Samples were stored at -70 ˚C prior to analysis.

Plasma and packed erythrocytes were simultaneously analysed for copper, zinc, selenium and iron using a 7500 CE inductively-coupled plasma mass spectrometer (Agilent, USA) operated in reaction cell mode. Prior to analysis plasma was diluted 1 in 10 with a solution containing 2% butan-1-ol, 0.05% EDTA, 0.05% Triton-X-100, 1% ammonia and 25 ug/L of germanium which served as internal standard. Packed erythrocytes were prepared for analysis by drying a 50 µl aliquot at 80˚C for 90 minutes. The dried sample was then digested in 500 µl of 60% ultrapure nitric acid at 70˚C for 4 hours and diluted with 4.5 mL of 1% nitric acid containing 25 ug/L of germanium. Plasma and whole blood internal quality controls (Seronorm, Billingstad, Norway) and plasma and whole blood external quality assurance samples (TEQAS, Guildford, UK) were analysed. No external quality assurance scheme was available for erythrocyte trace elements or GSH-Px. Erythrocyte trace elements were reported as a ratio to haemoglobin concentration to correct for inaccuracies associated with pipetting packed red blood cells. Iron was measured as a surrogate for haemoglobin (Oakes et al. 2008) whose concentration was calculated using the following equation where 64,456 is the molecular weight of haemoglobin in g and the denominator is the number of atoms of iron per haemoglobin molecule.

$$\text{Hb (g/L)} = \frac{\text{Iron (mol/L)} \times 64\,456}{4}$$

GSH-Px activity in plasma and erythrocytes was measured on an analytical platform (Sapphire 350, Audit Diagnostics, Ireland) using a commercially available kit (Randox, Co. Antrim, UK). Haemoglobin was measured colorimetrically on a Sapphire 350 by Drabkin's method (Drabkin and Austin 1935). Glutathione peroxidase activities, measured as glutathione peroxidase 1, were reported as IU/g Hb.

Albumin and CRP were measured in plasma by bromocresol purple and turbidimetric methods respectively using an automated analyzer (Architect; Abbot Diagnosis, Maidenhead, UK).

10.2.3. Statistics

Data was presented as median and range. Correlations between variables in the critically ill group were performed using the Spearman's rank correlation. Data from different time points in the patient group were tested for significance using Friedman's signed-rank test. Data from survivors and non-survivors were tested for statistical significance using the Mann-Whitney U-test. Outcome data were analysed by binary logistic regression analysis. Because of the large number of statistical comparisons, a p value of <0.01 was considered to be significant. Analysis was performed with the use of SPSS software (version 19; SPSS Inc, Chicago, IL).

10.3. Results

125 critically ill patients were recruited; 81 (66%) were male, the median age was 60 (range: 18 to 100), and the medical / surgical proportion was 55/70 (44%/56%). The median (lower and upper 2.5th percentiles) APACHE II score, SOFA score and length of stay and mortality were 21 (16 to 26), 7 (4 to 9), 3.7 days (1.5 to 11.1) and 19% respectively.

Laboratory results on admission are shown in Table 10-1. Median concentrations of albumin were low compared to the reference range whilst those of CRP and glucose were high. Median plasma concentrations of zinc and selenium were low while copper and glutathione peroxidase concentrations in plasma and concentrations of zinc, selenium, copper and glutathione peroxidase in erythrocyte were within the laboratory reference range. Plasma zinc and selenium concentrations were below the lower limit of the reference interval in 86% and 90% of patients respectively. Only plasma zinc and selenium concentrations were inversely associated with CRP (r_s= -0.266, P = 0,004, r_s = -0.322, P <0.001 respectively).

Thirty-one of the 125 patients had two further samples taken (Table 10-2); the remainder did not have additional samples due to discharge or death (n = 70). Plasma zinc (P = 0.004), plasma selenium (P = 0.003) increased significantly during the ICU stay although they remained below the reference interval. There were no significant changes in the SOFA score or in erythrocyte analytes or any of the remaining plasma analytes. Plasma selenium concentrations were directly associated with plasma glutathione peroxidase activity (r_s = 0.325, P = 0.001) and red cell selenium concentrations (r_s= 0.582, P = 0.001). Red cell selenium concentrations were directly associated with red cell glutathione peroxidase activities (r_s= 0.567, P = 0.001).

Twenty-four (19%) patients died in ICU following their admission. Compared with survivors, non-survivors had significantly longer (P <0.001) ICU lengths of stay and lower plasma albumin concentrations: median length of stay (upper and lower 2.5% percentile): 2.9 days (1.0 to 8.9) in survivors and 11.6 days (7.1 to 17.3) in non-survivors; and albumin concentrations: 19 g/L (14 to 26) in survivors and 13 g/L (10 to 17) in non-survivors. No statistically significant difference of plasma zinc or selenium between survivors and non-survivors was found (P <0.01) although selenium concentrations tended to be lower in non-survivors (P =0.04) (Table 10-3).

When admission patient SOFA and APACHE II scores and analyte concentrations were compared to mortality by univariate analysis, only length of stay and albumin concentrations showed significant differences, Odd Ratio (95% confidence intervals), length of stay: 1.05 (1.01-1.08, P = 0.007); and albumin concentration: 0.89 (0.82- 0.96). On multivariate logistic regression analysis neither of these significant variables was independently associated with mortality (Table 10-4).

10.4. Discussion

The results of the present study showed profoundly low plasma concentrations of selenium and zinc in ICU on admission compared with our Laboratory reference intervals. These results are in accordance with previous findings in critically ill patients (Berger et al. 1998; Heyland et al. 2006). Rather than suggesting deficiency of these trace elements, this finding can be explained by the existence of the systemic inflammatory response in these patients as evidenced by the high CRP concentrations. In a recent study it was demonstrated that plasma concentrations of essential trace elements are strongly directly associated with the systemic inflammatory response; as measured by CRP concentrations, the plasma concentrations of zinc and selenium element fall while copper initially rises and only falls with more a severe systemic inflammatory response. These changes are largely predictable when the metabolic dynamics of the systemic inflammatory response are considered and are unlikely to represent tissue status to any appreciable degree. Cytokines produced during the systemic inflammatory response increase capillary permeability allowing albumin, selenoprotein P (SePP) and other low molecular weight proteins to redistribute into the interstitium. As the plasma concentrations of these proteins fall any trace elements carried by them such as selenium and zinc will also fall (Fleck et al. 1985). It is generally accepted that plasma albumin concentration is determined by rates of synthesis and breakdown, and its distribution across fluid compartments (Buerk et al. 1973). Approximately 70 % of serum zinc is bound to albumin while approximately 53% and 9% of plasma selenium is bound to SePP or albumin respectively (Buerk et al. 1973; Deagen et al. 1993; Harrison et al. 1996; Nichol et al. 1998; Mostert et al. 1998). In addition, whilst selenium and copper may be bound to albumin, these elements are actually integral components of SePP and caeruloplasmin, respectively. The reduction in SePP

may be a result of down-regulation of synthesis by the liver as well as protein redistribution (Dreher et al. 1997; Mostert et al. 1998).

In the present study unlike plasma selenium and zinc concentrations, we found plasma copper concentrations remained within the reference interval at admission. Around 95% of plasma copper is bound to caeruloplasmin which is a positive acute phase reactant. Its concentration initially increases in response to the systemic inflammatory response although as its severity increases concentrations fall (Duncan et al. 2012b) as the capacity of the liver to synthesise caeruloplasmin falls. So again the finding of unchanged plasma copper concentrations may be explained as a manifestation of the systemic inflammatory response (DiSilvestro 1989).

Our conclusion that low plasma zinc and selenium concentrations in ICU patients can be largely explained by the systemic inflammatory response is corroborated by a similar response in well-nourished patients admitted for elective knee surgery. As the inflammatory response develops in these individuals plasma zinc and selenium fall abruptly but normalise in approximately 7 days without dietary intervention or supplementation (Oakes et al. 2008).

Plasma glutathione peroxidase activity can be used as an alternative to plasma selenium as a marker of selenium status. It is a measure of functional catalytic activity that, in one study was unaffected by the systemic inflammatory response (Defi et al. 2011) and in another study produced a small but significant increase (Nichol et al. 1998). The findings in the present study of normal activities of plasma glutathione peroxidase on admission to ICU and unchanging activities during the patients' stay in ICU, serve as a reliable indication that the nutrition status of selenium remained normal throughout. This finding consequently provides additional confirmation that the changes of plasma selenium

concentrations observed are due to the systemic inflammatory response rather than nutrition status.

There is strong evidence to recommend the use of erythrocyte selenium as a marker of status. Erythrocyte concentrations fall as deficiency develops (Daniels et al. 1996) and increase on supplementation (Jacobson and Plantin 1985; Neve et al. 1988). In healthy volunteers and patients who are not critically ill, there is a close correlation with plasma selenium concentrations and glutathione peroxidase activity (Rea et al. 1979) and this correlation is maintained in individuals who have sub-normal and supra-normal selenium intakes (Stefanowicz et al. 2013b). Therefore, the finding in the present study of normal red cell selenium concentrations on admission would tend to suggest that the patients' selenium status was normal in the previous 6 to 12 weeks.

The same cannot be inferred with respect to zinc; in several studies in which zinc deficiency is produced in healthy individuals, erythrocyte concentrations show little (Buerk et al. 1973) or no change (Prasad et al. 1978; Baer and King 1984) whereas plasma zinc levels fall quickly and significantly. Fewer studies have investigated the response of erythrocyte copper during depletion, however, in a hypocupraemic patient erythrocyte concentrations returned to normal after replacement therapy (Okahata et al. 1980).

An unexpected finding of this study was the slight improvement of plasma zinc and selenium concentrations with duration of stay in ICU; although both remained severely low at day 7, their concentrations had increased significantly whereas markers of the systemic inflammatory response remained unchanged. This may be due to faster recovery rates of plasma trace elements, or more specifically proteins which carry or are complexed with trace elements, compared with CRP, albumin and other markers of the systemic inflammatory response. Unfortunately only 31 patients remained in ICU for follow-up samples, and this low number is a limitation of this aspect of the study.

The early literature reports of low plasma selenium concentrations in ICU patients were taken to represent deficiency and prompted several trials of selenium supplementation (Berger et al. 1998; Berger et al. 2001; Berger et al. 2004). This hypothesis was supported by the anti-oxidant properties of selenium-dependent enzymes and the proposal that critically ill patients have significant reductions in body antioxidant stores, reduced intracellular cofactor concentrations, reduced activities of enzymes involved in detoxification of reactive oxygen species, and that the severity of critical illness may be exacerbated by such reductions of the antioxidant. Given our findings that low plasma selenium concentrations in ICU patients do not represent deficiency, this concept may in retrospect be misguided. Indeed, a recent meta-analysis of selenium supplementation and a Cochrane Review (Avenell et al. 2004) were unable to demonstrate convincing data supporting a beneficial effect of supplementation (Andrews et al. 2011). Despite the lack of evidence, zinc supplementation is still routinely given in some UK ICUs (Duncan et al. 2012b).

In summary, the low plasma zinc and selenium concentrations and elevated plasma copper concentrations observed in the present study on admission of critically ill patients to ICU are in keeping with our knowledge of the systemic inflammatory response and are a consequence of it rather than an indicator of trace element status.

Table 10-1: Laboratory concentrations on admission (day 1).

Analyte	Median Concentration (Range)	Reference Interval
Albumin (g/l)	18 (9-47)	32-45
CRP (mg/l)	108 (<1-565)	<10
Lab-Glucose (mmol/l)	6.4 (2.8-30.6)	3.5-5.5
Plasma Copper (µmol/l)	12 (4.5-28.5)	10-22
Red cell Copper (nmol/g Hb)	33.3 (13.1-46.2)	27.9-53.4
Plasma Zinc (µmol/l)	4.5 (0.6-27.0)	12.0-18.0
Red cell Zinc (nmol/g Hb)	581 (399-812)	423-781
Plasma Selenium (µmol/l)	0.31 (0.01-5.68)	0.8-2.0
Plasma GSH-Px (IU/L)	346 (30-740)	180-760
Red cell Selenium (nmol/g Hb)	5.3 (2.48-10.62)	3.66-10.56
Red cell GSH-Px (IU/g Hb)	27.0 (11.6-42.8)	20-55

Table 10-2: Characteristics, clinical scores and laboratory concentrations on admission (day 1) and follow-up (day 4 and day 7).

Characteristic Clinical Score	Numbers or Medians (ranges)			
	Admission day 1	Follow-up day 4	Follow-up day 7	P-value [a]
Number of recruits	31			
Sex (male/female)	23/9			
Age (years)	63 (20-81)			
Patients (medical/surgical)	14/18			
APACHE II score	24 (7-38)			
ICU length of stay (days)	16.1 (5.1-76.4)			
ICU mortality (%)	45.2 (18/14)			
SOFA score	7 (1-13)	8 (1-12)	6 (2-11)	0.067
Albumin (g/l)	14 (9-32)	14 (9-29)	13 (9-31)	0.085
CRP (mg/l)	111 (<6-565)	132 (20-356)	121 (15-455)	0.529
Lab-Glucose (mmol/l)	6.6 (2.8-30.6)	6.2 (3.7-12.5)	6.2 (2.3-12.0)	0.839
Plasma Copper (µmol/l)	10.5 (10.5-28.5)	10.8 (5.3-22.0)	13.3 (6.5-21.5)	0.295
Red cell Copper (nmol/g Hb)	32 (22-41)	32 (22-49)	32 (26-48)	0.962
Plasma Zinc (µmol/l)	4.0 (1.5-18.5)	5.0 (1.5-12.5)	6.5 (2.0-12.5)	0.004
Red cell Zinc (nmol/b Hb)	606 (493-779)	611 (482-777)	622 (477-791)	0.241
Plasma Selenium (µmol/l)	0.24 (0.01-0.83)	0.26 (0.01-5.65)	0.35 (0.04-0.99)	0.003
Plasma GSH-Px (IU/L)	344 (30-739)	316 (17-7831)	365 (74-874)	0.310
Red cell Selenium (nmol/g Hb)	5.3 (3.7-9.6)	5.5 (3.2-10.4)	5.6 (3.94-9.97)	0.080
Red cell GSH-Px (IU/g Hb)	28 (12-43)	25 (14-48)	27.0 (12-43)	0.236

[a] Friedman's signed-rank test

Table 10-3: Characteristics and trace element concentrations in survivors and non-survivors on admission to ICU

Characteristic/ Clinical score/ Analyte	Number / Median (range)		
	Survivors	Non-survivors	P-value*
Number	101	24	
Age (years)	56 (22-100)	67 (18-81)	0.042
Sex (male/female)	65/36	16/8	0.807
Patients (medical/surgical)	44/57	11/13	0.802
APACHE II score	20 (3-38)	25 (10-34)	0.014
SOFA score	6 (0-18)	8 (1-14)	0.022
ICU length of stay (days)	2.9 (0.2-76.2)	11.6 (1.8-59.5)	<0.001
Albumin (g/l)	19 (9-47)	13 (9-33)	<0.001
CRP (mg/l)	84.5 (0.6-438)	122 (2-565)	0.406
Lab-Glucose (mmol/l)	6.4 (2.8-30.6)	6.8 (4.1-26.4)	0.480
Plasma Copper (µmol/l)	12.0 (4.5-113.5)	12.0 (4.5-22.0)	0.255
Red cell Copper (nmol/g Hb)	32.4 (13.1-46.2)	30.31 (13.8-45.3)	0.655
Plasma Zinc (µmol/l)	5.0 (1.0-27.0)	3.5 (0.60-19.0)	0.306
Red cell Zinc (nmol/L Hb)	574 (399-812)	631 (492-779)	0.016
Plasma Selenium (µmol/l)	0.35 (0.01-5.68)	0.22 (0.04-0.72)	0.043
Plasma GSH-Px (IU/L)	376.3 (29.7-739.2)	272.8 (36.3-456.5)	0.286
Red cell Selenium (nmol/g Hb)	5.26 (2.48-10.62)	5.27 (2.63-9.62)	0.868
Red cell GSH-Px (IU/g Hb)	28.4 (13.9-37.1)	24.3 (11.6-42.8)	0.086

* Mann-Whitney U-test

Table 10-4: The relationship between admission characteristics, trace elements and ICU mortality.

	Univariate analysis		Multivariate analysis	
	OR (95%CI)	P-value	OR (95%CI)	P-value [a]
Age (years)	1.02 (0.99-1.05)	0.104	1.02 (0.97-1.06)	0.497
Sex (male/female)	0.89 (0.35-2.28)	0.806		
Patients (medical/surgical)	0.89 (0.36-2.18)	0.802		
APACHE II score	1.09 (1.02-1.16)	0.014		
SOFA score	1.14 (1.00-1.29)	0.440		
ICU length of stay (days)	1.05 (1.01-1.08)	0.007	1.04 (1.003-1.076)	0.035
Albumin (g/l)	0.89 (0.82- 0.96)	0.003		
CRP (mg/l)	1.00 (1.00-1.01)	0.446		
Lab-Glucose (mmol/l)	1.06 (0.95-1.17)	0.295		
Plasma Copper (µmol/l)	0.93 (0.84-1.03)	0.159		
Red cell Copper (nmol/g Hb)	0.98 (0.90-1.06)	0.584		
Plasma Zinc (µmol/l)	1.00 (0.89-1.12)	0.990		
Red cell Zinc (nmol/g Hb)	1.01 (1.001-1.011)	0.026		
Plasma Selenium (µmol/ l)	0.10 (0.01-1.09)	0.059		
Plasma GSH-Px (IU/L)	0.997 (0.992-1.002)	0.204		
Red cell Selenium (nmol/ g Hb)	1.00 (0.69-1.44)	0.984		
Red cell GSH-Px (IU/g Hb)	0.931 (0.824-1.053)	0.255		

[a] Binary logistic regression analysis.

11. The relationship between markers of the systemic inflammatory response and red cell vitamins B1, B2 and B6 concentrations.

11.1. Introduction

Vitamins B1, B2 and B6 serve as cofactors for several enzymes important in energy metabolism. Vitamin B1, also known as thiamine, was the first B vitamin to be identified. The B1 is an important cofactor for enzymes involved on carbohydrate metabolism, also it is a cofactor for several enzymes, such as pyruvate dehydrogenase, oxoglutarate dehydrogenase and transketolase (Gray et al. 2004; Fattal-Valevski A 2011). It also important for the biosynthesis of neurotransmitters and for the production of reducing substances used in oxidant stress defences (Fattal-Valevski A 2011). Vitamin B2 and its two cofactors flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD). FAD is mainly found in red blood cells, FMN and FAD act as cofactors in oxidation-reduction reaction and in the respiratory chain and are thus involved in energy production (e.g. cytochrome, glutathione reductase, glutathione peroxidase, xanthine oxidase and methylene tetrahydrofolate reductase) (Talwar et al. 2003b; Talwar et al. 2005). Vitamin B2 is required for normal immune function, by maintenance of glutathione status (Grimble 1997). It also has a suppressive effect on the production of tissue inflammatory mediators and decreases plasma elevated nitric oxide levels (Kodama K et al. 2005). Vitamin B6, also known as pyridoxal phosphate, acts as cofactor for a wide variety of enzymes of intermediary metabolism, particularly proteins, carbohydrates, fats and homocysteine and synthesis of several neurotransmitters and heam (Leklem 1991; Quasim et al. 2005). These vitamins have an essential biological function, which acts as a cofactor for many enzymes that regulate glucose, lipid and amino acid metabolism and neurotransmitter synthesis (O'Connell 2001).

In contrast to functional tests, direct measurements of thiamine, FAD and PLP concentrations in blood are thought to more reliably reflect nutritional status of vitamin B1, B2 and B6 (Bates 1977). However, as part of the systemic inflammatory response, plasma concentrations, in contrast to red cells, are reduced such that the relationship between plasma and red cell B1, B2 and B6 concentrations are disturbed (Talwar et al. 2003b; Gray et al. 2004; Quasim et al. 2005; Duncan et al. 2012b). Duncan and co-workers (2012) have recently shown in a large cohort that plasma concentrations of B6 may be confounded in the presence of a systemic inflammatory response as evidenced by CRP (Duncan et al. 2012b).

The aim of the present study was to examine the effect of the systemic inflammatory response, as evidenced by both CRP and albumin, on red cell measurements of vitamins B1, B2 and B6 and also examine these effects in patients with critical illness.

11.2. Patients and methods

11.2.1. Nutrition screen cohort

A total of 553, 251 and 313 consecutive heparin-treated whole-blood samples that had B1, B2 and B6 respectively with CRP and albumin measurements were received from hospitals throughout Scotland between January2008 and March 2013 for routine analysis of red cell measurements. As a regional centre blood samples were sent for analysis if the patient was considered at nutritional risk and was often secondary to a number of disease states. In addition, measurement of albumin and CRP was also recorded for these patients.

11.2.2. Critical illness cohort

Data and stored samples were available from cohorts which had been defined previously. The critical illness cohort comprised of medical or surgical patients ≥ 18 years admitted to the ICU of the Glasgow Royal Infirmary (GRI) between September 2006 and December 2008, and who had evidence of the systemic inflammatory response syndrome as per Bone's criteria (Bone et al. 1992; Levy et al. 2003), were studied. Briefly, APACHE II score (Knaus et al. 1985) and predicted hospital mortality and SOFA scores, CRP and albumin were recorded. This cohort has been described previously (Vasilaki et al. 2008).

This investigation was conducted with the intent of developing local guideline to aid in the interpretation of vitamin B1, B2 and B6 results. There were two cohorts studied. The first (a large unselected retrospective cohort) arose from an audit of patients who had a sample sent to a regional laboratory for a nutrition screen. The second (a small selected prospective cohort) arose from a prospective study of patients with critical illness. The patient data from the two cohorts was anonymised and de-identified prior to analysis (Andrew Duncan and Donald C McMillan respectively). In line with local ethical

procedures written informed consent was obtained for the latter cohort only. The latter study was approved by the ethics committees of the North Glasgow NHS Trust and Multicentre Research Ethics Committee (MREC) Scotland. Where patients were unable to give signed informed consent, consent was obtained from the patients' next of kin or welfare guardian in accordance with the requirements of the Adults with Incapacity Scotland (2000) Act (Appendix 1, 2).

11.2.3. Analytical methods

Consecutive heparin-treated whole-blood samples were received for routine analysis of Vitamin B1, B2 and B6 status was assessed by measuring TDP, FAD and PLP respectively in red blood cells by HPLC using established routine laboratory methods. Within- and between-assay precision <10% for all methods.

Albumin was measured by a BCP dye-binding method and C-reactive protein was measured using an automated analyser (Architect, Abbott Diagnostics, USA). For C-reactive protein the limit of detection was 5 mg/l. The inter-assay coefficient of variation was less than 3% and 5% over the sample concentration range for albumin and C-reactive protein respectively. The limit of detection for albumin was 10 g/L.

11.2.4. Statistical analysis

Data was presented in median and (range) value. Correlations between variables in the convenience sample were carried out using the Spearman rank correlation. The cohorts were divided into three groups according to CRP concentrations≤10, 11-80 and >80 mg/L as previously described (Marsik et al. 2008). The concentration of individual vitamins B1, B2 and B6 were grouped according to 3 categories of albumin concentrations ≥ 35, 25-34,< 25 g/Las previously described (Goldwasser and Feldman 1997). A P- value < 0.05

was considered significant and the analysis of the data was carried out using SPSS software (version 19; SPSS Inc, Chicago, Ill).

11.3. Result

11.3.1. Nutrition screen cohort

The characteristics of the convenience sample for vitamin B1 (n=553) are shown in Table 11-1. The majority were older than 50 years (median 54 years), male (50%) and had plasma albumin were below the normal range and plasma CRP and whole blood vitamin B1were in the normal range. Vitamin B1 was significantly associated with sex (r_s=-0.094, P =0.027), CRP (r_s= 0.290, P <0.001) and albumin (r_s= -0.265, P <0.001). CRP was significantly associated with age (r_s=0.253, P <0.001), sex (r_s=-0.123, P =0.004) and albumin (r_s=-0.651, P <0.001). Albumin was significantly associated with age (r_s=-0.255, P <0.001).

The characteristics of the convenience sample for red cells vitamin B2 (n=251) are shown in Table 11-1. The majority were older than 50 years (median 51 years), female (56%) and had plasma albumin were below the normal range and plasma CRP and red cell vitamin B2 in the normal range. Red cells vitamin B2 was significantly associated with sex (r_s=-0.335, P <0.001), CRP (r_s= -0.318, P <0.001), sex (r_s= -0.123, P =0.004) and albumin (r_s= -0.651, P <0.001). Albumin was significantly associated with age (r_s= - 0.255, P <0.001).

The characteristics of the convenience sample for red cells vitamin B6 (n=313) are shown in Table 11-1. The majority were older than 50 years (median 51 years), female (60%) and had plasma albumin were below the normal range and plasma CRP and red cells vitamin B6 in the normal range. Red cells vitamin B6 was significantly associated with sex (r_s=-0.254, P =0.013). CRP was significantly associated with age (r_s= 0.253, P <0.001), sex (r_s=-0.123, P =0.004) and albumin (r_s=-0.651, P <0.001). Albumin was significantly associated with age (r_s=-0.255, P <0.001).

11.3.2. Nutrition screen cohort for red cell vitamin B1

The effects of the magnitude of the systemic inflammatory response, as evidenced by CRP concentrations and albumin, on vitamin B1 are shown in Figure 11-1a and 11-1b.

The median concentrations of vitamin B1 (Table 11-2) grouped according to CRP concentrations ≤10, 11-80 and >80 mg/L were 542.5, 664.0 and 765.5 ng/g Hb respectively (P <0.001) with an overall elevation of 41%. The median whole blood concentrations of vitamin B1 grouped according to albumin concentrations ≥35, 25-34 and <25 g/l were 547.0, 664.0 and 701.0 ng/g Hb respectively (P <0.001) with an overall elevation of 28%.

When albumin concentrations were ≥ 35 g/L, the median concentrations of vitamin B1 grouped according to CRP concentrations ≤10, 11-80 and >80 were 535.0, 619.0 and 811.5 ng/g Hb respectively (P =0.003) with an overall elevation of 52%. When albumin concentrations were 25-34 g/L, the median concentrations of vitamin B1 grouped according to CRP concentrations ≤10, 11-80 and >80 mg/L were 611.0, 693.0 and 776.0 ng/g Hb respectively (P =0.011) with an overall elevation of 27%. When albumin concentrations were < 25 g/L, the median concentrations of vitamin B1 grouped according to CRP concentrations ≤10, 11-80 and >80 mg/L were 574.0, 671.0 and 745.0 ng/g Hb respectively (P =0.118) with an overall elevation of 30%. Median concentrations of vitamin B1 / albumin x100 ratio were significantly increased from 1450 to 2250 to 4030 respectively (P <0.001).

11.3.3. Nutrition screen cohort for red cell vitamin B2

The effects of the magnitude of the systemic inflammatory response, as evidenced by CRP concentrations and albumin, on vitamin B2 were shown in Figure 11-2a and 11-2b.

The median concentrations of red cell vitamin B2 (Table 11-3) grouped according to CRP concentrations ≤10, 11-80 and >80 mg/L were 2.2, 2.3 and 2.4 nmol/g Hb respectively (*P* <0.001) with an overall elevation of 9%. The median red cell concentrations of vitamin B2 grouped according to albumin concentrations ≥35, 25-34 and <25 g/l were 2.1, 2.4 and 2.3 nmol/g Hb respectively (*P* <0.001).

When albumin concentrations were ≥ 35 g/L, The median red cell concentrations of vitamin B2 grouped according to CRP concentrations ≤10 and 11-80 were 2.1 and 2.2 nmol/g Hb respectively (*P* =0.147) with an overall elevation of 4%. When albumin concentrations were 25-34 g/L, the median red cell concentrations of vitamin B2 grouped according to CRP concentrations ≤10 and 11-80 mg/L were 2.4 and 2.4 nmol/g Hb respectively (*P* =0.265). When albumin concentrations were < 25 g/L, the median red cell concentrations of vitamin B2 grouped according to CRP concentrations ≤10, 11-80 and >80 mg/L were 2.5, 2.3 and 2.5 nmol/g Hb respectively (*P* =0.334). Median red cell concentrations of vitamin B2 / albumin x100 ratio were significantly increased from 6 to 8 to 14 respectively (*P* <0.001).

11.3.4. Nutrition screen cohort for red cell vitamin B6

The effects of the magnitude of the systemic inflammatory response, as evidenced by CRP concentrations and albumin, on vitamin B6 were shown in Figure 11-3a and 11-3b.

The median concentrations of red cell vitamin B6 (Table 11-4) grouped according to CRP concentrations ≤10, 11-80 and >80 mg/L were 534, 548 and 767 pmol/g Hb respectively (*P* <0.001) with an overall elevation of 9%. The median red cell concentrations of vitamin B2 grouped according to albumin concentrations ≥35, 25-34 and <25 g/l were 462, 644 and 840 pmol/g Hb respectively (*P* <0.001).

When albumin concentrations were ≥ 35 g/L, the median red cell concentrations of vitamin B6 grouped according to CRP concentrations ≤10 and 11-80 were 478 and 413 pmol/g Hb respectively were not significantly different (P =0.286). When albumin concentrations were 25-34 g/L, the median red cell concentrations of vitamin B6 grouped according to CRP concentrations ≤10 and 11-80 mg/L were 818, 547 and 669 pmol/g Hb respectively (P =0.056). When albumin concentrations were < 25 g/L, the median red cell concentrations of vitamin B6 grouped according to CRP concentrations ≤10, 11-80 and >80 mg/L were 1037, 822 and 81 pmol/g Hb respectively (P =0.802). Median red cell concentrations of vitamin B6/ albumin x100 ratio were significantly increased from 1380 to 2030 to 3560 respectively (P <0.001).

11.3.5. Patients with critical illness

The characteristics of the critically ill cohort (n=94) are shown in Table 11-1. The majority were older than 50 years (median 60 years), male (64%), an APACHE II score of 21 and SOFA score of 7 and the associated median predicted mortality was 34%. The majority of patients were surgical (55%) and had a median length of ICU stay of 5 days and median hospital stay of 22 days. The median CRP and albumin concentrations were above and below the normal reference intervals respectively. Approximately half had some renal impairment as evidence by eGFR of <60ml/min.

The median whole blood vitamin B1 was elevated compared with the normal reference interval (783.5 ng/g Hb); whereas patients with critical illness have also extremely high whole blood vitamin B1 concentrations compared with general population of this nutrition screen cohort. Age was significant associated with albumin (r_s = -0.212, P =0.021). CRP was significantly associated with albumin (r_s=-0.374, P <0.001).The median whole blood concentrations of B1 grouped according to CRP concentrations ≤10, 11-80 and >80 mg/L

were 665.5, 800.0 and 768.0 ng/g Hb respectively (15%, *P* =0.348). The median whole blood concentrations of B1 grouped according to albumin concentrations ≥35, 25-34 and <25 g/l were 851.0, 789.0 and 727 ng/g Hb respectively (15%, *P* =0.361).

The median red cell concentrations of vitamin B2 was below the normal reference interval (19.7 nmol/g Hb), whereas patients with critical illness have higher vitamin B2 concentrations compared with general population of this nutrition screen cohort.red cell vitamin B2 was significantly associated with age (r_s =-0.179, *P* =0.053), sex (r_s =-0.322, *P* <0.001), CRP (r_s =-0.353, *P* <0.001) and albumin (r_s =0.334, *P* <0.001). Age was significantly associated with albumin (r_s=-0.212, *P* =0.021).CRP was significantly associated with albumin (r_s =-0.347, *P* <0.001). The median red cell concentrations of vitamin B2 grouped according to CRP concentrations ≤10, 11-80 and >80 mg/L were 40.8, 23.5 and 13.1 nmol/g Hb respectively (*P* <0.001). The median red cell concentrations of vitamin B2 grouped according to albumin concentrations ≥35, 25-34 and <25 g/l were 36.7, 29.8 and 16.9 nmol/g Hb respectively (*P* =0.010).

The median red cell concentrations of vitamin B6 was below the normal reference interval (23.5 pmol/g Hb), whereas patients with critical illness have lower vitamin B6 concentrations compared with general population of this nutrition screen cohort.red cell vitamin B6 was significantly associated with sex (r_s =-0.240, *P* =0.019). Age was significantly associated with albumin (r_s=-0.212, *P* =0.021). CRP was significantly associated with albumin (r_s =-0.347, *P* <0.001). The median red cell concentrations of vitamin B6 grouped according to CRP concentrations ≤10, 11-80 and >80 mg/L were 21.4, 24.2 and 24.1 pmol/g Hb respectively (*P* =0.250). The median red cell concentrations of vitamin B6 grouped according to albumin concentrations ≥35, 25-34 and <25 g/l were 14.6, 24.2 and 22.9 pmol/g Hb respectively (*P* =0.525).

11.4. Discussion

The results of the present study show that, in contrast to previous reported plasma values, in three cohorts of patients referred for a nutrition screen, red cell concentrations of vitamin B1, B2 and B6 were not lower with an increasing magnitude of the systemic inflammatory response as evidenced by both CRP and albumin concentrations. Indeed, there was a relatively small but significant increase in red cell B1, B2 and B6 concentrations with an increasing magnitude of the systemic inflammatory response. Therefore, in contrast to plasma measurements, red cell measurements prevent misclassification of vitamin B1, B2 and B6 deficiency in the presence of systemic inflammatory response.

These results of the present study are consistent with those of Vasilaki and co-workers (2008, 2010) who reported that plasma and red cell concentrations of B2 and B6 in patients with critical illness, red cell concentrations were less perturbed (Vasilaki et al. 2008; Vasilaki et al. 2010). Therefore, it would appear that during the systemic inflammatory response there is mobilisation of vitamin B1, B2 and B6 from the plasma into the cells, including red cells.

The results of the present study are consistent with previous reports. Louw and co-workers (1992) reported that in patients undergoing elective surgery vitamin B2 concentrations in the plasma were affected by albumin redistribution due to the systemic inflammatory response. Furthermore, in patients with critical illness, supplementation with pyridoxine was associated with an increase in concentrations of vitamin B6 in the red cell, but not in the plasma (Quasim et al. 2005). Indeed, it is recognised that, in plasma, riboflavin is extensively bound to proteins such as albumin and globulins, primarily immunoglobulins (Innis et al. 1986). This is consistent with the observation that plasma concentrations of vitamin B6 are transiently decreased in subjects undergoing elective surgery (Gray et al.

2004) and that vitamin-B6 supplementation in patients with critical illness was unable to increase plasma vitamin B6 concentration (Louw et al. 1992; Huang et al. 2002; Quasim et al. 2005).

Patients admitted to intensive care are under severe metabolic stress and may have increased utilization and consumption of vitamin B1, B2 and B6 and therefore some workers have advocated supplementation of these vitamins in these patients, which appears to have a beneficial effect on immune responses (Huang et al. 2005; Cheng et al. 2006). However, the results of the present study do not address the question of increased utilization and metabolic turnover of plasma vitamin ns B1, B2 and B6.

The present study has a number of limitations. In particular there was no information on the presence of chronic disease, co-morbidity or on body composition. Nevertheless, it is likely that the impact of these clinical factors on metabolic vitamins will involve the systemic inflammatory response, at least in part.

In summary, the results of the present study show that, unlike plasma concentrations, red cell concentrations of vitamin B1, B2 and B6 were not significantly affected by the systemic inflammatory response as a major confounding factor in determining their status. Therefore, red cell B1, B2 and B6 concentrations are likely to be a more reliable measure of status in patients with evidence of a systemic inflammatory response.

Table 11-1: Characteristics of nutrition screen cohort and vitamins B1, B2 and B6 concentrations.

	Reference interval	Nutrition screen cohort Whole blood vitamins B1 (n=553)	Nutrition screen cohort vitamins B2 (n=251)	Nutrition screen cohort vitamins B6 (n=313)	Critical illness cohort (n= 94)	P-value
Age (years)	NA	54 (18-100)	51 (16-82)	51 (16-82)	60 (18-100)	0.001 a
Sex (Male/Female)	NA	276 (50%)/ 277 (50%)	111 (44%)/ 140 (56%)	125 (40%)/ 188 (60%)	60 (64%)/ 34 (36%)	<0.001 a
C-reactive protein (mg/l)	<10	14.0 (<10- 565.0)	5.0 (<10- 565.0)	4.3 (<10- 301.0)	105 (<10- 565.0)	<0.001 a
Albumin (g/l)	35-55	32 (6-50)	36 (9-49)	35 (9-49)	18 (9-45)	<0.001 a
B1 (ng/g Hb)	275- 675	613 (175-3650)			783.5 (196-3650)	<0.001 b
B2 (nmol/g Hb)	1.0 – 3.4		2.2 (1.2-27.3)		19.7 (1.2-332.6)	<0.001 b
B6 (pmol/g Hb)	250 – 680			540 (79-100755)	23.5 (8.7-2140.3)	<0.001 b

(Median and range) a Kruskal-Wallis Test, b Mann-Whitney Test

Table 11-2: Distribution of whole blood vitamin B1 according to C-reactive protein and albumin concentrations (n=553).

whole blood vitamin B1 (ng/g Hb)	CRP ≤10 (mg/l)	11-80 (mg/l)	>80 (mg/l)	whole blood vitamin B1 according to albumin
Albumin ≥35 (g/l)	535 (211-2067) (n=175)	619 (201-1508) (n=61)	812 (805-818) (n=2)	547 (201-2067) (n=238)
34-25 (g/l)	611 (175-1781) (n=56)	693 (260-2552) (n=77)	776 (385-3650) (n=18)	664 (175-3650) (n=151)
<25 (g/l)	574 (233-1248) (n=19)	671 (288-2129) (n=71)	745 (196-3524) (n=74)	701 (196-3524) (n=164)
whole blood vitamin B1 according to CRP	543 (175-2067) (n=250)	664 (201-2552) (n=209)	766 (196-3650) (n=94)	

Median (range) (number of observation).

Table 11-3: Distribution of red cell B2 according to CRP and albumin concentrations (n=251).

Red cell B2 (nmol/g Hb	CRP ≤10 (mg/l)	11-80 (mg/l)	>80 (mg/l)	Red cell B2 according to albumin
Albumin ≥35 (g/l)	2.1 (1.2-5.9) (n=121)	2.2 (1.9-3.1) (n=15)	- (n=0)	2.1 (1.2-5.9) (n=136)
34-25 (g/l)	2.4 (1.2-27.3) (n=38)	2.4 (1.6-5.8) (n=38)	- (n=1)	2.4 (1.2-27.3) (n=77)
<25 (g/l)	2.5 (2.1-5.0) (n=6)	2.3 (1.3-3.5) (n=21)	2.5 (2.0-5.5) (n=11)	2.3 (1.3-5.5) (n=38)
Red cell B2 according to CRP	2.2 (1.2-27.3) (n=165)	2.3 (1.3-57.8) (n=74)	2.4 (1.6-5.5) (n=12)	

Median (range) (number of observation).

Table 11-4: Distribution of red cells B6 according to CRP and albumin concentrations (n=312).

Red cells B6 (pmol/g Hb)	CRP ≤10 (mg/l)	11-80 (mg/l)	>80 (mg/l)	Red cells B6 according to albumin
Albumin ≥35 (g/l)	478 (138-72374) (n=155)	413 (181-2812) (n=18)	- (n=0)	462 (138-72374) (n=173)
34-25 (g/l)	818 (79-61870) (n=47)	547 (164-9245) (n=42)	669 (320-1017) (n=2)	644 (79-61870) (n=91)
<25 (g/l)	1037 (206-3036) (n=12)	822 (183-100755) (n=24)	881 (291-23294) (n=12)	840 (183-100755) (n=48)
Red cells B6 according to CRP	534 (79-27374) (n=214)	548 (164-100775) (n=85)	767 (291-23294) (n=14)	

Median (range) (number of observation)

Figure 11-1a: The relationship between CRP (log 10) and whole blood vitamin B1 (ng/g Hb) (r_s= 0.290, *P*<0.001)) and in the critical illness cohort (r_s= 0.051, *P*=0.581).

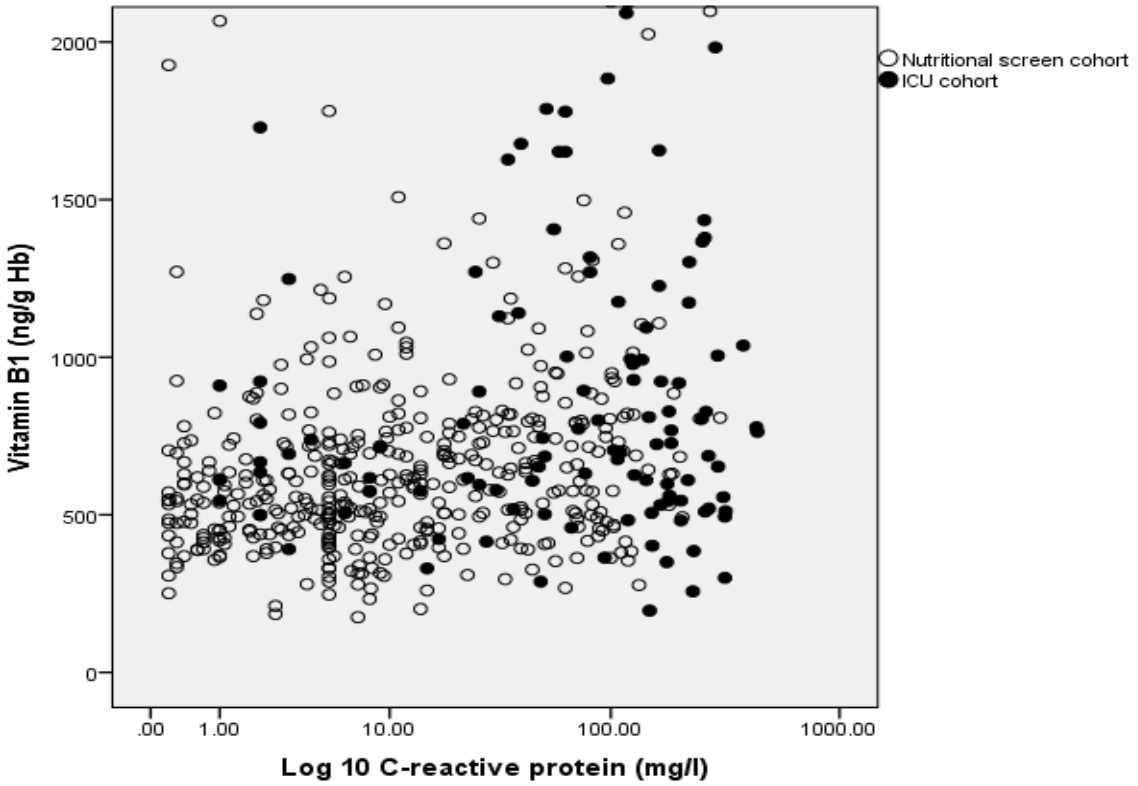


Figure 11-1b: The relationship between albumin and whole blood vitamin B1 (nmol/l) (r_s= - 0.265, *P*<0.001) and in the critical illness cohort (r_s= -0.567, *P*<0.001).

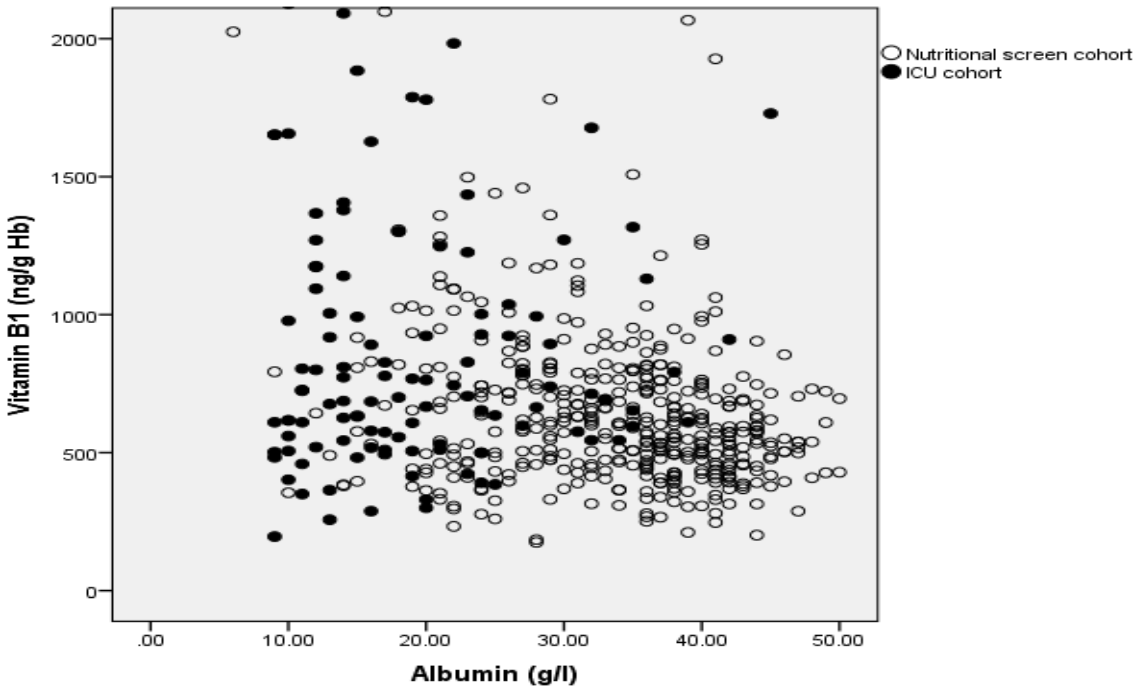


Figure 11-1c: The relationship between whole blood vitamin B1 adjusted for albumin and CRP (r_s= 0.583, P<0.001) and in the critical illness cohort (r_s= 0.275, P=0.003).

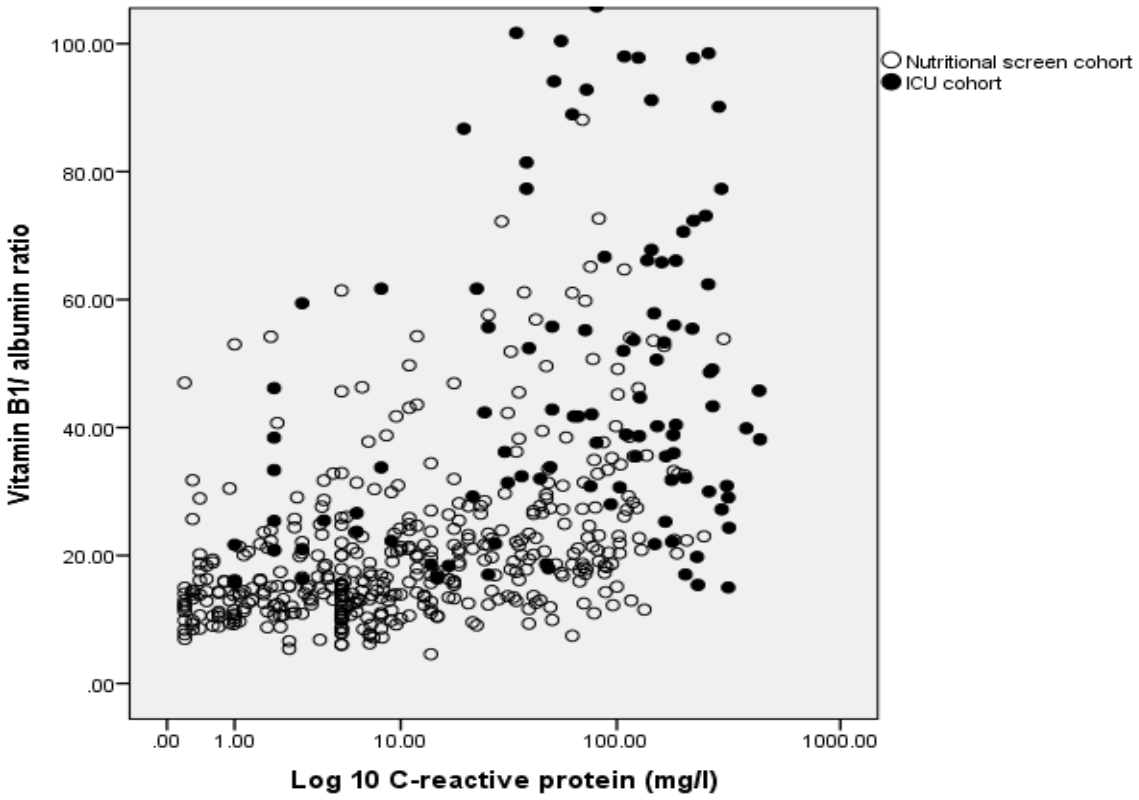


Figure 11-2a: The relationship between CRP (log 10) and red cell vitamin B2 (nmol/g Hb) (r_s= 0.189, *P*=0.003) and in the critical illness cohort (r_s, = 0.229, *P*=0.023).

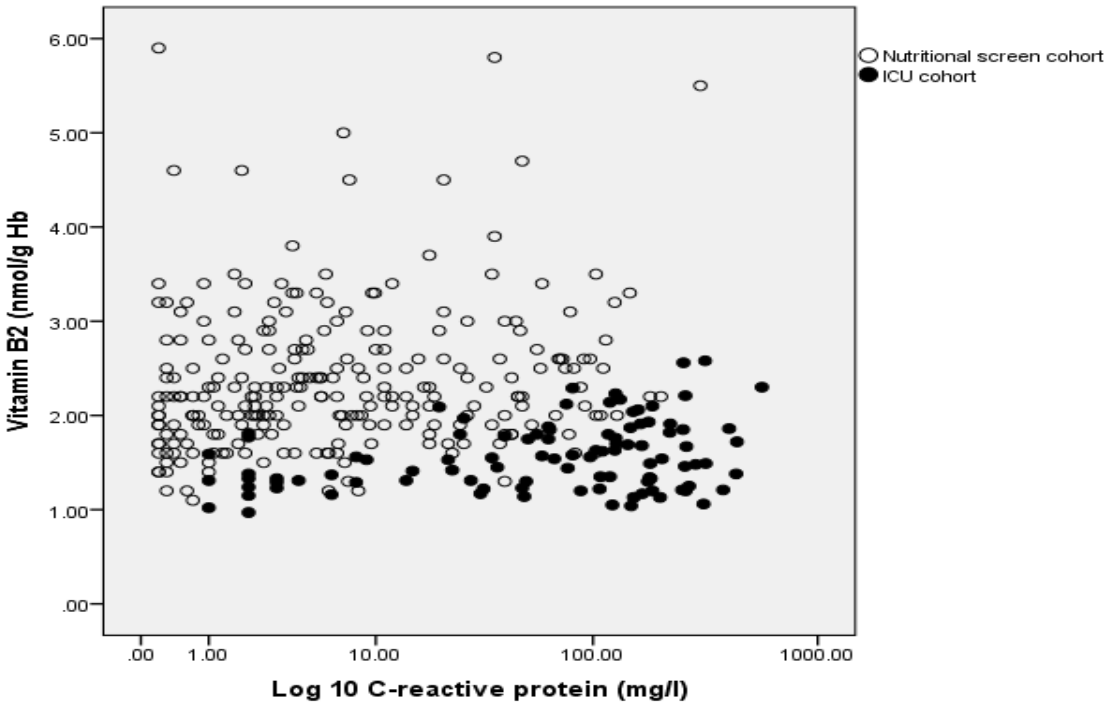


Figure 11-2b: The relationship between albumin and red cell vitamin B2 (nmol/g Hb) (r_s= 0.186, *P* <0.001) and in the critical illness cohort (r_s= - 0.139, *P*=0.173).

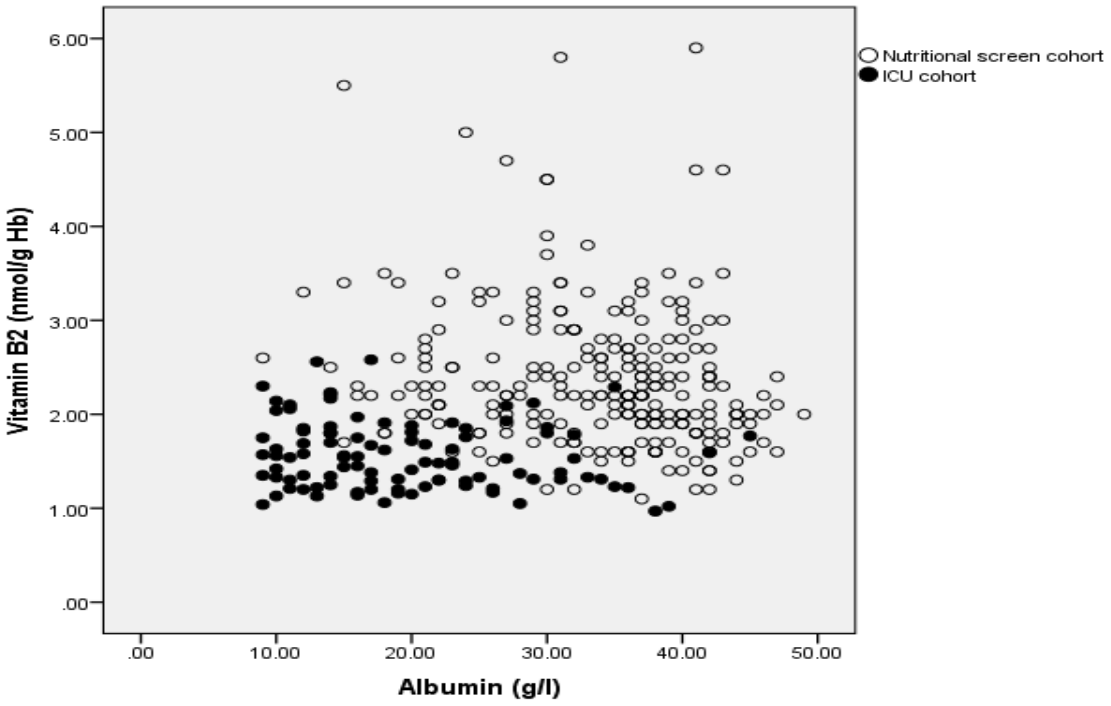


Figure 11-2c: The relationship between red cell vitamin B2 adjusted for albumin and CRP (r_s= 0.543, *P* <0.001) and in the critical illness cohort (r_s= 0.447, *P* =0.017).

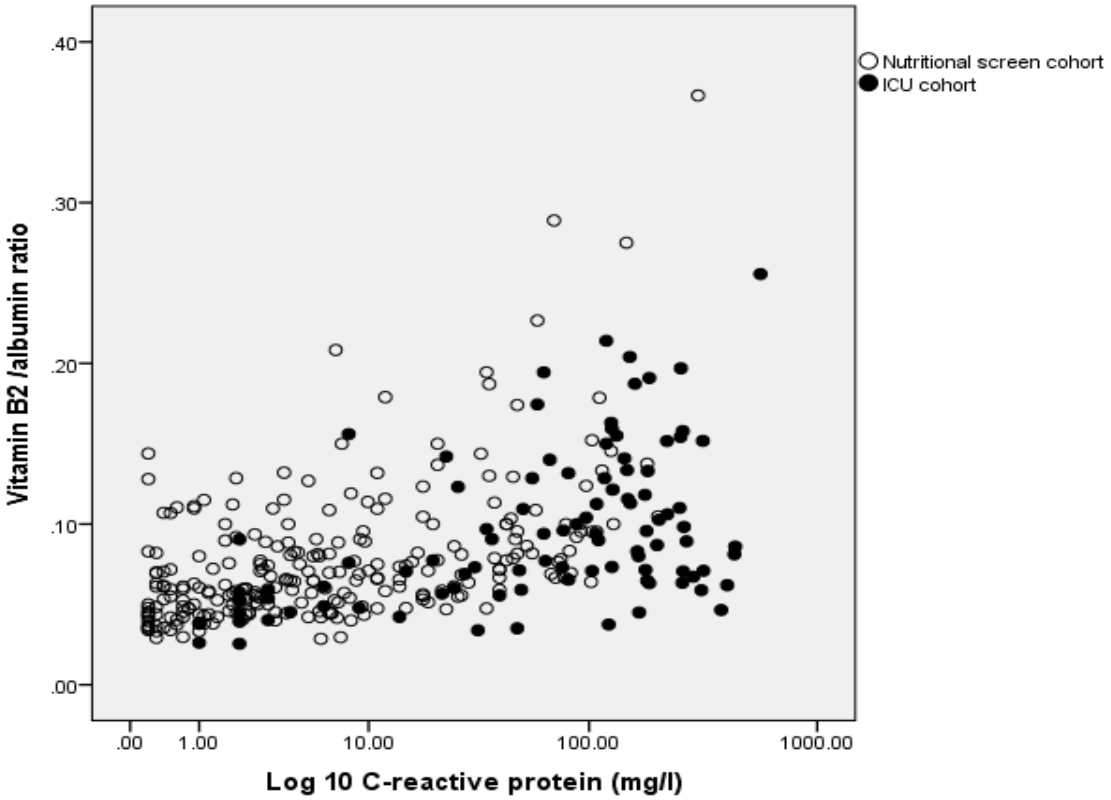


Figure 11-3a: The relationship between CRP (log 10) and red cells vitamin B6 (pmol/g Hb) (r_s= 0.079, *P* =0.161) and in the critical illness cohort (r_s= 0.084, *P* =0.411).

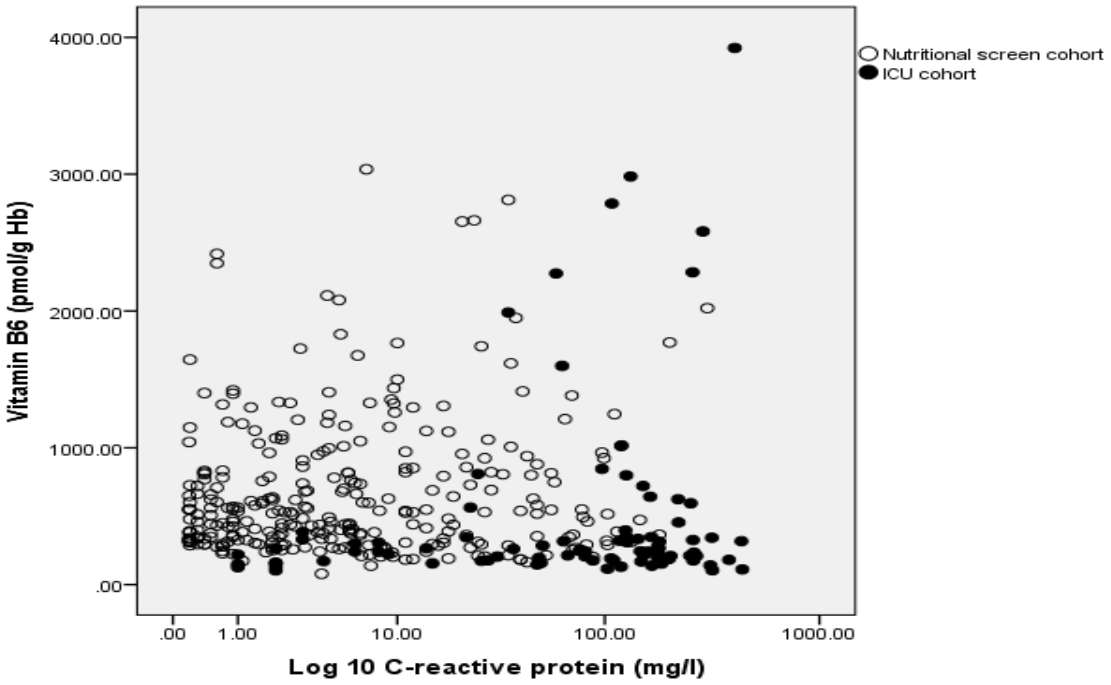


Figure 11-3b: The relationship between albumin and red cells vitamin B6 (pmol/g Hb) (r_s= - 0.217, *P* <0.001) and in the critical illness cohort (r_s=- 0.091, *P* =0.375).

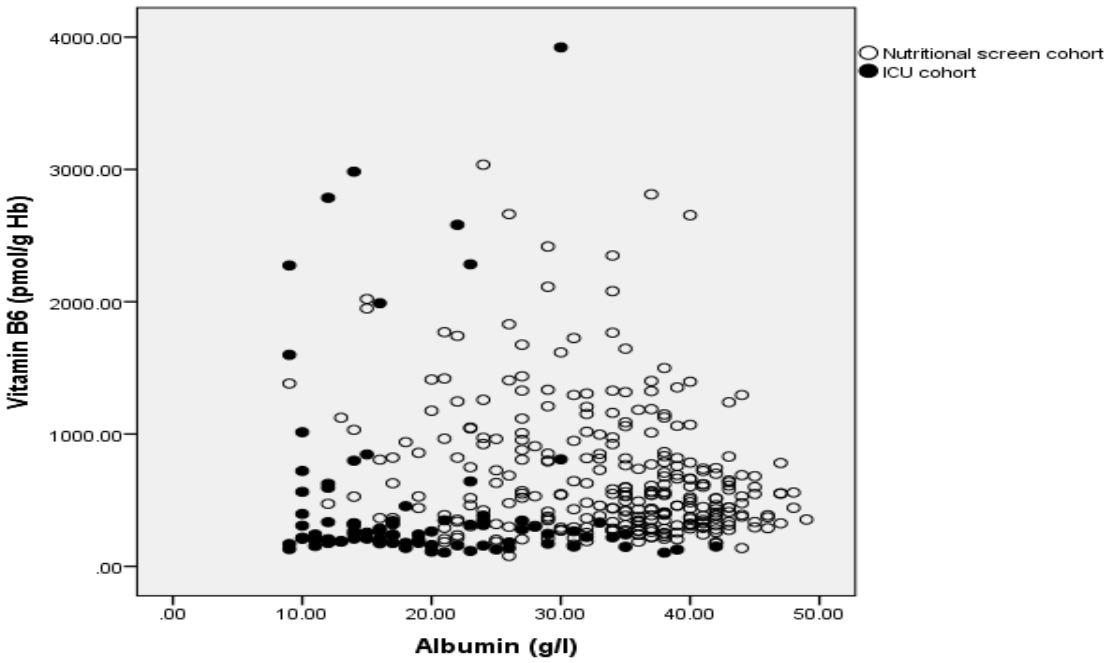
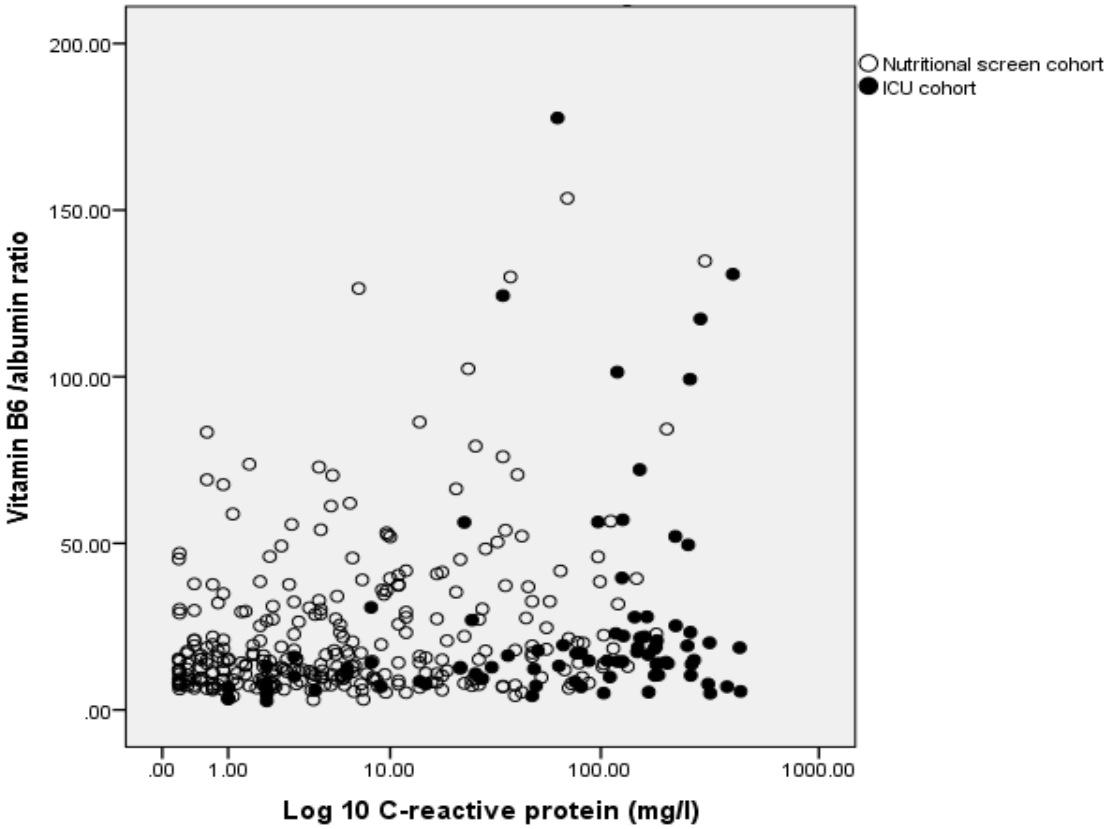


Figure 11-3c: The relationship between red cell vitamin B6 adjusted to albumin and CRP (r_s= 0.238, *P* <0.001) and in the critical illness cohort (r_s= 0.248, *P* =0.014).



12. Discussion

The aim of the thesis was:

To examine the relationship between the systemic inflammatory response, as evidenced by both CRP and albumin, on plasma glucose, plasma and red cell measurements of vitamins and trace elements in a nutrition screen cohort and a critical illness cohort.

12.1. The major findings of the study were:

In the critical illness cohort, insulin administration only accounted for a small part of the daily fluctuation of plasma glucose concentrations. In the nutrition screen cohort plasma concentrations of glucose appeared to be independently associated with both CRP and albumin and consistent with the systemic inflammatory response as a major confounding factor in determining its status. However, when this was examined in patients with critical illness, within the context of an insulin protocol, the relationship was more complex and multifactorial. Nevertheless, the combination of CRP and albumin concentrations during critical illness was independently associated with outcome. Compared with the complex APACHE II score and predicted mortality, the simple combination of CRP and albumin effectively predicted actual ICU and hospital mortality.

In the nutrition screen cohort and the critical illness cohort, the systemic inflammatory response was associated with the lower plasma values of a number of vitamins and trace elements. The basis of this association is not clear and may not be causal and so caution should be exercised in clinical interpretation. Therefore, it can be concluded that clinical interpretation of such plasma micronutrients only be made when the CRP and albumin concentrations are known or where the presence of a systemic inflammatory response can be excluded. The present data suggests that, in the presence of a moderate or severe

systemic inflammatory response (as indicated by a CRP concentration >20 mg/L), plasma concentrations of lutein, lycopene and β-carotene are not readily interpretable. Similarly, plasma concentrations of 25 (OH) D may not be reliably interpreted in the presence of abnormal CRP (>10mg/l) and albumin concentrations (<35g/l). It may be that plasma concentrations of 25 (OH) D in the presence of normal CRP and albumin concentrations reliably indicate nutritional status of vitamin D and the need for supplementation. Plasma concentrations of α-tocopherol/ cholesterol ratio and ascorbic acid were independently associated with both CRP and albumin and consistent with the systemic inflammatory response as a major confounding factor in determining their status. In particular, vitamin C concentrations were more profoundly affected by the presence of systemic inflammatory response. These results provide a basis for the better understanding of the impact of the systemic inflammatory response on plasma vitamin E and C. Similarly, plasma concentrations of vitamin E and C may not be reliably interpreted in the presence of abnormal CRP (>10mg/l) and albumin concentrations (<35g/l). It may be that plasma concentrations of vitamin E and C in the presence of normal CRP and albumin concentrations reliably indicate nutritional status of vitamin E and C and the need for supplementation.

Plasma concentrations of zinc were associated with both CRP and albumin. Moreover, that the impact of the systemic inflammatory response (as evidenced by elevation of CRP) on plasma zinc concentration may be adjusted by the albumin concentrations. Plasma concentrations of selenium were associated with both CRP and albumin. However, the impact of the systemic inflammatory response (as evidenced by elevation in CRP) may not be readily adjusted by the albumin concentration. Similar relationships were also observed in the cohort of patients with critical illness. The low plasma zinc and selenium concentrations and elevated plasma copper concentrations observed on admission of critically ill patients to ICU are in keeping with our knowledge of the systemic

inflammatory response and are a consequence of it rather than an indicator of trace element status. Similarly, plasma concentrations of zinc and selenium may not be reliably interpreted in the presence of abnormal CRP (>10mg/l) and albumin concentrations (<35g/l). It may be that plasma concentrations of zinc and selenium in the presence of normal CRP and albumin concentrations reliably indicate nutritional status of zinc and selenium and the need for supplementation.

Unlike plasma concentrations, red cell concentrations of zinc, selenium, copper, vitamin B1, B2 and B6 were not significantly affected by the systemic inflammatory response as a major confounding factor in determining their status. Taken together the results of the present thesis also provide a basis for the better understanding of the impact of the systemic inflammatory response on plasma and red cell micronutrients. This work, to my knowledge, is the most detailed systematic examination of the relationship between plasma glucose, micronutrients and the systemic inflammatory response. These results have led to changes in the methodology and practice of vitamin analysis in the Scottish Trace Elements and Micronutrients Reference Laboratory at Glasgow Royal Infirmary.

12.2. Limitations of the study

Limitations of the present studies were, principally, that they were observational studies and with reference to the critical illness cohort, represented a relatively small sample size.

The results of the present study, although clear because of their cross-sectional nature, cannot prove a causal effect of the systemic inflammatory response on vitamins and trace elements concentrations. There may be an underlying physiologic process that alters both the vitamins and trace elements concentrations, and the systemic inflammatory response, so that the observed relations could at times be indirect. In particular, there was no information on the presence of chronic disease, co-morbidity, or body composition.

Nevertheless, it is likely that the impact of these clinical factors on metabolic vitamins and trace elements will involve the systemic inflammatory response, at least in part.

12.3. Future studies

Future research is required both to confirm these results in nutrition screen and critical illness cohorts and also to carryout longitudinal studies across these cohorts. It will also be important to examine the clinical value of the intracellular micronutrients measurements in patient outcome. The red cell measurements presented in this thesis are an example of such an approach.

If such studies prove to be consistent then it will be important to incorporate these findings into future treatments with micronutrients. Taken together such new investigations will improve our understanding of the status of micronutrients in health and treatment in disease.

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14. Appendices

14.1.Consent form for participation in the study

Patient Identification Number for this study:

19th June 2006

CONSENT FORM

The relationship between the systemic inflammatory response, micronutrient status and outcome in patients with critical illness

(Version 2A)

Name of Researcher: Dr John Kinsella

I confirm that I have read and understand the information sheet dated 5th June 2006 (version 2A) for the above study and have had the opportunity to ask questions.

I understand that my participation is voluntary and that I am free to withdraw at any time, without giving a reason, without my medical care or legal rights being affected.

I give my permission for my GP to be informed about my participation in the above study.

I understand that the blood samples taken from me for routine purposes will be kept and analysed for research purposes which include vitamin and trace element concentrations in different blood compartments. I understand that these samples will not be returned to me.

I understand that the sections of any of my medical notes may be looked at by responsible individuals from the research team where it is relevant to my taking part in research. I give permission for these individuals to have access to my records.

I agree to take part in the above study.

_____	_____	_____
Name of patient	Date	Signature
---	---	---
Name of welfare guardian or Next of kin	Date	Signature
Nearest relative: Yes / No Welfare guardian: Yes / No	If yes, relationship to patient:_____	
_____	_____	_____
Name of person taking consent (if different from researcher)	Date	Signature
_____	_____	_____
Researcher	Date	Signature

1 for patient, 1 for researcher, 1 to be kept with hospital notes

14.2.Appendix 3 Information leaflet for patients and relatives

19th June 2006

PATIENT INFORMATION SHEET

The relationship between the systemic inflammatory response, micronutrient status and outcome inpatients with critical illness

(Version 2A)

You are being invited to take part in a research study. Before you decide 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 friends, relatives and your GP 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.

Consumers for Ethics in Research (CERES) publish a leaflet entitled 'Medical Research and You.' This leaflet gives more information about medical research and looks at some questions you may want to ask. If you would like to have a copy we will provide one.

Thank you for reading this.

What is the Purpose of the Study?

We wish to investigate the changes that occur in the concentrations of vitamins and other micronutrients in the blood during and after critical illness. This will require the further analysis of some of the blood samples that were routinely taken during your illness and a further blood sample after you have left intensive care.

We will also ask you some questions about your state of health and your diet. From this information we intend to establish the best method of assessing the vitamin and trace element nutritional status in critically ill patients.

Why have I been chosen?

All patients admitted to Glasgow Royal Infirmary ICU are being considered for admission into this study.

Do I have to take part?

It is up to you to decide whether or not to take part. If you 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. This will not affect the standard of care you receive.

What will happen to me if I take part?

Your treatment will not be altered by taking part to the study.

Phase 1 - When in the intensive care unit we would like to run some extra tests on your blood samples that have already been taken from you as part of routine. We will not ask you for anything else other than your consent to measure the vitamin and trace element concentrations in different compartments of your blood. This assessment is not done routinely and it will help us better assess the health of other patients admitted in the intensive care unit.

Phase 2 - If you agree to that we will also ask you to give us one more blood sample at clinic, and an interview about your health 3 – 6 months after your discharge from the intensive care unit. At this time we would also like you to provide us with a 7 day food diary that you will complete before coming to see us.

What do I have to do?

You don't have to do anything while in hospital (phase 1). If you agree to take part in phase 2 (at clinic) you only have to complete a 7 day food diary before visiting the surgical clinic. Please try not to change your usual eating habits when completing the diary. You should also follow your doctor's instructions and take your medication as prescribed.

Will any of my travel expenses be covered?

Travel expenses for routine hospital visits will not be covered. However, if you have to come to the hospital just for the purposes of the study your travel expenses will be covered in full.

What are the side effects of taking part?

There are no side effects of taking part in this study. It is purely an observational study does not include drug or nutritional intervention. In addition, if at any point you become concerned in any way please contact Dr John Kinsella, Consultant in Critical Care, tel. 0141 211 4625.

What are the possible disadvantages and risks of taking part?

There are no disadvantages or risks of taking part to this study.

What are the possible benefits of taking part?

As your treatment will be unchanged, there is no intended clinical benefit from taking part in the study. However, the information that we collect may help us to improve the treatment of future patients admitted to the intensive care unit.

What happens when the study stops?

Your treatment and follow up will continue as normal.

What if something goes wrong?

We have seen that the study will be absolutely safe for the patient as being observational in nature. If you are harmed by taking part in this study, there are no special compensation arrangements. Standard NHS indemnity does, however, apply in any case of adverse event. If you are harmed due to someone's negligence, then you may have grounds for 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 should be available to you.

Will my taking part in this study be kept confidential?

Yes. All information, which is collected about you during the course of research, will be kept strictly confidential. All the information required for the study will be kept strictly within the hospital departments. We have also set up a coding system so that your name will be replaced by a unique number and all of your information and results will be matched to this unique number and not your name.

If you agree, we will write to your GP and inform them of your participation in the study.

What will happen to the results of the research study?

The results of the study will be in large part to PhD thesis studies and may be published in scientific journals. Moreover, some of the results may also be presented in scientific meetings, e.g. conferences. We expect to publish our first results in 2007. If you wish you can obtain a copy of the published material from the University Department of Anaesthesia.

It is important to understand that at no publication or presentation of the results your name and/or personal details about your health will be identified.

Who is organizing and funding the research?

The funding of this research is from collaborative group of clinicians and scientists in the Glasgow Royal Infirmary.

Who has reviewed the study?

The study has been reviewed and approved by the Local Research Ethics Committee of the Royal Infirmary of Glasgow and the MREC for Scotland, Committee A.

Contact for Further Information

If you have any further questions or wish to obtain more information, please do not hesitate to contact the chief investigator –

Dr John Kinsella

Consultant

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