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CYTOKINES ASSOCIATED WITH INSULIN RESISTANCE IN CRITICALLY ILL PATIENTS.



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A research report submitted to the faculty of Health Sciences, University of the Witwatersrand, in the partial fulfillment of the requirements for the degree

of

Master of Medicine (Chemical Pathology)

Johannesburg, 2007

Declaration

I, Urs Wilgen, hereby declare that this research report is my own, unaided work. It is being submitted for the degree of Master of Medicine (Chemical Pathology) at the University of the Witwatersrand. It has not been previously submitted for any other degree at any other university.

This 20th day of December, 2007.

.....

Urs Wilgen

University approval was obtained from the University of the Witwatersrand Medical School, and ethical approval was given by the Human Research Ethics Committee (Medical) of the University of the Witwatersrand (Protocol M040214).

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Abstract

Mortality of patients requiring intensive care treatment for greater than 5 days has been shown to be about 20% worldwide. Hyperglycaemia is common in critically ill patients. Strict glucose control with insulin in critically ill patients was shown to reduce mortality and morbidity significantly. Several interrelated mechanisms are involved in the development of “stress hyperglycaemia” in critically ill patients. These include dextrose containing intravenous infusions and total parenteral nutrition; the counter regulatory hormones (catecholamines, cortisol, glucagon and growth hormone) which oppose the effects of insulin; nervous system signaling; increased insulin clearance; and excess production of cytokines that interfere with intracellular insulin signaling pathways.

Aim of study: To determine if the cytokines TNFα, IL-6 and adiponectin are significant determinants of insulin resistance in critically ill patients.

Methods: The study was a prospective observational study conducted in the intensive care unit (ICU) at the Chris Hani Baragwanath hospital. Forty sequential adult ICU admissions that met with the inclusion criteria were enrolled. Blood specimens were drawn for adiponectin, TNFα, and IL-6 at the time of ICU admission, on day 3, day 7 and on discharge from the ICU. Demographic data and clinical data were recorded, and body mass index (BMI) and APACHE II scores were calculated on admission. Blood glucose was measured every 2 to 4 hours, recorded and a mean value was calculated over the 24 hour period. Insulin infusions were started when the blood glucose values exceeded 6.0mmol/l. Administration of insulin was according to a fixed sliding scale. The total amount of insulin administered intravenously over that 24 hour period was recorded. Other factors known to be related to insulin sensitivity, such as inflammation (as indicated by C-reactive protein),

triglycerides, insulin, C-peptide and cortisol levels were also drawn in addition to the blood drawn for routine investigations.

Results: Duration of stay in ICU correlated with severity of illness as assessed by the APACHE II score ($r = 0.44$, $p = 0.004$). There was no significant difference in the mean 24 hour plasma glucose concentration throughout the duration of stay in ICU, there were however significant differences in the amount of insulin administered to maintain normoglycaemia. The amount of administered insulin required was found to peak on day 3 and decline thereafter. The main determinant of insulin administered was mean glucose ($r = 0.79$, $p < 0.00001$).

The measured insulin concentrations on admission correlated with mean plasma glucose ($r = 0.41$, $p = 0.009$) and C-peptide ($r = 0.45$, $p = 0.004$) levels. The main determinants of mean plasma glucose levels on admission were BMI ($r = 0.38$, $p = 0.013$) and serum cortisol ($r = 0.41$, $p = 0.008$) levels.

Serum triglycerides levels showed a significant difference from admission to discharge, with values increasing from admission levels.

Adiponectin levels showed a significant increase from admission to discharge. IL-6 levels showed a significant decrease. TNFα levels did not show statistically significant changes. No statistically significant correlations were found between the levels of TNFα or IL-6 and administered insulin. Adiponectin concentrations showed a negative correlation with amount of administered insulin on discharge ($r = -0.457$, $p = 0.0049$).

There were significant gender differences in BMI, administered insulin on admission, serum cortisol and C-peptide concentrations, with females having higher values than males. BMI was shown to account for the gender differences in administered insulin and C-peptide levels.

There were significant differences in IL-6 and TNFα concentrations between the survivors and non-survivors, with higher levels being seen in non-survivors. Adiponectin levels were lower in non-survivors, but this did not reach statistical significance.

Conclusion: Although there was a demonstrable change in insulin sensitivity during the stay in ICU, there was no statistically significant association between the cytokines TNFα or IL-6 and insulin administration. There was a negative correlation between adiponectin concentrations and administered insulin on discharge. This data also demonstrates that mortality is associated with increased levels of proinflammatory cytokines.

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List of abbreviations

ACTH	Adrenocorticotrophic Hormone
APACHE II	Acute Physiology And Chronic Health Evaluation II
ATP	Adenosine Triphosphate
CRP	C-reactive Protein
ET-1	Endothelin – 1
FFA	Free Fatty Acids
GH	Growth Hormone
GLUT	Glucose Transporter
ICAM	Intercellular Adhesion Molecule
ICU	Intensive Care Unit
IGF-1	Insulin-like Growth Factor – 1
IL-6	Interleukin – 6
IRS-1	Insulin Receptor Substrate - 1
MnSOD	Manganese Superoxide Dismutase
$NF_{\kappa}B$	Nuclear Factor Kappa B
NO	Nitric Oxide
NOS	Nitric Oxide Synthetase
eNOS	endothelial Nitric Oxide Synthetase
iNOS	inducible Nitric Oxide Synthetase
nNOS	neuronal Nitric Oxide Synthetase
$PPAR_{\gamma}$	Peroxisome Proliferator Receptor Gamma
PYK2	Proline rich tyrosine Kinase 2
ROS	Reactive Oxygen Species

RNS	Reactive Nitrogen Species
TGFβ	Transforming Growth Factor beta
TNFα	Tumour Necrosis Factor alpha
VEGF	Vascular Endothelial Growth Factor

1. Literature survey

1.1 Stress hyperglycaemia

Mortality of patients requiring intensive care treatment for greater than 5 days has been shown to be about 20% worldwide (Takala et al., 1999). Significant morbidity as a result of complications such as acute renal failure, nosocomial infections, anaemia, muscle weakness and critical illness polyneuropathy further prolong intensive care unit (ICU) stay (Van den Berghe, 2004). Hyperglycaemia and insulin resistance are common in critically ill patients, even in non-diabetic patients (Van den Berghe et al., 2001). In the study by Van den Berghe and co-workers, blood glucose concentration on admission to ICU was >6.1mmol/l (the upper limit of the reference range for non-diabetic fasting subjects) in 75% of patients, and >11.1mmol/l (the limit used for the diagnosis of diabetes mellitus in random blood glucose samples) in 12% of all ICU admissions (Van den Berghe et al., 2001). In a separate study of all hospitalized patients, 12% of all non-diabetic admissions were found to be hyperglycaemic (Umpierrez et al., 2002).

Insulin resistance is defined as a state of impaired disposal of glucose by insulin sensitive tissues in the presence of elevated concentrations of insulin in the bloodstream (Hunter & Garvey., 1998). Insulin resistance has been well demonstrated in both surgical (Thorell et al., 2004) and medical (Zauner et al., 2007) ICU patients.

“Stress hyperglycaemia” was initially viewed as a physiological adaptation to ensure a metabolic fuel supply for the central nervous system and phagocytic cells during trauma and injury, to allow the injured animal to sustain itself until its injuries are healed and as a result was left untreated (Mizock, 2001).

In a landmark study Van den Berghe and co-workers showed that strict glucose control with insulin in critically ill patients reduced mortality and morbidity significantly. An almost 50% reduction (from 20.2% to 10.6%) in overall mortality, and a substantial reduction in complications by such a simple intervention was a major advance in ICU management (Van den Berghe et al., 2001). A linear correlation between the risk of death and the degree of hyperglycaemia, as well as a linear correlation between the blood glucose concentration and the risk of development of critical illness polyneuropathy has been demonstrated (Van den Berghe et al., 2005).

This naturally posed the question whether it was the improvement in glycaemic control that was having the beneficial effect on mortality and morbidity, or an effect of the insulin that was administered. The linear correlation between risk of mortality and degree of hyperglycaemia was shown to persist even after correction for insulin dose and severity of illness. Van den Berghe and co-workers also showed in their study on critically ill patients, that a higher daily dose of insulin was associated with a less favourable outcome (Van den Berghe et al., 2002). It thus appears to be the prevention of hyperglycaemia rather than the amount of exogenous insulin administered that accounts for the reduction in mortality associated with intensive insulin therapy in critically ill patients (Finney et al., 2003).

It is clear that several interrelated mechanisms are involved in the development of “stress hyperglycaemia” in critically ill patients. These include dextrose containing intravenous infusions and total parenteral nutrition; the counter-regulatory hormones (catecholamines, cortisol, glucagon and growth hormone) which oppose the effects of insulin; nervous system signaling; and excess cytokine production (Hirsch, 2002). In addition, an increase in the rate of insulin clearance in critically ill patients has also been demonstrated (Hoshino et al., 2003).

1.2 Hyperglycaemia and increased mortality in critically ill patients

Mechanisms by which hyperglycaemia predisposes to complications and mortality in critically ill patients are not yet fully elucidated. Two possible mechanisms have been proposed: i) cellular glucose overload, and ii) toxic side effects of oxidative phosphorylation.

i) Cellular glucose overload.

Glucose uptake via insulin-*independent* glucose transporters such as glucose transporter 1 (GLUT-1), GLUT-2 and GLUT-3 occurs in the nervous system, hepatocytes, endothelial cells, epithelial cells (e.g. renal tubular cells, gastrointestinal enterocytes), pancreatic β cells and immune cells. Cytokines (e.g. transforming growth factor β (TGFβ)), endothelin-1 (ET-1), angiotensin II, and hypoxia result in upregulation of GLUT-1 and GLUT-3 allowing cellular glucose overload, which may be directly toxic to the cell (Klip et al., 1994).

In contrast, skeletal muscle and cardiac muscle have insulin-*dependent* glucose uptake via GLUT-4 transporters, and are thus thought to be spared the toxic effects of glucose (Klip et al., 1994).

ii) Toxic side effects of oxidative phosphorylation.

Intracellular glucose is metabolized to pyruvate via glycolysis, which is further metabolized to generate adenosine triphosphate (ATP) via the citric acid cycle and oxidative phosphorylation in the mitochondria. A small amount of superoxide is concurrently produced during this process by the mitochondrial respiratory chain complexes. This superoxide is a reactive oxygen species (ROS) that is harmful to the cell, and is normally detoxified by manganese superoxide dismutase (MnSOD). In the event of increased intracellular glucose concentrations, more pyruvate is utilized in oxidative phosphorylation, and more superoxide is produced, which exceeds the cellular ability to detoxify it by MnSOD, resulting in increased

superoxide concentrations. Superoxide reacts with nitric oxide (NO) to form peroxynitrate (a reactive nitrogen species (RNS)), which can damage mitochondrial oxidative phosphorylation protein complexes, impairing cellular ATP generation, and resulting in cell injury (Aulak et al., 2004).

1.3 Endothelial dysfunction in critically ill patients

Endothelial cell dysfunction has also been implicated in the development of complications such as multiple organ failure in critically ill patients. The vascular endothelium plays an important role in the control of vasomotor tone, and as a result blood flow to tissues through the microcirculation. The vascular endothelium is also important in coagulation and retention of fluid within the vascular compartment (Aird, 2003). Endothelial dysfunction will thus predispose to coagulopathy, capillary leakage and hypoperfusion of tissues. ROS, cytokines and growth factors (e.g. vascular endothelial growth factor (VEGF)) which are released from injured tissues contribute to endothelial cell dysfunction (Langouche et al., 2005).

Increased vascular permeability will result in fluid loss from the intravascular compartment resulting in decreased intravascular volume; cytokine stimulated upregulation of endothelial adhesion molecules e.g. vascular cellular adhesion molecule (VCAM), intercellular adhesion molecule (ICAM), E-selectin and P-selectin, results in increased white blood cell adhesion and obstruction of blood flow; and coagulopathy results in intravascular thrombosis further impairing perfusion. Impaired delivery of glucose to skeletal muscle as a result of decreased perfusion will also result in decreased glucose removal from the circulation (Langouche et al., 2005).

Nitric oxide is a vasodilator produced by endothelial cells from L-arginine, a reaction catalyzed by nitric oxide synthetase (NOS). There are 3 isoforms of NOS; neuronal NOS (nNOS) present in the central nervous system and skeletal muscle; endothelial NOS (eNOS) expressed in endothelial cells;

and inducible NOS (iNOS) an inducible form that can be induced by proinflammatory cytokines (e.g. interleukin-6 (IL-6) and tumour necrosis factor alpha (TNFα)) and is expressed in several tissues. Low concentrations of NO produced by eNOS have beneficial effects by acting as an anti-adhesive substance for leukocytes preventing their adhesion to the endothelium, whereas high concentrations of NO produced by iNOS has detrimental effects by promoting inflammation and upregulating cellular adhesion molecules (Aktan, 2004). Nitric oxide concentrations have been shown to be significantly elevated in critically ill patients when compared to healthy controls (Papathanassoglou et al., 2003).

Glucose has been shown to have proinflammatory effects (Aljada et al., 2004), and as discussed above, endothelial cell uptake of glucose is insulin-independent via GLUT-1, GLUT-2 and GLUT-3. Prevention of hyperglycaemia may thus protect the endothelium by preventing intracellular glucose overload, and indeed studies have shown evidence of decreased endothelial dysfunction in critically ill patients treated with intensive insulin therapy, and reduced circulating concentrations of intercellular adhesion molecules (ICAM-1 and E-selectin) (Langouche et al., 2005).

1.4 The counter-regulatory hormones in critically ill patients

Cortisol has numerous metabolic effects not only on carbohydrate metabolism, but also on protein and fat metabolism. Cortisol production from the adrenal gland is stimulated by adrenocorticotropic hormone (ACTH) released from the anterior pituitary in response to stress and cytokine stimulation. Cortisol stimulates glycogenolysis and gluconeogenesis from amino acids derived from skeletal muscle breakdown, thus increasing hepatic glucose production and output to increase blood glucose concentrations. In addition cortisol stimulates lipolysis with a resultant increase in blood triglyceride and free fatty acid (FFA) concentrations. The glycerol liberated from triglyceride hydrolysis is utilized as a gluconeogenic substrate (Desborough, 2000). Cortisol administration, in the form of hydrocortisone infusions, has been shown to improve survival in critically ill patients with severe sepsis (Annane, 2001). Hydrocortisone infusions however result in elevation of blood glucose

concentrations, and this will in turn require insulin infusions to maintain normoglycaemia (Groeneveld et al., 2002). Hydrocortisone has been shown to exert its beneficial effects through the enhancement of the inotropic effects of vasopressors such as the catecholamines (adrenalin and noradrenalin) and improved intravascular fluid retention, which offers haemodynamic advantages. Cortisol is known to inhibit the immune response caused by acute disease or trauma which is thought to indicate a negative feedback mechanism to dampen the inflammatory cascade and prevent an excessive response (Van den Berghe et al., 1998).

Apart from the metabolic effects on carbohydrate metabolism, abnormalities of lipid metabolism are also seen in critically ill patients. As a result of the counter-regulatory hormones, cortisol and the catecholamines, elevations in the concentrations of triglycerides are also observed (Messotten et al., 2004). In addition, serum triglycerides have been shown to be useful markers of insulin resistance (McLaughlin et al., 2003).

Growth hormone (GH) is secreted from the anterior pituitary gland in response to stress, and promotes hepatic gluconeogenesis and glycogenolysis, thus increasing the glucose output from the liver, and stimulates lipolysis and protein synthesis. It also inhibits cellular uptake of glucose thus decreasing glucose removal from the circulation, as a means to spare glucose for use by glucose dependent tissues such as the central nervous system and erythrocytes. Its effects are mediated directly as well as through the stimulation of hepatic production of insulin like growth factor-1 (IGF-1) (Desborough, 2000). In a study evaluating the possible beneficial effects of GH in critically ill patients to prevent the muscle and organ catabolism, the group receiving GH infusions was shown to have a mortality rate almost double that of the placebo group (39% vs 20%) (Takala et al., 1999). The reason for this increased mortality is still unexplained. Possible explanations involve immune function modulation, and enhanced proinflammatory cytokine production together with increased reactive oxygen species production. The study by Takala and co-workers however also demonstrated higher

blood glucose concentrations in the group receiving growth hormone, and hyperglycaemia, as previously mentioned, has been associated with an increased risk of death, sepsis and organ dysfunction (Takala et al., 1999).

Glucagon is produced by the α cells of the pancreas and stimulates glycogenolysis and gluconeogenesis in the liver and lipolysis of triglycerides in adipose tissue. Although blood glucagon concentrations increase in response to trauma, this response appears not to be a large contributor in the development of stress hyperglycaemia (Desborough, 2000).

1.5 The inflammatory process and insulin resistance

A number of cytokines have been shown to decrease insulin sensitivity by interfering in the intracellular insulin signaling pathways.

Tumour necrosis factor alpha (TNFα) is a polypeptide produced by neutrophils, activated T and B lymphocytes, natural killer cells, astrocytes, endothelial cells (Vilcek & Lee, 1991), and adipocytes (Mohamed-Ali et al., 1998) and has been implicated as a causative agent in the reduction of insulin sensitivity seen in obesity and type-2 diabetes mellitus. This cytokine induces lipolysis and enhances FFA release from adipose tissue; inhibits insulin signaling; and alters gene expression of adipocyte genes through the activation of the nuclear factor kappa B (NFκB), as well as synthesis of adipocyte specific proteins including adiponectin which has been shown to be an insulin sensitizing agent (Ruan & Lodish, 2003). Thus, TNFα is a potent inducer of insulin resistance, with increased TNFα expression associated with impaired glucose disposal. Endothelin-1 stimulated glucose transporter-4 (GLUT-4) translocation into the cell membrane and resultant glucose uptake into adipocytes has also been shown to be desensitized as a result of TNFα. This is due partially to decreased expression of Galpaq/11, leading to suppression of tyrosine phosphorylation of proline rich tyrosine kinase 2 (PYK2) (Rachdauoi & Nagy, 2003). In addition TNFα has been shown to inhibit insulin receptor substrate-1

(IRS-1) phosphorylation, thereby impairing this pathway of intracellular insulin signaling related to glucose metabolism in skeletal muscle, and this may induce compensatory hyperinsulinaemia (Yamaguchi et al., 2003).

Interleukin-6 is a polypeptide produced by lymphocytes, monocytes, fibroblasts, hepatocytes, endothelial cells (Kishimoto et al., 1992) and adipocytes (Mohamed-Ali et al., 1998) and may be an important local and systemic factor participating in insulin resistance in obesity (Bastard et al., 2002). Several studies have demonstrated a relationship between IL-6 and insulin resistance. Like TNFα, IL-6 exerts long-term effects inhibiting gene transcription of insulin receptor substrate-1 (IRS-1) and GLUT-4 and peroxisome proliferator activated receptor gamma (PPARγ). Rotter et al (2003) demonstrated that insulin stimulated glucose transport is significantly reduced by IL-6. It has also been shown to have antagonistic effects to insulin on both adipocytes (Lagathu et al., 2003) and hepatocytes (Klover et al., 2003).

Adiponectin is a 244 amino acid protein exclusively produced by adipose tissue (Scherer et al., 1995), and is involved in the regulation of insulin sensitivity (Filippi et al., 2004). Decreased adiponectin levels are found in insulin resistant states such as obesity and type-2 diabetes mellitus (Lebovitz et al., 2003). In addition, adiponectin knock-out mice were shown to have severe insulin resistance and impaired glucose metabolism (Matsuzawa et al., 2003). Adiponectin mRNA production and secretion of the protein are inhibited by TNFα. Adiponectin in turn negatively regulates TNFα levels (Keller et al., 2003), and subsequent TNFα induced expression of endothelial adhesion molecules (Ouchi et al., 2003). Hypoadiponectinaemia is closely associated with increased levels of inflammatory markers such as C-reactive protein (CRP) and IL-6, thus adiponectin appears to have additional anti-inflammatory properties as well (Ouchi et al., 2003).

Although the hypothesis that proinflammatory cytokines are the cause of the insulin resistance seen in critically ill patients seems attractive, there is still minimal evidence to support this. Kremen et al. (2006) demonstrated an increase in TNFα and IL-6 levels and showed that the rate of insulin infusion required to maintain normoglycaemia was increased up to 7 fold in patients admitted to the ICU following cardiac surgery. This increased insulin resistance was seen in conjunction with elevated TNFα and IL-6 concentrations. Also, proinflammatory cytokine levels have been shown to be elevated in critically ill patients with an adverse outcome when compared to patients who survived (Friedland et al., 1996). Thus, IL-1, IL-6, IL-8 and TNFα levels on admission were higher in patients who did not survive (Friedland et al., 1996).

Insulin is also known to have anti-inflammatory properties. Intensive insulin therapy has been shown to lower levels of inflammatory markers such as CRP (Hansen et al., 2003).

1.6 Indicators of mortality in critically ill patients

Prognostic factors for early mortality are likely to be of value to clinicians involved in making clinical decisions regarding intensity of further management and care of critically ill patients.

There are a multitude of different scoring systems, each using slightly different parameters to calculate a severity of illness score and predict mortality. The present study was restricted to adult patients and therefore scoring systems were confined to adult scoring systems. Trauma scores such as the Injury Severity Score (ISS), Revised Trauma Score (RTS) and Trauma Injury Severity Score (TRISS) were excluded because not all patients were trauma patients. Other severity of illness scores such as the Sepsis related Organ Failure score (SOFA), Multiple Organ Dysfunction Score (MODS) and Organ Dysfunction and/or Infection score (ODIN) were not used because not all patients were septic, or suffered from multiple organ dysfunction. Therefore, from the general adult severity of illness scores, we chose to grade the severity of illness using the Acute Physiology And Chronic

Health Evaluation (APACHE II) score, instead of the new Simplified Acute Physiology Score (SAPS II), because of its better performance at predicting mortality (Castella et al., 1995), and because of its successful and long-standing use in the ICU, where the present study was conducted.

The APACHE II score is a score based on current physiologic measurements (body temperature; heart rate; respiratory rate and mean arterial pressure), measured biochemical and haematological parameters (arterial pH; PaO_2; serum sodium, potassium and creatinine concentrations; haematocrit and white cell count), neurological status (Glasgow Coma Score), age and previous health condition to give a general measure of disease severity. The score can help in the assessment of disease severity in patients to determine the level and degree of diagnostic and therapeutic intervention. The minimum score is 0 with a maximum score of 71. A higher score is associated with increased risk of hospital death (Knaus et al., 1985). The APACHE II score is commonly used as a score of severity of illness, with higher scores indicating more severe illness and a worse prognosis (*See appendix A).*

A complete understanding of the causes of hyperglycaemia in critically ill patients, as well as the mechanisms by which hyperglycaemia predisposes to morbidity and mortality will ultimately shed light on possible interventions aimed at improving survival of critically ill patients.

The present study set out primarily to investigate the possible role of inflammatory and anti-inflammatory cytokines in the aetiology of insulin resistance in critically ill patients. The full aims of this study are explained in the following section.

2. Study Objectives

1. To determine the correlation between disease severity, as assessed by the APACHE II score, and insulin requirements.
2. To determine if IL-6, TNFα, adiponectin and CRP levels are related to insulin resistance and/or insulin requirements in critically ill patients.
3. To correlate the individual cytokine levels (IL-6, TNFα and adiponectin) with outcome at ICU discharge.

3. Materials and methods

3.1 Patient enrollment

The study was a prospective observational study conducted in the intensive care unit (ICU) at the Chris Hani Baragwanath hospital, which is an academic multi-disciplinary unit caring for medical and surgical patients.

University approval was obtained from the University of the Witwatersrand Medical School, and ethical approval was given by the Human Research Ethics Committee (Medical) of the University of the Witwatersrand (Protocol M040214).

All sequential adult ICU admissions that were not excluded by the exclusion criteria were enrolled in the period from the 1st of July 2004 until the end of August 2004, until a total sample number of forty (40) was obtained. The patients were used as their own controls.

Exclusion criteria were:

1 Known type 1- or type 2-diabetic patients were excluded because of the pre-existing insulin requirements in type 1 diabetes mellitus, and pre-existing insulin resistance in type 2 diabetes mellitus.

2 Patients with known liver failure were excluded because of the function of the liver in glycogen production and storage, gluconeogenesis, and insulin degradation.

3 Patients with known chronic renal failure were excluded because of the gluconeogenic function of the renal cortex.

Consent was obtained initially from the primary care physician or next of kin, and later from the patients themselves once they were able to give informed consent for the use of the blood specimens and demographic and clinical data collected during their stay in ICU.

3.2 Specimen collection

Blood specimens were drawn for adiponectin, TNFα, IL-6, C-reactive protein (CRP), triglycerides, C-peptide, insulin and cortisol levels in addition to the blood drawn for routine investigations at the time of ICU admission. These samples were drawn before any insulin infusions were administered. Further blood specimens were drawn on day 3, day 7 and on discharge from the ICU. The blood was drawn either from central venous lines or arterial lines, after discarding the first 5ml of blood, into vacutainer tubes with no additives. The blood was allowed 30 minutes to clot, and promptly centrifuged at 4000rpm for 5 minutes and the serum separated into cryo-tubes and frozen at -70ºC.

Demographic data and clinical data were obtained from the recorded data on ICU charts and the patients' bed-letters.

The patients were weighed on admission using a digital hanging scale, where the patients were fully suspended in a harness in the supine position, with only a sheet covering them. The heights (in centimeters) of the patients were measured lying supine and fully extended in the ICU bed. A ruler was used to laterally extend the inferior aspect of the heel and the vertex of the head, and the distance between the two points was measured using a tape measure.

Body mass index (BMI) was calculated using the formula: $BMI = \frac{\text{weight (kg)}}{\text{height (m)}^2}$

APACHE II scores were also calculated on admission, day 3, day 7 and discharge from the ICU (*See appendix A*).

Arterial blood gas analysis was performed routinely every 2 to 4 hours, and blood glucose was analyzed on each arterial blood sample. The blood glucose values were recorded and a mean value was calculated over the 24 hour period to give a mean plasma glucose concentration.

Insulin infusions were started when the blood glucose values exceeded 6.0mmol/l, with an incremental increase in the rate of insulin infusion with higher blood glucose concentrations. Administration of insulin was according to a sliding scale (*See appendix B*). The total amount of insulin administered intravenously over that 24 hour period was recorded in a data base. No changes to ICU protocols were required.



Figure 1. Photograph of a patient in one of the isolation cubicles in the Chris Hani Baragwanath Hospital Intensive Care Unit. The photograph shows an intubated and sedated patient requiring mechanical ventilation (far right of picture) and renal replacement therapy in the form of continuous veno-venous haemodialysis (CVVHD) (far left of picture). Multiple infusion pumps are required to control the rate of intravenous fluid, total parenteral nutrition (TPN), and medications (e.g. inotropes, insulin etc) administered.

3.3 Biochemical assays

The serum specimens were kept in storage at -70ºC until the total sample size of 40 patients was obtained, and then assayed using commercially available kits for adiponectin, TNFα and IL-6 available from R&D systems and distributed by Whitehead Scientific. The serum samples were thawed overnight in the refrigerator at 4ºC before analysis.

3.3.1 Adiponectin, TNFα and IL-6

These cytokine assays employ a quantitative sandwich enzyme linked immunoassay technique using a monoclonal antibody specific for the cytokine pre-coated onto a microplate. Standards and samples are pipetted into the wells and any analyte present is bound by the immobilized monoclonal antibody. After washing away any unbound substances, an enzyme-linked monoclonal antibody specific for the analyte is added to the wells. Another wash step removes unbound antibody-enzyme reagent, and a substrate reagent is added to the wells and a colour develops in proportion to the amount of analyte bound in the initial step. The colour development is stopped by changing the pH with sulfuric acid, and the intensity of the colour is measured spectrophotometrically.

Adiponectin assays were run in 1:100 dilution, as stipulated in the kit insert, by adding 5µl serum to 495µl of calibrator diluent which is a buffered protein base supplied with the kit. The TNFα and IL-6 assays used undiluted serum samples. The wash buffer which consists of a concentrated buffered surfactant solution with preservatives was prepared by diluting 20ml wash buffer concentrate into distilled water to prepare 500ml wash buffer.

The standards were prepared by reconstituting the cytokine standard with 2.0ml calibrator diluent to produce a stock solution. Serial dilutions produce standards of known concentrations, with the calibrator diluent serving as the zero standard. All reagents, samples, and controls were brought to room temperature before use. A 100µl aliquot of assay diluent, which consists of a buffered protein

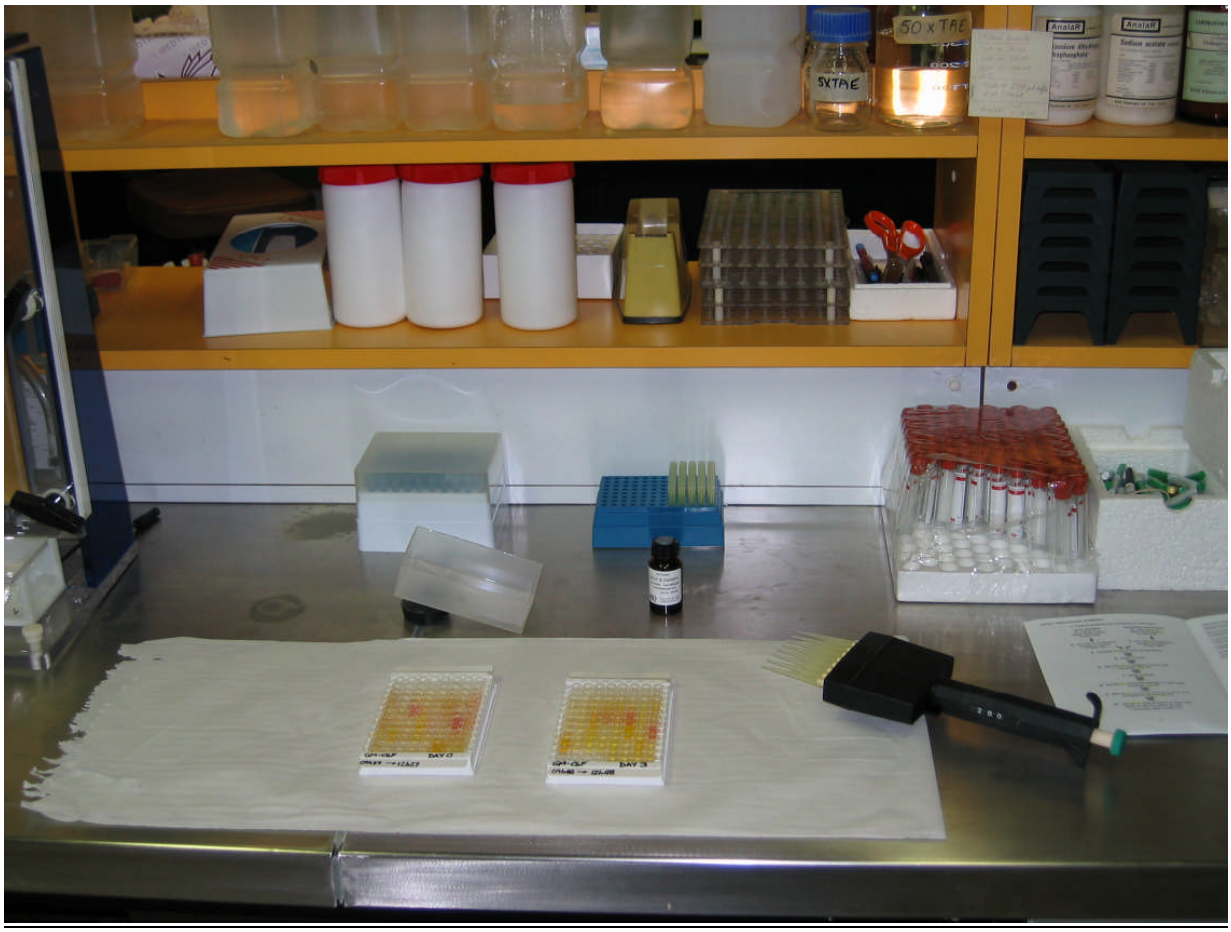
base with preservatives, was added to each well using a calibrated multi-tip pipette, and 50µl standard or sample was added to each well and left to incubate at room temperature for 2 hours. The microplate was then subjected to 4 aspiration and wash cycles using an automated plate washer Elx50 from BioTek instruments using the prepared wash buffer. A 200µl aliquot of conjugate which contains the mouse monoclonal antibody specific to the analyte and is linked to horseradish peroxidase was added to each well, and again incubated for 2 hours at room temperature. The microplate was again subjected to 4 cycles of aspiration and washing in the automated plate washer using the prepared wash buffer, and 200µl substrate solution prepared by adding equal quantities of colour reagent A and B (containing hydrogen peroxide and tetramethylbenzidine as chromogen, respectively) was added to each well. The microplate was protected from light in a cupboard and incubated at room temperature for 30 minutes. A 50µl volume of stop solution containing 2N sulfuric acid was added to each plate and the colour intensity read at 450nm immediately, and λ correction was performed at 540 nm on the Elx800 universal microplate reader from BioTek instruments.

A standard curve was generated from the standards, plotting absorbance on the y-axis and analyte concentration on the x-axis. The analyte concentrations of the samples were read from the standard curve.

All samples and controls were run in duplicate and the average of the 2 values were taken and multiplied by the dilution factor (for adiponectin only) to obtain the final result.

The analytical sensitivity of the adiponectin assay is 0.246ng/ml and with the upper limit of linearity being 250ng/ml for adiponectin. The adiponectin assay was performed using 1:100 dilution, due to the reference ranges in the normal population being 3900 – 10 200ng/ml (Schalkwijk et al., 2006.).

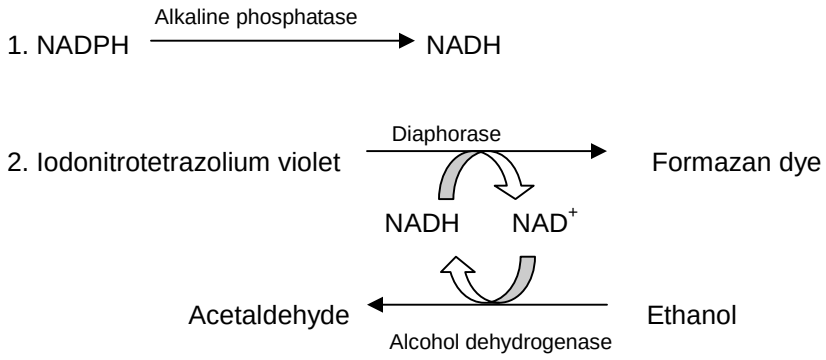
The analytical sensitivity for the IL-6 assay is 0.70pg/ml, with an upper limit of linearity being 300pg/ml. No significant cross-reactivity or interference has been observed for either assay.



<u>Figure 2.</u> Photograph of the 96 well ELISA plates that were used to measure adiponectin, TNFα and IL-6 levels in patient serum samples.

Most of the results obtained by using the TNFα assay were below the measurable limit of detection for the kit. A **high sensitivity TNFα assay** was purchased from R&D and all samples were re-analyzed. The high sensitivity TNFα assay employs the same quantitative sandwich enzyme immunoassay technique, except that the sample was allowed to incubate in the microtitre wells for 3 hours, and an amplifier step is included. A 30 second soak period was also introduced between washes to improve sensitivity.

The amplification step in this high sensitivity TNFα assay employs an alkaline phosphatase reaction in which alkaline phosphatase dephosphorylates the reduced form of nicotinamide adenine dinucleotide phosphate (NADPH) to reduced nicotinamide adenine dinucleotide (NADH). The NADH subsequently serves as a cofactor in a redox cycle reaction catalyzed by a second enzyme system consisting of alcohol dehydrogenase and diaphorase. Diaphorase catalyses a reduction of a tetrazolium salt (iodonitrotetrazolium violet), which consumes NADH, to produce an intensely coloured formazan dye and NAD^+ . NAD^+ is in turn reduced by ethanol in a reaction catalyzed by alcohol dehydrogenase to regenerate NADH, which re-enters the redox cycle. Thus the rate of reduction of the tetrazolium salt and formation of coloured compound is directly proportional to the amount of TNFα bound in the initial step.



The absorbance of the wells was read at 490nm with λ correction at 650nm to correct for background absorbance and imperfections in the wells.

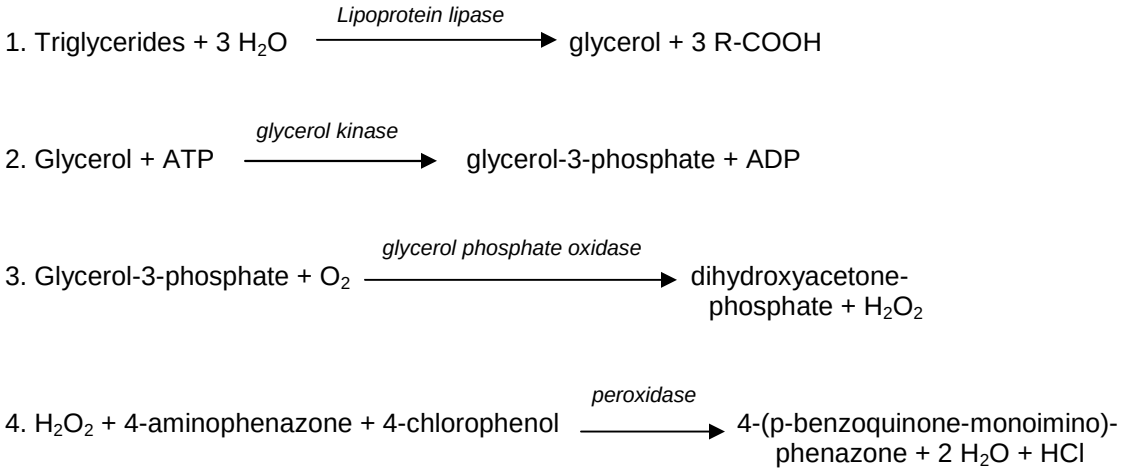
The analytical sensitivity of the high sensitivity TNFα assay is 0.12pg/ml, with the upper limit of linearity being 32pg/ml. No significant cross-reactivity or interference has been observed for the assay.

3.3.2 C-reactive protein (CRP) and Triglycerides

The CRP and triglycerides were analyzed on the Hitachi® Modular P800™ automated chemistry analyzer.

C-reactive protein is measured via an immunoturbidimetric assay, in which anti-CRP antibodies react with CRP in the sample to form an antigen-antibody complex, which is measured turbidimetrically. The analytical sensitivity of the CRP assay is 1mg/L, with a limit of linearity of 258mg/L, which is automatically extended to 515mg/L following a 1:2 dilution and rerun. No significant interference or cross-reactivity has been documented.

Triglycerides are measured via an enzymatic colorimetric assay in which triglycerides are hydrolyzed by lipoprotein lipase to glycerol, followed by oxidation to dihydroxyacetone phosphate and hydrogen peroxide. The hydrogen peroxide then reacts with 4-aminophenazone and 4-chlorophenol under the catalytic action of peroxidase to form a red dyestuff which is measured photometrically.



The analytical sensitivity of the triglyceride assay is 0.05mmol/l with an upper limit of linearity being 11.3mmol/l. The extended measuring range after automatic dilution and rerun is 62.2mmol/l.

3.3.3 Cortisol, C-peptide and Insulin

Cortisol was measured on the Advia® Centaur™ immunoassay system by Bayer instruments and insulin and C-peptide on the Immulite® by DPC.

Cortisol is measured via a competitive immunoassay using chemiluminescence. The cortisol in the patient sample competes with an acridinium ester labeled cortisol in the reagent for binding to a polyclonal rabbit anti-cortisol antibody in the solid phase. A monoclonal mouse antibody coupled to a paramagnetic particle binds the polyclonal rabbit anti-cortisol antibody. Thus an inverse relationship exists between the amount of cortisol present in the patient sample and the amount of light detected by the system. The concentration is read off a master curve on the system.

The analytical sensitivity of the cortisol assay is 5.5nmol/l, with an upper limit of linearity of 2069nmol/l. Cross-reactivity with hydrocortisone is not detectable.

Insulin and C-peptide were measured by a two-site enzyme labeled immunometric assay using chemiluminescence. A polystyrene bead enclosed within the Immulite test unit is coated with a monoclonal antibody specific for the analyte. The patient sample is incubated together with an alkaline phosphatase-polyclonal antibody conjugate in the test unit to form an analyte-antibody sandwich. Unbound conjugate is removed in a wash step, after which substrate is added and a further incubation takes place for 10 minutes. Alkaline phosphatase hydrolyzes the chemiluminescent substrate (a phosphate ester of adamanyl dioxetane) to produce an unstable intermediate, which results in a sustained emission of light, which is measured by a luminometer. The light emitted is directly proportional to the concentration of the analyte in the patient sample.

The analytical sensitivity of the C-peptide assay is 0.09µg/L, with an upper limit of linearity of 15µg/L. The assay is highly specific for C-peptide with no cross-reactivity with insulin, glucagon, or secretin, and 10.5% cross-reactivity with proinsulin.

The analytical sensitivity of the insulin assay is 2mU/L with an upper limit of linearity of 300mU/L The assay is highly specific for insulin with no cross-reactivity with C-peptide or glucagon, and 8.5% cross-reactivity with proinsulin.

3.3.4 Blood glucose

Blood glucose measurements were performed using 2ml syringes with added lyophylised heparin which were analyzed immediately after collection on a Radiometer Copenhagen™ ABL700 series blood gas analyzer using an amperometric measurement of pO_2. Glucose oxidase is immobilized in a gel on the outer-surface of a pO_2 electrode. The comsumption of O_2 when glucose is oxidized is measured at the pO_2 electrode, and the change in pO_2 is related to the glucose concentration in the sample.

$$\text{Glucose} + O_2 \xrightarrow{\textit{Glucose oxidase}} \text{gluconic acid} + H_2O_2$$

The analytical sensitivity is 1.11mmol/l, with an upper limit of linearity of 27.7mmol/l. No interference from substances other than halothane has been documented. Halothane may interfere at high concentrations, giving falsely low results. Only one patient in the study sample received halothane for the management of a severe acute asthmatic attack.

All analytes measured on automated instruments in the laboratory were recorded after the instrument quality control and calibration steps had been performed, and the method performance was judged to be in control using universally applied quality control rules.

3.4 Statistical analysis

Statistical analysis was performed with the aid of STATISTICA version 7.0 (StatSoft, Tulsa Oklahoma USA).

A Shapiro-Wilk W test was used to assess the distribution of the data. The majority of data was not normally distributed, and was transformed either by using the logarithm or square root of the variable to obtain a Gaussian distribution. Parametric statistical analysis was performed on data which had a normal distribution, or could be normalized. When values of an analyte could not be normalized, non-parametric statistical analysis was performed.

The mean, standard deviation (SD) and standard error of the mean (SEM) were calculated for each analyte with a normal distribution. For data with a non-Gaussian distribution, the median and interquartile ranges were calculated.

Spearman rank order correlation coefficients were calculated to determine the correlations between different parameters, with gender coded as male = 1 and female = 2. This same coding was used for multiple regression analysis.

The Student paired and unpaired T-tests were used to determine whether the change of analyte between the different days of admission to the ICU were statistically significant, when the data had a normal distribution, or on the log-transformed or square root-transformed data. The Mann-Whitney U-test and Wilcoxon matched pairs tests were used to determine whether the change of analyte between the different days of admission were statistically significant, when the data could not be normalized.

Sub-group analysis, on the basis of gender, duration of ICU stay less than or greater than 7 days, race, and outcome at discharge from the ICU was also performed for all variables.
An analysis of co-variance (ANCOVA) was used to determine the effect of confounding variables, and multiple regression analysis was also performed.

In graphs showing how variables change during the stay in ICU, time points shown are admission, day 3, day 7 and discharge later than 7 days. Correlation analyses were run for variables measured on admission, day 3, day 7 and discharge later than 7 days (ranging from 8 – 43 days).

Percentage change data was calculated as the difference between the discharge concentration and admission concentration expressed as a percentage of the value on admission.

In all calculations a p value of less than 0.05 was taken to represent statistical significance.

4. Results

4.1 General study population data

Forty patients were enrolled in the study and followed up until discharge from the ICU. The median age was 35.5 years, mean age of 37.95 years, with a minimum age of 18 and a maximum age of 66 years (*see figure 3*).

The majority of patients (54%) were between the ages of 25 and 52 years of age. Two thirds of the patients were male.

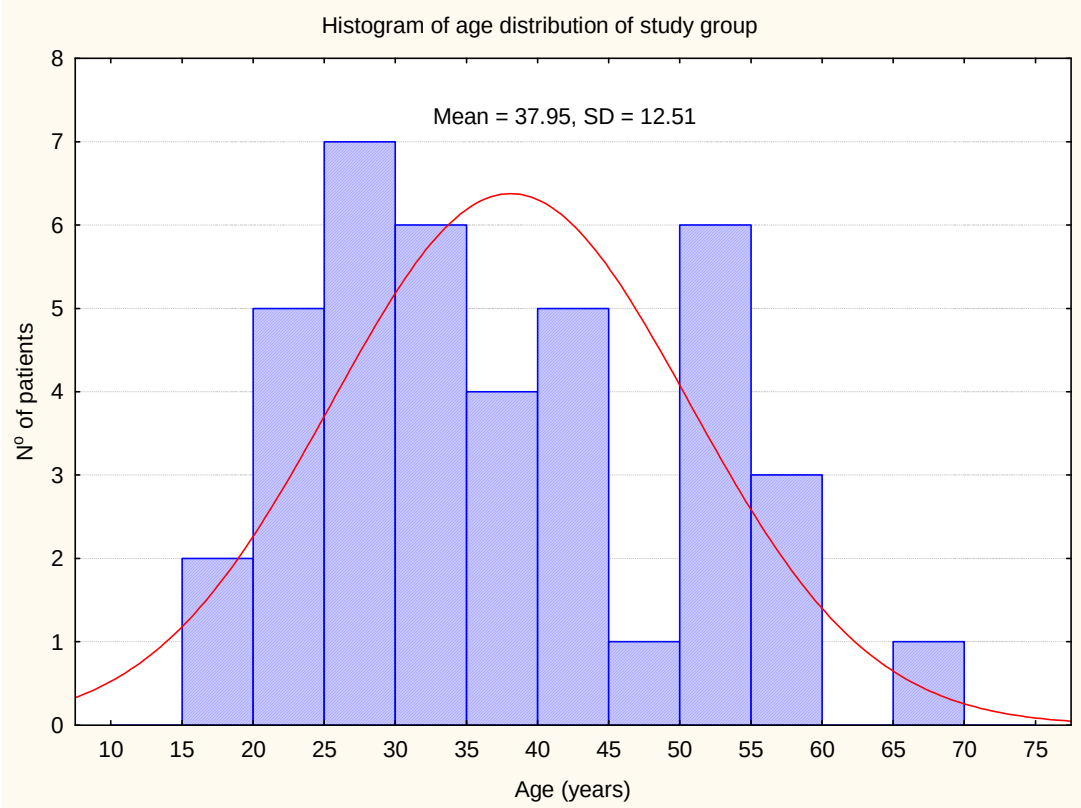


Figure 3. Histogram showing the age distribution of the study group.

Of all study patients 92.5% were surgical patients (general surgical, vascular surgery, urology, otorhinolaryngology, plastic and reconstructive surgery, gynaecology and obstetrics), with the remaining 7.5% being medical patients (*see table 1*).

Table 1. Diagnoses of patients admitted to the ICU.

	Diagnosis/condition	**Number of patients**
Trauma	Gunshots	9
	Motor vehicle accidents	6
	Blunt trauma	3
	Stab	2
	Electrocution	1
Abdominal surgery	Bowel obstruction/perforation	2
	Strangulated hernia	1
	Mesenteric vein thrombosis	1
	Necrotizing fasciitis	1
	Cholecystectomy	1
Vascular surgery	Abdominal aortic aneurysm	1
Malignancies	Oesophagus	1
	Pancreas	1
	Lip	1
Caustic ingestion	Oesophagectomy	2
Anaesthetic complications	Negative pressure pulmonary oedema	1
Gynaecological/Obstetric	Post partum haemorrhage	1
	Eclampsia	1
Medical	Asthma	1
	Guillian Barre	1
	Myesthenia gravis	1
	Drug overdose	1

Black patients made up the vast majority of the study group (80%), with the remainder being white (12.5%), Indian (2.5%), and coloured (5%) (s*ee figure 4*).

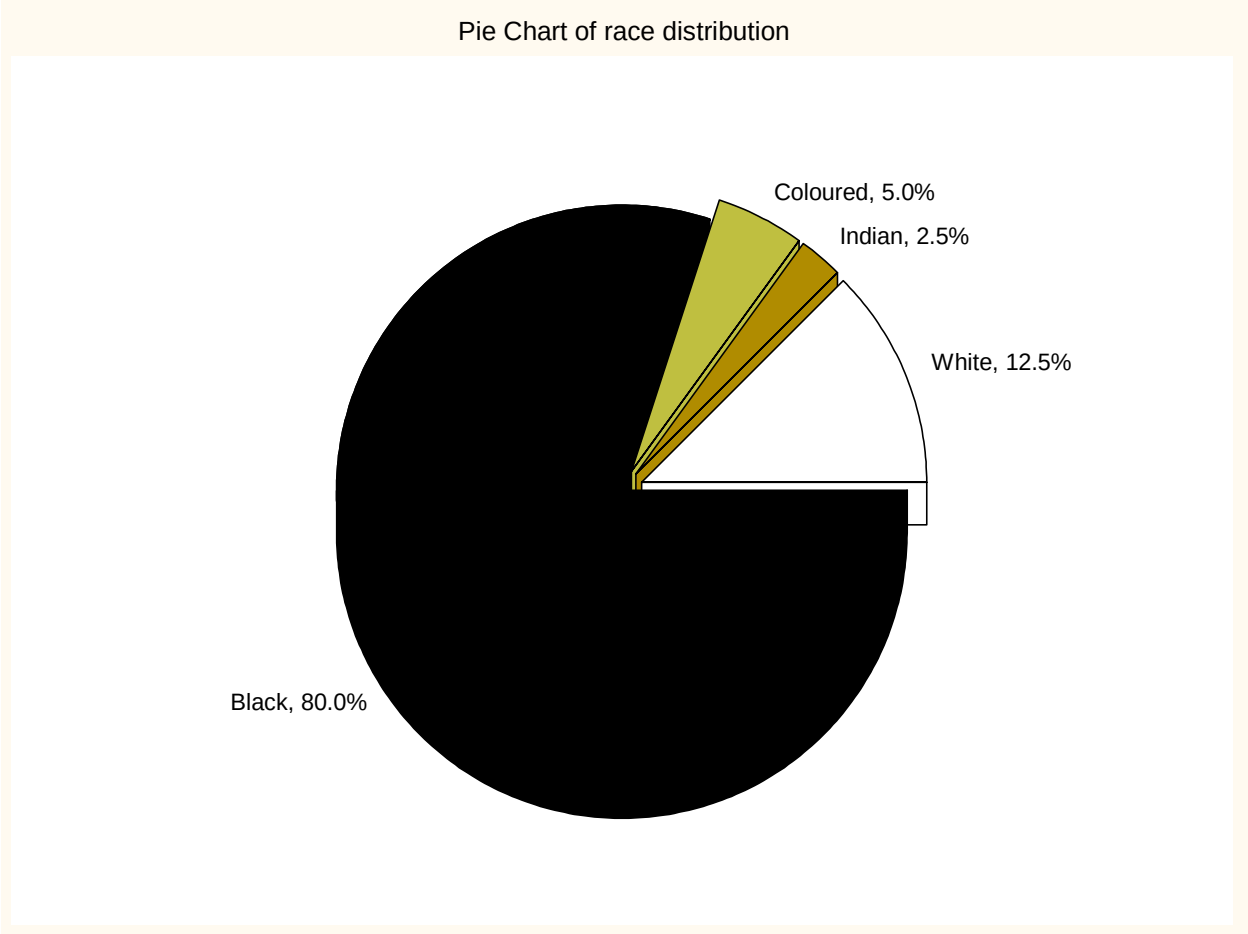


Figure 4. Pie chart showing the population group distribution of the study group.

The mean APACHE II score was 10.7, with a minimum score of 2 and a maximum score of 28 (s*ee figure 5*).

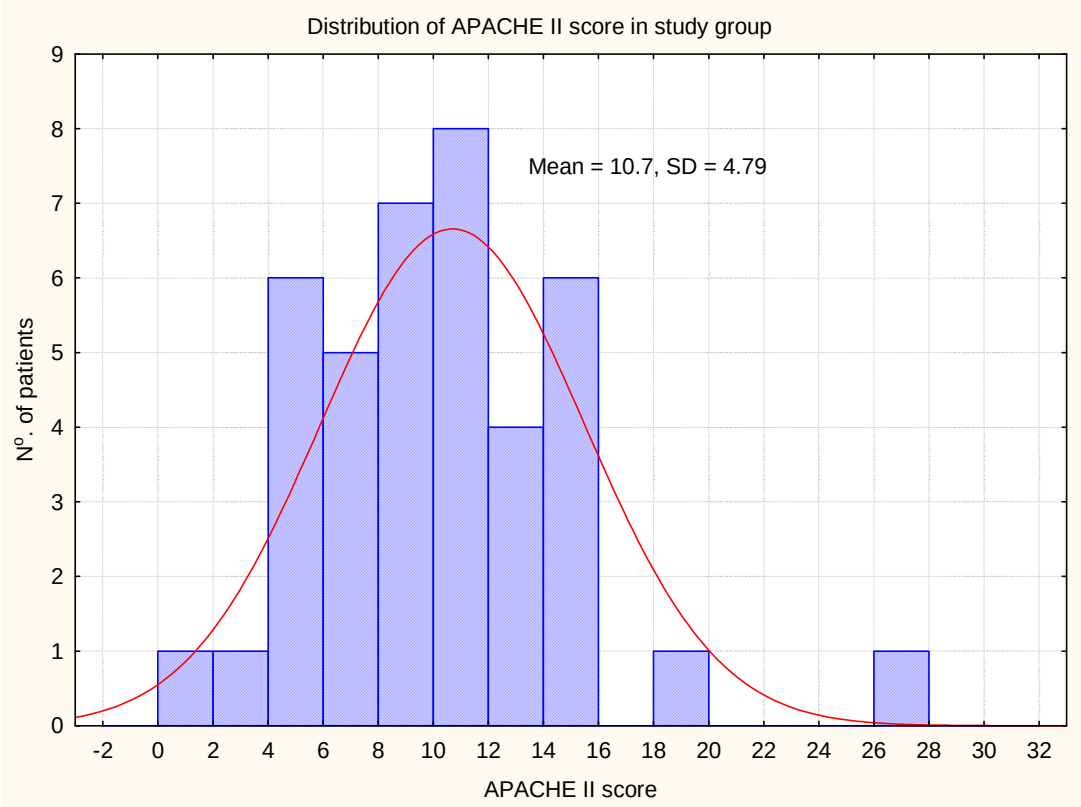


Figure 5. Histogram showing the distribution of APACHE II scores in the study group.

The body mass index (BMI) was calculated on admission .The mean calculated BMI of the study group was 24.5 kg/m^2, with a minimum of 14.6 kg/m^2 and a maximum of 35 kg/m^2 (*see figure 6*). The BMI was used to assess the degree of adiposity in the study group. Within the study group, 14 patients (35%) would be classified as being overweight (BMI >25 kg/m^2), and 5 patients (12.5%) would be classified as obese (BMI >30 kg/m^2) according to the World Health Organization (WHO) definitions (WHO expert committee, 1995). The remainder had BMI's <25 kg/m^2.

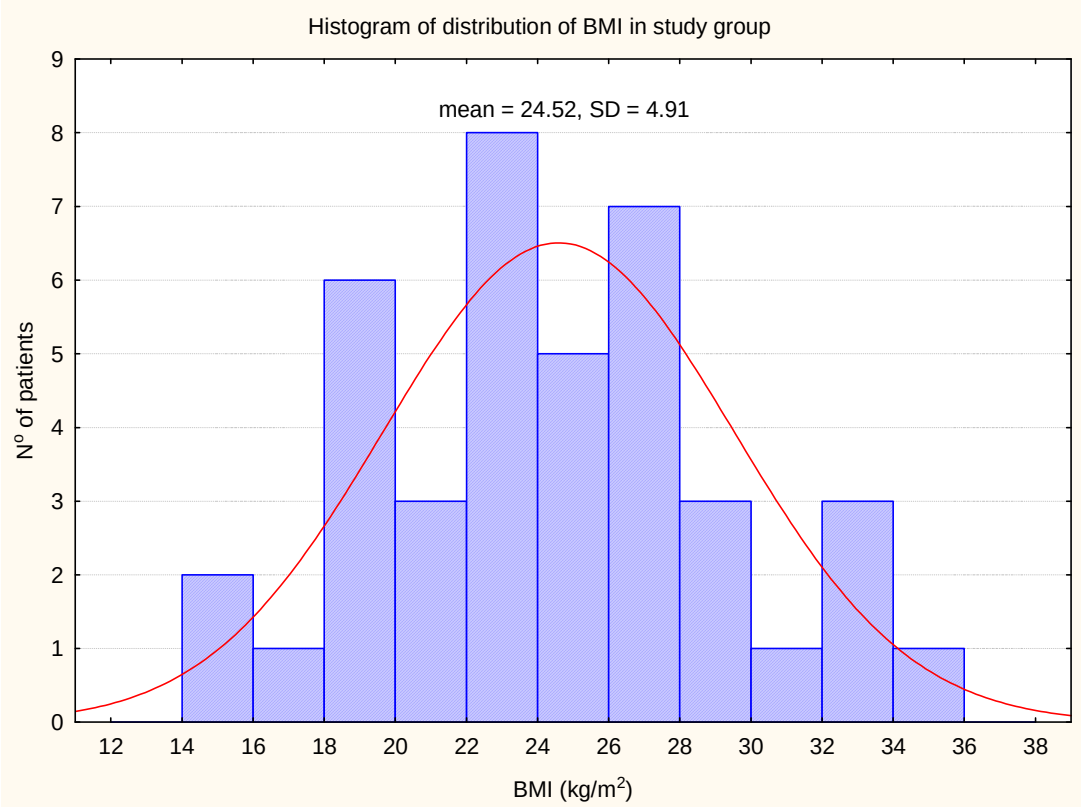


Figure 6. Histogram showing the distribution of BMI in the study group.

The median duration of stay in the ICU was 6 days, with a minimum stay of 1 day, and a maximum of 43 days.

The duration of stay in ICU (measured in days) correlated positively with the severity of illness, as measured by the APACHE II score (r = 0.44, p = 0.004, Spearman rank order) (*see figure 7*). There was no correlation between the severity of illness as measured by the APACHE II score and mean blood glucose, amount of administered insulin, TNFα, IL-6 or adiponectin levels.

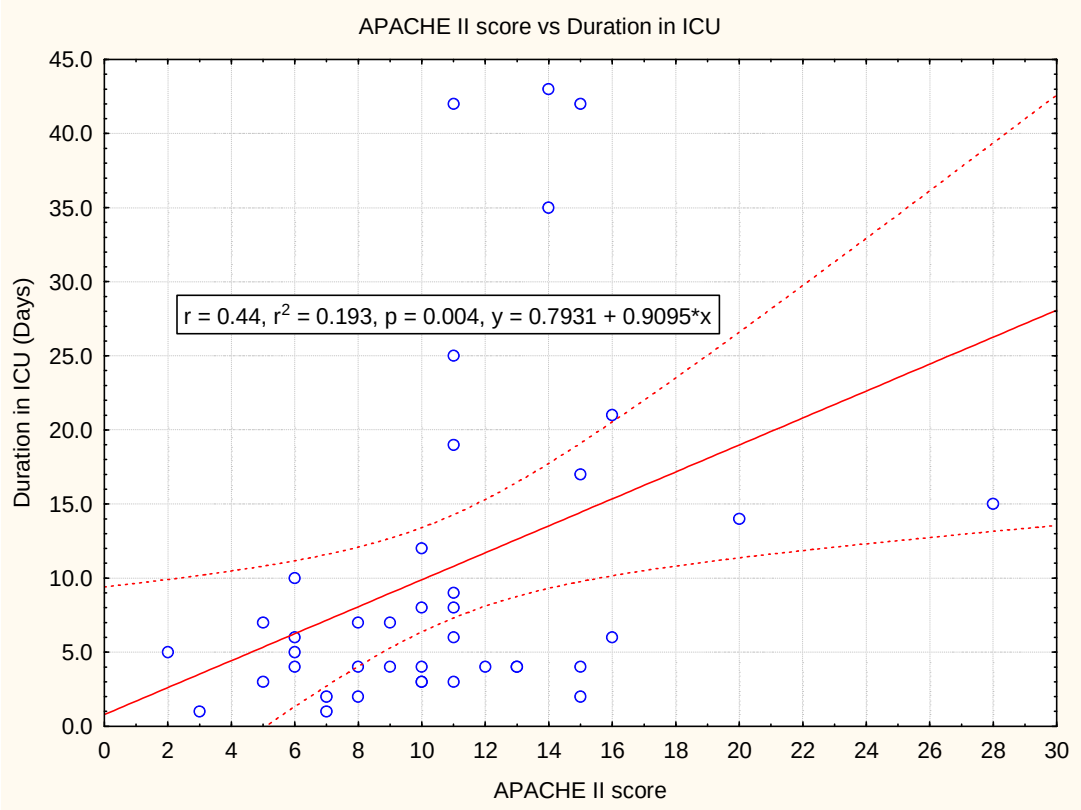


Figure 7. Graph showing the relationship of severity of illness as measured by the APACHE II score, and the duration of stay in ICU. Dotted lines indicate 95% confidence intervals.

4.2 Evidence of insulin resistance in the study population

The mean blood glucose concentrations (mmol/l) on admission, day 3, day 7 and discharge did not show a statistically significant difference from one another (s*ee table 2*). There was however a difference in the requirements for administered insulin over the 24 hour period to maintain normoglycaemia (s*ee table 2*).

*Table 2. Mean plasma glucose, administered insulin, administered insulin: mean glucose ratio and C-peptide concentrations on admission, day 3, day 7, discharge >7 days and all discharges from ICU. Data expressed as median [interquartile range]; #n numbers for C-peptide are 22 at day 3, 14 at day 7 and 35 for all discharges; *p <0.05 versus admission value (Mann-Whitney U test). Statistical analyses also carried out using Wilcoxon matched pairs test and this data is given in the text. Reference ranges given from the package inserts of the analytical methods used by the laboratory where the assays were performed.*

Variable	Admission (n = 40)	Day 3 (n = 25)	Day 7 (n = 16)	Discharge >7 days (n = 15)	All discharges (n = 38)	Reference ranges
Glucose (mmol/l)	6.13 [1.87]	6.20 [1.03]	6.70 [1.27]	6.80 [1.77]	5.88 [1.46]	4.1 – 5.9 (fasting) <11.0 (random)
Insulin administered (IU)	8.00 [23.0]	24.0 [38.0]	18.0 [47.0]	0.00 [44.0]	0.00 [32.0]	
Insulin: glucose ratio	1.00 [3.20]	4.21 [5.95]*	3.20 [7.23]*	0.00 [5.63]	0.00 [4.00]	
C-peptide (µg/l)#	1.88 [2.35]	2.10 [2.00]	2.80 [1.50]	3.30 [2.60]	2.50 [3.00]	1.1 – 5.0

Glucose levels did not vary significantly during stay in ICU, however the levels of administered insulin did rise to a peak on day 3 and fell to 0 at discharge (*see table 2*). However, when using the Mann-Whitney U test statistically significant differences in levels of administered insulin were not observed but the level on day 7 approached significance ($p = 0.08$) when compared to the admission level. When using the Wilcoxon matched pairs test, significant differences were noted for administered insulin. Thus, the admission level (8 [20] IU) was significantly lower than the level on day 3 (24 [38] IU; $p = 0.02$, $n = 25$) and the admission level (3 [10] IU) was also lower than that on day 7 (18 [47]; $p = 0.02$, $n = 16$). Furthermore the level of insulin administered on day 7 (20 [52] IU) was greater than that seen at discharge after >7 days (0 [44]; $p = 0.04$, $n = 13$).

To attempt to correct for the small variations in mean plasma glucose concentrations on the different days of ICU admission, a ratio of the total amount of administered insulin in a 24 hour period to the mean plasma glucose over that same 24 hour period (calculated as the mean of all glucose measurements taken at 2 to 4 hourly intervals) was calculated. This ratio was used as an indication of insulin sensitivity.

The insulin: glucose ratio also varied during stay in ICU, following a similar pattern to that observed for administered insulin (*see table 2*). Thus, levels were higher on days 3 and 7 compared to admission ($p < 0.05$ for both comparisons using Mann-Whitney U test; table 2) and there was a tendency for the day 7 level to be higher than that seen at discharge after >7 days ($p = 0.08$ by Mann-Whitney U test). The Wilcoxon matched pairs test also gave similar results with both day 3 ($p = 0.003$) and day 7 ($p = 0.013$) levels greater than admission levels, and the day 3 level (5.31 [3.57]) was higher than that observed at discharge from ICU after >7 days (0.0 [5.63]; $p = 0.06$, $n = 15$). The ratio on day 7 (3.49 [7.05]) was also significantly higher than that seen at discharge after >7 days (0.0 [5.63]; $p = 0.03$, $n = 13$).

When the administered insulin: mean glucose ratio was compared with the amount of administered insulin per day, the correlations were found to be very good (r = 0.94) (s*ee figure 8*). It was thus decided that using the calculated ratio did not offer significant benefit over using the amount of administered insulin as a measure of insulin resistance, and the ratio was not used in further statistical analysis.

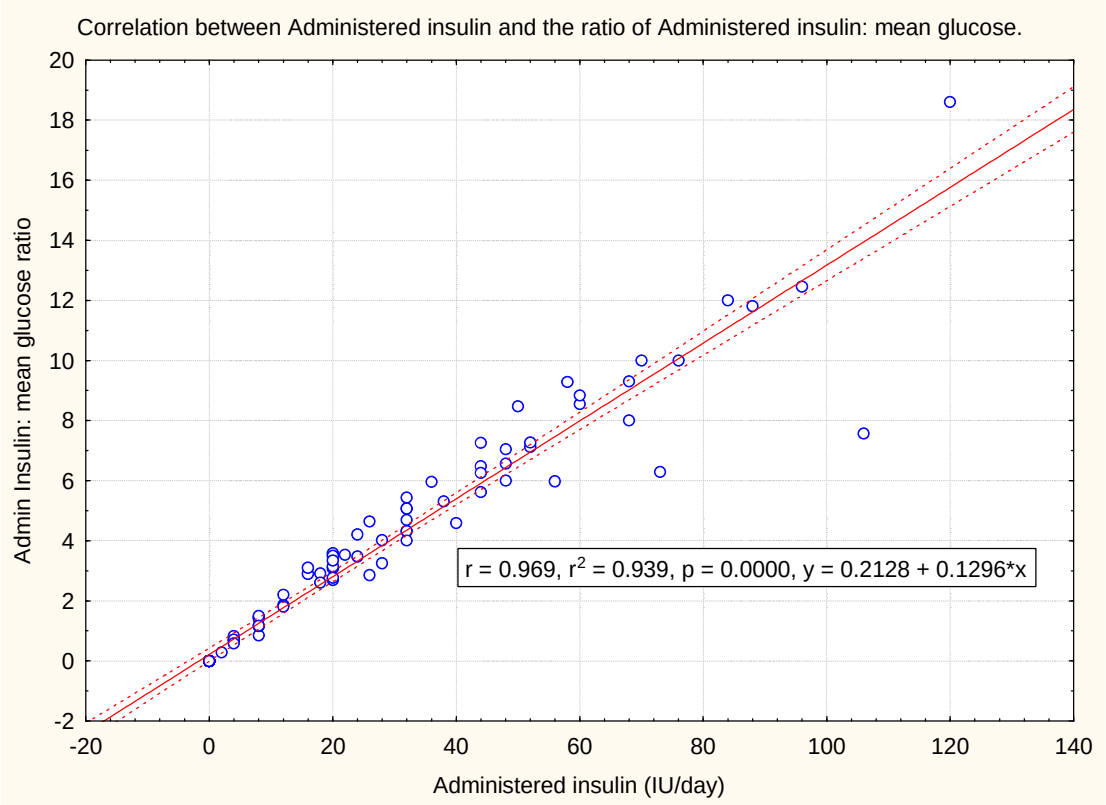


Figure 8. Scatter plot showing the correlation between the amount of administered insulin per day and the ratio of the administered insulin: mean 24hr glucose. Dotted lines indicate 95% confidence interval.

In the study population, 33 (82.5%) of the patients required insulin infusions during their ICU admission to maintain normoglycaemia, with only 7 (17.5%) of the patients being able to maintain normoglycaemia without the aid of insulin infusions.

The administered insulin on admission correlated very strongly with mean plasma glucose ($r = 0.79$, $p < 0.00001$) using Spearman rank order correlations, and weakly with BMI ($r = 0.31$, $p = 0.053$) and gender ($r = 0.32$, $p = 0.045$). Multiple regression analysis with administered insulin as the dependent and glucose, BMI and gender as the independent variables gave $r = 0.83$, $p < 0.0001$, with glucose as the major determinant ($\beta = 0.78$, $p < 0.0001$) with a small input from gender ($\beta = 0.20$, $p = 0.055$) and a minimal input from BMI ($\beta = -0.01$, $p = 0.88$).

The C-peptide levels did not differ significantly during stay in ICU (*see table 2*). Serum insulin level was measured on admission before insulin administration and the median value was 7.2 [9.0] mIU/l.

Measured insulin concentrations on admission showed statistically significant correlations (using Spearman rank order correlations) with mean plasma glucose ($r = 0.41$, $p = 0.009$) and C-peptide ($r = 0.45$, $p = 0.004$) on admission. Multiple regression analysis with measured insulin as dependent and glucose and C-peptide as independent variables gave $r = 0.48$, $p = 0.008$ with glucose ($\beta = 0.29$, $p = 0.06$) and C-peptide ($\beta = 0.32$, $p = 0.04$) contributing to a similar extent. The mean plasma glucose levels on admission correlated with BMI ($r = 0.38$, $p = 0.013$), serum cortisol levels ($r = 0.41$, $p = 0.008$), and C-peptide levels ($r = 0.34$, $p = 0.03$). Multiple regression analysis with glucose as dependent and BMI, cortisol, C-peptide and measured insulin as independent variables gave $r = 0.54$, $p = 0.016$ with BMI ($\beta = 0.30$, $p = 0.06$) and cortisol ($\beta = 0.30$, $p = 0.07$) as the main determinants, and insulin ($\beta = 0.25$, $p = 0.13$) and C-peptide ($\beta = -0.08$, $p = 0.62$) as insignificant role players.

C-peptide levels on admission also correlated positively with admission levels of triglyceride ($r = 0.41$, $p = 0.007$,) and cortisol ($r = 0.47$, $p = 0.001$), and weakly with BMI ($r = 0.29$, $p = 0.06$). Multiple regression analysis with C-peptide as dependent and triglyceride, cortisol, BMI, glucose and measured insulin as independent variables gave $r = 0.63$, $p = 0.003$ with triglyceride ($\beta = 0.34$, $p =$

0.031) and insulin (β = 0.33, p = 0.03) as the principal determinants and cortisol (β = 0.21, p = 0.20), BMI (β = 0.12, p = 0.43) and glucose (β = -0.06, p = 0.72) as non-significant contributors.

4.3 Cytokines and their relationships with insulin resistance and CRP

*Table 3. Cytokine and CRP concentrations on admission, day 3, day 7, discharge >7 days and all discharges from ICU. Data expressed as median [interquartile range].*p < 0.05, **p <0.005, ***p < 0.0001 vs admission levels (Student's non-paired T-test). CRP reference range given from the package insert of the analytical method used by the laboratory where the assays were performed. Cytokine reference ranges were not supplied with the kit, and were taken from published reference ranges (Adiponectin: Schalkwijk et al., 2006. TNFα: Elias et al., 2004. IL-6: Song & Sung, 2006).*

Variable	Admission (n = 40)	Day 3 (n = 23)	Day 7 (n = 14)	Discharge >7 days (n = 14)	All discharges (n = 38)	Reference ranges
Adiponectin (ng/ml)	4413.8 [3519.0]	6585.4 [6201.6]	10774.3 [8490.5]**	11585.3 [755.3]***	8504.6 [7137.2]**	3900 - 10200
TNFα (pg/ml)	3.25 [3.42]	4.31 [4.90]	5.13 [3.63]	4.23 [5.03]	4.00 [3.34]	1.20 – 15.30
IL-6 (pg/ml)	337.0 [788.8]	127.0 [160.4]*	32.2 [236.0]**	47.8 [143.9]**	46.9 [222.5]***	<1.4
CRP (µg/l)	101.5 [253.8]	231.8 [177.9]	83.2 [82.2]	121.3 [81.2]	137.0 [151.8]	<10

Table 4. Spearman rank order correlations of the cytokines with administered insulin on admission, day 3, day 7, discharge >7 days and all discharges.

	Valid N	r	p
TNFα			
Admission	40	0.004	0.98
Day 3	23	-0.057	0.80
Day 7	14	-0.15	0.59
Discharge >7 days	13	-0.12	0.67
All discharges	37	0.086	0.62
Adiponectin			
Admission	40	-0.007	0.96
Day 3	23	-0.13	0.55
Day 7	14	-0.42	0.12
Discharge >7 days	13	-0.66	**0.01**
All discharges	37	-0.457	**0.0049**
IL-6			
Admission	40	-0.17	0.29
Day 3	23	0.25	0.27
Day 7	14	-0.109	0.70
Discharge >7 days	13	0.41	0.13
All discharges	37	0.26	0.12

4.3.1 Tumour necrosis factor α

TNFα reached peak concentration on day 7 (5.13pg/ml) and then started to decrease in concentration, however the concentrations of TNFα did not show a statistically significant change during the period in ICU.

When comparing TNFα concentrations to the amount of administered insulin required to maintain normoglycaemia, no statistically significant correlation was found (s*ee table 4*).

4.3.2 Adiponectin

Adiponectin levels increased from the time of admission and peaked at discharge >7 days.

Thus, using both Student's non-paired (*see table 3*) and paired T-tests, adiponectin levels on day 7 ($p = 0.004$ and $p < 0.0001$ respectively) on discharge >7 days ($p < 0.0001$ and $p = 0.0002$ respectively) and on all discharge dates ($p = 0.003$ and $p < 0.0001$ respectively) were higher than admission levels.

Adiponectin concentrations showed a statistically significant negative correlation with the amount of insulin administered per day to maintain normoglycaemia on discharge from the ICU ($r = -0.457$, $p = 0.0049$).

4.3.3 Interleukin-6

IL-6 levels showed statistically significant changes during the stay in ICU, with peak levels being seen on the day of admission, and declining thereafter. Thus using both Student's non-paired (s*ee table 3*) and paired T-tests admission levels were higher than those on day 3 ($p = 0.02$ and $p = 0.02$ respectively), day 7 ($p = 0.0006$ and $p < 0.0001$ respectively), discharge >7 days ($p = 0.002$ and $p = 0.006$ respectively) and all discharge days ($p < 0.0001$ and $p = <0.0001$ respectively).

When comparing IL-6 levels with the amount of administered insulin required to maintain normoglycaemia, no significant correlation could be demonstrated (s*ee table 4*).

4.3.4 C-reactive protein

CRP levels showed statistically significant changes during the stay in ICU, with peak levels being seen on day 3 of admission, and declining thereafter to its lowest levels at discharge >7 days. Using both Student's non-paired and paired T-tests admission levels were lower than those on day 3 ($p = 0.03$ and $p = 0.059$ respectively), day 3 levels were higher than those on day 7 ($p = 0.018$ and $p =$

0.0005 respectively), and levels on day 3 were higher than those at discharge >7 days ($p = 0.017$ and $p = 0.0008$ respectively).

IL-6 levels were positively correlated with CRP levels on day 3 ($r = 0.48$, $p = 0.024$), day 7 ($r = 0.83$, $p = 0.0003$), discharge >7 days ($r = 0.80$, $p = 0.0003$) and all discharge data ($r = 0.65$, $p < 0.0001$). Although there was an associated decrease in the levels of the inflammatory markers IL-6 and CRP with the increase in adiponectin, these correlations did not reach statistical significance.

4.4 The relationships of the measured cytokines to one another

When comparing the cytokines chronologically, adiponectin reached peak concentration on discharge >7 days whilst TNFα reached a peak on day 7. There was an inverse relationship of TNFα with IL-6 levels, but this did not reach statistical significance (s*ee figure 9*).

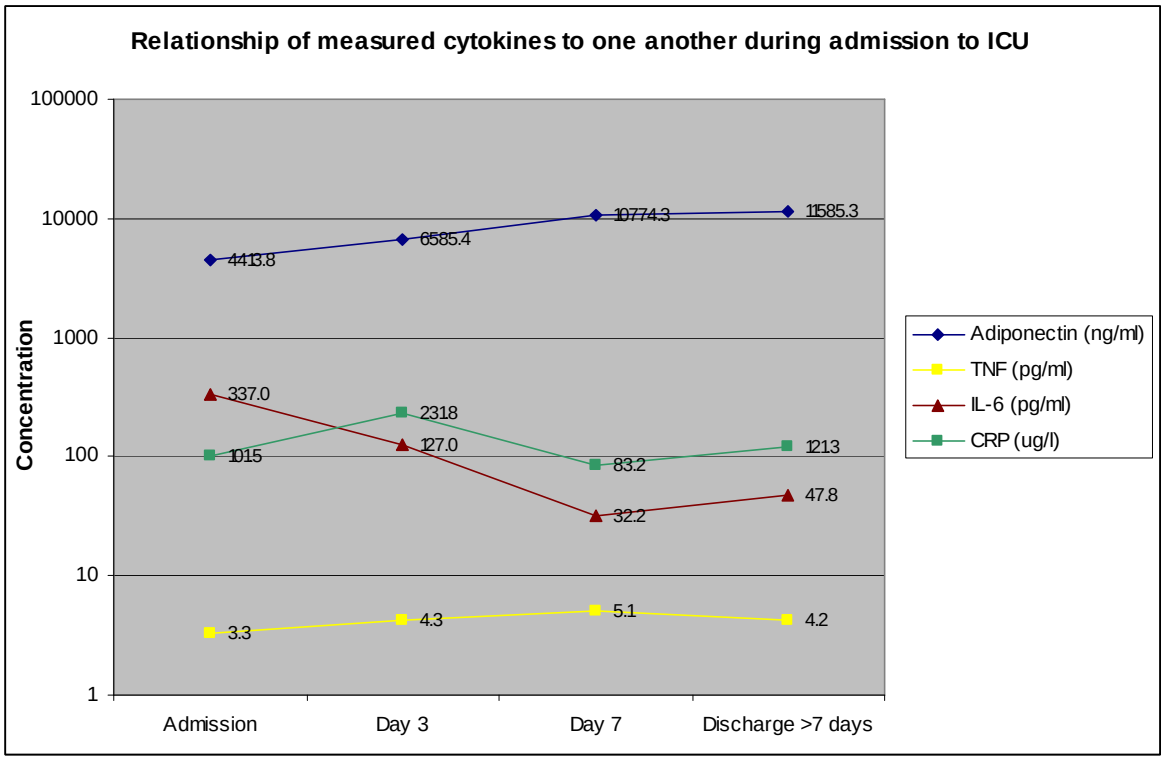


Figure 9. Graph showing the relationships of adiponectin, IL-6, TNFα and CRP over the duration of stay in ICU from admission until discharge. Median values plotted. Note logarithmic scale used for concentration.

4.5 Other variables associated with insulin resistance

We also looked at different parameters known to be useful in determining insulin sensitivity, namely serum triglyceride levels; or to influence insulin sensitivity, namely the counter-regulatory hormone cortisol.

*Table 5. Triglyceride and cortisol concentrations on admission, day 3, day 7 and discharge >7 days and all discharges from ICU. Data expressed as median [interquartile range]. *p < 0.05, **p < 0.0001 vs admission (Student's non-paired T-test). Reference ranges given from the package inserts of the analytical methods used by the laboratory where the assays were performed.*

Variable	Admission (n = 40)	Day 3 (n = 23)	Day 7 (n = 11)	Discharge >7 days (n = 15)	All discharges (n = 36)	Reference ranges
Triglycerides (mmol/l)	0.70 [0.75]*	1.10 [0.30]*	1.00 [0.30]*	1.30 [0.80]	1.30 [0.50]**	0.5 – 1.5
Cortisol (nmol/l)	439 [311]	415 [525]	375 [288]	366 [217]	373 [303]	160 - 620

4.5.1 Triglycerides

Serum triglyceride levels showed a trend of increasing gradually from admission until discharge, with lowest levels being found on admission (s*ee figure 10*). Thus, using both non-paired (*see table 5*) and paired Student's t-tests admission levels were lower than those on day 3 ($p = 0.006$ and $p = 0.006$ respectively), day 7 ($p = 0.006$ and $p = 0.001$ respectively), discharge >7 days ($p = 0.005$ and $p = 0.009$ respectively) and all discharge dates ($p < 0.0001$ for both).

Neither adiponectin nor TNFα showed any correlation with serum triglyceride levels, and the correlation of IL-6 with triglycerides was only significant on admission (n = 40, r = -0.36, p = 0.022). The percentage change in adiponectin however correlated well with the percentage change in triglycerides (n = 37, r = 0.378, p = 0.025) (*see figure 10*). The percentage change of IL-6 and triglycerides from admission to discharge was not significant.

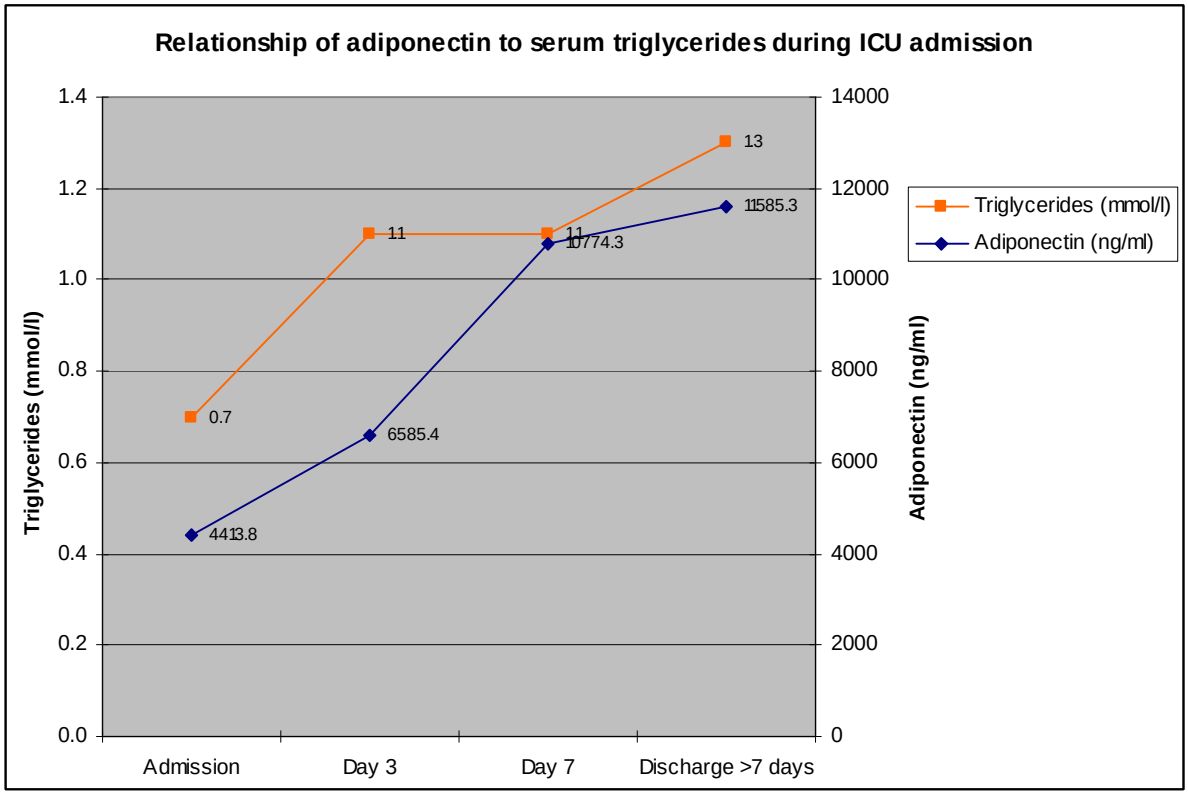


Figure 10. Graph showing the change in adiponectin and serum triglyceride concentrations during the ICU admission. Median values plotted.

4.5.2 Cortisol

Serum cortisol concentrations showed a trend of decreasing from their highest levels seen on admission until discharge, but this was not statistically significant (*see table 5*). Serum cortisol concentrations did not show statistically significant correlations with the amount of administered insulin.

There was no correlation between serum cortisol levels and adiponectin or TNFα, but there were statistically significant correlations of cortisol with IL-6 on admission ($r = -0.32$, $p = 0.04$) and discharge ($r = -0.35$, $p = 0.038$), using Spearman rank order correlations. Serum cortisol levels also showed a positive correlation with serum triglycerides on admission ($r = 0.43$, $p = 0.001$), but not thereafter.

4.6 Gender differences

There were statistically significant gender differences in a number of variables measured on admission: BMI, administered insulin, cortisol and C-peptide concentrations (s*ee table 6*).

Females had a higher mean BMI of 27.14kg/m^2 when compared with the mean BMI of 23.26kg/m^2 of males ($p = 0.017$). Females also had higher serum cortisol concentrations than males ($p = 0.035$).

There were differences in baseline adiponectin levels between the males and females of the study group, with females having higher levels than males, but this did not reach statistical significance ($p = 0.09$).

The confounding effects of BMI were analysed using an analysis of covariance (ANCOVA), where BMI was included as a co-variate. The only values that showed a significant change were C-peptide

(p went from 0.04 to 0.14) and administered insulin (p went from 0.04 to 0.18). Adjusting for BMI when analyzing adiponectin concentrations in males and females using ANCOVA with BMI as a co-variate showed that the p value changed to 0.02.

Table 6. Gender differences in variables on admission in the study population, with p values given for males versus females using Student's t-test for data with or data that could be transformed to a Gaussian distribution, and Mann-Whitney U-test for data that could not be transformed to a Gaussian distribution. Data given as mean ± standard deviation for normally distributed variables, and as median [interquartile range] for data with a non-Gaussian distribution.

Variable on admission	**Male (n = 27)**	**Female (n = 13)**	**p value male vs female**
Age (y)	38.25 ± 11.26	37.30 ± 15.27	0.82
APACHE II	11 [7]	10 [6]	0.88
BMI (kg/m^2)	23.26 ± 4.27	27.14 ± 5.26	**0.017**
Mean glucose (mmol/l)	6.05 [1.69]	6.96 [2.86]	0.29
Administered Insulin (IU)	4 [18]	20 [36]	**0.041**
TNFα (pg/ml)	3.38 [3.30]	4.52 [4.19]	0.69
Adiponectin (ng/ml)	4068 [2834]	6850 [4785]	0.09
IL-6 (pg/ml)	286 [516.6]	140 [571]	0.24
CRP (mg/l)	95 [238.5]	201.6 [264.3]	0.81
Cortisol (nmol/l)	413 [184]	661 [273]	**0.035**
C-peptide (µg/l)	1.6 [2.1]	2.7 [0.5]	**0.042**
Triglycerides (mmol/l)	0.6 [0.8]	0.9 [0.7]	0.23
Measured insulin (mU/l)	6.9 [4.6]	14.6 [14.5]	0.11

At discharge, no gender differences were noted for any of the metabolic variables (s*ee table 7*).

Table 7. Gender differences in variables on discharge from the ICU in the study population, with p values given for males versus females using Student's t-test for data with or data that could be transformed to a Gaussian distribution, and Mann-Whitney U-test for data that could not be transformed to a Gaussian distribution. Data given as mean ± standard deviation for normally distributed variables, and as median [interquartile range] for data with a non-Gaussian distribution.

Variable on discharge	**Male (n = 27)**	**Female (n = 13)**	**p value male vs female**
Mean glucose (mmol/l)	6.01 [1.54]	5.82 [1.09]	0.37
Administered Insulin (IU)	4 [44]	0 [8]	0.09
TNFα (pg/ml)	4.07 [3.34]	4.34 [4.72]	0.75
Adiponectin (ng/ml)	8115 [6486]	8504 [12773]	0.21
IL-6 (pg/ml)	46.95 [326.8]	38.32 [141.9]	0.76
CRP (mg/l)	143.5 [202.5]	132.0 [106.1]	0.85
Cortisol (nmol/l)	381 [319]	316 [334]	0.41
C-peptide (µg/l)	3.15 [3.2]	1.7[0.9]	0.94
Triglycerides (mmol/l)	1.35 [0.7]	1.2 [0.3]	0.87
Length of stay in ICU	7 [11]	4 [6]	0.07
%change in glucose	4.37 [19.07]	-9.4 [35.04]	0.19
% change in admin insulin	68.75 [512.5]	-100 [183.5]	0.09
%change in TNFα	11.2 [87.6]	22.7 [55.1]	0.33
%change in Adiponectin	63.8 [166.6]	18.4 [125.4]	0.46
%change in IL-6	-84.3 [165.5]	-76.9 [137.0]	0.81
%change in CRP	19.1 [549.7]	-34.3 [1782.3]	0.19
%change in Triglycerides	100 [81.3]	37.5 [142.3]	0.35
%change in cortisol	7.12 [54.9]	-54.3 [63.9]	0.63
%change in C-peptide	42.8 [253.3]	-21.4 [45.6]	0.09

Using multiple regression analysis with gender and BMI as independent variables, the mean plasma glucose levels on admission were found to correlate with BMI independent of gender ($p = 0.03$, $r^2 = 0.14$). When examining the determinants of administered insulin using gender, BMI and mean glucose on admission as independent variables, only mean plasma glucose was found to be a determinant of the amount of insulin administered ($p < 0.00001$, $r^2 = 0.69$).

4.7 Racial differences in some variables

When examining whether there were any racial differences in the variables between the different population groups, IL-6 and CRP concentrations on discharge were found to show statistical significance using Student's t-test. Whites had higher IL-6 (p = 0.002) and CRP (p = 0.018) levels than blacks. The percentage change in adiponectin levels from admission to discharge was also found to be different between whites and blacks (p = 0.01) (s*ee table 9*). No racial differences were found for variables measured on admission (*see table 8*).

There were no differences found between the other racial groups of the study because of the low patient numbers (coloured 2, Indian 1).

Table 8. Racial differences in variables on admission in the study population, with p values given for blacks versus whites using Student's t-test for data with or data that could be transformed to a Gaussian distribution, and Mann-Whitney U-test for data that could not be transformed to a Gaussian distribution. Data given as mean ± standard deviation for normally distributed variables, and as median [interquartile range] for data with a non-Gaussian distribution.

Variable on admission	Black (n = 29)	White (n = 5)	p value black vs white
Age (y)	37.3 ± 12.5	38.2 ± 12.9	0.88
APACHE II	10 [5]	12 [3]	0.52
BMI (kg/m^2)	24.2 ± 5.19	27.1 ± 1.41	0.21
Mean glucose (mmol/l)	6.08 [1.91]	6.48 [0.93]	0.76
Administered Insulin (IU/day)	6 [24]	18 [12]	0.82
TNFα (pg/ml)	3.83 [3.37]	3.36 [0.16]	0.42
Adiponectin (ng/ml)	5216 [3537]	4068 [345]	0.94
IL-6 (pg/ml)	337.1 [721.2]	345.9 [849.0]	0.29
CRP (mg/l)	94.6 [254.4]	268.6 [98.1]	0.15
Cortisol (nmol/l)	439 [340]	443 [184]	0.49
C-peptide (µg/l)	2.03 [2.25]	1.6 [0.3]	0.69
Triglycerides (mmol/l)	0.65 [0.65]	1.5 [0.9]	0.22
Measured insulin (mU/l)	7.4 [8.0]	5.8 [2.0]	0.93

Table 9. Racial differences in variables on discharge from the ICU in the study population, with p values given for blacks versus whites using Student's t-test for data with or data that could be transformed to a Gaussian distribution, and Mann-Whitney U-test for data that could not be transformed to a Gaussian distribution. Data given as median [interquartile range].

Variable on discharge	**Black (n = 29)**	**White (n = 5)**	**p value black vs white**
Mean glucose (mmol/l)	5.87 [1.45]	6.78 [1.2]	0.60
Administered Insulin (IU/day)	0 [20]	8 [60]	0.32
TNFα (pg/ml)	4.22 [3.43]	4.07 [7.29]	0.15
Adiponectin (ng/ml)	8541 [7137]	5283 [2557]	0.72
IL-6 (pg/ml)	33.1 [66.6]	356.1 [549.6]	**0.002**
CRP (mg/l)	132.3 [133.9]	301.1 [31.6]	**0.018**
Cortisol (nmol/l)	373 [337]	289 [98]	0.62
C-peptide (µg/l)	2.4 [2.2]	2.7 [2.5]	0.67
Triglycerides (mmol/l)	1.2 [0.4]	1.4 [0.6]	0.55
Length of stay in ICU (days)	5 [8]	6 [15]	0.20
%change in glucose	-3.08 [16.2]	7.53 [2.92]	0.99
%change in admin insulin	-81.1 [220]	86.6 [621.6]	0.82
%change in TNFα	11.2 [65.6]	26.2 [136.5]	0.25
%change in Adiponectin	76.7 [129.3]	18.7 [17.0]	**0.01**
%change in IL-6	-89.0 [167.6]	-68.8 [186.7]	0.34
%change in CRP	-6.35 [1399.7]	12.1 [17.1]	0.08
%change in Triglycerides	85.7 [137.8]	18.75 [120]	0.71
%change in cortisol	-6.1 [78.9]	-2.6 [26.9]	0.08
%change in C-peptide	-2.7 [211]	42.8 [92.8]	0.55

4.8 The associations of metabolic variables with outcome

There were no significant differences in demographic or metabolic data on admission between survivors and non-survivors (s*ee table 10*).

Table 10. Comparison of admission data between ICU survivors and non-survivors. P values given for survivors vs non-survivors using Student's t-test for data with or data that could be transformed to a Gaussian distribution, and Mann-Whitney U-test for data that could not be transformed to a Gaussian distribution. Data given as mean ± standard deviation for normally distributed variables, and as median [interquartile range] for data with a non-Gaussian distribution.

Variable on admission	**Survivors (n = 36)**	**Non-survivors (n = 4)**	**p value survivors vs non-survivors**
Age (y)	36.7 ± 12.3	49.0 ± 9.0	0.06
APACHE II	10 [5.5]	13 [6.5]	0.65
BMI (kg/m^2)	24.6 ± 5.05	23.1 ± 3.48	0.54
Mean glucose (mmol/l)	6.13 [2.17]	6.09 [0.82]	0.69
Administered Insulin (IU/day)	6 [23]	13 [19]	0.95
TNFα (pg/ml)	3.39 [3.40]	4.55 [11.75]	0.94
Adiponectin (ng/ml)	4413 [3519]	5353 [4134]	0.10
IL-6 (pg/ml)	307.5 [775.9]	810.2 [4944.4]	0.09
CRP (mg/l)	101.5 [251.6]	173.6 [233.3]	0.81
Cortisol (nmol/l)	439 [348]	448 [235]	0.49
C-peptide (µg/l)	2.03 [2.3]	1.1 [3.5]	0.48
Triglycerides (mmol/l)	0.7 [0.6]	0.95 [1.4]	0.80
Measured insulin (mU/l)	7.2 [8.9]	9.3 [25.1]	0.48

Table 11. Comparison of discharge data between ICU survivors and non-survivors. Data expressed as median [interquartile range]. P values given for survivors vs non-survivors using Student's t-test for data with or data that could be transformed to a Gaussian distribution, and Mann-Whitney U-test for data that could not be transformed to a Gaussian distribution .

Variable on discharge	Survivors (n = 36)	Non-survivors (n = 4)	p value survivors vs non-survivors
Mean glucose (mmol/l)	5.78 [1.46]	6.79 [[1.0]	0.32
Administered Insulin (IU/day)	0 [20]	34 [46]	0.08
TNFα (pg/ml)	4.0 [3.4]	7.85 [157.8]	**0.005**
Adiponectin (ng/ml)	8597 [7280]	4483 [4515]	0.10
IL-6 (pg/ml)	35.2 [70.9]	1056.4 [307.2]	**0.001**
CRP (mg/l)	132.7 [151.8]	178.0 [172.9]	0.93
Cortisol (nmol/l)	373 [279]	481 [353]	0.84
C-peptide (µg/l)	2.5 [3.0]	2.15 [3.55]	0.86
Triglycerides (mmol/l)	1.3 [0.7]	1.05 [0.65]	**0.009**
Length of stay in ICU (days)	5.5 [7.0]	11.5 [25.0]	0.29
%change in glucose	-3.08 [22.43]	3.7 [18.5]	0.47
%change admin insulin	-82.3 [220]	100 [319.0]	0.42
%change in TNFα	11.2 [65.6]	71.0 [4734.8]	**0.05**
%change in Adiponectin	70.4 [166.3]	9.9 [2.3]	**0.02**
%change in IL-6	-83.1 [26.4]	158.9 [174.3]	**0.01**
%change in CRP	-6.35 [750.3]	19.9 [74.2]	0.08
%change in Triglycerides	100 [135]	-10 [90.9]	**0.01**
%change in cortisol	-6.1 [63.3]	43.7 [104.2]	0.40
%change in C-peptide	0 [208.4]	96.4 [381.0]	0.15

Four patients in this cohort died and they had higher median IL-6 (1056 vs 35.2pg/ml, p = 0.001) and TNFα (7.85 vs 4.0pg/ml, p = 0.005) levels than survivors on discharge (Student's t-test). The serum triglyceride concentrations on discharge were also significantly lower in the non-survivors than in the survivors (1.05 vs 1.3mmol/l, p = 0.009).

When comparing survivors and non-survivors, the percentage change in TNFα (11.2% vs 71.0%, p = 0.05), adiponectin (70.4% vs 9.9%, p = 0.02), IL-6 (-83.1% vs 158.9%, p = 0.01) and triglycerides (100% vs -10%, p = 0.01) was also statistically significantly different.

5. Discussion

Significant morbidity and mortality is seen in patients requiring intensive care treatment (Takala et al., 1999). “Stress hyperglycaemia”, which is common in ICU patients even if they are not diabetic (Van den Berghe et al., 2001), is multifactorial in origin and is due to a combination of glucose containing intravenous infusions, total parenteral nutrition, neural signaling, counterregulatory hormones (catecholamines, cortisol, glucagon and GH) (Hirsch, 2002), increased insulin clearance rates (Hoshino et al., 2003), and resistance to the effects of insulin possibly induced by interference in intracellular insulin signaling pathways by proinflammatory cytokines such as TNFα (Ruan & Lodish, 2003) and IL-6 (Rotter et al., 2003). Prevention of hyperglycaemia has been shown to significantly reduce the mortality and morbidity associated with ICU management (Van den Berghe et al., 2001).

In this prospective observational study we aimed to determine whether the cytokines TNFα and IL-6, and the adipocyte derived insulin sensitizing peptide adiponectin, were significant determinants of insulin resistance in critically ill patients.

As expected, we found that duration of stay in ICU correlated well with severity of illness as assessed by the APACHE II score ($r = 0.44$, $p = 0.004$). The mean APACHE II score in the study population was 10.7, which is lower than the average APACHE II score of approximately 16 seen in the Chris Hani Baragwanath hospital ICU routinely. Interestingly the mortality rate in the Chris Hani Baragwanath ICU is also usually higher, about 20%, than the 10% mortality rate observed in our study population (Monthly Chris Hani Baragwanath ICU morbidity and mortality data). The reason for this is unclear, but may be due to a “good patch” where the severity of illness of the ICU admissions was less than usual over the 2 month period during which the study sample was collected.

Reduced insulin sensitivity was demonstrated in these critically ill patients by increased amounts of administered insulin being required to maintain normoglycaemia. Thus, of the patients included in this study 82.5% required insulin infusions during their ICU admission to maintain normoglycaemia, with only 17.5% being able to maintain normoglycaemia without the aid of insulin infusions. The mean glucose concentrations over the 24 hour periods did not change significantly. This is as a result of patients receiving graduated increases of insulin infusions according to a sliding scale to prevent hyperglycaemia as part of standard ICU management protocols (s*ee Appendix B)*. When a ratio of administered insulin to mean 24 hour blood glucose was calculated, there were significant changes seen during the ICU admission*,* however this ratio was shown to correlate extremely well with the amount of administered insulin (r = 0.93), and as a result did not offer additional benefit, and was not utilized further*.*

The severity of illness as measured by the APACHE II score did not correlate with insulin administration. Previous studies have found a significant positive correlation between the severity of illness scores using APACHE III scores and the degree of insulin resistance as measured by the euglycaemic hyperinsulinaemic clamp technique (Holzinger et al., 2007). The APACHE III score is a development of the APACHE II score, designed to improve the statistical predictive power of the scoring system to predict patient outcome. The differences between the APACHE II score and the APACHE III score include modifications to the acute physiology score component with the addition of measurements of serum albumin, urea, bilirubin, blood glucose and urine output; incorporation of information regarding patient origin before being admitted to ICU; and inclusion of more disease categories in the chronic health evaluation component of the score (Wong & Knaus, 1991). Whether these modifications alter the ability of the score to predict insulin resistance remains to be studied. Possibly the disparate findings between the study of Holzinger and co-workers (2007) and the present study can be attributed to the different methods of assessing insulin resistance, with the euglycaemic hyperinsulinaemic clamp procedure being the more accurate method.

Because of the well described associations of obesity with insulin resistance (McLaughlin, 2003), the body mass index (BMI) was used to assess the degree of adiposity in the study group. Within the study group, 35% would be classified as being overweight (BMI >25 kg/m^2), and 12.5% would be classified as obese (BMI >30 kg/m^2) according to the World Health Organization (WHO) definitions (WHO expert committee, 1995). Obesity has long been known to be associated with reduced insulin sensitivity, and the present study did demonstrate positive correlations between BMI and glucose and weak relationships between BMI and C-peptide and administered insulin levels.

The main determinant of administered insulin on admission was mean plasma glucose (r^2 = 0.69), with gender and BMI also exerting minor effects. As the dose of administered insulin given is determined on the basis of the blood glucose concentration, this result is not unexpected.

Measured insulin concentrations showed statistically significant correlations with mean plasma glucose (r = 0.41, p = 0.009) and C-peptide (r = 0.45, p = 0.004) on admission. Glucose is the primary stimulus for insulin secretion from the β-cell of the pancreas, and insulin is secreted in equimolar amounts with C-peptide (Sacks, 2005), thus this correlation is not unexpected. After admission to ICU, insulin infusions were commenced, and serum insulin concentrations were not measured. The mean blood glucose levels on admission correlated with BMI (r = 0.38, p = 0.013), and serum cortisol levels (r = 0.41, p = 0.008), independently of each other when analysed by multiple regression. The positive correlation between blood glucose and BMI can be explained by the known association between obesity and insulin resistance (McLaughlin, 2003), and the correlation between serum cortisol levels and blood glucose most likely stems from the glycogenolytic and gluconeogenic effects of cortisol (Desborough, 2000).

It was found that C-peptide levels on admission correlated positively with admission levels of insulin (r = 0.45, p = 0.004) and triglyceride (r = 0.41, p = 0.007). These relationships were maintained in a

multiple regression analysis. The correlation with triglycerides may be due to the relationship between triglycerides and insulin resistance (Taniguchi et al., 2003)

There was an interesting, although not statistically significant positive correlation between adiponectin and C-peptide concentrations. Staiger and co-workers found a positive correlation between the expression of adiponectin receptor (AdipoR1) mRNA and insulin and C-peptide levels, as well as serum triglyceride levels (Staiger et al., 2004). Adiponectin receptors (AdipoR1 and AdipoR2) have also been found on pancreatic β cells, and this may indicate that adiponectin plays a role in endogenous insulin secretion (Kharrhoubi et al., 2003).

Adiponectin showed a statistically significant negative association with insulin resistance at discharge as measured by the amount of administered insulin ($r = -0.457$, $p = 0.0049$). Thus higher adiponectin concentrations were associated with less insulin resistance. This is in keeping with the literature of the insulin sensitizing effect of adiponectin (Kadowaki et al., 2006). Why this negative association could only be demonstrated on discharge, and not on admission or the subsequent days of admission remains uncertain. Possibly the severity of physiological derangement and the compounding effects of other inflammatory cytokines and counter-regulatory hormones on the regulation of insulin sensitivity during the early stages of ICU admission interfere with the normal role that adiponectin plays. As these derangements normalize and normal physiology is restored adiponectin is again able to regulate insulin sensitivity by the time of discharge from the ICU.

IL-6 and TNFα concentrations have been correlated with insulin resistance in obese and type 2 diabetic patients (Bastard et al., 2002; Ruan & Lodish, 2003). Proinflammatory cytokines such as TNFα and IL-6 have also been shown to be associated with the severity of illness in critically ill patients as measured by the APACHE III score (Presterl et al., 1997), and as mentioned previously, APACHE III scores have been shown to correlate with insulin resistance (Holzinger et al., 2007).

Thus, one may assume that IL-6 and TNFα concentrations would correlate with insulin resistance. Proinflammatory cytokines have been suggested to be one of the factors involved in the development of insulin resistance in critically ill patients (Van den Berghe, 2004), and indeed support for this comes from a study by Kremen and co-workers who demonstrated and increase in both IL-6 and TNFα concentrations together with increased insulin requirements to maintain normoglycaemia in postoperative patients (Kremen et al., 2006). However, neither TNFα nor IL-6 levels showed the expected association with insulin administration or measured insulin levels on admission in our study. Fluctuations in cytokine concentrations in critically ill patients have been demonstrated, and a poor correlation between proinflammatory cytokine levels and physiological variables has also been shown (Friedland et al., 1996). Changes in cytokine concentrations in critically ill patients may be affected by different medications administered e.g. hydrocortisone, which has an immunomodulatory effect, and this may affect the relationship between cytokine levels and insulin resistance. Alternatively, these cytokines may have a lesser effect in critically ill than obese or type 2 diabetic patients, or the study population was too small to demonstrate a correlation.

The concentrations of TNFα in our study unexpectedly remained within the reference range throughout the duration in ICU. With other evidence supporting the presence of inflammation and an acute phase response i.e. elevated CRP and IL-6 levels, this is a puzzling finding. Another study showed similar findings with TNFα levels increasing to only 6pg/ml 6 hours postoperatively and thereafter remaining approximately 3pg/ml (Kremen et al., 2006).

The concentrations of IL-6 were markedly elevated above the reference range indicating the presence of inflammation and an acute phase response. The concentration of IL-6 concentrations fell sharply from admission to discharge. This reproduces a similar finding of IL-6 levels increasing to reach a peak 12 hours after surgery, and declining thereafter, with levels 5 days postoperatively still being about double that of the preoperative concentration (Kremen et al., 2006).This molecule is a

proinflammatory cytokine, and levels would be expected to decrease as the patients condition improved sufficiently to be discharged from ICU.

Adiponectin concentrations are inversely related to adiposity (Lebovitz, 2003). In our study adiponectin levels increased from admission to discharge. Kremen and co-workers also showed a decrease in adiponectin levels following surgery, with a return to preoperative concentrations 5 days postoperatively (Kremen et al., 2006). This may represent a reduction in adipose tissue of these patients due to the elevated metabolic demands of illness or insufficient nutrition. A recognized shortcoming of this study is the failure to reweigh the patients before discharge from the ICU. However, due to the fluid sequestration which occurs in critically ill patients due to a “capillary leak” of fluid from the intravascular space, and the usual delay before the patients were admitted to ICU from casualty or the wards, the admission weight may be falsely elevated as a result of sequestered fluid from intravenous fluid administration. As the patient’s condition improves, a diuresis occurs which will result in removal of oedema fluid so that discharge weight will normally be lower than admission weight, but this does not reflect a change in fat mass. Therefore fat mass must be measured specifically by dual emission X-ray absorbtivity (DEXA) or computerized tomography (CT) scans or bioelectrical impedence. Adiponectin levels rose by 92.6% (from a median concentration of 4413.8ng/ml on admission, to 8504.6ng/ml on discharge), and IL-6 levels fell by 86% (from a median concentration of 337.0pg/ml on admission, to 46.9pg/ml on discharge) and it is unlikely that this would be due only to a fall in body fat mass. Also, the increase in adiponectin, and fall in IL-6 levels did not correlate with changes in other metabolic variables. It is possible that the increase in adiponectin levels is a reflection of restoration to health. Indeed, adiponectin production has been shown to be inhibited by inflammatory cytokines in cell culture (Fasshauer et al., 2002), and adiponectin may serve a function in binding to and neutralizing lipopolysaccharide, thereby protecting the organism from its harmful effects (Tsuchihashi et al., 2006). The present study demonstrated a greater

percentage change in adiponectin in survivors (70.4%) than non-survivors (9.9%) substantiating this hypothesis.

In this study, serum triglyceride concentrations showed a trend of increasing gradually from admission until discharge. Triglycerides are an alternate metabolic energy substrate to glucose and are metabolized when there is no glucose available for the cells to utilize. This occurs in the absence of insulin, or the absence of the actions of insulin i.e. insulin resistance (Whalmsley & White, 1994). Therefore when there is elevated cellular metabolic demand such as occurs in illness, more triglycerides are utilized lowering the serum triglyceride concentration. When this metabolic demand returns to normal as occurs with convalescence, the reduced utilization of triglycerides will result in serum triglyceride concentrations increasing. This hypothesis is supported by the greater increase in triglycerides in survivors compared to non-survivors. However, this is in contrast to the study of Mesotten and co-workers who found that elevations in the serum triglyceride levels were associated with a higher mortality rate. In their study they compared a group receiving intensive insulin therapy to maintain a blood glucose <6.1mmol/l to a control group receiving insulin infusions only if their blood glucose concentrations exceeded 12mmol/l. They showed that serum triglycerides increased from admission until day 8 in the control group, but that the increase in serum triglycerides was attenuated in the intensive insulin therapy group (Mesotten et al., 2004). They showed a mean increase of 49% in serum triglycerides from admission to day 8 in their intensive insulin therapy group, as compared to a mean increase of 111% in the control group. It is difficult to compare the present study with that of Mesotten and co-workers, as in our study all patients were receiving intensive insulin therapy, and they selected only those patients that remained in the ICU for longer than 7 days.

Adiponectin levels were higher in females than in males and this reached statistical significance after adjusting for BMI. This gender difference in adiponectin concentrations, with females having higher

serum concentrations than males, has been documented, and may be related to differences in subcutaneous versus visceral fat mass (Combs et al., 2003).

There were also notable gender differences in the amount of administered insulin on admission, serum cortisol and C-peptide concentrations, with females having higher levels than males. The gender differences in administered insulin and C-peptide levels were found to be due to the difference in BMI when performing an analysis of co-variance. Furthermore, females also had higher glucose levels on admission than males, but this did not reach a statistically significant level. The differences in serum cortisol could not be explained on the basis of BMI. There are documented gender differences in cortisol secretion in the response to stress, however these studies showed that males had higher cortisol levels than females (Kirschbaum et al., 1992; and Takai et al., 2007).

Other studies have demonstrated ethnic differences in CRP, with higher CRP levels found in black patients compared to white patients (Khera et al., 2005 and Patel et al., 2006), however in the present study CRP levels were higher in white than black patients, as were IL-6 levels. There was a strong correlation between IL-6 and CRP levels on discharge in the whole study group ($r = 0.66$), and the association of these two variables is most likely due to IL-6 stimulating hepatic CRP production (Li & Goldmann, 1996). The higher IL-6 levels in white than black patients may therefore explain the higher CRP levels. The reason for these ethnic differences is not known, however the small number of white subjects make the data more difficult to interpret and must be confirmed in a larger study.

Although there was no significant difference in adiponectin levels on admission or discharge between whites and blacks, there was a significant difference in the percentage change in adiponectin, with levels increasing significantly more in blacks than in whites (76.7% vs 18.7%). A study by Ferris and co-workers demonstrated lower adiponectin levels in African-Americans than Caucasians (Ferris et

al., 2005). However the small n number for white patients in the present study makes interpretation of our data uncertain.

There were four non-survivors in the study group (10%), and although these numbers are too small to provide powerful statistical interpretation, there were nonetheless statistically significant differences in TNFα and IL-6 levels between the survivors and non-survivors, with higher levels being seen in non-survivors. Calandra and co-workers demonstrated an inverse correlation of TNFα levels with fatal outcome (1990). Other studies have also found IL-6 levels to be predictors of mortality, with higher levels being associated with an adverse outcome (Meduri et al., 1995; and Martin et al., 1997). In the present study we also demonstrated a significant difference in the percentage change between survivors and non-survivors of the proinflammatory cytokines TNFα and IL-6 (11.2% vs 71.0%, $p = 0.05$; and -83.1% vs 158.9%, $p = 0.01$ respectively), and adiponectin (70.4% vs 9.9%, $p = 0.02$). Thus while survivors showed improvement with a minimal increase (TNFα) or reduction (IL-6) in their serum levels, non-survivors showed an increase in the levels of these cytokines. Adiponectin levels increased to a greater degree in survivors than non-survivors, indicating that a return to health is associated with an increase in the levels of this peptide, in conjunction with an improvement in the level of proinflammatory cytokines when compared to non-survivors.

6. Recognized shortcomings of this study

1. The number of patients enrolled in this study was relatively small (40), which affects the statistical analysis of the results, and the power of the study. The n number was chosen based on logistical constraints. Despite this, statistically significant trends were still observed.

2. The cohort consisted mainly (80%) of surgical patients and as a result the study findings may not apply to critically ill medical patients.

3. The study cohort was young, with a mean age of 35.5 years and as a result the study findings may not apply to older critically ill patients.

4. The study cohort was not severely ill with a mean APACHE II score of 10.7, and this may affect the findings of this study.

5. The patients were not re-weighed before discharge from the ICU, and BMI was not recalculated. However, as discussed above, this may not affect interpretation of IL-6 or adiponectin concentrations.

6. We did not use an established score of insulin sensitivity such as HOMA (HOmeostasis Model Assessment) or QUICKI (QUantitative Insulin sensitivity Check Index). This is because these scores provide a measure of insulin sensitivity in the fasting state, and the patients in the ICU were being continuously fed either enterally or parenterally. In addition, these indices of insulin resistance have been shown to correlate poorly with the accepted gold standard measure of insulin sensitivity, the euglycaemic hyperinsulinaemic clamp technique in critically ill patients (Holzinger et al., 2007). Moreover, serum insulin measurements would also be

affected by administered insulin, and would thus not be an accurate reflection of endogenous insulin secretion. In addition, the altered clearance rate of insulin in critically ill patients (Hoshino et al., 2003) would make measured insulin concentrations difficult to interpret. Using the euglycaemic hyperinsulinaemic clamp procedure, was not feasible in the setting of critically ill patients. Using an oral glucose tolerance test in critically ill patients is also not a reliable method to determine insulin sensitivity, due to the limitations of gastrointestinal delivery of the glucose load. Critically ill patients often have delayed gastric emptying due to gastroparesis (Heyland et al., 1996). Other studies have used insulin requirements and the ratio of insulin requirement to blood glucose as measures of insulin sensitivity (Pidcoke et al., 2007). Therefore, the administered insulin dose was used as a measure of insulin sensitivity in the present study.

7. Some patients were on inotropic support using adrenalin or dobutamine infusions. Being β agonists, these drugs would be expected to have metabolic effects, and may hinder the interpretation of serum triglyceride, and glucose measurements. Measurement of serum catecholamine concentrations to determine the association between mean plasma glucose and catecholamine levels may have been helpful, but the measurement of catecholamines in serum is analytically difficult due to their extremely short half-life, and requires rigorous collection procedures to prevent preanalytical variation.

8. Many of the patients were receiving corticosteroids which have immunomodulatory effects and counterregulatory effects to insulin, and this variable was not taken into account in the analysis of data.

9. The different feeding protocols (i.e. enteral or parenteral), and the different nutritional composition of the feeds were not taken into account in the analysis of the data.

10. Critically ill patients in an ICU setting are a notoriously difficult study population. Variability in disease aetiology and severity, and the multitude of continuously varying clinical interventions to maintain physiological variables such as blood pressure, volume status, and renal function; surgical interventions; and antibiotics and drug administration further complicate the overall picture.

Despite these drawbacks, this study has shown that:

1. Although there was a demonstrable increase in insulin resistance during the stay in ICU, serum concentrations of the cytokines TNFα and IL-6 did not show the expected correlations with insulin resistance. Adiponectin did show a negative correlation with administered insulin on discharge.
2. Duration of stay in ICU correlated with severity of illness as assessed by the APACHE II score. APACHE II score however did not show a correlation with the amount of administered insulin.

3. Serum adiponectin and triglyceride concentrations showed significant differences from admission to discharge, with values increasing from admission levels, and IL-6 levels decreased significantly from admission to discharge. TNFα levels did not change significantly.

4. The main determinants of glucose levels on admission were BMI and serum cortisol levels. The main determinant of administered insulin were mean glucose and gender.

5. There were notable gender differences in BMI, the amount of administered insulin on admission, serum cortisol and C-peptide concentrations, with females having higher levels than males. There were no gender differences in the measured cytokine levels. The gender differences in administered insulin and C-peptide levels were found to be accounted for by the difference in BMI. The differences in serum cortisol could not be explained on the basis of BMI.

6. There were significant differences in TNFα and IL-6 levels between the survivors and non-survivors, with higher levels being seen in non-survivors.

7. Conclusions

The proinflammatory cytokines TNFα and IL-6 do not appear to be determinants of insulin resistance in critically ill patients. Adiponectin was shown to be a determinant of insulin sensitivity, but only on discharge. This was probably on the basis of the severe metabolic and physiological derangements seen in critically ill patients, and as normalization of these parameters occurred by the time of ICU discharge, adiponectin was again able to exert its metabolic effects.

Poor outcome at ICU discharge is associated with the elevation of the proinflammatory cytokines TNFα and IL-6, but lower levels of serum triglycerides.

8. Appendices

Appendix A

Generating the APACHE II score

The APACHE II score is a general measure of disease severity based on current physiologic measurements, age and previous medical condition. The score can help in the assessment of patients to determine the level and degree of diagnostic and therapeutic intervention.

Components:

1. Acute Physiology Score (APS)
2. Age points
3. Chronic health points

Data collection:

The data for the acute physiology score is collected during the initial 24 hour period after ICU admission, and can be calculated every 24 hours. The worst (most deranged) physiologic value is selected for grading.

1. Acute Physiology score:

Parameter	Finding	Points
rectal temp (°C)	≥ 41	+4
	39-40.9	+3
	38.5-38.9	+1
	36-38.4	0
	34-35.9	+1
	32-33.9	+2
	30-31.9	+3
	≤ 29.9	+4
mean arterial pressure (mmHg)	≥ 160	+4
	130-159	+3
	110-129	+2
	70-109	0
	50-69	+2
	≤ 49	+4
heart rate (beats/minute)	≥ 180	+4
	140-179	+3
	110-139	+2
	70-109	0
	55-69	+2
	40-54	+3
	≤ 39	+4
respiratory rate (breaths/min)	≥ 50	+4
	35-49	+3
	25-34	+1
	12-24	0
	10-11	+1
	6-9	+2
	≤ 5	+4
oxygenation	A-aDO2 ≥ 500 and FIO2 ≥ 0.5	+4
	A-aDO2 350-499 and FIO2 ≥ 0.5	+3
	A-aDO2 200-349 and FIO2 ≥ 0.5	+2
	A-aDO2 < 200 and FIO2 ≥ 0.5	0
	PaO2 > 70 and FIO2 < 0.5	0
	PaO2 61-70 and FIO2 < 0.5	+1
	PaO2 55-60 and FIO2 < 0.5	+3
	PaO2 < 55 and FIO2 < 0.5	+4
arterial pH	≥ 7.7	+4
	7.6-7.69	+3
	7.5-7.59	+1
	7.33-7.49	0
	7.25-7.32	+2
	7.15-7.24	+3
	< 7.15	+4

serum sodium (mmol/l)	≥ 180	+4
	160-179	+3
	155-159	+2
	150-154	+1
	130-149	0
	120-129	+2
	111-119	+3
	≤ 110	+4
serum potassium (mmol/l)	≥ 7.0	+4
	6.0-6.9	+3
	5.5-5.9	+1
	3.5-5.4	0
	3.0-3.4	+1
	2.5-2.9	+2
	< 2.5	+4
serum creatinine (µmol/l)	≥ 309 and not acute renal failure	+4
	177-308 and not acute renal failure	+3
	132-176 and not acute renal failure	+2
	53-133 and not acute renal failure	0
	< 53 and not acute renal failure	+2
	≥ 309 and acute renal failure	+8
	177-308 and acute renal failure	+6
	132-176 and acute renal failure	+4
	53-133 and acute renal failure	0
	< 53 and acute renal failure	+4
haematocrit (percent)	≥ 60	+4
	50-59.9	+2
	46-49.9	+1
	30-45.9	0
	20-29.9	+2
	< 20	+4
WBC count ($x10^3$/l)	≥ 40	+4
	20-39.9	+2
	15-19.9	+1
	3-14.9	0
	1-2.9	+2
	< 1	+4
Glasgow Coma Score		15 - (Glasgow Coma Score)

The score for serum creatinine is doubled if the patient has acute renal failure.

Mean arterial pressure (MAP) = $\frac{\text{(Systolic blood pressure) + 2 (Diastolic blood pressure)}}{2}$

If no blood gas data is available, the serum bicarbonate can be used, in place of pH.

Parameter	Finding	Points
serum bicarbonate (mmol/l)	≥ 52.0	+4
	41.0 – 51.9	+3
	32.0 – 40.9	+1
	22.0 – 31.9	0
	18.0 – 21.9	+2
	15.0 – 17.9	+3
	< 15.0	+4

2. Age points:

Age (y)	Points
≤ 44	0
45-54	2
55-64	3
65-74	5
≥ 75	6

3. Chronic health points:

Operative Status	Health Status	Points
Non-operative patient	history of severe organ insufficiency OR immunocompromised	5
	no history of severe organ insufficiency AND immunocompetent	0
Emergency postoperative patient	history of severe organ insufficiency OR immunocompromised	5
	no history of severe organ insufficiency AND immunocompetant	0
Elective postoperative patient	history of severe organ insufficiency OR immunocompromised	2
	no history of severe organ insufficiency AND immunocompetant	0

Organ insufficiency or immunocompromised state must have preceded the current admission.

The patient is considered immunocompromised if:

1. the patient is receiving therapy that reduces host defenses (e.g. immunosuppressive therapy, chemotherapy, radiation therapy, long term corticosteroid use or high dose corticosteroids).
2. has a disease severe enough to interfere with immune function, such as lymphoma, leukaemia, or AIDS.

Liver insufficiency is considered if the patient has:

1. biopsy proven cirrhosis
2. portal hypertension
3. episodes of upper gastrointestinal bleeding due to portal hypertension
4. prior episodes of hepatic failure, coma, or encephalopathy.

Cardiovascular insufficiency is considered if the patient is classified as having New York Heart Association Class IV congestive cardiac failure.

Respiratory insufficiency is considered if the patient has:

1. severe exercise restriction due to chronic restrictive, obstructive or vascular disease
2. documented chronic hypoxia or hypercapnoea, secondary polycythemia, or severe pulmonary hypertension
3. ventilator dependency

Renal insufficiency is considered if the patient is on chronic dialysis.

APACHE II score = Acute physiology score + Age points + Chronic health points

Interpretation:

Minimum score = 0

Maximum score = 71

An increasing score is associated with increasing risk of hospital death.

Appendix B

Sliding scale of insulin administration.

200IU rapid acting insulin is added to 200ml 0.9% saline and administered via an infusion pump at a rate according to the regimen below and adjusted every 2 hours with every blood glucose measurement.

Blood glucose concentration (mmol/l)	Insulin infusion rate (ml/hr or IU/hr)
0 – 6.0	0
6.1 – 7.0	2
7.1 – 9.0	4
9.1 – 11.0	6
11.1 – 13.0	8
13.1 – 15.0	10
15.1 – 17.0	12
> 17.1	14

When blood glucose concentrations fall below 3.5mmol/l, the insulin infusions are stopped, and 50ml of 50% dextrose water is given intravenously immediately, and the blood glucose is measured again in 10 minutes.

Appendix C

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

Division of the Deputy Registrar (Research)

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)
R14/49 Wilgen

CLEARANCE CERTIFICATE	**PROTOCOL NUMBER M040214**
PROJECT	Cytokines associated with insulin resistance in critically ill ptients.
INVESTIGATORS	Dr U Wilgen
DEPARTMENT	Chemical Pathology
DATE CONSIDERED	04.02.27
DECISION OF THE COMMITTEE*	Approved unconditionally

Unless otherwise specified this ethical clearance is valid for 5 years and may be renewed upon application.

DATE 04.04.07 **CHAIRPERSON** .....
(Professor PE Cleaton-Jones)

*Guidelines for written 'informed consent' attached where applicable

cc: Supervisor : Prof N Crowther

DECLARATION OF INVESTIGATOR(S)

To be completed in duplicate and **ONE COPY** returned to the Secretary at Room 10005, 10th Floor, Senate House, University.
I/We fully understand the conditions under which I am/we are authorized to carry out the abovementioned research and I/we guarantee to ensure compliance with these conditions. Should any departure to be contemplated from the research procedure as approved I/we undertake to resubmit the protocol to the Committee. **I agree to a completion of a yearly progress report.**

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

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