The prognostic value of cortisol measured by liquid chromatography coupled mass spectrometry in critically ill patients

Ph.D. Thesis

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Abbreviations

ACTH	adrenocorticotropic hormone
AIDS	acquired immune deficiency syndrome
APACHE	acute physiology and chronic health evaluation
AUC	area under the (ROC) curve
BNP	brain natriuretic peptid
CBG	corticosteroid binding globulin
CI	confidence interval
CRH	corticotropin releasing hormone
CV	coefficient of variation
D1	type 1 deiodinase
D2	type 2 deiodinase
D3	type 3 deiodinase
ECLIA	electrochemiluminescent immunoassay
EIA	enzyme immunoassay
EIC	extracted ion chromatogram
ELISA	enzyme-linked immunosorbent assay
ESI	electrospray ionisation
FC	free cortisol
FC0	free cortisol at 0 hour
FC6	free cortisol at 6 hours
FC24	free cortisol at 24 hours
FC48	free cortisol at 48 hours
FC96	free cortisol at 96 hours
FiO_2	fraction of inspired oxygen
GH	growth hormone
HPLC-MS	high performance liquid chromatography coupled mass spectrometry
ICU	intensive care unit
IGF-1	insulin-like growth factor 1
IGFBP-3	insulin-like growth factor-binding protein 3
IS	internal standard

LC-MS	liquid chromatography coupled mass spectrometry
LH	luteinizing hormone
LOD	limit of detection
LOQ	limit of quantification
MRM	multiple reaction monitoring
PaO_2	partial pressure of oxygen in arterial blood
PBS	phosphate-buffered saline
PRL	prolactin
QTOF	quadrupole time of flight
RAI	relative adrenal insufficiency
ROC	receiver operating characteristic
rT_3	reverse triiodothyronine
SAPS	simplified acute physiology score
SC	salivary cortisol
SPE	solid-phase extraction
SRM	selected reaction monitoring
T_3	triiodothyronine
T_4	thyroxin
TC	total cortisol
TC0	total cortisol at 0 hour
TC6	total cortisol at 6 hours
TC24	total cortisol at 24 hours
TC48	total cortisol at 48 hours
TC96	total cortisol at 96 hours
TOF	time of flight
TSH	thyroid-stimulating hormone

1 Introduction

1.1 Measurement of cortisol

Cortisol is a glucocorticoid hormone produced in the zona fasciculata of the adrenal gland. Its synthesis is regulated by the adrenocorticotrophic hormone of the pituitary gland [Pretorius, 2011]. Cortisol is secreted in a diurnal pattern with early morning peak levels. The investigation of the diurnal rhythm is an important diagnostic tool because it disappears in hypercorticism. Approximately 90 % of circulating cortisol is bound to proteins, whereas the remaining 10 % is present in an unbound, free form [Kirchhoff, 2011]. The bound fraction is distributed between corticosteroid binding globulin (CBG) and albumin [Kley, 1977; Rhen, 2005]. Only the free fraction is physiologically active [Tomlinson, 2004; Hamrahian, 2004]. Cortisol is present in blood, urine [Wear, 2007] and saliva; the latter clearly reflects blood free-cortisol status [Galbois, 2010; Restituto, 2008]. Cortisol has a diagnostic value in Cushing's syndrome [Sereg, 2011; Guaraldi, 2012], Addison's disease [Ross, 2013], renal and adrenal dysfunction [Arregger, 2008; Holst, 2007] and tumour diagnostics of the hypothalamic-pituitary-adrenal axis [Ronchi, 2012; van Waas, 2012].

Most routine laboratory methods measure total cortisol (TC) concentration, but the measurement of free cortisol (FC) provides more informative results. There is a large individual variation in the binding characteristics of CBG. Alterations in albumin and CBG concentrations might adversely affect results [Pretorius, 2011; Ho, 2006]. Possible ways of estimating FC cover two different approaches. One is to calculate FC mathematically, by quantifying TC and cortisol-binding proteins and then evaluating FC using the free serum cortisol index [Bonte, 1999] or the Coolens formula [Coolens, 1987]. The main problems with these approaches are that they do not consider inter-individual variations in binding characteristics, and that the possible errors of the measurements might accumulate [Kirchhoff, 2011]. The other option is to measure FC directly. The assay starts with the mechanical removal of the protein fraction to obtain a protein free preparation: this is commonly performed by equilibrium dialysis [Durber, 1976] or, as a faster alternative, by ultrafiltration. The latter requires an ultrafiltration device equipped with a membrane having a molecular weight cut-off value more than the weight of the analyte, but less than the weight of

CBG and albumin [McWhinney, 2010]. A simpler method of establishing unbound, circulating cortisol status is measuring salivary cortisol (SC), because this does not require the removal of any protein fraction [De Palo, 2009].

Analytical tools used for FC and SC estimation are similar to those used for the measurement of TC. The only difference is that TC assays contain a hormone displacement to liberate the protein-bound fraction. The routinely used immunoassays, for example enzyme immunoassays (EIA) and electrochemiluminescence assays (ECLIA) [Kominami, 1980; Kohen, 1980], have high sensitivity and are used on different matrices [Chiu, 2003]. The major challenge of immunoassays is specificity, because steroid compounds have high structural similarity, resulting in potential cross reactivity. This is especially problematic if the patient receives a common prednisolone or methylprednisolone treatment, which can lead to fals results [Kobayashi, 2002; Nishiyama, 2000]. Kobayashi et al. [Kobayashi, 2002] described a series of further steroids that can react with antibodies raised against cortisol. Immunoassays carry further analytical problems, namely the antibody interferences (heterophilic antibodies or autoantibodies).

Mass spectrometry coupled with high-performance liquid chromatography (HPLC-MS) has higher specificity than immunoassays because identification is on the basis of the compound's mass-to-charge ratio, instead of structural characteristics. This enables the differentiation and specific quantification of structurally highly similar compounds. Recent studies describe tandem mass spectrometry [Pretorius, 2011; McWhinney, 2010; Han, 2011; Kushnir, 2004] using triple-quadrupole instruments, where specificity is achieved by the fragmentation of cortisol, requiring a multiple reaction monitoring (MRM) transition. During the analysis, the mass of the analyte and the masses of two or three characteristic molecular fragments are scanned simultaneously. No analytical error from cross reactivity and heterophilic antibodies are expected using liquid chromatography (LC-MS). It allows analysis of multiply metabolites from small sample volume. However, LC-MS requires rigorous validation [Briegel, 2009; Clark, 2011].

1.2 Endocrine changes in critical illness

Critical illness is a life-threatening condition due to serious illnesses, infections, surgery or trauma manifested in vital organ failure and severe physical

stress. Support of insufficient organ systems is essential for survival. These patients might not survive without the new therapeutic options of critical care medicine.

The critical illness is associated with neuroendocrine and metabolic alterations such as central and peripheral endocrine stress reactions. The life-threatening condition is the ultimate form of severe physical stress. The endocrine and metabolic adaptations presumably provide energy for vital organ functions to serve the fight-or-flight response. The changes in lipolysis, proteolysis and gluconeogenesis help survival. The critical condition may persist for weeks and the patient may require long-term intensive care. The prolonged critical illness increases the risk of mortality. The endocrine characteristics of the chronic phase of critical illness are different from the acute phase and not solely beneficial. For example, the protein breakdown from skeletal muscles may impair the recovery. In recent years novel data accumulated about the pathomechanism of endocrine responses in critical illness [Sharshar, 2011; Van den Berghe, 2003].

1.2.1 Pituitary-adrenal axis

The strong relationship between stress and adrenocortical function was first described in 1923 by Scott [Scott, 1923] and then was investigated in detail by Selye in 1936 [Selye, 1998]. Since then it is universally accepted that stress is associated with elevated cortisol levels. Later human studies demonstrated the connection between the severity of stress and the magnitude of adrenocortical response [Melby, 1958; Chernow, 1987]. Stress reaction was believed to be mainly regulated by hypothalamic corticotropin releasing hormone (CRH) but vasopressin is also a weak adrenocorticotropic hormone (ACTH) secretagog, works in synergy with CRH and may have role in critical conditions [Schuster, 2012]. Proinflammatory cytokines have been also shown to activate the hypothalamic-pituitary-adrenal axis. Moreover, they can modulate the activity of 11-beta-hydroxysteroid dehydrogenases and affinity of glucocorticoid receptors influencing the effects of glucocorticoids. The term “relative adrenal insufficiency” (RAI) was introduced many decades ago and was later applied to critically ill patient populations [Annane, 2000; Annane, 2003; Beishuizen 1999; Beishuizen, 2001b; Cooper, 2003; Loriaux, 2009; Knapp, 2004; Kwon, 2009]. A serum total cortisol increase of 250 nmol/L following administration of 250 μg ACTH was used as the diagnostic criteria of normal glucocorticoid

response [Beishuizen, 2001b; Marik, 2008; Norasyikin, 2009]. However, others used 1 μg ACTH in the stimulation test and no consensus has been reached about the cortisol increment after stimulation. A threshold for minimal baseline cortisol was also suggested between 276 and 938 nmol/L without final conclusion [Mesotten, 2008]. The concept of RAI was primarily based on total serum cortisol measurements [Annane, 2003; Beishuizen, 2001b; Cooper, 2003; Venkatesh, 2011; Rothwell 1991]. However, the total serum cortisol and the response to ACTH probably are not the most reliable indicators of adrenal function in critically ill patients. No correlation was found between total serum cortisol and mortality in patients with septic shock [Dimopoulou, 2007; Loriaux, 2009; Dickstein, 2005; Rady, 2005; Udelsman, 1986]. Others found that high total cortisol levels were associated with increased mortality [Melby, 1958; Kehlet, 1973; Sam, 2004]. This observation debated the hypothesis of RAI. Moreover, total serum cortisol values measured by commonly used immunoassays showed a high degree of variability [Cohen, 2006] and the classification of patients markedly differed based on the assay used [Briegel, 2009]. The random measurement of cortisol further increased the uncertainty of evaluation [Venkatesh, 2011; Venkatesh 2005].

The measurement of serum total cortisol by immunoassay is markedly influenced by the concentrations of the CBG and albumin. In critically ill patients there is a decrease in CBG concentration due to elevated cytokines or insulin resistance. The mechanisms of CBG depletion are the decreased hepatic production and the elastase-induced cleavage which is a mechanism of cortisol delivery to tissues. Albumin levels are also markedly reduced in critical illness. CBG is saturated at higher cortisol levels. The concentration of free cortisol rises exponentially at higher concentrations due to the cleavage from CBG [Beishiuzen, 2001a; Venkataraman, 2007; Clark, 2011]. The relationship of total and free cortisol became nonlinear in critically ill patients, so free cortisol levels may be more informative. Free cortisol was reported being a good marker of inflammatory response in septic shock [Beishuizen, 2001a; Beishuizen, 2001b]. While free cortisol levels corresponded to severity of illness, the elevation of total cortisol did not [Rady, 2005; Hamrahain, 2004]. Others confirmed that free cortisol is a better marker of adrenal response in critical illness than total cortisol measurement [Beishuizen, 2001b; Hamrahain, 2004; Burt, 2013]. Thresholds for baseline free cortisol levels of 49.7 nmol/L and stimulated cortisol of 85.6 nmol/L have been

suggested [Hamrahian, 2004]. The effect of cortisol is regulated in tissue level by activity of 11-beta-hydroxysteroid dehydrogenase and the real cortisol effect on cellular level is difficult to predict [Beishuizen, 2001a; Gatti, 2009]. The concept of RAI has been further challenged based on the lack of survival benefit with supranormal dose glucocorticoid administration [Venkatesh, 2011; Oppert, 2000].

Nowadays a new concept about the dramatic increase of cortisol levels in critical illness has been proposed by Boonen et al. [Boonen, 2013; Boonen, 2014]. It is known from pioneering work from Vermes et al. [Vermes, 2001] that ACTH concentrations are just transiently elevated after trauma or sepsis. Plasma ACTH levels were rather suppressed in heterogeneous critically ill patient population at intensive care unit (ICU) admission [Mesotten, 2008]. The low plasma ACTH levels parallel to high serum cortisol concentrations may be due to non-ACTH-driven cortisol secretion or caused by reduced cortisol breakdown. The elevated proinflammatory cytokines may play a role in the stimulation of adrenocortical response. The decreased cortisol metabolism was proven and it is attributed to the low activity of A-ring reductases of the liver and the suppressed activity of 11-beta-hydroxysteroid dehydrogenase type 2 in the kidney. The potential role of the bile acids was proposed in mediating the suppression of these enzymes [Boonen, 2013]. This new concept of hypercortisolism in critical illness due to decreased cortisol metabolism may explain the concomitantly low plasma ACTH concentrations because high cortisol levels exert negative feedback inhibition in the pituitary gland and hypothalamus. The results of Boonen et al. change our explanation about cortisol responses to ACTH stimulation in critical illness and the concept of RAI. It was shown that cortisol responses in ACTH stimulation test correlated positively with both cortisol production rate and cortisol plasma clearance. So patients with the lowest response to ACTH were the ones with the most suppressed cortisol metabolism. The low cortisol response to ACTH presumably reflects the negative feedback inhibition by the already elevated cortisol levels and not the relative adrenal insufficiency [Boonen, 2013].

Recently, major development has been achieved in the measurement of cortisol levels by the technique of LC-MS [Clark, 2011; Montskó, 2014]. LC-MS has greater analytical specificity in detecting total and free serum cortisol than immunoassays and is able to separate and quantify serum total cortisol in the

presence of other steroids and metabolites [Burt, 2013; Gatti, 2009; Shackleton, 2010].

Despite the obvious advantages of free cortisol measurements by LC-MS in critically ill patients, literature data are sparse in this field [Vassiliadi, 2013], partly because free cortisol determination is time-consuming.

1.2.2 Somatotropic axis

The growth hormone (GH) secretion is very sensitive to stress. At the beginning of critical illness, GH levels become elevated, there are increased pulse frequency and higher interpulse concentrations [Ross, 1991; Voerman, 1992]. It is unknown which factors are responsible for the stimulation of GH release in response to stress. Insulin-like growth factor 1 (IGF-1) and insulin-like growth factor-binding protein 3 (IGFBP-3) concentrations decrease and an acquired peripheral resistance to GH is presumed. The role of cytokines is hypothesized behind the reduced GH receptor expression. It was suggested that the primary event is the reduced peripheral GH receptor expression resulting low IGF-1 levels and the reduced negative feedback is responsible for elevated GH release in stress. The elevated GH - low IGF-1 constellation may be beneficial in critical illness exerting direct lipolytic, insulin antagonizing and immune stimulating effects while the IGF-1 mediated metabolic consequences are decreased. This situation would shift the consumption of essential substrates to vital organs. GH secretion in the prolonged critical illness is reduced but still higher than in non-stressed conditions [Sharshar, 2011; Van den Berghe, 2003]. The question of relative GH insufficiency raised and high dose GH treatment was tried in a multicenter study. Unfortunately, GH treatment did not improve outcome but doubled mortality and increased morbidity [Takala, 1999]. No clear explanation is available for the unexpected outcome.

1.2.3 Thyrotropic axis

It is well known that acute illnesses and fasting result in decreased plasma concentration of triiodothyronine (T_3) and elevated reverse T_3 (rT_3) levels. This condition is called acute “low T_3 syndrome” or “non-thyroidal illness” or “sick euthyroid syndrome” [Van den Berghe, 2003]. The changes in thyroid hormone

levels are due to the inhibition of the thyroid hormone conversion from thyroxin (T_4) to T_3 by type 1 deiodinase (D1) and inactivation of thyroid hormone in peripheral tissues mediated by the increased activity of the type 3 deiodinase (D3). [Wajner, 2011; Peeters, 2005]. Concentrations of thyroid-stimulating hormone (TSH) and T_4 have been found to be increased immediately after surgery and then often return to normal levels [Michalaki, 2001]. The possible mediators of acute low T_3 syndrome in critically ill patients are the lack of nutrition, the increased levels of cytokines and hypoxia. It was found that proinflammatory cytokines are able to evoke the acute stress induced alterations of the thyroid axis. The inhibition of thyroid hormone binding and transport by elevated levels of free fatty acids may also be involved [Lim, 1993]. The beneficial or deleterious role of low T_3 syndrome is questionable. It may be adaptive by protecting the critically ill patient from hypercatabolism and consequences of malnutrition reducing energy expenditure. The decrease of thyroid hormone activation also occurs during fasting in healthy subjects and regarded as an adaptive process. However, the magnitude of rT_3 elevation and the T_3/rT_3 ratio was found to be associated with the severity of illness and risk of death [Peeters, 2005]. Beyond the serum T_3 concentrations, the thyroid hormone action on tissue level is determined by the increased D3 activity which may have a role in optimizing inflammatory responses [Lim, 1993; Boelen, 2008, Boelen, 2011]. In the prolonged phase of critical illness low serum T_3 concentrations are accompanied by low T_4 and low TSH concentrations which similar to central hypothyroidism [Boonen, 2014]. It was demonstrated that bacterial lipopolysaccharides stimulate type 2 deiodinase (D2) in the mediobasal hypothalamus and are responsible for the development of central hypothyroidism by suppressing thyrotropin-releasing hormone neurons in the paraventricular nucleus. The upregulation of D2 expression and activity in the prolonged phase of the critical illness was detected not just in the hypothalamus but in the skeletal muscles, lung and liver [Sánches, 2008].

1.2.4 Lactotropic axis

Prolactin (PRL) was one of the first hormones known to increase in response to stress. It was suggested that the elevated PRL level contributes to the altered immune functions. PRL receptors are present on T- and B-lymphocytes. The inhibition of PRL secretion by bromocriptine was found to modify the immune

response in humans after heart transplantation [Carrier, 1990]. In animal models, inhibition of PRL secretion impaired lymphocyte function, decreased macrophage activation and coping with bacterial infections. In the prolonged phase of critical illness, the PRL secretion is blunted and the secretory pattern is changed, the pulsatility is reduced. Dopamine administration as an inotropic drug has been found the further suppress PRL secretion and may be involved in impaired immune response [Van den Berghe, 2003; Devins, 1992]. As PRL is not available for therapy, it is theoretical question whether PRL might be used for optimizing immune functions in critical illness. It is also unknown that bromocriptine treatment should be interrupted or continued during intercurrent diseases.

1.2.5 Luteinizing hormone-testosterone axis

Testosterone is the most important anabolic steroid, so stress-induced changes in the luteinizing hormone-testosterone axis may influence the catabolic condition in critical illness. Decreased testosterone concentration was found during starvation, after myocardial infarction and surgical procedures [Klibanski, 1981; Wang, 1978a; Wang 1978b]. Low testosterone and high luteinizing hormone (LH) levels were found in the immediate postoperative stage and acute phase of myocardial infarction presuming the suppression of Leydig-cell function. The pathomechanism of Leydig-cell dysfunction is not known. The potential role of proinflammatory cytokines were suggested based on animal studies [Guo, 1990]. The rationale for decreased anabolic androgen levels would be to conserve energy and save metabolic substrate for vital functions.

The secretory pattern of LH is crucial for its biologic activity. In prolonged critical illness, high LH pulse frequency and low pulse amplitude has been reported resulting in impaired LH effect. The contributing role of endogenous and exogenous dopamine and opioids was presumed in the pathogenesis of hypogonadotropism [Van den Berghe, 1994]. Estradiol secretion is preserved and may further diminish LH secretion. Increased aromatization of adrenal androgens was suggested behind the normal estrogen levels. The effect of inflammatory cytokines in the hypothalamus was intensively studied to clarify the pathomechanism of hypogonadotrop hypogonadism. Interleukin-1 (IL-1) has a role in the suppression of luteinizing hormone-releasing hormone production. The adaptive or maladaptive

feature of hypogonadism is questionable, similarly to the low T_3-syndrome [Van den Berghe 2003]. The suppression of reproductive axis in critical illness was found to be related to diseases severity [Spratt, 1993].

1.2.6 Endocrine predictors of poor prognosis in critically ill patients

In the clinical practice, validated scoring systems are used in the critical care medicine to predict the outcome of individual patients: acute physiology and chronic health evaluation (APACHE), simplified acute physiology score (SAPS), etc. These scores use multiple parameters of vital functions, laboratory tests and patient characteristics. There were numerous attempts to find biomarkers of adverse outcome. Many hormones were investigated from this point of view. In the acute phase of critical illness, low cortisol response to ACTH stimulation and high baseline serum cortisol levels were reported as indicators of poor prognosis. The elevated rT_3 and T_3/rT_3 ratio were also found as markers of high mortality risk [Peeters, 2005]. Estradiol concentrations at 48 hour after admission were significantly elevated in non-survivors regardless of gender [Dossett, 2008]. Estradiol levels at admission and later also independently associated with mortality in a large population of critically ill patients [Kauffmann, 2011]. The possible prognostic role of ghrelin was investigated and high ghrelin levels were found as positive predictor at ICU-survival in septic patients. Ghrelin levels were associated with requirement of mechanical ventilation [Koch, 2010]. ICU-survivors had significantly lower brain natriuretic peptid (BNP) concentrations when compared to non-survivors in a large unselected cohort of critically ill patients. SAPS II and BNP levels were independently associated in a logistic regression model [Meyer, 2007]. Increased plasma adiponectin level was also reported being associated with mortality in patients with respiratory failure [Walkey, 2010]. Median GH concentrations at admission increased about 7-fold in non-survivors as compared with survivors in the study of Schuetz et al. As the GH plasma concentrations were independent predictors for mortality, the authors suggested to use GH measurement to complement the existing risk prediction scores [Schuetz, 2009].

It should be concluded from many studies that the magnitude of expected endocrine changes is higher in patients with more severe disease and high risk of

mortality, and a number of hormonal parameters can be useful in predicting the prognosis of individual patients.

2 Aims

2.1 Validation of a new method for cortisol measurement

Our objective was to develop a sensitive and specific HPLC-MS-based cortisol assay using a Bruker micrOTOF high-resolution mass spectrometer. Specificity was achieved using accurate mass identification instead of an MRM transition. The development of the assay was planned for the measurement of

a) total serum cortisol
b) free serum cortisol
c) salivary cortisol

2.2 Cortisol response measured by LC-MS in critical illness

The aim of this study was

a) to analyze the total and free cortisol concentrations measured by LC-MS in a mixed population of critically ill patients with medical emergencies
b) to determine the time course of cortisol response in critical illness
c) to estimate the prognostic role of cortisol levels in comparison to APACHE II and SAPS II mortality scores which are the best known predictors of mortality
d) to evaluate serum total and free cortisol concentrations as new prognostic biomarkers in critical illness

3 Patients and Methods

3.1 Chemicals and standards

All analytical standards (cortisol, cortisone, dexamethasone, prednisolone, prednisone, and methylprednisolone) were purchased from Sigma-Aldrich, Budapest, Hungary, except the deuterated internal standard (IS), (9,12,12-D3 cortisol), which was purchased from Cambridge Isotope Laboratories Inc. (USA). Two different internal standard solutions were prepared in 20 % methanol. One was used during the TC assay and had a concentration of 1.1 μmol/L, and the other was used for FC and SC analyses and had a concentration of 0.11 μmol/L. All other standard solutions were prepared in 20 % methanol in water, and all were kept at 4 °C. Calibrators used for the quantification of SC and FC measurements were prepared in phosphate-buffered saline (PBS) pH=7.4, to cover the concentration range 400 pmol/L to 100 nmol/L. The calibration series used during the TC assay was prepared in 6 % bovine serum albumin dissolved in PBS, to cover the concentration range 10-4000 nmol/L. All solvents were of LC-MS grade, purchased from Molar Chemicals (Molar Chemicals, Hungary). Ammonium acetate (Sigma-Aldrich, Hungary) and formic acid (Molar Chemicals, Hungary) were used as HPLC additives.

3.2 Patients and/or volunteers during the validation of LC-MS cortisol measurement

The total number of patients and/or volunteers involved was 292, with a gender distribution of 175 females and 117 males. The mean age of the female patients was 59.1 years (23-82 years) and that of the males was 53.4 years (24-76 years).

All blood samples were collected in anticoagulant-free Vacutainer (Becton Dickinson, Hungary) plastic tubes. Sampling was performed from 8:30 am to 10:00 am. After centrifugation at 2200g for 10 min, sera samples were transferred to plastic container tubes and were kept frozen at -24 °C until sample preparation. Salivary samples were collected from healthy volunteers among laboratory staff by using Salivette Cortisol devices (Saarstedt, Hungary) according to the manufacturer's procedure. Samples were stored frozen until the measurement. Blood samples were

randomly chosen; only patients receiving steroidal therapy were excluded. The authors have confirmed in writing that they have complied with the World Medical Association Declaration of Helsinki regarding ethical conduct of research involving human subjects and/or animals.

3.3 Sample preparation

TC samples were treated as follows: 20 μL internal standard solution (1.1 μmol/L) was added to 100 μL serum and the mixture was vortex mixed for 1 min. Afterwards, 300 μL acetonitrile was added for protein precipitation and the sample was vortex mixed vigorously for 1 min, followed by centrifugation at 14000g for 10min. 50 μL upper phase was added to 50 μL water in an autosampler vial and was vortex mixed. 20 μL of this mixture was injected onto the HPLC column.

To analyze FC, 500 μL serum was ultrafiltrated, using 30000 Da molecular weight cut-off Amicon Ultra-0.5 mL centrifugal filters (Merck, Hungary). After filtration according to the manufacturer's procedure, 400 μL ultrafiltrate was transferred to solid-phase extraction (SPE) cartridges.

Saliva samples and serum ultrafiltrate were further processed with SPE. 20 μL internal standard solution (0.11 μmol/L) was added to 400 μL saliva or ultrafiltrate. Extraction was performed on Strata-X (60 mg) polymeric reversed-phase extraction cartridges (Phenomenex, USA). The phase was activated with 1 mL methanol, and then equilibrated with 1 mL water. Samples were washed with 1 mL 20 % methanol in water; elution was performed using 2×500 μL acetonitrile. The eluate was dried under vacuum, and the residue was redissolved in 20 % methanol in water containing 2 mmol/L ammonium acetate and 0.05 % formic acid. The injection volume was 20 μL.

3.4 Instrumentation

A Dionex Ultimate 3000 (Dionex, USA) analytical HPLC equipped with an autosampler and a column thermostat set at 30 °C was used. Separation was performed on a Kinetex C8 2.6 μm, 2.1×100 mm analytical column (Phenomenex, USA) with a multi-step gradient elution at a flow of 200 μL/min. HPLC solvents contained 2 mmol/L ammonium acetate and 0.05 % formic acid in 5 % (A) and 95 %

(B) acetonitrile. The gradient profile is described in Table 1; the total runtime was 13 min.

Table 1. Description of the HPLC gradient used in the LC-MS run

Time (minute)	A (%)	B (%)
0.0	85	15
5.0	30	70
6.5	15	85
8.0 [a]	15	85
13.0	85	15

[a] *At the end of every chromatographic run a five minute re-equilibration phase was inserted to start the new run at 15 % „B" solvent concentration.*

The mass spectrometer coupled to the HPLC was a Bruker micrOTOF accurate mass instrument, equipped with an electrospray ionization (ESI) source operated in the positive mode. Main source settings were: capillary voltage: 4500 V, nebuliser pressure: 2.4 bar, drying gas flow (nitrogen): 8 L/min, and drying temperature: 210 °C. Mass spectra were collected between m/z 200 and m/z 500. Internal mass calibration was performed at the beginning of every run, using the peaks of Na^+ formate clusters.

3.5 Critically ill patients

The prospective, observational study was carried out in 69 non-selected patients admitted to the Intensive Care Unit of the 1st Department of Internal Medicine, University of Pécs, Hungary. During the enrollment period, 108 patients were treated at our intensive care unit and 79 were enrolled to the study (73%). In cases when informed consent was not feasible and those who died within six hours were not enrolled. Ten patients were excluded from the final evaluation because of missing samples at critical time points. Gender distribution was the following: 39 males and 30 females, median age was 74 (23-87) years. Patients' characteristics and

diagnosis at admission are shown in Table 2. It was a mixed population of patients with medical emergencies, no surgical or trauma patients were included (Table 2). Five patients had complete cardiopulmonary resuscitation and three patients were defibrillated prior to admission. Vital signs, clinical status and routine laboratory parameters were monitored. The treatment of patients was thoroughly evaluated and blood samples disturbed by glucocorticoid treatment were excluded from the further analysis (at admission 3 samples, at 6 hours 9 samples, 24 hours 7 samples and 48 hours 6 samples). The major indication for glucocorticoid treatment was the unresponsive septic shock. None of the patients received etomidate, ketoconazole or any other drug influencing the steroid metabolism. The severity of the diseases was scored according to the SAPS II [Le Gall, 1993] and the APACHE II [Knaus, 1985] (Table 3).

Our study was performed in accordance with the ethical guidelines of the 2003 Declaration of Helsinki and we obtained the permission of Regional Research Ethical Committee of University of Pécs. When feasible, informed consent was obtained prior to enrollment in conscious patients; otherwise the consent was obtained from the patients' next of kin.

Blood samples were taken for the measurement of free and total cortisol levels at admission (0 hour), 6, 24, 48 and 96 hours after admission. Blood samples were collected in anticoagulant free Vacutainer (Becton Dickinson, Hungary Kft., Környe, Hungary) plastic tubes. Routine laboratory parameters were determined by the Institute of Laboratory Medicine, University of Pécs accredited according to ISO 15189. Concentrations of free and total cortisol were measured by high performance liquid chromatography (HPLC) coupled high resolution electrospray ionization-time of flight (ESI-TOF) mass spectrometry.

Table 2. Patients' characteristics

Age (median, interquartiles) years	74.0 (60.5/79.0)
Gender (female/male)	30/39
Mortality rate (30-day)	26.1%
Mechanical ventilation	33.3%
Catecholamine treatment	34.8%
APACHE II score (median, interquartiles)	21.0 (16.5/29.0)
SAPS II score (median, interquartiles)	36.0 (25.0/55.5)
Diagnosis	
Sepsis	20
Heart failure	14
Pulmonary embolism	8
Acute myocardial infarction	7
Respiratory failure	6
Atrial fibrillation	3
Ventricular tachycardia	1
Complete atrioventricular block	2
Drug intoxication	3
Acute kidney failure	2
Diabetic ketoacidosis	1
Gastrointestinal bleeding	1
Hypothermia	1

Table 3. APACHE II and SAPS II clinical scoring systems to classify the severity of illness

APACHE II	SAPS II
Age	Age
Organ insufficiency or immunocompromised state	Type of admission: nonsurgical/surgical (scheduled/unscheduled)
Temperature	Temperature
Mean arterial pressure	Systolic blood pressure
Heart rate	Heart rate
Glasgow Coma Scale	Glasgow Coma Scale
PaO_2/FiO_2	PaO_2/FiO_2
White blood cell count	White blood cell count
Respiratory rate	Urine output
Hematocrit	Blood urea nitrogen
Creatinine	Bilirubin
Potassium	Potassium
Sodium	Sodium
Arterial pH	Bicarbonate
	AIDS
	Metastatic cancer
	Hematological malignancy

3.6 Statistics

Statistical analysis was performed using the IBM SPSS Statistics Version 20 (IBM Magyarország Kft. Budapest, Hungary) and SPSS Statistics Version 22.0 (SPSS, Inc., Chicago, IL, USA) softwares.

Kolmogorov-Smirnov test was used to determine the distribution of the data. All normally distributed data are presented as mean ± SD. Medians and interquartile ranges are reported for data that were not normally distributed. Significant results were those with P values < 0.05. Total and free cortisol levels of survivors and non-survivors were compared using Mann-Whitney U test. Relationships between non-normally distributed quantitative variables were evaluated by bivariate correlation (Spearman correlation). The diagnostic value of cortisol levels to predict mortality was determined by receiver operating characteristic **(**ROC) analysis. The optimal cut-off point was assessed using Youden's J statistic. Kaplan-Meier survival curves were created to compare the mortality risk in cortisol quartiles. Independent determinants of mortality were investigated by binary logistic regression analysis using backward method.

4 Results

4.1 Establishment of a new method for cortisol measurement using LC-MS

4.1.1 HPLC coupled ESI-TOF detection

The retention time of cortisol and the IS was consistent at 6 min (±0.1 min). In Figure 1a, extracted ion chromatograms (EIC) of a patient FC sample run are presented; the measured concentration is 12.1 nmol/L. The EIC of cortisol is at m/z 363.2099, and the EIC of the IS at m/z 366.2287. The mass width was set at ±0.001 Da (1 mDa). The same mass width was used for all the other measurements. The m/z value of the EIC was chosen according to the observed masses of cortisol (m/z 363.2099) and the IS (m/z 366.2287), as seen in Figure 1b.

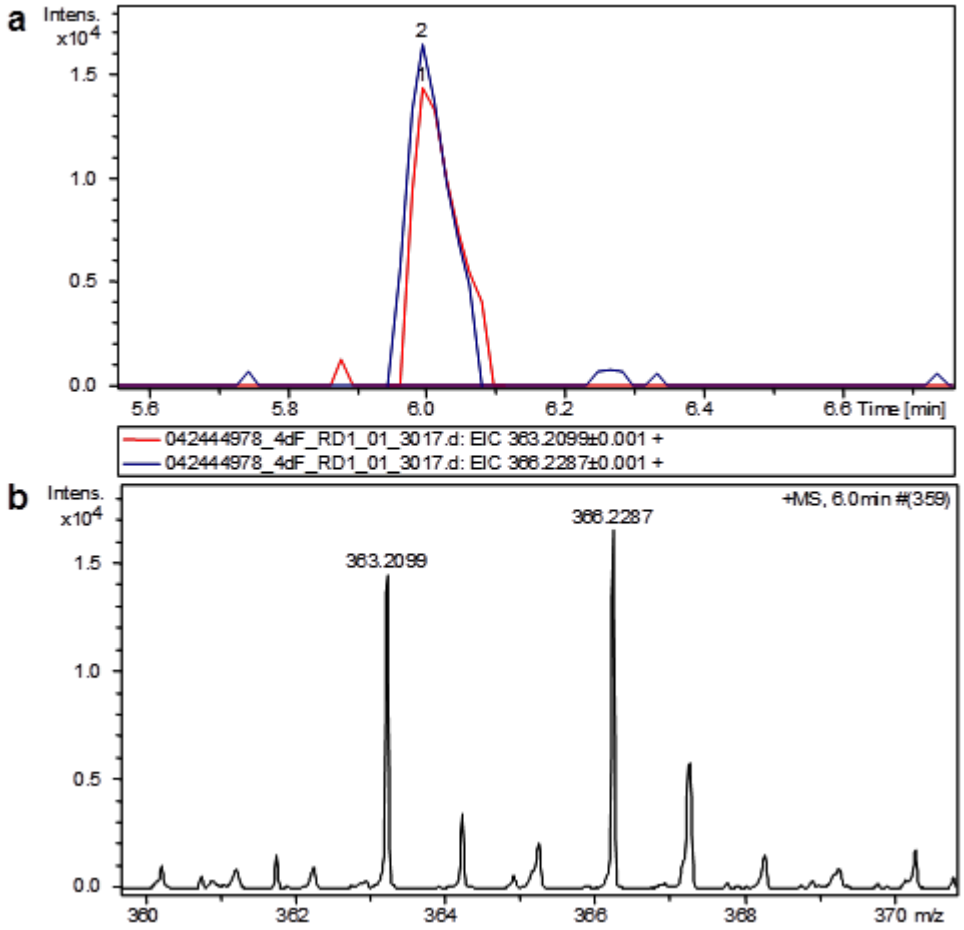

Figure 1. Extracted ion chromatograms **(a)** and average mass spectrum at half maximum **(b)** of a typical serum free cortisol sample run. Peak number 1 **(red)** corresponds to the proton adduct ion of cortisol $[M+H]^+$ at m/z 363.2099, while peak number 2 **(blue)** to the $[M+H]^+$ of the internal standard at m/z 366.2287. Mass accuracy is 1.6 ppm for cortisol and 1.4 for the internal standard

Both masses correspond to the proton adduct ions $[M+H]^+$. Ion chromatograms were selected according to the above-discussed rules for all the other sample runs. A mass accuracy of 1-2.5 ppm was achieved throughout the whole set of measurements. Because of the high mass accuracy achieved, the narrowest mass window (±1 mDa) was used on all chromatograms.

During the study the following concentration ranges were established from the processed samples. TC concentrations ranged from 35.6-1088 nmol/L, with an average of 372 nmol/L, whereas FC levels were in the range 0.5-12.4 nmol/L, with an average of 3.7 nmol/L. SC levels were in the range 0.7-10.4 nmol/L; the average value was 3.2 nmol/L.

4.1.2 Method validation

Intra-assay and inter-assay variations were determined using a total of nine pooled sample lots constructed for the three assays (TC, FC, and SC). Every pooled lot was composed of ten randomly chosen samples. One was constructed in the low range (<100 nmol/L for TC and <3 nmol/L for FC and SC), one in the middle range (100-500 nmol/L and 3-8 nmol/L), and one in the high range (>500 nmol/L and >8 nmol/L). Every material was assayed 20 times. Interassay variation was estimated by six repeated analyses of the respective material on consecutive days.

Limit of detection (LOD) and limit of quantification (LOQ) were determined using sera and saliva samples diluted with PBS till the achievement of a three (LOD) or ten (LOQ) times average signal-to-noise ratio. The values of LOQ and LOD were determined from the results of 10 repeated analyses of the corresponding material.

Recovery was calculated by determining the cortisol concentration of pooled samples (n=5) before and after addition of a known amount of cortisol. The spiking solution was prepared in 20 % methanol in water, with concentrations of 200, 500, and 1000 nmol/L for TC, and 2, 5, and 10 nmol/L for FC and SC. 20 µL spiking solution was added to every pool.

For evaluation of ion suppression, known amounts of IS were spiked into 100 µL of five different extracted TC, FC, and SC samples, and the same amount of IS was also spiked into 100 µL 20 % methanol. IS peak areas measured in the spiked solvents were compared with those measured in extracted samples.

The limit of detection and limit of quantification were similar for FC and SC samples, but much higher for TC. The values were determined by ten repeated

measurements of the corresponding diluted sample. Coefficient of variation (CV) values of the ten repeated LOD and LOQ measurements were also calculated. The LOD was 140 pmol/L (CV 8.9 %) for serum ultrafiltrate, and 190 pmol/L (CV 9.1%) for saliva. The LOD for TC assay was 9 nmol/L (CV 7.8 %), as a result of the dilution during sample treatment. LOQ for FC was 440 pmol/L (CV 6.6 %), and for SC the LOQ was 600 pmol/L (CV 7.1 %). For TC the LOQ was 12.5 nmol/L (CV 6.5 %). The assay was linear from 400 pmol/L-4000 nmol/L nominal concentration.

Results of the intra-assay and inter-assay study, and mean concentrations of the constructed sample pools, are summarized in Table 4.

Table 4. Results of the HPLC-MS method validation. The pooled TC, FC, and SC samples were constructed of ten randomly selected patient serum, serum ultrafiltrate, and saliva samples containing cortisol at the desired concentration interval

	Intra-assay (n=20)			**Inter-assay (n=6)**		
	Mean (nmol/L)	**SD**	**CV %**	**Mean (nmol/L)**	**SD**	**CV %**
Total cortisol	45.8	3.4	7.4	43.4	3.7	8.5
	254.5	4.5	1.7	242.6	22.9	9.6
	720	21.2	3.2	685.6	41.1	6.2
Free cortisol	1.6	0.11	6.9	1.5	0.12	8.0
	7.5	0.29	3.9	7.8	0.39	5.0
	10.5	0.49	4.6	11.1	0.57	5.1
Salivary cortisol	2.5	0.14	5.6	2.6	0.17	6.5
	8.4	0.26	3.1	8.8	0.36	4.1
	12.5	0.76	6.1	11.9	0.81	6.8

Intra-assay CV was best with the 254.5 nmol/L TC pool, at 1.7 %, and worst with the 45.8 nmol/L TC pool, at 7.4 %. We observed the worst inter-assay CV with the 254.5 nmol/L TC pool, at 9.6 %, and the best with the 8.4 nmol/L SC pool, at 4.1%. Overall, the best intra or inter-assay CV was 1.7 % and the worst was 9.6 %. The average intra-assay CV was 4.7 %, and the average inter-assay variance was 6.6%.

During the recovery studies, we observed a mean recovery of 101.2 % for TC, and of 98.9 % and 94.6 % for FC and SC, respectively. Calculated recovery values ranged from 94.6-107.8 %. No ion suppression was observed except in the saliva analysis, where the matrix effect resulted in an average suppression of the added IS signal to 96 % of the average signal measured in 20 % methanol.

4.1.3 Investigation of method interference with frequently used steroid drugs

Interference studies were performed using pooled samples spiked with solutions of frequently used steroidal drugs (cortisone, dexamethasone, prednisolone, prednisone, and methylprednisolone) to achieve a final concentration of 5000 nmol/L of the added drug. Every spiking experiment was repeated ten times. Cortisol concentrations of the samples were measured before and after the addition of the corresponding drugs.

Five commonly administered steroidal drugs were tested to establish whether they interfered with the HPLC-MS cortisol assay. Of the five drugs, cortisone, dexamethasone, prednisone, and methylprednisolone were chromatographically separated from cortisol and no interference was observed. Prednisolone, however, had the same retention time as cortisol and the IS. We observed an average 3 % (2.2-4.1 %, n=10) overestimation of cortisol concentration, which is less than the average CV observed during the study.

With our HPLC-ESI-TOF method, after internal calibration of each sample run a ±0.001 Da (1 mDa) mass width was obtainable on the EICs. For the masses of cortisol (362.2093 Da) and the IS (365.2282 Da), the calculated mass accuracy was always <2.5 ppm. Conventionally, a measurement is regarded as an "accurate mass measurement" if the maximum mass error is less than or equal to 2.5 mDa [Zhang, 2012] and the mass accuracy is not higher than 5 ppm [Hogenbooma, 2009], or, more

recently, 3 ppm [Polgár, 2012]. Figure 2 shows an example, where mass accuracy for the detected compounds is in the range 1.4-1.6 ppm.

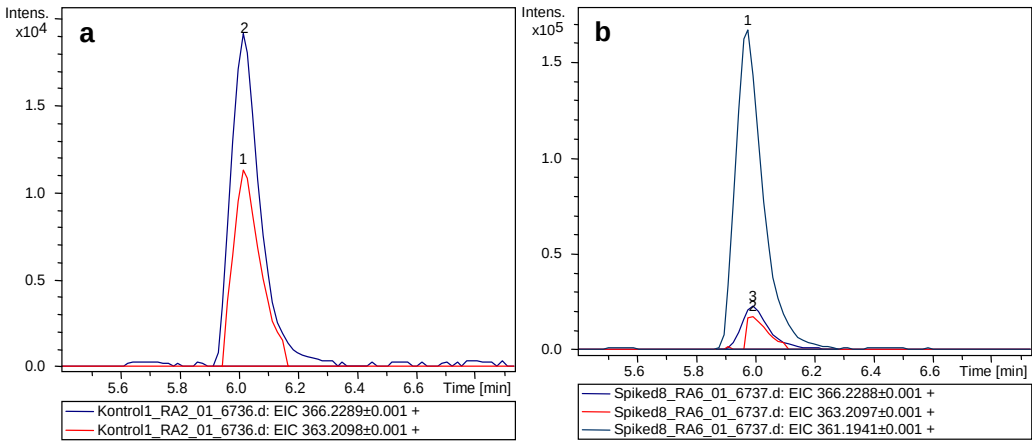

Figure 2. Example of the result of an interference study using prednisolone. The measured cortisol concentration of the sample was 246 nmol/L **(a)**. After the addition of prednisolone spiking solution to achieve a 5000 nmol/L final prednisolone concentration a 252 nmol/L cortisol level was estimated **(b)**. Chromatogram number 1 **(blue)** at 361.1941 m/z corresponds to the proton adduct ion $[M+H]^+$ of prednisolone

4.1.4 Comparison of the LC-MS method with commonly used immunoassays in non-critically ill patient population

Method comparison was performed using three different immunoassays. Results of the TC measurements were compared with the Roche Modular Analytics E 170 ECLIA assay (n=96), the validated method used in our laboratory. Results of the FC and SC measurements (n=96 in both cases) were compared with the results of two commercially available immunoassay kits. The Enzo Cortisol EIA kit (Biomarker, Budapest, Hungary) was used for parallel FC measurements, and the IBL International Cortisol ELISA kit (Diagnosticum, Budapest, Hungary) for SC measurements. Results of the LC-MS assay were compared with the results of the reference methods by Pearson correlation and linear regression analysis. Results are displayed on scattergrams (Figure 3a, b, c). The corresponding regression equations are described in the caption of Figure 3.

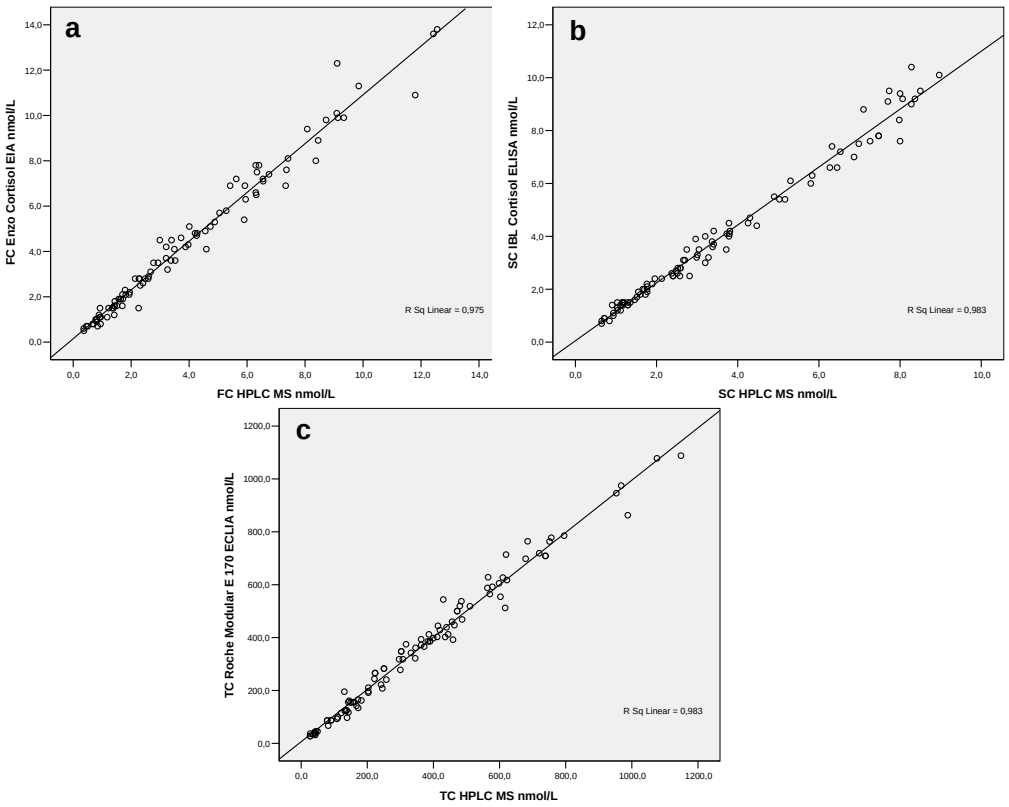

Figure 3. Scattergrams of the linear regression analyses of the three method comparison study. **(a)** Enzo Life Sciences Cortisol EIA assay (free cortisol, HPLC-MS=0.149+0.906×EIA), **(b)** IBL International Gmbh Cortisol ELISA assay (salivary cortisol, HPLC-MS =0.05+0.898×ELISA) and **(c)** Roche Modular Analytics E 170 ECLIA assay (total cortisol, HPLC-MS=0.0738+0.994×ECLIA)

A notable positive bias was observed in the higher concentration ranges for FC and SC measurements; however, we did not observe this when comparing results of the TC analysis. The value of the Pearson correlation coefficient was similar for the TC and SC results (0.991 and 0.992 $p<0.001$); however, the FC results were lower than those estimated using the EIA kit (0.987 $p<0.001$). The calculated statistical variables are listed in Table 5.

Table 5. Statistical parameters (linear regression and correlation) of the HPLC-MS method comparison

	Comparative method	Slope (95 % CI)	R^2	Pearson correlation	p (corr.)
Total cortisol	ECLIA	0.994 (0.967-1.02)	0.983	0.991	p<0.001
Free cortisol	EIA	0.906 (0.876-0.936)	0.975	0.987	p<0.001
Salivary cortisol	ELISA	0.898 (0.874-0.922)	0.983	0.992	p<0.001

4.2 Total and free cortisol measurements in critically ill patients

4.2.1 Cortisol response in critical illness and the correlation of total and free cortisol levels

The range of total cortisol varied between 49.9 and 8797.8 nmol/L (normal values: 138-690 nmol/L) with a median (interquartile ranges) of 583.5 (381.5/855.8) nmol/L, free cortisol between 0.4 and 759.9 nmol/L (reference range: 1-8 nmol/L) with a median (interquartile ranges) of 13.4 (4.3/60.1) nmol/L. The maximal elevation of total cortisol was 13 times, of free cortisol was 95 times of the upper limit of reference range.

The median of total cortisol of hydrocortisone treated septic patients (13 samples at different time points) was 3880 nmol/L, at the upper range of untreated patients. The median of free cortisol due to hydrocortisone treatment was supraphysiological high, 801 nmol/L. These values were not included in the statistical analyses.

The cortisol levels did not show normal distribution based on the Kolmogorov-Smirnov test so the groups were expressed as median and interquartile range. Total and free cortisol levels significantly correlated (P<0.001) (R=0.710) (Figure 4).

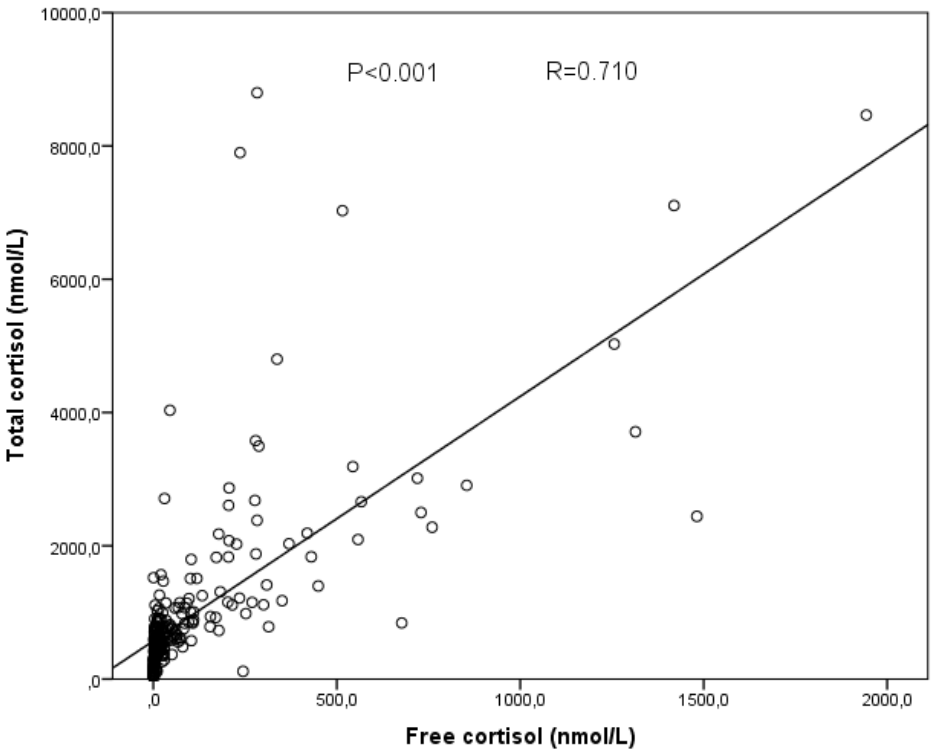

Figure 4. Correlation of free cortisol (FC) and total cortisol (TC) (Spearman correlation, $p<0.001$, correlation coefficient = 0.710)

4.2.2 Time course of adrenocortical response in critical illness

The median, interquartiles and 95% confidence interval of total and free cortisol at different time points can be seen at Figure 5 and 6. Total cortisol at admission was significantly higher than later and at 6 hours was also elevated compared to later time points (Figure 5). Free cortisol was significantly elevated only at admission compared to later measurements (Figure 6).

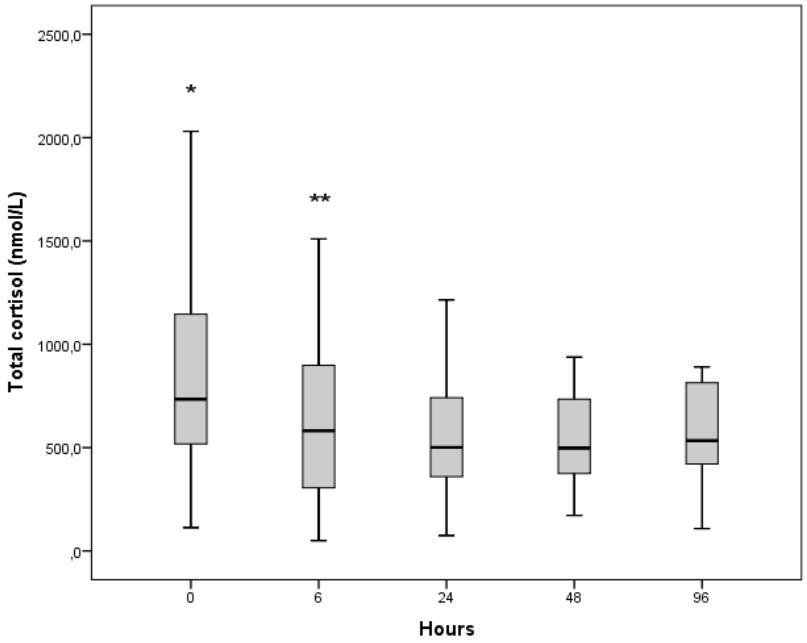

Figure 5. Total cortisol (TC) concentrations at different time points expressed as median, interquartiles and 95 % confidence interval (CI)
*Total cortisol at admission (TC0) is significantly higher than all the later medians
** Total cortisol at 6 hour (TC6) is significantly higher than the total cortisol at 24 hour (TC24)

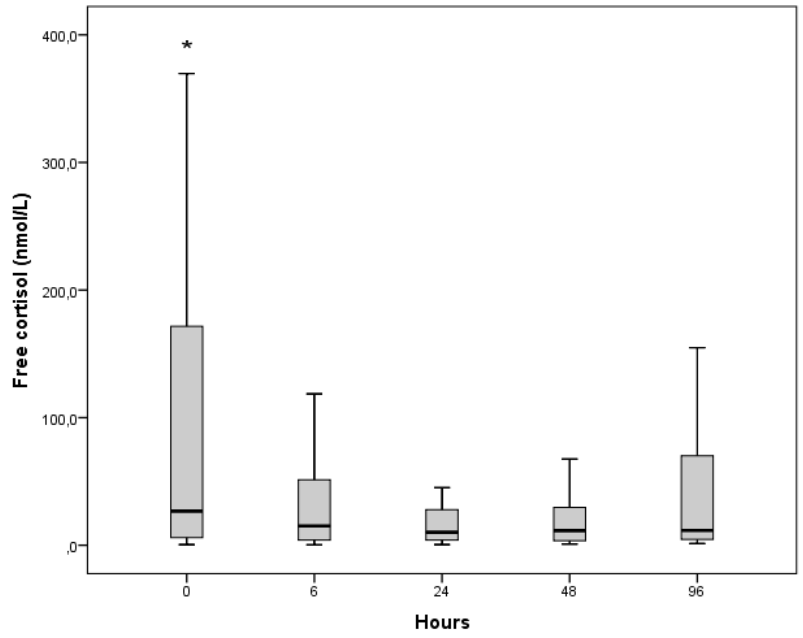

Figure 6. Free cortisol (FC) concentrations at different time points expressed as median, interquartiles and 95 % confidence interval
*Free cortisol at admission (FC0) is significantly higher than the other values

4.2.3 Comparison of cortisol values in survivor and non-survivor population

The total and free cortisol levels of survivors (n=51) and non-survivors (n=18) (30-day mortality) were compared. Elevated total cortisol was found in non-survivors at 0, 6 and 48 hours (Figure 7). Free cortisol was significantly higher in non-survivors at admission and 6, 24 and 48 hours after admission (Figure 8).

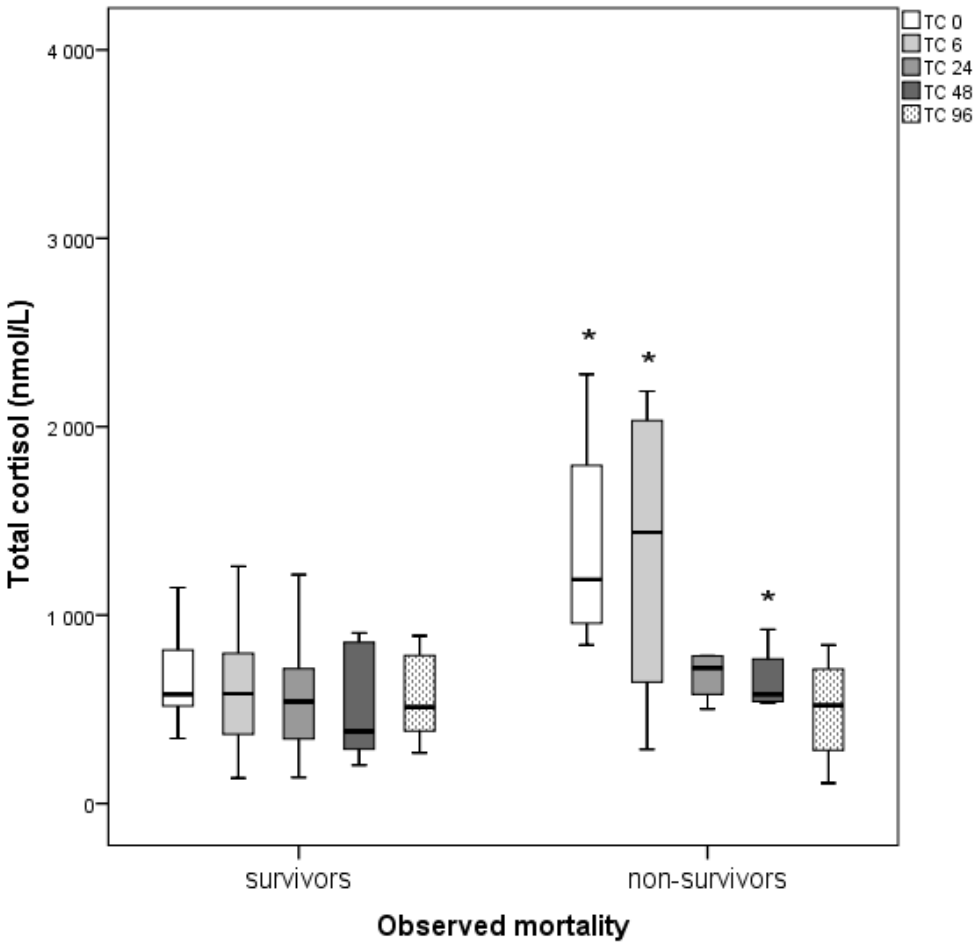

Figure 7. Total cortisol concentrations expressed as medians, interquartiles and 95% confidence intervals in survivors (n=51) and non-survivors (n=18) at different time points

* Significantly elevated cortisol levels in non-survivors analyzed by Mann-Whitney U-test. P values for TC0:0.001, TC6:0.029, TC48:0.017

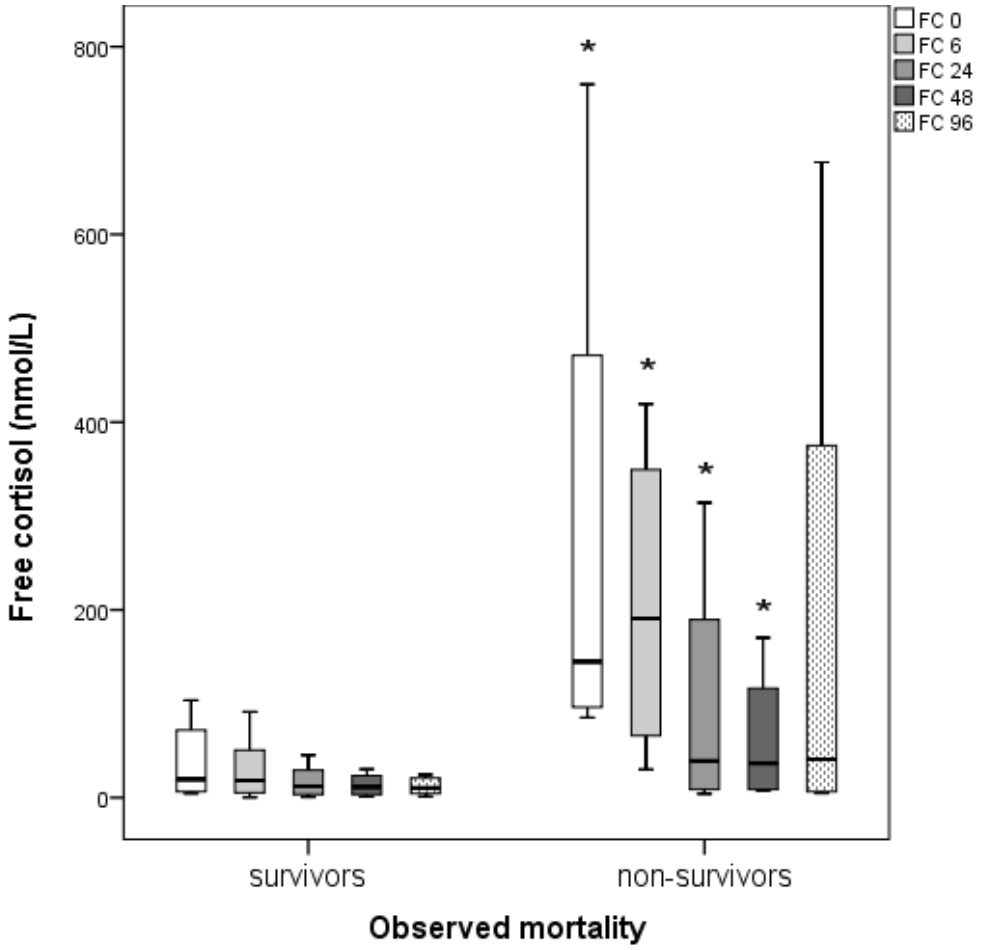

Figure 8. Free cortisol concentrations expressed as medians, interquartiles and 95% confidence intervals in survivors (n=51) and non-survivors (n=18) at different time points
*Significantly elevated cortisol levels in non-survivors analyzed by Mann-Whitney U-test. P values for FC0:0.000, FC6:0.001, FC24:0.018, FC48:0.002

Because intubation and mechanical ventilation may increase cortisol levels and may be associated with bad prognosis, the cortisol levels of ventilated and not ventilated patients were also compared at every time points. TC0 (p=0.001), FC0 (p<0.001) and FC6 (p=0.001) were significantly higher in ventilated patients. The mortality rate was also significantly elevated in cases that required ventilation (p<0.001).

4.2.4 The predictive role of cortisol concentrations at different time points for mortality

The predictive role of cortisol levels was investigated in comparison to the well accepted clinical scores, APACHE II and SAPS II mortality (Figure 9).

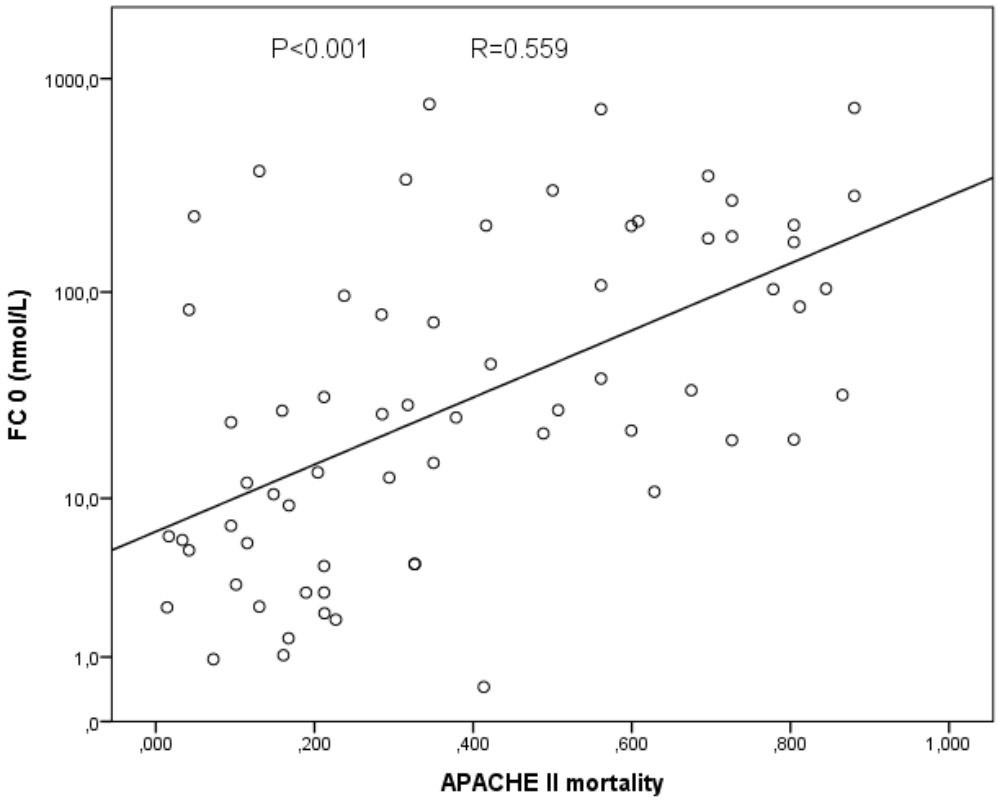

Figure 9. Relationship between free cortisol at admission (FC0) and APACHE II mortality (Spearman correlation, p<0.000, correlation coefficient: 0.559)

The results of these correlations can be seen in Table 6. Free cortisol at 0 (FC0), 6 (FC6), 24 (FC24), 48 (FC48) and 96 hours (FC96) significantly correlated with predicted mortalities. Total cortisol at 0 (TC0) and 6 hours (TC6) also correlated with mortality scores but the correlation disappeared from total cortisol at 24 hour (TC24).

Table 6. Correlations of free cortisol (FC) and total cortisol (TC) levels to APACHE II and SAPS II mortalities (Spearman correlation)

	APACHE II mortality		**SAPS II mortality**	
	p value	Correlation coefficient	p value	Correlation coefficient
FC0	**0.000**	**0.559**	**0.000**	**0.584**
FC6	**0.000**	**0.479**	**0.000**	**0.474**
FC24	**0.011**	**0.330**	**0.029**	**0.284**
FC48	**0.008**	**0.421**	**0.002**	**0.480**
FC96	**0.029**	**0.489**	**0.030**	**0.487**
TC0	**0.000**	**0.455**	**0.000**	**0.542**
TC6	**0.009**	**0.332**	**0.029**	**0.283**
TC24	0.125	0.202	0.272	0.145
TC48	0.435	0.130	0.132	0.249
TC96	0.206	0.295	0.195	0.302

The diagnostic value of cortisol concentrations to predict mortality was next evaluated by ROC analysis. ROC curve for serum total and serum free cortisol levels at admission are shown in Figure 10.

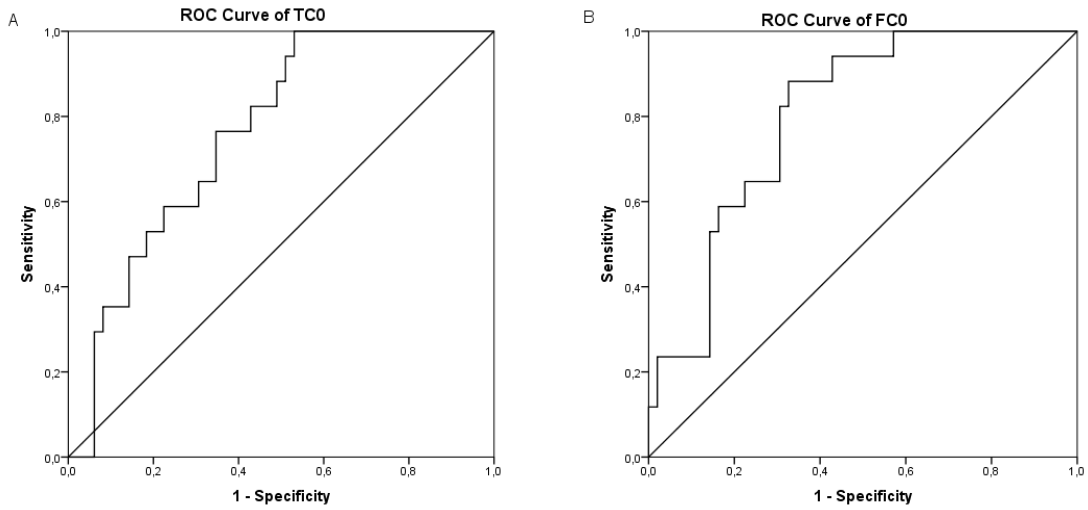

Figure 10. Receiver operating characteristic (ROC) curve for serum total cortisol levels at admission (TC0). AUC=0.762 **(A)** Receiver operating characteristic (ROC) curve for serum free cortisol levels at admission (FC0). AUC=0.801 **(B)**

The cut-off values of cortisol with optimal diagnostic accuracy can be seen at Table 7. For example, free cortisol level ≥ 28.2 nmol/L at admission has 88.2 % sensitivity and 67.3 % specificity to predict mortality. The FC0 and TC0 had higher sensitivity and lower specificity than the later values. The sensitivity and specificity of APACHE II and SAPS II mortality was similar to the diagnostic value of FC6, FC24 and FC48.

Table 7. Cut-off values of free cortisol (FC), total cortisol (TC) and mortality scores (APACHE II and SAPS II mortality) with optimal diagnostic accuracy (based on ROC analysis)

	AUC*	Cut-off value	Sensitivity (%)	Specificity (%)
FC 0 (nmol/L)	0.801	28.2	88.2	67.3
FC 6 (nmol/L)	0.769	51.0	62.5	87.0
FC 24 (nmol/L)	0.702	21.1	56.3	81.4
FC 48 (nmol/L)	0.847	29.7	75.0	87.1
TC 0 (nmol/L)	0.762	583.6	100.0	46.9
TC 6 (nmol/L)	0.694	886.5	57.1	82.6
APACHE II Mortality (%)	0.814	53.4	77.8	82.4
SAPS II Mortality (%)	0.843	54.1	66.7	88.2

** AUC: area under the curve*

The best separation of survival in Kaplan-Meier curves was found at FC0 analyzed in quartiles. Patients with the lowest 25 % of free cortisol all survived while 60 % of patients died in the highest quartile (>75 %) (Figure 11).

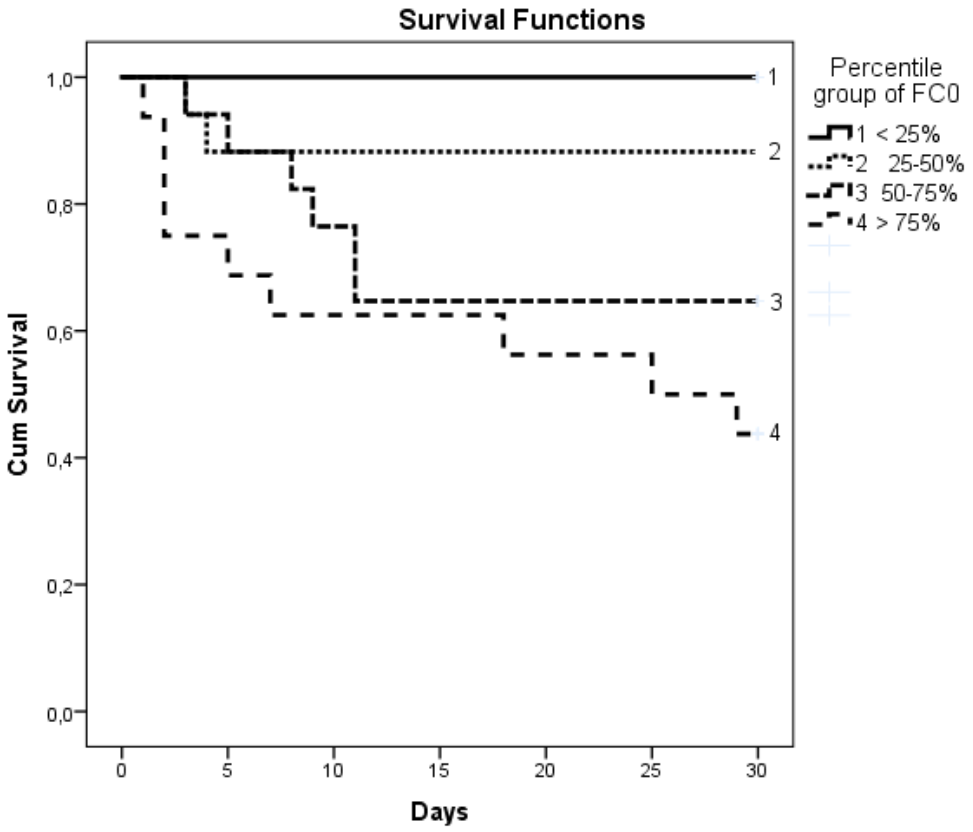

Figure 11. Kaplan-Meier curves of survival depending on free cortisol quartiles at admission (FC0). Patients belonging to the lowest quartile all survived and the mortality increased with increasing free cortisol level. Free cortisol 25%: 6.0 nmol/L, 50%: 26.7 nmol/L, 75%: 173.3 nmol/L

4.2.5 Mortality predicting models using total and free cortisol concentrations

Based on this excellent prognostic role of cortisol levels, they were included in mortality predicting models together with gender, age and the complex clinical scores, APACHE II and SAPS II mortality. FC6, FC24 and FC48 turned to be independent determinants of mortality beside APACHE II and SAPS II. The sensitivity, specificity and validity of these models were surprisingly high (Table 8). It is notable that the clinical scores created by 12 or 17 parameters and the cortisol as a single parameter are comparable in their predictive roles.

Table 8. Independent determinants of mortality in binary logistic regression analysis using six predictive models - observed mortality was used as a dependent variable

	Model 1	**p value**	**Model 2**	**p value**	**Model 3**	**p value**
	Gender Age APACHE II-mortality FC6		Gender Age APACHE II-mortality FC24		Gender Age APACHE II-mortality FC48	
independent determinant	FC6 APACHE II-mortality	0.058 0.006	FC24 APACHE II-mortality	0.004 0.001	FC48 age	0.021 0.056
Cox & Snell R^2	0.267		0.394		0.419	
Nagelkerke R^2	0.393		0.572		0.658	
sensitivity	87		86		96.8	
specificity	37.5		56.3		62.5	
validity	74.2		78		89.7	

	Model 4	**p value**	**Model 5**	**p value**	**Model 6**	**p value**
	Gender Age SAPS II - mortality FC6		Gender Age SAPS II - mortality FC24		Gender Age SAPS II - mortality FC48	
independent determinant	FC6 SAPS II - mortality	0.024 0.001	FC24 SAPS II - mortality	0.003 <0.001	FC48 SAPS II - mortality	0.023 0.051
Cox & Snell R^2	0.331		0.477		0.470	
Nagelkerke R^2	0.486		0.692		0.738	
sensitivity	89.1		90.7		96.8	
specificity	56.3		81.3		75.0	
validity	80.6		88.1		92.3	

The contribution of intubation and mechanical ventilation per se to cortisol elevation is difficult to estimate. To approach this question, the role of ventilation, mortality scores, gender, age and cortisol levels were investigated in binary logistic regression models. The need for ventilation was very strong determinant of mortality, even stronger than the mortality scores. However, FC6, FC24 and FC48 remained independent determinant of mortality beside ventilation. These data confirms that the intubation and ventilation themselves may, at least partly be responsible for the significant differences in cortisol levels of survivor and non-survivor patient population.

5 Discussion

5.1 Establishment of a new method for cortisol measurement using LC-MS

Commonly, targeted mass-spectrometry analysis is carried out using triple-quadrupole mass spectrometers. The advantage of these instruments is their ability to perform fragmentation of the analyte, providing a significant fragmentation pattern. Specificity is achieved by comparing the chromatographic retention time of the IS and the analyte and by scanning the previously identified molecular fragments - commonly two or three. Specific quantification is performed by integrating the fragment-ion peaks instead of the parent mass.

The other way of achieving specific mass-spectrometry molecular identification is using the accurate monoisotopic mass of the molecule instead of performing fragmentation before detection. The accurate theoretical monoisotopic mass of a molecule is a result of its elemental composition. If we can improve mass accuracy we can determine lower mass differences between two molecules. As a result, specificity increases, because we are able to exclude molecules having similar molecular mass but different elemental compositions. The other consequence of high mass accuracy is the possibility of using a narrow mass window on the EIC. If the accurate mass cannot be determined, a wide EIC mass window must be used, and there will be an increasing chance that the integrated chromatographic peak will also contain other compounds.

Quantification in our method was performed using a 5.5 ppm-wide mass window, with no interference observed even with coeluting compounds of similar mass. This is a very important aspect when developing an alternative method to immunoassays, because the major advantage of mass spectrometry over immunoassays is the higher specificity. Steroids administered as therapeutic drugs often cross-react with antibodies raised against cortisol. A work published by Han et al. [Han, 2011] described the use of a quadrupole time of flight (QTOF) instrument, where a 25 ppm mass accuracy was achieved and a 50 ppm-wide mass window was used for quantification. However, the authors did not publish data on interference studies.

Compound identification and quantification on the basis of accurate mass, instead of including an MRM or selected reaction monitoring (SRM) transition during method development, has several advantages. It may simplify transferring the

method to another instrument, because there is no need to develop an MRM transition. The other important advantage of an ESI-TOF instrument is that it is possible to scan a wide mass range without losing specificity, because accurate mass identification is possible for every compound present in the chromatographic run. This makes it possible to search for any additional compounds (hormone metabolites, therapeutic drugs, etc.) in sample data after the analysis. To achieve specificity using common triplequadrupole instruments, an MRM or SRM transition has to be developed before analysis for each potentially interesting compound. This means it is not possible to do a specific search for new compounds after the run is completed.

During the validation of the HPLC-MS method, LOD and LOQ values were first established. The LOQs for FC (440 pmol/L), SC (600 pmol/L) and TC (12.5 nmol/L) are well below the ranges commonly observed under physiological conditions (1-8 nmol/L FC and SC, 100-800 nmol/L TC) [McWhinney, 2010]; however, that for TC is much higher than for the two other assays. This is because TC has a relatively high concentration range, well within the sensitivity of mass spectrometry, so there was no need to use SPE sample concentration. Instead, a simple protein precipitation with acetonitrile was suitable, as seen from the results of the recovery, variation, and ion suppression measurements. This step results in dilution of the sample, because acetonitrile has to be added in a 1:3 ratio to achieve an adequate degree of precipitation. When higher sensitivity is required for TC (e.g. for applications related to adrenal insufficiency or Addison's disease), reversed-phase SPE concentration can be used; however, this means protein precipitation by organic solvents is not possible. Use of heavy metal salts [Hempen, 2012], for example $ZnSO_4$, which was tested in our laboratory, is a possible option (data not shown). The precipitation salt is removed from the sample during the washing step of the SPE.

The results of the intra- and inter-assay studies are detailed in the previous section. The CV value for TC, where no sample clean-up was involved, was in a range comparable with the variation of FC and SC measurements, where the use of SPE resulted in the removal of some undesired compounds. The averaged intra-assay CV value for TC is 4 %, and for FC and SC the value is 5 %. The averaged inter-assay CV value for TC is 8.1 %, whereas for FC and SC the value is 6 %. These results concur with those described in literature [Pretorius, 2011; Perogamvros, 2009]. Comparison with the immunoassays resulted in a positive bias in the higher

concentration ranges for TC and SC, as described elsewhere [Pretorius, 2011; Miller 2013]. We assume that this is caused by cross-reactions of the immunoassays with other matrix compounds. Usually our method underestimated cortisol compared with the results of all three immunoassays; this was less notable when comparing the results with the validated clinical Roche Modular E 170 ECLIA assay. We observed the best correlation of our results with the results of this immunoassay.

During the analysis of the TC, FC, and SC samples, observed concentration ranges were similar to the values established in other studies. However, the mean concentration of our samples was slightly higher than those described in other papers [Pretorius, 2011; Kushnir, 2004]: we found these values to be 372 nmol/L (TC), 3.7 nmol/L (FC), and 3.6 nmol/L (SC), whereas in other studies these values were found to be under 300 nmol/L (TC) and 3 nmol/L (FC, SC). This might be because our sampling was performed in the morning hours.

5.2 Free and total serum cortisol levels are useful prognostic markers in critically ill patients

In this prospective observational study, total and free cortisol levels were determined by LC-MS in the serum of critically ill patients with medical emergencies. An extremely wide range of cortisol concentrations were found upward exceeding the upper limit of normal by 13 times in total and 95 times in free cortisol levels. Both total and free cortisol levels were elevated at admission compared to later time points. Free cortisol within 2 days and total cortisol within 6 hours correlated with the observed mortality. Patients with higher cortisol levels had higher risk to die. The requirement for ventilation may be partly responsible for the higher cortisol levels in non-survivors. The prognostic role of cortisol concentration was compared to the routinely used clinical mortality scores APACHE II and SAPS II. Vital signs, clinical parameters, laboratory values are included in these scores systems. The diagnostic value of cortisol concentrations to predict mortality was similar to APACHE II and SAPS II. Furthermore, FC6, FC24 and FC48 proved to be independent determinants of mortality even in predicting models including – beyond gender and age – these complex clinical scores. Information provided by these cortisol levels as single parameters about the prognosis were similar to complex mortality scores that have been formed by 12 and 17 parameters, respectively.

There are some key reports investigating the total serum cortisol in critically ill patients [Cohen, 2006; Clark, 2011; Arafah, 2006]. Free cortisol levels were measured in few papers and sparse data are available about free cortisol determined by LC-MS [Clark, 2011; Burt, 2013; Shackleton, 2010; Vassiliadi, 2013; Cohen, 2013]. Only one paper is available where both total and free cortisol levels were measured by LC-MS in critically ill patients [Cohen, 2012]. Cortisol measurements using immunoassays are disturbed by many analytical errors, especially in critically ill patients. The changes in CBG and albumin concentrations, the cross-reactivity of antibodies to similar chemical structures and heterophilic antibodies all influence the results. These potential errors are eliminated by LC-MS method [Briegel, 2009; Clark, 2011; Burt, 2013]. Our cortisol method using LC-MS was previously validated in normal subjects [Montskó, 2014]. The method is capable of specific cortisol quantification in different matrices on the basis of accurate mass identification. The measurement of total cortisol is quite simple and accurate; therefore nowadays this method is used routinely for this purpose in our institute. The measurement of free cortisol requires more complex sample preparation which is a disadvantage in the everyday routine. Otherwise all LC-MS methods should be carefully validated [Clark, 2011]. Considering all these factors, free cortisol measured by LC-MS is the best method to evaluate the adrenal response in critically ill patients but for prognostic purposes total cortisol measured by LC-MS seems to be more available in the daily practice. Commonly, targeted mass-spectrometry analysis is carried out using triple-quadrupole mass spectrometers. The method and the way of analysis are different compared to our measurement but both assessments give similar results without the disadvantages of IAs [Montskó, 2014]. We believe that our results are fully transferable to cortisol levels determined by triple-quadrupole MS.

Beyond the practical consequences, our results have theoretical aspects. The cortisol response to medical emergencies seems to be proportional to the severity of illness. It can be presumed that the adrenal response is appropriate and maximal in life-threatening conditions [Melby, 1958; Chernow, 1987; Arafah, 2006]. It is not clear whether the cortisol level should be further increased by ACTH stimulation. According to a new concept of cortisol homeostasis in critical illness, ACTH is not elevated, even suppressed due to the high cortisol level. It is possible that ACTH stimulation test does not result in significant cortisol elevation in those patients who

already have high cortisol level. They are regarded having relative adrenal insufficiency [Dimopoulou, 2007; Knapp, 2004; Kwon, 2009; Norasyikin, 2009]. The overproduction of cortisol is also debated. The primary cause of cortisol elevation probably is the decreased metabolism of glucocorticoids [Van den Berghe 2003]. The concept of RAI should be reappraised based on these new studies. Our results did not support the existence of RAI and its role in the poor prognosis of critically ill patients. Further investigations are essential in patients with unresponsive septic shock where the high dose hydrocortisone treatment is a routine therapy [Sprung, 2008; Toma, 2011; Arafah, 2006].

Limitation of our study is the relatively small patient population as multiple cortisol measurements restricted the size of study. However, the investigated prognostic markers including cortisol had high statistical power. Although the heterogeneity of underlying diseases might be regarded as another potential weakness on the investigation, severity of various diseases were similar, therefore the same prognostic models could be applied to all of them.

6 Conclusions

In conclusion, our HPLC-MS assay based on accurate ESI-TOF-MS mass detection proved to be a real alternative to the common triple-quadrupole MS assays, with less complicated method development. The performance of the method was demonstrated on three clinically important laboratory variables. The only potential disadvantage of our method is that a triple-quadrupole instrument usually offers higher sensitivity. However, the LOD and LOQ achieved in this work are much lower than accepted and described physiological ranges. The use of a simpler mass-spectrometry tool might reduce instrument-to-instrument or laboratory-to-laboratory variation. Furthermore, with a high-resolution instrument we have the ability to search with high specificity for any new potentially interesting compounds (drugs, metabolites) present in the sample run, even after the measurement is completed. This feature enables us to overcome the main disadvantage of triple-quadrupole mass spectrometry, which cannot specifically detect compounds present in the sample but excluded from the process of MRM-transition development. Using an accurate mass spectrometry instrument, however, a high number of different compounds can be monitored in a single analytical run.

The total and free cortisol concentrations in critically ill patients varied in so wide range, that the highest total cortisol exceeded 13 times, free cortisol 95 times of the upper limit of reference range. Both total and free cortisol levels measured by LC-MS were useful prognostic markers, patients with higher cortisol levels had higher mortality risk. Free cortisol had an advantage compared to total cortisol being predictive for mortality in the first 2 days after admission. Free cortisol at 6, 24 and 48 hours proved to be independent predictor of mortality in prognostic models even including the SAPS II and APACHE II mortality scores. Cortisol levels in critical illness probably reflect the severity of disease.

7 Novel findings

7.1 We have validated a novel LC-MS method for measurement of

a) serum total cortisol
b) serum free cortisol
c) salivary cortisol.

7.2 Our method is capable of specific cortisol quantification in different matrices on the basis of accurate mass identification. The measurement of total cortisol is quite simple, accurate and cost-effective; therefore nowadays this method is used routinely in our institute.

7.3 Our study was the first where both total and free serum cortisol levels were measured by LC-MS in a mixed population of critically ill patients with medical emergencies.

7.4 We have proven that serum total and free cortisol concentrations measured by LC-MS are able to predict morality and reflect the severity of disease. Consequently both of them could be a useful prognostic marker in critical illness.

7.5 Our data support that the prognostic value of cortisol concentrations to predict mortality was similar to the routinely used clinical mortality scores (APACHE II and SAPS II) which are the best known predictors of mortality.

7.6 Our data also demonstrated that critically ill patients with higher cortisol levels had higher mortality risk and that cortisol levels at different time points of the critical condition proved to be independent determinants of mortality.

7.7 These findings could help the application of new prognostic biomarkers in critical illness.

Bibliography

1. Annane D, Sébille V, Troché G, Raphaël JC, Gajdos P, Bellissant E: A 3-level prognostic classification in septic shock based on cortisol levels and cortisol response to corticotropin. *JAMA* 2000;23:1038-1045
2. Annane D: Time for a consensus definition of corticosteroid insufficiency in critically ill patients. *Crit Care Med* 2003;31:1868-1869
3. Arafah BM: Hypothalamic pituitary adrenal function during critical illness: limitations of current assessment methods. *J Clin Endocrinol Metab* 2006;91:3725-3745
4. Arregger AL, Cardoso EM, Tumilasci O, Contreras LN: Diagnostic value of salivary cortisol in end stage renal disease. *Steroids* 2008;73:77-82
5. Beishuizen A, Thijs L, Vermes I: Patterns of corticosteroid-binding globulin and the free cortisol index during septic shock and multitrauma. *Intensive Care Med* 2001;27:1584-1591
6. Beishuizen A, Thijs LG: Relative adrenal failure in intensive care: an identifiable problem requiring treatment? *Best Pract Res Clin Endocrinol Metab* 2001;15:513-531
7. Beishuizen A, Vermes I, Hylkema BS, Haanen C: Relative eosinophilia and functional adrenal insufficiency in critically ill patients. *Lancet* 1999;353:1675-1676
8. Boelen A, Boorsma J, Kwakkel J: Type 3 deiodinase is highly expressed in infiltrating neutrophilic granulocytes in response to acute bacterial infection. *Thyroid* 2008;18:1095-1103
9. Boelen A, Kwakkel J, Fliers E: Beyond low plasma T_3: local thyroid hormone metabolism during inflammation and infection. *Endocr Rev* 2011;32:670-693
10. Bonte HA, van den Hoven RJ, van der Sluijs Veer G, Vermes I: The use of free cortisol index for laboratory assessment of pituitary-adrenal function. *Clin Chem Lab Med* 1999;37:127-132
11. Boonen E, Van den Berghe G: Endocrine responses to critical illness: novel insights and therapeutic implications. *J Clin Endocrinol Metab* 2014;99:1569-1582

12. Boonen E, Vervenne H, Meersseman P: Reduced cortisol metabolism during critical illness. *N Engl J Med* 2013;368:1477-1488
13. Briegel J, Sprung CL, Annane D: Multicenter comparison of cortisol as measured by different methods in samples of patients with septic shock. *Intensive Care Med* 2009;35:2151-2156
14. Burt MG, Mangelsdorf BL, Rogers A, Ho JT, Lewis JG, Inder WJ, Doogue MP: Free and total plasma cortisol measured by immunoassay and mass spectrometry following $ACTH_{1-24}$ stimulation in the assessment of pituitary patients. *J Clin Endocrinol Metab* 2013;98:1883-1890
15. Carrier M, Wild J, Pelletier C: Bromocriptine as an adjuvant to cyclosporine immunosuppression after heart transplantation. *Ann Thorac Surg* 1990;49:129-132
16. Chernow B, Alexander HR, Smallridge RC, Thompson WR, Cook D, Beardsley D, Fink MP, Lake CR, Fletcher JR: Hormonal responses to graded surgical stress. *Arch Intern Med* 1987;147:1273-1278
17. Chiu SK, Collier CP, Clark AF, Wynn-Edwards KE: Salivary cortisol on ROCHE Elecsys immunoassay system: pilot biological variation studies. *Clin Biochem* 2003;36:211-214
18. Clark PM, Gordon K: Challenges for the endocrine laboratory in critical illness. *Best Pract Res Clin Endocrinol Metab* 2011;25:847-859
19. Cohen J, Smith ML, Deans RV, Pretorius CJ, Ungerer JP, Tan T, Jones M, Venkatesh B. Serial changes in plasma total cortisol, plasma free cortisol, and tissue cortisol activity in patients with septic shock: an observational study. *Shock* 2012;37:28-33
20. Cohen J, Venkatesh B, Tan T: Comparison of the diagnostic accuracy of measured and calculated free cortisol in acutely ill patients using the Coolens equation. *Crit Care Resusc* 2013;15:39-41
21. Cohen J, Ward G, Prins J: Variability of cortisol assays can confound the diagnosis of adrenal insufficiency in the critically ill population. *Intensive Care Med* 2006;32:1901-1905
22. Coolens JL, van Baelen H, HeynsW: Clinical use of unbound plasma cortisol as calculated from total cortisol and corticosteroidbinding globulin. *J Steroid Biochem* 1987;26:197-202

23. Cooper MS, Stewart PM: Corticosteroid insufficiency in acutely ill patients. *N Engl J Med* 2003;348:727-734
24. De Palo EF, Antonelli G, Benetazzo A, Prearo M, Gatti R: Human saliva cortisone and cortisol simultaneous analysis using reverse phase HPLC technique. *Clin Chim Acta* 2009;405:60-65
25. Devins SS, Miller A, Herndon BL: Effects of dopamine on T-lymphocyte proliferative responses and serum prolactin concentrations in critically ill patients. *Crit Care Med* 1992;263:9682-9685
26. Dickstein G: On the term "relative adrenal insufficiency" - or what do we really measure with adrenal stimulation tests? *J Clin Endocrinol Metab* 2005;90:4973-4974
27. Dimopoulou I, Stamoulis K, Ilias I, Tzanela M, Lyberopoulos P, Orfanos S, Armaganidis A, Theodorakopoulou M, Tsagarakis S: A prospective study on adrenal cortex responses and outcome prediction in acute critical illness: results from alarge cohort of 203 mixed ICU patients. *Intensive Care Med* 2007;33:2116-2121
28. Dossett LA, Swenson BR, Evans HL, Bonatti H, Sawyer RG, May AK: Serum estradiol concentration as a predictor of death in critically ill and injured adults. *Surg Infect (Larchmt)* 2008;9:41-48
29. Durber SM, Daly JR: A simple method for the determination of free cortisol in plasma, with normal levels in men and women, including women taking low oestrogen contraceptives. *Clin Chim Acta* 1976;68:43-48
30. Galbois A, Rudler M, Massard J, Fulla Y, Bennani A, Bonnefont-Rousselot D, Thibault V, Reignier S, Bourrier A, Poynard T, Thabut D: Assessment of adrenal function in cirrhotic patients: salivary cortisol should be preferred. *J Hepatol* 2010;52:839-845
31. Gatti R, Antonelli G, Prearo M, Spinella P, Cappellin E, De Palo EF: Cortisol assays and diagnostic laboratory procedures in human biological fluids. *Clin Biochem* 2009;42:1205-1217
32. Guaraldi F, Salvatori R: Cushing syndrome: maybe not so uncommon of an endocrine disease. *J Am Board Fam Med* 2012;25:199-208
33. Guo H, Calkins JH, Sigel MM: Interleukin-2 is a potent inhibitor of Leydig cell steroidogenesis. *Endocrinology* 1990;127:1234-1239

34. Hamrahian AH, Oseni TS, Arafah BM: Measurements of serum free cortisol in critically ill patients. *N Engl J Med* 2004;350:1629-1638
35. Han J, Kalyan S, Prior JC, Borchers CH: Quantitation of urinary 6ß-hydroxycortisol and free cortisol by ultra-performance liquid chromatography-quadrupole time-of-flight mass spectrometry. *Clin Exp Pharmacol* 2011;doi:10.4172/2161-1459.S5-005
36. Hempen C, Elfering S, Mulder AH, van den Bergh FA, Maatman RG: Dexamethasone suppression test: development of a method for simultaneous determination of cortisol and dexamethasone in human plasma by liquid chromatography/tandem mass spectrometry. *Ann Clin Biochem* 2012;49:170-176
37. Ho JT, Al-Musalhi H, Chapman MJ, Quach T, Thomas PD, Bagley CJ, Lewis JG, Torpy DJ: Septic shock and sepsis: a comparison of total and free plasma cortisol levels. *J Clin Endocrinol Metab* 2006;91:105-114
38. Hogenbooma AC, van Leerdama JA, de Voogta P: Accurate mass screening and identification of emerging contaminants in environmental samples by liquid chromatography-hybrid linear ion trap Orbitrap mass spectrometry. *J Chromatogr A* 2009;1216:510-519
39. Holst JP, Soldin SJ, Tractenberg RE, Guo T, Kundra P, Verbalis JG, Jonklaas J: Use of steroid profiles in determining the cause of adrenal insufficiency. *Steroids* 2007;72:71-84
40. Kauffmann RM, Norris PR, Jenkins JM, Dupont WD, Torres RE, Blume JD, Dossett LA, Hranjec T, Sawyer RG, May AK: Trends in estradiol during critical illness are associated with mortality independent of admission estradiol. *J Am Coll Surg* 2011;212:703-712
41. Kehlet H, Binder C: Adrenocortical function and clinical course during and after surgery in unsupplemented glucocorticoid-treated patients. *Br J Anaesth* 1973;45:1043-1048
42. Kirchhoff F, Briegel J, Vogeser M: Quantification of free serum cortisol based on equilibrium dialysis and isotope dilution liquid chromatography-tandemmass spectrometry. *Clin Biochem* 2011;44:894-899
43. Kley HK, Bartmann E, Krüskemper HL: A simple and rapid method to measure non-protein-bound fractions of cortisol, testosterone and oestradiol

by equilibrium dialysis: comparison with centrifugal filtration. *Acta Endocrinol* 1977;85:209-219

44. Klibanski A, Beitens IZ, Badger TM: Reproductive function during fasting in man. *J Clin Endocrinol Metab* 1981;53:258-66
45. Knapp PE, Arum SM, Melby JC: Relative adrenal insufficiency in critical illness: a review of the evidence. *Curr Opin Endocrinol Diabetes* 2004;11:147-152
46. Knaus WA, Draper EA, Wagner DP, Zimmerman JE: APACHE II: a severity of disease classification system. *Crit Care Med* 1985;13:818-829
47. Kobayashi N, Sun P, FujimakiY, Niwa T, Nishio T, Goto J, Hosoda H: Generation of a novel monoclonal antibody against cortisol-[C-4]-bovine serumalbumin conjugate: application to enzyme-linked immunosorbent assay for urinary and serum cortisol. *Anal Sci* 2002;18:1309-1314
48. Koch A, Sanson E, Helm A, Voigt S, Trautwein C, Tacke F: Regulation and prognostic relevance of serum ghrelin concentrations in critical illness and sepsis. *Crit Care* 2010;14:R94
49. Kohen F, Pazzagli M, Kim JB, Lindner HR: An immunoassay for plasma cortisol based on chemiluminescence. *Steroids* 1980;36:421-437
50. Kominami G, Fujisaka I, Yamauchi A, Kono M: A sensitive enzyme immunoassay for plasma cortisol. *Clin Chim Acta* 1980;103:381-391
51. Kushnir MM, Neilson R, Roberts WL, Rockwood AL: Cortisol and cortisone analysis in serum and plasma by atmospheric pressure photoionization tandem mass spectrometry. *Clin Biochem* 2004;37:357-362
52. Kwon YS, Kang E, Suh GY, Koh WJ, Chung MP, Kim H, Kwon OJ, Chung JH: A prospective study on the incidence and predictive factors of relative adrenal insufficiency in Korean critically-illpatients. *J Korean Med Sci* 2009;24:668-673
53. Le Gall JR, Lemeshow S, Saulnier F: A new Simplified Acute Physiology Score (SAPS II) based on a European/North American multicenter study. *JAMA* 1993;270:2957-2963
54. Lim CF, Docter R, Visser TJ: Inhibition of thyroxine transport into cultured rat hepatocytes by serum of nonuremic critically ill patients: effects of bilirubin and nonesterified fatty acids. *J Clin Endocrinol Metab* 1993;76:1165-1172

55. Loriaux DL, Fleseriu M: Relative adrenal insufficiency. *Curr Opin Endocrinol Diabetes Obes* 2009;16:392-400
56. Marik PE, Pastores SM, Annane D, Meduri GU, Sprung CL, Arlt W, Keh D, Briegel J, Beishuizen A, Dimopoulou I, Tsagarakis S, Singer M, Chrousos GP, Zaloga G, Bokhari F, Vogeser M: Recommendations for the diagnosis and management of corticosteroid insufficiency in critically ill adult patients: consensus statements from an international task force by the American College of Critical Care Medicine. *Crit Care Med* 2008;36:1937-1949
57. McWhinney BC, Briscoe SE, Ungerer JP, Pretorius CJ: Measurement of cortisol, cortisone, prednisolone, dexamethasone and 11-deoxycortisol with ultrahigh performance liquid chromatography-tandem mass spectrometry: application for plasma, plasma ultrafiltrate, urine and saliva in a routine laboratory. *J Chromatogr B Anal Technol Biomed Life Sci* 2010;878:2863-2869
58. Melby JC, Spink WW: Comparative studies on adrenal cortical function and cortisol metabolism in healthy adults and in patients with shock due to infection. *J Clin Invest* 1958;37:1791-1798
59. Meyer B, Huelsmann M, Wexberg P, Delle Karth G, Berger R, Moertl D, Szekeres T, Pacher R, Heinz G: N-terminal pro-B-type natriuretic peptide is an independent predictor of outcome in an unselected cohort of critically ill patients. *Crit Care Med* 2007;35:2268-2273
60. Michalaki M, Vagenakis AG, Makri M, Kalfarentzos F, Kyriazopoulou V: Dissociation of the early decline in serum T(3) concentration and serum IL-6 rise and TNFα in nonthyroidal illness syndrome induced by abdominal surgery. *J Clin Endocrinol Metab* 2001;86:4198-4205
61. Miller R, Plessow F, Rauh M, Gröschl M, Kirschbaum C: Comparison of salivary cortisol as measured by different immunoassays and tandem mass spectrometry. *Psychoneuroendocrinology* 2013;38:50-57
62. Montskó G, Tarjányi Z, Mezősi E, Kovács GL: A validated method for measurement of serum total, serum free, and salivary cortisol, using high-performance liquid chromatography coupled with high-resolution ESI-TOF mass spectrometry. *Anal Bioanal Chem* 2014;406:2333-2341

63. Nishiyama M, Makino S, Suemaru S, Nanamiya W, Asaba K, Kaneda T, Mimoto T, Nishioka T, Takao T, Hashimoto K: Glucocorticoid effects on the diurnal rhythm of circulating leptin levels. *Horm Res* 2000;54:69-73
64. Norasyikin AW, Norlela S, Rozita M, Masliza M, Shamsul AS, Nor Azmi K: Adrenal insufficiency in acute coronary syndrome. *Singapore Med J* 2009;50:962-966
65. Oppert M, Reinicke A, Gräf KJ, Barckow D, Frei U, Eckardt KU: Plasma cortisol levels before and during "low-dose" hydrocortisone therapy and their relationship to hemodynamic improvement in patients with septic shock. *Intensive Care Med* 2000;26:1747-1755
66. Peeters RP, Wouters PJ, van Toor H, Kaptein E, Visser TJ, Van den Berghe G: Serum 3,3',5'-triiodothyronine (rT_3) and 3,5,3'-triiodothyronine/ rT_3 are prognostic markers in critically ill patients and are associated with postmortem tissue deiodinase activities. *J Clin Endocrinol Metab* 2005;90:4559-4565
67. Perogamvros I, Owen LJ, Newell-Price J, Ray DW, Trainer PJ, Keevil BG: Simultaneous measurement of cortisol and cortisone in human saliva using liquid chromatography-tandem mass spectrometry: application in basal and stimulated conditions. *J Chromatogr B Anal Technol Biomed Life Sci* 2009;877:3771-3775
68. Polgár L, García-Reyes JF, Fodor P, Gyepes A, Dernovics M, Abrankó L, Gilbert-López B, Molina-Díaz A: Retrospective screening of relevant pesticide metabolites in food using liquid chromatography high resolution mass spectrometry and accuratemass databases of parent molecules and diagnostic fragment ions. *J Chromatogr A* 2012;1249:83-91
69. Pretorius CJ, Galligan JP, McWhinney BC, Briscoe SE, Ungerer PJ: Free cortisol method comparison: ultrafiltration, equilibrium dialysis, tracer dilution, tandem mass spectrometry and calculated free cortisol. *Clin Chim Acta* 2011;412:1043-1047
70. Rady MY, Johnson DJ, Patel B, Larson J, Helmers R: Cortisol levels and corticosteroid administration fail to predict mortality in critical illness: the confounding effects of organ dysfunction and sex. *Arch Surg* 2005;140:661-668

71. Restituto P, Galofré JC, Gil MJ, Mugueta C, Santos S, Monreal JI, Varo N: Advantage of salivary cortisol measurements in the diagnosis of glucocorticoid related disorders. *Clin Biochem* 2008;41:688-692
72. Rhen T, Cidlowski JA: Antiinflammatory action of glucocorticoids-new mechanisms for old drugs. *N Engl J Med* 2005;353:1711-1723
73. Ronchi CL, Leich E, Sbiera S, Weismann D, Rosenwald A, Allolio B, Fassnacht M: Single nucleotide polymorphism microarray analysis in cortisol-secreting adrenocortical adenomas identifies new candidate genes and pathways. *Neoplasia* 2012;14:206-218
74. Ross IL, Levitt NS, Van der Walt JS, Schatz DA, Johannsson G, Haarburger DH, Pillay TS: Salivary cortisol day curves in Addison's disease in patients on hydrocortisone replacement. *Horm Metab Res* 2013;45:62-68
75. Ross R, Miell J, Freeman E, Jones J: Critically ill patients have high basal growth hormone levels with attenuated oscillatory activity associated with low levels of insulinlike growth factor-1. *Clin Endocrinol* 1991;35:47-54
76. Rothwell PM, Udwadia ZF, Lawler PG: Cortisol response to corticotropin and survival in septic shock. *Lancet* 1991;337:582-583
77. Sam S, Corbridge TC, Mokhlesi B, Comellas AP, Molitch ME: Cortisol levels and mortality in severe sepsis. *Clin Endocrinol (Oxf)* 2004;60:29-35
78. Sánchez E, Singru PS, Fekete C, Lechan RM: Induction of type 2 iodothyronine deiodinase in the mediobasal hypothalamus by bacterial lipopolysaccharide: role of corticosterone. *Endocrinology* 2008;149:2484-2493
79. Schuetz P, Müller B, Nusbaumer C, Wieland M, Christ-Crain M: Circulating levels of GH predict mortality and complement prognostic scores in critically ill medical patients. *Eur J Endocrinol* 2009;160:157-163
80. Schuster KM, Macleod JB, Fernandez JB, Kumar M, Barquist ES: Adrenocorticotropic hormone and cortisol response to corticotropin releasing hormone in the critically ill-a novel assessment of the hypothalamic-pituitary-adrenal axis. *Am J Surg* 2012;203:205-210
81. Scott WJ: The influence of the adrenal glands on resistance: I. The susceptibility of adrenalectomized rats to morphine. *J Exp Med* 1923;38:543-560

82. Selye H: A syndrome produced by diverse nocuous agents. 1936. *J Neuropsychiatry Clin Neurosci* 1998;10:230-231
83. Sereg M, Toke J, Patócs A, Varga I, Igaz P, Szücs N, Horányi J, Pusztai P, Czirják S, Gláz E, Rácz K, Tóth M: Diagnostic performance of salivary cortisol and serumosteocalcin measurements in patients with overt and subclinical Cushing's syndrome. *Steroids* 2011;76:38-42
84. Shackleton C: Clinical steroid mass spectrometry: a 45-year history culminating in HPLC-MS/MS becoming an essential tool for patient diagnosis. *J Steroid Biochem Mol Biol* 2010;121:481-490
85. Sharshar T, Bastuji-Garin S, Polito A, De Jonghe B, Stevens RD, Maxime V, Rodriguez P, Cerf C, Outin H, Touraine P, Laborde K; Groupe de Réflexion et d'Etude des Neuromyopathies En Réanimation: Hormonal status in protracted critical illness and in-hospital mortality. *Crit Care* 2011;15:R47
86. Spratt DI, Cox P, Orav J: Reproductive axis suppression in acute illness is related to disease severity. *J Clin Endocrinol Metab* 1993;76:1548-1554
87. Sprung CL, Annane D, Keh D, Moreno R, Singer M, Freivogel K, Weiss YG, Benbenishty J, Kalenka A, Forst H, Laterre PF, Reinhart K, Cuthbertson BH,Payen D, Briegel J, CORTICUS Study Group: Hydrocortisone therapy for patients with septic shock. *N Engl J Med* 2008;358:111-124
88. Takala J, Ruokonen E, Webster NR: Increased mortality associated with growth hormone treatment in critically ill adults. *N Engl J Med* 1999;341:785-792
89. Toma A, Stone A, Green RS, Gray S: Steroids for patients in septic shock: the results of the CORTICUS trial. *CJEM* 2011;13:273-276
90. Tomlinson JW, Walker EA, Bujalska IJ, Draper N, Lavery GG, Cooper MS, Hewison M, Stewart PM: 11-beta-hydroxysteroid dehydrogenase type 1: a tissue-specific regulator of glucocorticoid response. *Endocr Rev* 2004;25:831-866
91. Udelsman R, Ramp J, Gallucci WT: Adaptation during surgical stress. A reevaluation of the role of glucocorticoids. *J Clin Invest* 1986;77:1377-1381
92. Van den Berghe G, de Zegher F, Lauwers P: Luteinizing hormone secretion and hypoandrogenemia in critically ill men: effect of dopamine. *Clin Endocrinol* 1994;41:563-569

93. Van den Berghe G: Endocrine evaluation of patients with critical illness. *Endocrinol Metab Clin North Am* 2003;32:385-410
94. Van Waas M, Neggers SJ, van Eck JP, van Noesel MM, van der Lely AJ, de Jong FH, Pieters R, van den Heuvel-Eibrink MM: Adrenal function in adult long-term survivors of nephroblastoma and neuroblastoma. *Eur J Cancer* 2012;48:1159-1166
95. Vassiliadi DA, Ilias I, Tzanela M, Nikitas N, Theodorakopoulou M, Kopterides P, Maniatis N, Diamantakis A, Orfanos SE, Perogamvros I, Armaganidis A, Keevil BG, Tsagarakis S, Dimopoulou I: Interstitial cortisol obtained by microdialysis in mechanically ventilated septic patients: correlations with total and free serum cortisol. *J Crit Care* 2013;28:158-165
96. Venkataraman S, Munoz R, Candido C, Witchel SF: The hypothalamic-pituitary-adrenal axis in critical illness. *Rev Endocr Metab Disord* 2007; 8:365-373
97. Venkatesh B, Cohen J: Adrenocortical (dys)function in septic shock - a sick euadrenal state. *Best Pract Res Clin Endocrinol Metab* 2011;25:719-733
98. Venkatesh B, Mortimer RH, Couchman B: Evaluation of random plasma cortisol and the low dose corticotropin test as indicators of adrenal secretory capacity in critically ill patients: a prospective study. *Anaesth Intensive Care* 2005;33:201-209
99. Vermes I and Beishuizen A: The hypothalamicpituitary-adrenal response to critical illness. *Best Pract Res Clin Endocrinol Metab* 2001;15:495-511
100. Voerman HJ, Strack van Schijndel RJM, de Boer H: Growth hormone: secretion and administration in catabolic adult patients, with emphasis on the critically ill patient. *Neth J Med* 1992;41:229-244
101. Wajner SM, Goemann IM, Bueno AL, Larsen PR, Maia AL: IL-6 promotes nonthyroidal illness syndrome by blocking thyroxine activation while promoting thyroid hormone inactivation in human cells. *J Clin Invest* 2011;121:1834-1845
102. Walkey AJ, Rice TW, Konter J, Ouchi N, Shibata R, Walsh K, deBoisblanc BP, Summer R: Plasma adiponectin and mortality in critically ill subjects with acute respiratory failure. *Crit Care Med* 2010;38:2329-2334
103. Wang C, Chan V, Tse TF: Effect of acute myocardial infarction on pituitary testicular function. *Clin Endocrinol* 1978;9:249-253

104. Wang C, Chan V, Yeung RTT: Effect of surgical stress on pituitary-testicular function. *Clin Endocrinol* 1978;9:255-266

105. Wear JE, Owen LJ, Duxbury K, Keevil BG: A simplified method for the measurement of urinary free cortisol using LC-MS/ MS. *J Chromatogr B Anal Technol Biomed Life Sci* 2007;858:27-31

106. Zhang F, Yu C, Wang W, Fan R, Zhang Z, Guo Y: Rapid simultaneous screening and identification of multiple pesticide residues in vegetables. *Anal Chim Acta* 2012;757:39-47

List of Publications of the author

Publications

Publications related to the thesis

Montskó G*****, **Tarjányi Z***, Mezősi E, Kovács GL: A validated method for measurement of serum total, serum free, and salivary cortisol, using high-performance liquid chromatography coupled with high-resolution ESI-TOF mass spectrometry. *Anal Bioanal Chem* 2014;406:2333-2341
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Tarjányi Z, Montskó G, Kenyeres P, Márton Zs, Hágendorn R, Gulyás E, Nemes O, Bajnok L, Kovács GL, Mezősi E: Free and total cortisol levels are useful prognostic markers in critically ill patients: a prospective observational study.
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Publications not discussed in the thesis

Nemes O, Kovács N, Czeiter E, Kenyeres P, **Tarjányi Z**, Bajnok L, Büki A, Dóczi T, Mezősi E: Predictors of post-traumatic pituitary failure during long term endocrine follow-up
Accepted for publication in Hormones
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Nemes O, Csiszár A, Rucz K, Bajnok L, Bódis B, Gulyás E, **Tarjányi Z**, Nagy Zs, Dóczi T, Mezősi E: A hypophysisadenomás betegek gondozásával szerzett tapasztalataink. *Magyar Belorvosi Archívum* 2011. (64. évf.), 5. sz., 273-278. p.

Presentation and poster abstracts

Presentations and posters related to the thesis

Tarjányi Z, Montskó G, Mezősi E, Kovács GL: Összkortizol szint mérés LC-ESI TOF tömegspektrometriával humán szérumban. *24th Congress of the Hungarian Society of Endocrinology and Metabolism,* Szolnok, 2012.

Montskó G, **Tarjányi Z**, Mezősi E, Kovács GL: Vér össz-, szabad, valamint nyál kortizol hormon szintek meghatározása LC-ESI TOF tömegspektrometriával. *42nd Membrane Transport Conference,* Sümeg, 2012.

Tarjányi Z, Montskó G, Mezősi E, Kovács GL: Measurement of serum total cortisol using high performance liquid chromatography coupled ESI-TOF mass spectrometry. *From Medicine to Bionics, 1st European PhD Conference,* Budapest, 2013.

Tarjányi Z, Montskó G, Mezősi E, Kovács GL: Measurement of serum total cortisol using HPLC coupled ESI-TOF mass spectrometry. *16th European Congress of Endocrinology,* Wrocław, 2014.

Tarjányi Z, Montskó G, Kenyeres P, Márton Zs, Hágendorn R, Gulyás E, Nemes O, Bajnok L, Kovács GL, Mezősi E: A kortizol hasznos prognosztikai marker kritikus állapotú betegekben. *25th Congress of the Hungarian Society of Endocrinology and Metabolism,* Pécs, 2014.

Other presentations and posters

Schmidt E, Mezosi E, **Tarjanyi Z**, Molnar TF, Szabo Z, Szekeres S, Rucz K, Nemes O, Zambo K: Successful localization of bronchial carcinoid causing ectopic Cushing's syndrome by SPECT/CT fusion imaging. *European Journal of Nuclear Medicine and Molecular Imaging* 37:(Suppl. 2) p. S349., 2010

Tarjányi Z, Peti A, Kőszegi T, Bajnok L, Bódis B, Rucz K, Karádi O, Kovács GL, Mezősi E: A kromogranin A meghatározás tapasztalatai. *Endocrine Spring Training Course,* Velence, 2011.

Nemes O, Csiszár A, Rucz K, Bajnok L, Bódis B, Gulyás E, **Tarjányi Z,** Nagy Zs, Dóczi T, Mezősi E: Hypophysis adenomás betegek gondozásával szerzett tapasztalataink. *24th Congress of the Hungarian Society of Endocrinology and Metabolism,* Szolnok, 2012.

Nemes O, Kovács N, Czeiter E, Kenyeres P, **Tarjányi Z**, Bajnok L, Büki A, Dóczi T, Mezősi E: Traumás koponyasérülést követő hypophysis funkciózavar előrejelzői hosszútávú endokrin követés során. *25th Congress of the Hungarian Society of Endocrinology and Metabolism,* Pécs, 2014.

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