3. MATERIAL AND METHODS

3.1. INTRODUCTION TO THE CLINICAL METHOD

Present study entitled “A Prospective Biochemical Study of Systemic Stress Responses during Cholecystectomy & Abdominal Hysterectomy” included total of 63 patients who were operated in the Jawaharlal Nehru Medical College Hospital, A. M. U., Aligarh (UP), India. The study was conducted over a period of four years from October 2004 to September 2008. Patients were divided in three groups viz. the Laparoscopic Cholecystectomy (LC) group, Open Cholecystectomy (OC) group and Open Abdominal Hysterectomy (OH) group. Each group comprised of twenty one patients. The perioperative systemic stress response was evaluated with the help of changes in Biochemical Stress Markers and Clinical Parameters. The biochemical markers used for the above study were Serum Glucose, Serum Cortisol, Serum Adrenaline, Serum Noradrenaline, C-reactive protein and Tumour Necrosis Factor-alpha (TNF-alpha). Haemodynamic Responses were analysed with the help of perioperative changes in Pulse Rate (PR), Systolic Blood Pressure (S.B.P.), Diastolic Blood Pressure (D.B.P.) and Mean Arterial Pressure (M.A.P.), as clinical parameters to compare the stress involved to the patients during period of surgery in the three groups.

In each patient five blood samples (Pre-operative, ½ hour after surgery, end of surgery, 1st post-operative day and 4th post-operative day) were timely collected and analysed.

3.2. ETHICAL CONSIDERATIONS

Study protocol was approved by the Board of Studies (BOS) and Committee for Advanced Scientific Research (CASR) of our University. All patients have given written informed consent for the study before operation.

3.3. PATIENT METHOD
MATERIAL AND METHODS

After detailed informed consent, elective cases (ASA grade I and II) with symptomatic uncomplicated cholecystolithiasis, fit for procedure were considered. Patients with acute cholecystitis, pancreatitis, choledocholithiasis, malignancy, jaundice, history of allergy, steroid intake, cytotoxic chemotherapy, pregnancy or hypoproteinaemia and patients requiring intraoperative blood transfusion were excluded from the present study. For control group patients undergoing total abdominal hysterectomy were considered for the study. Patients for pan hysterectomy, carcinoma cervix, uterine malignancy, or on cytotoxic drug were also excluded from the study.

3.4. SAMPLE SIZE

In consultation with our statistician, the sample size was calculated taking into account the reported prevalence of stress during anaesthesia and surgery. After considering the prevalence of stress in surgical patients to 100% from previous studies and past records, if we take it 95% and allowed an error of 10% of the prevalence rate of stress rate on either side and wished to correct our estimate in 95 out of 100 patients. Hence if p is the prevalence of stress rate in percentage and q is the non stress rate in percentage, \( (p + q = 100) \), then the standard error is \( \sqrt{pq/n} \) where n is the sample size. Here in the p=95%, q=5%, hence the sample found to be \( 2\sqrt{pq/n} = 10\% \) of p; therefore: \( (4 \times 95 \times 5)/n = (10 \times 95/100) \times (10 \times 95/100) \); and as a result \( n = 21 \). Hence, each group comprised of 21 patients, with total of 63 patients.

3.5. SELECTION CRITERIA FOR LAPAROSCOPIC VS. OPEN PROCEDURE

This study is a prospective randomized study, where patients were consented and discussed for specific procedures before surgery. Surgical procedure opted depends on many factors-

- **Patient’s finances:** The existing financial circumstances of the patients including patients’ inability to expend extra money for laparoscopic procedure (The high cost involvement in LC procedure (LC is 40% more expensive than open procedure (Barkun et al, 1995))).
MATERIAL AND METHODS

- **Patient's preference:** for specific general surgeon who lack suitable expertise in Laparoscopic technique.

- **Lack of facilities:** de novo absence or malfunctioning of existing gazette. Furthermore, being a government hospital, equipment failure do occurs in the form of lack of CO₂ cylinder supply or broken endoscopic instrument which takes long time to get repaired.

- **Patient's insistence:** patient's phobia of complications after laparoscopic cholecystectomy based on bad experience of his/her kith & kin.

- **Lack of expertise:** as laparoscopic surgeon were not available.

3.6. SAMPLE COLLECTION

5ml of venous blood was withdrawn from antecubital vein in plain sterile glass vial for every sample. The samples were sent immediately to biochemical laboratory for centrifugation at 3000 G for 10 min. for separation of serum in the department of Biochemistry. The sera were put in plastic tubes and transported in ice box for storage in deep freezer at -60°C in our nearby Institute of Microbiology where the biochemical analysis was done by the competent laboratory personnel who were unaware of the study, test serum and surgical intervention given to patients.

3.7. SAMPLE TIMES

Serial measurement of various biomarkers were done by following blood sampling:

**First** baseline sample was collected preoperatively after overnight fasting

**Second** sample at half an hour after start of surgery (mostly correspond to the time of separation of gall bladder from liver bed)

**Third** sample at the time of completion of surgery

**Fourth** sample on 1st post-operative day

**Fifth** sample on 4th post-operative day.

3.8. PERIOPERATIVE PROTOCOL
MATERIAL AND METHODS

All cases were first screened through pre-anaesthetic clinic including detailed information about relevant history, clinical examination; related investigations were recorded in the pre-anaesthetic card and then planned for the surgery. Details of the procedure were explained to all the patients in PAC clinic itself. Diazepam (5 mg) orally in the night before the operation and advice for overnight fasting was given to all patients.

3.9. ANAESTHESIA

Patients received midazolam (0.04 mg kg\(^{-1}\)) intravenously in the preoperative room 30 minutes before induction of anaesthesia and later on, injection metoclopramide (0.2 mg kg\(^{-1}\)) and fentanyl (2 \(\mu\)g kg\(^{-1}\)) bolus were given as pre-emptive adjuvant to maintain surgical analgesia; fentanyl was repeated 1 \(\mu\)g kg\(^{-1}\) h\(^{-1}\) till the surgery was completed. All the patients were anaesthetized with injection thiopentone sodium (6-7 mg kg\(^{-1}\)). Tracheal intubation was facilitated with the help of suxamethonium (1.5 mg kg\(^{-1}\)). Surgical relaxation was maintained with vecuronium bromide (0.08 mg kg\(^{-1}\)) as bolus and then intermittent repeat doses, titrated with train-of-four response of 0-1 twitches. To maintain the systolic blood pressure within 20% of the basal level, halothane (0.5-1%) was used. The inspired oxygen fraction (\(\text{FiO}_2\)) was adjusted to 0.4 percent in 60% nitrous oxide to maintain the \(\text{SpO}_2\) above 98%. All the patients were ventilated mechanically at the rate of 12 breaths min\(^{-1}\). Perioperative monitoring includes HR, MAP, EKG, pulse oximetry and end-tidal carbon dioxide (ET\(\text{CO}_2\)) which was maintained in the range of 32 to 35 mmHg. Before reversal of anaesthesia, deep intramuscular injection of diclofenac sodium (1.5 mg kg\(^{-1}\)) was given to all patients as part of our routine anaesthetic practice. The neuromuscular block was reversed with neostigmine (0.05 mg kg\(^{-1}\)) and glycopyrrrolate (0.008 mg kg\(^{-1}\)) at end of surgery.

3.10. FLUID THERAPY

Intravenous fluid regime comprised of 8-10 ml kg\(^{-1}\) hr\(^{-1}\) of Ringer’s lactated solution during intraoperative period and this regime was continued for 6 to 24 hours at the rate of 80-100 ml hr\(^{-1}\) depending upon the climatic conditions. Usually oral intake was
allowed in the same evening in LC group and in the next morning in OC group subject to patients’ tolerance.

3.11. SURGERY

The surgical procedures were performed by consultant trained surgeons. Open cholecystectomy was performed through right subcostal incision (6-9 cms) and laparoscopic cholecystectomy by 4-trocar technique with electrocautery dissection. Pneumoperitoneum was achieved with carbon dioxide insufflation, maintaining intra-abdominal pressure of 12-14 mmHg. Patients who required per-operative cholangiography or conversion to laparotomy were excluded from the study.

3.12. POST-OPERATIVE CARE

Deep intramuscular injection of diclofenac sodium 1.5mg.kg-1 at every 8 hour was advised in the postoperative order as analgesic protocol. On the 1st day of surgery, injection Pentazocine (0.5mg.kg-1) or injection Tramadol (2 mg.Kg-1) was given through intramuscular route SOS on demand if patient has significant pain inspite of administration of diclofenac sodium.

3.13. EXCLUSION CRITERIA

Female patients who were pregnant or lactating were not included in the present study.

Patients developing significant alterations in their baseline preoperative investigations (Haemoglobin, total and differential leukocytes, serum urea, serum creatinine, serum sodium and potassium, platelets and prothrombin time) during intra-operative or post-operative period were also not taken into consideration for the study. Patients with incomplete blood sampling either due to refusal by the patient or his/her attendant for blood sampling in post-op period or forgetfulness or early discharge from the hospital were also excluded from the present study. Patients found to have intra-operative injury to adjacent organ/structure including bile duct injury were also excluded from the study.
3.14. SERUM GLUCOSE ESTIMATION

Method for Serum glucose analysis was (DPEC- GOD /POD)
For quantitative determination of serum glucose -glucose oxidase (GOD) and glucose peroxidase (POD) method (GOD/POD) was used. Test principle is as follows:

TEST PRINCIPLE:
Following equation occurs on addition of glucose oxidase and peroxidase

\[ \text{Glucose} + \text{O}_2 + \text{H}_2\text{O} \rightarrow \text{Glucoronid acid} + \text{H}_2\text{O}_2 \]

Peroxidase

\[ \text{H}_2\text{O}_2 + \text{Phenol} + 4\text{Aminoantipyrine} \rightarrow \text{Coloured complex} + \text{H}_2\text{O} \]

The red coloured complex formed is Measured at 505 nm and the intensity of the colour formed is directly proportional to the concentration in the sample.

KEY CONTENTS:

Reagent 1: Enzyme powder
- Glucose oxidase \( \geq 6.7 \)
- Horseradish peroxidase \( \geq 6.2 \)
- 4Aminoantipyrine 0.2 mM
- Phosphate buffer 8 mM, pH 7.0

Reagent 2:
- Phenol 86 mM

Reagent 3:
- Glucose 100 mg / dl
- Insert 01 No.

PREPARATION OF WORKING SOLUTION:
1 bottle of enzyme powder was dissolved with distilled water as indicated on the label to get enzyme solution. For daily requirement, reagent 2 was add (phenol) to the enzyme solution as indicated on the label to obtain the Working Solution.
The enzyme solution is stable for six weeks at 2-8 °C. It should remain refrigerated and be taken out only for short periods to prepare the Working Solution.

**TEST PROCEDURE: END POINT**

<table>
<thead>
<tr>
<th>Pipette in to test tube</th>
<th>Blank</th>
<th>standard</th>
<th>Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Working Solution</td>
<td>1.0 ml</td>
<td>1.0 ml</td>
<td></td>
</tr>
<tr>
<td>Standard</td>
<td>--</td>
<td>10 μg</td>
<td>--</td>
</tr>
<tr>
<td>Sample</td>
<td>--</td>
<td>--</td>
<td>10 μg</td>
</tr>
<tr>
<td>Distilled water</td>
<td>10 μg</td>
<td>--</td>
<td>--</td>
</tr>
</tbody>
</table>

Mixed and incubated at 37 °C for 15 min. or at room temperature (21 - 25 °C) for 30 min. Mixed and read the absorbance of the test (AT), standard (AS) and reagent Blank (A) at 505 nm or with green filter against distilled water.

**CALCULATIONS**

Glucose (mg/dl) = (AT - AB) / (As -A) ×100 (O: Standard Concentration)

To convert mg / dl to mmol –

1 mmol= 1 mg/dl × 0.056

LINEARITY : 700 mg / dl

**TEST PROCEDURE KINETIC**

Wavelength: 505 nm

Reaction Temperature: 37 C

<table>
<thead>
<tr>
<th>Pipette in to test tube</th>
<th>Standard</th>
<th>Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Working Solution</td>
<td>1.0 ml</td>
<td>1.0</td>
</tr>
<tr>
<td>Standard</td>
<td>10 μg</td>
<td>--</td>
</tr>
<tr>
<td>Sample</td>
<td>--</td>
<td>10 μg</td>
</tr>
</tbody>
</table>

Mixed and aspirated. After 1 min. incubation, measure the change in absorbance (Δ Abs) for one min. for Standard and test and use this Δ Abs for calculation.

**CALCULATIONS**

Glucose (mg / dl) = (Δ Abs of test)/ (Δ Abs of std.)×100 (Standard Concentration)
MATERIAL AND METHODS

LINEARITY: 1000 mg/dl

NORMAL VALUES
Fasting: 70 – 100 mg / dl
Postprandial (2 hrs): Upto 140 mg / dl

INTERESTING SUBSTANCES:
Estimation of glucose by this method is relatively free of interference from lipids, bilirubin, uric acid, ascorbic acid and anti-diabetic drugs.

END POINT METHOD

<table>
<thead>
<tr>
<th>INPUT PARAMETERS</th>
<th>VALUES</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type of reaction</td>
<td>End point</td>
</tr>
<tr>
<td>Wavelength</td>
<td>505 nm</td>
</tr>
<tr>
<td>Reagent volume</td>
<td>1.0 ml</td>
</tr>
<tr>
<td>Standard / sample volume</td>
<td>10 µg</td>
</tr>
<tr>
<td>Incubation time</td>
<td>15 min</td>
</tr>
<tr>
<td>Temperature</td>
<td>37 C</td>
</tr>
<tr>
<td>Standard concentration</td>
<td>100 mg/dl</td>
</tr>
<tr>
<td>Upper Normal value</td>
<td>140 mg/dl</td>
</tr>
<tr>
<td>Lower Normal value</td>
<td>70 mg / dl</td>
</tr>
<tr>
<td>Linearity</td>
<td>700 mg / dl</td>
</tr>
</tbody>
</table>

KINETIC METHOD

<table>
<thead>
<tr>
<th>INPUT PARAMETERS</th>
<th>VALUES</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type of reaction</td>
<td>Initial rate kinetic</td>
</tr>
<tr>
<td>Wavelength</td>
<td>505 nm</td>
</tr>
<tr>
<td>Incubation time</td>
<td>60 sec.</td>
</tr>
<tr>
<td>Interval time</td>
<td>60 sec.</td>
</tr>
<tr>
<td>Interval No.</td>
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</tr>
<tr>
<td>Reagent volume</td>
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</tr>
<tr>
<td>Standard / sample volume</td>
<td>10 µg</td>
</tr>
<tr>
<td>Standard Concentration</td>
<td>0 100 mg dl</td>
</tr>
</tbody>
</table>
MATERIAL AND METHODS

<table>
<thead>
<tr>
<th>Temperature</th>
<th>37°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Upper Normal value</td>
<td>140 mg/dl</td>
</tr>
<tr>
<td>Lower Normal value</td>
<td>70 mg/dl</td>
</tr>
<tr>
<td>Linearity</td>
<td>1000 mg/dl</td>
</tr>
</tbody>
</table>

3.15. CORTISOL

The Cortisol is determined by the EIAgen CORTISOL assay kit REF No.L14003K Adaltis Italia S.p.a.-Italy. The quantitative determination of cortisol in the serum and plasma was done by the competitive colorimetric immuno-enzymatic method.

PRINCIPLE OF THE METHOD

The DRG Cortisol ELISA Kit is a solid phase enzyme-linked immunosorbent assay (ELISA), based on the principle of competitive binding. The micro titer wells are coated with a monoclonal antibody directed towards a unique antigenic site on the Cortisol molecule.

Endogenous Cortisol of a patient sample competes with a cortisol horseradish peroxide conjugate for binding to the coated antibody. After incubation the unbound conjugate is washed off. The amount of bound peroxide conjugate is reverse proportional to the concentration of cortisol in the sample. After addition of the substrate solution, the intensity of colour developed is reverse proportional to the concentration of cortisol in the patient sample (Adaltis EIAgen CORTISOL Guideline).

Contents of the Kit:

Microtiter wells, 12x8 (break apart) strips, 96 wells. Wells coated with anti-Cortisol monoclonal antibody

Standard (Standard 0-6), 7 vials, 1 ml, ready to use. Concentrations: 0, 20, 50, 100, 200, 400, 800 ng/ml, thus corresponding to 0, 55.2, 138, 276, 552, 1104, 2208 nmol/l.

Conversion factor: 1 ng/ml = 2.76 nmol/l.

Enzyme Conjugate, 1 vial, 25 ml, ready to use Anti-Cortisol antiserum conjugated to horseradish peroxidase.

Substrate Solution, 1 vial, 14 ml, ready to use TMB
MATERIAL AND METHODS

Stop Solution, 1 vial, 14 ml, ready to use contains 0.5M H2SO4. Contact with the stop solution may cause skin irritations and burns.

Wash Solution, 1 vial, 30 ml (40X concentrated)

**Other equipment and material required for cortisol estimation**

A microtiter plate calibrated reader (450±10 nm) (Microtiter plate Reader).
Calibrated variable precision micropipettes
Absorbent paper
Aqua distilled

**Preparation of Reagents**

All reagents and required number of strips should reach room temperature prior to use.

Wash Solution: Dilute 30 ml of concentrated Wash Solution with 1170 ml deionised water to a final volume of 1200 ml.

Specimen Storage: Specimens should be capped and may be stored for up to 5 days at 2-8°C prior to assaying. Specimens held for a longer time should be frozen only once at -20°C prior to assay. Thawed samples should be inverted several times prior to testing.

Specimen Dilution: If in an initial assay, a serum specimen is found to contain more than the highest standard, the specimens can be diluted 10 fold or 100 fold with Standard 0 and reassayed as described in Assay Procedure. For the calculation of the concentrations this dilution factor has to be taken into account.

Dilution 1:10: 10 µl Serum + 90 µl Standard 0 (mix thoroughly)
Dilution 1:100: 10µl dilution a) 1:10+ 90 µl Standard 0 (mix thoroughly).

**TEST PROCEDURE:**

**General Remarks**

All reagents and specimens were allowed to come to room temperature before use and all reagents were mixed without foaming.

Once the test has been started, all steps were completed without interruption.

New disposable plastic pipette tips for each standard, control or sample was used in order to avoid cross contamination.
As the absorbance is a function of the incubation time and temperature, to ensure equal elapsed time for each pipetting step without interruption. Hence, before starting the assay, all reagents were ready, caps removed, all needed wells secured in holder. As a general rule the enzymatic reaction is linearly proportional to time and temperature.

**Assay Procedure**

All standards, samples, and controls were run in duplicate to ensure that all testing conditions were same. Assay was done as follows:

1. Secured the desired number of Microtiter wells in the holder.
2. Dispensed 20 µl of each Standard, controls and samples with new disposable tips into appropriate wells.
3. Dispensed 200 µl Enzyme Conjugate into each well.
4. Thoroughly mix for 10 seconds, ensure complete mixing in this step.
5. Incubated for 60 minutes at room temperature without covering the plate.
6. Contents of the wells were briskly shaken out.
7. The wells were rinsed 3 times with diluted Wash Solution (400 µl per well). And wells were stricken sharply on absorbent paper to remove residual droplets. As the sensitivity and precision of this assay is markedly influenced by the correct performance of the washing procedure!
8. 100 µl of Substrate Solution was added to each well.
9. Incubated for 15 minutes at room temperature.
10. The enzymatic reaction was stopped by adding 100 µl of Stop Solution to each well.

The OD at 450±10 nm was read with a microtiter plate reader within 10 minutes after adding the Stop Solution.

**Calculations of Results**

Average absorbance value was calculated for each set of standards, controls and patient samples.

A standard curve was constructed by plotting the mean absorbance obtained from each standard against its concentration with absorbance value on the vertical(Y) axis and concentration on the horizontal (X) axis by Biochemistry Instant software.

Using the mean absorbance value for each sample we determined the corresponding concentration from the standard curve.
EXPECTED VALUES

It is strongly recommended that each laboratory should determine its own normal and abnormal values.

Cortisol values in serum or plasma ranges from according to Tietz's Textbook:
50 to 230 ng/ml (138-635 nmol/L) between 8:00 - 10:00 a.m., and
30 to 150 ng/ml (82.8-414 nmol/L) at 4:00 p.m.

Assay Dynamic Range

The range of the assay is between 0 - 800 ng/ml.

Analytical Sensitivity

The analytical sensitivity was calculated from the mean plus two standard deviations of twenty (20) replicate analyses of Standard 0 and was found to be 2.5 ng/ml (6.9 nmol/L).

Precision

Intra Assay Variation

The within assay variability is shown below:

<table>
<thead>
<tr>
<th>Sample</th>
<th>n</th>
<th>Mean (ng/ml)</th>
<th>CV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>20</td>
<td>43.5</td>
<td>8.1</td>
</tr>
<tr>
<td>2</td>
<td>20</td>
<td>226.5</td>
<td>3.2</td>
</tr>
<tr>
<td>3</td>
<td>20</td>
<td>403.6</td>
<td>5.6</td>
</tr>
</tbody>
</table>

Inter Assay Variation

The between assay variability is shown below:

<table>
<thead>
<tr>
<th>Sample</th>
<th>Mean (ng/ml)</th>
<th>CV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>55</td>
<td>6.6</td>
</tr>
<tr>
<td>2</td>
<td>209</td>
<td>7.7</td>
</tr>
<tr>
<td>3</td>
<td>361</td>
<td>6.5</td>
</tr>
</tbody>
</table>
3.16. CATAChOLAMINES

Quantitative measurement of epinephrine and norepinephrine in the serum or plasma was done by BI-CAT-ALISA Enzyme Immuno Assay kit the DLD DIAGNOSTICA GM&H which based on ELISA technique.

Principle of Assay:
Norepinephrine and epinephrine are extracted using a cis-diol-specific affinity gel and acylated to N-acylnoradrenaline and N-acyladrenaline and then converted enzymatically during the detection procedure into N-acylnormetanephrine and N-acylmetanephrine.

The competitive Catecholamine assay ELISA kit contains micro titer plate. Epinephrine and norepinephrine present in the plasma bound to the solid phase of the micro titer plate. Acylated catecholamine from the sample and solid phase bound catecholamine compete for a fixed number of antiserum binding sites. When the system is in equilibrium, free antigen and free antigen-antiserum complexes are removed by washing. The antibody bound to the solid phase catecholamine is detected by anti-rabbit IgG / peroxidase. The substrate TMB / peroxidase reaction is monitored at 450 nm. The amount of antibody bound to the solid phase catecholamine is inversely proportional to the catecholamine concentration of the sample.

Content of the Kit
Reagents for Extraction of epinephrine and norepinephrine
Macro titer Plate - wells coated with boronate affinity gel
Extraction-Buffer
HCl -0.025 M HCl
Assay Buffer - 1 M HCl

Standards (A-F) and Concentrations:

<table>
<thead>
<tr>
<th>STANDARD</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
<th>F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Epinephrine (ng/ml)</td>
<td>0</td>
<td>1</td>
<td>4</td>
<td>16</td>
<td>64</td>
<td>256</td>
</tr>
<tr>
<td>Norepinephrine (ng/ml)</td>
<td>0</td>
<td>4</td>
<td>16</td>
<td>64</td>
<td>256</td>
<td>1,024</td>
</tr>
</tbody>
</table>
MATERIAL AND METHODS

Control 1 & 2
Acylation reagent
Acylation buffer

(B.) Reagents for ELISA:
Enzyme – lyophilized catechol-O-methyl transferase
Co-enzyme – S-adenosyl-L-methionine
Enzyme buffer
Epinephrine-Anti-serum (Color coded blue)
Noradrenaline-Anti-serum(Color coded yellow)
MT-Strips: Precoated with derivatized adrenaline (color coded blue) coated with derivatized noradrenaline (color coded yellow).
POD conjugate Anti-Rabbit IgG-POD conjugate/peroxidase (color coded green)
Wash Buffer – 50 ml was supplied as concentrated solution which was diluted with dist. water to make 500ml total volume vials)
Substrate – TMB solution 11ml (2 vials)
Stop Solution – 0.5M sulphuric acid, not corrosive. 11ml (2 vials)

Additional material used during estimation:
Pipettes (10, 20, 100, 300 μl)
Repeating dispenser for 25, 50, 100, 150, 200, 250 μl and 1ml
Horizontal shaker
Microplate washing device
Microplate photometer
Distilled water
Preparation of Wash Buffer
Dilute the content of the bottle with dist. water to a total volume of 500 ml

TEST PROCEDURE:
Preparation of the Reagents: Enzyme Solution
Freshly prepared enzyme solution was used for the assay and prepared 10-15 minutes before testing. Vial labelled "Enzyme" was reconstituted with 1 ml distilled water and mix thoroughly. Added 0.3 ml Coenzyme and 0.7 ml Enzyme buffer to the freshly prepared enzyme to make it 2ml.
3.17. ADRENALINE OR EPINEPHRINE ELISA

Reagents and samples were allowed to reach at room temperature. Steps were as follows:

Pipetted 25 µl of freshly prepared Enzyme solution into all wells.
Pipetted 100 µl patient samples into the respective wells.
Incubated for 30 minutes at 37 °C.
Pipetted 50 µl Adrenaline-Antiserum into all wells.
Incubated for 2 hour at room temperature on an orbital shaker (600-900 r/min).
Discarded the contents of the wells and washed it thoroughly with each 300 µl Wash Buffer. Removed the residual liquid by tapping the inverted plate on clean absorbent paper. Repeated the washing procedure 3 times.
Pipetted each 100 µl POD-Conjugate into all wells.
Incubated for 30 minutes at room temperature on an orbital shaker (600-900 r/min).
Washing: Repeated the step 6.
Pipetted each 100 µl Substrate into all wells.
Incubated 20 to 30 minutes at room temperature on an orbital shaker (600-900 r/min), preferably in the dark.
Pipetted 100 µl Stop Solution into all wells.
Optical density was read at 450 nm (reference wavelength between 570 and 650 nm) in a microplate photometer within 10 minutes.

3.18. NORADRENALINE OR NOREPINEPHRINE ELISA

All the reagents and samples were allowed to reach at room temperature.
Steps were as follows:
Pipetted each 25 µl of freshly prepared Enzyme solution into all wells.
Pipetted each 20 µl extracted patient samples into the respective wells.
Incubated for 30 minutes at 37 °C.
Pipetted each 50 µl Noradrenaline-Antiserum into all wells.
Incubated for 2 hour at room temperature on an orbital shaker (600-900 r/min).
MATERIAL AND METHODS

Discarded the contents of the wells and wash thoroughly with each 300 µl Wash Buffer. Removed the residual liquid by tapping the inverted plate on clean absorbent paper. Repeat the washing procedure 3 times.

Pipetted each 100 µl POD-Conjugate into all wells.

Incubated for 30 minutes at room temperature on an orbital shaker (600-900 r/min).

Washing: Repeat step 6.

Pipetted each 100 µl Substrate into all wells.

Incubated 20 to 30 minutes at room temperature on an orbital shaker (600-900 r/min), preferably in the dark.

Pipette 100 µl Stop Solution into all wells.

Optical density was read at the 450 nm (reference wavelength between 570 and 650 nm) in a microplate photometer within 10 minutes.

CALCULATION OF RESULTS

On a semi logarithmic graph paper the concentration of the standards (x-axis, logarithmic) are plotted against their corresponding optical density (y-axis, linear). Alternatively, the optical density of each standard and sample can be related to the optical density of the zero standard expressed as a ratio (OD/OD)\(_{\text{max}}\), and then plotted on the y-axis.

The concentration of the controls was read off by the standard curve directly.

Reference Ranges

<table>
<thead>
<tr>
<th></th>
<th>Adrenaline</th>
<th>Noradrenaline</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urine</td>
<td>&lt; 20 µg/day</td>
<td>&lt; 90 µg/day</td>
</tr>
<tr>
<td>Plasma</td>
<td>&lt; 100 pg/day</td>
<td>&lt; 600 pg/day</td>
</tr>
</tbody>
</table>

3.19. C-REACTIVE PROTEINS

High Sensitivity CRP (hs-CRP) Microplate ELISA Product Code: BC 1119M from CALIBIOTECH The Quantitative determination of CRP (C-reactive protein) concentration in Human Serum was done by a Microplate Immunoenzymometric assay.
PRINCIPLE:

Immunoenzymometric assay:

The essential reagents required for an immunoenzymometric assay include high affinity and specificity antibodies (enzyme and immobilized), with different and distinct epitope recognition, in excess, and native antigen. In this procedure, the immobilization takes place during the assay at the surface of a microplate well through the interaction of streptavidin coated on the well and exogenously added biotinylated monoclonal anti-CRP antibody.

Upon mixing monoclonal biotinylated antibody, the enzyme labelled antibody and a serum containing the native antigen, reaction results between the native antigen and the antibodies, without competition or steric hindrance, to form a soluble sandwich complex. The interaction is illustrated by the following equation,

\[ E_{\text{iiZAb(p)}} + A_{\text{gCRP}} + B^m_{\text{Ab(m)}} \rightleftharpoons E_{\text{Ab(p)}} - A_{\text{gCRP}} - B^m_{\text{Ab(m)}} \]

\[ k_a \]

\[ B^m_{\text{Ab(m)}} \rightarrow \text{Biotinylated Monoclonal Antibody (Excess Quantity)} \]

\[ A_{\text{gCRP}} \rightarrow \text{Native Antigen (Variable Quantity)} \]

\[ E_{\text{Ab(p)}} \rightarrow \text{Enzyme labeled Antibody (Excess Quantity)} \]

\[ E_{\text{Ab(p)}} - A_{\text{gCRP}} - B^m_{\text{Ab(m)}} \rightarrow \text{Antigen-Antibodies Sandwich complex} \]

\[ k_a \rightarrow \text{Rate Constant of Association} \]

\[ k_{-a} \rightarrow \text{Rate Constant of Dissociation} \]

Simultaneously, the complex is deposited to the well through the high affinity reaction of streptavidin and biotinylated antibody. This interaction is illustrated below:

\[ E_{\text{Ab(p)}} - A_{\text{gCRP}} - B^m_{\text{Ab(m)}} + \text{Streptavidin}_{c.w} \rightarrow \text{Immobilized complex} \]

\[ \text{Streptavidin}_{c.w} \rightarrow \text{streptavidin immobilized on well} \]

\[ \text{Immobilized complex} \rightarrow \text{sandwich complex bound to the solid surface} \]

After equilibrium is attained, the antibody bound reaction is separated from unbound antigen by decantation or aspiration. The enzyme activity in the antibody bound fraction is directly proportional to the native antigen concentration. By utilizing several different serum references of known antigen values, a dose response curve can be generated from which the antigen concentration of an unknown can be ascertained.
MATERIAL AND METHODS

REAGENTS:

Provided:
CRP Calibrators – 1 ml vial - A-F. Six (6) vials of references CRP Antigen at levels of 0 (A), 0.5 (B), 2.0 (C), 50 (D), 15 (E) and 30 (F) µg/ml
CRP Enzyme Reagent – 13 ml / vial. One (1) vial containing Biotin labeled monoclonal mouse IgG and Anti-CRP HRP in buffer, dye, and preservative.
Streptavidin Coated Microplate - 96 wells. One 96-well microplate coated with streptavidin and packaged in an aluminum bag with a drying agent
Serum Diluent Concentrate - 20 ml. One (1) vial of serum diluent containing buffer salts and a dye.
Wash Solution Concentrate - 20 ml. One (1) vial containing a surfactant in buffered saline. A preservative has been added.
Substrate A - 7 ml / vial. One (1) vial containing tetramethylbenzidine (TMB) in buffer. Substrate B - 7 ml / vial containing hydrogen peroxide (H₂O₂) in buffer.
Stop Solution - 8 ml vial containing a strong acid (1N HCl).

Other things required: were as follows:
Pipette capable of delivering 25 µL & 50 µL volumes with a precision of better than 1.5%
Dispenser for repetitive deliveries of 0.100 ml and 0.300 ml volumes with a precision of better than 1.5%.
Microplate washers or a squeeze bottle (optional).
Microplate Reader with 450nm and 620nm wavelength absorbance capability
Absorbent Paper for blotting the microplate wells
Plastic wrap or microplates cover for incubation steps.
Vacuum aspirator (optional) for wash steps.
Timer.
Quality control materials.

REAGENT PREPARATION:
Serum Diluents: Serum was diluted to 200 ml in a suitable container with distilled water.
MATERIAL AND METHODS

Wash Buffer: Dilute contents of Wash Concentrate to 1000ml with distilled or deionised waters

Working Substrate Solution Prepared by mixing equal portions of Substrate A and Substrate B in a suitable container.

TEST PROCEDURE (based on the Tietz's procedure, 1995):
Before proceeding with the assay, all reagents, serum references and controls were brought to room temperature (20 - 27° C).
Microplates' wells were formatted for each serum reference, control and patient specimen to be assayed in duplicate.
Pipetted 0.025 ml (25μl) of the appropriate serum reference into the assigned wells.
Added 0.100 ml (100μl) of the CRP enzyme reagent 10 in each well. All reagents were dispensed close to the bottom of the coated well.
Microplate was swirl gently for 20-30 seconds to mix and cover.
Incubated for 15 minutes at room temperature.
Discarded the contents of the microplate by decantation or aspiration and blotted the plates dry with absorbent paper.
Added 300 μl of wash buffer and total of three (3) washes.
Added 0.100 ml (100 μl) of working substrate solution to all wells
Incubated at room temperature for fifteen (15) minutes.
Add 0.050ml (50 μl) of stop solution to each well and mix gently
Absorbance was read at 620-630nm in each well at 450nm (using a reference wavelength to minimize well imperfections) in a microplate reader. The results were read within thirty (30) minutes of adding the stop solution.

RESULTS:
A dose response curve is used to ascertain the concentration of HSCRP in unknown specimens.
Absorbance was obtained and recorded from the printout of the microplate reader
Absorbance was plotted the for each duplicate serum reference versus the corresponding CRP concentration in ng/ml on a linear graph paper.
Curve was drawn through the plotted points.
Concentration of CRP for an unknown, was determined by locating the average absorbance of the duplicates for each unknown on the vertical axis of the graph,
intersecting point was found on the curve, and the concentration (in ng/ml) was read from the horizontal axis of the graph.

We calculated the absorbance from the absorbance obtained from the print out of the microplate Elisa reader, was plotted in duplicate serum reference versus the corresponding CRP concentration in µg/ml with help of a software Microbiology-Instant. The hs-CRP (high sensitivity CRP) microplate Elisa procedure has sensitivity of 0.2µg/ml. The intra-assay and inter-assay coefficient of variation for CRP within run precision were 7.5%-2.3% and 4.1%-2.5% respectively. Expected normal value = <3.0 µg/ml

3.20. TNF-A ELISA

Diaclone Immunoassay, product code no. 850 090 096.

PRINCIPLE OF THE TEST:

A monoclonal antibody specific for TNF-α has been coated onto the wells of the microtiter strips provided.

![Coated Micro Well](image)

During the first incubation, TNF-α present in the sample or standard and a monoclonal anti TNF-α antibody conjugated to biotin are simultaneously incubated.

![First Incubation](image)
MATERIAL AND METHODS

Following incubation unbound biotinylated anti TNF-α is removed during a wash step.

Streptavidin-HRP is added and binds to the biotinylated anti TNF-α. After incubation and a wash step a substrate solution reactive with HRP is added to the wells.

Second Incubation
A coloured product is formed in proportion to the amount of TNF-α present in the sample. The reaction is terminated by addition of acid and absorbance is measured at 450 nm.

Third Incubation
## MATERIAL AND METHODS

### REAGENTS PROVIDED:

<table>
<thead>
<tr>
<th>REAGENTS (STORE AT 2-8°C)</th>
<th>Colour Code</th>
<th>Quantity 1x96 tests</th>
<th>Quantity 2x96 tests</th>
<th>RECONSTITUTION</th>
</tr>
</thead>
<tbody>
<tr>
<td>96-wells precoated microtiter plate</td>
<td></td>
<td>1</td>
<td>2</td>
<td>Ready to use</td>
</tr>
<tr>
<td>Plate cover</td>
<td></td>
<td>2</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>TNF-α standard: 800 pg/ml</td>
<td>Yellow</td>
<td>2 vials</td>
<td>4 vials</td>
<td>Reconstitute with the volume of standard diluent indicated on the vial</td>
</tr>
<tr>
<td>Control</td>
<td>Silver</td>
<td>2 vials</td>
<td>4 vials</td>
<td>Reconstitute with the volume of standard diluent indicated on the vial</td>
</tr>
<tr>
<td>Standard diluent buffer</td>
<td>Black</td>
<td>1 bottle</td>
<td>1 bottle</td>
<td>(25 ml) 10X concentrate. dilute in distilled water</td>
</tr>
<tr>
<td>Standard diluent: human serum</td>
<td>Black</td>
<td>1 bottle</td>
<td>2 bottles</td>
<td>(7 ml) Ready to use</td>
</tr>
<tr>
<td>Biotinylated anti TNF-α</td>
<td>Red</td>
<td>1 vial</td>
<td>2 vials</td>
<td>(0.4 ml) dilute in biotinylated antibody diluent</td>
</tr>
<tr>
<td>Biotinylated antibody diluents</td>
<td>Red</td>
<td>1 bottle</td>
<td>1 bottle</td>
<td>Ready to use</td>
</tr>
<tr>
<td>Streptavidin – HRP</td>
<td></td>
<td>2 vials</td>
<td>4 vials</td>
<td>(5 µl) add 0.5 ml of HRP-diluent before further dilutions</td>
</tr>
<tr>
<td>HRP diluent</td>
<td>Red</td>
<td>1 bottle</td>
<td>1 bottle</td>
<td>(23 ml) Ready to use</td>
</tr>
<tr>
<td>Washing buffer</td>
<td>White</td>
<td>1 bottle</td>
<td>2 bottles</td>
<td>(10ml) 200X concentrate. Dilute in distilled water</td>
</tr>
<tr>
<td>Substrate solution; chromogen TMB</td>
<td></td>
<td>1 bottle</td>
<td>1 bottle</td>
<td>Ready to use</td>
</tr>
</tbody>
</table>
MATERIAL AND METHODS

| Stop reagent H₂SO₄ | Black | 1 bottle | 2 bottles | (11 ml) Ready to use |

ADDITIONAL MATERIALS REQUIRED:
1. 5 ml and 10 ml graduated pipettes
2. 5 μl to 1,000 μl adjustable single channel micropipettes with disposable tips.
3. 50 μl to 300 μl adjustable multichannel micropipette with disposable tips
   Multichannel micropipette reservoir.
4. Vortex, Mixer.
5. Beakers, flasks, cylinders necessary for preparation of reagents.
6. Device for delivery of wash solution (multichannel wash bottle or automatic wash system)
7. Microwell strip reader capable of reading at 450 nm (620 nm as optional reference wave length)
8. Glass-distilled or deionized water
9. Statistical calculator with program to perform linear regression analysis.

PREPARATION OF REAGENTS:

1. Washing Buffer
Dilute the Washing Buffer Concentrate (200X) in a clean graduated cylinder. Mix gently to avoid foaming.

Entire content (10 ml) of the Washing Buffer Concentrate was poured into a clean 2,000 ml graduated cylinder to bring final volume to 2,000 ml with glass-distilled or deionized water. Mix gently to avoid foaming.

2. Preparation of Standard Diluent Buffer
Add the content of the vial (10X) to 225 ml distilled water before use.

3. Preparation of TNF-α Standards
Depending on the type of samples assayed, the kit includes two standard diluents. Because biological fluids might contain proteases or cytokine-binding proteins that could modify the recognition of the cytokine

Reconstitute TNF-α Standard by addition of appropriate Standard Diluent. Reconstitute volume is stated on the label of the standard vial. This reconstitution produces a stock solution of 800 pg/ml TNF-α. Standard was allowed to stand for 5
minutes with gentle swirling prior to making dilutions. Serial dilutions of standard must be made before each assay and cannot be stored.

4. Preparation of Controls
Freeze-dried control vials should also be reconstituted with the most appropriate Standard Diluent.
Controls have to be reconstituted with the volume of Standard Diluent indicated on the vial. Reconstitution of the freeze-dried material with the indicated volume, will give a solution for which the TNF-α concentration is stated on the vial. Control was allowed to stand for 5 minutes with gentle swirling prior to distribute in control wells.

5. Preparation of biotinylated anti TNF-α
Preparation immediately before use is recommended. Dilute the biotinylated anti-TNF-α with the biotinylated antibody diluent in a clean glass vial. Biotinylated anti TNF-α may be prepared as needed according the following table.

<table>
<thead>
<tr>
<th>No. of strips</th>
<th>Biotinylated antibody concentrate (μl)</th>
<th>Biotinylated antibody diluent (μl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>40</td>
<td>1060</td>
</tr>
<tr>
<td>3</td>
<td>60</td>
<td>1590</td>
</tr>
<tr>
<td>4</td>
<td>80</td>
<td>2120</td>
</tr>
<tr>
<td>6</td>
<td>120</td>
<td>3180</td>
</tr>
<tr>
<td>12</td>
<td>240</td>
<td>6360</td>
</tr>
</tbody>
</table>

6. Preparation of Streptavidin – HRP
Dilute the Streptavidin-HRP 1:100 just prior to use by adding 0.5 ml of HRP diluent to the vial containing Streptavidin-HRP concentrate.
MATERIAL AND METHODS

Further dilution with HRP-Diluent in a clean glass vial was needed according to the following table:

<table>
<thead>
<tr>
<th>No. of strips</th>
<th>Pre-diluted streptavidin-HRP(μl)</th>
<th>HRP diluent(μl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>30</td>
<td>2</td>
</tr>
<tr>
<td>3</td>
<td>45</td>
<td>3</td>
</tr>
<tr>
<td>4</td>
<td>60</td>
<td>4</td>
</tr>
<tr>
<td>6</td>
<td>75</td>
<td>5</td>
</tr>
<tr>
<td>12</td>
<td>150</td>
<td>10</td>
</tr>
</tbody>
</table>

TEST PROTOCOL

a. A. Mix all reagents thoroughly without foaming before use.

b. 100 μl of appropriate Standard Diluent (see preparation of reagents) was added to standard wells B1, B2, C1, C2, D1, D2, E1, E2, F1, and F2. Reconstituted the standard vial with the appropriate volume. Pipetted 200 μl of standard into wells A1 and A2 (see Figure 1 and 2), Transfer 100 μl from A1 and A2 to B1 and B2 wells. Mixed the contents by repeated aspirations and ejections. Taken care not to scratch the inner surface of micro wells. Repeated this procedure from the wells B1, B2 to wells C1, C2 and from wells C1, C2 to D1, D2 and so on creating two parallel rows of TNF-α standard dilutions ranging from 800 to 25 pg/ml. Discarded 100 μl from the content of the last micro wells used (F1, F2).

c. Prepare and arrange the standards as shown below:

![Figure 1: Preparation of TNF-α standard dilutions](image)

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MATERIAL AND METHODS

**Figure 2:** Diagram depicting an example of the arrangement of blanks, standards, samples and controls in the Microwell strips:

<table>
<thead>
<tr>
<th>Standard Concentrations</th>
<th>Sample wells</th>
</tr>
</thead>
<tbody>
<tr>
<td>pg/mL</td>
<td></td>
</tr>
<tr>
<td>A 800 800</td>
<td></td>
</tr>
<tr>
<td>B 400 400</td>
<td></td>
</tr>
<tr>
<td>C 200 200</td>
<td></td>
</tr>
<tr>
<td>D 100 100</td>
<td></td>
</tr>
<tr>
<td>E 50 50</td>
<td></td>
</tr>
<tr>
<td>F 25 25</td>
<td></td>
</tr>
<tr>
<td>G Blank Blank</td>
<td></td>
</tr>
<tr>
<td>H Control Control</td>
<td></td>
</tr>
</tbody>
</table>

**d.** Added 100 μl of appropriate Standard Diluent in duplicate, to the blank wells (G1, G2).

**e.** Added 100 μl of Sample to sample wells, in duplicate, to the designated wells and 100 μl of reconstituted control vial, in duplicate, to control wells (H1, H2).

**f.** Prepared biotinylated anti TNF-α

**g.** Added 50 μl of diluted biotinylated anti TNF-α to all wells.

**h.** Covered it with a Plate Cover and incubate at room temperature (18° to 25°C) for 3 hours.

**i.** Removed the cover and washed the plate as follows:

   a. 1. Aspirated the liquid from each well;
   b. 2. Dispensed 0.3 ml of washing solution into each well;
   c. 3. Aspirated again the content of each well;
   d. 4. Repeated step 2. and 3. two times

**j.** Prepared Streptavidin-HRP solution just before use.

**k.** Distributed 100 μl of Streptavidin-HRP solution to all wells, including blanks.
MATERIAL AND METHODS

l. Covered the plate and incubate the plate at room temperature (18°C to 25°C) for 30 min.

m. Removed the cover and empty wells. Wash micro well strips according to step i. Proceed immediately to the next step.

n. Pipetted 100 μl of ready-to-use TMB Substrate Solution to all wells, including the blank wells and incubated in the dark for about 12-15 minutes at room temperature. Avoided direct exposure to light by wrapping the plate in aluminium foil.

o. Incubation time of the substrate solution was determined by the ELISA reader performances. Many ELISA readers record absorbance only up to 2.0 0.0. Therefore the colour development within individual microwells was watched by the person running the assay, and the substrate reaction stopped before positive wells are no longer properly recordable.

p. The enzyme-substrate reaction is stopped by quickly pipetting 100 μl of H2S04. Stop Reagent into each well, including the blank wells to completely and uniformly inactivate the enzyme. Results were read immediately after the H2S04. Stop Reagent was added.

q. Absorbance of each microwell was read on a spectrophotometer using 450 nm as the primary wave length (optionally 620 nm as the reference wave length; 610 nm to 650 nm is acceptable).

CALCULATION OF RESULTS:
Results were read on spectrophotometer using 450 nm as primary wave length. Average absorbance was calculated for each set of duplicate standards, samples and controls. The linear standard curve was created by plotting the mean absorbance for each standard concentration on the ordinate against the TNF-α standard concentration on the abscissa. The overall intra-assay coefficient of variation was calculated to be 3.3%. The inter-assay coefficient of variation was 9%. Spike recovery ranged from 74%-90% and expected normal serum value was taken < 8 pg/ml.
MATERIAL AND METHODS

Fig. 3: Representative standard curve for TNF-α ELISA ranging from 25 to 800 pg/mL. A standard curve was plotted for each group of micro well strip assayed.

Typical data using the TNF-α ELISA

<table>
<thead>
<tr>
<th>Standard</th>
<th>TNF-α (pg/mL)</th>
<th>O.D (450 nm)</th>
<th>O.D (mean)</th>
<th>C.V (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>800</td>
<td>1.834</td>
<td>1.833</td>
<td>3.7</td>
</tr>
<tr>
<td></td>
<td>800</td>
<td>1.932</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>400</td>
<td>0.993</td>
<td>1.076</td>
<td>10.8</td>
</tr>
<tr>
<td></td>
<td>400</td>
<td>1.158</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>200</td>
<td>0.702</td>
<td>0.724</td>
<td>4.3</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>0.746</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>100</td>
<td>0.407</td>
<td>0.430</td>
<td>7.4</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>0.452</td>
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<td></td>
</tr>
<tr>
<td>5</td>
<td>50</td>
<td>0.276</td>
<td>0.277</td>
<td>0.3</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>0.277</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>25</td>
<td>0.167</td>
<td>0.180</td>
<td>10.2</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>0.193</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blank</td>
<td>0</td>
<td>0.105</td>
<td>0.102</td>
<td>4.2</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>0.099</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
REPRODUCIBILITY

A. Intra-Assay

Intra-assay coefficient of variation has been calculated to be 3.3%.

<table>
<thead>
<tr>
<th>Session</th>
<th>Sample</th>
<th>Assay 1 [TNF-α] pg/ml</th>
<th>Assay 2 [TNF-α] pg/ml</th>
<th>Assay 3 [TNF-α] pg/ml</th>
<th>mean intra assay</th>
<th>SD intra assay</th>
<th>CV intra assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>837.0</td>
<td>870.0</td>
<td>781.0</td>
<td>829.3</td>
<td>45.0</td>
<td>5.43</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>531.0</td>
<td>518.0</td>
<td>539.0</td>
<td>529.3</td>
<td>10.6</td>
<td>2.00</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>199.0</td>
<td>203.0</td>
<td>201.0</td>
<td>201.0</td>
<td>2.0</td>
<td>1.00</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>817.0</td>
<td>814.0</td>
<td>790.0</td>
<td>807.0</td>
<td>14.8</td>
<td>1.8</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>447.0</td>
<td>470.0</td>
<td>449.0</td>
<td>455.3</td>
<td>12.5</td>
<td>2.8</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>168.0</td>
<td>165.0</td>
<td>182.0</td>
<td>171.7</td>
<td>9.1</td>
<td>5.3</td>
</tr>
<tr>
<td>3</td>
<td>1</td>
<td>827.0</td>
<td>845.0</td>
<td>827.0</td>
<td>833.0</td>
<td>10.4</td>
<td>1.2</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>501.0</td>
<td>492.0</td>
<td>476.0</td>
<td>489.7</td>
<td>12.7</td>
<td>2.6</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>170.0</td>
<td>175.0</td>
<td>173.0</td>
<td>172.7</td>
<td>2.5</td>
<td>1.5</td>
</tr>
</tbody>
</table>

B. Inter-Assay

Overall coefficient of variation was 9%.

<table>
<thead>
<tr>
<th>Operator</th>
<th>Session</th>
<th>Sample 1</th>
<th>Sample 2</th>
<th>Sample 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>1</td>
<td>837</td>
<td>531</td>
<td>199</td>
</tr>
<tr>
<td></td>
<td></td>
<td>870</td>
<td>518</td>
<td>203</td>
</tr>
<tr>
<td></td>
<td></td>
<td>781</td>
<td>539</td>
<td>201</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>817</td>
<td>446</td>
<td>168</td>
</tr>
<tr>
<td></td>
<td></td>
<td>814</td>
<td>469</td>
<td>164</td>
</tr>
<tr>
<td></td>
<td></td>
<td>790</td>
<td>449</td>
<td>182</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>827</td>
<td>501</td>
<td>170</td>
</tr>
<tr>
<td></td>
<td></td>
<td>845</td>
<td>492</td>
<td>175</td>
</tr>
<tr>
<td></td>
<td></td>
<td>827</td>
<td>476</td>
<td>173</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>843</td>
<td>429</td>
<td>149</td>
</tr>
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### MATERIAL AND METHODS

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#### 3.21. STATISTICAL ANALYSIS

Data were stored and analyzed with standard computer software (SPSS v17.0 Inc). Intra-group and inter-group comparisons were done by paired sample statistics and independent sample t-tests respectively. Values were expressed as means with standard deviation (SD), when normally distributed, or medians with the 25th and 75th percentiles inter-quartile range, when skewed. The changes in the same group (intergroup) with reference to varying time point of sampling during perioperative period were calculated with the help of student’s t-test using group statistics, the paired sample t-test. As all the data are satisfying the assumption of equality and homogeneity of variance (Levene’s Test), the parametric Independent sample t-test was used to compare the two groups together. The results are reported as the means plus or minus the standard error of the mean (SEM).

Comparison among groups was computed with the help of ANOVA and for further two-sided multiple comparisons among groups, the Bonferroni test was applied. A two-tailed significance or p-values were reported and probability value of $P < 0.05$ was considered statistically significant.

The correlation between two biomarkers for example epinephrine versus cortisol or one biomarker with clinical parameter such as epinephrine vs. heart rate was computed with Pearson correlation to know the linear correlation including direction
and strength of relationship between two test pairs. Recently, the value of Pearson correlation is interpreted as follows: the correlation between -0.7 to -0.3 indicate weak negative association, while coefficient value between -0.3 to +0.3 little or no association, +0.3 to +0.7 weak positive association, and +0.7 to +1.0 strong positive association.