# CHAPTER THREE

# MATERIALS AND METHODS

## 3.1 Study participants and design

This was a cross-sectional, observational study conducted in the Faculty of Medicine, Universiti Teknologi MARA (UiTM). A total of 74 FH patients attending the Lipid Specialists' Clinics who fulfilled the set criteria and 77 normolipaemic controls (NC) were recruited for this study. All patients were screened through a protocol consisting of medical history, physical examination and laboratory tests. Diagnosis of FH was made based on the Simon Broome's criteria (Ose, 1999) (Appendix A). Patients with diabetes mellitus, renal, liver, endocrine diseases or any other causes of secondary hypercholesterolemia, those with recent febrile illness, concomitant neoplasm, inflammatory disease or immunosuppressive therapy including steroid usage and those taking vitamin supplements were excluded from this study. This study protocol was approved by the Ethics Committee of Faculty of Medicine, UiTM. Written informed consent was obtained prior to commencement of this study (Appendix B).

## 3.2 Anthropometry Measurements and Data Collection

Blood pressure (BP), body mass index (BMI), waist hip ratio (WHR), smoking habits and history of personal CAD were measured and documented.

Systolic and diastolic BP was measured by an automated BP reader (Omron HEM-712C, Japan) with the patient in a seated position and after 5 to 10 minutes rest. BP readings were taken from the average of the last 2 readings obtained. The systolic (SBP) and diastolic blood pressure (DBP) was measured to the nearest 1 mmHg. BMI was calculated using the formula: BMI = weight (kg) / height$^2$ (m$^2$). WHR was

calculated using the formula waist circumference / hip circumference. Waist circumference was measured to the nearest 0.5cm using a measuring tape at midway between the inferior margin of the last rib and the iliac crest in a horizontal plane. Hip circumference measurement was taken around the pelvis at the point of maximal protrusion of the buttocks (WHO, 1995). Presence of CAD was assessed based on the clinical history, previous medical records and exercise tolerance test reports. (Appendix C).

### 3.3 Sample Collection

Twenty millilitres (ml) overnight fasting venous blood samples were collected into plain tubes and EDTA tubes following non-traumatic venepuncture in the morning between 0800 and 1000 hours. Serum was separated within 1 hour of collection. Four ml of whole blood in EDTA tubes for DNA analysis were stored at -80 $^{o}$C until DNA extractions were performed.

### 3.4 Biochemical Analysis

Routine biochemical analyses that were performed on all subjects consisting of fasting serum lipid (FSL), fasting plasma glucose (FPG), liver function tests (LFT), renal profile (RP) and thyroid function test (TFT). FSL, FPG, LFT and RP were performed on automated analysers (Cobas Integra 800 and Cobas Integra 400 plus, Roche Diagnostics, Switzerland). TFT was performed using automated analysers (Axsym, Abbott Diagnostic, USA and Elecsys 2010, Roche Diagnostics, Switzerland).

### 3.4.1 Fasting Plasma Glucose

Fasting blood glucose levels were analyzed using enzymatic reference method with hexokinase on automated analysers (Cobas Integra 800 and Cobas Integra 400 plus, Roche, Switzerland). In this method, hexokinase catalysed the phosphorylation of glucose by ATP to form glucose-6-phosphate. Following this reaction, the second enzyme, glucose-6-phosphate dehydrogenase (G6PD) was used to catalyse oxidation of glucose-6-phosphate by NADP to form NADPH. The concentration of the NADPH formed was direcly proportional to the glucose concentration. The glucose concentration was determined by measuring the increase in absorbance at 340 nm (Roche Diagnostic Method Manual for Cobas Integra System).

### 3.4.2 Fasting Serum Lipid (FSL)

FSL consists of total cholesterol (TC), triglycerides (TG), low-density lipoprotein cholesterol (LDL-c) and high-density lipoprotein cholesterol (HDL-c).

TC levels were measured by enzymatic, colorimetric method with cholesterol esterase, cholesterol oxidase and 4-aminoantipyrine. Cholesterol esters were cleaved by the action of cholesterol esterase to yield free cholesterol and fatty acids. Cholesterol oxidase then catalysed the oxidation of cholesterol to cholest-4-en-3-one and hydrogen peroxide. In the presence of peroxidase, the hydrogen peroxide formed effects the oxidation coupling of phenol and 4-aminoantipyrine to form a red quinine-imine dye. The colour intensity of the dye formed was directly proportional to the cholesterol concentration. The cholesterol concentration was measured spectrophotometrically at 520 nm.

TG was measured by an enzymatic, colorimetric method (GPO-PAP method) with glycerol phosphate oxidase and 4-aminophenazone. Triglycerides were hydrolysed by lipoprotein lipase (LPL) tioglycerol and fatty acids. Glycerol was then phosphorylated

to glycerol-3-phosphate by ATP in a rection catalysed by glycerol kinase. The oxidation of glycerol-3-phosphate was catalysed by glycerol phosphate oxidase to form dihydroxyacetone phosphate and hydrogen peroxide. In the presence of peroxidase, hydrogen peroxide effects the oxidation coupling of 4-chlorophenol and 4-aminophenazone to form a red-coloured quinon-emine dye, which was measured spectrophotometrically at 512 nm.

HDL-c concentration was measured by a homogenous enzymatic colorimetric assay. The colour intensity of the blue quinon-emine dye formed was directly proportional to the HDL-c concentration, which was measured spectrophotometrically at 483 nm.

LDL-c concentration was derived by calculation using the Friedwald equation (LDL in mmol/L = TC – HDL – TG /2.17) (Friedwald *et al*., 1972) except when TG concentration is more or equal to 4.5 mmol/L.

### 3.4.3 Liver Function Tests (LFT)

LFT consists of total protein (TP), albumin (ALB), total bilirubin (TB), alanine aminotransferase (ALT) and alkaline phosphatase (ALT).

TP was measured by the biuret reaction. In the TP method, divalent copper reacts with peptide bonds of proteins under alkaline conditions to form pink to purple biuret complex. Sodium potassium tartrate prevented copper hydroxide precipitation and potassium iodide prevented autoreduction of copper. The colour intensity is directly proportionate to the protein concentration and the absorbance was measured at 552 nm.

TB was measured using Diazo method and was measured spectrophotometrically at 552 nm.

ALB was measured by colorimetric assay with end-point method. Albumin at pH 4.1 was sufficiently cationic to bind with the anionic bromocresol green dye to form a blue-

green coloured complex. The colour intensity was directly proportional to albumin concentration, which was measured at 583 nm.

ALT was measured according to the International Federation of Clinical Chemistry (IFCC) guideline with phyridoxal-5'-phosphate. The reaction between L-alanine and 2-oxoglutarate was catalysed by ALT. The pyruvate formed was reduced by NADH in a reaction catalysed by lactate dehydrogenase to form L-lactate and $NAD^+$. Pyridoxal phosphate served as a coenzyme in the amino transfer reaction. The rate of NADH oxidation is directly proportional to the catalytic ALT activity, which was measured at 340 nm.

ALP was measured using colorimetric assay according to International Federation of Clinical Chemistry (IFCC) guideline. In the presence of magnesium and zinc ion, p-nitrophenyl phosphate was cleaved by phosphatases into phosphate and p-nitrophenol. The p-nitrophenol released was directly proportional to the catalytic ALP activity, which was measured at 409 nm.

### 3.4.4 Renal Profile (RP)

RP consists of sodium, potassium, chloride, creatinine and urea. Sodium, potassium and chloride were measured using ion-selective electride (ISE) indirect method.

Creatinine was measured using buffered kinetic Jaffe reaction without deproteinisation method. In this method, a yellow red adduct was produced as a results from the reaction of alkaline creatinine solution with picrate. The rate of dye formation is directly proportional to the creatinine concentration in the specimens. The creatinine concentratin was measured spectrophotometrically at 512 nm.

Urea in the serum was measured using with urease and glutamate dehydrogenase method. Urea was hydrolysed by urease to form ammonium and carbonate. In the second reaction, 2-oxoglutarate reacts with ammonium in the presence of glutamate

dehydrogenase (GLDH) and coenzyme NADH to produce L-glutamate. In this reaction, two moles of NADH were oxidised to NAD for each mole of urea hydrolysed. The rate of decrease in the NADH concentration is directly propotional to the urea concentration and was determined by measuring the absorbance at 340 nm.

### 3.4.5 Thyroid Function Test (TFT)

TFT consists of free thyroxine (FT4), free triiodothyronine (FT3) and thyroid stimulating hormone (TSH). Those tests were measured using microparticle enzyme immunoassay method on Abbott Diagnostic Axsym analyser and using electrochemiluminescent assay method on Roche Diagnostic Elecsys 2010.

## 3.5 Molecular Analysis

### 3.5.1 DNA Extraction

DNA extractions were performed on 74 FH and 77 NC whole blood specimens using Maxwell® 16 Blood DNA Purification kit. The DNA extractions were done on an automated extraction system (Promega Maxwell® 16 Automated DNA Extraction System).

Four hundred microliters (µl) of whole blood from EDTA tubes was added into well no 1 of the Maxwell® 16 Blood DNA Purification kit reagent cartridge which contained lysis buffer. Purification plunger was place into well no 7 of the cartridge which contained wash buffer. The cartridge was then placed into the automated instrument. Three hundred µl elution buffers were added into an elution tube that has been labelled with sample identification number. The elution tube was placed into the elution tube slots at the front of platform of the automated instrument. When the extraction was completed, the elution tube was removed from the elution tube slots and placed into the

magnetic elution tube rack. The eluted DNA sample was transferred into sterilized 1.5 ml microcentrifuge tube and stored at 4 $^{o}$C.

### 3.5.2 DNA Quantification

The extracted DNA was quantified using NanoDrop ND-1000 (Thermo Fisher Scientific) to determine the concentration and purity of the sample. The purity of the DNA was measured at 260/280 absorbance ratio reading. Pure DNA has expected ratio of more than 1.8. Samples with ratio of more than 1.8 were selected for this study and subjected to amplification step.

### 3.5.3 Amplification of LDLR gene by Polymerase Chain Reaction (PCR)

The extracted DNA of 74 FH and 77 NC specimens were subjected to PCR to amplify the promoter region and all exons of LDLR gene. The primers used to amplify the target regions (promoter and Exon 1 until Exon 18 of LDLR gene) were adapted from Bodamer *et al*., 2002 (Table 3.1).

**Table 3.1: List of primers sequence for amplification of LDLR gene (adapted from Bodamer *et al*., 2002)**

| Exon | Forward (5' to 3') | Reverse (5' to 3') | Size, bp |
|---|---|---|---|
| Promoter | cagctcttcaccggagaccc | acctgctgtgtcctagctgg | 275 |
| Exon 1 | actcctccccctgctagaaacctca | ctattctggcgcctggagcaagcc | 244 |
| Exon 2 | ttgagagacccttctcctttcc | gcatatcatgcccaaagggg | 189 |
| Exon 3 | ttcctttgagtgacagttcaatcc | gataggctcaatagcaaaggcagg | 196 |
| Exon 4 5' end | ggtctcggccatccatccctg | ctgttgcactggaagctggcgg | 187 |
| Exon 4 mid | agacgaggcctcctgcccggt | gagcaggggctactgtcc | 186 |
| Exon 4 3' end | cgactgcgaagatggctcg | ggaacccagggacaggtgataggac | 240 |
| Exon 5 | agaaaatcaacacactctgtcctg | ggaaaaccagatggccagcg | 182 |
| Exon 6 | tcctccttcctctctctggc | tctgcaagccgcctgcaccg | 180 |
| Exon 7 | ggcgaagggatgggtagggg | gttgccatgtcaggaagcgc | 242 |
| Exon 8 | cattggggaagagcctcccc | ccacccgccgccttcccgtgctcac | 206 |
| Exon 9 | tccatcgacgggtcccctctgaccc | agccctcatctcacctgcgggccaa | 275 |
| Exon 10 5' end | agatgagggctcctgctggtgcgatgcc | gcccttggtatccgcaacagagaca | 213 |
| Exon 10 3' end | gatccacagcaacatctactggacc | agccctcagcgtcgtggata | 166 |
| Exon 11 | cagctattctctgtcctcccaccag | gctgggacggctgtcctgccaccag | 177 |
| Exon 12 | gcacgtgacctctccttatccactt | cacctaagtgcttcgatctcgtacg | 213 |
| Exon 13 | gtcatcttccttgctgcctg | gtttccacaaggaggtttcaaggtt | 217 |
| Exon 14 | gaatcttctggtatagctgat | gcagagagaggctcggagg | 284 |
| Exon 15 | gaagggcctgcaggcacgtggcact | gtgtggtggcgggcccagtcttt | 255 |
| Exon 16 | cctcactcttgcttctctcctgca | cgctgggggaccggcccgcgcttac | 134 |
| Exon 17 | gggtctctggtctcgggcgc | ggctctggctttctagagaggg | 245 |
| Exon 18 | gcctgtttcctgagtgctgg | tctcaggaagggttctgggc | 140 |

### 3.5.3.1 PCR set up

A master mixture of PCR reagents (Qiagen) was prepared for PCR analysis as shown in Table 3.2. For each reaction tubes, 100 ng of genomic DNA was mixed with 0.2 µM of each primers, 1.5 mM of $MgCl_2$, 200 µM of each dNTPs, 1X PCR Buffer and 2.5 U of *Taq* Polymerase (Qiagen). The PCR analysis was performed in a final volume of 50µl. Seventy four samples from FH patients and 77 samples from NC subjects were amplified with the use of the Mastercycler Gradient (Eppendorf). PCR reactions were performed at 95 $^{o}$C for 5 minutes, followed by 35 cycles at 95 $^{o}$C for 1 minute, 57 $^{o}$C for 1 minute (with the exception of exon 16, which was run at 65 $^{o}$C), and 72 $^{o}$C for 1 minute, respectively, and with final elongation step at 72 $^{o}$C for 7 minutes. A reaction tube without DNA template was included as negative control in all PCR analysis. The conditions for PCR for promoter region and all exons were tabulated in Table 3.3 with exception for exon 16 was shown in Table 3.4

**Table 3.2: PCR master mixture for amplification of LDLR gene**

| Component | Reaction concentration | Volume (µL) |
|---|---|---|
| 10X PCR buffer | 1X | 5 |
| $MgCl_2$ solution, 25mM | 2.0mM | 1 |
| dNTP mix, 10mM | 200µM | 1 |
| *Taq* DNA polymerase, 5U/µl | 2.5U | 0.5 |
| Primers Forward | 0.2µM | 1 |
| Primers Reverse | 0.2µM | 1 |
| Template DNA | 100ng | 10 |
| Nuclease-free water | - | Top up to 50.0 |
| Total volume | | 50.0 |

**Table 3.3: PCR protocols for the amplification of LDLR gene for promoter region and all exons with exception of exon 16.**

| Step | Temperature ($^{o}$C) | Time (Minute) | Number of cycles |
|---|---|---|---|
| Initial denaturation | 95 | 5 | 1 |
| Denaturation | 95 | 1 | 35 |
| Annealing | 57 | 1 | 35 |
| Elongation | 72 | 1 | 35 |
| Final Elongation | 72 | 7 | 1 |

**Table 3.4: PCR protocols for the amplification of LDLR gene for exon 16.**

| Step | Temperature ($^{o}$C) | Time (Minute) | Number of cycles |
|---|---|---|---|
| Initial denaturation | 95 | 5 | 1 |
| Denaturation | 95 | 1 | 35 |
| Annealing | 65 | 1 | 35 |
| Elongation | 72 | 1 | 35 |
| Final Elongation | 72 | 7 | 1 |

### 3.5.3.2 Analysis of PCR product

The analysis of PCR product was performed using DNA 1000 kit (Agilent) on an automated on-chip capillary gel electrophoresis (Agilent 2100 Bioanalyzer). The DNA 1000 kit contained chips and reagents designed for sizing and analysis of DNA fragments. Twenty five microliters of DNA dye concentrate was added into 500µl DNA gel matrix and the mixture was vortexed vigorously. The mixture was spun down briefly and transferred to a spin filter provided by the kit. The gel-dye mixture in the spin filter was centrifuged at 4400 revolutions per minute (rpm) for 15 minutes. The filtrate was covered with aluminium foil to protect from light and stored at 4 $^{o}$C until use. The gel-dye mix must be equilibrated to room temperature for 20 minutes before use. A syringe provided with the kit was attached to the luer lock adapter and screwed tightly to the priming station (Figure 3.1).

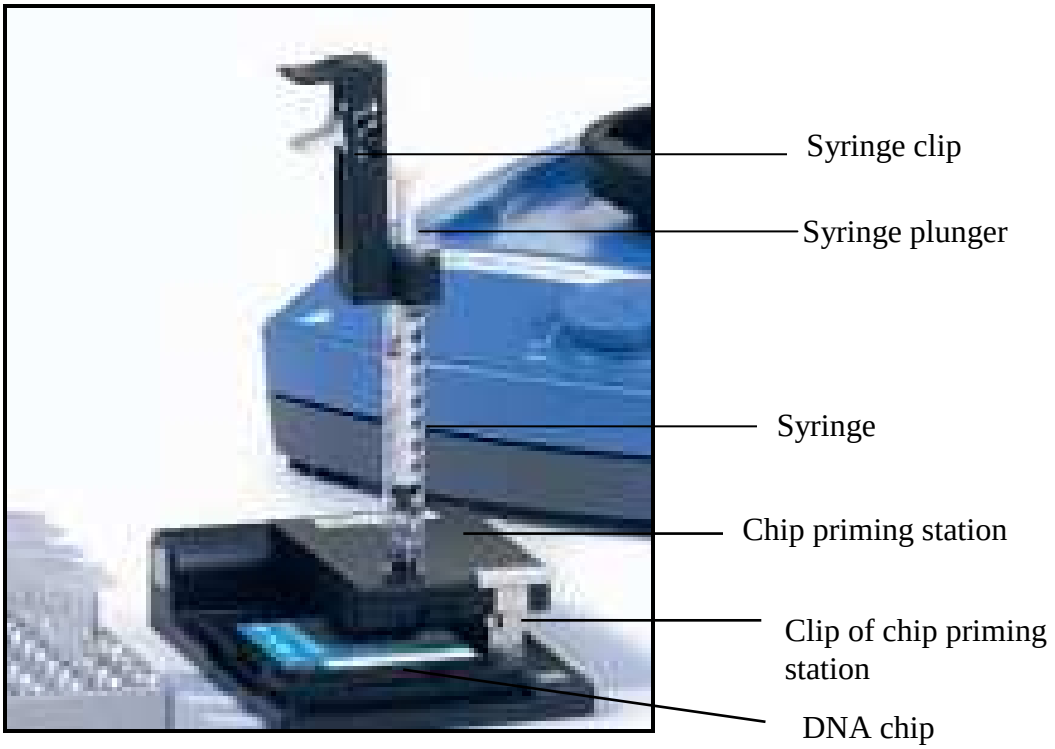


**Figure 3.1: Chip priming station of Agilent Bionalyser**

Syringe clip was adjusted to the lowest position in order to hold the syringe. The DNA chip was placed on the chip priming station and 9 µl of gel-dye mix was pipetted into well marked with G. The plunger of the syringe was pulled to 1-ml position and then the chip priming station was closed. The plunger was pressed down from the 1-ml position until was held by the clip of chip priming station for 1 minute. The clip of chip priming station was released after 1 minute and the plunger was pulled back to 1-ml position. The chip priming station was opened and 9 µl of gel-dye mix was pipetted into well marked with G. Five µl of marker was pipetted into all 12 sample wells and ladder well. One µl of ladder was pipetted into the well marked as ladder. One µl of amplified samples was pipetted into each of 12 samples wells. The DNA chip was placed horizontally in the vortex adapter and vortexed for 1 minute at 2400 rpm. The chip was analysed using Agilent 2100 Bioanalyser (Figure 3.2) within 5 minutes.

**Figure 3.2: Agilent Bioanalyser System for PCR product analysis**

### 3.5.3.3 PCR Product purification

The PCR products were purified using MinElute PCR purification kit (Qiagen) before subjected to DNA sequencing. Five volume of Buffer PB or binding buffer containing guanidine hydrochloride and isopropanol were added to 1 volume of PCR sample and mixed well. The MinElute column was placed in 2 ml collection tube. The sample mixture was applied to the column and centrifuged at 13000 rpm for 1 minute to bind the DNA. The flow-through was discarded and the column was placed back into the same 2 ml collection tube. Seven hundred and fifty microliters buffer PE (wash buffer) was added to the MinElute column and centrifuged at 13000 rpm for 1 minute. The flow-through was discarded and the column was placed into the same 2 ml collection tube. The column was centrifuged for an additional 1 minute to remove residual ethanol from buffer PE. The MinElute column was placed in another sterilized 1.5 ml microcentrifuge tube. Ten µl of Buffer EB (10 mM Tris-Cl, pH 8.5) was added to the centre of the membrane of MinElute column. The column was incubated at room temperature for 1 minute and then centrifuged at 13000 rpm for 1 minute.

## 3.5.4 Denaturing High Performance Liquid Chromatography (DHPLC)

Mutation screening was performed using partial denaturation mode of DHPLC on Wave Nucleic Acid Fragment Analysis System (Transgenomic, USA). Equal volumes of unpurified PCR products from 74 FH patient samples and 77 NC samples were mixed in a PCR tubes. The mixtures were placed into Mastercycler Gradient (Eppendorf). The mixtures were denatured at 95 $^{o}$C for 5 minutes, followed by a slow reduction in temperature at a rate of 0.1 $^{o}$C per seconds until a temperature of 25 $^{o}$C is reached. These steps were necessary for the formation of heteroduplex. Five µl were injected into preheated $C_{18}$ reverse-phase column of DHPLC. DNA samples were eluted within a linear acetonitrile gradient consisting of Buffer A (0.1 mol/L

triethylammonium acetate) and Buffer B (250 ml/L acetonitrile). The analysis of melting profile of each DNA fragment for each LDLR exons were deducted using Transgenomic Wave software based on the temperature at which heteroduplex detection occurred. The DHPLC temperature profiles for each LDR exon were listed in Appendix D.

### 3.5.5 DNA Sequencing

Forty six of FH samples showing heteroduplex peaks on DHPLC chromatogram were sent for sequencing in order to confirm the mutations. The purified PCR products of 46 FH samples were sequenced on an ABI 3730xl DNA Analyser (Applied Biosystem, USA) at 1$^{st}$Base Laboratory in Seri Kembangan, Selangor. Ten nanogram (ng) of purified PCR products were mixed with 4 µl of Ready Reaction Mix and 2 µl BigDye Sequencing Buffer (BigDye$^{TM}$ Terminator Cycle Sequencing Kit, Applied Biosystem, USA). Five picomol (pmol) for each set of sequencing primers were added into the mixture and finally deionised water was added to make a total volume of 20 µl. In another sterile microtube, a control reaction was prepared by adding 200 ng/µl of control DNA (M13mp18) into 4 µl Ready Reaction Mix, 2 µl BigDye Sequencing Buffer and 2 pmol/µl control primer. Deionised water was added into the control reaction microtube to make a total volume of 20 µl. Both control and patient samples mixture were mixed thoroughly, centrifuged and placed into the thermal cycler. Cycle sequencing was carried out using a Mastercycler Gradient (Appendorf) which was programmed as 95 $^{o}$C for 20 sec, 50 $^{o}$C for 15 sec and 60 $^{o}$C for 1 min. The cycle was repeated for 35 cycles. The product of this cycle sequencing process was then precipitated using ethanol/ammonium acetate precipitation protocol.

#### 3.5.5.1 Ethanol/Ammonium Acetate Precipitation

For 1x reaction, 20 µl of each cycle sequencing reaction product was transferred into a fresh reaction tube containing 2 µl of 7.5M ammonium acetate. Sixty microliters of cold 95 % ethanol was added to the reaction tube and mix well. The mixture was centrifuged for 15 minutes at 12,000 rpm. The supernatant was aspirated and discarded. The pellet was washed by adding 200 µl 70 % ethanol and centrifuged for 5 minutes at 12,000 rpm. The supernatant was drawn off and air dried for 10 to 15 minutes. The pellet was resuspended in 10 µl of injection buffer and injected into ABI 3730 DNA Analyzer (Applied Biosystem, USA). Finally, the sequence results were analysed and aligned with the reference sequences of LDLR gene obtained from nucleotide database of the National Centre for Biotechnology Information (NCBI) using Invitrogen Vector NTI Advance Version 10 software.

### 3.5 Statistical Analysis

Demographic variables are presented as mean ± 1 standard deviation (SD) for continuous normally distributed variables, as mean $\pm$ SEM for continuous non-normally distributed data, and as percentages for categorical data. Analysis of normality was performed with the Kolmogorov–Smirnov test. For continuous normally distributed variables, comparisons between the two groups were performed by use of Student's t-test. While non-normally distributed data were compared between the groups by using Mann-Whitney test. Categorical data and proportions were analysed using Chi-square test. Pearson's or Spearman's correlation coefficient was used to analyze correlation between two variables with normal distribution or non-normal distribution respectively. A p-value $<0.05$ was considered statistically significant. All statistical analysis was performed on the Statistical Package for Social Sciences (SPSS version 16.0) software.