3. Materials and Methods

3.1 Study design:
This is a quantitative, analytical case control study.

3.2 Study area, period and population:
The study was done in Khartoum state, the study was carried during the period between March 2013 to July 2013, and the Study was conducted on Sudanese smokers as a test group and healthy volunteers nonsmokers (age and sex were matched) were involved as a control group.

3.5 Selection criteria:
3.5.1 Inclusion criteria:
Test group: Sudanese smokers.
Control group: Healthy Sudanese nonsmokers.

3.5 Exclusion criteria:
Patients with type I and type II diabetes mellitus, hypertension, hyperlipidemia and patients with signs of any acute or chronic conditions, affecting cognitive functions, was considered as exclusion criteria.

3.6 Ethical consideration:
The objectives of the study were explained to all individuals participating in this study.
An informed consent was obtained from all participants.
Health education was provided to all interested participants.

3.7 Data collection and analysis:
3.7.1 Interview with a questionnaire:
An interview with a questionnaire to obtain clinical data was used for each participant in this study.

3.7.2 Blood sample collection:
After informed consent, a local antiseptic (70% ethanol) was used to clean the skin.
Venous blood (4 mL) were taken from each participant by standard procedure, and were
put in plain containers for 30 minutes, and then centrifuged at 3000 rpm for 15 minutes and serum was separated and obtained in a darkened Plain eppendorf’s tubes for being kept at minus 20 degrees Celsius (-20°C) until they are used.

3.8 Biochemical measurements and instruments used:
Atomic absorption spectrophotometric method ‘using Buck Scientific Atomic Absorption Spectrophotometer Model: 210VGP’ was used for measuring serum levels of copper (Cu), magnesium (Mg) and zinc (Zn). The method used in this study had been selected because all are available in Sudan, easy to perform, sensitive and specific method.

3.8.1 Principle:
While a sample is being aspirated into a flame, a light-beam is directed through the flame into a monochromator and onto a detector that measures the amount of light absorbed by the atomized element in the flame. A source lamp composed of the element of interest is used because each element has its own characteristic wavelength. This makes the method relatively free from spectral or radiation interferences. The amount of energy at the characteristic wavelength absorbed in the flame is proportional to the concentration of the element in the sample over a limited concentration range. Most atomic absorption instruments are also equipped for operation in an emission mode.

3.8.2 Composition of Reagents:
Metal-free water was essentially used for the preparation of all reagents. Hydrochloric acid and nitric acid, HNO₃, (analytical grade) was used for standard preparation and for digestion methods.

3.8.3 Procedure:
3.8.3.1 Adjustment of the Instrument:
1. A hollow cathode lamp for the desired element was installed in the instrument and the wavelength dial was set to the appropriate setting for the element.
2. The slit width was set according to the manufacturer’s suggested value for the element being measured.
3. The instrument then turned on and the lamp current was adjusted to the level suggested by the manufacturer.
4. The instrument then warmed up, 10-20 minutes, and current readjusted as necessary.
5. The wavelength dial was adjusted until optimum energy gain is obtained.
6. The lamp was aligned in accordance with the directions in the operating manual.
7. The suitable burner head was installed and its position was adjusted.
8. The air was then turned on and its flow was adjusted to the rate recommended to give maximum sensitivity for the metal being measured.
9. Acetylene then turned on and its flow was adjusted to recommended rate, then ignited and the flame was allowed a few minutes to stabilize.
10. A blank of deionized water that has been given the same treatment and acid concentration as the standards and samples was aspirated and the reading adjusted to zero.
11. A Standard solution was aspirated and the aspiration rate was adjusted to obtain maximum sensitivity.
12. The burner was adjusted horizontally and vertically to obtain maximum response.

3.8.3.2 Preparation of the calibration curve

1. At least five concentrations of each metal ion standard solutions were selected to perform a calibration curve. There should be one concentration greater and one less than that expected in the sample(s).
2. A blank was aspirated and adjusted to the zero value.
3. Each standard was aspirated in turn into the flame and the absorbance was recorded.
4. A calibration curve was performed by plotting the absorbance of the standards against their concentrations. This step is not necessary for instruments with direct concentration readout.
3.8.4 Copper analysis:

3.8.4.1 Preparation of standards:
Standard solutions of known metal concentrations were prepared in water with a matrix similar to the tested samples. Copper standards are prepared by diluting stock standard solution with 10% glycerol (v/v). A 10% glycerol solution must be used as a blank solution when determining copper.

3.8.4.2 Preparation of Samples:
The nebulizer was rinsed by aspirating with water containing 1.5 ml HNO3 per liter. The blank was atomized and set to the zero value. For determination of serum copper dilute the sample with an equal volume of deionized water. Allow plasma samples to come to room temperature and then mix each sample by gently inverting the tube six times. Prepare working standards as previously described. Establish instrumental and gas-flow settings and aspiration rate precisely, to optimize signal and minimize background noise.

3.8.4.3 Normal serum levels:

<table>
<thead>
<tr>
<th>Element</th>
<th>Unit</th>
<th>µg%</th>
<th>mg/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Copper</td>
<td></td>
<td>70 – 140</td>
<td>0.7 – 1.4</td>
</tr>
</tbody>
</table>

3.8.5 Magnesium analysis:

3.8.5.1 Preparation of standards:
Standard solutions of known metal concentrations were prepared in water with a matrix similar to the tested samples. Magnesium standards are prepared by diluting stock standard solution with 0.1% (w/v) lanthanum as chloride. Deionized water can be used as a blank solution when determining Magnesium.

3.8.5.2 Preparation of Samples:
The nebulizer was rinsed by aspirating with water containing 1.5 ml HNO3 per liter. The blank was atomized and set to the zero value. For determination of serum magnesium
dilute the sample 1:50 with deionized water ‘or 0.1%(w/v) lanthanum as chloride can be used as diluent’ Allow plasma samples to come to room temperature and then mix each sample by gently inverting the tube six times. Prepare working standards as previously described. Establish instrumental and gas-flow settings and aspiration rate precisely, to optimize signal and minimize background noise.

3.8.5.3 Normal serum levels:

<table>
<thead>
<tr>
<th>Element</th>
<th>µg%</th>
<th>mEq/L</th>
<th>mg/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Magnesium</td>
<td>1700 – 2800</td>
<td>1.5 – 2.3</td>
<td>17.0 – 28.0</td>
</tr>
</tbody>
</table>

3.8.6 Zinc analysis:

3.8.6.1 Preparation of standards:
Standard solutions of known metal concentrations were prepared in water with a matrix similar to the tested samples. Zinc standards are prepared by diluting stock standard solution with 5% glycerol. A 5% glycerol solution must be used as a blank solution when determining zinc.

3.8.6.2 Preparation of Samples:
The nebulizer was rinsed by aspirating with water containing 1.5 ml HNO3 per liter. The blank was atomized and set to the zero value. For determination of serum zinc dilute the sample 1:5 with deionized water.
Allow plasma samples to come to room temperature and then mix each sample by gently inverting the tube six times. Prepare working standards as previously described. Establish instrumental and gas-flow settings and aspiration rate precisely, to optimize signal and minimize background noise.

3.8.6.3 Normal serum levels:

<table>
<thead>
<tr>
<th>Element</th>
<th>µg%</th>
<th>mg/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zinc</td>
<td>50 – 120</td>
<td>0.5 – 1.2</td>
</tr>
</tbody>
</table>
3.8.7 Calculations:
Concentrations are read directly from instruments with a direct readout capability. If a sample has been diluted, appropriate dilution factor were applied. The recommended wavelengths are 324.8, 285.2 and 213.9 for copper, magnesium and zinc respectively.

3.9. Quality Control:
The use of standard to calculate results to obtain accuracy independent of the system and instrument used. To ensure adequate quality control (QC), each run included a set of controls.

3.10 Data processing and analysis:
Data were collected manually in a master sheet and analysis was performed using Statistical packages for social sciences program (SPSS version 18), using Pearson correlations.