CHAPTER 5: DATA RECORDED

5.1 Demographic and clinical data

The demographic data recorded included age, gender, occupation, race, suburb of residence, alcohol and smoking history, and nutritional history (meat and fruit intake) of the patients. Clinical parameters documented and recorded included duration and severity of symptoms namely shortness of breath, cough, pleuritic pain, fever and night sweats and weight loss for the study patients, and admission diagnosis for the control group. Examination findings recorded included blood pressure, pulse rate, temperature, presence or absence of oral thrush, lymphadenopathy, wasting, skin changes, weight, height (BMI calculated), chest and abdominal findings.

5.2 Laboratory data

The laboratory data recorded were all results of routine blood tests performed on the patients by their respective doctors. This was not part of the investigational data in the study. It included haematological and biochemical parameters. The haematological data recorded included white cell count, haemoglobin, mean cell volume, platelet count and the erythrocyte sedimentation rate. The biochemical data included urea, electrolytes, creatinine, total protein, albumin, billirubin, alkaline phosphates, γ Glutamyl transferase, aspartate aminotransferase, alanine
aminotransferase and C reactive protein. HIV status was recorded if this information was available. Sputum results for acid-fast bacilli by the Ziehl Nielsen technique were recorded. Gram stain results as well as sputum culture for tuberculosis were recorded, if available.

5.3 Chest radiography

Abnormalities on chest radiographs were recorded for all patients. These abnormalities included cavitation, nodulation, consolidation, hilar adenopathy and pleural effusions in the study group and pneumothoraces, haemo-pneumo-thoraces or pulmonary contusion in the study group.

5.4 Core research laboratory data

The core research-related laboratory data performed in both study and control patients included white cell vitamin C levels, plasma vitamin C levels, serum cortisol, plasma catecholamines levels (dopamine, epinephrine, norepinephrine), serum C- reactive protein levels, ferritin levels and urine cotinine levels.
5.5 Sample collection and analysis for core research laboratory data parameters

Blood was collected by peripheral venipuncture between 8:00am and 9:00am after an eight-hour overnight fast.

5.5.1 Measurement of plasma vitamin C

Blood collected was separated and serum stored at -70°C until sufficient numbers were obtained and were analysed for vitamin C by the 2, 4 – dinitro phenyl hydrazine (DNPH, Sigma Chemical Co.) spectro-photometric method (Attwood et al 1974). Plasma proteins were precipitated with 25% trichloro acetic acid (12.5% final concentration) in a volume of 4ml to give a final serum dilution of a half. The precipitated serum proteins were deposited by centrifugation and the supernatants removed for the vitamin C assay. 1ml of plasma/trichloro acetic acid supernatant was mixed with 0.3ml of the DNPH/thiourea/CuSO₄ solution or with 0.3ml distilled H₂O (plasma background control system) in clean 5ml glass test tubes. The tubes were then incubated for four hours at 37°C in a water bath after which the tubes were held on ice and 1.5ml of 65% H₂SO₄ added to each tube (to extract the chromogens), vortexed, and assayed spectrophotometrically at 520 nm. Plasma concentrations of vitamin C were calculated from a standard curve constructed from known concentrations (2.5 – 20
μg/ml) of the vitamin. The results are expressed as μg vitamin C/ml plasma.

5.5.2 White cell vitamin C

This was analysed by white cell vitamin C assay (modified from Marchand and Pelletier 1977). After collecting whole blood in ethylenediamine tetraacetate (EDTA) tubes, 0.6 ml of 1% CMC solution (0.9 gm NaCL, 1.0 gm Methyl Cellulose, 2 ml Glycerol, made up to 100 ml with distilled water) was added, mixed well and allowed to settle. 2 ml plasma was taken off and placed in a 15 ml conical plastic tube, filled up with normal saline to 12 ml mark and mixed well. 0.5 ml was taken off for a white cell count. The rest was spun for 10 minutes at approximately 2000rpm, supernatant decanted and pellets stored at -20ºC until sufficient were obtained. Pellets were thawed and 1.3 ml 5 % TCA (Trichloracetic acid) was added and spun for 10 minutes at 2600rpm. One ml supernatant was taken off and 0.3 ml of 2,4-dinitrophenylhydrazine (DNPH) reagent mixture added. This was incubated for 4 hours at 37ºC, placed on ice and 1.5 ml 65% H₂SO₄ added and allowed to stand for 30 min. on ice. A blank solution consisting of 1 ml 5% TCA and 0.3 ml DNPH reagent mixture was made. Values were read at 520 nm, using the blank as zero and
concentrations calculated from a standard curve. Concentration expressed as μg Vitamin C × 10⁸ leucocytes.

5.5.3 Cortisol

This was analysed by Radioimmunoassay, ADVIA Centaur cortisol assay, (Bayer Diagnostic USA). The ADVIA Centaur Cortisol assay is a competitive immunoassay using direct chemiluminescent technology. Cortisol in the patients sample competes with a cradiniun ester- labeled cortisol in the Lite Reagent for binding to polyclonal rabbit anti-cortisol antibody in the Solid Phase. The polyclonal rabbit anti-cortisol antibody is bound to monoclonal mouse anti-rabbit antibody, which is covalently coupled to paramagnetic particles in the Solid Phase. The system automatically performed the following:

- Dispensed 20μL of sample into a cuvette.
- Dispensed 50μL of Lite Reagent and 250μL of Solid Phase and incubated for 5 minutes at 37°C.
- Separated, aspirated and washed the cuvettes with reagent water.
- Dispensed 300μL of Acid Reagent and 300μL of Base Reagent to initiate the chemiluminescent reaction.

Results reported as nmol/L. The ADVIA Centaur Cortisol assay is standardized analytically and confirmed by gas chromatography-mass spectroscopy.
5.5.4 Catecholamines

These were analysed by high performance liquid chromatography (HPLC) method (BIO-RAD Laboratories Diagnostics Group, California USA).

A small quantity of alumina (30-40mg) is placed in conical centrifuge tubes, to which 2ml of plasma, 200μL working internal standard and 1ml TRIS buffer are added. Plasma catecholamines are absorbed onto the lumina with vigorous shaking of the tubes. The tubes are then centrifuged, the supernatant removed and the alumina washed with two 1ml aliquots of water. 150μL of dilute phosphoric acid were added to the alumina and thoroughly mixed to extract the catecholamines from the alumina. The tubes were centrifuged once more and the supernatant carefully removed from the alumina. 50μL of the supernatant were injected onto a Plasma Catecholamine (PCAT) Analytical Column using an Isocratic mobile phase. Aquantition was based on comparison to the standards. Results reported in ρmol/L.

5.5.5 C Reactive protein

Serum levels of CRP were quantified by an automated nephelometric procedure using reagents and equipment supplied by Behring Diagnostics (Marburg, Germany). Serum (50 μl) was
incubated with N-CRP reagent (60 μl, Behring Diagnostics). Six minutes after mixing, concentration of serum CRP was determined using a Behring Nephelometer Analyser 11, according to the extent of dispersion of the light beam. The results are expressed as mg CRP/ml serum.

5.5.6 Ferritin

Serum ferritin levels were measured using a commercial sandwich ELISA/Immuno magnetic system (Technicon Immuno – 1 system, Miles Inc., Diagnostic Division, Tarrytown, NY, USA). Serum (30 μl) was mixed with 65 μl of alkaline phosphatase – conjugated murine anti-human ferritin monoclonal antibody and 65 μl of a fluorescein – conjugated murin anti-human ferritin monoclonal antibody in reaction cuvettes and incubated for two hours at 37°C on the Immuno – 1 system. Following incubation, 25μl of immuno magnetic particles anti flourescein murine monoclonal antibody – coated magnetic particles were added to the cuvettes, which were re-incubated for two hours at 37°C, during which the antigen/antibody complexes bind to the particles. The particles are then washed in assay buffer before addition of 300 μl of p-nitrophenyl phosphatase to form a coloured reaction product, p-nitrophenoxide, which is detectable spectrophotometrically at 405 - 450 nm. The intensity of the colour is in direct proportion to the
amount of ferritin in the serum sample. Results are expressed as μg ferritin/L serum.

5.5.7 Urine cotinine

Cotinine concentrations in urine specimen were measured using a commercial, competitive binding radioimmunoassay procedure (Nicotine Metabolite Kit, Diagnostic Products Corp., LA, California, USA). Urine samples (50 μl) were incubated with 100 μl of I\textsuperscript{125} cotinine and 100 μl of nicotine metabolite anti-serum incubated for 30 minutes at room temperature. One microlitre of cold precipitating solution (16% polyethylene glycol) was added following incubation. The complexes were then collected, centrifuged and the supernatants containing unbound I\textsuperscript{125} cotinine decanted. The amount of radio activity in the precipitates (inversely related to urine cotinine concentration) was measured using a LKB1261 multi gama (Turku, Finland), gama counter. Concentrations of cotinine in the urine specimens were calculated from a standard curve constructed from a series of known standards provided by the manufacturer. Results were expressed as μM cotinine/L urine.
5.6 **Statistical analysis**

Demographic data, habits, clinical data and laboratory data were captured and analysed using SPSS 7.5 software. Continuous variables were analysed using students t-test, categorical data by non-parametric analysis and a linear regression model was used to examine effects of, and correlation, between vitamin C and other variables in the two groups. Statistical significance was defined as P less than 0.05.

5.7 **Ethical considerations**

Consent for the study was obtained from the University of Witwatersrand committee for research on human subjects- **PROTOCOL NUMBER: M01-11-14**. Informed written consent was obtained from the patients and confidentiality of all results was upheld by restricting access to investigations and using codes to identify patients.