

**UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG
SCHOOL OF CLINICAL MEDICINE**

**THE RELATIONSHIP OF ZINC AND COPPER WITH
STAGE IN NON-SMALL CELL CANCER OF THE
LUNG**

by

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**A RESEARCH REPORT (RASE7018) SUBMITTED TO THE SCHOOL OF
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DECLARATION

I, Dinoshan N Chetty, declare that this research report is my own work. It is being submitted for the degree of Master of Medicine (Radiation Oncology) at the University of the Witwatersrand, Johannesburg. It has not been submitted previously for any degree or examination at this or any other university.

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Full Name: Dinoshan Namasivayan Chetty

May 2011

DEDICATION

To my mother, Panji, who both inspired and persuaded me to complete this report.

ABSTRACT

Objective

Several studies have shown that serum copper concentrations are higher in various carcinomas when compared to a healthy population; owing to their role as an inflammatory marker. Zinc acts as a cellular growth protector and it has been demonstrated that its deficiency is involved in several stages of malignant cell transformation. However, the usefulness of the serum zinc and copper determinations in cancer prevention, detection, treatment monitoring and prognosis require further investigation.

The aim of this study is to demonstrate the diagnostic and prognostic significance of serum zinc levels (SZL) and serum copper levels (SCL), and the copper/zinc (Cu/Zn) ratio, in non-small cell lung cancer (NSCLC).

Materials and Methods

Thirty-four patients with NSCLC were prospectively identified, prior to treatment, over a period of one calendar year (February 2003 - January 2004) at the Department of Radiation Oncology, Johannesburg General Hospital (now Charlotte Maxeke Johannesburg Academic Hospital) and the University of the Witwatersrand. SCL and SZL were measured using atomic absorption spectroscopy (AAS) and the Cu/Zn ratio was calculated.

Results

SCL shows an increase (mean SCL were 0.66mg/L, 0.74mg/L and 0.76mg/L for stage II, III and IV respectively) (P=0.0897); and SZL shows a decrease (0.70mg/L, 0.63mg/L and 0.62mg/L for stage II, III and IV respectively) (P=0.199) with advancing stage. The levels of both these trace elements are much lower than the reference range for a normal population. The Cu/Zn ratio increases with stage (0.995, 1.308 and 1.441 for stage II, III and IV respectively). The results were not statistically significant, but a definite trend could be observed. In addition, marked differences were noted between early stages (II) and advanced stages (III and IV) of the disease.

Conclusion

The lower levels of both trace elements, when compared to a reference range, are an indication of the low levels of immunity and poor general condition of patients with NSCLC (with particular reference to the author's institution). A clear trend could be demonstrated of increasing SCLs and decreasing SZLs with progressive stages in NSCLC. The Cu/Zn ratio also reflects similar findings in relation to stages of the disease.

The results were not statistically significant, although this can be attributed to a small sample size. While trace element levels and the Cu/Zn ratio cannot be advocated as a tumour marker and prognostic variable for NSCLC at present, they do merit further study, especially in a resource constrained environment, as a simple and inexpensive diagnostic and prognostic test.

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DEFINITION OF TERMS

Performance Status

In the clinical discipline of oncology, two main classifications are used in the assessment of a patient's general physical and functional condition, viz.:

1) Eastern Co-operative Group (ECOG) Staging (a simple grading system of functional status from 0-5).

0	Asymptomatic (Fully active, able to carry on all pre-disease activities without restriction.)
1	Symptomatic but completely ambulatory (Restricted in physically strenuous activity but ambulatory and able to carry out work of a light or sedentary nature. For example, light office work).
2	Symptomatic, <50% in bed during the day (Ambulatory and capable of all self-care but unable to carry out any work activities. Up and about more than 50% of waking hours.)
3	Symptomatic, >50% in bed, but not bedbound (Capable of only limited self-care, confined to a bed or chair 50% or more of waking hours.)
4	Bedbound (Completely disabled. Unable to carry out any self-care. Totally confined to a bed or chair.)
5	Death

(Also known as the WHO scoring.)

This can be summarized as 0-2 being good general condition and probably suitable for radical treatment; and 3-4 being poor general condition and probably suitable for palliative treatment.

2) Karnofsky Scoring - a more extensive scoring system (from 100 - 0)

100%	normal, no complaints, no signs of disease
90%	capable of normal activity, few symptoms or signs of disease
80%	normal activity with some difficulty, some symptoms or signs
70%	caring for self, not capable of normal activity or work
60%	requiring some help, can take care of most personal requirements
50%	requires help often, requires frequent medical care
40%	disabled, requires special care and help
30%	severely disabled, hospital admission indicated but no risk of death
20%	very ill, urgently requiring admission, requires supportive measures or treatment
10%	moribund, rapidly progressive fatal disease processes
0%	Death

Early Stage and Advanced Disease

The general clinical opinion in oncology (NCCN level 1 consensus) with regard to NSCLC is that Stage I & II of the disease (AJCC 2002) be viewed as early stage, and Stage III & IV as advanced disease stage (in terms of clinical decision making).

LIST OF ACRONYMS AND ABBREVIATIONS

AAS:	Atomic absorption spectroscopy
AJCC:	American Joint Committee on Cancer
CT:	Computerized tomography
NHLS:	National Health Laboratory Services
NSCLC:	Non-small cell lung cancer
QC:	Quality control
SCLC:	Small cell lung cancer
SZL:	Serum zinc levels
SCL:	Serum copper levels
ECOG:	Eastern Co-operative Oncology Group
NCCN:	National Comprehensive Cancer Network

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CHAPTER ONE: INTRODUCTION AND LITERATURE REVIEW

Lung cancer accounts for 18% of deaths from cancer, making it the cancer with the highest mortality rate in the world, in both men and women.¹

Cigarette smoking increases the risk of getting lung cancer and dying from it, with approximately 80% of cases caused by smoking.² Other causes of lung cancer are genetic factors³, exposure to asbestos⁴, atmospheric pollution and passive smoke inhalation.^{5,6,7}

Lung carcinogenesis is known to occur from an accumulation of several genetic alterations, most commonly with p53 mutations and deletions on chromosomes 3p, 5q, 9p, 11p and 17p.⁸ These alterations are more frequently seen in smokers than in non-smokers⁸ and peak incidence occurs in the 7th and 8th decades.

Changes in smoking prevalence and patterns have produced changes in the incidence of lung cancer globally. While there is a substantial decline in the incidence of lung cancer in the developed world⁹ a rise in incidence is noticed in the populations of developing countries and among women.^{10,11} Socio-economic factors and level of education (both individual and awareness campaigns) show a strong correlation with lung cancer incidence and prevalence.

Lung cancer can be categorised histologically into two groups, viz.: small cell lung cancer (SCLC); and non-small cell lung cancer (NSCLC). SCLC is usually

disseminated at presentation, owing to its predisposition to early metastatic spread, thus conferring a poorer prognosis. SCLC is almost always associated with smoking, though its aetiology and pathophysiology are much less clear.¹²

Treatment of lung cancer is dependent on cell type, stage of the disease and the patient's performance status. Small cell lung cancer (SCLC) is usually considered a systemic disease at the time of diagnosis, owing to its propensity for widespread haematogenous spread (more than 75% of patients with SCLC present with metastatic disease).

Associated paraneoplastic syndromes include: the syndrome of inappropriate antidiuretic hormone (SIADH), most commonly, hypercoagulable states (common); ectopic adrenocorticotrophic hormone (ACTH) syndrome (uncommon); and Eaton-Lambert (myasthenia) syndrome (rarely seen with any other tumour). Hypercalcaemia occurs rarely in SCLC, even in the presence of extensive bony metastases.

SCLC is chemo-sensitive, which makes chemotherapy the most appropriate first line treatment. Radiotherapy may also be administered, usually as a palliative treatment,^{13, 14} or for prophylactic brain irradiation in selected cases. Relapse almost always occurs in SCLC in sites previously affected, as well as in previously uninvolved ones, after both chemotherapy and radiotherapy.

Non-small cell lung cancer (NSCLC) accounts for by far the majority of cases of lung cancer worldwide (80% - 85%). The histological variants (squamous, adenocarcinoma, large cell) are grouped together as NSCLC because of similarities in presentation, treatment and natural history.

Squamous cell carcinoma (20% - 25% of NSCLC) was previously thought to occur in a predominantly central location, whereas adenocarcinoma occurred peripherally. Recent studies show a changing radiographic presentation, with the two cell types having similar patterns of presentation.¹⁵ Compared with other kinds of lung cancers, squamous cell lung cancers are most likely to remain localised early in the disease and to recur locally after surgery or radiotherapy.

Hypercalcaemia, resulting from ectopic production of parathyroid hormone-related peptide (PTH–RP), is the most frequent associated paraneoplastic syndrome.

Hypertrophic osteoarthropathy (occasional), paraneoplastic neutrophilia (sometimes associated with hypercalcaemia), prominent joint syndromes (occasional), or hypercoagulability are also seen.

While adenocarcinoma is the most commonly associated sub-type in both non-smokers and women, most cases are related to smoking. More than half of all patients with adenocarcinoma initially localised as a peripheral nodule, have regional nodal metastases.¹⁵ Adenocarcinoma, and large cell carcinomas have similar natural histories and spread widely outside the thorax by haematogenous dissemination, commonly involving the bones, liver and brain.¹⁵ Associated paraneoplastic syndromes include: hypertrophic osteoarthropathy, hypercoaguable states, hypercalcaemia due to PTH–RP or cytokines, and gynaecomastia (large cell).

Although surgical excision offers the best prospect of cure for NSCLC, only about 20% of patients present at a resectable stage.¹⁶ This figure is probably lower in a South

African context. For NSCLC patients who are not eligible for surgery, radiotherapy or chemo-radiotherapy is the most appropriate treatment option .^{14, 17}

Survival outcome with lung cancer is one of the lowest when compared to other solid tumour cancers; this is largely due to late diagnosis in the majority of the patients, when curative treatment is no longer possible.^{14, 17} The reasons for these dismal statistics are essentially two-fold, viz.: lack of a cost-effective screening technique (with consequent late stage at presentation); and sub-optimal treatment for advanced stage disease. Both prevalence and survival have been correlated with socio-economic factors .^{10, 11}

Five year survival estimates in the USA, are 13% for men and 17% for women (overall). In the UK it is 7.4 % and 7.9% for men and women respectively.^{18, 19, 20} Lung cancer survival is related to stage at presentation, with survival declining at higher stages of malignancy. Five year survival rates for NSCLC for respective stages at presentation are:

- stage IIa : 36% - 46%
- stage IIb : 25% - 36%
- stage IIIa : 19% - 24%
- stage IIIb : 7% - 9%
- stage IV : 2 % - 13%¹⁸

There is a paucity of accurate statistics in the developing world, but consensus of opinion and crude statistics suggest that patients in a middle income developing country like South Africa would have a higher stage at presentation and a lower survival rate by stage when compared to patients in the USA .^{11, 12}

Trace elements are known to play a vital role in many biological processes by their action as competitors with other elements and proteins for binding sites (competitive inhibition). They also serve as molecular activators and inhibitors and in other processes of cell regulation.^{21, 22}

The biological role of trace metals, especially of copper and zinc, in different physiologic and pathological conditions has been the subject of many studies over the past two to three decades. Interest in the study of serum levels of trace elements has increased recently owing to research findings that suggest that these elements could be of value in the diagnosis, staging and monitoring of malignancies.^{20, 23, 24} In essence, the levels of these elements in serum, and their ratio to one another, could be used as a tumour marker.²⁵ Further impetus to this possible use has been added by the probability of reduced laboratory and other costs associated with testing, owing to advanced technology.

Zinc is an essential trace element in humans and is constituent of many enzymes. It is involved in many metabolic pathways and acts as a co-enzyme and a cellular growth protector. It plays an important role in nucleic acid metabolism, cell replication, tissue repair and growth through its function as a nucleic acid polymerase. It is essential for the synthesis of RNA and DNA.²⁶ Serum zinc levels (SZL) are shown to decline in malignant disease and its deficiency has been demonstrated to be involved in several stages of malignant disease progression²⁷⁻²⁹, with the level declining with advancing disease stages.^{26, 30-35}

Zinc, along with copper, represents a vital cog in the machinery of the cell.³⁶⁻⁴⁰ Zinc is an important component of enzymes, principally oxidases, and is associated with immune suppression, probably as a result of altered absorption. Through the stabilization of the structure of deoxyribonucleic acid (DNA), ribonucleic (RNA) and ribosome, zinc plays an anticarcinogenic role⁴¹, by also protecting cells from free-radical injury.⁴²

Clinical manifestations of zinc deficiency include growth retardation, retarded skeletal maturation, testicular atrophy and increased susceptibility to infections. The association of a zinc deficiency with malignancy is not clearly understood⁴¹, even though zinc deficiency is associated with advanced malignancy.⁴³⁻⁴⁶ Recently, emphasis has been placed on the study of tumour markers in human cancer.⁴⁶⁻⁵⁰ Disease perturbation of the homeostasis of serum concentrations of trace elements has been demonstrated to be a useful adjunct to diagnosis, as well as prognosis in specific diseases.⁴⁶⁻⁵⁰

Copper is an essential trace element that is widely distributed in food and water. Copper is a component of metalloenzymes such as ceruloplasmin, cytochrome oxidase, dopamine hydroxylase and tyrosinase.³⁵ Ceruloplasmin, which carries 96% of total serum copper, is responsible for oxidizing DOPA, dopamine, adrenaline, noradrenaline and serotonin.³⁶ In the extracellular space, ionised copper induces the synthesis of adhesive fibronectin and collagen, which stimulates the migration of neoplastic cells.³⁷
³⁸ In addition, ionized copper binds with heparin and fibroblast growth factor, an angiogenic protein that is also involved in cell migration.³⁹ Copper plays a key role in the absorption and metabolism of iron.⁴⁰ Copper deficiency states are rare and may

manifest clinically as anaemia or pancytopenia and, very rarely, as a neurodegenerative disorder.

Copper is found in a variety of enzymes and is closely related with haematopoiesis and the metabolism of osseous and connective tissue.²⁷ Copper has also been shown to be a vital angiogenic co-factor.^{27, 28} Serum copper levels (SCL) can be interpreted as an acute phase protein that increases in malignancy and in other causes of inflammation. Increases of SCL in malignant disease are shown to be much higher than in benign disease.²⁹

Previous reports have shown abnormal serum levels of zinc and copper in a number of malignancies, including lung cancer^{25, 29}, brain tumours³⁰ and ovarian cancer.³¹ However, contradictory data exist on the usefulness of copper and zinc measurements in the diagnosis and prognosis of cancer.²⁵⁻³¹ Furthermore, the ratio of serum copper to zinc has been shown to be a more reliable and accurate parameter for prognosis than individual serum copper or serum zinc levels (SZL). There is a trend to higher ratios in more advanced disease.³²

This study aimed to investigate the use of serum levels of copper (SCL), SZL and the serum copper to zinc (Cu/Zn) ratio as a diagnostic and prognostic tool as well as a possible tumour marker in NSCLC in the South African population.

CHAPTER TWO: AIM and OBJECTIVES

AIM

To assess the feasibility of biochemical parameters as a cost-effective staging and prognostic tool in non-small cell lung cancer (NSCLC), with specific reference to a developmental setting.

SPECIFIC STUDY OBJECTIVES

The objective of this study is to analyse pre-treatment levels of serum zinc (SZL) and copper (SCL) in NSCLC and to correlate these levels, as well as the Cu/Zn ratio, to help ascertain their diagnostic and prognostic value with regard to:

- diagnosis of NSCLC
- disease progression and stage, and thus usefulness as a prognostic variable
- use as a tumour marker

CHAPTER 3: METHODOLOGY

- This is a prospective cohort study, which was done at the Radiation Oncology Department of the Johannesburg General Hospital [now Charlotte Maxeke Johannesburg Academic Hospital]. Recruitment of participants was done at the Charlotte Maxeke Johannesburg Academic Hospital over a one-year period from February 1, 2003 to January 31, 2004.

Inclusion criteria were:

- Study participants were patients with NSCLC.
- Eligible patients had a cytological or histological confirmation of NSCLC
- All patients who volunteered to participate in this study were 18 years or older.
- All participants signed an Informed Consent Form (see Appendix 3).
- All the study participants were HIV negative at the time of enrolment.

Exclusion criteria were:

- Participants taking trace element and/or vitamin supplements, or with diseases of malabsorption.
- HIV infected patients.

NB:

Age, general condition and performance status (ECOG and Karnofsky Performance Status scale ratings) were NOT used as inclusion or exclusion criteria.

Participants were included in the study prior to treatment and their management was not affected in any way.

Cytological and histological diagnosis of NSCLC was carried out by the National Health Laboratory Services (NHLS). Histological sub-types of NSCLC viz. squamous cell lung cancer, adenocarcinoma and large cell carcinoma were specified.

Participants were counselled by a professional nurse. No financial incentives were offered and participation or refusal in the trial did not affect the medical management of participants.

All patients in the Department of Radiation Oncology have a known HIV status. HIV Elisa testing upon diagnosis of malignancy was standard departmental protocol during the period of the study. HIV infected patients were excluded, owing to the unknown effect of the infection on trace element absorption and secretion. To ensure accuracy in the serum levels of zinc and copper, patients taking trace element and/or vitamin supplements, or with diseases of malabsorption, were excluded.

Atomic absorption spectroscopy (AAS) was employed in measuring serum levels of copper and zinc.³³

3.1 Sample Size

Thirty-four (34) newly diagnosed, histologically or cytologically proven patients with NSCLC were prospectively included in the study. The study group comprised twenty-five (25) males and nine (9) females.

Owing to funding and logistic constraints, patient inclusion was limited to patients presenting at the Department of Radiation Oncology, Charlotte Maxeke Johannesburg Academic Hospital over a period of one calendar year.

None of the patients had undergone chemotherapy or radiotherapy before the commencement of the study.

Patients were staged according to the departmental protocol at the time. Cancer stage was established based on the results of routine investigations done at the department, viz.: chest x-rays, CT scans of the chest and abdomen, and/or bronchoscopy and mediastinoscopy. Bone scans were done when indicated, depending on the symptoms of the patients. The patients were staged according to the American Joint Committee on Cancer (AJCC) 2002 classification. Standard laboratory tests for lung cancer were also performed.

There were no patients with stage I of the disease. The studied patients were grouped into 3 stages, i.e. stages II to IV.

3.2 Blood Sample Collection and Measurement

Patients' blood samples were obtained by venepuncture to avoid external contamination and haemolysis. The blood was centrifuged and serum frozen until analysis. Blood sample collection was done by trained and registered phlebotomists at the Charlotte Maxeke Johannesburg Academic Hospital.

3.3 Sample Analysis and Measurement

The determination of serum levels of copper and zinc was carried out using AAS. AAS is a method of determining the concentration of a particular metal constituent in a sample. AAS uses the absorption of light to measure the concentration of gas-phase atoms.

The AAS process involves two major steps, i.e.:

- A) Atomisation of the sample: Samples in liquid or solid form are vaporized to convert atoms into a free state by exposing the sample to a flame. This converts the analysed sample into a form that can be measured. Atomisation involves the following steps:
- Desolvation: the liquid solvent is evaporated and the dry sample remains.
 - Vaporisation: the solid vaporises to a gas.
 - Atomisation.
 - The compounds making up the sample are broken up into free atoms.
- B) Absorption: The atoms in their free state are then exposed to light of appropriate wavelength, which causes the atoms to transition to higher electronic energy

levels. The greater the number of atoms there is in the vapour, the greater is the amount of radiation is absorbed. The amount of light absorbed is proportional to the number of atoms.

The analyte concentration is determined from the amount of light absorption, which is measured against a standard curve. A standard curve is constructed by running several samples of known metal concentration under the same conditions as used when running the unknown metal. The amount of the known absorption is compared with the standard curve and this enables the calculation of the unknown concentration in a sample.

Due to the unique configuration of electrons in its outer shell, every atom has its own distinct pattern of wavelength at which it will absorb energy. The wavelength absorbed corresponds to the energy needed to promote electrons from one energy level to another, higher, energy level.

In order to measure elements using the AAS technique, an atomic absorption spectrometer with the following components: a light source; a sample cell to produce gaseous atoms; and a means of measuring the specific light absorbed.

The light source

The source of light is a 'hollow cathode lamp', which contains a tungsten anode, and a cylindrical hollow cathode made of the element to be determined. These are sealed in a glass tube filled with an inert gas, such as neon or argon.

By applying a potential difference of about 300–400V between the anode and the cathode, ionization of atoms takes place. These gaseous ions bombard the cathode and eject metal atoms from the cathode in a process called sputtering. Some sputtered atoms are in an excited state and emit radiation characteristic of the metal as they fall back to the ground state. The shape of the cathode concentrates the radiation into a beam, which passes through a quartz window, and the shape of the lamp is such that most of the sputtered atoms are re-deposited on the cathode.

The atomic absorption instrument holds several lamps, each for a different element. These lamps are housed in a rotating turret so that the correct lamp can be selected quickly.

The optical system and detector

A monochromator is used to select the specific wavelength of light – the spectral line – that is absorbed by the sample and to exclude other wavelengths. The selection of the specific light allows the determination of the selected element in the presence of others. The light selected by the monochromator is directed onto a detector (typically a photomultiplier tube). This produces an electrical signal proportional to the light intensity.

Double beam spectrometers

Modern spectrometers incorporate a beam splitter so that one part of the beam passes through the sample cell and the other is the reference. The intensity of the light source

may not stay constant during an analysis. If only a single beam is used to pass through the atom cell, a blank reading containing no analyte (substance to be analysed) would have to be taken first, setting the absorbance at zero. If the intensity of the source changes by the time the sample is put in place, the measurement will be inaccurate.

In the double beam instrument, there is constant monitoring between the reference beam and the light source. To ensure that the spectrum does not suffer from loss of sensitivity, the beam splitter is designed so that as high a proportion of the energy of the lamp beam as possible passes through the sample.

Atomisation of the sample

Two systems are commonly used to produce atoms from the sample, i.e.:

- Aspiration: this involves sucking a solution of the sample into a flame.
- Electro-thermal atomisation: drop of sample is placed into a graphite tube that is then heated electrically.

The aspiration technique was employed in this study.

Background absorption

It is possible that other atoms or molecules, apart from those of the element being determined, will absorb or scatter some radiation from the light source. These species could include unvaporised solvent droplets, or compounds of the matrix (chemical species, such as anions, which tend to accompany the metals being analysed) that are

not removed completely. This means that there is background absorption as well as absorption by the sample. One way of measuring and correcting this background absorption is to use two light sources, one of which is the hollow cathode lamp appropriate to the element being measured. The second light source is a deuterium lamp.

The deuterium lamp produces broad band radiation, not specific spectral lines as with a hollow cathode lamp. By alternating the measurements of the two light sources – generally at 50 –100 Hz – the total absorption (absorption due to analyte atoms plus background) is measured with the specific light from the hollow cathode lamp and the background absorption is measured with the light from the deuterium lamp. Subtracting the background from the total absorption gives the absorption arising from only analyte atoms.

Calibration

A calibration curve is used to determine the unknown concentration of an element in a solution. The instrument is calibrated using several solutions of known concentrations.

A calibration curve is produced that is continually re-scaled as more concentrated solutions are used – the more concentrated solutions absorb more radiation up to a certain absorbance level. The calibration curve shows the concentration against the amount of radiation absorbed.

The sample solution is fed into the instrument and the unknown concentration of the element is then displayed on the calibration curve. In this study, zinc and copper were studied.

The use of special light sources and specific wavelength selection allows the quantitative determination of individual components of a multi-element mixture.^{33,34}

3.4) Measurement of Copper

Principle:

Diluted serum samples are aspirated into a flame. The atoms at ground state absorb incident light emanating from a copper hollow cathode lamp at the resonance wavelength of 324.8nm. Absorbance of energy at this wavelength is specific for copper and proportional to its concentration.

Specimen:

A fresh 5ml sample of clotted blood was collected into a plastic-topped tube. The use of rubber stoppers was avoided, as these stoppers may affect the results.

Materials and equipment:

1. Atomic absorption spectrophotometer
2. Assorted pipettes
3. A copper hollow cathode lamp - Varian Cat No. 56-101233-00
4. BDH Spectrosol 1000mg/l copper solution_BDH Cat No. 141392N
5. Glycerol-Merck Cat No. 104092

6. Milli-Q water
7. Sterile disposable urine containers
8. 500ml volumetric flask

All glassware was washed by soaking the glassware in 6N nitric acid overnight; it was rinsed using Milli-Q water. All reagents were transferred to a plastic container immediately after preparation.

Reagents:

1. Sample and 50ml of standard diluent 10% glycerol was measured into a 500ml volumetric flask.
2. Water was added to make up volume to 500ml.

Calibration:

1. Stock standard-BDH spectrosol 1000mg/l copper solution.
2. Intermediate stock standard - 1000 μ mol/L transferred into a universal container (plastic urine bottle). 14.7ml of Milli-Q water was added using a pipette. 1000 μ l of stock copper solution was then added.
3. Working standards - The following was measured out into four urine containers:

Copper concentration	gm of water	μl of standard
5.00	19.90	100
10.00	19.80	200
20.00	19.60	400
40.00	19.20	800

Quality Control (QC)

Internal QC:

1. Bio Rad QCS 2 was used as control 1 and Humatrol P as control 2 for accuracy controls.
2. The sample was reconstituted with 5ml Milli-Q water and allowed to stand for 30 minutes. The results of QC were recorded.

Procedure - Stepwise:

1. The atomic absorption spectrophotometer is switched on.
2. Set to wavelength - 324.8nm
Lamp current - 5
Acetylene - 19-22
Slit width - 0.5
Program - 4
Air flow - 35
3. Allow lamp to warm up for 30 minutes.
4. Light flame and aspirate using Milli-Q water.

Method:

1. Diluted all standards as follows: 1.0ml standard + 1.0ml glycerol
2. Diluted all serum samples and controls: 0.5ml serum + 0.5ml water
3. Standards were aspirated and calibrated.
4. After calibration, samples and controls were run on the instrument using water as a blank between each sample.

5. Results were taken from the printout.

3.5) Measurement of Zinc

Principle:

Diluted serum samples are aspirated into a flame. The atoms at ground state absorb incident light emanating from a zinc hollow cathode lamp at the resonance wavelength of 213.9nm. Absorbance of energy at this wavelength is specific for zinc and proportional to its concentration.

Specimen:

5ml of fresh blood was collected pre-prandial into a plastic topped tube. This was because zinc levels may fall by 20% after meals. The use of rubber stoppers was avoided because these affect the results markedly.

Materials and equipment:

1. Atomic absorption spectrophotometer
2. Assorted pipettes
3. A zinc hollow cathode lamp - Varian Cat No. 56-101-00
4. BDH Spectrosol 1000mg/l zinc solution
5. Glycerol-Merck
6. Milli-Q water
7. Sterile disposable urine containers
8. 500ml volumetric flask

All glassware was washed by soaking it in 6N nitric acid overnight. It was rinsed using Milli-Q water. All reagents were transferred to a plastic container immediately after preparation.

Reagents:

1. The sample and 50ml of standard diluent 10% glycerol was measured into a 500ml volumetric flask.
2. Water was added to make up volume to 500ml.

Calibration:

1. Stock standard - BDH spectrosol 1000mg/l zinc solution
2. Intermediate stock standard - 1000 μ mol/L
3. A universal container (plastic urine bottle) was used and 14.3ml of Milli-Q water was added using a pipette.
4. 1000 μ l of stock zinc solution was then added.
5. Working standards - The following was measured out into four urine containers:

Zinc concentration	gm of water	μl of standard
5.00	19.90	100
10.00	19.80	200
20.00	19.60	400
40.00	19.20	800

Quality Control (QC)

Internal QC:

1. Serosods and Serosods plus were used for accuracy controls.
2. The sample was reconstituted with 5ml Milli-Q water and allowed to stand for 30 minutes. The results of QC were recorded.

Procedure-Stepwise:

1. The atomic absorption spectrophotometer is switched on.
2. Set to wavelength – 213.9nm
 - Lamp current - 10
 - Acetylene - 19-22
 - Slit width - 0.5
 - Program - 4
 - Air flow - 35
3. Allow lamp to warm up for 30 minutes.
4. Light flame and aspirate using Milli-Q water.

Method:

1. Diluted all standards as follows: 1.0ml standard + 1.0ml glycerol.
2. Diluted all serum samples and controls: 0.5ml serum + 0.5ml water.
3. Standards were aspirated and calibrated.
4. After calibration, samples and controls were run on the instrument using water as a blank between each sample.
5. Results were taken from the printout.

AAS is a technique that has been found to be reliable and valid for routine analysis of patient sample serum zinc.²⁸

Serum analysis of zinc and copper for this study was done at the National Atomic Centre (NAC) Laboratory, Witwatersrand branch (Now iTemba Labs). iTemba is an academic laboratory affiliated to the University of the Witwatersrand. It is accredited to carry out serum analysis of zinc, copper and molybdenum using the AAS technique.

3.6 Data Analysis

The study is a prospective trial with three groups of participants divided according to stage (II-IV). In order to describe the demographic characteristics of the patients, frequency, means and standard deviations were used.

Normality of distribution was assessed using the Shapiro-Wilk test. The serum levels of zinc and copper zinc ratios were normally distributed, but the serum level of copper did not have a normal distribution. Values are expressed as a mean with standard deviations for zinc and copper zinc ratio and median for copper levels.

Comparisons were made using students' t-test for zinc and copper zinc ratios and Wilcoxon rank-sum test for zinc levels. Comparisons were done between early stage NSCLC (stage II patients) and advanced stage NSCLC (stage III and IV patients).

Corresponding p-values were calculated to test for statistical significance at the 5% level. Data analysis was done using STATA version 10 statistical software (StataCorp, 2008, Texas, USA).

Ethical Clearance

Ethical clearance was obtained from the University of Witwatersrand Committee for Research on Human Subjects (Medical) for permission to carry out this study (Protocol number **PROTOCOL M02-10-33**). See Appendix 1 and Appendix 2.

Written consent was obtained from all patients who participated in this study (see Appendix 3).

CHAPTER FOUR: RESULTS

Clinical Endpoints

Relevant clinical histories, e.g. smoking history (active and passive), family history of cancer or lung cancer and employment history, were not specifically evaluated in this study. However, such data could provide useful information in a future, expanded, prospective study.

Demographic Characteristics

Table 1: Demographic characteristics of study participants

Variable	N= 34 Frequency (%)
Age in years	(mean=61.1, std= 8.50)
40-50	3 (8.82)
50-60	9 (26.47)
60-70	17 (50.00)
>70	5 (14.7)
Gender	
Female	9 (26.5)
Male	25 (73.5)
Race	
Black	26 (76.5)
White	8 (23.5)

A total of thirty-four patients participated in this study and males (73.5 %; 25) accounted for the majority. The mean age of patients was 61.1 years (standard

deviation = 8.50 years). Table 1 shows the demographic pattern, with 17 patients (50%) belonging to 60-70 year age group. More than three-quarters of the patients were Blacks (26) and the rest were Whites (8). There were no patients of Asian/Indian and Coloured (mixed race) origin.

Figure 1: Distribution of participants by gender (males 25; females 9)

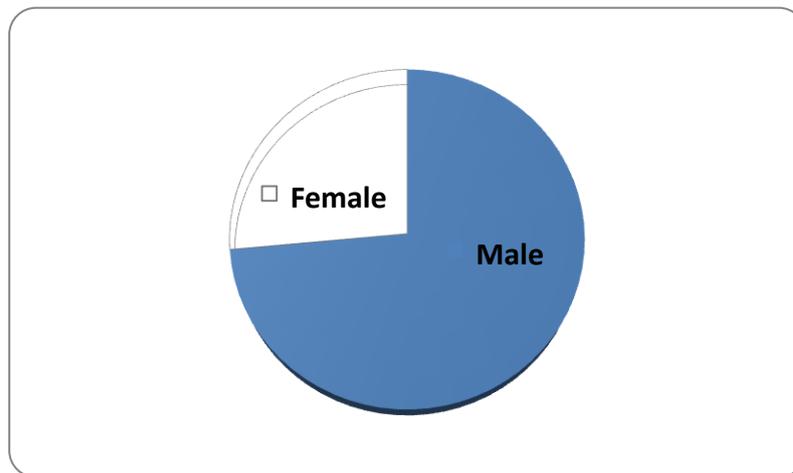


Figure 1 shows the gender distribution of the 34 patients: 25 patients are male (74%); 9 patients were female (26%)

Data analysis was done using STATA version 10 statistical software (StataCorp, 2008, Texas, USA).

Cancer Stage Analysis

Sixteen patients (47%) had Stage IV disease, while 14 (41.2%) and 4 (11.8%) were grouped into stage III and stage II respectively.

In this study, patients aged 60–70 years were in the majority at all stages of NSCLC. Patients aged 50-60 years were the next most affected group for late stage NSCLC (stage III and IV); patients more than 70 years old were the third most affected group for early stage NSCLC (stage II). (See Table 2).

There were more cases of NSCLC among males than females at all the stages of NSCLC. There is a male to female ratio of 3:1 (stage II) and 7:1 (stage IV). There was a more equal number of cases of male and female cases (8 vs. 6) at stage III.

More Black patients had NSCLC in the late stage than White patients. There were no cases of stage II NSCLC among Black patients.

Table 2: Demographic characteristics of study participants by cancer stage

	Stage II Frequency (%)	Stage III Frequency (%)	Stage IV Frequency (%)
Age (years)			
40-50	0 (0)	2 (14.29)	1 (6.25)
50-60	0 (0)	3 (21.43)	6 (37.50)
60-70	3 (75.00)	7 (50.00)	7 (43.75)
>70	1 (25.00)	2 (14.29)	2 (12.50)
Gender			
Male	3 (75.00)	8 (57.14)	14 (87.50)
Female	1 (25.00)	6 (42.86)	2 (12.50)
Race			
Black	0 (0)	12 (85.71)	14 (87.50)
White	4 (100)	2 (14.29)	2 (12.50)

Data analysis was done using STATA version 10 statistical software (StataCorp, 2008, Texas, USA).

Serum levels of Copper, Zinc and the Copper Zinc Ratio

Figure 2-6 illustrate the overall levels of the above-mentioned trace elements and their ratio, as well as individual elements and their ratio vs. stage.

Figure 2: The mean serum copper, zinc and copper zinc ratios of all 34 participants.

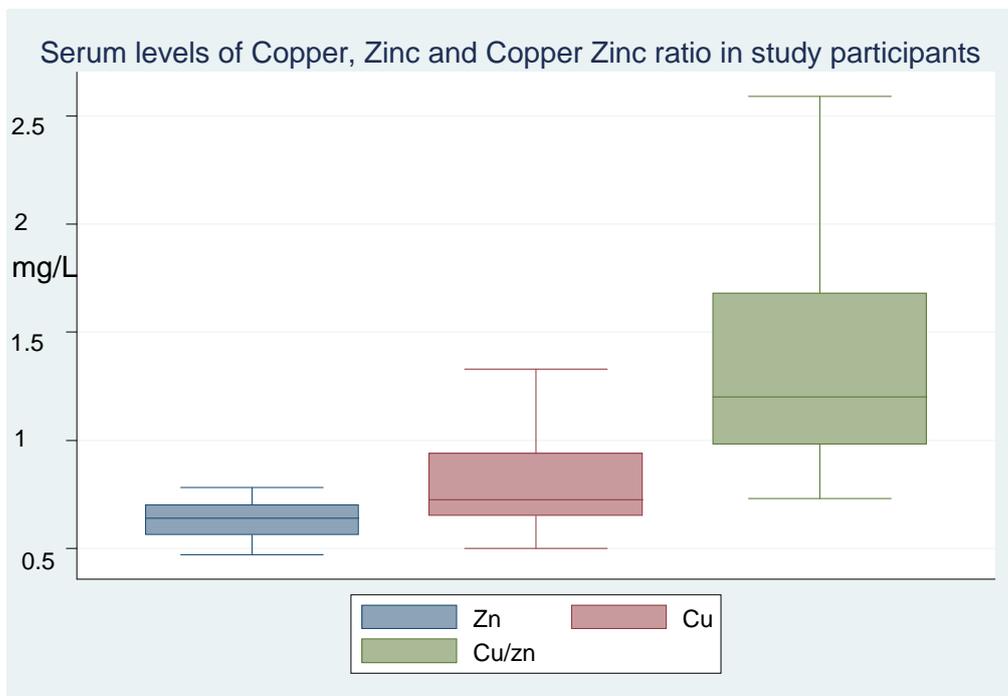
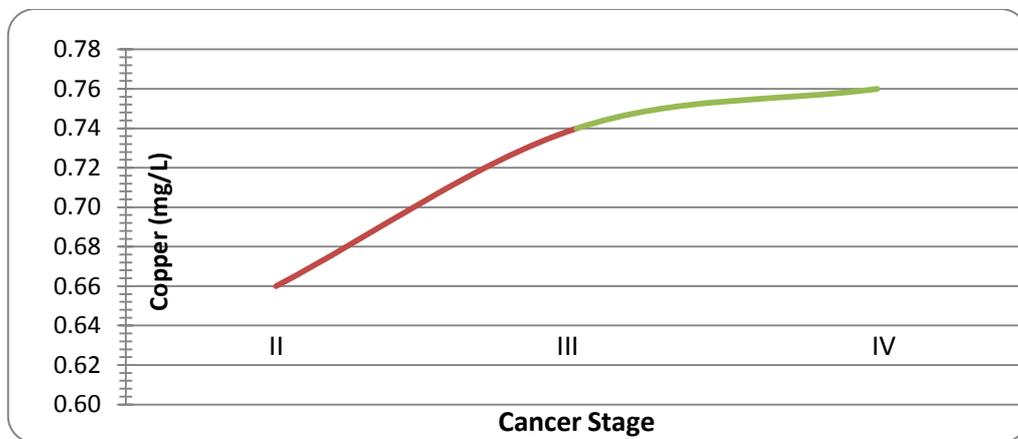


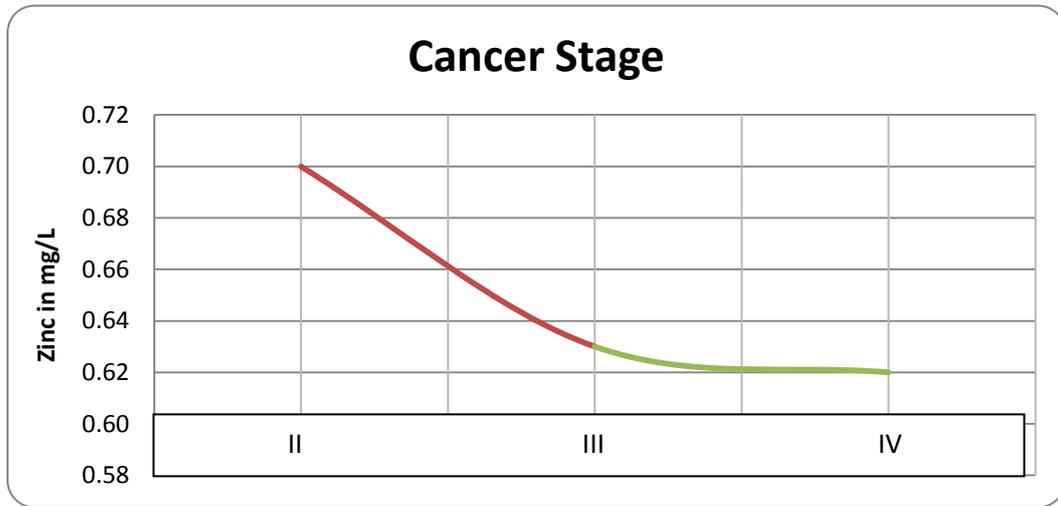
Figure 3: Participants' serum level of copper by NSCLC stage



Mean SCL at different stages of NSCLC were 0.66mg/L, 0.74mg/L and 0.76mg/L for stage II, III and IV patients respectively.

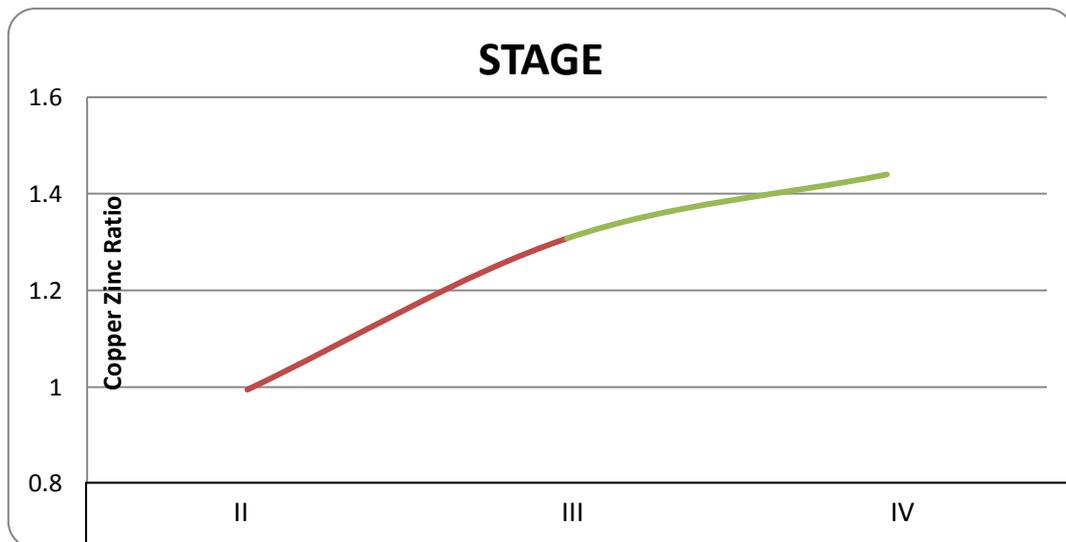
Data analysis was done using STATA version 10 statistical software (StataCorp, 2008, Texas, USA).

Figure 4: Participants' serum level of zinc by NSCLC stage



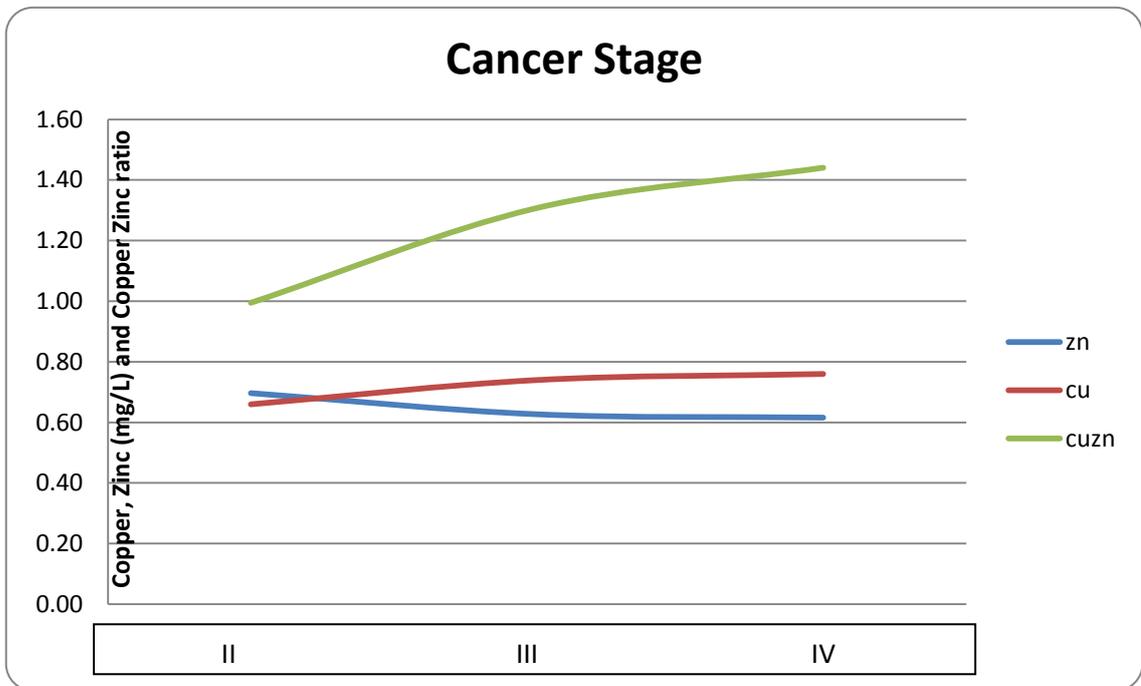
Mean serum zinc levels (SZL) at different stages of NSCLC were 0.70mg/L, 0.63mg /L and 0.62mg/L for stage II, III and IV patients respectively.

Figure 5: Participants' serum level of copper zinc ratio by NSCLC stage



Copper zinc ratios at different stages of NSCLC were 0.995, 1.308 and 1.441 for stage II, III and IV patients respectively.

Figure 6: Combined copper, zinc levels and copper zinc ratio vs. stage in NSCLC



Data analysis was done using STATA version 10 statistical software (StataCorp, 2008, Texas, USA).

CHAPTER FIVE: DISCUSSION AND CONCLUSION

In this study, the data confirms previous reports of decreased SZL and elevated SCL in patients with NSCLC,^{28, 33, 41} and provides useful local demographic information.

5.1 Demographics

Demographic findings show some correlation with established trends in the literature.^{10,}

¹¹ We know that lung cancer predominates in males and this is reflected in the study, with 71% of patients being male.^{10, 11} It is also in keeping with lung cancer accounting for a greater percentage of total cancers in males.

The ratio of males to females (7:1) is at its highest in stage IV disease, which reflects later presentation in male patients. The aetiology of lung cancer is, to a large extent, associated with socio-economic factors, rather than phenotypical or genotypical factors, as reflected by the exaggerated proportion of males in this study.^{51, 52, 53} It also suggests that access to tertiary health care facilities still has a patriarchal bias in South African society.^{51, 52, 53}

Advanced age at presentation is a marker of NSCLC. This is in keeping with its most common aetiology of accumulated and persistent mutations that are secondary to smoking. This study shows peak incidence in the 7th decade, with a significant proportion in the 6th and 8th decades. This age distribution largely mimics data from Western studies, which is contrary to the usual finding of earlier age at presentation that

is commonly found at South African state institutions. A possible reason for this is that all patients were HIV negative, which alludes to a higher baseline medical and socio-economic level in the South African context.^{51, 52} This participant sample would closer resemble a sample, using similar criteria, in a Western context. This finding also reflects the inadequate screening and therapeutic interventions for lung cancer even in resource-rich environment.⁶⁻¹¹

Racial demographics closely resembled population statistics, though it should be noted that only White patients presented with early stage lung cancer. This is in keeping with the findings of American studies and is probably attributable to socio-economic factors,^{10, 11} rather than to genetic factors. In the South African context, this variable could be studied more accurately in a co-operative study between a private and public health facility, although its relevance is declining over time.^{52, 53}

5.2 Serum Copper and Zinc Levels in all Participants

In this study, the mean serum level for copper measured in all 34 patients was 0.81 mg/L (mean in healthy individuals 1.17mg/L).⁴¹ The mean serum copper was statistically lower than the reference mean serum level in healthy individuals ($P < 0.001$).

These results are contrary to findings from previous studies, which reported a higher serum level of copper in cancer patients when compared to healthy individuals.^{41, 44}

One of the conclusions of these studies was the role of serum copper as an acute phase marker.^{41, 44} This result should be interpreted with caution as this study did not have controls that would have made for a more reliable and accurate comparison. It should

also be noted that the general condition of patients presenting at a South African state hospital oncology department would represent a low overall state of health and immunity baseline when compared to population samples in other studies.^{52, 53} The proportion of patients with early stage disease (11.8%) was also disproportionately low.

The mean SZL in all patients was 0.63 mg/L (the mean in healthy individuals is 0.94mg/L).³⁹ The mean serum zinc was statistically lower than the reference mean serum level in healthy individuals (P<0.001). The lower serum zinc level seen in this study correlates with other studies that compared SZL in patients with malignant conditions with serum levels in healthy individuals or individuals with benign conditions.^{25, 41, 44} The reduced serum level of zinc observed in patients with NSCLC may have led to the loss of the DNA and RNA stabilization function of zinc, thereby increasing the risk of developing lung cancer. This finding highlights the protective (anticarcinogenic) role of zinc. A diminished serum level of zinc might be a marker for the development of NSCLC.

5.3 Serum Copper, Zinc and Copper Zinc Ratio by NSCLC Stage

Table 3: Serum level of Copper, Zinc and Copper Zinc ratio by NSCLC stage among study participants

	Stage II	Stage III	Stage IV
* Copper (mg/L)	0.66 (0.18)	0.74 (0.22)	0.76 (0.42)
** Zinc (mg/L)	0.70 (0.067)	0.63 (0.072)	0.62 (0.09)
** Copper Zinc ratio	0.99 (0.24)	1.31 (0.43)	1.44 (0.55)

* Median (inter-quartile range)

** Mean (standard deviation)

Although the serum level of copper demonstrated an increasing pattern with advancing stage of NSCLC in this study, this trend was not statistically significant. This is illustrated by the fact that patients with early stage NSCLC (stage II) had a mean level of 0.66mg/L, compared to patients with late stage NSCLC (stage III and IV), who had a mean level of 0.75 mg/L (P=0.199).

Similar studies reported elevated serum levels of copper in neoplastic conditions (when compared to a healthy control) and an increasing level of copper with advancing lung cancer.^{25, 29} In this study, the serum zinc level was shown to decline in patients with advancing stages of NSCLC. The decreasing serum levels of zinc with advancing stages of lung cancer in this study were not statistically significant; this was probably attributable to the small sample size. Patients with early stage NSCLC have a higher SZL when compared to patients with late stage NSCLC - 0.70mg/L mean stage II versus 0.62 mg/L mean stage III & IV, P=0.0897.

Previous studies have reported statistically significant decreasing levels with advancing lung cancer .^{25, 40} These findings suggest a prognostic significance of declining SZLs.

Copper zinc (Cu/Zn) ratio is considered to be a better discriminator than serum copper or SZL alone in the diagnosis and prognosis of malignancies.^{25, 35} As a diagnostic tool with 1.72 as a cut-off value, when comparing controls and lung cancer patients, copper zinc ratio had: a sensitivity of 89%, specificity of 84%, positive predictive value of 78%, and a negative predictive value of 92%.²⁴ Patients' eligibility for curative

resection and survival were also shown to be better in cancer patients with lower copper zinc ratios .²⁵

Even though the difference in copper zinc ratios in advanced NSCLC were not significantly different from early stage NSCLC, the mean value was higher in advanced NSCLC in this study, with 0.995, 1.308, 1.441 for stage II, III and IV patients respectively and a definite trend of an increasing ratio with progressive disease. This suggests that the copper zinc ratio could be used as a prognostic marker in NSCLC. Sensitivity, specificity, positive and negative predicative values for this study could not be calculated; this was because there was no control arm.

Further studies in the South African population may be needed to provide evidence in support of these findings, which have elaborated the diagnostic and prognostic values of SCL and SZL and the copper zinc (Cu/Zn) ratio in cancer patients.^{24, 32, 37}

To conclude, the routine use of SCL, SZL and Cu/Zn ratios cannot be advocated as a tumour marker and prognostic variable for NSCLC as a result of this study. Further studies however, with a larger sample size, could demonstrate the role of the copper to zinc ratio as a cost-effective staging tool in NSCLC.

Limitations

- 1) The non-inclusion of a comparison group of patients (a healthy control group) from the same population as the NSCLC patients in this study limits the correlation and comparison of the findings with other studies of a similar nature.

The sample size (34 patients) was also relatively small when compared to other trials and the protocol of the trial limited data collection to a period of one calendar year (12 months). These factors were a result of logistic and funding limitations.

- 2) The staging system used was AJCC 2002, which was the standard at the time. There has subsequently been a newer staging system (AJCC 2009), and caution is required when interpreting the data using the new system to prevent the effect of ‘stage migration’. The author is, however, not aware of any similar or related study using the newer staging system.

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APPENDIX 1: ETHICS APPROVAL

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

Division of the Deputy Registrar (Research)

COMMITTEE FOR RESEARCH ON HUMAN SUBJECTS (MEDICAL)

Ref: R14/49 Ray

CLEARANCE CERTIFICATE **PROTOCOL NUMBER** M02-10-33

PROJECT Relationship of Copper and Zinc with Staging of Lung Cancer

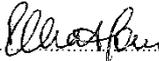
INVESTIGATORS Dr S Ray

DEPARTMENT School of Clinical Medicine, Johannesburg Hospital

DATE CONSIDERED 02-10-25

DECISION OF THE COMMITTEE Approved unconditionally

Unless otherwise specified the ethical clearance is valid for 5 years but may be renewed upon application
This ethical clearance will expire on 30 July 2007.

DATE 02-11-13 CHAIRMAN..........(Professor P E Cleaton-Jones)

* Guidelines for written "informed consent" attached where applicable.

c c Supervisor: Dr ES Haddad
Dept of Physics Department Wits University
Works2\lain0015\HumEth97.wdb\W 02-10-33

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DECLARATION OF INVESTIGATOR(S)

To be completed in duplicate and ONE COPY returned to the Secretary at Room 10001, 10th Floor, Senate House, University.

I/we fully understand the conditions under which I am/we are authorized to carry out the abovementioned research and I/we guarantee to ensure compliance with these conditions. Should any departure to be contemplated from the research procedure as approved I/we undertake to resubmit the protocol to the Committee. I agree to a completion of a yearly progress form. I/we agree to inform the Committee once the study is completed.

DATE 25/10/02 SIGNATURE 

PLEASE QUOTE THE PROTOCOL NO IN ALL QUERIES : M 02-10-33

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES



UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

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15 January 2003

The Postgraduate Committee
Wits Medical School
Johannesburg Academic Complex
Parktown
Johannesburg
2193

Dear Sir/madam,

TRANSFER OF ETHICAL CLEARANCE

I, Dr Saddhasattwa Ray-student no.9908401Y, a postgraduate MMED student in Radiation Oncology obtained an ethics clearance till 30 July 2007.

PROJECT: RELATIONSHIP OF ZINC AND COPPER STAGING IN LUNG CANCER
REF: R 14/49 RAY
ETHICS CLEARANCE NO: M02-10-33

I give my unconditional and full consent to my colleague, Dr Dinoshan N. Chetty-student no 9202615 P, a postgraduate MMED student in radiation oncology to proceed with this project under his name.

Yours faithfully,

[Signature of Dr S. Ray]
Dr S. Ray

[Signature of Dr D. Chetty]
Dr D. Chetty

Witness 1: [Signature] A. M. Isaacs

Witness 2: [Signature] B. D. Dunde

FOR ATTENTION:
Mrs. McLean (Wits Postgraduate Office)
Professor PE Cleaton Jones (Chairman-Ethics Committee)
Professor Feldman (Chair: Higher Academic Committee)

APPENDIX 2: PROTOCOL APPROVAL



Faculty of Health Sciences
UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

7 York Road PARKTOWN Johannesburg 2193 Telegrams WITSMED Telex 4-24655.SA
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E-MAIL healthpg@health.wits.ac.za

DR DN CHETTY
303 BRACKENHURST
14 COLLET DRIVE
ILLOVO
2196

APPLICATION NUMBER 9202615P
STATUS (DEG 73) (MMU00) PZZ

2003-06-24

Dear Dr Chetty

Approval of protocol entitled The relationship of zinc and copper with stage in non-small cell cancer of the lung

I should like to advise you that the protocol and title that you have submitted for the degree of Master Of Medicine (In Radiation Oncology) have been approved by the Postgraduate Committee at its recent meeting. Please remember that any amendment to this title has to be endorsed by your Head of Department and formally approved by the Postgraduate Committee.

Prof B Donde has/have been appointed as your supervisor/s. Please maintain regular contact with your supervisor who must be kept advised of your progress.

Please note that approval by the Postgraduate Committee is always given subject to permission from the relevant Ethics Committee, and a copy of your clearance certificate should be lodged with the Faculty Office as soon as possible, if this has not already been done.

Yours sincerely

A handwritten signature in black ink, appearing to be 'ME Fick'.

ME Fick (Mrs)
Faculty Registrar
Faculty of Health Sciences

Telephone 717-2075/2076

Copies - Head of Department _____ Supervisor/s

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The University seeks to serve South Africa by furthering access to equal opportunity while striving for excellence in teaching, learning and research

APPENDIX 3: INFORMED CONSENT

INFORMED CONSENT FORM (STUDY GROUP)

Name of trial: *Relationship of Copper and Zinc with staging of Lung Cancer*

CONSENT

I have read the subject information sheet and understood its contents/it has been translated and explained to me by an interpreter.

I _____ understand that I have cancer of the lungs and will be receiving radiotherapy/chemotherapy. I am willing to participate in the above study and volunteer on my own free will.

You are free to withdraw at any stage without prejudice.

I agree to have blood drawn from me as described above.

Should I have any concerns regarding participating in the study, I can contact the Investigator, *Dr. Suddhasattwa Ray on 082 404 3922 /011 488-4032 or Dr. Dinoshan Chetty 084-5864414/ 4884032.*

Signature of patient: _____ Date: _____

Witness (1): _____ Date: _____

Witness (2): _____ Date: _____

Doctor's Signature: _____ Date: _____