Comparative Evaluation of Metal Imbalances in the Blood of Breast Cancer Patients in Comparison with Controls

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Comparative Evaluation of Metal Imbalances in the Blood of Breast Cancer Patients in Comparison with Controls

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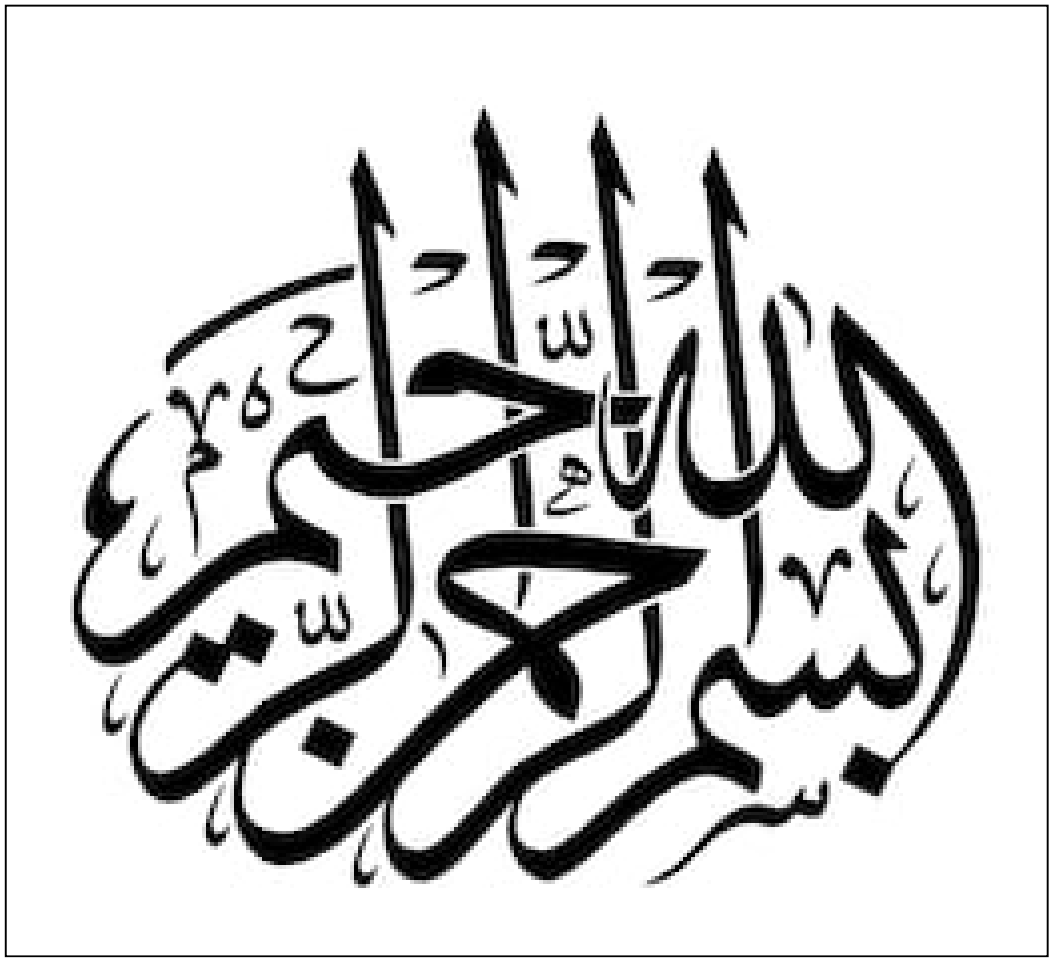

IN THE NAME OF ALLAH,

THE MOST MERCIFUL,

THE MOST KIND

Dedication

Every challenging work needs self efforts as well as guidance of elders especially those who are very close to our hearts

Father and Mother,

whose affection, love, encouragement and prays of day and night make me able to get such success and honour,

Along with all hardworking and respected Teachers

And above all to the Almighty Allah!

DECLARATION

This is to certify that this dissertation entitled ***"Comparative Evaluation of Metal Imbalances in the Blood of Breast Cancer Patients in Comparison with Controls"*** by ***Shabnam Amin*** is accepted in its present form by the Department of Chemistry, Quaid-i-Azam University, Islamabad, Pakistan as satisfying the dissertation requirements for the degree of ***Master in Philosophy*** in ***Analytical/Inorganic Chemistry.***

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ABSTRACT

Cancer incidence and morality rates have been increasing rapidly worldwide. It has been reported that exposure to trace metals plays important role in the development of cancer. Therefore, the present study was designed to evaluate the imbalances of selected essential and toxic metals (Ca, Cd, Co, Cr, Cu, Fe, K, Mg, Mn, Na, Pb, Sr and Zn) in the blood of newly diagnosed breast cancer patients in comparison with their counterpart healthy donors. Concentration of the metals was qualified by flame atomic absorption spectrometry by employing nitric acid and perchloric acid based wet digestion method. Average levels of Ca, Cd, Co, Fe, Mg, Mn and Pb were found to be higher in blood of the patients compared with the controls. Correlation studies showed significantly strong relationships ($r > 0.500$) between Pb-Co, Zn-Co, Zn-Pb and Ca-K in the blood of breast cancer patients while Mg-K, Zn-Fe and Mn-Co showed strong correlations in the blood of healthy donors. Significant variations in the trace metal levels were observed with the habitat, food habits, employment status and smoking habits of both donor groups. Metal levels exhibited considerable differences with the stages of breast cancer and the age-groups of the subjects. Principal component analysis and cluster analysis of metal data manifested significantly divergent apportionment of the metals in the blood of both the donor groups.

Chapter 1

INTRODUCTION

1.1 Cancer Incidences and Mortality

Cancer is not a single disease but a large group of diseases, all of which can be characterized by the uncontrolled growth of an abnormal cell to produce a population of cells that have acquired the ability to multiply and invade surrounding and distant tissues (Smart, 2004). According to World Health Organization (WHO) deaths due to cancer are projected to continue to increase with an estimation of almost 11.4 million by 2030. With more than one million new cancer cases diagnosed every year, breast cancer has attained the global status of fastest growing and most common form of cancer among women (Bray *et al*., 2004). According to the WHO, almost 7.6 million people died of cancer all over the globe in 2005, which accounts for 13% of all deaths in that year. Cancer is a leading cause of death all over the globe. The number of cancer cases/cancer incidence and mortality is expected to rise rapidly as populations grow and adopt lifestyle behaviours that elevate cancer risk (Torre *et al*., 2015). One in four deaths in the United States is due to cancer (Siegal *et al*., 2013). In 2012, approximately 3.45 million new cancer incidences and 1.75 million cancer patients died in Europe, 53% cases (1.8 million) occurred in male and 47% cases (1.6 million) occurred in female. In 2012, it was reported that Breast cancer is the most common cancer in women in Europe (464,000 cases, 13.5% of all cancer cases), followed by colorectal cancer (447,000, 13.0%), prostate cancer (417,000, 12.1%) and lung cancer (410,000, 11.9%). According to an estimate from the International Agency for Research on Cancer (IARC), approximately 14.1 million new cancer incidences and 8.2 million cancer patients died all over the globe in 2012 (Ferlay *et al*., 2012,Torre *et al*., 2015).

Breast cancer is common cause of cancer-related mortality among women (Shibuya *et al*., 2002). One in ten of all new cancer cases diagnosed all over the globe each year is breast cancer and it is the most common cancer in women in both developing and developed areas worldwide (Bray *et al*., 2004). According to an estimate, breast cancer is the most common type of cancer and the most common cause of cancer-related mortality among women globally. However, the burden is not evenly distributed worldwide and according to the available data, there are large variations in the incidence,

mortality and survival between different countries and different regions of the world and even within specific regions (Hortobagyi *et al*., 2012). In western countries, 1 out of 8 women have the lifetime risk of developing breast cancer (Ries *et al*., 2004). Since 1975, the 5-year relative survival rates with breast cancer increased steadily for women over time in United States, the 5-year relative survival rate for women diagnosed between 1995 and 2000 was 87.7% compared with 74.9% between 1975 and 1979 (Ries *et al*., 2004). According to an estimates, the age-standardized incidence per 100,000 population ranges from 13.2 in Tajikistan to 50.1 in Pakistan while average rate for South Central Asia is 21.8. Similarly, the age-standardized mortality from breast cancer per 100,000 population ranges from 6.2 in Tajikistan to 22 in Pakistan and the average rate for South Central Asia is 11.1. In India, age-standardized breast cancer–related mortality rates are relatively low (10.4 per 100,000) as compared to other regions of the world (Ferlay *et al*., 2004). During 1990-2008, breast cancer was the most common cancer in Indians/Pakistanis, Chinese, Filipinos, Kampucheans, Koreans, Laotians and Vietnamese, Japanese women followed by colorectal/lung cancers. From 1990 to 2008, Pakistanis and Indians experienced a statistically significant 3% per year increase in breast and uterine cancer. Chinese women experienced a statistically significant 1.2% annual increase in breast cancer from 1990 to 2008 (Gomez *et al*., 2013).

1.2 Causes and Stages of Breast Cancer

The causes of breast cancer are not yet fully known although a number of risk factors have been identified. There are many risk factors that increase the chance of developing breast cancer which include age, family history, personal history, genetic risk factors, etc. The chances of breast cancer increase with age. The risk of breast cancer is higher among women who have a close relative with the disease. Having been diagnosed with breast cancer in one breast increases the risk of cancer in the other breast or the chance of an additional cancer in the original breast. The most common causes of breast cancer are mutations in the genes. Inheriting a mutated gene from a parent means that one has a significantly elevated risk of developing breast cancer in their lifetime. Women had an increased risk of breast cancer which were diagnosed with certain benign breast conditions. These include atypical hyperplasia, a condition in which there is abnormal proliferation of breast cells but no cancer has developed. Women who started their menstrual cycle at a younger age (before 12) or went through menopause later (after 55)

have a slightly increased risk of breast cancer in their life. Exposure to radiation or use of diethylstilbestrol increases the risk of breast cancer. The risk of breast cancer increases in women having no children or the first child after age 30. Breast cancer risk lowers by breast feeding for one and a half to two years. Being overweight increases the risk of breast cancer both in pre- and postmenopausal women but at different rates. The risk of breast cancer increases with alcohol use, and this seems to be proportional to the amount of alcohol used. A recent study concluded that all levels of alcohol use even light drinking are associated with an increased risk for breast cancer.

There are many types of breast cancer that differ in their capability of spreading (metastasize) to other body tissues. Some of the most common types of cancer are; '*Ductal carcinoma*' that is the most common type of non-invasive breast cancer that has not spread and therefore usually has a very high cure rate, '*Invasive ductal carcinoma*' is most common form of breast cancer and it starts in a duct of the breast and grows into the surrounding tissue, '*Invasive lobular carcinoma*' which starts in the glands of the breast that produce milk. Determining the stage helps determine the best way to eliminate the breast cancer.

The stage is based on the many factors that includes size of the tumour within the breast, number of lymph nodes affected, the nearest lymph nodes are found under the arm, known as the axillary area, signs indicating whether or not the breast cancer has invaded other organs within the body. If breast cancer has spread or metastasized, evidence may be found in the bones, liver, lungs, or brain. Stage is usually expressed as a number on a scale of 0 through IV - with stage 0 describing non-invasive cancers that remain within their original location and stage IV describing invasive cancers that have spread outside the breast to other parts of the body. Stage-0 is used to describe non-invasive breast cancer, there is no evidence of cancer cells or non-cancerous abnormal cells breaking out of the part of the breast in which they started. Stage-I describes invasive breast cancer. It is divided into subcategories known as IA and IB; former describes invasive breast cancer in which the tumour measures up to 2 centimetres and the cancer has not spread outside the breast while the latter describes invasive breast cancer in which there is no tumour in the breast or there is a tumour in the breast that is no larger than 2 centimetres, there are small groups of cancer cells (larger than 0.2 millimetre but not larger than 2 millimetres) found in the lymph nodes. Stage-II breast cancer is still in the earlier stages, but there is evidence that the cancer has begun to grow or spread. It is still contained to the breast area and is generally very effectively treated. Stage-III is divided into subcategories known as III-A,

III-B, and III-C. Stage-IIIA describes invasive breast cancer in which no tumour is found in the breast or the tumour may be any size; cancer is found in 4 to 9 auxiliary lymph nodes or in the lymph nodes near the breastbone. Stage-IIIB describes invasive breast cancer in which the tumour may be any size and has spread to the chest wall and/or skin of the breast and caused swelling or an ulcer. Stage-IIIC describes invasive breast cancer in which there may be no sign of cancer in the breast or if there is a tumour it may be any size and may have spread to the chest wall and/or the skin of the breast. Stage-IV describes invasive breast cancer that has spread beyond the breast and nearby lymph nodes to other organs of the body.

1.3 Trace Elements and Health

The importance of trace elements in health and disease is irrefutable because of their essential function in specific concentration ranges and shows toxic effects at relatively higher levels. Trace elements perform many significant functions as stabilizers, elements of structure, essential elements for hormonal function and cofactors in enzymes. Lower levels of essential elements influences the structure through numerous processes (Feinendegen *et al.*, 1980). Individual hormonal levels and metabolism are affected by environmental contaminants such as metals (Martin *et al.*, 2003). Human metabolism is significantly affected by the certain toxic metals even in trace levels, which disturb the body functions (Pasha *et al.*, 2007). Human health can be only maintained by intake of optimal doses of all essential elements (Graneroa and Domingo, 2002; lyengar, 1989).

Cancer was considered to be genetically linked, however it is now well recognized that diet and environmental exposure has a significant effect on cancer incidences (Bowen, 2000). In 1993, the International Agency for Research on Cancer (IARC) reported cadmium as a human carcinogen (IARC, 1993) which may result in different types of cancers (Drasch *et al.*, 2005; Schrauzer, 2000). Manganese, copper, zinc, arsenic, nickel, chromium, cadmium, and iron content in serum and blood have been used to diagnose lung cancer and gastric cancer (Bihui, 1990; Zhengxian, 1987; Guangwei 1990). Biochemical mechanism of these elements for the cause of cancer in human body is not very clear; more research work has to be done to get a better understanding of the relationship. Physiological roles of some of the important essential and trace elements are discussed below.

1.3.1 Cadmium

Cadmium is one of the toxic elements to human body. Main sources of cadmium are cigarette smoke, paint additives, cadmium batteries, water, air and food (Cuypers *et al*., 2010). Smoking tobacco is thought to double the life time body burden of cadmium in non-occupationally exposed persons. Environmental exposure is also common (Satarug *et al*., 2003; Chattopadhyay *et al*., 1990). It can also be introduced in environment through pesticides, fertilizers, electroplating and fossil fuels. Cadmium has long half life in human body and low level exposure of long term may cause chronic diseases (Godt *et al*., 2006; Orlowski, 2003). People living near to hazardous sites, factories and metal refining industries are under threat of higher exposure of cadmium (Lenntech, 2010; Cope *et al*., 2004; Stoeppler, 1991). Cadmium exposure causes damage to kidney, bronchitis, bone marrow cancer, high blood pressure, lung cancer, prostate cancer, pulmonary cancer, liver cancer, stomach cancer and cardiovascular disease/heart failure (Singh *et al*., 2012; Liu *et al*., 2009; Durham and Snow, 2006; Waalkes, 2000; IARC 1993). The adverse physiological effects associated with high exposure to Cd involve depressed growth rate, anaemia and hypertension (Nordberg, 2009). Cadmium is toxic at extremely low level; it is also associated with bone defects like osteomalacia. Smoking has also been reported to be a contributing factor to higher bioaccumulation of cadmium which can cause prolonged urinary calcium loss. It is also a nephrotoxicant (Satarug and Moore, 2004) and contributes towards diabetes (Akesson *et al*., 2005).

1.3.2 Chromium

Chromium (Cr) is ubiquitous in the environment. It is found naturally in soils, rocks and living organisms (Krebs, 2006). It occurs in many valence states; however only the trivalent (Cr^{3+}) and hexavalent (Cr^{6+}) forms have significant environmental stability (IETEG, 2005). It is found in higher amount in places near landfills, hazardous waste disposal sites, chromate industries (welding, chrome plating, chrome pigmenting, ferrochrome industry and leather tanning) and highways. Epidemiological studies have reported that work place exposure resulted in an elevated risk of respiratory disease, fibrosis, perforation of the nasal septum and lung cancer (Amdur *et al*., 1991). Although it is an essential element for health in trivalent form but in hexavalent form it causes severe disorders (Anderson *et al*., 1985). Deficiency of Cr(III) is principal cause of diabetes, obesity, poor growth and tiredness (Rajpathak *et al*., 2004; Anderson, 2000). International agency for research on cancer and USEPA classified hexavalent chromium to be

carcinogenic (IRAC, 1990; USEPA, 1998). Elevated exposure of Cr leads to stomach ulcer, vomiting, nausea, damage to liver/kidney and various cancers (Smith and Steinmaus, 2009; Durham and Snow, 2006). It can also damage the nervous system and may cause irritation of respiratory tract, pneumonia and bronchospasm (Dayan *et al*., 2000). Reduction of Cr(VI) to its lower oxidation states and related free-radical reactions play an important role in carcinogenesis (Shi *et al*., 2010). It also induces germ cell mutagenicity and causes DNA deletions in developing embryos (McCarroll *et al*., 2009).

1.3.3 Calcium

Calcium is present in human body in large amount and it accounts for about 2% of total body weight. Major sources of calcium include milk, meat, grain, cheese, vegetables, nuts, fruits and fish (Subar *et al*., 1998; Weaver, 2000). According to National Academy of Science, Food and Nutrition Board dietary reference intake recommended adult dietary allowance of calcium is 1000 mg per day (Institute of Medicine, 1997). Calcium along with magnesium play significant role in enzymatic systems in the myocardium and in maintaining the electrolyte balance (Tubek, 2006). Calcium also plays major role in muscle contraction and nerve transmission (Theobald, 2005). Low intake of Ca is likely to be associated with hypertension (Hajjar and Kitchen, 2003). Deposition of Ca in arteries leads towards atherosclerotic plaque formation. Cholesterol and its oxidation process along with other risk factors including hypertension and smoking may accelerate coronary calcification (Hemelrijick *et al*., 2013). Hypercalcaemia is abnormal increase in concentration of serum ionized calcium which can lead to nephrogenic diabetes insipidus and may cause acute renal failure. Hypocalcaemia is low ionized calcium concentration in serum; it is major cause of tetany (Maziarka and Pasternak, 2013). Calcium and vitamin-D play important role in lowering the risk of diabetes (Pitas *et al*., 2007). Decrease in Ca concentration is one of the causes of colon cancer (Newmark *et al*., 1984).

1.3.4 Cobalt

Cobalt (Co) is considered essential as it is the part of Vitamin B-12. It resembles in chemical properties with iron and nickel. Mostly cobalt compounds occur in two valence states; cobaltous (Co^{2+}) and cobaltic (Co^{3+}). Former is most commercially and environmentally available (Barceloux, 1999; Paustenbach *et al*., 2013a). Major sources of Co include contaminated air, water, soil, electronic devices (Kang *et al*., 2013), cosmetics and jewellery (Bocca *et al*., 2014) while occupational exposure includes hard metal

industry (Klasson *et al*., 2016), construction industry (Wang *et al*., 2011), e-waste recycling industry (Julander *et al*., 2014), diamond industry (Barceloux, 1999), pigment production and paint industry (Christensen and Poulsen, 1994). Exposure to Co-containing dust has been considered as cause of increased risk of lung cancer; however it is certainly not the main causative agent in this context especially the combination with tungsten carbide (WC) is considered carcinogenic (IARC, 2006; Wild *et al*., 2009). The IARC categorizes the mixture Co/WC as 'probably carcinogenic to humans' (IARC, 2006). The average human adult contains about 1.1 g of Co and daily requirement is 0.0001 mg/day. Cobalt has been reported to be used as a treatment for anaemia. At higher levels, it also increases RBC production in healthy people (Paternain, 1988). It is responsible to induces erythropoietin and blocks iodine uptake by the thyroid. Deficiency of Co is principal cause of cardiomyopathy, congestive cardiac failure, pericardial effusion, polycythemia and thyroid enlargement (Barceloux, 1999). It is acutely toxic in larger doses; it can be cytotoxic, may induce apoptosis and considered as genotoxic (Simonsen, 2012). Inhaling cobalt in large amounts may cause damage to the lungs, leading to asthma, pneumonia, or lung cancer. Its carcinogenic properties have association with its ability to inhibit repair mechanisms and to cause DNA damage (Qayyum and Shah 2014).

1.3.5 Copper

Copper (Cu) is an essential trace element which can exists in two oxidation states; Cu (II) and Cu (I) (Harris, 1996). It plays a very important role in body metabolism and allows many critical enzymes to function properly (Rosenzweig and Sazinsky, 2006). Mainly copper is available in the liver, shellfish, dried fruit, milk/milk products, sunflower seeds, oysters, sesame seeds and sun dried tomatoes (Deleves, 2009). The recommended daily intake of Cu in healthy adults is 0.9 mg/day (Gaetke, 2003). Copper is frequently accumulated in the liver, brain and kidney more than rest of body (Walvetvens, 1980). Excess of Cu show deleterious effects on the proper functioning of the cell through the generation of highly reactive oxygen species which produce hydroxyl radicals that adversely modify proteins, lipids and nucleic acids (Armendariz and Vulpe, 2003; Rae *et al*., 1999). In Wilson's disease, there is a defective peptide that causes excessive accumulation of Cu in liver (LaFontaine *et al*., 1998; Brewer, 2001; Yamamoto *et al*., 2001). Elevated levels of serum copper have been reported in primary lung cancer (Mateo *et al*., 1979). Serum and tumour Cu levels have been reported to be significantly elevated in cancer patients compared to controls (Kuo *et al*., 2002). Elevated serum Cu was also

reported in patients with mammary carcinomas, bronchial carcinoma and gastric carcinomas (Ridge *et al*., 2008; Maloba *et al*., 2006). The elevated Cu levels observed in the breast cancer tissue possibly promote breast cancer through angiogenesis and oxidative DNA damage (Tapia *et al*., 2003). Deficiency of Cu for prolonged period leads to anaemia, growth retardation, defective keratinisation and pigmentation of hair, hypothermia, mental retardation, changes in skeletal system and degenerative changes in aortic elastin (Odell, 1982). High Cu intake for prolonged period causes increased percentages in serum and tissue that in turn causes oxidative stress and affects several immune functions (Turnland *et al*., 2004).

1.3.6 Iron

Iron (Fe) is an essential metal that plays a crucial role in many cellular processes including oxygen transport, respiration, tricarboxylic acid cycle, lipid metabolism, gene regulation and DNA synthesis (Cairo *et al*., 2006). Both iron deficiency and iron overload are deleterious and lead to disease development in many ways. Elevated levels of iron are associated to numerous chronic diseases, such as heart disease, diabetes, defective immune regulatory control and cancer (Tuomainen *et al*., 1998; Salonen *et al*., 1998; Walker, 2000; Stevens *et al*., 1998). Iron is causative agent and it promotes cancer; it acts as a catalyst which causes tissue damage in the conversion of hydrogen peroxide to free-radical ions which further attack cellular membranes and causes DNA strand breaks, inactivate enzymes, depolymerise polysaccharides and initiate lipid peroxidation (McCord, 1996). It promotes inflammation and cancer cell grows at faster rate (Weinberg, 1999; Lieu, 2001). The excess accumulation of Fe in humans is found to have a relationship with an increased risk of cancer (Cunzh, 2003; Reddy , 2004). Iron toxicity is also responsible for vomiting and diarrhoea, with subsequent effects on the cardiovascular and central nervous systems, kidney, liver, and blood (Goldhaber, 2003). Deficiency of Fe leads to numerous disorders, most important among them is iron deficiency anaemia which is associated with microcytic hypochromic RBC's, tiredness, achlorhydria, Plummer–Vinson syndrome, atrophy of epithelium, impaired attention, irritability, and lowered memory (Lieu *et al*., 2001). Iron deficiency anaemia can lead to heart failure (Gil, 2014).

1.3.7 Potassium

Potassium (K) is essential for the proper function of all cells, tissues, and organs in the human body. It is also crucial to heart function and plays a key role in skeletal and

smooth muscle contraction. It plays a key role in that potassium bicarbonate is the primary intracellular inorganic buffer. Potassium enters the cell more readily than sodium and initiates the brief sodium-potassium exchange across the cell membranes. Too much potassium (hyperkalemia) is characterized by irritability, nausea, decreased urine production and cardiac arrest. Fatigue is the most common symptom of chronic K deficiency. Early symptoms include muscle weakness, slow reflexes and dry skin or acne; these initial problems may progress to nervous disorders, insomnia, slow or irregular heartbeat, and loss of gastrointestinal tone. A sudden loss of potassium may lead to cardiac arrhythmia. Potassium is very important in cellular biochemical reactions and energy metabolism; it participates in the synthesis of proteins from amino acids in the cell. Potassium also functions in carbohydrate metabolism (REF).

1.3.8 Magnesium

Magnesium (Mg) is the 2nd most abundant cation within human cells after K. It controls structural integrity of the skin and plays a significant role in maintaining effective homeostatic regulation. It plays a significant role in phosphorylation reaction, energy transfer, protein synthesis, lipid and carbohydrate metabolism. Magnesium is present as cofactor and activator in more than 300 enzymes. It is also involved in stabilization of nucleic acid. Its deficiency can cause stress, neuromuscular hyper-excitability, hyper-irritability and cardiovascular manifestation in human. Excess of blood Mg levels lead to necrosis, hypertension and respiratory paralysis (Dechen and Kettler, 2012). High serum Mg concentrations are associated with nausea and vomiting. On the other hand, hypomagnesaemia is an electrolyte disturbance characterized by an abnormally low level of magnesium in the blood (Whang *et al.*, 1994). Normal serum Mg levels in human ranges between 1.5–2.5 mg/dL (or 1.0–1.2 mmol/L), when the serum Mg level is lower than 0.7 mmol/L, we refer to the condition as hypomagnesaemia.

1.3.9 Manganese

Manganese (Mn) is an essential trace metal and it is present approximately 0.1% of the earth's crust (ATSDR, 2012b). It enters in the environment via mining, smelting, refining, alloy manufacturing, fossil fuel combustion, dust, volcanic eruptions, vegetation and forest fires. The common source of Mn for general population is through ingestion of food/water or inhalation of dust particles. The daily intake levels of Mn are 2-3 mg/day (FNB/IOM, 2001). It plays vital role in amino acid, cholesterol and carbohydrate

metabolism and it also provides cellular protection from free radical damage (Goldhaber, 2003). Elevated intake of Mn can cause neurological disorder with symptoms that include tremors, difficulty walking and facial muscle spasms (ATSDR, 2012b). Its deficiency cause bleeding disorders due to increased prothrombin time while excess amount of Mn accumulate over a long period causes anorexia, apathy, headache impotence, leg cramps, speech disturbance, encephalitis like syndrome and Parkinson like syndrome. Psychosis may also occur due to excess of Mn (Van Rij *et al*., 1979).

1.3.10 Sodium

Sodium ions are the major cations of extra-cellular fluid in human body. To maintain internal fluid and electrolyte balance, water, sodium and potassium are in constant movement between the intra-cellular and extra-cellular body compartments. Potassium and sodium ions are particularly important in the renal regulation of acid-base balance because hydrogen ions are substituted for Na and K ions in the renal tubule. Abnormal levels of these electrolytes may result in a variety of pathological disorders (Ganong, 1991). Very high concentration of Na (a condition called hypernatremia) leads to edema thirst and lessened urine production while hyponatremia is usually characterized by headache, confusion, seizures, muscle spasms, nausea and vomiting. Long term use may leads to stroke and coronary heart diseases. Excess of Na may leads to osteoporosis, gastric cancer and bronchial reactivity and it also plays major role in cardiovascular issues (Caballero, 2009; Whelton *et al*., 2012).

1.3.11 Lead

Lead (Pb) is a potent toxicological agent which poses serious health risks to human (Canfield *et al*., 2003; Koller *et al*., 2004). It can substitute Ca in the body and its half-life in bones and teeth is several decades (Verstraeten *et al*., 2008). Even though Pb has been phased out from gasoline, a major present-day source of urban lead is suggested to be the presence of dust linked to roadside soil where long-term Pb deposition has occurred due to previous usage of leaded fuels. Other possible non-fuel sources of Pb exposure can be ascribed to small industrial activities and natural sources (Varrica *et al*., 2003). Major consumption of Pb is due to lead-acid storage batteries used in motor vehicles, electric powered vehicles and as emergency stationary power supply. It is also applied in shields against radiation, glass, glaze, plastic, functional ceramics, building and construction industries, electronic technologies, smelters and welding (Thornton *et al*., 2001; ILZSG,

2010). Pb poisoning may cause inhibition of the synthesis of haemoglobin, dysfunctions in the kidneys, joints and reproductive systems, acute and chronic damage to the central nervous system (Ogwvegbu and Muhanga, 2005). Adverse health effects of Pb included deleterious effects on the haematological and cardiovascular systems (ATSDR, 2007). International Agency for Research on Cancer (IARC) has described it as probably carcinogenic to humans, group 2A (IARC, 2006). The effects of Pb exposure on the immune system have not been well documented but some immunotoxic abnormalities induced by Pb have been suggested (Mishra, 2009).

1.3.12 Strontium

Strontium (Sr) has almost similar behaviour to Ca in human body; it can substitute Ca in the bones, as they both have the same charge and similar ionic radius. It enters the human body usually in small quantities through breathing, eating and drinking. Burton *et al*., (2003) showed that barium and strontium enter skeletal tissues in proportion to diet and hence to local environmental levels. Sr is an essential metal and it plays a major role in endocrinology. It mimics the action of calcium in many cases (Nielsen, 2004). It is effective for bones and enhances the bone health. As it performs many functions which are similar to calcium so it can be used as marker for assessing the Ca absorption. Absorption of both Ca and Sr is stimulated by vitamin D and they also share analogous physical characteristics (Holick, 2004).

1.3.13 Zinc

Zinc (Zn) is an essential metal for human as it is part of more than 300 enzymes (Dreosti, 2001; Selinus and Alloway, 2005). It also plays an important role as a part of proteins involved in genetic transcriptions and in metallothionine to which it is bound. Many researchers have reported that Zn inhibits the development of cancer and that low level of Zn is associated with several forms of cancers (Fong *et al*., 1978; Rensburg *et al*., 1980). It is an essential component of the enzymes involved in DNA synthesis and repair. Zinc deficiency affects the immune system, anorexia nervosa, wound healing, the senses of taste/smell, growth retardation, impairing DNA synthesis, hypogonadism with impaired reproductive capacity and dermatitis (ATSDR, 2005b; Saper and Rash, 2009; Salgueiro *et al*., 2000). It prevents tumour development through its role in the cell immunity (Wellinghausen and Rink, 1998). It plays an important role in maintaining anti-tumour immunity by assisting the recognition of cancer cells and attacking the cancerous host

cells (Millos *et al*., 2008). Exposure to the elevated levels of Zn may cause chest pain, cough, reduced lung volumes, nausea, chills, malaise, leukocytosis, vomiting, abdominal cramps, and diarrhoea (ATSDR, 2005b). It is mainly used as metal coatings for iron or other metals for rust protection, alloy manufacturing, dry cell batteries, dyes, paint coatings and as a catalyst. It enters into the environment by natural as well as anthropogenic sources including alloy manufacturing, smelting, ore processing, mining operations and fossil fuel combustion (ATSDR, 2005b). General population is exposed to Zn through dietary sources and the recommended daily intake of Zn for adults is 8-11 mg/day (FNB/IOM, 2006). It is essential for normal spermatogenesis and maturation, genomic integrity of sperm, for normal organogenesis, proper functioning of neurotransmitters, proper development of thymus, proper epithelialization in wound healing, taste sensation, and secretion of pancreas/gastric enzymes (Watson, 1998). It is also a component of DNA and RNA polymerase and has protective and modulatory effects on the growth of normal and cancer cells (Singh and Gary, 1998).

1.4 Biological Samples used for Trace Element Analysis

A variety of biological specimens have been used in various clinical investigations and every type of sample is associated with specific advantages and limitations. Generally, choice of the sample depends on the target analytes, available protocols, study population and methodology to be used. Although various samples have been reported for trace metal analysis but most frequently blood samples are preferably used. Blood is one of the widely used specimens for biological trace element research because of its natural significance and ease of sampling (Prange, *et al*., 1989). It is medium of transport of trace metals and other nutrients and provides direct evidence of metabolism about the trace metals concentrations (Schrauzer *et al*., 1977). Therefore whole blood, plasma and serum are convenient samples for determination of trace metal status of an individual (Smith *et al*., 2002). Moreover, it is in contact with all tissues and organs where metals are deposited. It does not require washing and it is free from external contamination. Blood sample has some limitations as well: blood tend to show current or recent body status. It reflects elemental exposure based on a very short or limited period (few hours). Storage and transport is difficult as it requires careful handling as it can be infectious. Patient may feel faint after blood drawing. Blood collection sometime ruptures the blood cells, producing results that are inaccurate.

1.5 Cancer Incidences in Pakistan

Pakistan is categorized as a 'lower-middle income country' by the World Bank, with its population estimated to be 185 million in the year 2014 and the life expectancy at birth being 66 years (65 years for males and 67 years for females). It is estimated that one in nine Pakistani women will develop breast cancer at some stage in their life. The number of registered breast cancer patients at Shaukat Khanum Memorial Cancer Hospital & Research Centre, Pakistan is highest amongst all reported cancer patients. Between 2010 and 2012, in Lahore a total of 15840 new cancers were diagnosed in 43% male patients and 57% female patients; 93.5% were microscopically confirmed and 6.5% non-microscopically. In females, the ASIR was 105.1 and in males, it was 66.7. ASIRs of leading cancers among women were breast 47.6, ovary 4.9 and corpus uteri 3.6, whereas among men they were prostate 6.4, bladder 5.0, and trachea, bronchus and lung 4.6. A total of 5134 deaths by cancer were recorded during this period (Badar *et al*., 2016).

Karachi Institute of Radiotherapy and Nuclear Medicine (KIRAN) is a comprehensive healthcare facility for diagnosis, treatment and research of all cancers. Analysis of the cancer patients of both genders of all age groups to determine frequencies of different cancers, from 1st January 2000 to 31st December 2008 showed that 16351 cancer patients were registered at KIRAN; male cancers accounted for 48.2% and female cancers 51.8%. In male patients the five most frequent malignancies were head and neck (32.6%), lung (15%), gastrointestinal tract (6.9%), lymphoma (6.1%), and bone and soft tissue (4.9%). In female patients breast cancer was the most common cancer accounting for 38.2%, followed by head & neck (15.1%), cervical (5.5%), ovarian (4.9%) and gastrointestinal tract cancer (4.9%), respectively (Hanif *et al*., 2009). The incidence of breast cancer in Karachi South for the period 1995-1997 was the third highest in Asia. A total of 709 cases of breast cancer incidence were reported. Breast cancer accounted for approximately one-third of the cancers in females. In Karachi South 60% of the newly diagnosed breast cancers were observed in women below 50 years. Invasive breast cancers predominated with 99.4%, with in-situ cancers contributing to 0.6% of the malignancies (Bhurgri *et al*., 2006).

1.6 Aim and Objectives of the Present Study

The aim of present study is to evaluate the concentrations of essential and toxic metals in the blood of breast cancer patients and their counterpart healthy donors having same age, gender, socioeconomic status, food habits and habitat. Following are the major objectives of the present study:

- To assess comparative distribution of selected essential and toxic metals (Ca, Cd, Co, Cr, Cu, Fe, K, Mg, Mn, Na, Pb, Sr and Zn) in the blood of breast cancer patients and matching healthy donors.
- To establish the mutual relationships among selected essential and toxic metals in cancer patients and healthy donors using correlation coefficients.
- To compare the concentrations of selected metals in different demographic groups of both donor categories.
- To evaluate the concentrations of selected metals in different stages of breast cancer.
- To determine the multivariate apportionment among the metal in the blood of the patients and controls by multivariate statistical models.
- To explore the role of selected metals towards the breast cancer so that it may be helpful in treatment of breast cancer.

Chapter 2

EXPERIMENTAL METHODOLOGY

2.1 Study Population

The subjects involved in present study were breast cancer patients and controls. All the participants were thoroughly briefed about objectives of the study and there was no compulsion on the subject to participate in this research and written consent was obtained from each subject before the blood collection. All the subjects were selected to participate in this research on volunteer basis. Every participant was assured that all the information recorded from them will remain confidential. The participants filled a questionnaire to record information such as name, gender, age, diet, nature of ailment, ailment duration, disease stage, medication, blood transfusion, any surgery, any other disease and smoking habits. Questionnaire sample is given as Annexre-1. The participants were selected on basis of comparable environmental exposure, habitat, socioeconomic status, diet, same gender and almost matching age.

2.1.1 Breast Cancer Patients

The blood samples were collected from breast cancer patients admitted in Nuclear Oncology and Radiotherapy Institute (NORI), Islamabad, on volunteer basis. Prior to the sample collection, protocol of the present study was approved by ethical review committee of the institute after comprehensive review. The blood samples were collected from newly diagnosed breast cancer patients prior to any treatment such as, surgery, chemotherapy or radiotherapy. All the donors were not taking any mineral supplements for at least last three months. Clinical diagnosis was confirmed by histopathological and radiological examination in the institute. Seventy blood samples were collected from newly diagnosed and previously untreated breast cancer patients during the present study.

2.1.2 Healthy Subjects

In the present study, seventy blood samples from healthy subjects/controls were collected on volunteer basis. In most of the cases, controls were family members or close relatives of the breast cancer patient, thus they had similar environmental exposure, diet, socioeconomic status, gender, age and residence. Healthy donors were not taking any

mineral supplements on a regular basis and they were not suffering from any type of major disease. Prior to the samples collection they went through a routine medical examination (Zowczak *et al*., 2001). The demographic characteristics related to breast cancer patients and healthy donors included in the present study are shown in Table 1.

Table 1. Demographic characteristics of the subjects

Characteristics	Breast Cancer Patients	Healthy Subjects
n	70	70
Age (years)		
Range	24-63	23-63
Mean	43.5	43.0
Gender		
Female	70 (100%)	70(100%)
Diet		
Vegetarian	42 (60%)	41 (59%)
Non-vegetarian	28 (40%)	29 (41%)
Habitat		
Urban	33 (47%)	32 (46%)
Rural	37 (53%)	38 (54%)
Tobacco Use		
No Use	60 (86%)	63 (90%)
Use	10 (14%)	07 (10%)
Family History		
Positive	10 (14%)	08 (11%)
Negative	60 (86%)	62 (89%)
Side of cancer		
Left	23 (33%)	--
Right	47 (67%)	--
Stages of the Cancer		
Stage-I	02 (3%)	--
Stage-II	24 (34%)	--
Stage-III	31 (44%)	--
Stage-IV	13 (19%)	--

2.2 Sample Collection and Storage

Blood samples are susceptible to external contaminations during blood collection. To avoid contaminations with exogenous metals, special precautions were followed during collection, storage and analysis of the blood samples. Origin of contamination may be subject's skin or hands of person collecting the sample. Generally, it is recommended that specially designed evacuated tubes should be used for blood collection purpose to avoid any external contamination. Separate syringes should be used to collect appropriate amount of blood sample from subjects after cleaning their skin. Few methods used to collect and store the blood samples reported in literature are described below:

- Venous blood samples (3–5 mL) were sampled by using metal-free vacutainer EDTA tubes and the samples were stored at –20°C until required for analysis. Thoroughly, mixed the whole blood sample and transferred to the storage tube at −4°C till further treatment (Cornelis *et al*., 1995).
- The blood sample (about 3 mL) was drawn from an antecubital vein. The blood sample was transferred to a vacutainer tube at room temperature. The blood sample was stored in a refrigerator until analysis (Qayyum and Shah, 2017).
- The blood samples were collected from an antecubital vein by using appropriate precautions to prevent contamination with exogenous trace metals. Venous blood was obtained in heparinized evacuated tubes. Each blood sample was softly shaken by hand and centrifuged at 2000 rpm for 15 min. The plasma was separated by using Finn pipette carefully into another polyethylene vial duly labeled with relevant codes related to the name of donor, age, diet, social and general health status, all information recorded and compiled on regular questionnaire at the time of sampling. Samples were stored at –70°C until analysis was performed (Chappuis *et al*, 1994; Subramanian, 1995).
- Fasting blood samples (approx. 10 mL) were collected from cancer patients and healthy donors under aseptic precautions by venopuncture method. The blood samples were centrifuged at 3000 rpm for 10 min at 4°C. Both the patients and healthy donors' sera were stored at 4°C in an ice chest for no longer than 24 h before freezing and were stored at –70°C until analysis. In order to avoid contamination, trace-metal free containers were used (Huang *et al*., 1999).
- Venous blood samples (5 mL) were collected by using heparinized vacutainer tubes. 2 mL of venous blood samples were stored at 20°C until elemental analysis,

while remaining (3 mL) were used for separating the sera. The blood is allowed to clot at room temperature for 15-30 min. When the blood has clotted completely then centrifuged for 5-10 min at 2500 rpm. The supernatant fluid is then separated by a Pasteur pipette, labelled and stored at 20°C until analysis (Kolachi *et al*., 2012).

- ❖ Blood samples were centrifuged to separate serum of the three groups at 1200 rpm for 5 min. Serum was diluted with 0.05% Triton X-100 and homogenized before analysis (Kuo *et al*., 2002).
- ❖ For serum analysis, blood samples were allowed to coagulate. Serum samples were prepared by centrifugation at 3000 g for 15 min using a universal centrifuge. The serum samples were stored at –80°C prior to the analysis. The samples were thawed at room temperature, then 1 mL sample was diluted with 9 mL deionised water. Deionised water was also used for blank throughout. The last dilutions of the samples were mixed on a shaker for 15 min just before analysis (Bursalioglu *et al*., 2017).
- ❖ About 4-mL blood sample was collected from each participant. Blood samples were collected in trace-metal free evacuated tubes containing heparin as an anticoagulant. Two mL of blood was then pipetted into an eppendorf tube (2 mL volume) previously cleaned and immediately frozen at −20°C before analysis. For plasma separation, 2 mL of blood samples were centrifuged (1000 ×g for 6 min). The plasma fraction was then pipetted into an eppendorf tube (2 mL volume) previously cleaned and was immediately frozen at –20°C before analysis (Rodrigues *et al*., 2008).

The method used for blood collection and storage in the present study is briefly described below: To avoid contamination of exogenous metals appropriate precautions were taken during the collection and storage of blood samples. Skin of each donor was cleaned and BD syringes (5 mL) were used to collect about 3 mL blood from forearm antecubital vein by vein puncture method. The collected blood sample was immediately transferred from syringes into specially designed 5 mL evacuated tubes (BD Vacutainer Ref. 366,430). The blood samples were carefully transferred to the laboratory and kept frozen at –4°C until further analysis.

2.3 Sample Preparation

Human blood contains large amount of organic contents which can cause interferences during elemental analysis, therefore digestion is done to completely remove the organic contents from blood. Proper precautions are followed during the sample preparation to avoid loss of sample and external contamination. Different methods of blood digestion have been reported in literature, some of which described below:

- Nitric acid–perchloric acid (10:1 v/v) mixture was used to digest plasma samples with subsequent heating to a soft boil until white dense fumes evolved. It was followed by cooling to room temperature and diluted the samples with doubly distilled water (Pasha *et al*., 2008; Sansoni and Panday, 1994; Ren *et al*., 1997).
- Blood samples were digested by using a mixture of HNO_3–$HClO_4$ (10:1 v/v). Each flask was subsequently heated (80°C) to a soft boil until dense white fumes evolved. After digestion, the flasks were left to cool at room temperature and then samples were diluted with doubly distilled water (Qayyum and Shah, 2016).
- Accurately 0.5 mL of whole blood was taking into Pyrex flask. 3 mL of freshly prepared mixture of concentrated nitric acid and hydrogen peroxide (2:1 v/v) was added into the blood sample and sample stood for 10 minutes. The flasks were covered with watch glass and then digested at 60-70°C for 1-2 hours. Then treated with 2 mL nitric acid and few drops of H_2O_2, while heating continued on hot plate at about 80°C until a clear digested solution was obtained. The excess acid mixture was evaporated to semi-dry mass and then cooled to room temperature and diluted with 0.1 M nitric acid (Memon *et al*., 2007).
- Blood samples (200 μL) were transferred into conical tubes (15 mL) with help of pipette. After this, the volume made up to 10 mL with a solution containing 0.5% (v/v) HNO_3 + 0.005% (v/v) Triton X-100. As internal standard, Rhodium was added to get a 10 μg/L final concentration (Palmer *et al*., 2006).
- About 2 mL of blood was placed in 25 mL glass tubes. After this, samples were vaporized to dry in the heating block at the temperature of 180–200°C and then the samples were mineralized in the stove at the temperature of 480°C for 14 h. After this, the samples were treated with 3 mL of pure nitric acid and then samples were placed in heating block, bringing the temperature to 200°C (1 h). Followed by increase in the temperature to 250°C (2 h). After that chilling, solutions were supplemented with redistilled water up to 5 mL (Wieloch *et al*., 2012).

- Precisely weighed blood sample was taken in the digestion flask and then 10 mL of HNO_3 was added to digestion flask and stood for 5 min. Then 10 mL of $HClO_4$ was added to the digestion flask. Then flasks were placed on a hot plate for about 5–6 h maintaining the temperature between 70 and 80°C until white dense fumes evolved. After this, sample was transferred to the 50 mL volumetric flask and the final volume was adjusted with 0.1 N HNO_3 up to the mark. The blank was also processed in the exactly same sequence along with each batch except sample (Pasha *et al*., 2010).
- About 0.5 mL of blood samples were directly placed into Teflon flasks to which 2 mL of freshly prepared mixture of HNO_3-H_2O_2 (2:1, v/v) was added. After that left the samples for 10 minutes at room temperature. Then flasks were placed in a PTEE container and heated at 80% of total power (900 W) for 2-4 minutes. Samples completely digested and flasks were left to cool to room temperature. After cooling, resulting solution was evaporated to a semi-dried mass to remove excess acid. About 5 mL of 0.1 M HNO_3 was added to the residue followed by filtration thorough a Whatman No.42 filter paper then diluted with deionised water (Kazi *et al*., 2008).

In the present study, blood samples of the patients and controls were digested by using following method: Blood sample was transferred from evacuated tube to a digestion flask and accurately weighed on an analytical balance. Then 15 mL of concentrated HNO_3 was added slowly into the sample and left for about one hour. After that 15 mL of concentrated $HCLO_4$ was added into the digestion flask and stood for overnight. To avoid any contamination of exogenous metals, flask was covered with watch glass. Afterwards the flask was placed on hot plate and heated for 7-8 h by keeping the temperature between 70 to 80°C until dense white fumes vanished. At this point it was observed that the blood sample was completely digested. The digested sample was cooled to room temperature and transferred to 50 mL volumetric flask and diluted with 0.1 N HNO_3 up to the mark and properly labelled with relevant codes. The blank containing all reagents except the sample was prepared and processed in same manner. One blank was processed along with a batch of 5 samples. This method was optimized by using different proportions of HNO_3-$HCLO_4$ and HNO_3-H_2O_2; however HNO_3-$HCLO_4$ (1:1 v/v) was selected because it was time saving, less expensive and resulted in quick/complete digestion of the blood sample.

2.4 Measurement of Selected Metals

Analysis of biological samples is a big challenge as most of the metals concentration is very low; so sensitive techniques are required to accurately and precisely measure the concentrations of different metals in the biological samples. Atomic absorption spectroscopy is a sensitive analytical technique which is used to measure the concentrations of essential and toxic metals at trace and ultra-trace levels. Atomic absorption spectroscopy is used to quantify more than sixty metals over a wide range of concentration in different biological, clinical, geological and environmental samples. Atomic absorption spectroscopy measures the concentration of metals based on absorption of radiations of specific wavelength by free atoms of metals which undergo transition from ground state to higher energy level. Amount of light absorbed is directly proportional to concentration of metal atoms in the sample.

Table 2. Optimum analytical conditions maintained on the instrument (Shimadzu AA-670, Japan) for analysis of selected elements using air-acetylene flame

Metal	Wavelength (nm)	HC lamp current (mA)	Slit width (nm)	Fuel-gas flow rate (L/min)	1% Absorption concentration (ppm)
Ca	422.7	6.0	0.5	2.0	0.08
Cd	228.8	4.0	0.3	1.8	0.02
Co	240.7	6.0	0.2	2.2	0.20
Cr	357.9	5.0	0.5	2.6	0.09
Cu	324.8	3.0	0.5	1.8	0.09
Fe	248.3	8.0	0.2	2.0	0.10
K	766.5	5.0	0.5	1.9	0.04
Mg	285.2	4.0	0.5	1.6	0.007
Mn	279.5	5.0	0.4	1.9	0.05
Na	589	6.0	0.5	1.6	0.02
Pb	217	7.0	0.3	1.8	0.20
Sr	460.7	4.0	0.5	1.6	0.10
Zn	213.9	4.0	0.5	2.0	0.02

In the present study, selected essential and toxic trace metals including Ca, Cd, Cr, Co, Cu, Fe, K, Mg, Mn, Na, Pb, Sr and Zn were quantified in the blood samples of cancer patients and healthy subjects using flame atomic absorption spectrophotometer (Shimadzu AA-670, Japan) with automatic background compensation and under optimum analytical

conditions such as hollow cathode lamp current, detection wavelength, slit width, flame type and fuel flow rates as shown in Table 2. The principal attractions of the technique of AAS are high attainable sensitivity for a wide range of elements and high selectivity for the analyte elements. Instrumentation for AAS is relatively inexpensive, small sample size is required and it is easy to operate the instrument. To ensure high analytical precision and accuracy, calibration lines are automatically recorded. To exclude deviant data, lamp position, beam balance and detector gain all are adjusted automatically. Atomic emission spectroscopy, fluorescence spectroscopy and inductively coupled plasma emission or mass spectrometry can also be used for analysis of biological samples such as serum, blood, urine, tissues etc.

2.5 Statistical Analysis

Statistical analysis of the quantified results of selected metals was carried out using MS-Excel and STATISTICA software (StatSoft, 1999). The data distribution and recognition tools used in this study include pre-treatment of data in order to achieve normalization by discarding the outliers employing Q test ($p < 0.05$). However, the overall outliers were less than 5% of the total measurements. Both multivariate and univariate statistical methods of analyses were employed in the current study. Basic statistical parameters included maximum, minimum, mean, median, standard deviation (SD), standard error (SD), skewness, kurtosis and correlation coefficients which showed the distribution pattern and mutual relationships among selected essential and toxic metals in the analysed samples. Two most widely used multivariate methods are principal component analysis (PCA) and cluster analysis (CA) which were used in the present study (Jobson, 1991). PCA and CA are successfully employed to quantified results of the metals in biological samples to distinguish between the diseased person and healthy subjects (Pasha *et al*., 2008; Qayyum and Shah, 2014). The multivariate methods have also been used for trace metal analysis of scalp hairs of patients and healthy donors (Shah *et al*., 2006). Multivariate statistical analyses have been also used to investigate trace elements level in the blood plasma and scalp hair of gastrointestinal cancer patients and controls (Pasha *et al*., 2010). Multivariate methods were used to determine the contribution of soil, water and food consumption to metal exposure of children from geological enriched environments (Okoth *et al*, 2013).

2.5.1 Principal Component Analysis

Principal component analysis (PCA) rotates the data-set such that maximum variables are projected onto the axes. In PCA a set of correlated variables is transformed into a set of uncorrelated variables which are ordered by reducing variability. The uncorrelated variables are linear combinations of the original variables and the last of these variables can be removed with minimum loss of real data. The main use of PCA is to reduce the dimensionality of a data-set while retaining as much information as possible. It computes a compact and optimal description of the data-set. The first principle component (PC) is the combination of the variables that explains the greatest amount of the variance. The second PC defines the next largest amount of the variation and it is independent to the first PC. There can be as many possible PCs as there are variables. It can be viewed as finding a projection of the observations onto orthogonal axes contained in the space defined by the original variables. The criteria being that the first axis contains the maximum variation, the second axis contains the maximum variation orthogonal to the first and the third axis contains the maximum variation orthogonal to the first and second axis and so on until one has the last new axis which is the last amount of variation left. PCA was applied to find out the multiple relationships among the analysed metals (Yongming, *et al*., 2006).

2.5.2 Cluster Analysis

Cluster analysis (CA) classifies a set of observations into groups based upon combinations of internal variables in the form of dendrogram. This technique is a classification procedure that involves a measurement of the similarity between the variables. The purpose of cluster analysis is to discover a system of organizing observation where member of the groups/variables share the properties in common. The variables are grouped in the cluster in terms of their nearness or similarity. The measurement of similarity is based on Pearson-*r* distance. The clustering method used in present study was Ward's method, which considers the heterogeneity or deviance (sum of square of distance of variables from the barycentre of the cluster) of every possible cluster that can be created by linking two existing clusters. Therefore, it is cognitively easier to predict the mutual properties based on overall group membership (Prystupa *et al*., 2016; Pasha *et al*., 2010).

Chapter # 3

RESULTS AND DISCUSSION

3.1 Distribution of Selected Metals

Various statistical parameters related to the distribution of selected essential and toxic metal levels in the blood samples of breast cancer patients are shown in Table 3. Most of the metals exhibited wide range of concentrations as shown by their minimum and maximum levels. On comparative basis, considerably higher mean levels were found for Na (1556 ppm) and K (1233 ppm), followed by moderately higher levels of Fe (117.5 ppm) , Ca (43.46 ppm), Mg (29.89), Zn (5.447 ppm), Pb (2.135 ppm) and Co (1.625 ppm) while the least concentration was found for Cd (0.146 ppm) and Mn (0.310 ppm) in the blood of breast cancer patients. Overall the average concentration of essential and toxic metals showed following decreasing order: Na > K > Fe > Ca > Mg > Zn > Pb > Co > Cu > Cr > Sr > Mn > Cd. Predominantly a random distribution pattern was shown by most of the metals as demonstrated by relatively higher SD and SE values on one hand and distinctly dissimilar mean and median levels on the other hand. Relatively larger spread of concentration was observed in the case of Na, whereas some of the metals (Cd, Mn, Sr, and Cr) exhibited moderately Gaussian distribution as supported by relatively lower SE and SD values. Large skewness and kurtosis value for Cu, Zn, Na, Fe, Mg, Mn, and Co demonstrate their significant asymmetric distribution while modest skewness values of Cd, K, Sr, Ca and Cr showed moderately symmetrical distribution of these metals in the blood of breast cancer patients (Table 3).

Basic statistical distribution parameters for selected essential and toxic metal levels in the blood of healthy donors are shown in Table 4. Very broad range of concentration was exhibited by most of the metals as demonstrated by their minimum and maximum concentrations in the blood of controls. On the average basis significantly higher concentrations were observed for Na (1585 ppm) and K (867.3 ppm), followed by moderately higher levels of Fe (96.38 ppm), Mg (24.31 ppm), Ca (16.03 ppm) and Zn (6.757 ppm). However, mean levels of Cr (0.796 ppm), Sr (0.560), Mn (0.194 ppm) and Cd (0.134 ppm) were found at sub-ppm levels. The selected metals in the blood of normal donors showed following decreasing order in their average concentrations: Na > K > Fe > Mg > Ca > Zn > Co > Pb > Cu > Cr > Sr > Mn > Cd.

Table 3. Statistical distribution parameters for the concentrations of selected metals (ppm) in the blood of breast cancer patients

	Min	Max	Mean	Median	SD	SE	Kurtosis	Skewness
Ca	8.222	113.8	43.46	41.12	22.05	2.674	0.900	0.970
Cd	0.016	0.451	0.146	0.111	0.104	0.014	0.408	1.075
Co	0.044	6.045	1.625	1.309	1.390	0.187	2.055	1.399
Cr	0.019	1.901	0.648	0.576	0.414	0.057	0.977	1.012
Cu	0.044	6.929	1.072	0.761	1.007	0.124	16.71	3.245
Fe	57.86	276.6	117.5	105.9	45.40	5.465	3.708	1.939
K	706.8	1233	889.0	875.7	115.8	13.84	0.469	0.873
Mg	10.53	44.95	29.89	29.77	5.601	0.669	2.590	-0.303
Mn	0.036	1.013	0.310	0.283	0.204	0.027	2.073	1.428
Na	1201	2256	1556	1547	162.2	19.39	3.958	0.911
Pb	0.132	7.384	2.135	1.629	1.841	0.244	1.019	1.204
Sr	0.018	1.528	0.457	0.358	0.381	0.056	0.660	1.073
Zn	0.568	31.27	5.447	3.644	5.349	0.708	9.169	2.583

Table 4. Statistical distribution parameters for the concentrations of selected metals (ppm) in the blood of healthy subjects

	Min	Max	Mean	Median	SD	SE	Kurtosis	Skewness
Ca	1.847	40.34	16.03	13.23	10.67	1.366	-0.334	0.842
Cd	0.014	0.421	0.134	0.121	0.102	0.015	0.283	0.927
Co	0.104	4.187	1.390	1.249	1.013	0.133	0.595	0.986
Cr	0.055	2.323	0.796	0.675	0.540	0.067	0.368	0.939
Cu	0.114	8.605	1.237	0.972	1.046	0.125	3.599	2.230
Fe	29.50	267.0	96.38	76.65	62.20	8.030	0.546	1.194
K	546.1	1292	867.3	867.2	115.7	13.83	2.596	0.645
Mg	9.623	34.26	24.31	24.96	5.167	0.618	0.738	-0.808
Mn	0.014	0.478	0.194	0.180	0.115	0.016	-0.461	0.396
Na	1226	1923	1585	1572	125.3	14.97	0.857	0.442
Pb	0.014	3.163	1.286	1.404	0.885	0.140	-1.027	0.212
Sr	0.039	1.311	0.560	0.522	0.368	0.056	-1.085	0.307
Zn	1.038	20.88	6.757	5.485	4.382	0.539	2.896	1.590

Mostly a random distribution pattern was displayed by the most of the metals as established by their elevated SD and SE values as well as markedly dissimilar mean and median levels in the blood of healthy donors (Table 4). Comparative larger dispersion was observed for Na, K, Fe and Ca, however some of the metals (Cr, Sr, Mn, and Cd) exhibited relatively normal distribution pattern supported by lower SE and SD values. Large skewness and kurtosis values for Cu, Zn, K, Sr, and Pb showed their predominantly asymmetric distribution while Cd, Ca, Cr and Mn showed relatively symmetrical distribution in the blood of healthy subjects. Nonetheless, the extent of randomness was found to be comparatively less in controls than those of the patients which showed reasonably higher randomness in their concentrations.

The quartile distribution of selected metals (in the form of box & whisker plot) in the blood of breast cancer patients and healthy donors is shown in Figures 1 and 2, respectively. Most of the metals demonstrated relatively broad and asymmetrical distribution in the blood of both donor groups; however, relatively narrow distribution was observed for Na, K, Fe, and Mg in blood samples of breast cancer patients. Conversely, measured levels of Co, Cr, and Sr showed rather broad and asymmetric variations in the blood samples of cancer patients while Cu, Pb, Mn, Zn, Ca, and Cd showed moderately asymmetric distribution. Such imbalances in the metal levels may be ascribed to the disproportions of the essential nutrients and toxic metals in the breast cancer patients. In the case of healthy donors, very broad range and asymmetric variations were noted for Cr, Mn, Pb, Sr, and Ca while least variations were observed for Na, K, Mg, and Fe. Somewhat symmetric distribution was noted for Cd, Cu, and Zn in the blood of healthy donors (Figure 2).

The average metal concentrations in the blood of breast cancer patients and healthy donors were also compared as shown by bar-graph in Figure 3. On comparative basis, average concentrations of Cr, Cu, Sr, and Zn were found to be evidently higher in the blood of healthy donors than the patients which showed relatively higher contributions of Ca, Co, Fe, Mg, Mn, and Pb in their blood samples. Nonetheless, average levels of Cd, K, and Na were marginally higher in the blood of the patients than the controls but the differences were not statistically significant. The comparative study thus indicated an imbalance of the metal levels in the blood of breast cancer patients in comparison with counterpart healthy subjects.

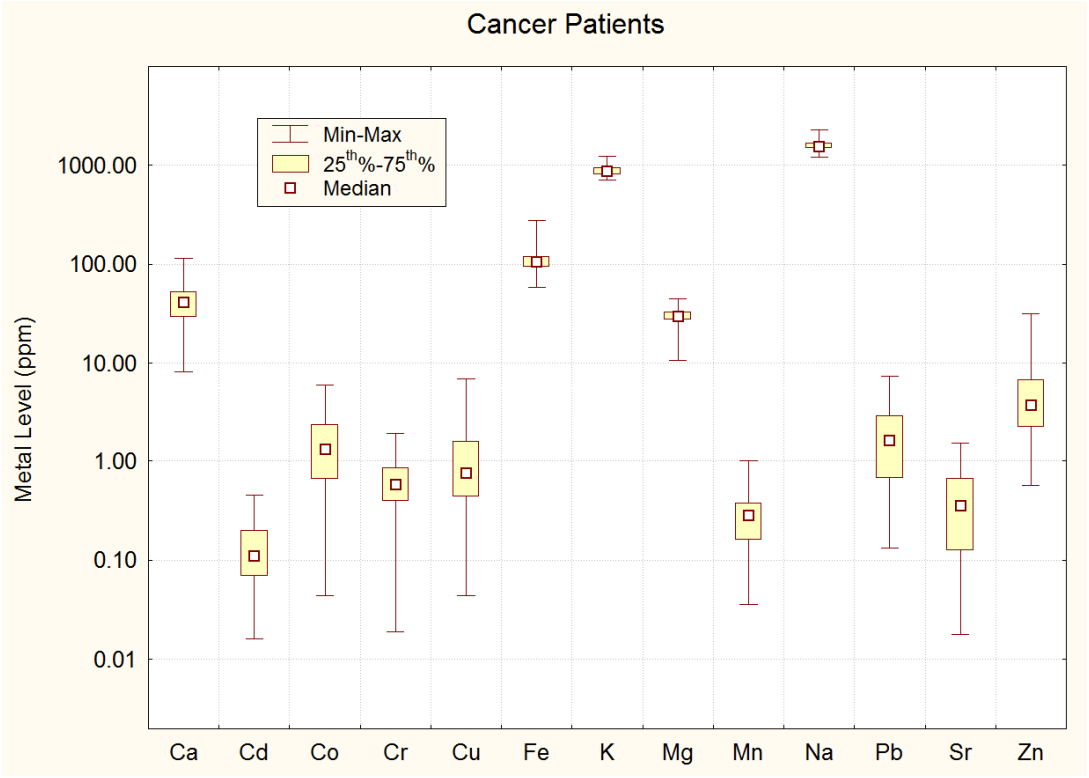

Figure 1. Quartile distribution of selected metal levels (ppm) in the blood of breast cancer patients

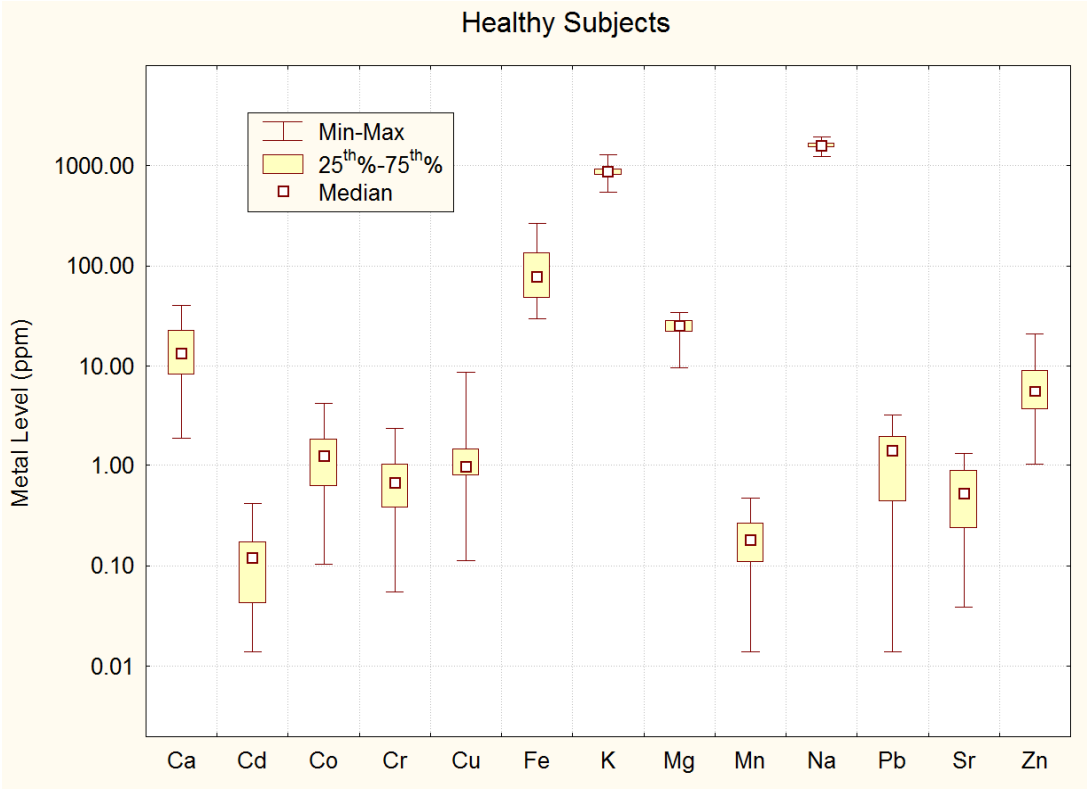

Figure 2. Quartile distribution of selected metal levels (ppm) in the blood of healthy subjects

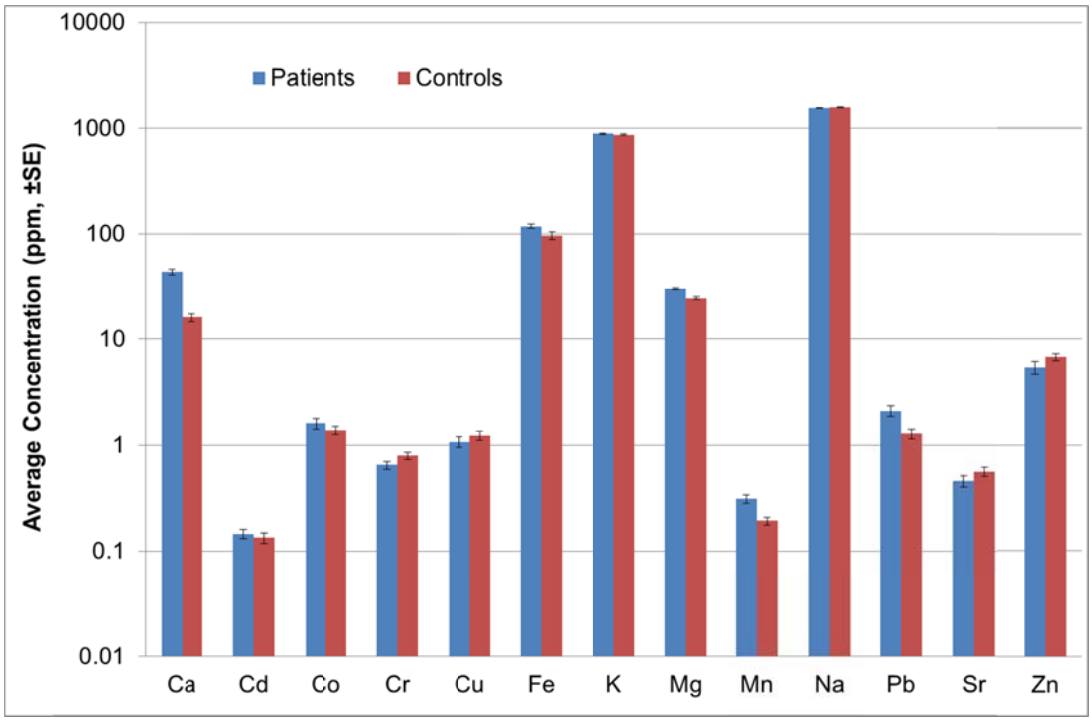

Figure 3. Comparison of the average metal concentrations (ppm) in the blood of breast cancer patients and healthy subjects

3.2 Correlation Study of Selected Metals

One of the important aspects of the present study was to investigate the mutual relationships among the essential and toxic trace metals in the blood of the patients and controls by Spearman correlation coefficients. The data on metal-to-metal correlations in the blood of breast cancer patients are shown in Table 5, wherein the bold *r*-values are significant at $p < 0.05$. Among the selected metals, K-Ca ($r = 0.352$), Na-Cd ($r = 0.276$), Mg-Co ($r = 0.327$), Pb-Co ($r = 0.414$) , Zn-Co ($r = 0.394$), K-Cr ($r = 0.308$), Zn-Cu ($r = 0.325$) Zn-Fe ($r = 0.366$), Mn-K ($r = 0.315$), Mg-K ($r = 0.297$), and Pb-Zn ($r = 0.430$) showed statistically significant positive correlations in the blood of cancer patients. Some of the metal pairs such as Fe-Ca ($r = -0.412$), Na-Ca ($r = -0.281$), Na-Cr ($r = -0.326$), Na-K ($r = -0.333$), and Sr-Mn ($r = -0.331$) exhibited inverse relationships and opposing distributions in the blood of breast cancer patients. Rest of the metal pairs exhibited insignificant and weak positive or negative relationships. The correlation study thus showed mutual association among some essential and toxic trace metals in the blood of the breast cancer patients while the major electrolytes Na-K showed negative relationship.

Table 5. Correlation coefficient (r)* matrix for selected metals in the blood of breast cancer patients

	Ca	Cd	Co	Cr	Cu	Fe	K	Mg	Mn	Na	Pb	Sr	Zn
Ca	1.000												
Cd	-0.074	1.000											
Co	-0.022	0.017	1.000										
Cr	0.098	-0.026	-0.012	1.000									
Cu	0.003	0.168	0.203	-0.172	1.000								
Fe	**-0.412**	0.037	0.247	-0.197	0.104	1.000							
K	**0.352**	0.097	0.240	**0.308**	-0.006	-0.015	1.000						
Mg	0.213	-0.204	**0.327**	0.101	0.082	**0.297**	**0.297**	1.000					
Mn	0.180	-0.040	0.248	0.129	0.219	0.008	**0.315**	0.238	1.000				
Na	**-0.281**	**0.276**	-0.033	**-0.326**	**0.258**	0.082	**-0.333**	-0.171	-0.185	1.000			
Pb	0.037	0.068	**0.414**	-0.083	-0.033	0.231	0.144	0.036	**0.266**	-0.048	1.000		
Sr	-0.102	-0.031	0.231	0.083	0.039	0.269	0.072	0.158	**-0.331**	0.059	0.075	1.000	
Zn	-0.153	-0.006	**0.394**	-0.104	**0.325**	**0.366**	0.077	0.124	0.199	0.093	**0.430**	0.201	1.000

*bold *r*-values are significant at $p < 0.05$

Table 6. Correlation coefficient (r)* matrix for selected metals in the blood of healthy subjects

	Ca	Cd	Co	Cr	Cu	Fe	K	Mg	Mn	Na	Pb	Sr	Zn
Ca	1.000												
Cd	0.064	1.000											
Co	0.010	0.100	1.000										
Cr	-0.155	-0.139	0.043	1.000									
Cu	-0.055	0.108	-0.096	0.071	1.000								
Fe	0.176	-0.012	-0.041	-0.142	0.015	1.000							
K	0.145	**0.263**	0.230	-0.009	0.095	-0.011	1.000						
Mg	0.116	0.063	0.094	0.074	0.143	-0.057	**0.661**	1.000					
Mn	-0.044	0.024	**0.292**	0.002	-0.093	-0.127	-0.039	-0.045	1.000				
Na	0.143	0.107	0.020	0.142	-0.040	-0.042	**0.261**	-0.173	0.037	1.000			
Pb	-0.159	0.167	-0.124	-0.124	-0.068	**-0.287**	-0.084	-0.072	0.109	0.120	1.000		
Sr	**-0.257**	-0.108	-0.069	0.016	**-0.257**	0.124	**-0.318**	-0.163	-0.108	-0.019	**-0.261**	1.000	
Zn	-0.063	-0.173	0.040	0.030	0.133	**0.313**	**-0.036**	-0.037	0.056	0.002	**0.264**	0.041	1.000

*bold *r*-values are significant at $p < 0.05$

The mutual associations therefore demonstrated the disproportion of the essential and toxic metals in the case of breast cancer patients in which the toxic metals are interfering with the essential metals.

The correlation coefficient matrix for selected essential and toxic trace metals pertaining to the blood of healthy subjects is shown in Table 6, wherein the significant *r*-values are shown in bold at $p < 0.05$. A strong positive correlation was observed between Mg-K ($r = 0.661$) while some significant positive correlations were observed between K-Cd ($r = 0.263$), Mn-Co ($r = 0.292$), Zn-Fe ($r = 0.313$), Na-K ($r = 0.261$), and Zn-Pb ($r = 0.264$). Nevertheless some of the metal pairs including Sr-Ca ($r = -0.257$), Sr-Cu ($r = -0.257$), Pb-Fe ($r = -0.287$), Sr-K ($r = -0.318$), and Sr-Pb ($r = -0.261$) exhibited inverse relationships and opposing distributions in the blood of healthy donors. In comparison to the patients, correlation study demonstrated mutual associations among most of the essential metals (with few exceptions) while mostly trace metals showed separate grouping in the blood of healthy donors. Consequently the correlation study pointed out noticeably diverse associations among the metals in the cancerous patients and healthy donors which may be associated with the initiation and progression of the disease in breast cancer patients. Communal variations of the elemental levels in both donor groups would be further explored by multivariate statistical methods in forthcoming section.

3.3 Comparative Evaluation of Selected Metals in the Blood based on Demographic Characteristics of the Subjects

Average concentrations of selected essential and toxic trace metals in the blood of the patients and controls were compared to explore any viable difference based on their demographic characteristics. Habitat-based comparison in the average concentrations of selected metals in the blood of breast cancer patients and controls is shown in the Figure 4. The comparative evaluation revealed elevated average concentrations of Cd, Co, Cu, and Sr in the blood of urban patients than the rural counterparts which exhibited elevated level of Fe in their blood. Nevertheless, average levels of Na, K, Cr, Mg, and Pb were found to be comparable in both rural and urban patients. In the case of controls, mean contents of Fe, and Pb were found to be higher in the blood of rural subjects than urban donors, whereas mean levels of K, Mg, Mn, and Na were nearly comparable in the blood of both rural and urban healthy donors. Average levels of Ca, Co, Cr, Cu, Sr, and Zn were found to be somewhat higher in the urban donors than the rural donors (Figure 4).

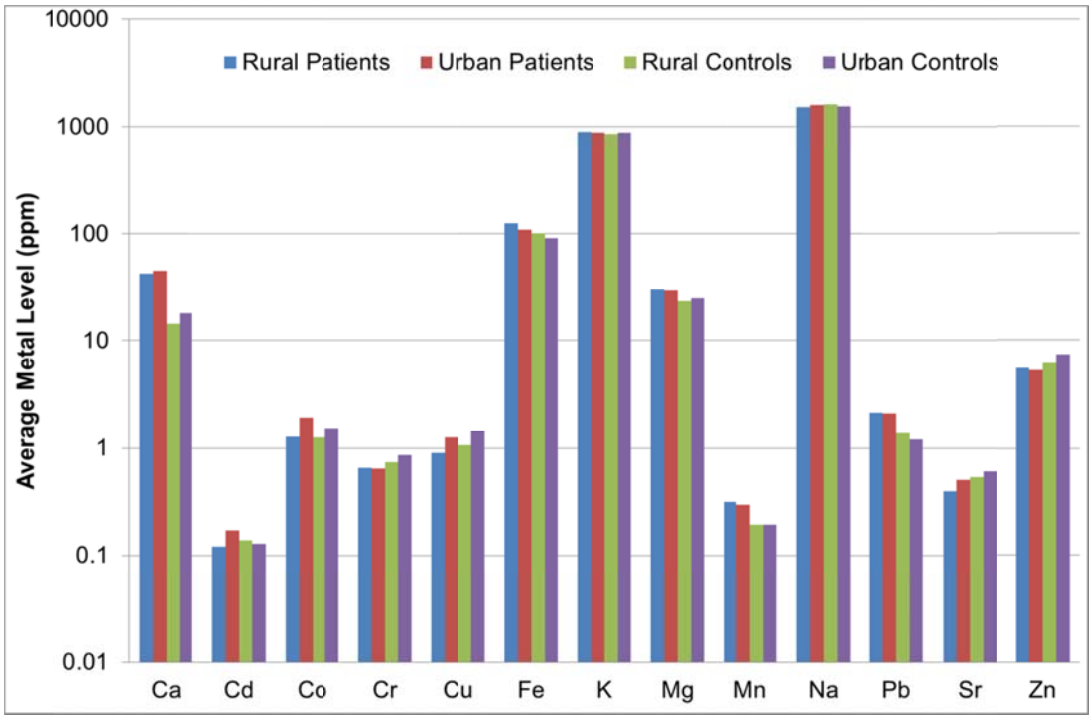

Figure 4. Comparison of the average concentrations of selected metals (ppm) in the blood of breast cancer patients and controls based on their habitat

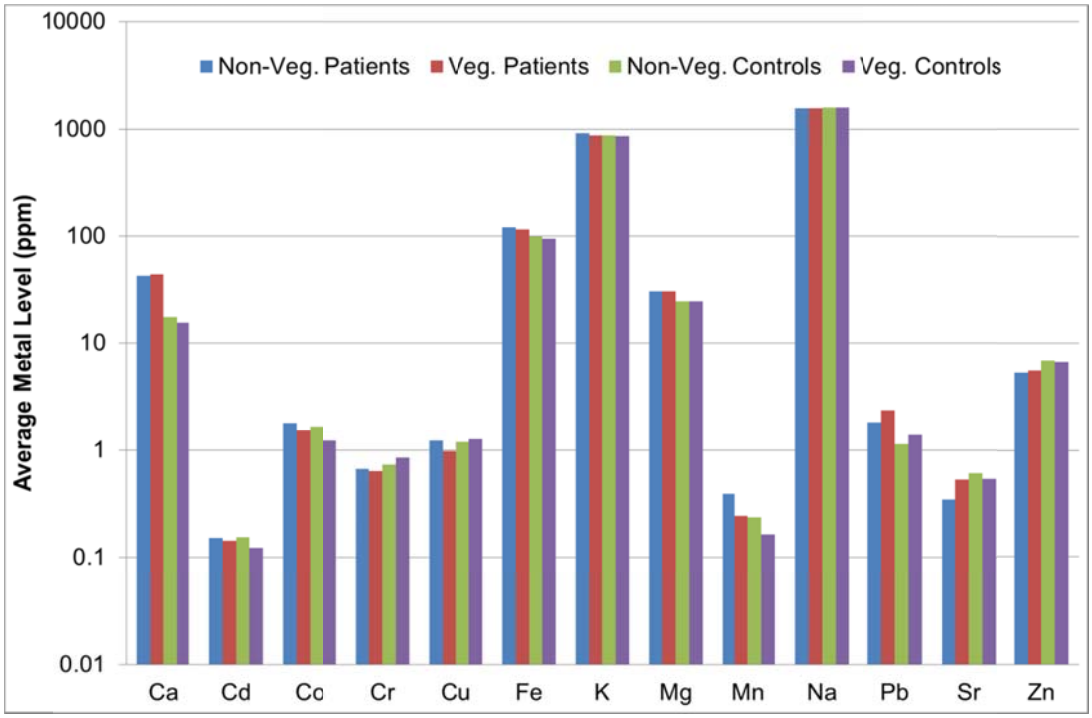

Figure 5. Comparison of the average concentrations of selected metals (ppm) in the blood of breast cancer patients and controls based on their food habits

Average concentration of selected metals in the blood of the patients and controls based on their food-habits are shown in Figure 5. Comparative assessment of the metals showed approximately equivalent levels of Ca, Cr, K, Mg, and Na in the blood of vegetarian and non-vegetarian patients. However, mean contents of Pb, Sr, and Zn showed relatively higher contribution in the blood of vegetarian patients, while Co, Cu, Fe, and Mn were found to be comparatively higher in the blood of non-vegetarian patients than the vegetarian patients. On the other hand, in the case of controls relatively higher concentration of Ca, Co, Cd, Mn, and Sr were observed in the blood of non-vegetarian subjects compared with the vegetarian donors. However, mean levels of Cr, and Pb were relatively higher in the blood of vegetarian controls than the non-vegetarian donors. Average concentrations of Cu, Fe, K, Mg, Na and Zn were found to be almost comparable in the blood of both control groups.

Tobacco addiction-based comparison of the selected metals in the blood of the patients and controls is depicted in the Figure 6, which showed that relatively higher concentrations of Co, Cu, and Mn were found in the blood of tobacco user patients than the non-user patients. Average contents of Fe, K, Mg, Na, and Zn were almost comparable in both tobacco user and non-user patients, while average levels of Ca, Cd, Cr, Pb and Sr were found to be relatively higher in the blood of non-tobacco user patients. In the case of controls, mean levels of Fe, Sr and Cd showed relatively higher contribution in the blood tobacco user subjects while mean level of K, Mg, and Na showed comparable levels in both donor groups. Besides, average concentrations of Ca, Co, Cr, Cu, Mn, Pb and Zn were found to be considerably higher in the blood of non-tobacco user controls than the tobacco user controls.

Employment-based comparison of the selected essential and toxic metals in the blood of the patients and controls is depicted in the Figure 7, which showed that relatively high concentrations of Ca, Cr, and Cu were found in the blood of non-working patients compared with the working patients. Average contents of K, Mg, Mn, Na and Pb were almost comparable in both working and non-working patients, while average concentrations of Cd, Co, Fe, Sr and Zn were found to be relatively higher in the blood of working patients than non-working counterparts. In the case of controls, mean levels of Ca, Fe, Mn, Sr and Zn showed relatively higher contribution in the blood of working subjects while mean level of Co, K, Mg and Na showed more or less comparable levels in both donor groups. In addition, average concentrations of Cd, Cu, Cr and Pb were found to be comparatively higher in the blood of non-working controls than the working controls.

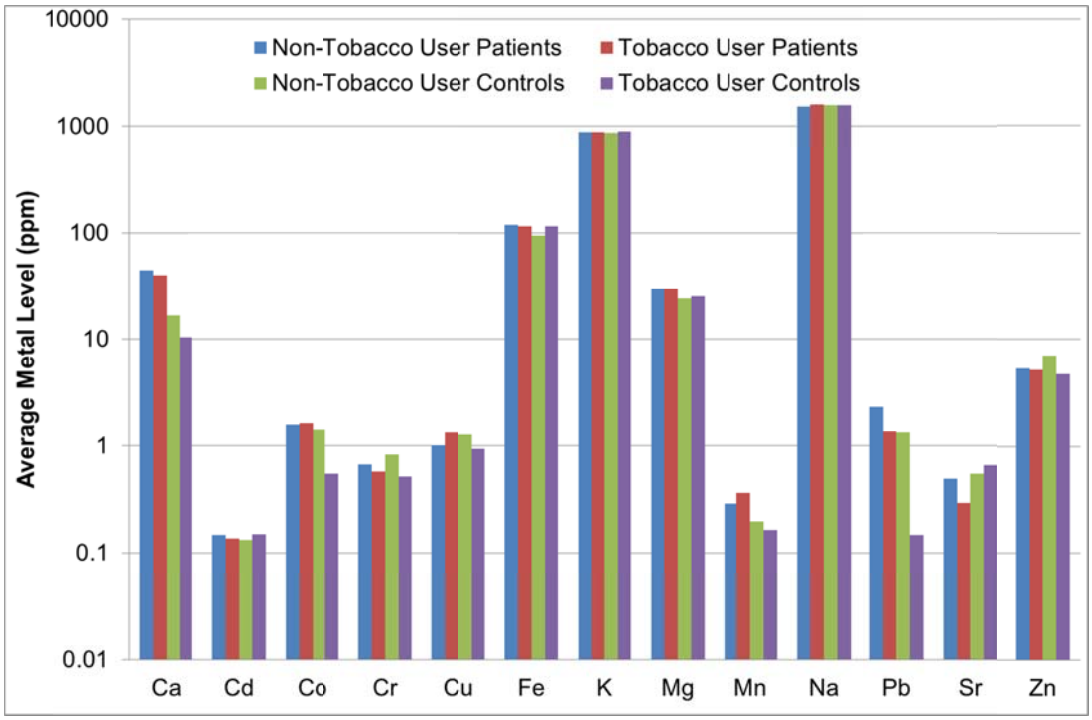

Figure 6. Comparison of the average concentrations of selected metals (ppm) in the blood of breast cancer patients and controls based on tobacco addiction

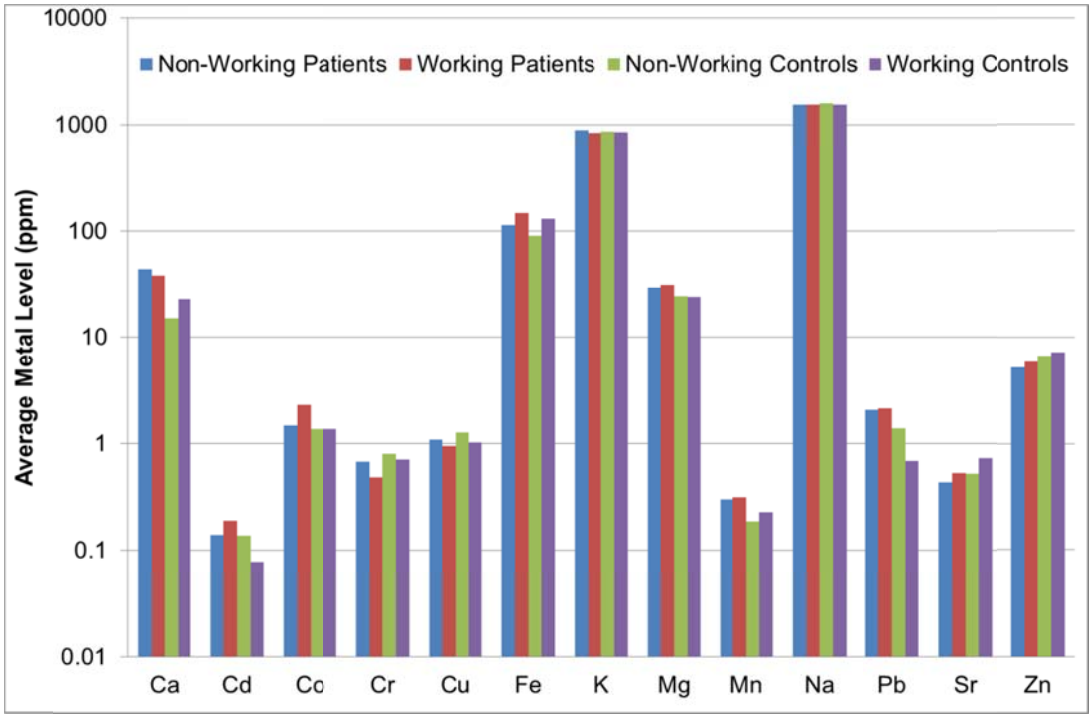

Figure 7. Comparison of the average concentrations of selected metals (ppm) in the blood of breast cancer patients and controls based on employment status

Mean concentrations of selected metals in the blood of the patients and controls were also compared to explore the age-based variations and for this purpose the donors were classified into three age groups; ≤ 40 years, 41-50 years and ≥ 51 years. Age-based comparison among the patients is shown in Figure 8, which revealed relatively higher concentrations of Cr, Cu and Sr in the patients of ≤ 40 years while 41-50 years age group showed higher contents of Co, Mn, Pb and Zn in their blood. Nonetheless, relatively higher level of Cd was found in the blood of the patients of ≥ 51 years age. Average levels of Ca, Fe, K, Mg and Na were almost comparable in three age groups of the patients. Similarly, age-based comparison for the controls is shown in Figure 9. Comparatively higher contribution of Fe was observed in the blood of ≤ 40 years of controls while those of 41-50 years showed higher concentrations of Co, Cu and Pb. However, relatively higher levels of Cd, Mn and Zn were shown by elderly controls (≥ 51 years). Average contents of Ca, K, Mg and Na were almost comparable in the blood of three age groups of controls. Overall, the demographic characteristics-based comparison manifested some significant and diverse variations of the essential and toxic trace metals in the blood of the patients and controls which may be ascribed to the imbalance of the metal levels due to the disease.

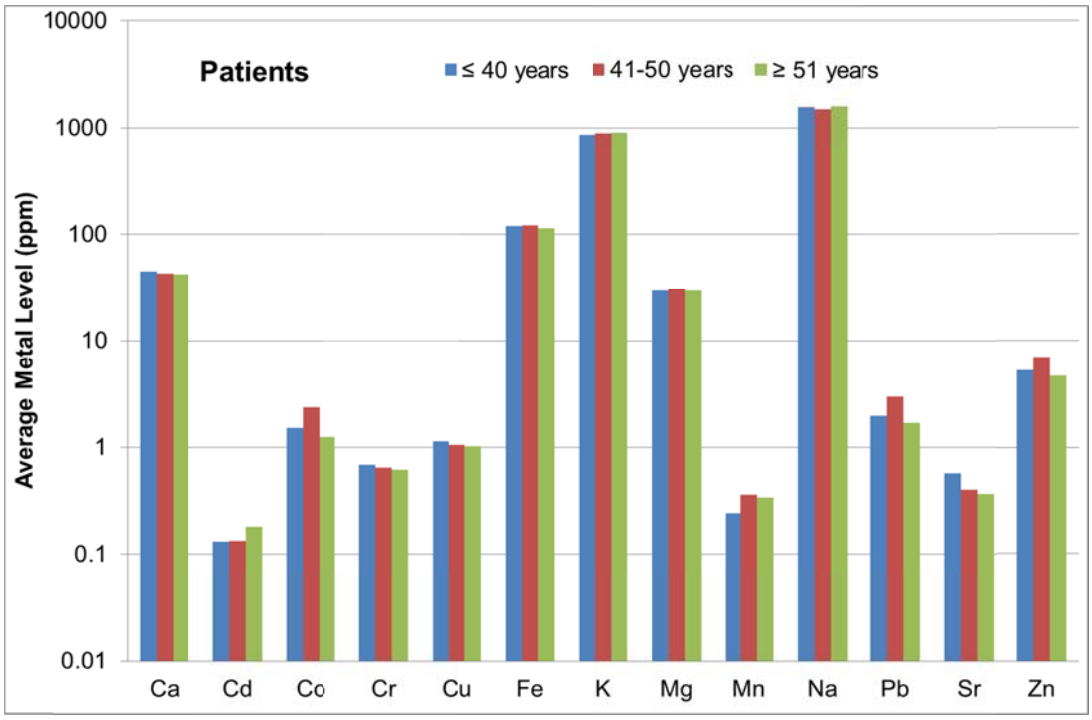

Figure 8. Comparison of the average concentrations of selected metals (ppm) in the blood of various age groups of breast cancer patients

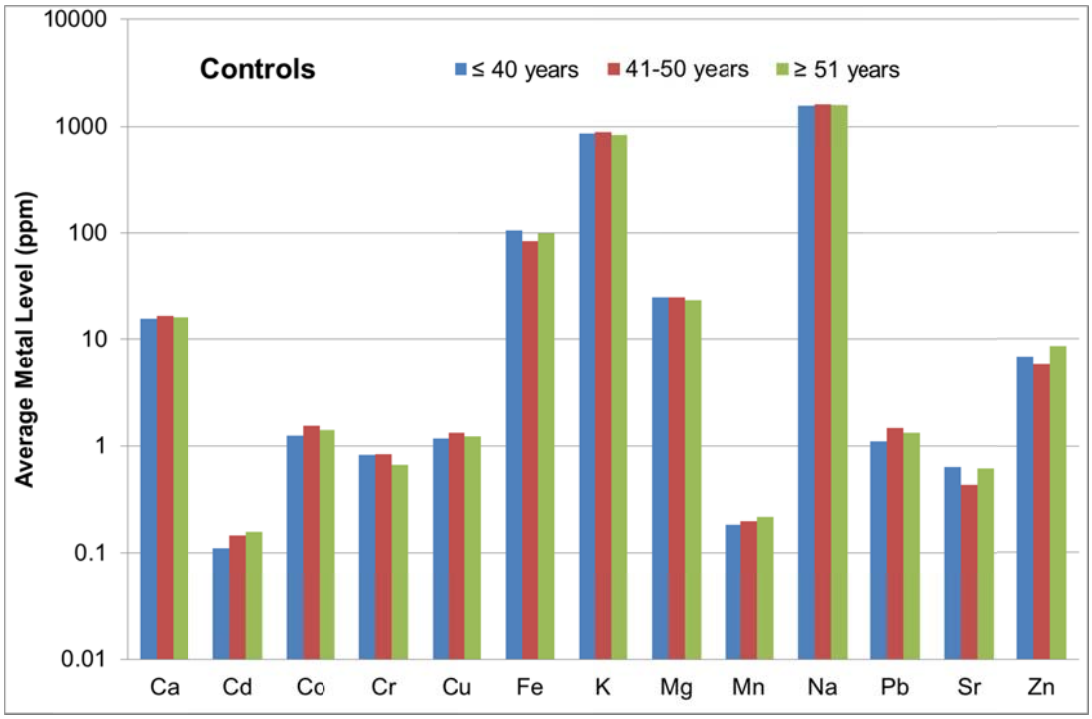

Figure 9. Comparison of the average concentrations of selected metals (ppm) in the blood of various age groups of healthy subjects

3.4 Comparative Evaluation of Selected Metals in the Blood based on Cancer Stages and Diagnosis Time

Stage of the disease can sometime considerably affect the balance of essential nutrients and trace elements; the effect is generally more pronounced with time and duration. In the present study, selected metal levels measured in the blood of the cancer patients were also compared based on the stages of breast cancer patients. The comparison of average concentrations of the metals in the blood at different cancer stages is shown in Figure 10. Comparative evaluation indicated that the mean levels of Fe, Mg, Mn, Pb, Sr, and Zn were found to be significantly elevated at stage-I, while moderately elevated level of Cr was found at stage-II of breast cancer. Average levels of Co, and Cu were found to be significantly elevated at stage-III. Likewise, significant increase in the concentration of Cd was observed in the blood of the patients at stage-IV whereas mean levels of K and Na were approximately comparable in the blood at all four stages. Overall, the comparative study revealed some significant variations in the metal levels at various stages of breast cancer (Figure 10).

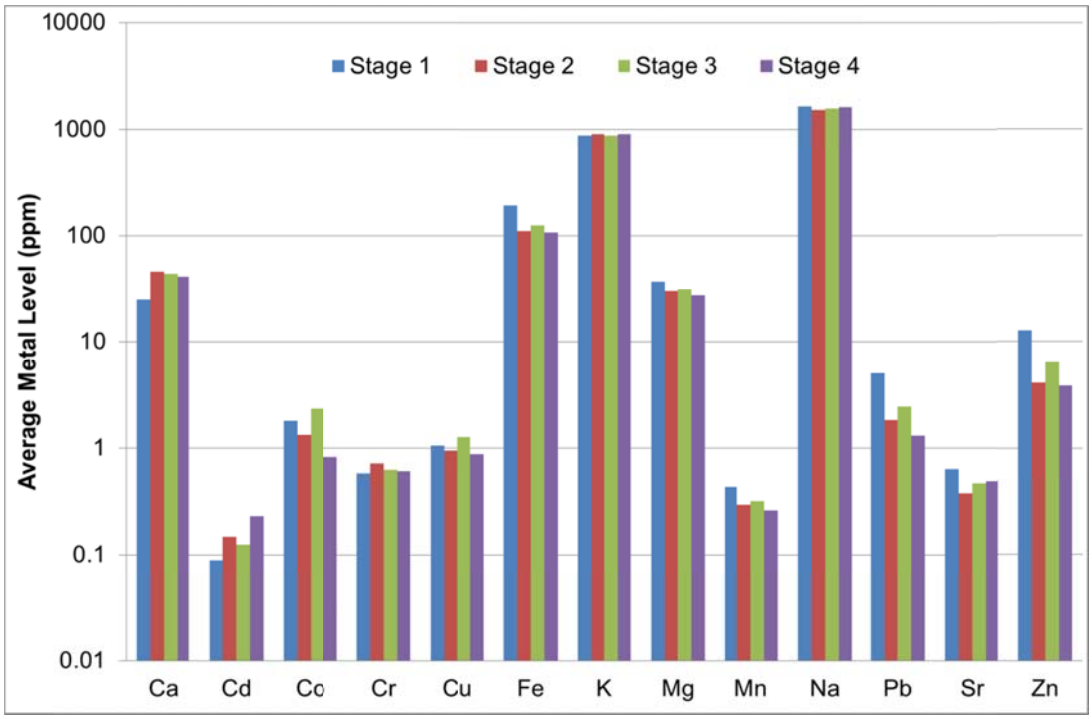

Figure 10. Comparison of the average concentrations of selected metals (ppm) in the blood of breast cancer patients at various stages

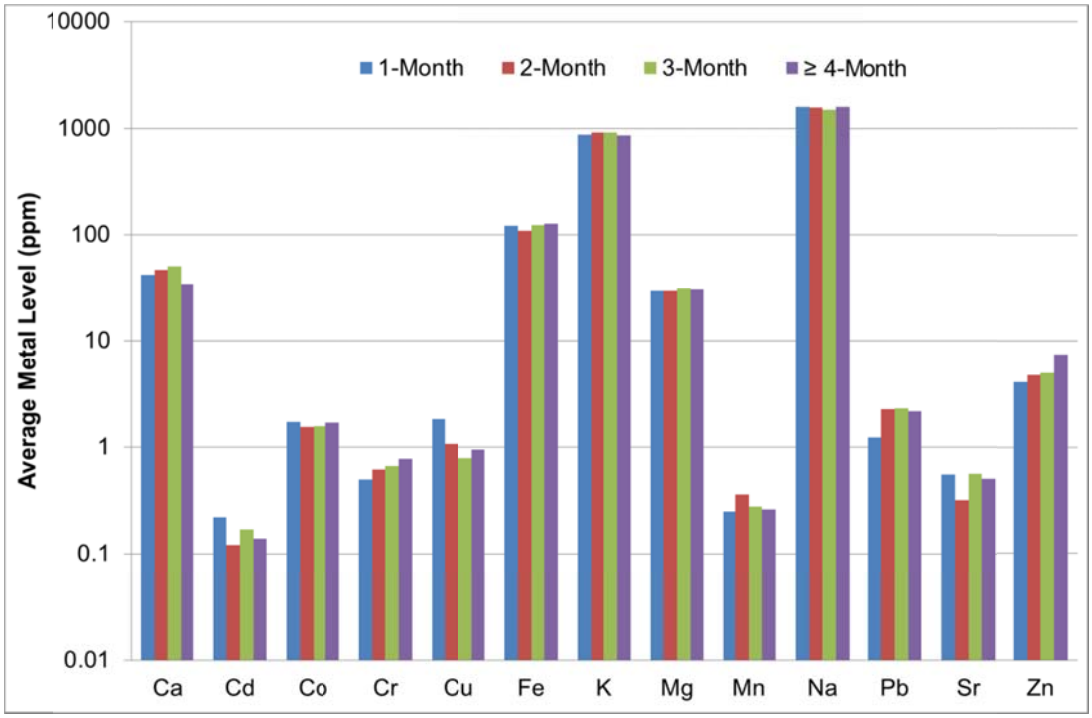

Figure 11. Comparison of the average concentrations of selected metals (ppm) in the blood of breast cancer patients based on diagnosed time

Selected metal levels measured in the blood of the cancer patients were also compared based on diagnosed time. The comparison of average metal levels at different diagnosed time is shown in Figure 11. Comparative assessment indicated that the average levels of Cd, and Cu were found to be significantly elevated in the patients diagnosed within 1-month while moderately elevated level of Mn was found in the patients diagnosed within 2-month. Similarly, mean levels of Ca and Sr were found significantly elevated in blood of the patients diagnosed within 3-months. Significant increase in the concentrations of Zn and Cr were observed in the blood of patients diagnosed ≥ 4-month whereas mean levels of Co, K, Mg, and Na were almost comparable in all four groups based on diagnosed time. Overall, the comparative study revealed significantly diverse variations in the blood metal levels at different diagnosed time (Figure 11).

3.5 Multivariate Analyses of Selected Metals

Another fascinating aspect of the present study was the multivariate apportionment of the metal levels in the blood of breast cancer patients and controls using principal component analysis (PCA) and cluster analysis (CA). The PC loadings extracted by varimax-normalized rotation on the metals data for the patients and healthy donors as shown in Tables 7 and 8, respectively. In case of the patients, PCA yielded five significant PCs with eigen value greater than 1 and commutatively explaining approximately 82% of the total variance of data (Table 7). The CA of metals data pertaining to the cancer patients is shown in Figure 12. PC 1 showed elevated loadings for Ca, Cr, and Fe with a similar cluster of the metals in CA. PC 2 showed maximum loadings for Mn, Sr, and Mg with a parallel cluster of the metals in CA. These two PCs showed the interference of toxic trace metals with the essential metals in the patients and they were believed to be mainly contributed by dietary sources. PC 3 indicated higher loadings for Cu, K, and Sr along with a similar cluster in CA. PC 4 showed higher loadings of Cd and Na while PC 5 indicated higher loadings of Co, Zn, and Pb with a similar cluster in CA. These metals were mostly derived from the nutritional habits of the subjects and environmental contaminants by various anthropogenic activities. The PCA and CA results were in very good agreement with each other and both multivariate methods showed the interferences in the role of essential metals by the toxic trace metals in the breast cancer patients.

In the case of controls, PCA of the metals data yielded five major PCs with eigen value > 1 and commutatively explaining approximately 100% of the total variance of data

(Table 8). The CA of the metals data pertaining to the blood of controls is shown as dendrogram in Figure 13. PC 1 showed higher loadings for Ca, K, Mg, Sr and Cr whereas PC 2 showed maximum loadings for Cu, Fe, and Zn with a similar cluster of the metals in CA. These metals were mostly associated with the food habits and therefore derived form the dietary sources of the subjects. PC 3 showed elevated loadings for Cd and Co while PC 4 showed elevated loadings for Mn and Pb. The CA also revealed strong clusters of these metals in the blood of controls. These metals were mostly regulated by internal body metabolism and affected by the anthropogenic exposure of the subjects. PC 5 showed higher loadings for Na only which indicated its independent behaviour in the blood of healthy donors. It may be associated with the excessive use of table salt in routine diet of healthy subjects.

Table 7. Principal component analysis of selected metal levels in the blood of breast cancer patients

	PC 1	PC 2	PC 3	PC 4	PC 5
Eigen value	3.672	2.781	1.850	1.291	1.123
Total Variance (%)	28.25	21.40	14.23	9.933	8.636
Cumulative Eigen value	3.672	6.453	8.303	9.594	10.72
Cumulative Variance (%)	28.25	49.64	63.87	73.80	82.44
Ca	0.887	-0.020	-0.263	-0.025	0.121
Cd	0.095	0.135	-0.060	0.879	0.231
Co	0.306	-0.004	-0.417	-0.053	0.662
Cr	0.770	-0.438	0.001	-0.300	0.064
Cu	-0.145	-0.017	0.866	0.170	-0.223
Fe	0.779	0.333	0.175	0.090	0.190
K	0.061	-0.082	0.742	-0.325	-0.197
Mg	0.157	0.725	-0.098	0.311	0.112
Mn	-0.163	0.897	-0.030	-0.128	-0.166
Na	-0.497	0.006	-0.007	0.756	-0.223
Pb	0.113	-0.145	-0.083	-0.614	0.689
Sr	0.165	0.506	0.438	0.320	0.469
Zn	0.090	0.020	-0.233	0.178	0.924

Table 8. Principal component analysis of selected metal levels in the blood of healthy subjects

	PC 1	PC 2	PC 3	PC 4	PC 5
Eigen value	4.952	2.959	1.999	1.965	1.126
Total Variance (%)	38.09	22.76	15.37	15.12	8.660
Cumulative Eigen value	4.952	7.910	9.909	11.87	13.00
Cumulative Variance (%)	38.09	60.85	76.22	91.34	100.0
Ca	0.809	0.265	0.113	0.513	0.009
Cd	0.042	-0.113	0.848	-0.395	-0.332
Co	0.090	0.264	0.877	0.255	0.295
Cr	0.641	-0.615	0.091	-0.061	-0.446
Cu	0.299	0.890	0.024	0.211	0.272
Fe	0.425	0.730	-0.296	0.399	-0.200
K	0.823	0.404	0.248	0.173	0.260
Mg	0.976	-0.030	-0.004	0.184	0.111
Mn	0.313	-0.229	-0.216	0.895	0.046
Na	0.137	0.238	0.029	-0.189	0.942
Pb	0.039	0.275	0.193	0.899	-0.277
Sr	0.746	0.303	-0.505	-0.298	-0.087
Zn	0.026	0.907	0.247	-0.283	0.189

It is important to note that in the case of controls, the toxic metals were not primarily associated with the essential metals as was the case in the cancer patients; consequently it indicated a disproportion and imbalance among the metals in breast cancer patients. Overall, PCA and CA showed significantly diverse apportionment of the essential and toxic metals in the blood of the cancer patients and healthy subjects which may be attributed to the imbalances of trace metals in the cancer patients. Consequently, the multivariate methods can be employed for diagnostic and prognostic purpose in clinical studies but they are required further validation by considering more variables on larger population groups from different geographical areas around the world.

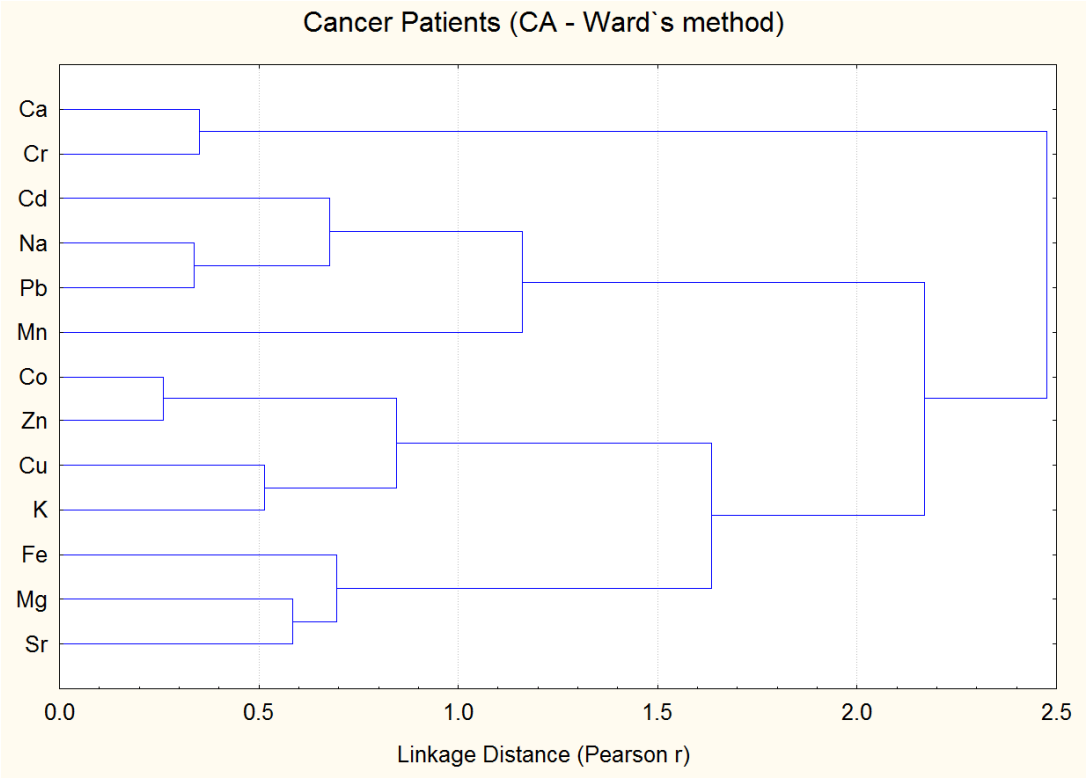

Figure 12. Cluster analysis of selected metal levels in the blood of breast cancer patients

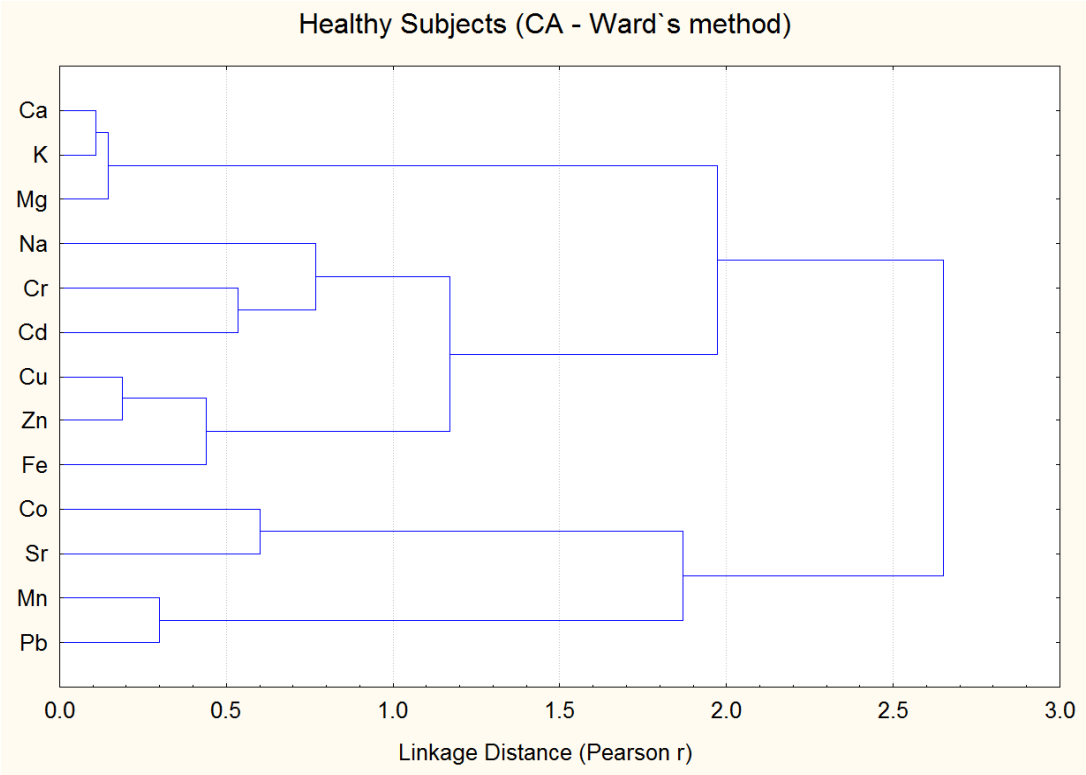

Figure 11. Cluster analysis of selected metal levels in the blood of healthy subjects

3.7 Salient Findings of the Present Study

Based on the deliberations stated in foregoing sections, following salient findings emerged from the present study:

- ❖ Most of the metals showed random distribution in the blood of both donor groups, however the dispersion and asymmetry was higher in the patient group.
- ❖ Average levels of Ca, Cd, Co, Fe, Mg, Mn and Pb were found to be considerably higher in the blood of the patients than controls.
- ❖ Comparative variations in the quartile distribution were considerably divergent in the patients compared with the controls.
- ❖ Correlation study showed significantly diverse associations among the metals in the blood of the patients and controls.
- ❖ Significant variations in the metal levels were observed with the habitat, food habits and smoking habits of both donor groups.
- ❖ Metal levels exhibited considerable differences with the age of the donors and stages of breast cancer.
- ❖ PCA and CA revealed considerably divergent apportionment of the metals in the patients and controls; it may be considered as a diagnostic tool in the clinical studies.
- ❖ Metals imbalance plays a critical role in the breast cancer patients; therefore regular monitoring should be ensured.

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