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ESTABLISHMENT OF REFERENCE RANGES FOR BIOCHEMICAL PARAMETERS IN CHILDREN AND ADOLESCENTS OF AGES 1-17 YEARS IN MERU COUNTY, KENYA

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Establishment of reference ranges for

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DECLARATION

I, Munene Rhoda Kainyu, duly declare that this thesis is my original work and has not been presented for a degree in any other university or any other award.

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DEDICATION

To my children Kanana and Mutuma.

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ABBREVIATIONS AND ACRONYMS

ALB	Albumin
ALP	Alkaline Phosphatase
ALT	Alanine Aminotransferase
AST	Aspartate Aminotransferase
D-BIL	Direct Bilirubin
CLSI	Clinical Laboratory Standard Institute
CV	Coefficient of Variation
GGT	Gamma-Glutamyl Transferase
HBsAg	Hepatitis B surface Antigen
IFCC	International Federation of Clinical Chemistry
ISE	Ion Selective Electrode
LFT	Liver Function Test
MCH	Mother and Child Health
ML5H	Meru level five hospital
NCCLS	National Committee for Clinical Laboratory Standards
QC	Quality Control
RPM	Revolutions per Minute
SD	Standard Deviation
SOP	Standard Operating Procedure
T-BIL	Total Bilirubin
VDRL	Venereal Disease Research Laboratories

ABSTRACT

Clinical Biochemistry (Clinical Chemistry/Chemical Pathology) is the study of the biochemical basis of disease, and the application of biochemical and molecular techniques in diagnosis. An understanding of the biochemical mechanisms of disease provides modern medicine with a rational basis for diagnosis and therapy. A reference range is a set of values used by a health professional to interpret a set of medical test results. The range is usually the set of values in which 95% of the normal population falls. Clinical chemistry reference ranges vary due to factors such as age, sex, diet, race, climate, altitude and genetics. As a result, International Federation of Clinical Chemistry (IFCC) recommends that every laboratory establishes its own reference intervals for biochemical parameters and not rely on those obtained from a different population. There is little information in the literature on biochemical reference values for children in Kenya and in particular those from Meru County. This study was aimed at determining age and sex-based reference ranges for thirteen routinely analyzed biochemical parameters for liver and renal function for the children population in Meru County. This was a population based cross-sectional study carried out at the Meru Level Five Hospital. 768 healthy males and females were recruited in this study and only 740 whose serum samples tested negative for HIV, hepatitis B, syphilis were used in the final analysis; 380 males and 360 females. 28 samples were excluded, out of which 6 were HIV positive and 22 were hemolyzed. DRI-CHEM NX 500I Clinical Chemistry analyzer (Fujifilm, Europe) was used to analyze thirteen clinical chemistry parameters including serum proteins, bilirubin, tissue enzymes and electrolytes. Clinical and Laboratory Standards Institute (CLSI) guidelines were followed to create study consensus intervals. Determination of reference ranges was done in order to estimate the lower 2.5 and upper 97.5 percentiles of distribution by use of parametric methods. The determined percentiles were considered as the lower and upper reference limits respectively. There were significant differences in relation to sex in children reference values for potassium ($p = 0.009$), total protein ($p = 0.039$) and sodium ($p = 0.003$). Other parameters did not show significant differences across the age groups and by gender. In conclusion, the findings of this study provide sex and age specific reference range values for children from Meru County in Kenya. From the study findings, recommendation is made to health care practitioners and facilities in Meru County to adopt the new reference values developed, particularly for the three parameters that exhibited significant differences in sex and for other regions in Kenya to carry out a similar study to determine their own reference values.

CHAPTER ONE

INTRODUCTION

1.1 Background Information

Reference value refers to the value or test result obtained through observation or measurement of a particular type of analyte on an adequate number of individuals selected to represent the general population. Clinicians order laboratory tests for a variety of reasons: - screening for disease, diagnosis of disease, monitoring levels of drugs and other endogenous substances like electrolytes, determining prognosis, confirming a previous abnormal test, clinician education and medical legal purposes. When a test is used for disease screening, diagnosis or prognosis, the test result is normally compared with a normal range that is defined as usual value for a healthy population (Harris *et al.*, 1980).

Clinical medicine practice requires that test results from a patient are compared against some pre-determined standard results so as to determine whether the patient is "normal" or is suffering from a certain pathological condition. Laboratories should therefore report test results along with the corresponding reference intervals since physicians and other health practitioners make their medical decisions based on available, appropriate and reliable reference intervals. Medical decision is also backed by information gathered during medical interview as well as clinical examination. In the laboratory, the word "normal" has several meanings other than being used to describe the usual range of laboratory data for

healthy populations. It is used to describe the health of individuals and is also synonymously used with the "Gaussian" when the shapes of distributions are described (Figure 1).

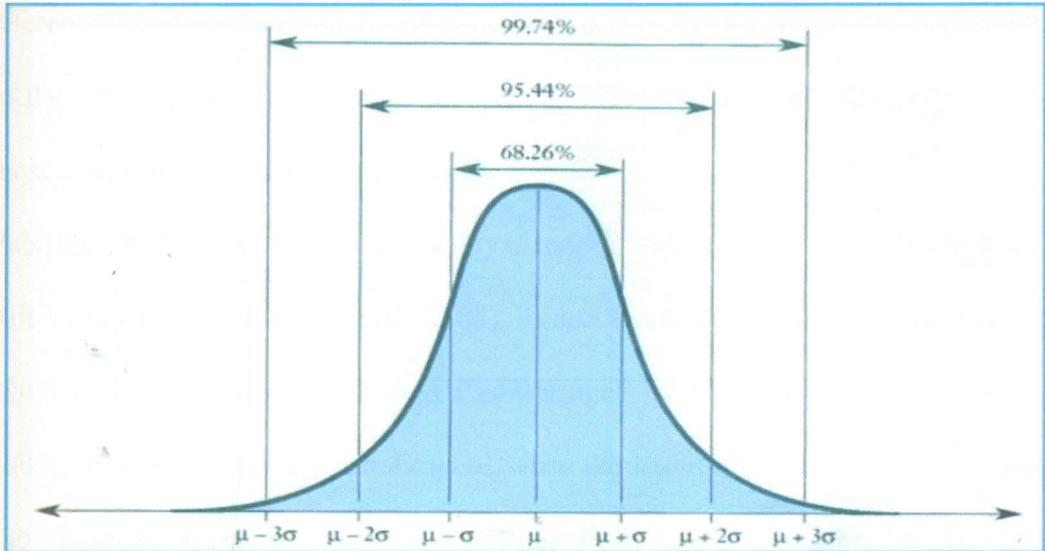

Figure 1: Gaussian distribution of Values
Source: Hyperphysics.*phy-astra.gsu.edu*

Factors specified when reference values are established include: (1) Make up of reference population in terms of age, gender and genetic and socio-economic factors. (2) The inclusion and exclusion criteria used. (3) The conditions, both physical and physiological under which the reference population is sampled and studied. (4) The procedure of collecting the specimen, including how the subject was prepared before collection and (5) the method of analyzing the sample used giving details of its precision and accuracy (Grasbeck *et al.*, 1979).

Measured laboratory parameters are affected not only by individuals' factors such as age, sex, diet but also by population and ecological factors such as ethnic background, climate, geography and altitude. As such, they are found to vary not only between individuals but also between populations (NCCLS, 2000). A few reference value studies have been carried out in East Africa in the recent past due to the ongoing AIDS vaccine trials and their results revealed differences with those being used at the health facilities.

Published reference values for a Kenyan population from Kericho in the south Rift Valley region (Kibaya *et al.*, 2008), in north Rift Valley, Kenya (Alice *et al.*, 2009) and the whole of Kenya for adults aged 18-55 years (Waithaka *et al.*, 2009), showed significant variations between the locally derived reference values and those provided in literature. Another study carried out on the children population of Iganga District of Uganda showed differences on the biochemical parameters between the Ugandan children and the Caucasian children found in literature (Kironde *et al.*, 2013).

These studies have shown that even within the African continent, substantial differences in reference values exist due to vast differences in human genetics, climate, diet and variations in laboratory techniques. All the studies recommended establishment of site-specific reference values at each health facility. IFCC also recommends that every laboratory establishes its own reference ranges for the biochemical parameters they analyze. It is not recommended to use reference

ranges reported in literature to interpret a clinical laboratory report of a patient in a different geographical region from where the reference ranges were established (NCCLS, 2000), because this may exclude healthy participants thus making the process of clinical trials more time-consuming, expensive and rendering the results less generalizable. Presently most clinical laboratories in Meru County as well as the whole republic of Kenya are either using the reference values as indicated on reagent kits or those published in medical or laboratory textbooks to interpret patient results, which is a big mistake bearing in mind that parameters vary from one region to another.

Published reference ranges in literature do not sometimes adequately represent the specific population from which the patient comes from based on age, sex, genetics, diet, and altitude (Waithaka *et al.*, 2009). The patient's results can also vary due to the methods used in analysis that is either manual or automation. Thus, it is important that clinical chemistry laboratories determine reference values that are specific to the populations they serve. For accurate and precise results, there is need to establish reference values following the standard operating procedures (SOP) (Ichihara *et al.*, 2008).

This study is designed to establish health associated reference ranges to be used by clinical chemistry laboratories for children and adolescents in Meru County and determine whether there are significant variations between the reference

values for the selected biochemical parameters routinely analyzed as provided by the reagent manufacturers and that of the population.

1.2 Statement of the Problem

In Meru Level Five Hospital, clinicians use reference ranges published in medical textbooks or those found on the manufacturers' reagents to interpret medical tests for their patients. These reference values are based on American, European or Asian populations where these reagents are manufactured. Since reference ranges vary depending on many factors including age, sex, climate, altitude and geographical location, there is need for laboratories in Kenya to establish reference values that are specific for the populations that they serve.

1.3 Justification of study

In Meru Level Five hospital, clinicians use either reference ranges from literature or those stated in laboratory diagnostic kits. These reference intervals for clinical laboratory parameters are obtained from European and North American populations. However, many variations have been reported between these values when compared with values obtained from healthy African populations due to the fact that age, sex, diet and geographical location affect reference ranges. Hence there is need to establish population based reference ranges for biochemical analytes determined in the clinical chemistry laboratory of Meru Level Five Hospital from healthy blood donors. Normal ranges of serum components for

adults differ from those of children yet information sources of serum biochemistry normal ranges for African children are scarce or missing altogether. This study attempts to solve this problem by determining normal reference range values of blood serum biochemistry for healthy Kenyan children and adolescents aged 1 to 17 years.

1.4 Research Questions

i) Are there biochemical reference range values for the Kenyan 1-17 years population of Meru County?

ii) Are there sex related significant differences in biochemical reference range values in the 1-17 years population in Meru County?

iii)Are there age related significant differences in biochemical reference range values in the under 17 years in Meru county?

iv) Are the thirteen biochemical parameters reference range values developed for the study population different from those found in literature?

1.5 Research Hypotheses

i) No significant sex and age related differences exist in biochemical reference values for children of 1-17 years of Meru County, Kenya and those found in literature.

ii) There is no significant variation between the reference ranges of the biochemical parameters of the Kenyan children aged between 1-17 years population and that of the Caucasian population quoted in literature.

1.6 Objectives of the study

1.6.1 General objective

To determine the reference values for some routinely analyzed biochemical parameters for children and adolescents of 1-17 years in Meru County, Kenya.

1.6.2 Specific objectives

i. To determine age specific reference values for thirteen routinely analyzed biochemical parameters in males and females of between 1-17 years of Meru County, Kenya.

ii. To determine sex specific reference values for thirteen routinely analyzed biochemical parameters in males and females of between 1-17 years of Meru County, Kenya.

iii. To determine whether the reference range values obtained for the parameters are different from those in found literature.

1.7 Significance of the study

This study emphasizes the need to use site specific reference values in interpreting biochemical results in clinical practice and clinical trials and determining eligibility and reporting adverse events during clinical trials. The use of inappropriate reference values obtained from Caucasian populations would result in the exclusion of otherwise healthy participants leading to unnecessary prolongation of clinical trials and inappropriate treatment of patients in routine healthcare practice. Upon establishing the reference values of commonly requested biochemical parameters for both genders at ages 1 - 5 years, 6 - 10 years, 11 - 15 years and over 15 years in Meru County, Kenya, recommendation will be made to all paramedics in the region to adopt them for accurate and precise patient management.

CHAPTER TWO

LITERATURE REVIEW

2.1 Use of reference ranges

Reference ranges form the basis for physicians and/or other health professionals to interpret medical test results for a particular patient. Reference range values serve as a monitor of good health because people whose values are found to be within range are generally considered to be in good physiological health. Reference ranges are also used in screening for physiological and pathological conditions, therefore valuable in routine health assessment (Clement *et al.*, 2012). Further, reference ranges enable clinicians interpret laboratory data accurately and appropriately thus provide assistance in creating a comprehensive clinical perspective for diagnosis and management of patients. The importance of clinical laboratory test results cannot be underestimated because nearly 80% of physicians' medical decisions are based on information provided by laboratory reports (Chan *et al.*, 2008).

A test result by itself may not be of much value to a clinician if it is not reported together with the appropriate information to enable for its interpretation; usually in the form of a reference interval (RI) or medical decision limit. According to the CLSI guidelines, reference intervals for a population should be established upon selecting a group of at least 120 healthy subjects within the population under study for the results to be considered statistically significant (NCCLS, 2000; Alex

et al., 2010). The guidelines however note that the greatest challenge in any study geared towards establishment of reference ranges is defining what is considered healthy, since health is generally a relative condition with no universal definition. This is because there will often be some degree of uncertainty that goes with a given selection criteria based on the definition of health that was adopted and also the fact that out of the selected subjects, some may actually have some form of subclinical disease (Ceriotti *et al.*, 2007).

Most laboratories also find it difficult in terms of time and cost to recruit a valid group of reference subjects on which reference range studies can be conducted. This challenge is complicated further when determining reference ranges for different age groups (e.g. pediatric patients and geriatric patients), when using uncommon sample types (e.g. cerebrospinal fluid and aspirations) or when doing timed collections, challenge tests and serial measurements. It is as a result of these difficulties that most laboratories choose to verify reference ranges that have been determined by other laboratories. In doing that, a small sample of only twenty healthy subjects is needed and it is assumed that the laboratory analytic system is calibrated and producing similar results as the method that was originally used to produce the published RIs. This may however turn out to be untrue since some details of the reference study such as its design, the inclusion and exclusion criteria used in the selection of participants and pre-analytic sources of variation may not be available (Ceriotti *et al.*, 2007).

A reference range is constructed to include a range of values (after transformation of data if necessary) found in 95% of a reference population of healthy individuals. Reference ranges are constructed either from an individual (subject-based reference ranges) or a group of individuals (population-based reference ranges).

Subject-based reference values are the values that are obtained from the same individual in different but defined states of health whereas population-based reference values refer to the values that are obtained from a group of well-defined reference individuals (Solberg, 1987). Population-based reference ranges are the type of reference values reported in this study.

Laboratory reference values for Caucasian populations are widely available in journals, textbooks and on the internet. This is however not the case for many populations living in Africa. Comparison of results from a study in Southern Tanzania with those available in literature revealed distinct variations in the reference ranges for clinical chemistry values, with average concordance of 80.9% and 86.7% thus concluding that reference values determined in developed countries are inappropriate for use in sub-Saharan Africa and suggested that laboratories in this region should either develop reference ranges that are specific for Africa or verify the values determined under similar conditions (Saathoff *et al.*, 2008).

A population-based reference interval study for common biochemical parameters in adults of 18 to 55 years old from Ghana showed higher values for liver function tests as compared with the reference values that were being locally used in the country. The variations observed could be as a result of differences in diet or environmental differences; and the study stressed the need to determine site specific reference range values that are applicable to a specific population, (Koram *et al.*, 2007). A similar study done in Rwanda revealed higher reference range values for serum proteins and electrolytes as compared to those from western populations (Gahutu and Wane, 2006).

A study in Kuwait to determine biochemical analytes reference intervals based on age and sex in people of 15 years and above found that the values differed from commercial reference values (Olusi and Al-Awadhi, 2002). Both race and geographical location affect reference values.

Nevertheless, Meru Level Five hospital and the surrounding health facilities in Meru County have been found to use reference range values found in medical textbooks and the manufacturers reagent kits. (Table 1)

Table 1: Reference range values used at ML5H

Test	Reference Range / Unit
Alanine aminotransferase	Male: 0 – 34 U/L; Female: 0 – 35 U/L; Child: 0 – 50 U/L
Aspartate aminotransferase	Male: 0 – 35 U/L; Female: 0 – 40 U/L; Child: 0 – 50 U/L
Alkaline Phosphatase	Adult: 56 – 112 U/L; Child: 47 – 406 U/L
Gamma glutamyl transferase	Adult: 0 – 55 U/L; Child: 1 – 132 U/L
Albumin	Adult: 35 – 52 g/L; Child: 30 – 48 g/L
Total Protein	Adult: 66 – 88 g/L; Child : 33 – 56 g/L
Direct Bilirubin	Adult: ≤3.4 μmol/L
Total Bilirubin	Adult: 1.7 – 21 μmol/L
Creatinine	Adult: 44 – 97 μmol/L; Child: 62 – 115 μmol/L
Urea	Adult: 2.8 – 7.2 mmol/L
Sodium	Adult: 135 – 150 mmol/L
Potassium	Adult: 3.5 – 5.0 mmol/L
Chloride	Adult: 97 – 105 mmol/L

Source: Abbott Diagnostics Clinical Chemistry by Roberta Reed, Ph.D (2011)

2.2 Biochemical Parameters

2.2.1 Total Protein (TP)

Total protein refers to the total amount of the two classes of proteins found in the fluid portion of blood, that is, albumin and immunoglobulins (principally IgG, IgA and IgM). Albumin prevents fluid from leaking out of blood vessels while globulins play a critical role in the immune system. Total protein measurement quantifies the concentration of all proteins present in serum (excluding clotting factors) (Lindsey, 1986). Proteins are useful in proper functioning of body cells and enzymes (Preejith *et al.*, 2003). Functions of proteins are transportation of hormones, drugs, vitamins, lipids, and regulation of extra cellular fluids. Protein loss is mostly via glomerulus, but it is restricted by the size of the pore and the negative charge of the protein molecules (Lindsey, 1986).

2.2.2 Albumin (ALB)

Albumin is synthesized by the liver and composes about 50 - 60% of blood plasma proteins. It is a transport protein for many molecules in the body including free fatty acids, certain ions (e.g. Ca^{2+}, Zn^{2+}), bilirubin and many drugs (Lindsey, 1986). It contributes to the oncotic pressure of plasma and maintaining the distribution of extracellular fluid between the vascular and extra vascular compartments. Albumin is also a buffer of hydrogen ions. A serum albumin test measures the concentration of this protein in the clear liquid portion of the blood.

This protein is easily and cheaply analyzed in the laboratory. Elevated levels of albumin in blood are indicative of dehydration or high protein diet.

Decreased levels of albumin are found in weight loss, malnutrition, inflammation and shock. Low levels may also reflect conditions in which the kidneys have lost their capacity to prevent it from leaking from the blood into urine and being lost. Low albumin results to oedema since the intravascular oncotic pressure is higher than the pressure in extra vascular space (Koller, 1984).

2.2.3 Bilirubin

Bilirubin is formed mainly when the liver breaks down old erythrocytes and other compounds that contain heme such as myoglobin and cytochromes. Heme, the component of hemoglobin that is responsible for binding oxygen so that it can be transported to the tissues, is detached from the globin and converted into biliverdin in the spleen. The biliverdin produced is converted further into bilirubin through reduction by biliverdin reductase (Fody, 2005).

$$\text{Hemoglobin} \longrightarrow \text{Biliverdin} \xrightarrow{\text{Biliverdin reductase}} \text{Bilirubin}$$

This bilirubin is referred to as indirect (unconjugated) bilirubin because it is insoluble in water and cannot be excreted in urine. It is bound to albumin and transported in the blood to the liver. In the hepatocytes, the enzymes UDP-glucose dehydrogenase and UDP-glucuronosyl transferase conjugate glucuronic acid to bilirubin forming bilirubin monoglucuronides (20%) and bilirubin diglucuronides (80%) which are soluble in water and excreted in urine (Balisteri and Shaw 1987).

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Bilirubin is present in plasma in three forms: albumin bound (unconjugated bilirubin) which is the major component; conjugated bilirubin and δ - bilirubin (conjugated bilirubin covalently bound to albumin). Unconjugated bilirubin is transported in the bloodstream to the liver where it is rendered water soluble by conjugation with glucuronic acid and is excreted in the bile. The presence of conjugated bilirubin in the plasma is an indication of a pathological process (Zucker *et al*., 2004). The physiological significance of bilirubin is not completely understood, but according to recent research, it is not only for the disposal of hemoglobin, but is also a powerful natural antioxidant that gives promise for the discovery of new treatments for cancer, cardiology and many other diseases.

2.2.3.1 Total Bilirubin (T-BIL)

This refers to both conjugated and unconjugated bilirubin. Conditions in which bilirubin is synthesized faster than it is metabolized in the liver cause an overall rise in the amount of unconjugated (indirect) bilirubin in circulation. Immaturity of the liver, hemolytic jaundice, Gilbert's disease, physiological jaundice and Dubin-Johnson syndrome (conditions that cause impairment in bilirubin conjugation) also lead to high levels of unconjugated bilirubin in circulation. Obstruction of the bile tract and damage to hepatocellular structure causes elevations of both direct and indirect bilirubin in the circulation (Balisteri and Shaw, 1987).

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2.2.3.2 Direct Bilirubin (DBIL)

This refers to bilirubin that is found unbound in the bloodstream. It is soluble in water and can therefore easily pass into the small intestines as well as the kidneys where it is excreted in urine. Direct bilirubin is formed when unconjugated bilirubin combines with glucuronic acid in the liver making it soluble therefore it can pass through the gall bladder into the bile for subsequent elimination via the digestive tract. An increase in conjugated (direct) bilirubin is observed in diseases affecting the liver or bile ducts. When more than half of the total bilirubin in circulation is conjugated bilirubin, this may indicate that there is hepatocellular injury or cholestasis (Fody, 2005).

2.2.4 Aminotransferases

Aminotransferases consist of two enzymes - aspartate aminotransferase (AST) and alanine amino transferase (ALT) that catalyze the transfer of the alpha amino acids of aspartate and alanine respectively to the keto group of ketoglutaric acid (Davis and McMillin, 2010).

ALT is primarily found in the liver while AST is widely distributed in many tissues including the heart, skeletal muscle, kidney, brain and liver. Whereas AST is present in both the mitochondria and cytosol of hepatocytes, ALT is only localized in the cytosol (Thapa and Walia, 2007). Increased amounts of

mitochondrial AST are observed in serum after extensive tissue necrosis and in chronic liver disease. Aminotransferases are not present in the urine or bile and hepatic sinusoids are the chief sites for their clearance (Rochling, 2001).

2.2.4.1 Aspartate Aminotransferase (AST)

AST is an intracellular enzyme found in both the cytoplasm and mitochondria of cells. It is widely distributed in the body, but is present mainly in cardiac muscle and skeletal muscle, liver, heart, and the kidneys. AST catalyzes the transfer of amino groups from aspartate to 2-oxoglutarate.

Aspartate + α-ketoglutarate $\xrightarrow{AST}$ Oxaloacetate + glutamate

AST that is found in plasma is as a result of the normal turnover of tissue cells. Elevated levels of the enzyme are found in hepatic, cardiac and skeletal muscle damage (Bethany and Krefetz, 1992). A large amount of AST is found in liver, kidney, cardiac muscles and skeletal muscles. Elevated values of AST are associated with liver damage (Chiasera and Xu, 2010).

After the liver cell damage, 24-36 hours are needed for a marked elevation to be noted, and the elevation takes 3-7 days to normalize. AST to ALT ratio values are useful in differentiating liver damage causes. Increase in AST alone is not specific to liver damage since the enzyme can be released from other organs (Rochling, 2001).

2.2.4.2 Alanine Aminotransferase (ALT)

ALT is an intracellular enzyme that is found in the cytoplasm of mainly liver and the kidney cells. ALT catalyzes the transfer of amino groups from alanine to 2-oxoglutarate.

$$\text{Alanine} + \alpha\text{-ketoglutarate} \xrightarrow{ALT} \text{Pyruvate} + \text{glutamate}$$

ALT is a key enzyme in gluconeogenesis. Presence of this enzyme in plasma is as a result of normal turnover of tissue cells but elevated amounts of the enzyme in plasma are an indication of tissue damage and particularly hepatic damage. ALT is therefore used as a marker of liver damage since significant increases in its plasma activity are observed in liver disease, (Bethany and Krefetz, 1992).

It may be assayed in patients exhibiting clinical features of liver disease or in those at greatest risk of developing liver disease, for example those suffering from alcoholism, obesity, diabetes, having consumed potentially hepatotoxic drugs or those with a family history of liver disease (Dufour *et al.*, 2000). Elevated levels are found in liver damage such as hepatitis (Cheesebrough, 2009). The ALT levels in serum and plasma are increased before any presentation. Some drugs such as lipid reducing and anti-diabetic drugs elevates the ALT levels thus the need to monitor them before making any decision in the laboratory (Pratt *et al.*, 2000).

2.2.5 Alkaline Phosphatase (ALP)

ALP is a zinc-containing metalloenzyme activated by Mg^{2+} and other divalent ions. It is part of a class of enzymes involved in the breakdown of phosphate esters in an alkaline medium, producing an organic radical and an inorganic phosphate.

The enzyme is present in many body tissues but significant amounts are found in the liver (cells of the biliary system) and bones (osteoblasts). Lesser amounts of the enzyme are found in the small intestines (mucosal cells), placenta, kidneys (proximal convoluted tubules) and leukocytes (Friedman *et al*., 1996).

In bone, ALP is important in mineralization by catalyzing the formation of phosphate from pyrophosphate while in the gut it is involved in lipid transport. In obstructive liver disease, ALP is the first enzyme to be elevated, while damage of liver cells causes the aminotransferases to be marked. This makes enzyme analysis useful to distinguish the disease condition, either cholestatic and hepatocellular. Under normal conditions, ALP is increased due to bone growth, healing of a bone that was broken or rickets disease. Germ cell tumors and inflammatory bowel (ulcerative colitis) disease also produce alkaline phosphate that leaks in to the blood stream (Fischbach, 1996).

2.2.6 Gamma-glutamyl transpeptidase (GGT)

Gamma-glutamyl transpeptidase (GGT) is found in the cell membranes of the kidneys, bile ducts, pancreas, gall bladder, spleen, heart, brain, prostate gland and seminal vesicles. GGT plays a critical role in the transfer of amino acids across

the cellular membranes as well as being involved in leukotriene metabolism (Ruttman *et al.*, 2005).

High amounts of GGT in serum are noted in diseases affecting the liver, biliary system, and pancreas. GGT is therefore used together with ALP when diagnosing diseases of the biliary tract but GGT is thought to have better sensitivity, though generally, ALP is the first enzyme to assay for presence of biliary disease (Dosoo *et al.*, 2014). Accumulation of GGT in atherosclerotic plaques suggests a potential role in pathogenesis of cardiovascular diseases (Kamisha and Gwen, 2006).

GGT is also elevated after ingestion of large quantities of alcohol (Schiele *et al.*, 1998) therefore isolated or disproportionate elevation of this enzyme in blood compared to other liver enzymes may be an indicator of alcohol abuse or alcoholic liver disease. Determination of GGT activity in serum is however not specific to alcohol intoxication but may indicate excess consumption of alcohol of up to three or four weeks prior to the test. Drugs such as barbiturates and phenytoin and the condition, congestive heart failure may also result to increase in GGT, (Ruttmann *et al.*, 2005).

2.2.7 Urea (UREA)

Urea is the chief nitrogen-containing metabolite of protein catabolism in the body. It accounts for more than 75% of non-protein eventually excreted. It is derived from the breakdown of amino acids in the liver by the process of deamination. In

this process, nitrogen in the amino acids is converted to ammonia (NH_3). Ammonia is a waste product produced in the body from the breakdown of unabsorbed protein by bacteria in the intestines. If not processed, excess ammonia accumulates in the blood and passes to the brain where it is toxic (Ruttmann *et al.*, 2005).

Accumulation of ammonia in the body would be fatal because of its high toxicity, thus it is quickly eliminated after production by the liver through a system of carrier molecules and enzymes.

$NH_3 + CO_2 \longrightarrow CO\ (NH_2)_2$ (urea)

Urea is a product of metabolism in the Krebs-Henseleit urea cycle in the liver. Conversion of NH_4^+ into urea, via urea cycle is essential because hyper-ammonemia (excess NH_3 in the cells) depletes α-ketoglutarate and inhibits TCA cycle, NADH and ATP production. This may cause malfunctioning of the central nervous system including blurred vision, tremor, muscular weakness and coma leading to death. The rate of ammonia synthesis depends on intake of exogenous nitrogen and endogenous protein catabolism. Up to 90% of urea is excreted by the kidney while the rest is eliminated via the gastrointestinal tract. Urea is used to detect any abnormality of the kidneys, (Rock *et al.*, 1986).

2.2.8 Creatinine (CRE)

Creatinine is a heterocyclic nitrogenous compound formed as a by-product of muscle metabolism in the body. It is produced when energy is used in the muscle

through a biological system involving creatine, phosphocreatine (also known as creatine phosphate), and adenosine triphosphate (ATP) (Newman and Price, 1993). A small amount of creatinine (about 10%) is derived from dietary sources (particularly cooked meat). CRE is excreted in its original form by the kidneys, principally by glomerular filtration and to a small extent by active secretion. Creatinine is a waste product with no known to have any physiological function (Verma *et al.*, 2006).

Measurement of serum creatinine is a superior renal function test in comparison to urea measurement since creatinine is not affected by non-renal factors for example turnover of proteins and the hydration state of the individual. Further, measurement of serum/plasma creatinine is less affected by age, dehydration or bleeding than urea levels; it is less influenced by diet e.g. protein intake and it can be used in the investigation of renal diseases associated with HIV as well as to monitor the prognosis of patients receiving nephrotoxic antiretroviral drugs such as tenofovir (Remuzzi *et al.*, 1997).Creatinine is excreted out of the body through filtration by the kidneys.

High CRE levels in blood are observed in cases of faulty filtering by the kidneys or in marked damage to the nephrons. Its levels in blood and urine may be used to calculate the creatinine clearance (CrCl), which reflects the glomerular filtration rate (GFR) (First, 1996). The clearance of a substance is the volume of plasma from which that substance is removed per unit time.

Therefore, $Clearance = \frac{UxV}{p}(ml\min^{-1})$

Where U = Urinary concentration of given substance, V = Volume of urine produced, P = Plasma concentration of given substance

The GFR is clinically important because it is a measurement of renal function (Kaplan and Pesce, 2003). Creatinine is however unsuitable for detecting early-stage kidney disease, creatinine clearance (CrCl) test or evaluating the kidney function (Frank, 2005).

2.2.9 Sodium (Na)

In human beings, sodium plays a critical role in the regulation of blood volume, blood pressure, osmotic pressure and helps in maintaining a constant physiological pH (DuBose, 2008). Sodium levels in plasma represent a balance between the sodium and water in the food and drinks consumed and the amount of the analyte in urine. Small amounts of sodium are lost through stool and sweat. The amount of sodium in the body is influenced by the renin–angiotensin hormone system that regulates blood pressure and fluid balance. Low blood pressure and low sodium levels in the kidneys causes the formation of renin. Presence of renin leads to the formation of angiotensin enzyme and mediates the regulation of extra cellular fluid volume and arterial vasoconstriction. Presence of renin also causes the production of aldosterone and angiotensin hormones that

cause excretion of sodium in urine. Increased concentration of sodium in plasma lowers renin formation and sodium levels return to normal (First, 1996).

Sodium is also essential for the proper functioning of neurons and osmoregulation between cells and the extracellular fluid through the Na^+ /K^+ pump (First, 1996). Sodium level in blood is analyzed in case of recent injury, surgery, or serious illness, consumption of large or small amounts of salt or fluid, treatment with water pills (diuretics) or certain other medications for example aldosterone and giving of intravenous (IV) fluids (Kleinman and Lorenz, 2003).

2.2.10 Potassium (K)

Potassium is the primary intracellular cation, with about 98% of the 120 grams of potassium contained in the entire body found within cells. Along with sodium, potassium is involved in the regulation of water balance and the acid-base balance in the blood and tissues (DuBose, 2008). Potassium instigates the brief sodium-potassium exchange across the cell membranes as it enters the cells more readily than sodium. In the nerve cells this sodium-potassium flux generates the electrical potential that assists in the conduction of nerve impulses across the cell membranes. The electrical potential gradient created by the sodium-potassium pump helps to generate muscle contractions, regulate the heart beat and prevent swelling of cells.

Other functions of potassium are: (1) energy metabolism (2) protein synthesis from amino acids in the cells (3) carbohydrate metabolism (4) glucose and glycogen metabolism (5) important for normal growth and muscle building. The disorders of potassium can be high or low levels of this analyte in blood. Causes of elevated levels of potassium in the body are renal dysfunction, metabolic acidosis, under-function of adrenal cortex, diabetes, massive tissue destruction, bradycardia and respiratory dysfunction. Low potassium level are as a result of diarrhoea or vomiting, over function of the adrenal cortex, anaemia, use of diuretics, paralysis, hypertension, metabolic alkalosis and anaerobic state (Fody, 2006).

2.2.11 Chloride (Cl)

Chloride is the principal anion in the blood, representing about 70% of the body's total negative ion content. Chloride, together with potassium and sodium is involved in the conduction of electrical impulses in the nerve cells as well as maintaining the body's acid-base balance and osmotic pressure. In addition to being an electrolyte, chloride plays a critical role in digestion by combining with hydrogen in the stomach to form hydrochloric acid that breaks down proteins, absorbs other metallic minerals and activates the intrinsic factor which in turn absorbs vitamin B12.

Once utilized in hydrochloric acid, the remaining chloride ions are reabsorbed back into the bloodstream by the intestines where it is required for maintenance of extracellular fluid volume (Polancic, 2006).

Chloride ions also play an important role in the central nervous system where the inhibitory action of glycine and Gamma-amino butyric acid (GABA) relies on the entry of chloride ions (Cl^-) into specific neurons. Chloride levels in the blood are increased as a result of dehydration, renal tubular acidosis, acute renal failure or metabolic acidosis associated with prolonged diarrhoea and loss of sodium bicarbonate. Chloride levels can be decreased by drugs such as acetazolamide, ammonium chloride, androgens, cortisone, estrogen and loop diuretics, thiazide diuretics and triamterene (DuBose, 2008).

2.3 Quality Control (QC) Assessment

Quality control in the clinical chemistry laboratory is a statistical process that is aimed at monitoring and evaluating the analytical process that is employed in generating the patient's results. Quality control involves regular testing of QC products along with patient samples and comparing the QC results obtained with specific statistical ranges. The procedures and processes under quality control are aimed at detecting and minimizing errors that may arise during the time of carrying out the tests and determining whether the instrument is operating within certain specifications in order to ensure the results are reliable, precise and accurate (Cheesebrough, 2009). QC involves all the procedures that are followed

from collection of specimen, analysis, reporting, and dispatch of the results. This is essential to countercheck the quality of the tests performed. This in turn assists in detection and ratification of different procedures that are responsible for the errors (Bolann and Omenas, 1997).

Control was evaluated by use of quality control chart (Levey Jennings control chart) that was prepared daily. Control values that are within ± 2 standard deviation are a good indicator that the results obtained are reliable and therefore can be reported with confidence. Developing countries have been found to have problems in issuing accurate results. This has necessitated the building of reliable quality control systems in order to report excellent results assured of quality and proper documentation of tests carried out on quality control material (Krishnan *et al.*, 1999).

Reliability and reproducibility of laboratory results have been found to be affected by the environment, laboratory materials, specimen handling, personnel, test methods, equipment, reading and reporting. External quality control is an objective system of assessing laboratory ability to produce reliable results. Inclusion of external quality assessment is regarded as an addition to internal quality control (Chesebrough, 2009).

External quality control is performed once in a while where the control material is from outside the laboratory setting from where the values are performed and the results compared with what is received from the laboratory after using the same

sample material which usually is lyophilized (Ricos *et al.*, 1996). The clinical chemistry laboratory of Meru Level Five Hospital complies with the principles of good clinical laboratory practice and has standard operating procedures (SOP) that are strictly followed.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study Site

This study was carried out in Meru County, Kenya. Meru County is located on the slopes of Mt. Kenya, approximately 225 kilometers north-east of Nairobi at an altitude of 5000 feet above sea level. Meru County covers an area of approximately 6936 square kilometers (2678 square miles) (Figure 2). From the 2009 report by Kenya National Bureau of Statistics (KNBS), the population of Meru County stood at 1,365,301 people with a population density of 200 people per square kilometer. The county shares its borders with four other counties: Isiolo County to the north, Nyeri County to the south- west, Tharaka-Nithi County to the south-east and Laikipia County to the west. Meru County is divided into nine sub-counties: Igembe south, Igembe central, Igembe north, Tigania east, Tigania west, North imenti, Central imenti, South imenti and Buuri.

The county is principally an agricultural county with most people engaged in subsistence farming of common food crops such as maize, beans, potatoes, sorghum, millet and cabbages. The county is also renowned for its wide-scale growing of Miraa (Khat) - an herbal plant which is a lucrative cash crop for the locals, and fetches millions of shillings in the export market. The climate in Meru is cool and warm, with temperatures ranging between 16°C during the cold season

and 23°C in the hot-warm season. The county receives an average rainfall of between 500mm and 2600 mm each year.

The most prevalent diseases in Meru County that affect this population of children and adolescents are malaria, diseases of the respiratory system including pneumonia and tuberculosis, HIV/AIDS and intestinal infectious diseases including diarrhoea. Other lifestyle diseases for example diabetes, heart diseases, kidney diseases and cancers are also some of the top causes of both outpatient and inpatient morbidity. Sampling was done at Kagaene, Kiirua, Maua, Kanyakine, Kaongo, Nchiru, Kangeta, Athiru ruujine and Kaaga and laboratory analysis done at the clinical chemistry laboratory of Meru Level Five Hospital.

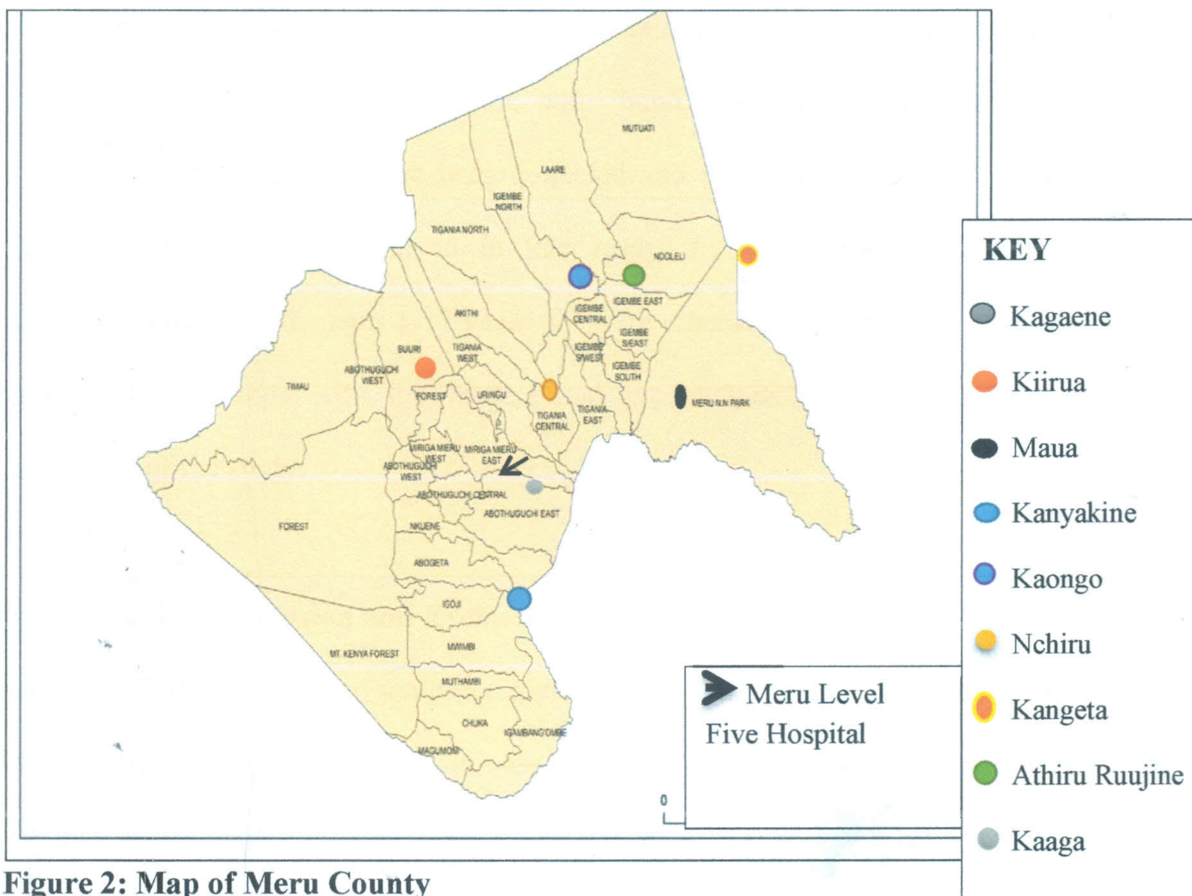

Figure 2: Map of Meru County
Source: Kenya Counties Map, lands.go.ke

3.2 Selection of Reference Population

The reference population was selected based on the guidelines described by the NCCLS/CLSI (2000). According to the guidelines, the reference individuals selected should be closely similar to the patient population under study and should be of the same age to be clinically significant.

There are two methods of selection for a reference population described in the guidelines: - a priori and a posteriori selection method (Zeh *et al.*, 2012). In a priori sampling, selection of an adequate number of subjects who serve as

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reference individuals takes place first and then samples are drawn for analysis. In a posteriori sampling method, on the other hand, the reference population is selected after the samples have been collected and the analytes tested. A priori sampling method was employed in this study. In this study, children and adolescents between the ages 1-17 years old were randomly selected after holding community meetings with the leaders and parents/guardians to explain the objectives of the study.

3.2.1 Sampling, Inclusion and Exclusion Criteria

Using a stratified random sampling method, one child/adolescent from every selected household was enrolled. In cases where a household had more than one eligible child, balloting was done in order to select one subject from the household. All the participants received a physical examination and a review of medical history by interviewing them and/or their parents/guardians by use of a standardized questionnaire (Appendix 28). Only apparently healthy children as assessed by the study physician and lacking any clinical manifestation of any illness were enrolled.

Inclusion into the study was purely by willingness of the child/adolescent and/or the parent/guardian to take part in the study, by signing the consent form (Appendix 27) and providing the sample required. They had to be residents in Meru County for a period of not less than six months.

Excluded from the study were subjects who had undergone either a blood donation or transfusion within three months prior to the study or those who were hospitalized within a month preceding the study; those on any form of medication; serum samples that were found to be HIV positive, HBsAg and Syphilis positive. Also excluded were serum samples from adolescent females that were found to be pregnant after undergoing a human chorionic gonadotrophin, HCG hormone test in urine.

3.3 Study Design

This was a population based cross-sectional study involving 740 healthy male and female subjects of age 1 to 17 years.

3.4 Ethical Approval

Ethical approval was obtained from Kenyatta University Review and Ethical Committee (Appendix 29) and the Ministry of Health, Meru County Kenya (Appendix 30).

3.5 Specimen Collection

Five milliliters (5ml) of blood was collected from children visiting the Mother and Child Health (MCH) clinic at the hospital and from children in selected households and schools. Blood was collected by venipuncture on the upper arm after sterilizing the area with 70% alcohol and dispensed into a plain vacutainer

(without anticoagulant) tube then transferred in 2 ml tubes (vacutainer TM, Becton Dickinson, Franklin Lakes, NJ). All the specimen tubes were labeled correctly with the subject's name, the study number and the date of collection of the sample. Specimens were arranged in Styrofoam cool boxes at 4°C and covered to protect them from heat and sunlight, awaiting transportation to the main analytical center.

3.5.1 Specimen Transportation, Processing and Storage

Collected specimens were ferried from the point of collection to the Clinical Chemistry Laboratory of Meru Level Five Hospital in cool boxes within two hours of collection at room temperature. This was done to avoid deterioration of the samples when exposed to sunlight, heat or fluorescent light, for example, un-conjugated bilirubin is decomposed by light. Decreases in serum bilirubin levels may be observed after delays in transporting blood samples due to breakdown of bilirubin by light (Poovendran *et al.*, 2010). Upon arrival at the Clinical Chemistry Laboratory, the blood specimens were centrifuged at a speed of 3000 rpm for two minutes to obtain serum. The serum was transferred to separate tubes labeled with subject's identification details. Laboratory analysis was done as soon as possible to avoid loss of sample viability since certain analytes such as potassium are known to leak slowly from the cells into the surrounding medium along a concentration gradient. If analysis was not done immediately, the samples were stored at -20°C for a period not exceeding seven days

3.6 Laboratory Analysis

Reference intervals were established for thirteen biochemical analytes including tissue enzymes, serum proteins, electrolytes, non-protein nitrogen and bilirubin. These analytes were: alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), gamma glutamyl transferase (GGT), total protein, albumin, sodium, potassium, chloride, urea, creatinine, direct bilirubin and total bilirubin. All the assays were carried out as per the standard operating procedures (SOPs) found at the hospital's laboratory.

3.7 Equipment for Analysis

The equipment used for the sample analysis was DRI-CHEM NX500I (Fujifilm, Europe) a dry Chemistry Clinical Analyzer that is able to carry out a wide range of chemical tests in a single run.

3.7.1 Reagent preparation

The machine uses coded slides that are commercially acquired and specifically tailored for the equipment. Each slide is impregnated with reagent for a specific parameter and is labeled clearly. The slides for the various tests being carried out were inserted in the machine along with the sample and once the patient details are entered and the machine set to start, the tests run automatically and a print - out of the results is obtained.

3.7.2 Calibration of the test

In order to make sure that the values obtained from the samples assayed were both accurate and precise, the machine was set to perform a calibration procedure for the tests. Calibration was done by passing the QC card through the QC card reader whenever slides from a new lot number were being used. Calibration procedure serves three purposes: (1) ensures readings from the instrument are consistent with other measurements. (2) To determine the accuracy of the instrument readings. (3) To establish the reliability of the instrument.

Results of measurements are reliable if they relate to similar measurements, made at a different time, a different place, by a different person using a different instrument.

3.7.3 Quality control (QC) materials

Assayed multisera whose values were predetermined and known (normal) were used for the quality control of the analytical work during the study period. The QC multisera were supplied in lyophilized form and were reconstituted as per the manufacturer's guidelines. Internal quality control assessment was done by analyzing the multisera daily or at any other time as deemed necessary.

3.8 Analytical Methods for Biochemical Parameters

3.8.1 Total protein (TP)

A timed end point Biuret method reaction, using the total protein reagent was done to determine the concentration of total protein in the sample (Koller, 1984).

During the reaction, the peptide bonds present in the protein sample bind to cupric ions in an alkaline medium to form a peptide/copper complex. 10 µL of sample was deposited on a FUJI DRI CHEM slide TP-PIII (Fujifilm, Europe). The sample was allowed to spread uniformly on the spreading layer thereby reacting with the reactive reagent that was released from the reagent layer to form a coloured product. The slide was incubated at 37°C for a fixed time in the analyzer and the optical reflection density measured at 540nm. The optical density was then converted into the total protein concentration using a calibration curve pre-installed in the analyzer.

Principle of the reaction

Protein + Cu^{2+} $\xrightarrow{OH-}$ Protein - Copper complex (Red Purple colour)

3.8.2 Albumin (ALB)

Albumin in plasma and serum is measured in clinical chemistry by dye-binding methods, usually with bromocresol green or bromocresol purple. Albumin concentration was measured by a timed end-point reaction by use of the albumin reagent. 10 µL of serum was deposited on a FUJI DRI-CHEM slide ALB-P and allowed to spread uniformly on the spreading layer. In the process, ALB reacts with bromocresol green (BCG) to form an albumin - BCG complex that diffuses onto the underlying layer. The slide was incubated at 37°C for a fixed time in the analyzer and the optical reflection density was measured at 625nm. The optical reflection density was then converted into the albumin concentration using calibration curves pre-installed in the analyzer.

Principle of the reaction

Albumin + BCG $\longrightarrow$ Albumin - BCG complex (blue colour dye)

3.8.3 Total bilirubin (T-BIL)

Bilirubin is measured in the blood through chromatography, capillary electrophoretic and photometric methods. 10 μL of serum sample was deposited on a FUJI DRI-CHEM slide TBIL-PIII. After depositing, the sample spreads uniformly on the spreading and reagent layer and indirect bilirubin is dissociated with dyphylline and undergoes diazo reaction together with direct bilirubin by 2, 4-dichloro benzene diazonium salt to form diazo dye. The slide was incubated at 37°C for a fixed time in the analyzer and the optical reflection density was measured at 540nm. The optical reflection density was then converted into the total bilirubin concentration using a calibration curve pre-installed in the analyzer.

Principle of the reaction

Direct Bilirubin + Diazonium salt of benzene sulfonic acid $\xrightarrow{Diazoreacton}$ Diazo dye

Indirect Bilirubin $\xrightarrow{Dyphylline}$ 2, 4-Dichlorobenzenediazonium salt

3.8.4 Direct bilirubin (D-BIL)

Conjugated and direct bilirubin reagent was used to measure the concentration of D-BIL by a timed end point reaction. 10 μL of serum sample was deposited on a FUJI DRI-CHEM slide DBIL-PII. After depositing, the sample spreads uniformly

on the special spreading layer and direct bilirubin reacts with diazonium salt of benzene-sulfonic acid to form diazo dye. The slide was incubated at 37°C for a fixed time in the analyzer and the optical reflection density was measured at 577nm. The optical reflection density was then converted into the direct bilirubin concentration using a calibration curve pre-installed in the analyzer.

Principle of the reaction

Direct Bilirubin + Diazonium salt of benzene sulfonic acid $\xrightarrow{\textit{Diazoreacton}}$ Diazo dye

3.8.5 Aspartate aminotransferase (AST)

In this reaction, AST reagent was used to measure the activity of the enzyme by enzymatic rate method. 10 μL of serum sample was deposited on a FUJI DRI-CHEM Slide GOT/AST-PIII. The slide was incubated at 37°C and the sample catalyzes the amino-transition reaction with the substrate of L- aspartic acid after spreading uniformly in the spreading layer. Oxaloacetic acid produced by the reaction is converted to pyruvic acid by oxaloacetate decarboxylase (OAC) and further by hydrogen peroxidase (POD) and forms a blue colour dye. The increase in absorbance by the generated dye was measured from 2.5 minutes to 4 minutes at 650nm by reflective spectrophotometry and the AST activity was calculated according to the pre-installed formula.

Principle of the reaction

L- Aspartic acid + α-ketoglutaric acid $\xrightarrow{AST}$ Oxaloacetic acid + L-glutamic acid

Oxaloacetic acid $\xrightarrow{OAC}$ Pyruvic acid + CO_2

Pyruvic acid + O_2 + Phosphoric acid + H_2O $\xrightarrow{POP}$ H_2O_2 + Acetyl Phosphate + CO_2

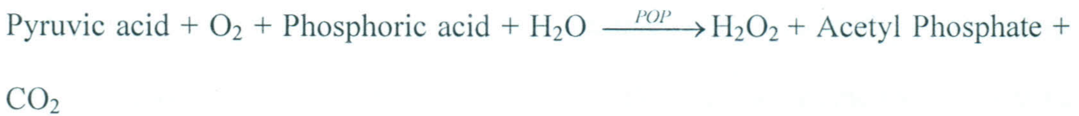
Diarylimidazole leuco dye + H_2O_2 $\xrightarrow{POD}$ Blue colour dye + $2H_2O$

3.8.6 Alanine aminotransferase (ALT)

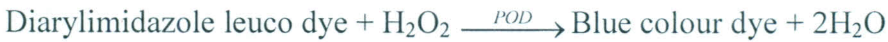
Alanine aminotransferase reagent was used to measure ALT activity by enzymatic rate method. 10 μL of serum sample was deposited on a FUJI DRI-CHEM Slide GPT/ALT-PIII. The slide was incubated at 37°C and the sample catalyzes the amino-transition reaction with the substrate of L-alanine after spreading uniformly in the spreading layer. Pyruvic acid produced by the reaction generates hydrogen peroxide by pyruvate oxidase (POP). Hydrogen peroxide oxidizes diary imidazole leuco dye by the catalytic reaction of peroxidase (POD) and forms a blue colour. The increase of absorbance by the generated dye was measured at 650nm by reflective spectrophotometry and the ALT concentration was calculated according to the installed formula.

Principle of the reaction

L- Alanine + α- ketoglutaric acid $\xrightarrow{AST}$ Pyruvic acid + L- glutamic acid



$$Pyruvic\ acid + O_2 + Phosphoric\ acid + H_2O \xrightarrow{POP} H_2O_2 + Acetyl\ Phosphate + CO_2$$



$$Diarylimidazole\ leuco\ dye + H_2O_2 \xrightarrow{POD} Blue\ colour\ dye + 2H_2O$$

3.8.7 Alkaline phosphatase (ALP)

Alkaline phosphatase reagent was used to measure ALP activity by kinetic method. 10 μL of serum sample was deposited on a FUJI DRI-CHEM Slide ALP-PIII.

The spotted sample was incubated at 37°C and catalyzes the hydrolyzing reaction of co-existing p-Nitrophenyl phosphate while spreading uniformly in the spreading layer. The p-Nitrophenyl dye formed with the start of the reaction diffused and was collected in the buffer layer. Increase in absorption by the generated dye was measured at 400 nm by reflective spectrophotometry and the ALP activity was calculated according to the pre-installed formula.

Principle of the reaction

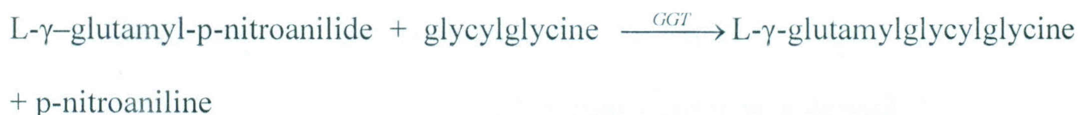
$$p\text{-}Nitrophenyl\ phosphate \xrightarrow{ALP} p\text{-}Nitrophenol\ dye + Phosphoric\ acid$$

3.8.8 Gamma- glutamyl transferase (GGT)

Gamma glutamyl transferase reagent was used to measure GGT activity by enzymatic rate method. 10 μL of serum sample was deposited on a FUJI DRI-CHEM Slide GGT-PIII. As the sample spreads, GGT present in specimen catalyzes the amino transition reaction with the substrate of L-γ-glutamyl-p-

nitroanilide. The GGT activity in the sample was measured by reflective spectrophotometry at 400nm and the amount calculated according to the installed formula.

Principle of the reaction



3.8.9 Urea (UREA)

BUN reagent was used to measure the concentration of urea in the sample by enzymatic rate reaction. A 10 μL serum sample was deposited on a FUJI DRI-CHEM Slide BUN-PIII. As the specimen spreads uniformly on the spreading layer, the large molecular components (protein and dye) are filtered out before penetrating into the reaction layer.

Urea in the sample is decomposed by urease releasing ammonia and carbon dioxide. The ammonia gas produced permeates through the porous layer to reach the detection layer where it changes Bromocresol green (BCG) contained in the layer from yellow to green. The change in colour is proportional to the concentration of urea nitrogen in the sample. The slide was incubated at 37°C for a fixed time in the analyzer. Optical reflection density was measured at 600nm and then converted into the urea nitrogen concentration using a calibration curve pre-installed in the analyzer.

Principle of the reaction

$$Urea + H_2O \xrightarrow{Urease} 2NH_3 + CO_2$$

$$\text{Bromocresol green} + NH_3 \longrightarrow \text{Green dye}$$

3.8.10 Creatinine (CRE)

Creatinine reagent was used to measure the concentration of CRE in the sample by a modified Jaffe reaction. A 10 μL serum sample was deposited on a FUJI DRI-CHEM Slide CRE-PIII. The sample was allowed to spread uniformly in the spreading layer while diffusing and penetrating into the reaction layer. Endogenous ammonia present in the sample was removed by the action of α-ketoglutaric acid, glutamate dehydrogenase (GLDH) and NADPH. Creatinine was decomposed by the action of creatinine deiminase (CD) in the reaction layer releasing ammonia gas that passes through the porous layer to the detection layer. This causes bromphenol blue in the detection layer to change colour from yellow to blue. The slide was incubated at 37°C for a fixed time in the analyzer and the optical reflection density was measured at 600nm. This was automatically converted into the concentration of creatinine using a calibration curve pre-installed in the analyzer.

Principle of the reaction

$$NH_3 + \alpha\text{- ketoglutaric acid} + NADPH \xrightarrow{GLDH} \text{L-Glutamic acid} + NADP^+$$

$$\text{Creatinine} + H_2O \xrightarrow{CD} \text{N-Methylhydantoin} + NH_3$$

$$\text{Bromophenol blue} + NH_3 \longrightarrow \text{Blue colour dye}$$

3.8.11 Electrolytes (Sodium, Potassium and Chloride)

The Fujifilm system determines sodium, potassium and chloride serum levels by measuring electrolytes ion activity in solution. 50µL of the reference fluid and 50µL of serum sample was deposited on a FUJI DRI CHEM slide Na-K-Cl at the same time on the reference side and the sample side respectively. After depositing, the reference fluid and sample spread along the distribution device and also towards each other on the special thread bridge to form a stable ionic junction. A potential difference proportional to the logarithm of each ionic concentration ratio of the two fluids was generated between the two half cells. The slide was incubated at 37°C for a fixed time in the analyzer and the potential difference between the reference and the sample measured. This value was then converted into the concentration of each of the electrolytes using a calibration curve pre-installed in the analyzer.

Principle of the reaction

For the three electrolytes, the change in potential voltage developed at the face of each specific electrode was calculated using Nernst equation.

Sodium $E = \text{Constant} + (\text{slope})(\log [Na^+])$

Potassium $E = \text{Constant} + (\text{slope})(\log [K^+])$

Chloride $E = \text{Constant} + (\text{slope})(\log [Cl^-])$

3.9 Screening for Human Immunodeficiency Virus (HIV)

A qualitative immuno-chromatographic assay (HIV 1/2 Stat-Pak®, Chembio Diagnostic Systems New York, USA) was used for the detection of antibodies to human immunodeficiency virus types 1 and 2 (HIV-1 and HIV-2) in human serum. A sample of 50 μL was deposited on to the sample pad and after one minute chase buffer was added. The sample was allowed to spread for 15 minutes. The test was positive results if two red lines appeared on the sample pad, one on the control window and another on the patient window and negative if there was appearance of only one red line at the control window.

3.10 Screening for Hepatitis B Surface Antigen (HBsAg)

The HBsAg one step hepatitis B surface antigen test strip (one step HBsAg rapid test kit, Kinghawk Pharmaceutical Co., Ltd, Beijing, China) was used for the screening of hepatitis by a qualitative lateral flow immunoassay test. The test strip was briefly immersed in the serum sample for screening, removed and placed on a non-absorbent flat surface. It was left to stand for fifteen minutes to allow the sample to spread. Results were positive if there appeared two distinct red lines, one on the control region and the other on the test region and negative if only one red line appeared at the control region.

3.11 Screening for Venereal Disease Research Laboratory (VDRL)

The syphilis ultra-rapid test immunoassay (TP antibody rapid test card, Kinghawk Pharmaceutical Co., Ltd Beijing, China) was used for the screening of *Treponema pallidum,* the causative agent of syphilis. A sample of 50 µL was deposited on the sample pad followed by one drop of buffer.

The sample was allowed to spread on the sample pad and the result read after 10 minutes. Results were positive if two red lines appeared; one on the control region and the other on the test region and negative if only one red line appeared on the control region.

3.12 Statistical Methods Used in the Establishment of Reference Values

In order to produce unbiased reference values for the 1 to 17 years population of Meru County, the data from 740 study subjects was statistically treated using the following steps; (1) Partitioning of reference values, (2) Inspection of data distribution, (3) Detection and handling of outliers, (4) Determination of reference limits (5) Selection of statistical method.

3.12.1 Partitioning of Reference Values

Partitioning of the data was done based on the sex and age of the participant. Partitioning according to age produced four age categories: 1-5 years, 6-10 years, 11-15 years and >15 years. This was done in order to determine the effect of age and sex on the reference ranges. The data from each age group was used to

construct reference values for the thirteen analytes studied and for comparisons with those available in literature.

3.12.2 Inspection of Data Distribution

Graphs and box plots for the analytes were prepared by the use of excel program and then examined visually to test fit to Gaussian distribution. This was to safeguard against the misapplication or misinterpretation of statistical methods as well as to provide valuable information about the data.

From the graphs, values that were highly deviating, also known as outliers were easily detected. This represented abnormal values in the collected data that would affect the determination of reference values.

3.12.3 Identification and Handling of Abnormal Values

The identification of abnormal values (outliers) was done by visual inspection of the graphs. According to Solberg (1987), there is no other statistical test for the identification of outliers, which is more sensitive or more reliable than the simple visual inspection of a histogram. The data that remained after removing the outliers from the two tails of the Gaussian curve (representing 95 % normal reference population) was used to construct the reference values.

3.12.4 Determination of Reference Limits

In this study, the upper and lower reference limits of each analyte were determined using the formula: mean ± 1.96 multiplied by standard deviation (x ± 1.96SD). All the values in between and including the two reference limits gives the reference range (interval) of the analytes. This reference interval is the central 95% interval bounded by 2.5 and 97.5 percentiles.

3.12.5 Selection of Statistical Method

The cleaned data obtained from the randomly selected individuals was subjected to normality distribution testing using Kolmogorov-Smirnov Test. The data having been found to assume a normal distribution was further subjected to parametric statistical methods. The lower and upper limits of the reference intervals were calculated using the following formula: mean ± 1.96 multiplied by standard deviation (x ± 1.96SD).

The data was entered into the Excel spread sheet, cleaned and then exported to the Statistical Package for Social Sciences (SPSS) for analysis. T-test was used for comparison of means. The tests were conducted at 95% confidence interval and significance level of 5%; p values of less than or equal to 0.05 ($p \leq 0.05$) were considered statistically significant. The performance of analytical instruments and methods to analyze the levels of the selected analytes were achieved using the paired T test. Quality control was observed throughout the study to make sure that all the results were in the recommended ranges of reporting. This was achieved

using normal and pathological (PNU and PPU) pre-determined values respectively.

3.12.6 Interpretation of Results

In laboratory medicine, different analyzed values are used to give different information that aids in patients management. When the determined values are within the normal ranges, this indicates that the patient is in good health. Values slightly above or below the normal ranges indicate that the patient has a problem, the problem being related to type of the parameters evaluated, but where certain parameters are found in more than one organ, a single parameter is not enough to make a decision, for example AST is found in the heart, liver and kidneys. Where the values are extremely high, this indicates poisoning which is important in forensic medicine and interpretation in case of legal cases.

It is imperative to note that in the case of clinical chemistry parameters, the reference values obtained are not definitive of presence or absence of a particular disease or condition but rather serve as markers and other specific tests should be carried out for definitive diagnosis.

Further, extensive tissue damage must have occurred for significant levels of some of these parameters to be identifiable in blood.

CHAPTER FOUR

RESULTS

4.1 Reference ranges by age and sex for biochemical parameters for males and females

A total of 740 samples were analyzed in this study out of the 768 initially collected. Twenty eight samples were excluded because 6 (21.4%) of them were HIV positive and 22 (78.6%) were hemolyzed. Reference values for thirteen biochemical parameters including tissue enzymes, bilirubin, non - protein nitrogen and electrolytes were established for both males and females of age 1 to 17 years. Reference values were constructed using 2.5^{th} and 97.5^{th} percentiles as lower and upper limits respectively at 95% confidence interval in accordance with CLSI guideline for determining reference intervals (NCCLS 2000).

Tables 2 and 3 show combined sex and/or age specific reference values for each parameter based on the p-values for the difference between male and female participants. Significant sex differences were observed for TP ($\rho = 0.039$), NA ($\rho = 0.003$) and K ($\rho = 0.009$). The separate reference intervals of these three parameters for males and females were: total protein (TP) 30.78-55.72 g/L for males versus 29.95-54.57 g/L for females; sodium (Na) 133.81-150.71 mmol/L for males versus 128.94-147.88 mmol/L for females; potassium (K) 3.10-6.06 mmol/L for males versus 3.13-4.97 mmol/L for females. The combined male and female reference intervals for other measured parameter were: 10.75-57.80 U/L

for ALT, 9.92-54.60 U/L for AST, 61.63-114.31 U/L for ALP, 13.51-128.33 U/L for GGT, 28.30-48.72 g/L for ALB, 0.46-3.88 μmol/L for D-BIL, 12.60-72.56 μmol/L for T-BIL, 2.55-6.50 mmol/L UREA, 56.04-106.22 μmol/L CREAT, and 96.11-106.11 mmol/L Cl. In the course of the study, everyday control value result and the standard deviation (SD) from the control target values were noted and recorded everyday (Table 7 and 8). The control results for each of the measured thirteen parameters were within the expected values.

Table 2: Reference intervals for ALB, AST, ALP, ALT, D-BIL, T-BIL, TP and GGT for children and adolescents of ages 1-17 years in Meru County

Analyte (unit)	Sex	N	Mean	Reference Interval	RI	Difference between M&F	
						F value	Sig
ALB (g/L)	M	380	38.47	27.98 - 48.96	20.98	0.120	0.949
	F	360	38.56	28.62 - 48.50	19.88		
	M&F	**740**	**38.51**	**28.30 - 48.72**	**20.42**		
AST (U/L)	M	380	32.07	10.16 - 54.30	44.14	0.167	0.919
	F	360	32.39	5.65 - 58.93	53.28		
	M&F	**740**	**32.20**	**9.92 - 54.60**	**44.68**		
ALP (U/L)	M	380	87.97	61.63 - 114.31	52.68	0.461	0.709
	F	360	87.97	58.21 - 114.23	56.02		
	M&F	**740**	**87.48**	**61.63 - 114.31**	**52.68**		
ALT (U/L)	M	380	32.23	11.18 - 57.20	46.02	0.896	0.443
	F	360	32.29	10.24 - 56.84	46.60		
	M&F	**740**	**33.11**	**10.75 - 57.80**	**47.05**		
D-BIL (μmol/L)	M	380	2.13	0.43 - 3.53	3.10	2.640	0.152
	F	360	2.20	0.21 - 4.19	3.98		
	M&F	**740**	**2.17**	**0.46 - 3.88**	**3.42**		
T-BIL (μmol/L)	M	380	42.17	12.98 - 71.78	58.80	0.158	0.924
	F	360	42.82	12.19 - 73.45	61.26		
	M&F	**740**	**42.48**	**12.6 - 72.56**	**59.96**		
TP (g/L)	M	**380**	**43.25**	**30.78 - 55.72**	**24.94**	2.809	**0.039**
	F	**360**	**42.26**	**29.95 - 54.57**	**24.62**		
	M&F	740	42.77	30.38 - 55.18	24.80		
GGT (U/L)	M	380	73.58	18.82 - 128.34	109.52	0.399	0.753
	F	360	68.10	8.44 - 127.76	119.32		
	M&F	**740**	**70.92**	**13.51 - 128.33**	**114.82**		

Results expressed as mean values for the number of subjects indicated under the column labelled N. The sex difference is significant at $p < 0.05$; Sig = significance; RI = reference interval

Table 3: Reference intervals for UREA, CRE, Na, Cl and K for children and adolescents of ages 1-17 years in Meru County

Analyte (unit)	Sex	N	Mean	Reference Interval	IV	Difference between M&F	
						F value	Sig
UREA (mmol/L)	M	380	4.46	2.51-6.67	4.16	-1.058	0.290
	F	360	4.18	2.42-5.94	3.52		
	M&F	**740**	**4.32**	**2.55-6.50**	**3.95**		
CRE (µmol/L)	M	380	81.57	56.29-105.13	48.84	-1.413	0.158
	F	360	80.71	55.52-107.32	51.80		
	M&F	**740**	**81.13**	**56.04-106.22**	**50.18**		
Na (mmol/L)	M	**380**	**138.72**	**133.81-150.71**	**16.90**	-2.945	**0.003**
	F	**360**	**138.41**	**128.94-147.88**	**18.94**		
	M&F	740	138.72	130.98-149.80	18.82		
K (mmol/L)	M	**380**	**4.08**	**3.10-6.06**	**2.96**	-2.64	**0.009**
	F	**360**	**4.05**	**3.13-4.97**	**1.84**		
	M&F	740	4.07	3.11-5.03	1.92		
Cl (mmol/L)	M	380	101.31	96.51-106.11	9.60	-1.343	0.179
	F	360	101.10	96.10-106.10	10.00		
	M&F	**740**	**101.21**	**96.11-106.11**	**10.02**		

Results expressed as mean values for the number of subjects indicated under the column labelled N. The sex difference is significant at $p < 0.05$; Sig = significance; RI = reference interval

4.2 Age dependent reference intervals for kidney and liver function tests for children and adolescents of age 1-17 years in Meru County

The participants were divided into four age categories as follows: (a) Category 1 (1-5 years), (b) Category 2 (6-10 years), (c) Category 3 (11-15 years), and (d) Category 4 (Above 15 years). Reference range differences between males and females were determined for each age category by using ANOVA and post ANOVA tests where p-values less than 0.05 were considered statistically significant. Where there was no significant difference observed across the age categories, the mean value of the total number of subjects was considered to be

the reference value. Tables 4 and 5 show combined age and/or sex specific reference values for each parameter based on the p-values for the difference between male and female participants.

Results of this study indicate that the reference intervals for CREAT, UREA, ALB, ALP, GGT, AST, ALT, D-BIL, T-BIL and Cl are age and sex independent. Further, for K, Na, and TP, the reference intervals for females are age dependent, while those of males are age independent. For the combined males and females, the reference intervals for TP and T-BIL are age dependent.

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Table 4: Age dependent reference intervals for CRE, UREA, Na, K and Cl for children and adolescents of ages 1-17 years in Meru County

Analyte	Sex	Age categories (years)									
		N	1-5	N	6-10	N	11-15	N	>15	N	ALL
CREAT	F	63	79.97±12.76	102	83.25±13.61	131	81.29±13.05	51	80.87±12.90	360	81.57±13.14
µmol/L	M	96	81.43±13.33	107	81.86±12.76	131	79.35±12.07	59	80.56±11.10	380	80.71±12.46
	M&F	159	80.85±13.09	209	82.75±13.19	262	80.32±12.58	110	80.73±12.05	740	81.13±12.80
UREA	F	63	4.11±0.97	102	4.23±0.85	131	4.16±0.84	51	4.21±1.05	360	4.18±0.90
µmol/L	M	96	4.15±1.41	107	5.05±9.04	131	4.26±1.01	59	4.38±1.48	380	4.46±4.80
	M&F	159	4.13±1.24	209	4.63±6.34	262	4.21±0.93	110	4.29±1.24	740	4.32±3.50
Na	F	63	138.87±4.66^{b}	102	139.13±4.27^{b}	131	138.31±4.98^{ab}	51	136.83±5.33^{a}	360	138.41±4.83
mmol/L	M	96	138.22±3.79	107	139.71±4.09	131	139.63±4.67	59	139.74±4.11	380	142.26±4.31
	M&F	159	138.49±4.16	209	139.42±3.94	262	138.97±4.87	110	138.18±5.00	740	140.39±4.80
K	F	63	4.06±0.40^{a*}	102	4.05±0.50^{ab}	131	4.13±0.48^{b}	51	4.05±0.43^{ab}	360	4.11±0.47*
mmol/L	M	96	4.25±0.54	107	4.08±0.51	131	4.09±0.48	59	4.00±0.48	380	4.22±0.50
	M&F	159	4.05±0.50	209	4.06±0.50	262	4.10±0.48	110	4.02±0.46	740	4.07±0.49
Cl	F	63	101.11±2.48	102	100.80±2.53	131	101.13±2.55	51	101.41±2.68	360	101.10±2.55
mmol/L	M	96	101.66±2.52	107	101.15±2.23	131	101.15±2.53	59	101.39±2.53	380	101.31±2.45
	M&F	159	101.44±2.51	209	101.02±2.39	262	101.44±2.53	110	101.40±2.60	740	101.21±2.50

Results expressed as Mean ± Standard deviation of the number of subjects indicated in the column labeled N.
Along the rows, values with the same superscript are similar
*Represents significant sex differences in each age category at $p<0.05$

Table 5: Age dependent reference intervals for TP, ALB, ALP and GGT for children and adolescents of ages 1-17 years in Meru County

Analyte	Sex	Age categories (years)								ALL	
		1-5		6-10		11-15		>15			
		N	Mean±SD	N	Mean±SD	N	Mean±SD	N	Mean±SD	N	Mean±SD
TP g/L	F	63	44.22±6.24[b]	107	41.49±6.33[a]	131	42.36±5.95[ab]	51	41.34±6.58[a]	360	42.26±6.28
	M	96	43.28±6.40	102	44.30±6.33	131	42.83±6.11	59	42.18±6.87	380	43.25±6.36
	M&F	159	43.65±6.33[b]	209	44.86±6.47[ab]	262	42.60±6.02[ab]	110	41.72±6.70[a]	740	42.77±6.33
ALB g/L	F	63	38.86±5.56	107	38.86±5.08	131	38.50±4.77	51	37.82±5.22	360	38.56±5.07
	M	96	38.91±5.26	102	38.36±6.17	131	38.46±4.84	59	37.85±5.09	380	38.47±5.35
	M&F	159	38.89±5.36	209	38.62±5.63	262	38.48±4.80	110	37.83±5.13	740	38.51±5.21
ALP U/L	F	63	88.29±12.35*	107	88.19±14.45	131	86.02±14.20	51	85.44±13.89	360	86.97±13.91
	M	96	88.86±14.59	102	88.39±13.58	131	86.54±12.56	59	89.18±13.15	380	87.97±13.44
	M&F	159	88.63±13.71	209	88.29±13.99	262	86.28±13.38	110	87.17±13.62	740	87.48±13.67
GGT U/L	F	63	66.75±31.03	107	71.41±28.25	131	67.73±30.99	51	64.37±32.52*	360	68.10±30.44*
	M	96	74.23±27.40	102	73.33±29.25	131	73.70±28.05	59	72.54±26.75	380	73.58±27.94
	M&F	159	71.26±29.03	209	72.35±28.69	262	70.72±29.65	110	68.16±30.13	740	70.92±29.29
AST U/L	F	63	32.39±12.52	107	32.94±11.42	131	31.98±11.03	51	31.73±12.05	360	32.29±11.55
	M	96	32.07±11.14	102	32.14±11.57	131	32.60±11.17	59	31.79±11.41	380	32.23±11.26
	M&F	159	32.20±11.67	209	32.55±11.48	262	32.26±11.08	110	31.76±11.71	740	32.26±11.40
ALT U/L	F	63	34.23±12.52	107	34.63±11.33	131	33.32±11.45	51	31.32±13.07	360	33.54±11.89
	M	96	31.36±11.92	102	31.98±11.63	131	34.84±28.45	59	31.09±12.95	380	32.69±19.34
	M&F	159	32.50±12.20	209	33.34±11.53	262	34.09±21.66	110	31.21±12.96	740	33.11±16.14
D-BIL µmol/L	F	63	2.53±2.54	107	2.35±2.77	131	1.98±1.03	51	2.05±0.92	360	2.20±1.99
	M	96	2.14±1.00	102	2.02±1.23	131	2.11±0.86	59	2.42±2.81	380	2.13±1.40
	M&F	159	2.30±1.78	209	2.19±2.16	262	2.05±0.95	110	2.22±2.03	740	2.17±1.71
T-BIL µmol/L	F	63	43.70±27.40	107	45.40±29.28	131	43.85±32.65	51	34.88±31.16	360	42.82±30.63
	M	96	44.25±29.16	102	43.73±28.44	131	41.59±30.27	59	36.61±27.93	380	42.17±29.19
	M&F	159	44.03±28.39[ab]	209	44.59±28.81[b]	262	42.72±31.44[ab]	110	35.69±29.59[a]	740	42.48±29.88

4.3 Comparison of established biochemical parameters reference intervals of 1-17 year olds with the adult reference intervals used in Meru Level Five Hospital (ML5H)

Table 6 compares the established reference intervals for liver and renal function tests with those used in ML5H. This was done by comparing the values for the lower and upper reference limits as well as the interval values for each analyte. From the study, it was noted that significant differences exist between the reference intervals developed for children and adolescents and those for adults in use at Meru Level Five Hospital. Out of the thirteen parameters studied, only nine parameters had reference intervals different from those used to interpret laboratory results for adults and children in ML5H. These were ALT, AST, ALP, GGT, D-BIL, T-BIL, UREA, CRE and K. The other four studied parameters had similar reference intervals for children and adolescents, as those used in ML5H for children and adults. These reference intervals are therefore suitable for use in interpreting test results for both children and adolescents, and adults in ML5H. These are ALB, TP, Cl and Na.

Table 6: Comparison of established reference intervals by sex of 1-17 year olds in Meru County with reference intervals in literature.

Parameter	**Reference interval (This study)**		Reference interval used in ML5H RI (M&F) [**ADULTS**]	
		IV		IV
ALB (g/L)	**28.30-48.72**	20.42	**30-48**	18
ALP (U/L)	61.63-114.31	52.68	47-406	359
ALT (U/L)	10.75-57.80	47.05	0-50	50
AST (U/L)	9.92-54.60	44.68	0-50	50
D-BIL(μmol/L)	0.46-3.88	3.42	≤ 3.4	3.4
T-BIL (mol/L)	12.60-72.56	59.96	1.7-21	19.3
GGT (U/L)	13.51-128.33	114.82	1.0-132	131
TP (g/L)	**30.78-55.72 (M)**	24.94	**33-56**	23
	29.95-54.57 (F)	24.62	**33-56**	23
UREA(mmol/L	2.55-6.50	3.95	2.8-7.2	4.4
Cl (mmol/L)	**96.11-106.11**	10.02	**97-105**	8
CRE(μmol/L)	56.04-106.22	50.18	62-115	53
K (mmol/L)	3.10-6.06 (M)	2.96	3.5-5.0	1.5
	3.13-4.97 (F)	1.84	3.5-5.0	1.5
Na(mmol/L)	**133.81-150.71 (M)**	16.90	135-150	15
	128.94-147.88 (F)	18.94	135-150	15

Table 7: Quality Control (QC) report for TP, ALB, ALP, ALT, AST, D-BIL and T-BIL values for children and adolescents of ages 1-17 years in Meru County

Analyte (unit)	QC Type	Assigned QC Report		Study QC Report	
		Mean ± SD	% CV	Mean ± SD	% CV
TP (g/L)	PPU	46±2	4.3	46.6±2.3	2.5
	PNU	68±3.4	5.07	67.5±3.4	5.03
ALB (g/L)	PPU	29.6±2	6.07	46±2.3	5
	PNU	48.8±2	4.1	102±5	**4.90**
ALP (U/L)	PPU	259±13	5	226.8±5.1	2.23
	PNU	102±5	4.9	83.8±2.6	3.16
ALT (U/L)	PPU	139±7	5.04	143.1±2.5	1.75
	PNU	51±3	5.88	49.3±2	4.09
AST (U/L)	PPU	122±6	4.92	145.5±1.9	1.32
	PNU	38±2	5.26	44.8±1.3	2.99
D-BIL (µmol/L)	PPU	33.7±2.5	7.42	36.32±0.75	2.06
	PNU	12.7±1.9	14.96	8.52±0.29	3.35
T-BIL (µmol/L)	PPU	66.3±4.9	7.39	93.5±3.1	3.27
	PNU	17.1±1	5.85	21.7±0.9	4.26
GGT (U/L)	PPU	259±13	5.02	264±10	3.77
	PNU	53±3	**5.66**	53±3	**6.30**

PPU: This is quality control for pathological parameters.
PNU: This was quality control for normal parameters (non- pathological)
% CV: coefficient of variation

Table 8: Quality Control report for UREA, CRE, Na, K and Cl for children and adolescents of ages 1-17 years in Meru County

Analyte (unit)	QC Type	Assigned QC Report		Study QC Report	
		Mean ± SD	% CV	Mean ± SD	% CV
UREA (mmol/L)	PPU	26.3±1.3	4.94	22.5±1.9	**8.31**
	PNU	7.4±0.4	5.40	7.0±0.3	4.83
CRE (mmol/L)	PPU	398±20	5.03	422±20	4.65
	PNU	92±5	5.43	93±5	5.56
Na (mmol/L)	PPU	144±4	2.80	144±2.5	1.7
	PNU	124.3±2.1	1.70	129±5	**3.87**
K (mmol/L)	PPU	5.2±0.6	3.00	6.5±0.1	2.0
	PNU	5.2±0.6	3.00	4±0.6	1.5
Cl (mmol/L)	PPU	116±3	2.60	115.8±2.9	2.5
	PNU	85.3±2.6	3.10	105±5	**4.76**

PPU: This is quality control for pathological parameters.
PNU: This was quality control for normal parameters (non-pathological)
% CV: Coefficient of variation

CHAPTER FIVE

DISCUSSION, CONCLUSIONS AND RECOMENDATIONS

5.1 DISCUSSION

From the study, it was noted that reference intervals that are used in management of children and adolescents differ with those that were established from the study, thus qualify the need to have each laboratory establishing reference values that are specific to its population as required by the NCCLS. Correct interpretation of biochemical data for patients is critical in the correct diagnosis of illnesses. In Meru County, accurate reference intervals for these biological data that closely relate to the subjects under investigation have not been reported previously and diagnosis is often based on data obtained from subjects/patients outside Kenya. This is therefore the pioneer clinical chemistry reference intervals study for the population of 1 to 17 years in Meru County, Kenya using 740 samples, 380 males and 360 females. The number of participants in each category exceeded the minimum number of 120 participants per subgroup required for 95% reference interval determination as recommended by CLSI (2000). The tests were done using the same analytical methods and results expressed in the same units as those found in the literature for easy comparisons. Eexternal and internal quality control methods were closely followed and monitored throughout the study so as to ensure accuracy and precision of the test results (Gahutu and Wane, 2006) in addition to following all the standard operating procedures at the Meru Level Five Hospital..

All the parameters studied portrayed differences in the reference intervals obtained. ALT, AST, ALP, GGT and T-BIL all showed higher lower reference range limits than those used in the hospital laboratory. ALT and AST had higher upper reference interval limits compared to those in use at the hospital (ALT upper reference interval: M=57.20, F=56.84; ML5H upper reference limit=50, AST: M=54.3, F =58.93 ML5H = 50). The lower reference range limits of the enzymes ALT and AST were observed to be as high as ten times more than the hospital's (ALT: M=11.18, F=10.24 ; AST: M=10.16, F=5.65; ML5H=0). The enzyme ALP showed a significantly higher lower limit and a significantly lower upper limit as compared to the hospital's range (lower limit: M= 61.63, F = 58.21, ML5H= 47; upper limit: M =114.31, F = 114.23, ML5H = 406). Thus, the reference interval for ALP was significantly shorter compared to that being used in the ML5H laboratory. This was also observed in GGT (lower limit: M = 18.82, F = 8.44, ML5H = 1.0; upper limit: M = 128.34, F = 127.76, ML5H = 132). CRE had both lower and upper reference limits that were lower than those in use at the hospital (lower limit: M=56.29, F=55.32, ML5H=62; upper limit: M = 105.13, F = 107.32, ML5H = 115).

Na and K showed significant differences in females across the four age categories. Male candidates in age categories 3 (11-15 years) and 4 (> 15 years) had higher values of Na than their female counterparts (11-15 years: M = 139.63, F = 138.31; > 15 years: M = 139.74, F = 136.83). This was also observed in K

where males in age category 1 (1 - 5years) had higher values of the analyte compared to the females (M= 4.25, F= 4.06). In age category 1 (1 - 5 years) males had a significantly higher level of CRE than their female counterparts (M= 81.43, F=79.97) while in age categories 2 (6 - 10 years) and 3 (11 - 15 years) females had higher values of the same analyte than males (6-10 years: M=81.86, F= 83.25; 11-15 years: M= 79.35, F= 81.29). All age categories showed high levels of UREA in males than in females. This difference in the levels of UREA was especially significant in the age category 2 (6 - 10 years) (M= 5.05, F= 4.23). Chloride was relatively constant across the age categories. However, males in age category 2 had a significantly higher values compared to females in the same category (M=101.15, F= 100.80).

All Liver Function Tests (LFTs) except ALB showed significant sex and age related differences. Females in age categories 1 (1 - 5 years) and 2 (6 - 10 years) had higher levels of D-BIL as compared to males (1-5 years: M=2.14, F= 2.53; 6-10 years: M=2.02, F=2.35). In age categories 3 (11 - 15 years) and 4 (> 15 years), males showed higher values of the analyte as compared to females (11-15 years: M=2.11, F=1.98; > 15 years: M=2.42, F=2.05). The levels of T-BIL decreased progressively with increase in age, with age category 4 (> 15 years) having the lowest values of the analyte for both males and females. However, females in age categories 2 (6 - 10 years) and 3 (11 - 15 years) had higher values than males in the same age categories (6-10 years: M= 43.73, F= 45.40; 11-15 years: M= 41.59, F= 43.85)

Female infants and children had higher values of the enzymes AST and ALT. The values of AST decreased progressively with increasing age for both males and females. Age differences were observed in the enzyme ALT with age categories 1 (1 - 5 years) and 2 (6 - 10 years) registering higher values in females than in males (1-5 years: M=31.36, F=34.23; 6-10 years: M=31.98, F=34.63). All age categories exhibited sex differences in TP, ALP and GGT with males showing higher values than females. Females in age category 4 (> 15 years) had significantly lower values of ALP and GGT as compared to their male counterparts and other age categories. Significant sex differences in GGT levels were observed in the age category 2 (6 - 10 years) (M=73.33, F= 71.41). ALB did not show any significant changes with increasing age.

In the study, it was established that there exists no reference ranges in Meru County for children and adolescents of 1-17 years for six out of the thirteen parameters studied. During clinical trials and routine assessment, this group of the population was found to be considered together with the adult population. Out of the thirteen parameters studied only seven were found to have reference ranges specific to children, though it does not define the ages covered by that category. These parameters include ALB, ALT, AST, GGT, ALP, TP and CRE. There were no reference range values available for T-BIL, D-BIL, UREA and electrolytes (Na, K, Cl) for this study population therefore the reference values developed in this study were compared against those for the adult population available. Assuming that adults and children exhibit the same reference ranges is a big

mistake since reference ranges are known to vary with age. Most studies carried out in Africa report reference intervals for most parameters (creatinine, direct bilirubin and albumin) that are similar with those published for populations from the United States (Zeh *et al.*, 2012). However, certain parameters such as total bilirubin (T-BIL) have upper intervals that are substantially higher (Abbott: 1.7 – 2.1 μmol/L; established: 12.6 – 72.56μmol/L). The reason for high T-BIL in the African populations may be due to a number of factors including hemolysis of red blood cells as a result of malaria infection or sickle cell disease, malnutrition or physical exertion. However, even within the African continent, substantial differences exist because of large differences in climate, location, diet and human genetics (Saathoff *et al.*, 2008).

Higher values of UREA, Na, ALT, D-BIL, AST, TP and T-BIL were observed in males than female adolescents. These gender differences were significantly greater for T-BIL and TP in both adolescents and children while for AST, the difference was significant only among the adolescents and older children. This could be due to differences in muscle mass which affects AST; older male children have developed muscles hence produce more AST. The sex difference observed in serum TP in this study is in contrast to the one found in literature where males and females have the same reference values but agrees with the study done in Rwanda for adult humans (Gahutu and Wane, 2006). ALB, ALT, AST, T-BIL, D-BIL and K have demonstrated higher values compared to the

ranges used in ML5H. This could be due to differences in diet, genetics and analytical methods. Males had higher values of GGT than females; this could be as a result of extra production of the enzyme from the prostate gland in males as compared to females who lack the organ. This result was reported in studies carried out in other East African states (Saathoff *et al.*, 2008; Eller *et al.*, 2008).

Increases or decreases of some biochemical analytes in one sex or the other as age progressed indicate that these parameters are influenced by age. Sex differences observed for Na (p=0.003) and K(p= 0.009) may be due to differences in response to dietary salts brought about by the effects of sex hormone patterns and sex-related genetic factors. Sex difference was also observed in TP (p=0.039) perhaps due to differences in muscle mass in adolescent males than females. Cl values remained relatively constant across the four age groups but exhibited higher upper reference limit values than those reported in literature. This could be attributed to diet and geographical location. ALT and AST have demonstrated higher values for both lower and upper reference values compared to those of manufacturer's range and other locations (ALT: 10.75 - 57.8 U/L; 0 - 50U/L ; AST: 9.92 - 54.6U/L; 0 - 50 U/L).

UREA and CRE exhibited variations both per age and sex across all age categories. This indicates that these analytes are both age and sex dependent. Different lifestyles and genetic composition of could also explain the differences

(Manolio et al., 1992). These differences have also been reported from other countries (Olusi and Al-Awadhi, 2002; Saathoff *et al.*, 2008; Eller *et al.*, 2008).

Generally, reference ranges have been shown to vary with populations due to differences in diet, genetics, physical, environmental and socio-economic conditions (Koram *et al.*, 2007). The reference values for the parameters analyzed in this study differ from those used to service the population. This clearly indicates that there is need to determine sex- and age-based reference values that are applicable to specific populations rather than take a set of reference values determined for one population and use them on another population. This will decrease the frequency of values reported as abnormal in otherwise healthy children and adolescents.

The study had the following limitations:

i) While physical examinations were carried out on all the participants in the study and anthropometric measures collected, for some children this information was not used during the analysis of serum biochemistry, thus it might be possible that some participants had mild illnesses.

ii) It was not possible to screen for all medical conditions that would have influenced the biochemical parameters under study for example micronutrient deficiency. However, the fact that a relatively large number of apparently healthy children and adolescents was used in the study makes it more likely to be representative.

5.2 CONCLUSIONS

The study has established the first age specific reference ranges for some routinely analysed biochemical parameters in the clinical chemistry laboratory of Meru level five hospital. These parameters are total bilirubin, direct bilirubin, total protein, albumin, ALP, AST, ALT and GGT. There was little or no information found in literature for this population thus comparisons were done with those of adult populations. This was also observed in the clinical setting for most of the biochemical parameters studied, with adult values being used to interpret the results for this group of the population. Out of the thirteen parameters studied, only seven of them had distinct values for adults and children as used in the hospital. These are ALT, AST, ALP, GGT, albumin, total protein and creatinine. The other six parameters studied didn't have distinct values for children and adults and the same reference values were being be used when interpreting test results for both children and adults. These are T-BIL, D-BIL, UREA, Na, K and Cl and were all found to be different from those used in the hospital particularly for T-BIL that had showed very high values (ML5H range: 1.7 - 2.1μmol/L, established range: 12.6 – 72.56μmol/).

The study also developed sex-specific reference ranges for the thirteen clinical chemistry parameters studied. It was observed that out of the thirteen parameters studied, only two-ALT and AST had different ranges for males and females. Reference ranges are known to vary between males and females because of

differences in sex hormones and muscle mass. This was clearly seen in three of the parameters studied, where there were significant differences for total protein (p=0.039), sodium (p=0.003) and potassium (p=0.009). In the case of these parameters, a different set of values for males and females should be used.

The results of the study show that reference values obtained vary with those from literature and those that are used at Meru Level Five Hospital. The upper limits of serum transaminases, bilirubin, potassium, urea, total protein and albumin for the Kenyan children were higher than those from the Caucasian children. Sex specific differences were observed in TP (p = 0.039), Na (p = 0.003) and K (p = 0.009). Most parameters like ALP had a much shorter reference range compared to that found for Caucasian population (Established: 61.63 – 114.31 U/L; manufacturer's: 47 – 406 U/L) (table 7). This clearly indicates the need to determine population specific ranges instead of using a general range developed using a different population.

Clearly, similar studies of children in Africa should be carried out so as to broaden the present findings thus enabling improved care and conduct of clinical trials. Population-specific reference ranges/values obtained will be useful to achieve accurate, clinically relevant results that will provide true information about the patient's state of health in the region.

5.3 RECOMMENDATIONS

i) Adoption of the study findings upon sufficient clinical trials on the reference population.

ii) Other health and medical research facilities in different counties and regions should develop laboratory specific reference values for the populations that they serve.

iii) The differences observed between the established reference ranges and those from the manufacturers should be investigated further.

iv) Similar studies should be done to determine reference values for other biochemical parameters and in other regions.

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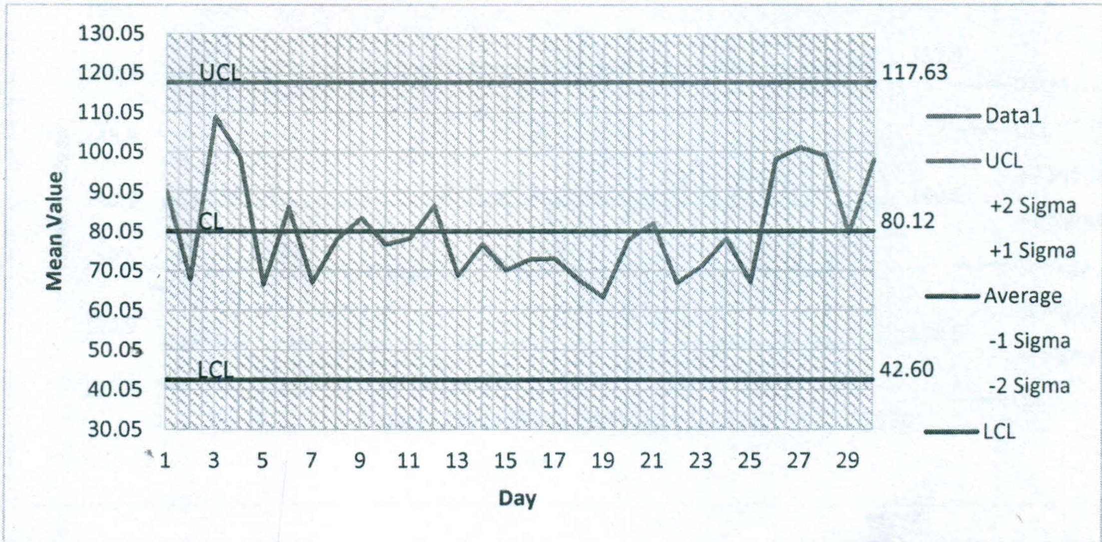
APPENDICES

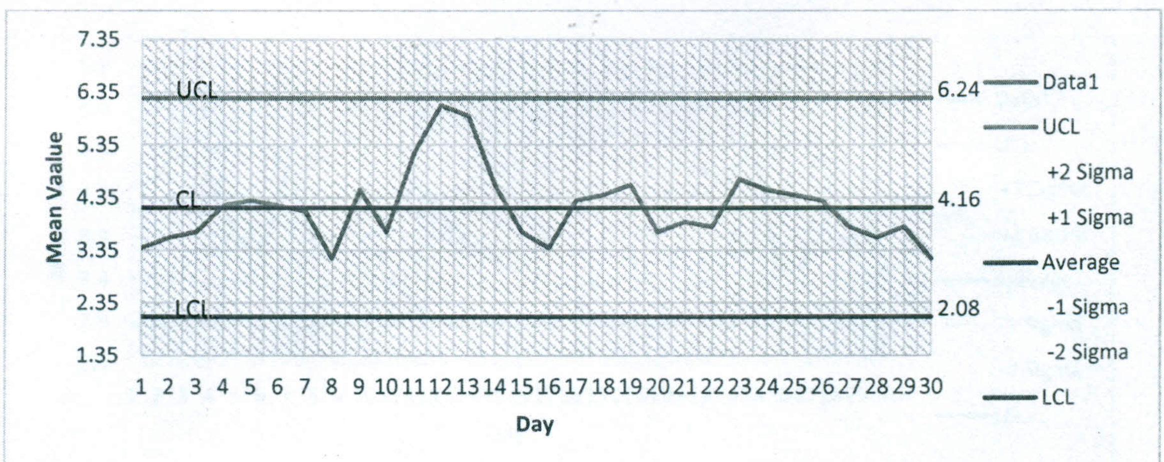
Appendix 1: Levey-Jennings Control Chart for Creatinine

Creatinine (µmol/L)						
-3SD	-2SD	-1SD	Mean	+1SD	+2SD	+3SD
42.73	55.33	68.33	81.13	93.93	106.73	119.53



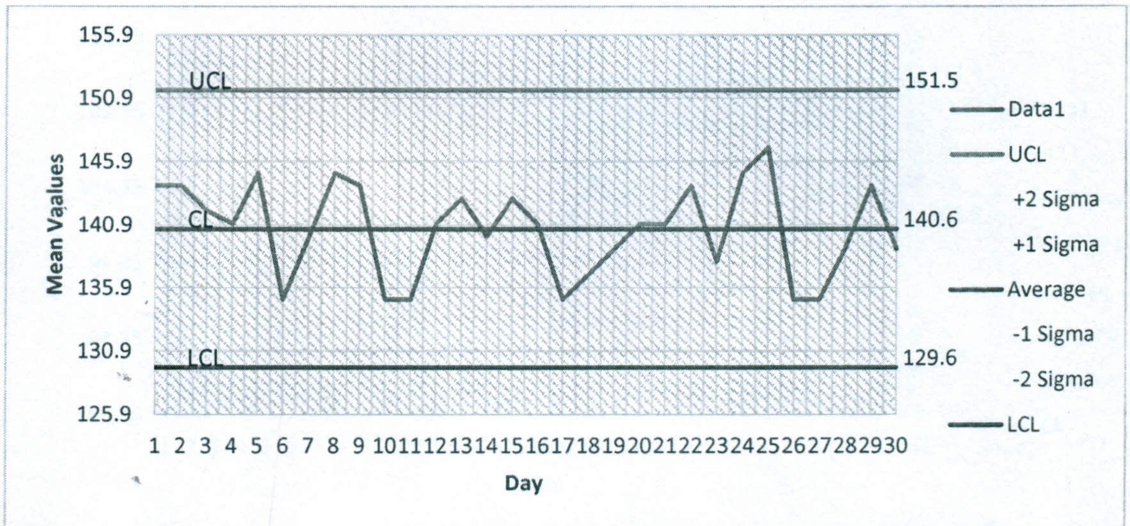
Appendix 2: Levey Jennings Control Chart for UREA

UREA (mmol/L)						
-3SD	-2SD	-1SD	Mean	+1SD	+2SD	+3SD
-6.18	-6.18	0.82	4.32	7.82	11.32	14.82



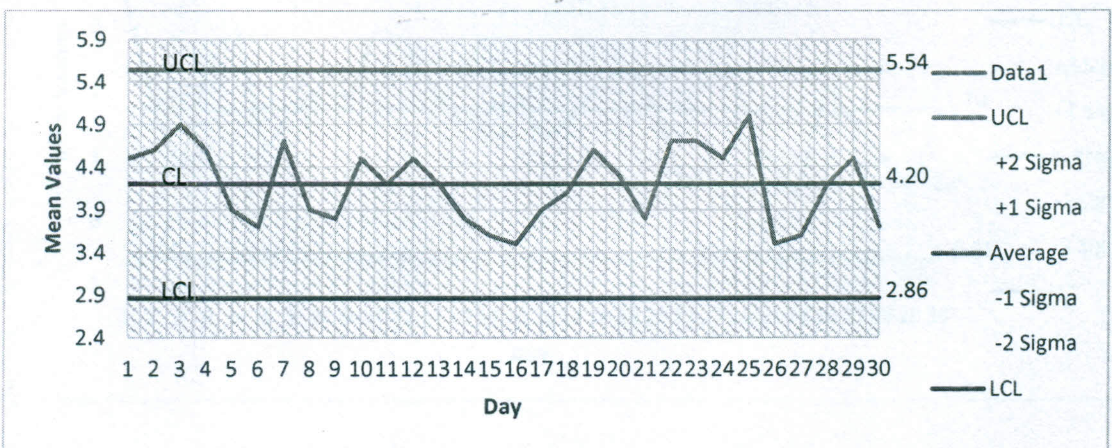
Appendix 3: Levey Jennings Control Chart for Sodium

NA (mmol/L)						
-3SD	-2SD	-1SD	Mean	+1SD	+2SD	+3SD
125.94	130.2	134.46	138.72	142.98	147.24	151.5



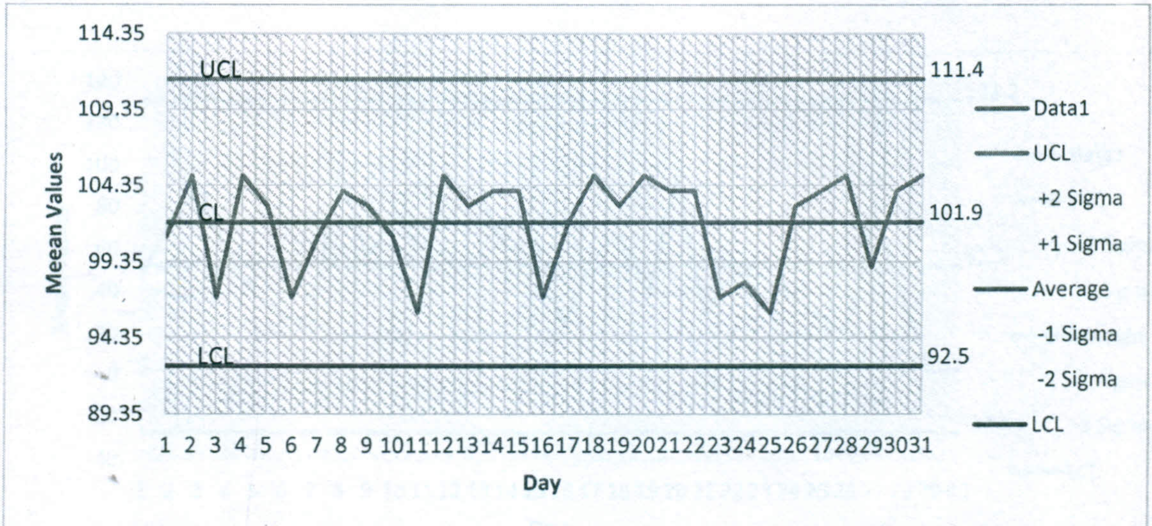
Appendix 4: Levey Jennings Control Chart for Potassium

K (mmol/L)						
-3SD	-2SD	-1SD	Mean	+1SD	+2SD	+3SD
2.6	3.09	3.58	4.07	4.56	5.05	5.54



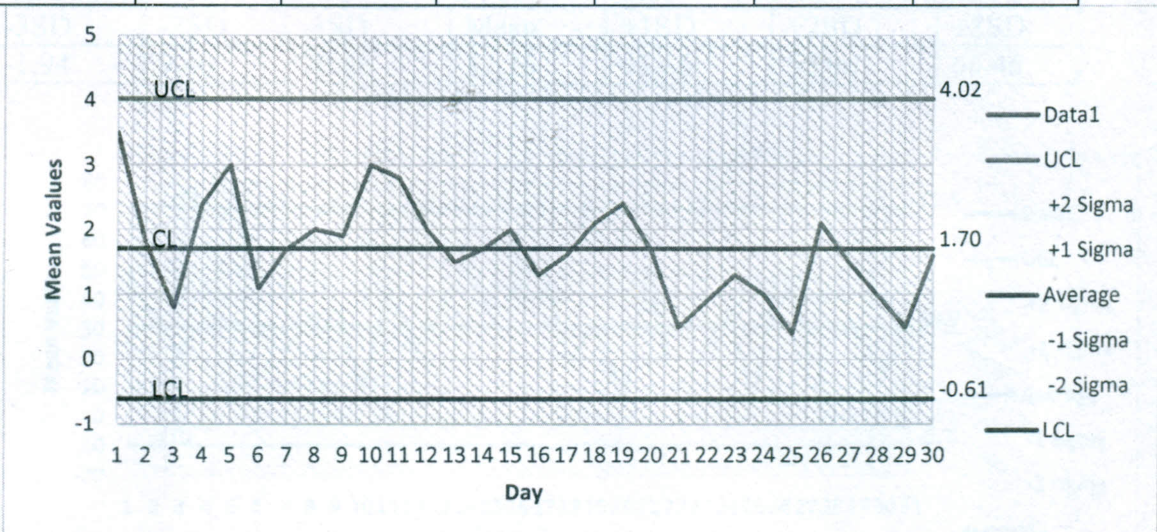
Appendix 5: Levey Jennings Control Chart for Chloride

CL (mmol/L)						
-3SD	-2SD	-1SD	Mean	+1SD	+2SD	+3SD
93.71	96.21	98.71	101.21	103.71	106.21	108.71



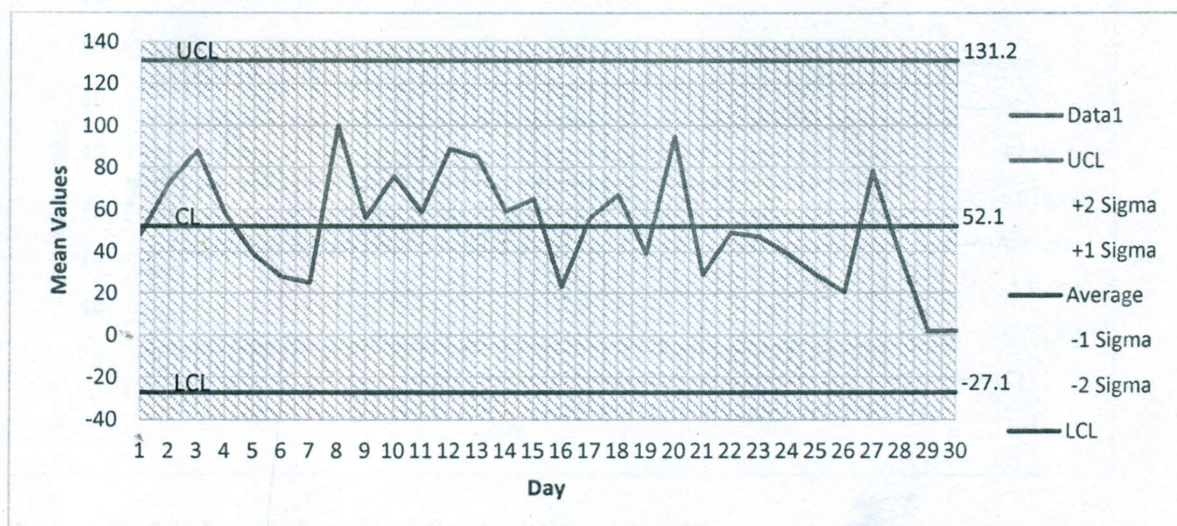
Appendix 6: Levey Jennings Control Chart for Direct Bilirubin

DBIL (µmol/L)						
-3SD	-2SD	-1SD	Mean	+1SD	+2SD	+3SD
-2.96	-1.25	0.46	2.17	3.88	5.59	7.3



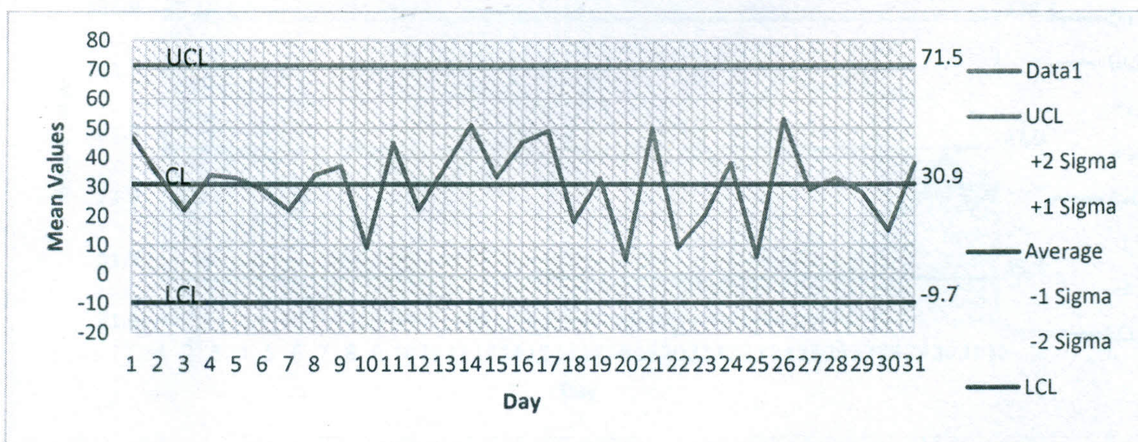
Appendix 7: Levey Jennings Control Chart for TBIL

TBIL (μmol/L)						
-3SD	-2SD	-1SD	Mean	+1SD	+2SD	+3SD
-47.16	-17.28	12.6	42.48	72.36	102.24	132.12



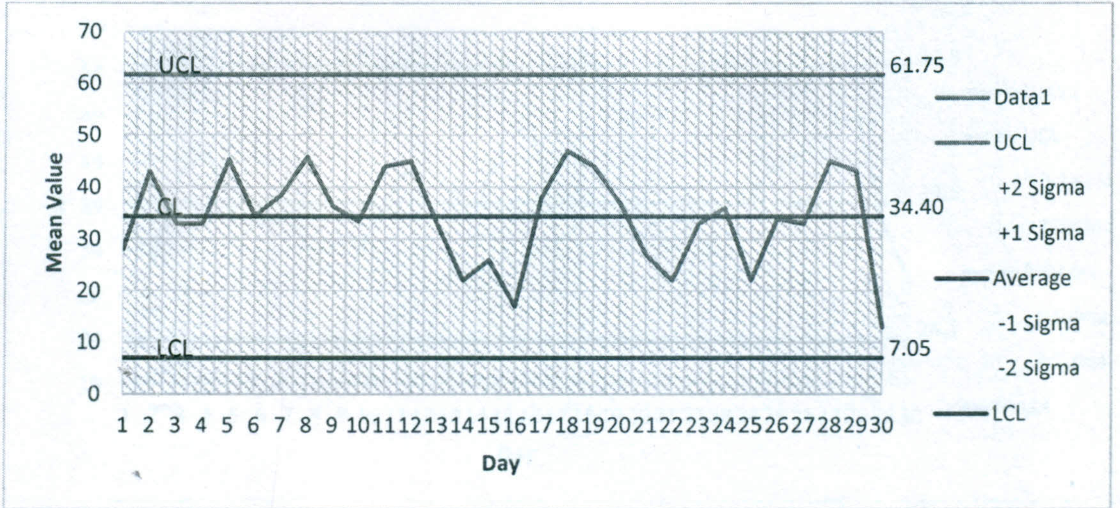
Appendix 8: Levey Jennings Control Chart for AST

AST (U/L)						
-3SD	-2SD	-1SD	Mean	+1SD	+2SD	+3SD
-1.94	9.46	20.86	32.26	43.66	55.06	66.46



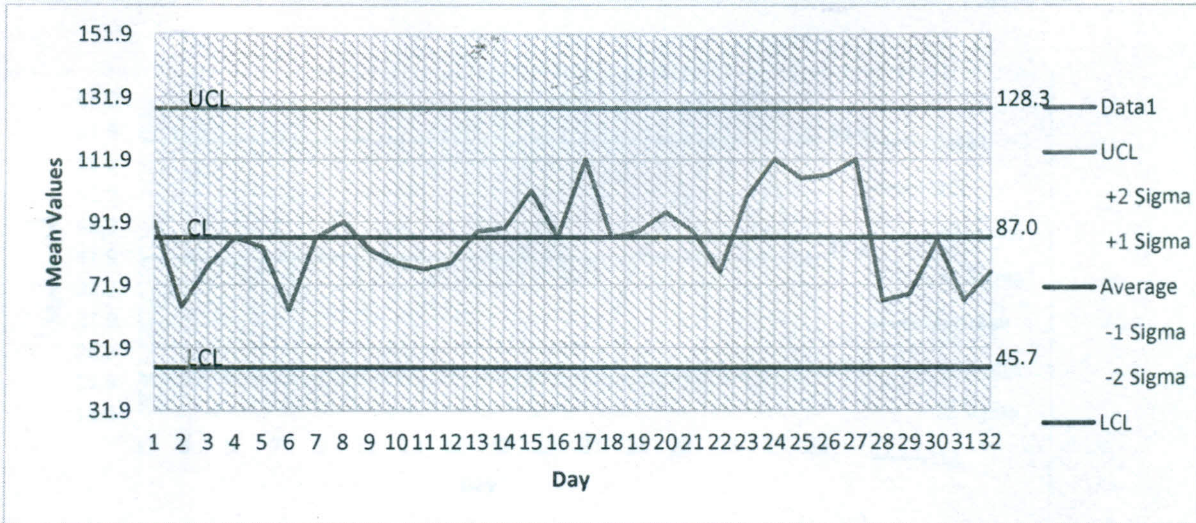
Appendix 9: Levey Jennings Control Chart for ALT

ALT (U/L)						
-3SD	-2SD	-1SD	Mean	+1SD	+2SD	+3SD
-11.11	3.63	18.37	33.11	47.85	62.59	77.33



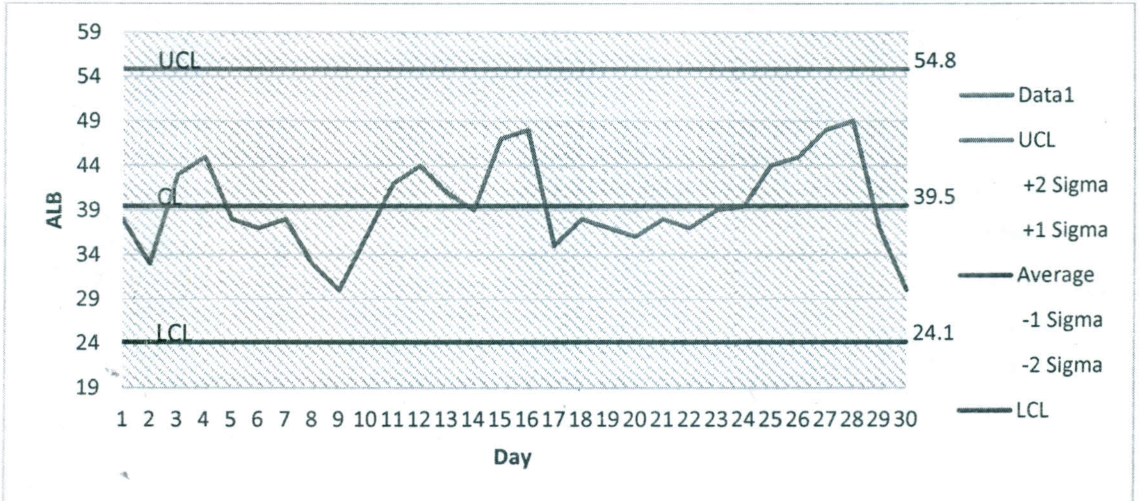
Appendix 10: Levey Jennings Control Chart for ALP

ALP (U/L)						
-3SD	-2SD	-1SD	Mean	+1SD	+2SD	+3SD
46.47	60.14	73.81	87.48	101.15	114.82	128.49



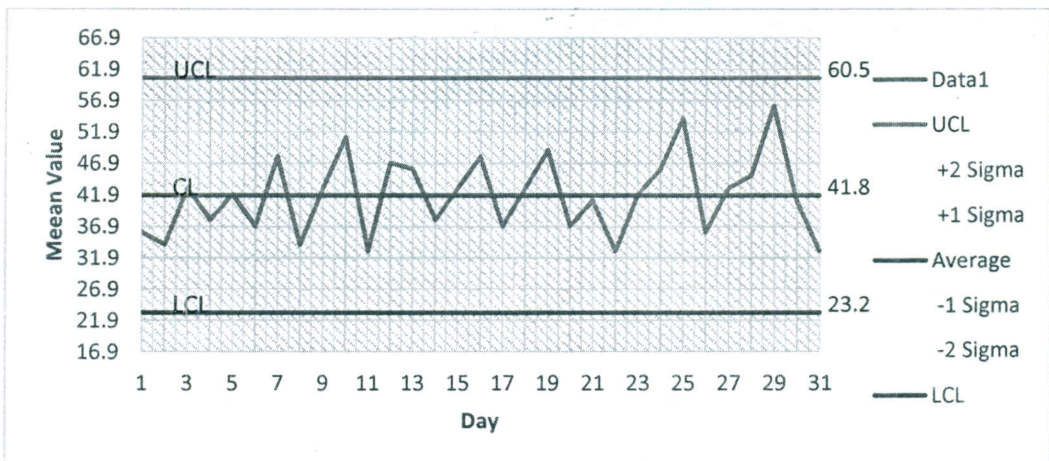
Appendix 11: Levey Jennings Control Chart for Albumin

ALB (g/L)						
-3SD	-2SD	-1SD	Mean	+1SD	+2SD	+3SD
22.88	28.09	33.3	38.51	43.72	48.93	54.14



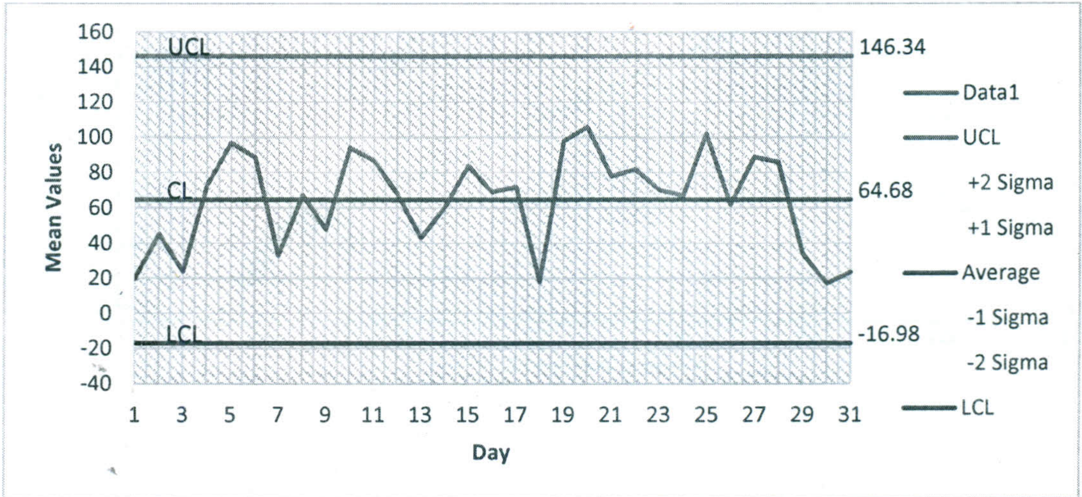
Appendix 12: Levey Jennings Control Chart for Total Protein

TP (g/L)						
-3SD	-2SD	-1SD	Mean	+1SD	+2SD	+3SD
23.78	30.11	36.44	42.77	49.1	55.43	61.76

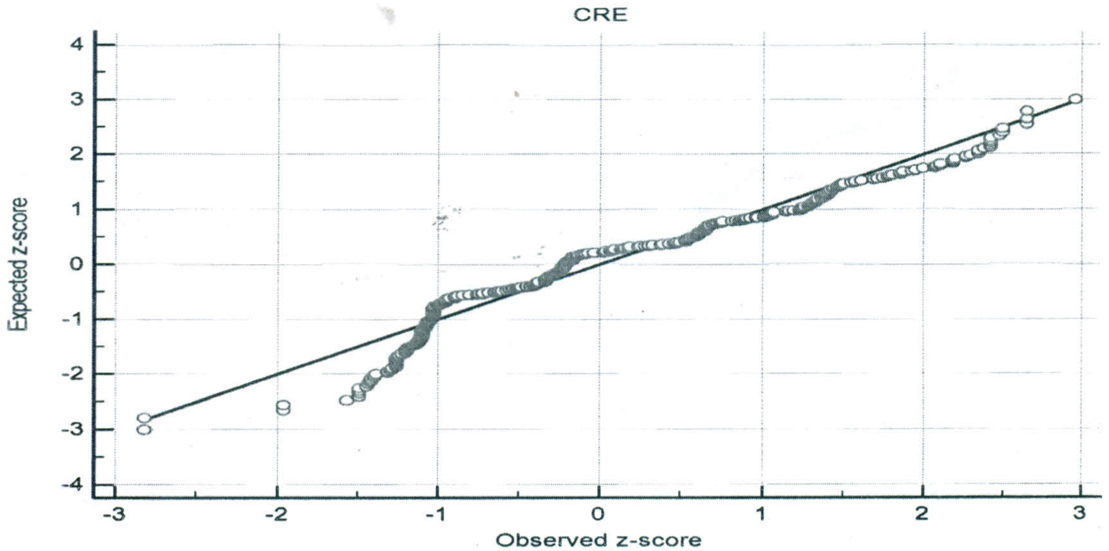


Appendix 13: Levey Jennings Control Chart for GGT

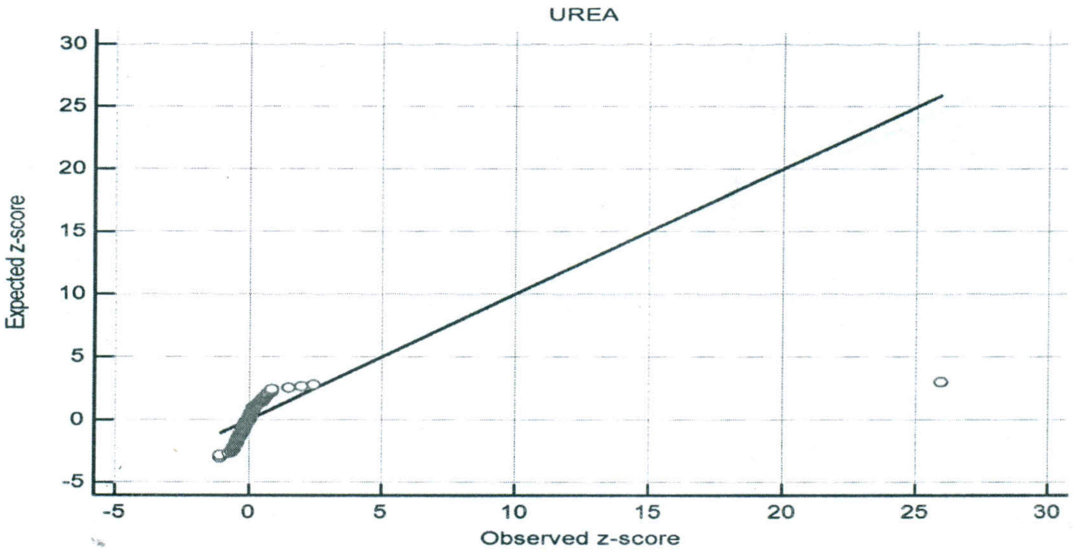
GGT (U/L)						
-3SD	-2SD	-1SD	Mean	+1SD	+2SD	+3SD
-16.95	12.34	41.63	70.92	100.21	129.5	158.79



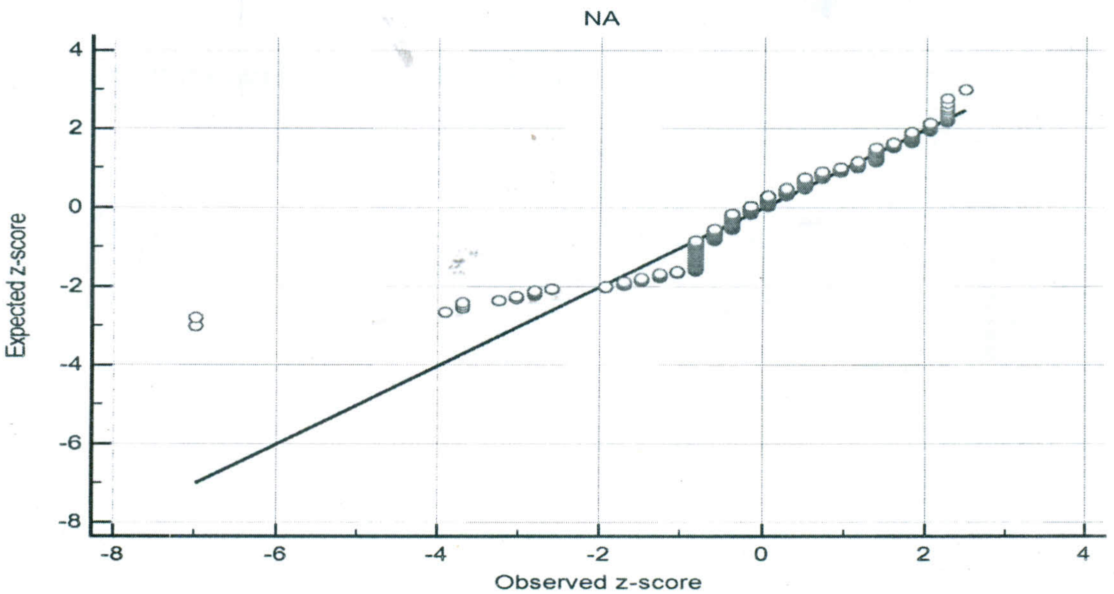
Appendix 14: Normal Q-Q Plot for Creatinine



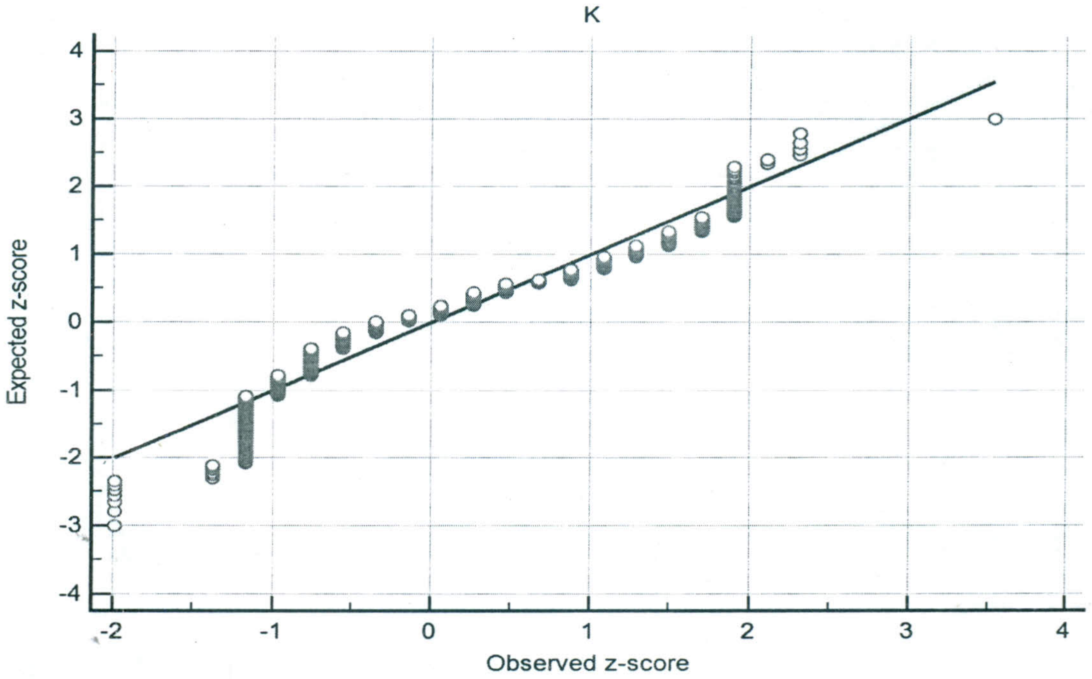
Appendix 15: Normal Q-Q Plot for Urea



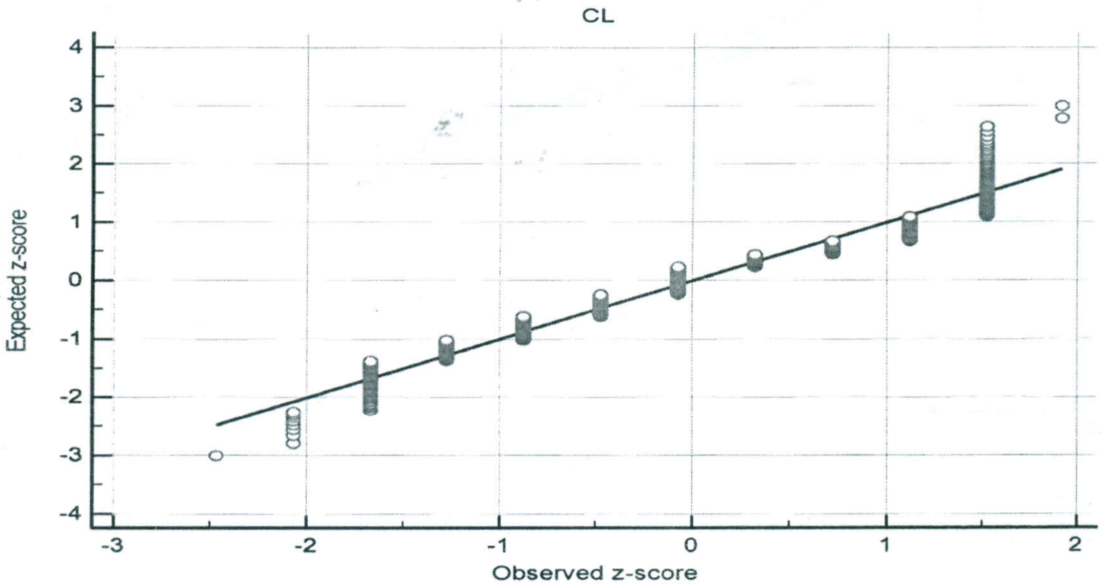
Appendix 16: Normal Q-Q Plot for Sodium



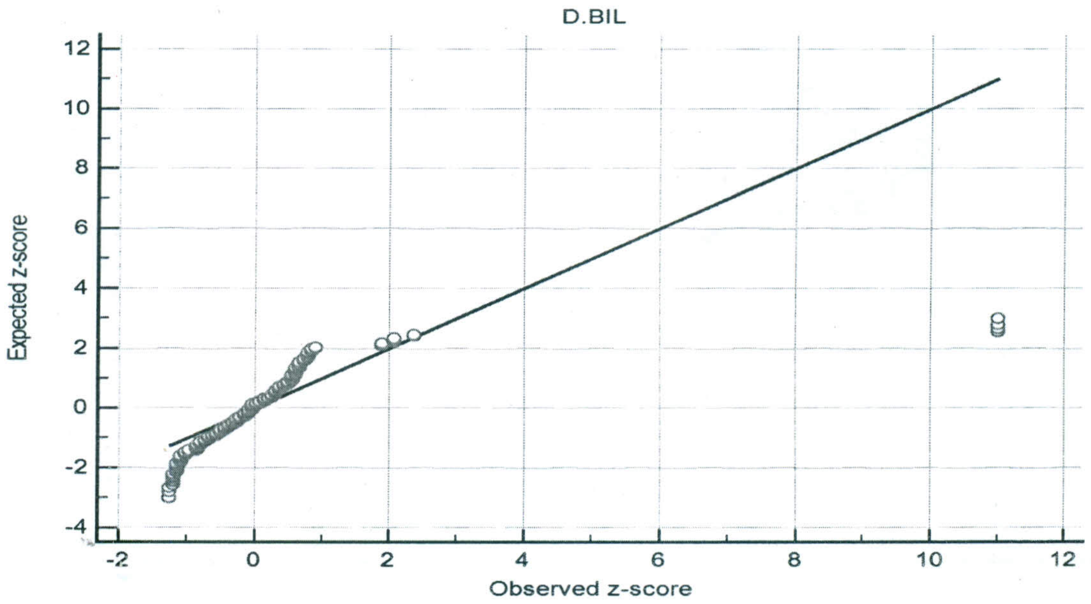
Appendix 17: Normal Q-Q Plot for Potassium



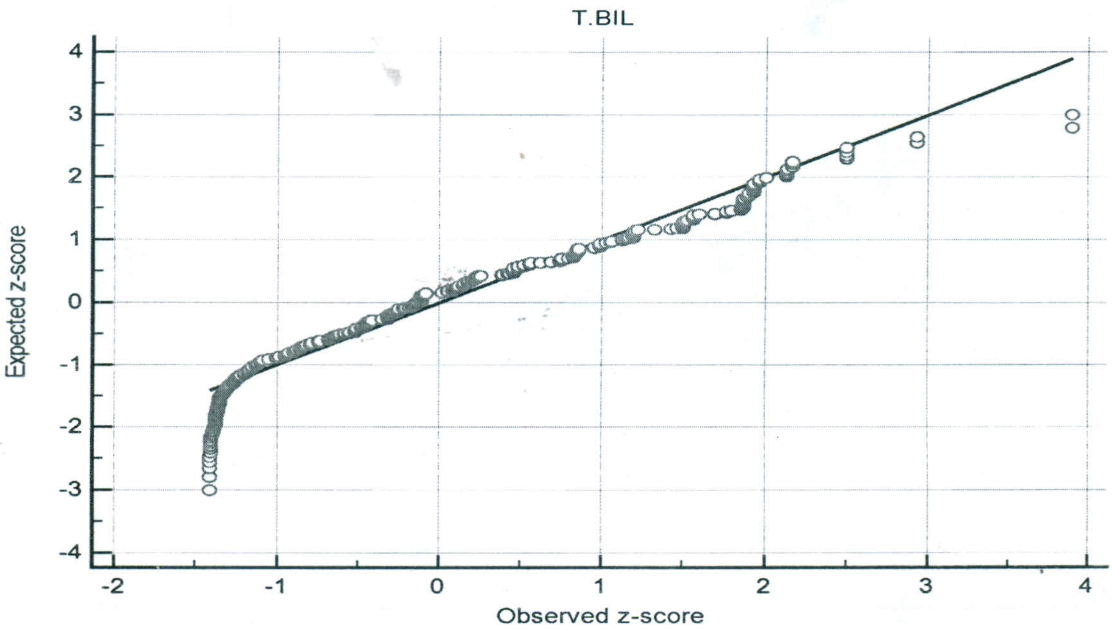
Appendix 18: Normal Q-Q Plot for Chloride



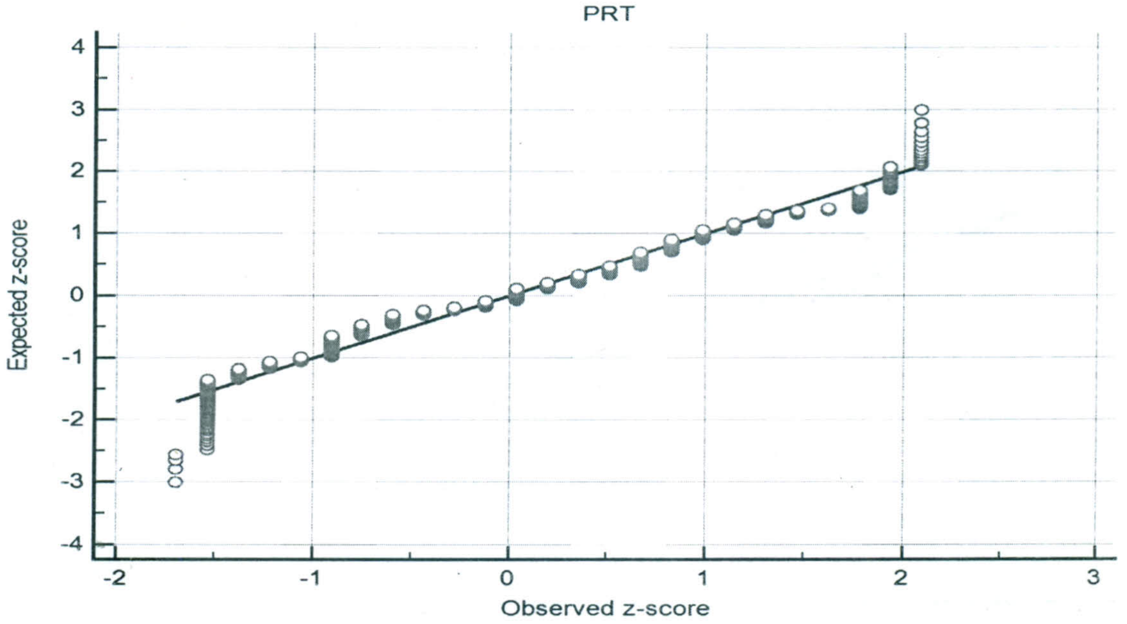
Appendix 19: Normal Q-Q Plot for Direct Bilirubin



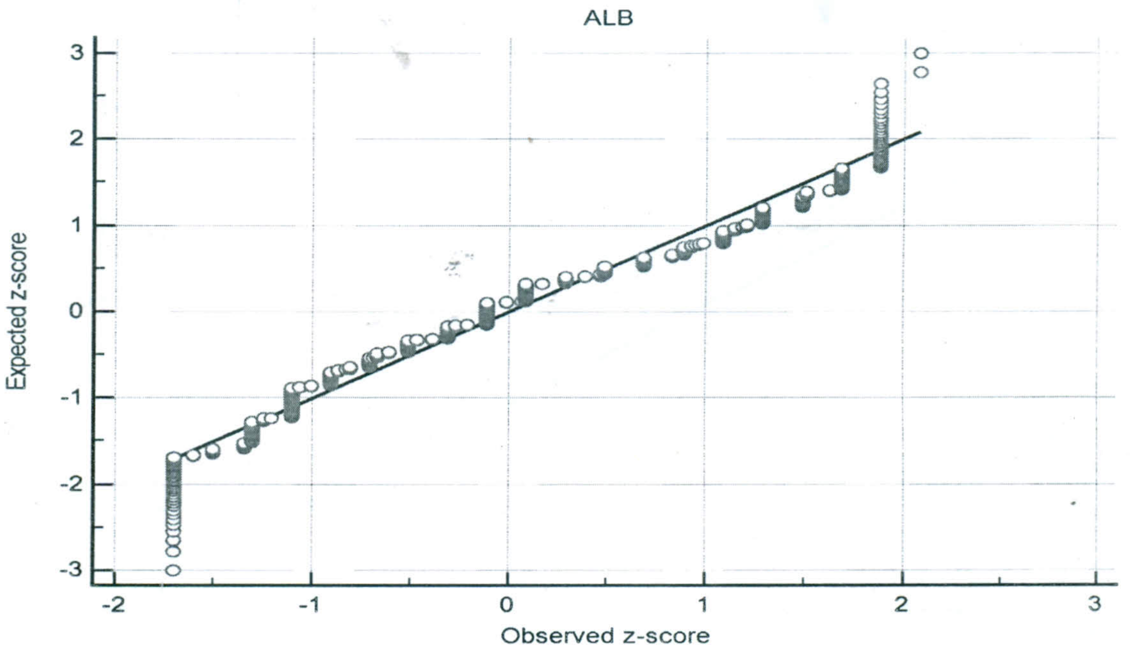
Appendix 20: Normal Q-Q Plot for Total Bilirubin



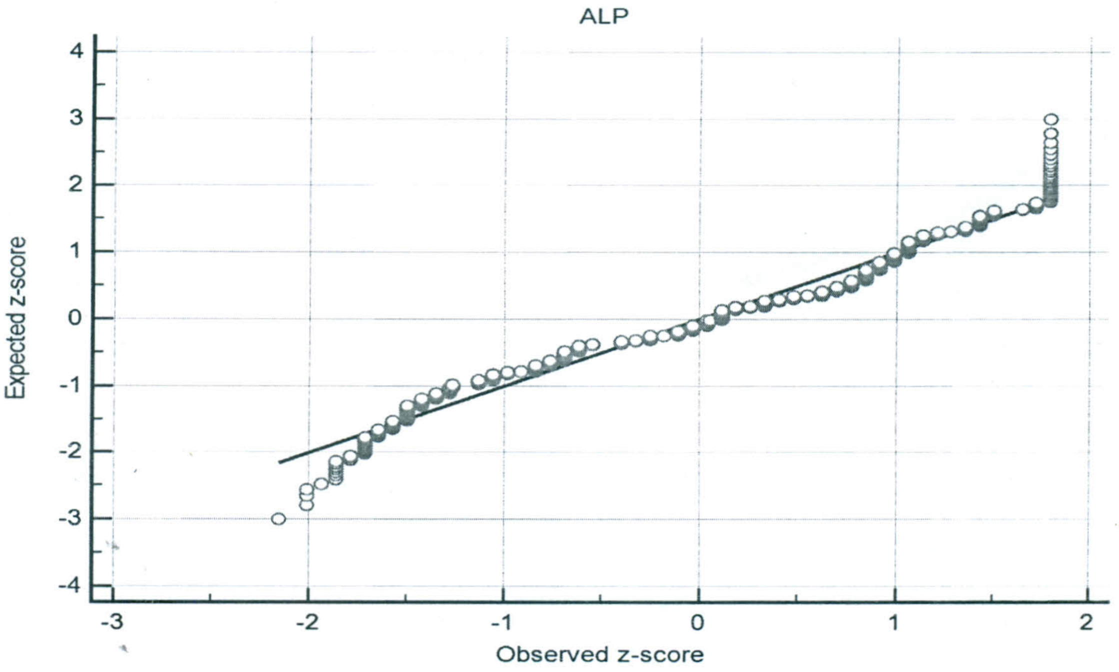
Appendix 21: Normal Q-Q Plot for Total Protein



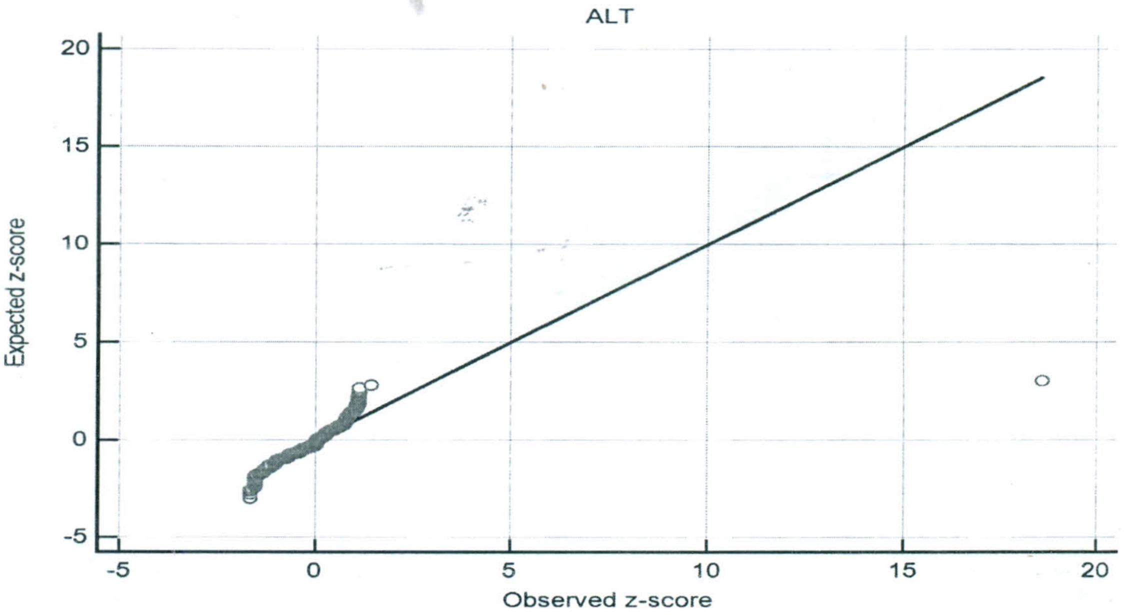
Appendix 22: Normal Q-Q Plot for Albumin



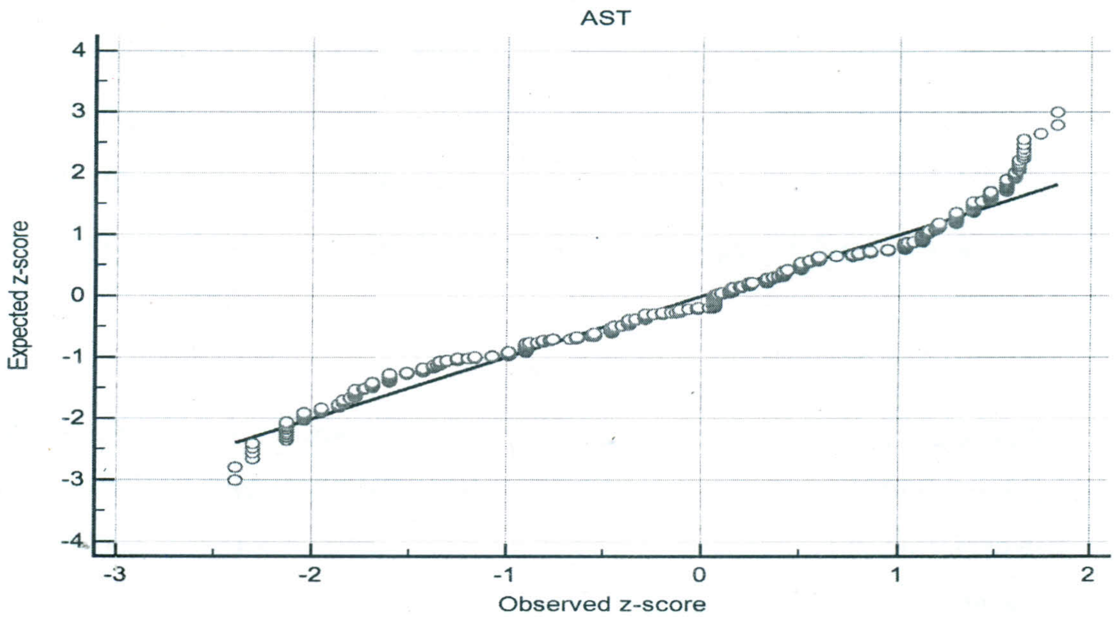
Appendix 23: Normal Q-Q Plot for ALP



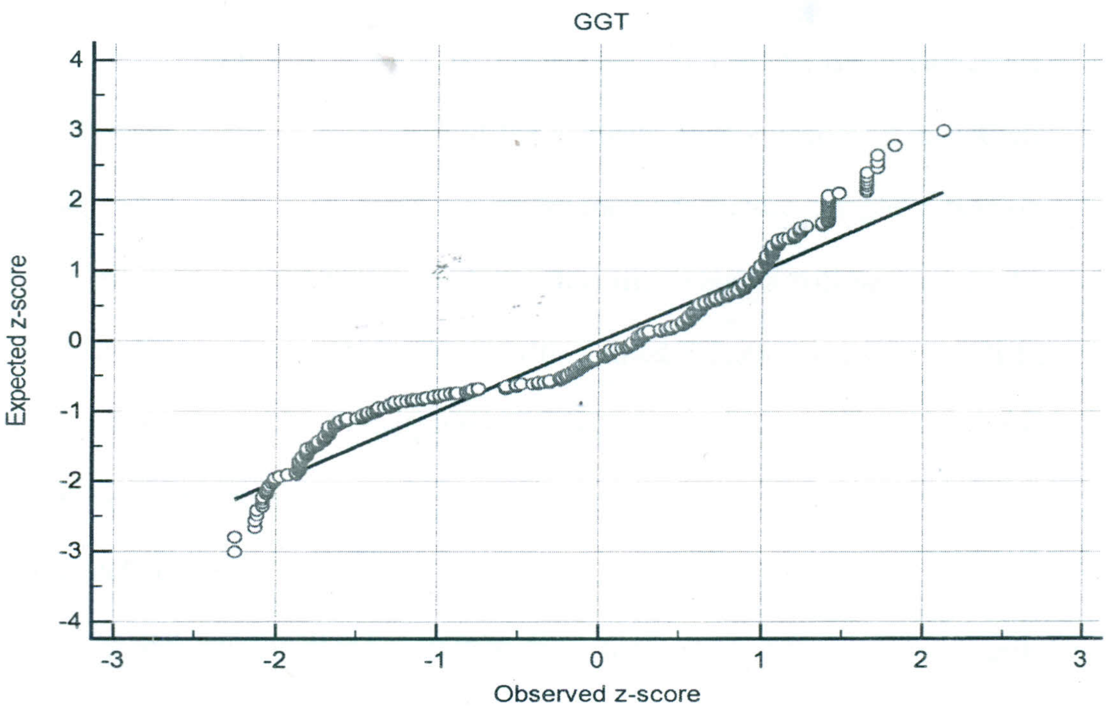
Appendix 24: Normal Q-Q Plot for ALT



Appendix 25: Normal Q-Q Plot for AST



Appendix 26: Normal Q-Q Plot for GGT



Appendix 27: CONSENT FORM

Name of Investigator: Rhoda Kainyu Munene

Postal Address: P.O. Box 1281, Meru.

Cell phone: 0712 360 035.

E-mail: munene.rhoda@gmail.com

Supervisors: Prof. Eliud N. M. Njagi, Dr. Orinda George, Dr. Silas Kiruki

Kenyatta University

P.O. Box 43844-00100, Nairobi

I am Master of Science student at Kenyatta University. I am carrying out a study on biochemical evaluation of children in Meru County to establish certain biochemical parameters. I am kindly requesting you to participate in the study which will also assist our health workers to manage our young children and adolescents better.

Participation

Participation is voluntary and one is free to reject. If you choose to participate, you will need to read and understand the contents of this document then I will provide you with a donor questionnaire to complete before sample collection. Sample will be obtained during blood donation after which it will be screened for HIV, Syphilis and Hepatitis B. Those with limited capacity to consent will be excluded from the study. Participants will include those who will have lived in Meru County for a period not less than six months.

Sample collection

This study will involve blood collection from the vein in the arm with a sterile needle and a syringe. This will only be done once for the entire study period.

Risks and Discomforts

Participants may experience some pain during venipuncture but which I will try as much as possible to minimize. There will be no risks involved since the study requires just 5ml of blood from volunteer blood donors.

Benefits and Costs

Participants will not spend any money on this study. In case the participant is found with any clinical condition he/she will be informed upon his/her own request. This study will be of benefit to the hospital and entire community since its success will aid in proper clinical decision making and treatment of patients.

Care and protection of participants

Great care will be taken to ensure that all the participants do not suffer any injuries during blood collection. This will be done by enlisting the services of a qualified phlebotomist and especially for the infants. For children who will test HIV positive or found to have any other condition, the parent and/or guardian will be informed of the results and will be referred to a health care provider of the parent's choice. Counseling will also be provided by an experienced individual for all participants with seropositive test results. Further, participants with any other abnormal test results will be referred for appropriate care and treatment.

Confidentiality

All information about you will be treated in the strictest confidence and will be given to you or your physician upon request.

I……………………………………..do agree that I have read, been explained to, allowed to ask questions concerning this study, understood and consented.

Signature ………………………………………..Date…………………………....

I……………………a parent / legal guardian of ………………………………do hereby consent for my child to participate in this research study which has been explained to me.

Signature…………………………………………..Date…………………………...

I have clearly explained the above study to the participant and he/she has understood and consented.

Signature of Researcher……………………………Date………………………….

Enquiries

For further explanation and queries please contact the investigator on the above address. For more about your rights contact the Ethical Committee of Kenyatta University P.O Box 43844 - 00100 Nairobi or the Ethical Committee of Meru Level Five Hospital, P.O. Box 8 - 60200 Meru, Kenya.

Appendix 28: Blood Sampling Questionnaire

Name .... Study case number..........................

Date of birth.............................. Place of birth...............................

Sex....................................... Date...

Whether suffered from any of the following ailments	Yes	No	Whether having any of the following conditions	Yes	No
High blood pressure			Pregnancy		
Diabetes mellitus			Lactation		
Renal disease			Family planning devices		
Tuberculosis			Menses		
Any allergy			On any medication		
Epilepsy			Drug abuse		
Stomach ulcers			Cigarette smoking		
Jaundice			Alcohol consumption		
Hepatitis B and C			Recent surgery/hospitalization		
Heart disease			Frequent blood donor		
Surgery					
Malaria					
Syphilis					
HIV/AIDS					

Appendix 29: Kenyatta University Ethics Review Committee Approval

KENYATTA UNIVERSITY
ETHICS REVIEW COMMITTEE

Fax: 8711242/8711575
Email: kuerc.chairman@ku.ac.ke
kuerc.secretary@ku.ac.ke
Website: www.ku.ac.ke

P. O. Box 43844,
Nairobi, 00100
Tel: 8710901/12

Our Ref: **KU/ERC/ APPROVAL/VOL.1** (142)

Date: 14th June, 2018

Rhoda Munene Kainyu
P.O Box 43844-00100
Nairobi

Dear Rhoda,

APPLICATION NUMBER: PKU/829/1895 "ESTABLISHMENT OF REFERNCE RANGES FOR BIOCHEMICAL PARAMETERS IN CHILDREN AGED BETWEEN 1-17 YEARS IN MERU COUNTY, KENYA"

1. IDENTIFICATION OF PROTOCOL

The application before the committee is with a research to **"Establishment of Reference Ranges for Biochemical Parameters in Children Aged Between 1-17 Years In Meru County, Kenya"** received on 15th January, 2018 and discussed on 12th June, 2018.

2. APPLICANT

Rhoda Munene Kainyu

3. SITE

Meru County, Kenya

4. DECISION

The committee has considered the research protocol in accordance with the Kenyatta University Research Policy (section 7.2.1.3) and the Kenyatta University Ethics Review Committee Guidelines and **APPROVED that the research may proceed for a period of ONE year from 12th June , 2018.**

Appendix 30: Ministry of Health Permit to Collect Blood Samples

MINISTRY OF HEALTH

Telegrams: "MEDICAL" Meru
Telephone: Meru 064-32370/1
Fax: 31242
Email: [illegible]
When replying should be sent to
Medical Superintendent
Ref: MRU/MED/GEN/F.6B

MERU LEVEL FIVE HOSPITAL
P.O BOX 8-60200
MERU

DATE: 27th November 2014

RHODA KAINYU MUNENE
P.O BOX 43844-00100
NAIROBI

Dear Madam,

RE: PERMIT TO COLLECT BLOOD SAMPLES

This is in reference to your letter dated 22nd September 2014 addressed to the County Ethical Committee. Having considered your request, I hereby advise you to go ahead with the planned exercise while considering the ethical regulations that you are privy to.

I also humbly request you to share the outcome of your planned research with this office.

Thank You

Yours faithfully

J. M. Muchiri
For: Medical Superintendent
Meru Level 5 Hospital

Appendix 31: Ministry of Education Permit to carry out research

Republic of Kenya

MINISTRY OF EDUCATION
SCIENCE & TECHNOLOGY
EDUCATION DEPARTMENT

Telegrams: " ELIMU " Meru
Telephone 06832372

When Replying please quote:

REF: IMN/EDUC/312/VOL 8/172

District Education Office
Imenti North District,
P.O. Box 61,
MERU.

11th July 2016

TO
ALL PRINCIPALS
PUBLIC SECONDARY SCHOOLS

RE: AUTHORIZATION TO CONDUCT RESEARCH

This is to inform you that Munene Rhodah Kainyu has been authorized by this office to conduct research in our public secondary schools.

Kindly accord her all the necessary assistance.

SUB-COUNTY DIRECTOR OF EDUCATION
IMENTI NORTH

JOHN MUTHEE
SUB-COUNTY DIRECTOR OF EDUCATION
IMENTI NORTH

KENYATTA UNIVERSITY LIBRARY