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Reference Values and Numerical Ratios of Total Plasma Protein and Hemoglobin Concentration among Apparently Healthy Prospective Blood Donors in Calabar Municipality, Cross Rivers State, Nigeria: A Comparative Study using Three Hemoglobinometers

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Authors' contributions

This work was carried out in collaboration among all authors. Author FJN did conceptualization of the manuscript. Authors FJN, IIE, AIS, EBD, OEO, KSV and OCJ designed the study. Authors FJN, EWO and did formal analysis of the study. Authors FJN, EWO and IIE did formal analysis of the study. Authors FJN, EWO, OJM, OPC, UVU and AIS did Statistical analysis of the study. Authors FJN, EWO, and OJM prepared the original draft of the manuscript. All authors read and approved the final manuscript.

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ABSTRACT

Background: Despite recent development and improvement made on blood donor's eligibility assessment and screening profile for blood donation, it is only in the last three decades that researchers in the primary, secondary, tertiary, federal and national levels have observed an unprecedented increase in blood donor's disqualification, rejection, and deferment .This may have been hypothesized to be caused by apparently low hemoglobin concentrations or false high hemoglobin concentrations thus leading to false acceptance of blood donors. This may also happen due to the effects of total plasma proteins.
Objective: This study aimed at determining the reference values and ratios of total plasma protein (TPP) levels and hemoglobin (Hb) concentration analyzed using three different types of hemoglobinometry methods in prospective blood donors recruited within Calabar Municipality, Nigeria.
Methodology: This cross-sectional one-year study (2021-2022) employed an experimental design with randomized simple sampling and purposeful sampling techniques. Participants (n = 430, 230 males, 200 females aged 20-60 years) completed an opened-ended, semi-structured self-administered questionnaire form after providing informed / written consent. Blood samples were analyzed for TPP levels using Biuret's spectrophotometric method, Hb concentration using Cyanmethemoglobin (HICN) method, Packed cell volume (PCV) using Micro-hematocrit, and specific gravity using copper sulphate ($CuSO_4$) gravimetric method).
Results: The mean TPP levels were 7.59 ± 0.83 g/dl (total), 7.71 ± 0.88 g/dl (male), and 7.48 ± 0.79 g/dl (female) with control sample of 7.70 g/dl. Mean Hb concentrations were 13.53 ± 1.91 g/dl (total), 14.43 ± 1.92 g/dl (male), and 12.77 ± 2.20 g/dl (female) with mean control sample value of 15 ±2.5 g/dl.The mean micro-hematocrit value in (%) were 39.16 ±8.34 , 43.51 ± 4.74 , and 41.36 ±6.73 for female, male and total participants respectively, and with control standard sample value of 45%.Mean specific gravity value using copper sulphate solution (female, male and total participants) were 1.055 ±0.009 (12.5 ±10 g/dl of Hb equivalent),1.058 ± 0.006 (equivalent to 13.60 ± 0.07g/dl of Hb), and 1.057 ± 0.0075 (equivalent to 13.50 ± 0.095 g/dl of Hb) respectively with mean control standard sample value of 1.058 (equivalent to 13.6 g/dl of Hb).The mean TPP: HICN

Ratio, TPP: PCV Ratio and TPP: $CuSO_4$ ratio for all three methods were = 0.55. While the total mean HICN:TPP ratio, PCV: TPP, $CuSO_4$: TPP ratio, for all three methods were 1.7- 1.9. There was no statistically significant difference between total, male and female genders respectively (*p >0.05).*

Conclusion: This study demonstrates that variations in TPP levels which can lead to inaccurate Hb concentration measurements, thus resulting in unnecessary acceptance or disqualification of blood donors. Understanding the reference range and ratio of TPP levels to Hb concentration is crucial for improving blood donor eligibility assessment and blood donor screening profile .

Keywords: Reference values; ratios; TPP levels; HB concentration; three types of hemoglobinometers; prospective blood donors; Calabar municipality.

1. INTRODUCTION

According to the Encyclopedia Britannica a hemoglobinometer can be defined as an instrument used to measure specifically hemoglobin concentration of the blood and usually involve colorimetric or spectrophotometric measurements while hemoglobinometry is defined as the procedure and techniques involved in carrying out this procedure [1,2,3]. Good examples are the point of care testing portable hemoglobinometers which provide easy and convenient measurements of hemoglobin concentrations and are particularly useful in areas where no standard clinical laboratories are available. These devices are also useful in accident and emergency centers due to their ease-of-usage , accuracy, and fast delivery of results [4]. Although the Cyanmethemoglobin (HiCN) and spun packed cell volume (PCV) micro-hematocrit methods are considered reference, gold standard and recommendable hemoglobinometers for routine screening and estimation of hemoglobin (Hb) concentration of blood donors, these former instruments however, are becoming highly expensive and useless where there is no steady power supply [5,6,7,8].The copper sulphate gravimetric method previously considered obsolete because of interference by other proteins which can be precipitated by the copper sulphate. but appear to be so relevant and are becoming very popular in some blood donors screening centers because of some advantages that it has over other forms of hemoglobinometers for routine screening of hemoglobin (Hb) for blood donation. It is precisely seven decades ago when [9] the copper sulphate solution method was first invented and elaborated as a method of hemoglobinometry. In its traditional form it consisted of a graded serial copper sulphate solution of known specific gravity whereby whole blood and plasma values could be obtained directly from pre-determined aqueous solutions of copper sulphate standard. Subsequently and overtime this discovery led to the development of a line chart where the concentrations of hemoglobin as well as plasma proteins could thus be obtained. Apart from a paper published by [10], there have been little published work documented on the use of this latter method. Despite this, the latter method has gained a lot of public interest, clinical relevance and wide applications in resource-poor setting where there is no electricity, massive casualties, emergency and large-scale disasters [11]. More so as a result of its inexpensiveness, economic nature, its simplicity to operate and the fact that it can be used in field work where there is large drive for blood donors and donation campaigns taking place [12]. For a very long-time researchers have considered that the copper sulphate method for measuring the specific gravity of serum gave satisfactory and consistent estimates of the protein concentration. However, in most recent times there have been increasing hypothesis suggesting that the copper sulphate ($CuSO_4$) solution method of hemoglobinometry has become inaccurate, obsolete and should be disused and replaced with second and third generations of hemoglobinometers [13,14].This is so because the copper sulphate method has been hypothesized to be wholly over-estimating or under-estimating blood donor's hemoglobin (Hb) concentration due to significant effects of increased or low plasma total proteins (TPP) levels, even in normal healthy states. If such hypothesis is ever correct it would mean that the true Hb concentration using copper sulphate solution method alone have never been known and, in this case, many blood donors may have been bled or deferred and rejected unnecessarily and unjustly [15,16].

1.1 Statement of the Problem

The determination of hemoglobin concentration is one of the most important parameter of full blood count profile , unfortunately it appears to be one of test which is frequently requested

routinely with and sometimes the results are inaccurate because of insensitivity of the instruments, equipment and method used and low power supply. Mannario and Mac-phensor, 1963 using only the copper sulphate solution method for screening blood donor's Hb concentration first observed that increase levels of plasma total protein could have some significant effects on Hb. Concentration [17]. Later, Pirofsky, et al, 1964 using the $CuSO_4$, Cyanmethaemoglobin (HiCN) and Micro-hematocrit (PCV) methods, also observed gross errors and discrepancies in the results of the three methods, and attributed the differences to the effects of TPP level on Hb concentration interaction and interrelationship [18,19].

1.2 Justification and Rationale of the Current Study

In reality,the safety and health of recipients of blood transfusion and other blood products or hemotherapy still relies heavily on blood donations from human blood donors, and their eligibility is determined by their hemoglobin (Hb) concentration. Therefore, selecting the appropriate hemoglobinometers before donation is crucial. This step is not only a rate-limiting factor but also a critical part of pre-donation counseling and screening of recruited blood donors [20-22]. Although HiCN and PCV methods are recommended for routine Hb concentration and screening, the copper sulfate solution method has gained popularity in many blood donor's screening centers, particularly during blood drives or large donation campaigns, due to its relevance and practicality in fieldwork [23]. Unfortunately, there is huge gap of knowledge in that the exact effect of total plasma protein on hemoglobin concentrations using both methods of hemoglobinometry is not well documented at this point in time in the study area and population.

1.3 Research Questions

1)What is the reference value of total plasma protein and hemoglobin concentration (estimated using three different types of hemoglobinometers) among apparently healthy prospective blood donors in the study area?
2)What is the numerical ratio of total plasma protein to hemoglobin concentration (estimated using three different types of hemoglobinometers) among apparently healthy prospective blood donors in the study area?
3)What is the numerical ratio of hemoglobin concentration (estimated using three different types of hemoglobinometers to total plasma protein) among apparently healthy prospective blood donors in the study area?

1.4 Research Hypothesis

Research null hypothesis:

1) There will be no statistically significant differences between the reference values of total plasma protein and hemoglobin concentration estimated using three different types of hemoglobinometers among male and female apparently healthy prospective blood donors.
2) There will be no statistically significant differences between the numerical ratios of total plasma protein to hemoglobin concentration estimated using three different types of hemoglobinometers among apparently healthy prospective blood donors in the study area
3) There will be no statistically significant differences between the numerical ratio of hemoglobin concentration estimated using three different types of hemoglobinometers to total plasma protein and among male and female apparently healthy prospective blood donors in the study area.

Research alternative hypothesis:

1) 1)There will be statistically significant difference between the reference value of total plasma protein and hemoglobin concentration estimated using three different types of hemoglobinometers among male and female apparently healthy prospective blood donors.
2) There will be statistically significant numerical ratio of total plasma protein to hemoglobin concentration estimated using three different types of hemoglobinometers among male and female apparently healthy prospective blood donors in the study area.
3) There will be statistically significant numerical ratio of hemoglobin concentration estimated using three different types of hemoglobinometers to total plasma protein and among male and female apparently healthy prospective blood donors in the study area.

1.5 Main Objective of this Study

The aim of the current study was to determine the reference and numerical ratio of total plasma protein (TPP) and hemoglobin concentration estimated using three different hemoglobinometry methods amongst male and female apparently healthy prospective blood donors within Calabar Municipality, Nigeria.

1.6 Specific Objectives of this Work Are

1) To determine or estimate or analyze the reference range of total plasma protein (TPP) of male and female blood donors using Biuret method (spectrophotometric method).
2) To estimate or analyze the Hemoglobin concentration of the male and female blood donors, using HiCN, PCV, and $CuSO_4$ methods and compare with control standard samples
3) To calculate the numerical ratio of the TPP levels to Hemoglobin Concentration of male and female using HiCN, PCV, and $CuSO_4$ methods.
4) To calculate the numerical ratio of the Hemoglobin concentration to TPP levels of male and female to using HiCN, PCV, and $CuSO_4$ methods.

1.7 Significance of the Study

The findings of this study are hoped to contribute to the ongoing quality control program within the blood transfusion department and to contribute to general scientific and research community and medical science as a whole.

2. LITERATURE REVIEW

Hemoglobin (Hb) is a metalloprotein and chromoprotein which made up the primary component of mature red blood cells (RBCs) in animals including humans [24]. As a crucial element of the respiratory system, hemoglobin plays a vital role in human physiology [25]. Despite extensive research, the complexity of its tertiary and quaternary structure and numerous physiological and biochemical functions remain partially understood. Hemoglobin was one of the first proteins studied using X-ray crystallography, earning Max Perutz the Nobel Prize in Chemistry in 1962 [26,27]. Recent studies have revealed hemoglobin's polyfunctionality which involves Catalytic activities (nitrite reductase, NO dioxygenase, monooxygenase, alkylhydroperoxidase, esterase, lipoxygenase), Nitric oxide metabolism, Metabolic reprogramming, pH regulation and Redox balance maintenance (Kosmachevskaya and Topunov, 2018) [28]. Hemoglobin's molecular weight is approximately 64,500 Dalton. Its primary function is transporting oxygen from lungs to tissues, binding and releasing oxygen cooperatively, as demonstrated by the oxygen equilibrium curve (OEC) [29].

Structure and Function: Hemoglobin's structure consists of two α-subunits (α1 and α2) and two β-subunits (β1 and β2), arranged around a 2-fold axis of symmetry [31-33]. The α-and β-clefts serve as entry points into the central water cavity. The interdimer interface (α1β1–α2β2) exhibits more salt-bridge/hydrogen-bond interactions in the T state than in the R state. Each subunit has a heme-binding pocket formed by the E and F helices. The heme consists of a ferrous ion coordinated by four nitrogen atoms of the porphyrin ring. The Fe is anchored to hemoglobin by an imidazole of a histidine residue (proximal histidine or His F8) [30,31,33].

Ligand Binding and Cooperativity: Ligand binding and unbinding events induce conformational changes in the globin E helix, CD and FG corners, affecting the size of the distal pocket, central water cavity, α- and β-clefts, and salt-bridge/hydrogen-bond interactions across the α1β1–α2β2 interface. This triggers cooperativity events and the T → R transition, giving rise to allostery [30,32,33].

Genetics and Evolution: Hemoglobin genes (HBA1, HBA2, and HBB) code for protein subunits. Alpha 1 and alpha 2 subunits are coded by genes HBA1 and HBA2 on chromosome 16, while the beta subunit is coded by gene HBB on chromosome 11. Amino acid sequences differ between species, with increasing differences corresponding to evolutionary distance [34,35,36,37,38].

Synthesis and degradation of Hemoglobin (Hb): There are series of complex enzymatic steps in the synthesis of Hemoglobin (Hb). The heme part of Hb is synthesized in a series of steps in the mitochondria and the cytosol of immature red blood cells, while the globin protein parts are synthesized by ribosomes in the cytosol [39]. Production of Hb continues in the cell throughout its early development from the pro-erythroblast to the reticulocyte in the bone marrow. At this point, the nucleus is lost in

mammalian red blood cells, even after the loss of the nucleus in mammals, residual ribosomal RNA allows further synthesis of Hb until the reticulocyte loses its RNA soon after entering the vasculature (this hemoglobin-synthetic RNA in fact gives the reticulocyte its reticulated appearance and name) [40].There are many steps involved in the metabolic pathway of the degradation of Heme from Hb molecule which normally leads to the formation of Bilirubin [41,42]. Heme released from the hemoglobin of red cells or from other hemoproteins is degraded by an enzymatic process involving heme oxygenase, the first and rate-limiting enzyme in a two-step reaction requiring NADPH and oxygen and resulting in the release of iron and the formation of carbon monoxide and biliverdin. Metalloporphyrins, synthetic heme analogues, can competitively inhibit heme oxygenase activity. Biliverdin is further reduced to bilirubin by the enzyme biliverdin reductase. Carbon monoxide can activate guanylyl cyclase (GC) and lead to the formation of cyclic guanosine monophosphate (cGMP). It can also displace oxygen from oxyhemoglobin or be exhaled. The bilirubin that is formed is taken up by the liver and conjugated with glucuronides to form bilirubin monoglucuronide or diglucuronide (BMG and BDG, respectively), in reactions catalyzed by uridine diphosphate and monophosphate glucuronosyltransferase. The bilirubin glucuronides are then excreted into the intestinal lumen but can be deconjugated by bacteria so that the bilirubin is reabsorbed into the circulation [43,44].

Definition of Total Plasma Protein: Total Plasma Protein (TPP) refer to the sum of all the proteins in the plasma. It has been defined as the measurement of the total concentration of proteins in blood plasma. Recent studies has shown that the normal composition of total plasma protein is made up of Albumin (60-70%) Globulins (20-30%) Fibrinogen (4-6%) and Lipoproteins (2-4%) Typical Functions of total plasma protein include:- Maintaining blood volume and osmotic pressure.Transporting nutrients, hormones, and waste products [75], Regulating blood clotting and fibrinolysis ,Supporting immune function and Maintaining acid-base balance. Studies have also consistently shown that normal range of total plasma protein in Adults varies from 6.4-8.3 g/dL (64-83 g/L).Total plasma protein Measurement Methods include Biuret assay , Bradford assay , Refractometry]. Its Clinical Significance and application includes abnormal TPP levels may indicate: - Liver or kidney disease, Malnutrition , Infection , Cancer and Immunological disorders.

3. METHODOLOGY

3.1Study Setting

Study area: The area where the current study was carried out is Calabar and it is the present capital of Cross River State in the south eastern part of the Federal Republic of Nigeria [45] Geographically, Calabar has a total land area surface of 142 km² while the total local government area population is estimated to be 320,826 of which 166,203 are males and 154,659 females [46]. The inhabitants are mainly of the Efiks, Quas, Ejagham, Efut, Ibibio, Annang by tribe and others– include the migrant workers and mixed multitudes. They are mainly civil servants, subsistence farmers, traders and fishermen. There are many important, primary, secondary, tertiary health facilities and educational centers belonging to either federal or state government in Calabar municipality [47].

3.2 Sites for Participant Recruitment and Pre-Counseling for Samples Collection

The participants of the current study were made up of apparently healthy individual who presented to the blood donors department of UCTH Calabar for eligibility assessment and screening profile for blood donation.

3.3 Sampling Techniques

This study utilizes the convenient and random sampling method in the selection and enrolment of participants who were found to serve as eligible voluntary apparently healthy prospective blood donors and who gave their written/ informed consent. This study adopted a cross-sectional approach which was conducted within a year period (2021 to 2022).

3.4 Study participants

The participants were enrolled at the University of Calabar Teaching Hospital, Calabar, Cross River State. The documentation of the study participant's demographics, blood transfusion history, risky behavioral conduct, number of sexual relationships, drug injection history and clinical background was done using the semi close- ended research questionnaire prepared, verified and adopted for this study.

3.5 Study Design

Experimental and analytical designed were adopted in this study and all collected samples for estimation of hemoglobin concentration and total plasma protein concentration was carried out in the Department of Hematology & Blood Transfusion Sciences and Department of Chemical Pathology,Faculty of Medical Laboratory Science, University of Calabar, Cross Rivers State ,Nigeria.

3.6 Calculation of Sample Size

The Formula of Cochran, 1977, for calculating the sample size (S) was adopted in current study and is denoted by formula viz: [48]**: S= t^2 p (1-p)/ e^2** , *Where* t= t value (The alpha level used in determining sample size in most educational research studies is either 0.05 % or 5% **.** In Cochran's formula, t-value for alpha level of .05 is 1.96 for 95% confidence level for sample sizes above 120.**P=** prevalence rate in percentage (%) from previous study of estimation of hemoglobin concentration in non-Caucasian population in Calabar and in this case it is taken to be 0.5 or 50% since someone had never worked on this population [49,50] While **e =** tolerance error or confidence interval expressed as decimal and it is taken to be 0.05.Therefore **S =** $(1.962)^2$ $(.5(1-0.5)/ (0.05)^2$, S = $(1.962)^2 (0.5)^2/ (0.05)^2$ = 384.16 ,hence S = ~ 400 subjects were used in cases of any loss data or specimen during the study or in cases of non-respondent individuals .

- ★ Correction for a small/finite population below 10,000 the formula viz: **n=no/1+(no-1)/N** was used.

where n is the corrected sample size, n_0 is the calculated sample size and N is the population size. Therefore n=384/1+(384-1)/5650=360= minimum sample size needed.

- ★ Non- respondent rate =384/1-0.1=384/.9=426 samples

Approximately 430 as maximum samples were collected by convenient sampling techniques after correction for missing or spoiled samples [51,52,53,54].

3.7 Inclusive and Exclusive Criteria for Selection of Participants

A total of 430 apparently healthy voluntary participants of both genders, aged between 20 to 50 years and who were randomly recruited from city and into General Hospital Calabar or University of Calabar Teaching Hospital Calabar, Cross River State, Nigeria. The participants were divided into two study groups according to their ages and sexes and a questionnaire form designed and prepared for this purposed, was used for both inclusive and exclusive criteria.

3.8 Ethical Approvals

These were sought and obtained from the Research Ethical Committee, Centre for Clinical Governance, Research & Training Ministry of Health Calabar, and Cross Rivers State, Nigeria.

3.9 Informed and Written Consent

These were also sought and obtained from these subjects before inclusion in the study.

3.10 Administration of Questionnaire

The harmless nature and advantage of the research was also explained to each participant in the form of pre-counselling in which the prepared questionnaire forms were administered on each of the subjects to obtain more medical information about the clinical history. After the Pre-counselling, informed consent forms were filled and signed by these participants for screening to start**.**

3.11 Study Population

A total of 430 apparently healthy prospective blood donors or both sexes, aged between 20 to 50 years were recruited within Calabar Municipality of Gross River State. Recruited participants were pre-counseled and screened in accordance with the Questionnaire form designed, validated and prepared to be adopted for this purpose.

3.12 Method for Collection and Treatment of Blood Samples

About five milliliters of venous blood samples was withdrawn from the antecubital vein of the arms of previously counseled and screened apparently healthy prospective blood donors of both sexes. By a mean of a disposable plastic five milliliters syringe fitted with 19 SWG needle. The area of venipuncture was first of all cleaned with 70% methylated spirit alcohol and allowed to dry. A tourniquet was tied just for a short time. The withdrawn samples were put into sample bottles containing 4mg of potassium ethylene dimethylamine tetra acetic acid (K_2 EDTA) and thoroughly mixed immediately. The samples

were used for determination of hemoglobin concentrations and those samples that were not analyzed immediately within 2 hours of collection were stored at 4^{o} C – 6^{o}C. The samples were usually spun at 4000 rpm for 10 minutes to harvest plasma which were used for estimation of total plasma protein stored at -20^{o}C and the screening was done within 7 days. Collection and preparation of blood samples were done from Monday to Friday of each week and between the hours of 7.00am and 5.00 pm.

3.13 Laboratory Methodology for Analyzing Various Parameters

Method and procedure for cyanmethaemoglobin (HiCH) method: The principle of cyanmethaemoglobin (HiCN) method is based on the fact that when whole blood is added to a solution containing potassium cyanide and potassium ferricyanide. The ferricyanide converts the hemoglobin iron from the ferrous state (Fe^{2+}) to ferric state (Fe^{3+}) to form the methaemoglobin, which then combines with potassium cyanide to form the stable pigment, cyanmethaemoglobin. The color intensity of this mixture is measured in a colorimeter at a wavelength of 540nm or using a yellow green filter. The absorbance of the solution is proportional to be concentration of hemoglobin in the whole blood sample. All forms of hemoglobin are measured with this method, except sulfhaemoglobin **[**55].

Method and procedure for microhaematocrit method: Whole blood is centrifuged for maximum red blood cell packing. The space occupied by the red cells in measured and expressed as percent of whole volume [55].

Method and procedure for biuret's method: The principle of Biuret method or Biuret reaction is based, on the fact all proteins contain a large number of peptide bonds. When a solution of protein is treated with Cu^{2+} in a moderately alkaline medium, a violet color chelating-complex is formed between the Cu^{2+} and the carbonyl (=COOH) and amino (=N-H) groups of the peptide bonds, the intensity of the color changed produced is proportional to the number of peptide bonds presence or (undergoing in the reaction), when measured calorimetrically at 540 nm [55].

Copper sulphate solution method: The principle of the copper sulphate solution method is based on the fact that, when whole blood is dropped into a solution of CuSO4, the CuSO4 reacts with the protein at the periphery of the drop to form copper proteinate which acts as a protective membrane. Thus, preventing the dispersion of the drop. Whether or not the drop will float or sink is dependent on the hemoglobin concentration in it. (Phillips et al, 1950, and Henry et al, 1974)

3.14 Method of Data Collection and Statistical Tools for Data Analysis

After codification and collation of the raw data for both sexes of the results were entered and subjected to statistical analysis using Statistical Package for Social Students software version 26 (SPSS Incorporation, Chicago, United State America). Data were represented with frequency and percentages while continuous data were expressed as mean plus or minus standard deviations (X±SD). One sample Kolmogorov-Smirnov test was used to assess the normality of the data. All data were normally distributed; hence, parametric procedure was used for the statistical analysis of the data. The prevalence rate formulae were used to calculate the prevalence rate. A two tailed p-value of <0.05 was considered indicative of a statistically significant difference. Comparison of the parameters and variables between the samples were performed using independent t-test while comparison among various age groups were analyzed using ANOVA. Association between variables was analyzed using Chi Square and Fischer exact test. Alpha value of 0.5 was used. Coefficient of Variation (CV) Formula given by CV=σ/μ, where: σ=standard deviation and μ=mean was used to calculate the coefficient of variation of the desire variables.

4. RESULTS

The results obtained for the current study are shown in the Tables 1, 2, 3, 4 and 5.

Table1 shows the results of the frequency distribution by demographic parameters and Age range of participants recruited within Calabar Municipality, Cross Rivers State, Nigeria. A total of 430 blood samples comprising of 230 (53.5%) and 200 (46.5%) from male and female participants and with ages between 20 to 60 years were collected using standard procedures. All apparently healthy individuals recruited within Calabar Municipality. participants have been consented and precanceled before recruitment into the study. The Mean age ± SD (years) for female was 24.99±1.01 and male 29.95±7.85 with statistically significant difference between ages (P<0.05, t=7.2822, P=0.0001).

Table 1. Distribution by demographic parameters and Age range of participants recruited within Calabar Municipality, Cross Rivers State, Nigeria

Participant type (Sex)	Age Range (Year)	Total Sample Frequency	Percentage (%)	t-value	*P*-value	remarks
Male	20 – 31	230	53.5	7.2822	0.0001	p>0.05 S*
Female	20 – 29	200	46.5			
Female + Male	20 – 31	430	100			

*S * denotes statistically significant difference*

Table 2. Results of Means Values of Parameters investigated among apparently healthy blood donors in Calabar Municipality, Cross River State, Nigeria

Parameter investigated			Male participants Mean Values	Female participants Means Values	Total participants Means Values	p-values	Remarks
Plasma Total Protein	PTP	(g/dl)	7.71 ± 0.88	7.48 ± .79	7.59 ± 0.83	0.000	(P>0.05) NS*
Hb. Concentration	(HiCN)	g/dl	14.43 ± 1.92	12.77 ± 2.20	13.53 ±1.91	0.000	(P>0.05) NS*
Micro haematocrit	(PCV)	%	43.51 ± 4.7	39.16 ± 8.24	41.36 ± 6.73	0.000	(P>0.05)NS*
(Hb. Equivalent in		(g/dl)	14.33 ± 1.58	13.05 ± 2.75	13.67 ± 2.24	0.000	(P>0.05)NS*
Specific gravity of copper sulphate solution	$CuSO_4$ (S.G.)		1.058± 0.006	1.055 ± 0.009	1.057 ± 0.0075	0.000	(P>0.05)NS*
(Hb. Equivalent In		g/dl)	13.60 ± 0.07	12.5 ± 0.11	13.50 ± 0.095	0.000	(P>0.05)NS*

*NS * denotes no statistically significant difference. There was no statistically significant difference between the results of total mean values and that of the male and female genders respectively (p >0.05). Using ANOVA, the association between rows (groups) and columns (outcomes) were not statistically significant (p >0.05).. While using Chi-square with Yates correction, the Chi squared equals 0.000 with 1 degree of freedom. The two-tailed P-value equals 0.996.*

In Table 2 the results of means values of parameters investigated among apparently healthy male and female blood donors in Calabar Municipality, Cross River State, Nigeria. The mean value plus one standard deviation (X±SD) of total plasma protein (TPP) levels were 7.59 ±0.83 g/dl for total participants ,7.71 ± 0.88 g/dl for male and 7.48 ±79 g/dl for female participants respectively. That of control samples was 7.70 g/dl for both participants. The mean value plus one standard deviation (X±SD) of hemoglobin concentration using (HiCN) methods were 12.77 ±2.20 g/dl, 14.43 ± 1.92 g/dl, and 13.53 ± 1.91g/dl for female, male, and total participants respectively. The control sample value was 15 ±2.5 g/dl. The mean value plus one standard deviation (X ± SD) for micro-hematocrit were 39.16 ±8.34 g/dl, 43.51 ±4.74 g/dl, and 41.36 ±6.73 g/dl for female, male and total participants. The control sample was 45%. Similarly, the mean value plus one standard deviation (X±SD) for specific gravity of the copper sulphate solution were 1.055 ±0.009 (12.5 ±10 g/dl of Hb equivalent), 1.058 ± 0.006 (13.60 ± 0.07g/dl of Hb equivalent), and 1.057 ±0.0075 (13.50 ± 0.095 g/dl of Hb equivalent) for female, male participants and total participants respectively, the control sample was 1.058 (13.6 g/dl of Hb equivalent). The mean value plus one standard deviation (X±SD) of PTP levels were 7.48±0.48 g/dl fo male subjects and 7.59 ±0.83 g/dl for total subjects.

Table 3 show he Mean value of control for PCV, PTP, HB, and $CuSO_4$ solution for female and male participants in Calabar Municipality, Cross River State, Nigeria. Mean control TPP in (g/dl) were 7.7 (total), 7.65 (male) and 7.65 (female), Hb in (g/dl) were 15.5 ± 2.6(total),14.0 ± 2.5 (female 15 ± 2.5 (male), PCV in (%) were ,47.0 (male),42 (female) 45, $CuSO_4$ (S.G.) in g/dl of Hb equivalent were 1.058 (total),1.055 (male) and 1.053 (female) participants in Calabar Municipality, Cross River State, Nigeria.

Table 3. Mean value of control for PCV, TPP, HB, and $CuSO_4$ solution for female and male participants in Calabar Municipality, Cross River State, Nigeria

Control parameters	units	Male	Female	Total control sample
TPP	(g/dl)	7.65	7.65	7.7
Hb	(g/dl)	15.5 ± 2.6	14.0 ± 2.5	15 ± 2.5
PCV	(%)	47.0	42	45
$CuSO_4$ (S.G.) Hb equivalent in	g/dl	1.055	1.053	1.058

Table 4. Comparative result of coefficient of variance cv (%) of parameters among apparently healthy blood donors in Calabar Municipality, Cross River State, Nigeria

Parameters Investigated			Coefficient Variation
	Code	Unit	(%)
Total plasma protein	PTP	g/dl	10.93
Haemoglobin conc.	(HiCN)	g/dl	14.11
Microhaematocrit	PCV	%	16.27
Copper sulphate solution	CuOS4		0.75

Table 5. Comparative results of the ratio of TPP to Hb concentration (estimated using three haemoglobinometers) for female and male participants in Calabar Municipality, Cross River State, Nigeria

Gender	Parameters Investigated				
	TPP: HICN Ratio	TPP: PCV Ratio	TPP: $CuSO_4$ ratio	p-value	remarks
Male	0.53	0.54	0.57	0. 000.	P>0.05 NS*
Female	0.59	0.57	0.59		
Total	0.55	0.547	0.55		

*NS * denotes no statistically significant difference*

Using Chi-square with Yates correction, the Chi squared equals 0.000 with 1 degrees of freedom.The two-tailed P-value equals 0.9961. The association between rows (groups) and columns (outcomes) not statistically significant. TPP: HICN Ratio,TPP: PCV Ratio and TPP: $CuSO_4$ ratio were = 0.55

In Table 4 the Comparative results of coefficient of variance CV (%) of parameters among apparently healthy blood donors recruited in Calabar Municipality, Cross River State, Nigeria are shown. The coefficient of variance CV (%) of Total plasma protein (TPP) was 10.93%, Hemoglobin concentration (HiCN) was 14.11%, that of Microhaematocrit (PCV) was 16.27% and that of Copper sulphate ($CuSO_4$ method) was 0.75%.

Table 5 Shows comparison results of the ratio of TPP to Hb concentration estimated using the cyanmethemoglobin (HiCN), TPP: PCV Ratio using the micro-hematocrit method (PCV) and TPP: $CuSO_4$ ratio using copper sulphate ($CuSO_4$) method .The total mean TPP:HICN ratio was 0.55 comprising of 0.534 male and for female 0.585, while the total mean TPP: PCV ratio was 0.547comprising of 0.538 for male and 0.57 for female and finally the total mean TPP: $CuSO_4$ ratio was 0.55 comprising of 0.566 for male and 0.598 for female respectively. There was no statistically significant different between the results of total mean values and that of the male and female genders respectively (*p <0.5).* Using Chi-square with Yates correction, the Chi squared equals 0.000 with 1 degrees of freedom. The two-tailed P-value equals 0.9961. The association between rows (groups) and columns (outcomes) not statistically significant.

5. DISCUSSION

Studies have shown that over the years blood donor's hemoglobin (Hb) estimation is an important pre-donation test that is performed prior to blood donation. This is because it plays the double role of protecting the donors' health against anemia and at the same time ensuring good quality of blood components, which has a direct implication on recipients' health [4]. Due to the fact that diverse cutoff criteria have been used for hemoglobinometry worldwide depending on the population characteristics, however, no testing methodology and sample requirement have been specified for hemoglobin screening. This is why the British Committee for Standards in Hematology (BCSH) [(1991)] [8] and the International Committee for Standardization in

Hematology (ICSH) and the European Society of Hematology (ESH) [23] have been instituted. Besides the technique, there are several physiological and methodological factors that can affect accuracy, precaution, reproducibility and reliability of hemoglobin estimation. The aim of the current study was to determine the reference and numerical ratios of total plasma protein (TPP) and hemoglobin concentration (estimated using three different hemoglobinometers) amongst apparently healthy prospective blood donors within Calabar Municipality, Nigeria.

Table 1 shows the results of the frequency distribution by demographic parameters and age range of participants recruited within Calabar Municipality, Cross Rivers State, Nigeria. A total of 430 blood samples comprising of 230 (53.5%) and 200 (46.5%) from male and female participants respectively and with ages between 20 to 60 years were collected using standard procedures. All apparently healthy participants recruited within Calabar Municipality were consented and counselled before recruitment into the study. The Mean age ± SD (years) for female was 24.99 ±1.01 and male was 29.95±7.85 respectively and with statistically significant difference between ages ($P<0.05$, $t=7.2822$, $P=0.0001$). From the results in Table 1 it is clearly seen that there was a gender difference in the response rate. There were more males than females who responded and turned out for the study. Therefore, using Chi Squared (X^2) statistical stool there was a statistically significant differences between the response rate in the results. This is in line with previous findings that have been published by others authors such as [56,57,58].

In Table 2 the results of the mean values of total plasma protein levels, hemoglobin concentration, the micro-hematocrits and copper sulphate methods are shown. These results were perfectly within the normal range or limit for blood donation and are in line with previous report that have been published early by WHO and others authors such as [59,60,61,62,63].

Table 3 show the Mean value of control for PCV, PTP, HB, and $CuSO_4$ solution for female and male participants in Calabar Municipality, Cross River State, Nigeria. Mean control standard TPP (g/dl) was 7.7 (total), 7.65 (male) and 7.65 (female), Hb (g/dl)15.5 ± 2.6(total),14.0 ± 2.5 (female), 15 ± 2.5 (male), PCV (%) ,47.0 (male),42 (female) 45, $CuSO_4$ (S.G.) Hb equivalent in g/dl, 1.058 (total),1.055 (male) and 053 (female) participants in Calabar Municipality, Cross River State, Nigeria.

The coefficient of variation (CV) is defined as the ratio of the standard deviation to the mean. Coefficient of Variation (CV) formula used was in the index study given by $CV=\sigma\mu$ where: σ=standard deviation and μ=mean [64]. The higher the coefficient of variation, the greater the level of dispersion around the mean. It is generally expressed as a percentage and without units, it allows for comparison between distribution of values whose scales of measurement are not comparable. In Table 4 the Comparative results of coefficient of variance CV (%) of parameters among apparently healthy blood donors recruited in Calabar Municipality, Cross River State, Nigeria are shown. The coefficient of variance CV (%) of total plasma protein (PTP) was 10.93%, Hemoglobin concentration (HiCN) was 14.11, that of Microhaematocrit (PCV) was 16.27% and that of Copper sulphate ($CuSO_4$ method) was 0.75%. These results are in line with those published by [65,66,67].

Table 5 shows comparative results of the ratio of TPP to Hb concentration estimated using the cyanmethaemoglobin (HiCN), TPP: PCV Ratio using the micro-hematocrit method (PCV) and TPP: $CuSO_4$ ratio using copper sulphate ($CuSO_4$) method .The total mean TPP:HICN ratio was 0.55 comprising of 0.534 male and for female 0.585, while the total mean TPP: PCV ratio was 0.547comprising of 0.538 for male and 0.57 for female and finally the total mean TPP: $CuSO_4$ ratio was 0.55 comprising of 0.566 for male and 0.598 for female respectively . There was no statistically significant difference between the results of total mean values and that of the male and female genders respectively (*$p > 0.05$).* Using Chi-square with Yates correction, the Chi squared equals 0.000 with 1 degree of freedom. The two-tailed P-value equals 0.9961. The association between rows (groups) and columns (outcomes) not statistically significant. TPP: HICN Ratio, TPP: PCV Ratio and TPP: $CuSO_4$ ratio were = 0.55. These results were considered lower when compared with those earlier published by other authors and documented in textbooks or quoted in literatures for other normal Caucasian populations [68,69,70,71]. The reason for these differences may be attributed to the fact that the reference range and normal ratio of total plasma proteins to hemoglobin concentration in human varies depending on factors such as sex and nutritional status as already documented by [72,73,74].

Table 6. Comparative results of the ratio of Hb concentration (estimated using three hemoglobinometers) for female and male apparently healthy blood donors to TPP in Calabar Municipality, Cross River State, Nigeria

Gender	Parameters Investigated				
	HICN: TPP Ratio	PCV: TPP Ratio	$CuSO_4$: TPP ratio	p-value	remarks
Male	1.9	1.9	1.8	0. 000.	(P<0.05) NS
Female	1.7	1.8	1.7		
Total	1.8	1.9	1.8		

In Table 6 the comparative results of the numerical ratio of Hb concentration (estimated using various methods for female and male participants) to TPP in Calabar Municipality, Cross River State, Nigeria. The total mean HICN:TPP ratio was 1.8 comprising of 1.9 male and for female 1.7, while the total mean PCV: TPP ratio was 1.8 comprising of 1.9 for male and 1.8 for female and finally the total mean $CuSO_4$: TPP ratio was 1.8 comprising of 1.8 for male and 1.7 for female respectively. There was no statistically significant difference between the results of total mean values and that of the male and female genders respectively (*p <0.5).* Using Chi-square with Yates correction, the Chi squared equals 0.000 with 1 degrees of freedom. The two-tailed P-value equals 0.9961. The association between rows (groups) and columns (outcomes) not statistically significant. HICN: TPP Ratio, PCV: TPP Ratio and $CuSO_4$: TPP ratio ranges from 1.7-1.9 for total, males and females. These results were considered lower when compared with those earlier published by other authors and documented in textbooks or quoted in literatures for other Caucasian normal populations [68,69,70,71].

6. CONCLUSION

This study demonstrates that variations in TPP levels can lead to inaccurate Hb concentration measurements, resulting in unnecessary acceptance or disqualification of blood donors. Understanding the reference value and normal ratio of TPP to Hb concentration is crucial for improving blood donor screening and eligibility assessment. This study has also demonstrated that samples from apparently healthy blood donors with dysproteinemic or hypoproteinemia or hyperproteinemia may contribute to false positive or false negative or low or high hemoglobin values by over-estimating the hemoglobin concentration as the in case of hemoconcentration state or under estimate the hemoglobin concentration as in the case of hemodilution state. Hence the study infers that effects of low or high total plasma protein level son hemoglobin concentration may lead to unnecessary acceptance or disqualification, rejection and deferment of blood donors on regular basis due to apparently false low or high hemoglobin concentrations.

7. RECOMMENDATIONS

1) Since the effects of low or high total plasma protein levels may also effects hemoglobin concentration leading to false low or high hemoglobin concentrations, and unnecessary acceptance or disqualification, rejection and deferment of blood donors o, it is therefore recommended that all apparently healthy blood donors' samples should be collected and analyze for total plasma proteins levels and numerical ratio to hemoglobin concentration.
2) This is to ensure that dysproteinemic or hypoproteinemia or hyperproteinemia that may contribute to false positive or false negative or low or high hemoglobin values by over-estimating the hemoglobin concentration in hemoconcentration or under estimating hemoglobin concentration in hemodilution individuals can be screened.
3) Future study in this area should use a larger samples size and larger population in order to compare the results of the current study or findings.

AVAILABILITY OF DATA AND MATERIALS

Datasets generated and analyzed in this study are available from the corresponding author on request.

CONSENT AND ETHICAL APPROVAL

It is not applicable.

DISCLAIMER (ARTICIAL INTELLIGENCE)

Author(s) hereby declare that No generative AI technologies such as Large Language Models, Chat GPT, COPILOT etc.) and text-to-image

generators have been used during the writing or editing of this manuscript .

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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