ADDIS ABABA UNIVERSITY

COLLEGE OF HEALTH SCIENCES

DEPARTMENT OF MEDICAL LABORATORY SCIENCES

Establishment of Reference Interval for Hemoglobin A1c and Fructosamine for Apparently Healthy Adults in Addis Ababa, Ethiopia.

By Tigist Getahun (BSc)

Advisors: Aster Tsegaye (MSc, PhD, Associate Professor)
Samuel Kinde (MSc, PhD Candidate)
Feyissa Challa (MSc, PhD Candidate)

A thesis submitted to the Department of Medical Laboratory Sciences, College of Health Science, Addis Ababa University, in Partial fulfillment of Master of Science Degree in Clinical Laboratory Sciences (Clinical Chemistry)

July, 2020

Addis Ababa, Ethiopia

Addis Ababa University

School of Graduate Studies

This is to certify that the thesis prepared by *Tigist Getahun*, entitled: Establishing reference interval for Hemoglobin A1c and Fructosamine for apparently healthy adults in Addis Ababa Ethiopia, submitted in partial fulfillment of the requirements for Master of Science degree in Clinical Laboratory Sciences (Clinical Chemistry) complies with the regulations of the University and meets the accepted standards with respect to originality and quality.

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Acknowledgments

I would like to express my deepest gratitude to Dr. Aster Tsegaye, Mr. Samuel Kinde, Mr. Feyissa Challa, Mr. Zeleke Geto, and Ms Anna for their valuable guidance and comment during this study.

I would like to thank also the study participants and those who participated in data collection. My sincere gratitude to my sponsor, Ethiopia Public Health Institute, especially the National Reference Laboratory for Clinical Chemistry and also Addis Ababa University, College of Health Sciences, Department of Medical Laboratory Sciences. I would like to thank also my family for their continuous support. Addis Ababa Health Bureau and health extension workers for facilitating the data collection and Ministry of Innovation and Technology for supporting the Reference Interval project are gratefully acknowledged.

Finally, my deepest gratitude goes to all of them who supported us in any aspect during the completion of the study.

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Abbreviations

ADA	American Diabetes Association
BMI	Body Mass Index
CKD	Chronic Kidney Disease
CLSI	Clinical and Laboratory Standards Institute
CVD	Cardiovascular disease
DCCT	Diabetes Control and Complication Trial
DM	Diabetes Mellitus
EPHI	Ethiopian Public Health Institute
HbA1c	Hemoglobin A1c
HBV	Hepatitis B Virus
HCV	Hepatitis C Virus
HIV	Human Immunodeficiency Virus
IDF	International Diabetes Federation
IFCC	International Federation of Clinical Chemistry and Laboratory Medicine
NCD	Non-Communicable Diseases
NGSP	National Glycohemoglobin standardization Program
RBC	Red blood cell
TDM	Therapeutic Drug Monitoring
WHO	World Health Organization

Abstract

Background: HbA1c and fructosamine used for diabetes monitor and control as a glycemic control. The absence of locally derived referenc intervals for these parameters from apparenetly health subjects for the local will impact on the physician, laboratories, and researchers.

Objective: to establish reference interval for hemoglobin A1c and fructosamine for apparently healthy adults in Addis Ababa, Ethiopia.

Methodology: A cross sectional study was conducted in four selected sub-cities (Akaki, Kirkos, Arada, Yeka), Addis Ababa, Ethiopia from December 2019 to April 2020. The study was including study participants with 18 and above years of age. Blood sample was collected and analyzed by using Cobas 6000(c 501) instrument. Both Kolmogorov–Sminorv, and Mann-Whitney test were analyzed to check the data normality distribution and partitions, respectively. Reference intervals, lower 2.5^{th} percentile and 97.5^{th} percentile for the upper, (overall, and specific for sex and age) were determined using non-parametric methods.

Result: - Out of the total 344 participants, 256 (121 males, 135 females,) study participants aged 18-60 years participated in this study. Reference interval was established from 4.70 – 6.27% and 203 - 321μmol/L for HbA1c, and fructosamine, respectively. There was weak association between HbA1c and age (r=0.1803, p=0.0378).

Conclusion: This study established reference intervals for both HbA1c and Fructosamine for adult population using the current available equipment platform. The established reference interval is applicable for the current practice in the country to diagnosis, treatment, and monitor of diabetics mellitus.

Key-words: *Hemoglobin A1c, fructosamine, reference interval, Ethiopia.*

1. Introduction

1.1 Background

Reference intervals sometimes referred to as “normative” or “expected” values, refer to ranges of upper and lower outside of which values would be considered abnormal (1). Reference intervals are fundamental tools used by healthcare and laboratory professionals to interpret patient laboratory test results, ideally enabling differentiation of healthy and unhealthy individuals (2)
In 1968 a paper was published with entitled ‘Normal values and Statistics’ as an initial study in the field of reference values (3). Other investigators have followed this by presenting ‘establishment and use of ‘normal values’. The term “normal value” was recognized as not appropriate and even partially incorrect. so that the term “reference interval ” came to be used (4).

HbA1c has been recommended by the American Diabetes Association (ADA) since 1988 to monitor glycemic control in patients with DM (5). HbA1c is the most commonly used standard biomarker to diagnose prediabetes and diabetes. Biochemically, HbA1c is formed through a non-enzymatic reaction of glycation to a valine amino acid of the hemoglobin beta chains of the red blood cells (RBCs) (6). A chemical reaction involved in the glycation of hemoglobin is shown in Figure 1 below.

The concentration of HbA1c reflects the average blood glucose level over the previous 3 months, depending on both prevailing glucose concentrations and the factors affecting the rate of glycation. As an example, obesity associated with oxidative stress is an independent factor for the hemoglobin glycation in non-diabetic patients (7, 8). About fifty percent of HbA1c level comes from the last first month of glycemia (9), 25% for the past 1-2 month, and the rest 25% accounts for the 2-4 months of glycemia. The level of hemoglobin value is affected by different factors some of them like altitude, vitamin B12, folate, and so on. Since HbA1c result is calculated as the ratio between glycated hemoglobin and total hemoglobin, the level of HbA1c is affected independent of hyperglycemia (10).

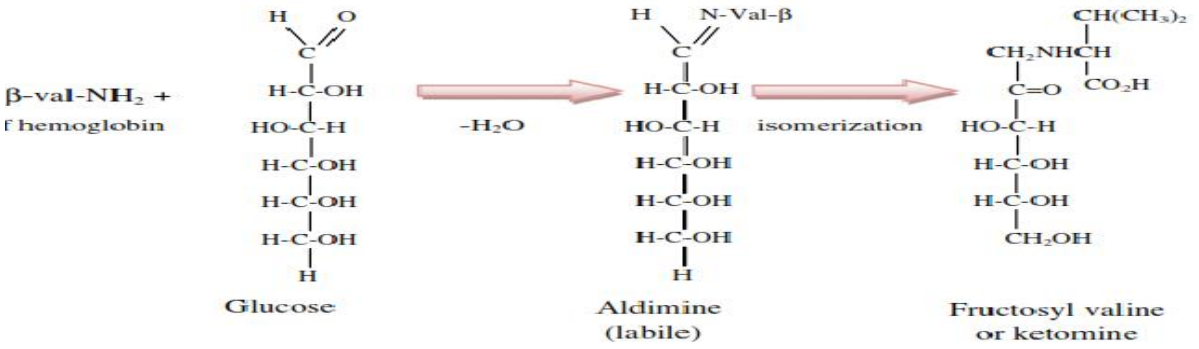

Figure 1. Chemical reactions involved in glycation of hemoglobin (Source: Determination of glycated hemoglobin with special emphasis on biosensing methods (11)

Fructosamine is the product of an irreversible non-enzymatic reaction, insulin-independent binding of glucose to serum proteins figure 2. Fructosamine is the common name for 1-amino-1-deoxy fructose and the generic name for plasma protein ketamine. The major component of fructosamine in plasma is glycated albumin (12).

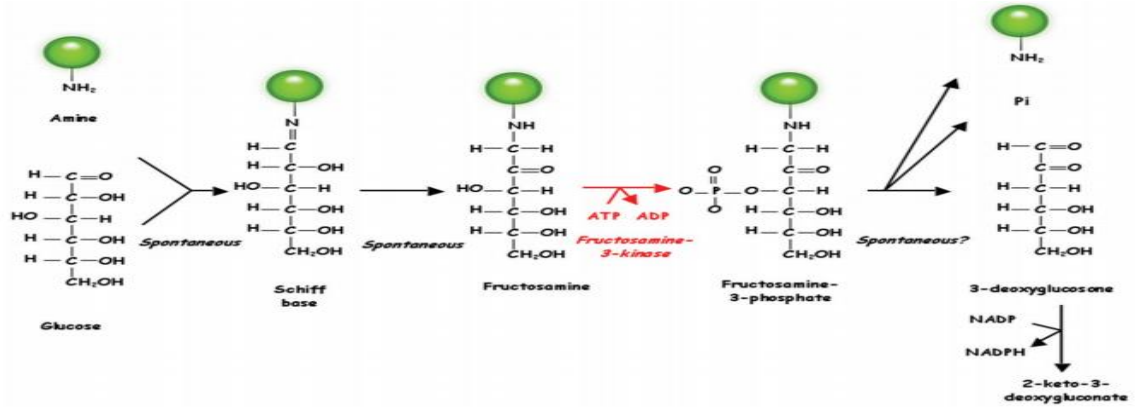

Figure 2. Mechanism of fructosamine-3-kinase formation (Source: Evaluation of glycated albumin (GA) and GA/ HbA1c ratio for diagnosis of diabetes and glycemic control: A comprehensive review)(13).

Fructosamine is both used for screening and may be potentially useful for diagnosing prediabetes. It is also an alternative glycemic marker for diabetes (14). Fructosamine is a ketoamine created by glycosylation of total serum proteins, primarily albumin. The formation of Fructosamine increases related to the level of blood glucose. Metallization occurs within 1 to 3 weeks, corresponding to the turnover of most proteins. Since it reflects average blood glucose concentrations over the previous 1–4 weeks, it can be a useful clinical marker of short-term

glycemic fluctuation and glucose control. Measurement of Fructosamine is quick, inexpensive, and technically simple (15, 16).

The lack of reliable reference intervals derived from apparently healthy and well defined local subjects impacts on patient management. It is enough to think of about 70 % of diagnosis done by physicians depends on the laboratory investigations (17). Thus the lack of such important evidenced-based values can mislead the diagnosis and management and often may lead us to unnecessary investigation and waste of scarce resources. In this contest, it is evident that the clinical laboratory plays a vital role in clinical decision making (18).

In few African countries the reference interval studies are established and enriched for other parameters (19, 20) but in the rest of the African countries such value might be nonexistent and when they are available these values have frequently being derived using either small sample size or using populations that have not been carefully selected (21).

In Ethiopia, a country with a highly diversified population and rich in geographical diversity, there is no well-established reference intervals from apparently healthy individuals for Hemoglobin A1c (HbA1c) and fructosamine yet, although efforts were initiated for other biochemical and hematological parameters.

1.2 Statement of the Problem

Previously infectious diseases were predominant in Africa. Nowadays, non-communicable diseases like in other low-income countries, are undergoing epidemiological transition in sub-Saharan countries like Ethiopia (22). According to the 2017 global estimate the prevalence of diabetes mellitus in adults population (age 18-99 years) is 451 million in the world. This prevalence will be expected to increase to 693 million by 2045 (23). The 2015 Ethiopia national survey for NCDs indicated that the national prevalence of diabetics mellitus was 9.1% with ADA criteria and 3.8% with WHO criteria (24).

Chronic hyperglycemia will be developed into long-term complications which involves multiple tissues to cause irreversible damage that will lead to significant morbidity, disability, and premature mortality if effective method of diagnosis and monitoring is not implemented (25). HbA1c and fructosamine are used as biomarkers for prediabetes, diabetes, and associated complications due to chronic variable blood glucose levels (26). The cut-off values of these biomarkers are variable among the population depending on race, age, gender, and other physiological and clinical conditions (6). A significant difference among the HbA1c values was found between Blacks, Hispanics, American Indians, Asians, and whites population in different studies (27). It has been suggested that using reference interval developed elsewhere could lead to misdiagnosis, inappropriate management of patients, and wastage of resources (28). In Ethiopia with cultural and geographical diversified populations, there is no such pertinent reference interval for HbA1c and Fructosamine, although it is recommended to have locally appropriate RI for each analyte considering that it depends on the analytical method (instrument, reagent, analytical principle), and on the population that belongs to the laboratory (29). However, a well-performed experiment to obtain reference interval is very demanding and very expensive so that usually the laboratories erroneously use the reference interval derived from the reagents kit, that do not feet for purpose. This study is trying to address such a gap in our setting.

1.3. Significance of the study

Nowadays, there is a rise in use of HbA1c and Fructosamine in medical care for diagnosis and monitoring of diabetes mellitus. These commonly used parameters are affected by different environmental and physiological factors such as age, gender, ethnic background, life-style, and dietary conditions (1). The establishment of reference intervals in local population is critical for the aforementioned tests. The RIs established by this study will aid in the interpretations of patient result for diagnosis and monitoring of treatment outcome. It can also help researchers to interpret data in any research measuring these parameters as well as in clinical trials.

2. Literature review

Diabetes mellitus (DM) is a group of metabolic diseases characterized by chronic high blood glucose concentration (hyperglycemia) resulting from insulin deficiency or resistance. DM has reached epidemic proportions across the globe with the largest increases seen in sub-Saharan Africa (30). The uncontrolled hyperglycemia develops several complications involving different tissues resulting in diseases like nephropathy, cardiovascular disease (CVD), neuropathy, and retinopathy (13). Most of the adverse complications are associated with specific protein glycation where the reducing sugars and/or their reactive degradation products react with N-terminus of protein (31). With an increasingly high burden of DM and complications in the globe and huge morbidity and mortality, maintaining good glycemic control is a cornerstone in diabetes care need (32). Effective methods of diagnosing pre-diabetes will be required to reduce the risks of progression to diabetes and its long term complication (26). Monitoring of glycemic control in diabetes and prediabetes is currently based on self-monitoring of blood glucose level with laboratory testing of HbA1c and fructosamine which is a marker of the glycemic medium level of the last 2-3 months (33).

2.1 Glycated Hemoglobin (HbA1c)

HbA1c values are also influenced by heterozygous and homozygous Hemoglobin A1c values that are affected by hemoglobin level and gender as demonstrated in a study amongnon-anemic Koreans. (HbAS, HbAE, HbAC, HbAD, HbF, depending on laboratory methodology (34). Moreover, HbA1c biological variability is dependent on several clinical factors (Red blood cell (RBC) life span, chronic kidney disease (CKD), and blood glucose concentration) and on physiological factors (i.e pregnancy)(35,36). Additionally, demographic factors such as race, age, and sex, seem to influence HbA1c values. With this multifaceted variability, the cut off values for HbA1c has not reached yet the same consensus agreement for screening and monitoring prediabetes and diabetes control (37).

2.1.1 HbA1c and Ethnicity

Even though the underlying mechanism is unclear, different scholars clearly reported that ethnic differences in HbA1c values among patients with diabetes and without diabetes(38,39).

Boltri et al. analyzed data from US National Health and Nutrition Examination Survey (1999-2000), and reported that the mean HbA1c value using HPLC with diagnosed diabetes were 7.1%, 8.1%, and 8.2% in whites, blacks and Hispanics, respectively (40). A meta-analysis done by Cavagnolli *et al.*, in 2017, considering data from 49,238 individuals, also found a significant difference between HbA1c levels in Blacks, Asians; and Latinos compared to Whites (41).

In another prospective observational study done in the USA by Bergenstal *et al.*, in 2017, the HbA1c mean value, coming from 104 White and 104 Black with type 2 diabetes for at least 2 years, was of 9.1% for Black people and 8.3% for white people (42). Further support about racial difference in HbA1c is demonstrated in the study done by Kahkoska and colleagues among 1313 youths with type 2 diabetes. The study assessed glycemic control for an average of 9 months, and showed that non-Hispanic black and Hispanic youths had elevated HbA1c when compared with Non-Hispanic white patients (43). Herman and Cohen also found that HbA1c levels were significantly higher in Black (6.2%), Hispanic (5.9%), American Indian (6.2%) and Asian participants (6.0%) as compared to Whites (5.8%) which was done among patients with impaired glucose intolerance, recent onset of type 2 diabetic and long-standing type 2 diabetic(27).

Furthermore, the cohort study conducted from December 2012 to April 2013 from non-diabetic subjects done by Shipman *et al.*, in UK showed that South Asians had higher HbA1c concentration than Whites (44). Many other studies confirm that the level of HbA1c is variable among race both in adults and children with diabetes and without diabetes, in which black people have higher HbA1c level than non-Hispanic white people (27,42,45,46). A Meta-analysis done by Kirk *et al.*, has found that a higher value of HbA1c in African Americans than in non – Hispanic withes people (47).

2.1.2 Gender, age, and HbA1c

Controversial reports are available regarding the effect of sex and age on HbA1c. For example, even though the author used latex immune-agglutinin method to measure HbA1c, a study indicated that the HbA1c values have been increased with increasing age in Japanese patients (48). Whereas, the study in Chinese adults showed no association between HbA1c and gender as well as age (49). However, another cross-sectional study using HPLC among 18,265 china

people without the diagnose of diabetes mellitus, reported that HbA1c levels were associated with gender and age (50).

A cross-sectional study conducted by Ibrahim Ali et al., in Sudan which recruited 444 volunteers with normal glycemic status (91 males, and 353 females) showed that the mean (±SD) HbA1c was 3.8±1.17% and with the range of 1.2-5.4%. In this study, there was week correlation between HbA1c value and age (r = 0.07)(51).

2.2 Fructosamine

A study conducted by William et al. (2019) established the reference range of the Fructosamine in the Brazilian population (n=466) using apparently healthy subjects. The established reference interval was significantly different between women and men respectively 186–248 µmol/L and 196–269 µmol/L with a negative correlation with the BMI (r = -0.117; p = 0.011) (56).

The study conducted in Asia by Xun Chen et al. in 2016, established the adult fructosamine reference intervals in Beijing, China using a large sample size (n=1497) of healthy subjects. The subjects were classified into subgroups: males, females and divided also by age. However, the study found no significant difference between males and females, and a gradual increase of fructosamine level proportional to age (57).

A cross-sectional study was conducted by P. Carson et al. in 2016, to determine average levels of glycemic markers (HbA1c, GA, and fructosamine) in adults with and without diagnosed diabetes, (n=2692). Racial differences were not observed for any of the glycemic markers in people with a diagnosis of diabetes. On the contrary, African-Americans with no diabetes had higher mean levels of Fructosamine (229.8±27.6µmol/l), HbA1c(5.6±0.7 %), GA (13.2±2.0 %) than whites people (fructosamine, 225.7±20.6, HbA1c, 5.3±0.5, Glycated albumin, 12.6±1.4). The analytes were analyzed using colorimetric method with Cobas 6000 chemistry analyzer (45).

Similar study supports the differences in Fructosamine levels depending on race as the study done by Elizabeth Selvin et al., among non-diabetic subjects with and without diabetes. Black persons had significantly higher Fructosamine levels of 238.6 µmol/l (235.3–241.9) than white persons 228.4 (226.9–230.0) (54). Conversely, another study was done by Nouya *et al.,* in 2015,

obtaining results from 437 healthy Cameroonians, which showed that the level of Fructosamine was not associated with neither age, sex nor ethnicity (55).

3. Objective

3.1 General Objective:

- To establish reference interval for Hemoglobin A1c and Fructosamine for apparently Healthy Adults in Addis Ababa, Ethiopia

3.2 Specific Objective:

- To establish a gender-based reference interval for HbA1c and fructosamine
- To compare the distribution of HbA1c and Fructosamine values by gender and age.

4. Hypothesis

The reference intervals for HbA1c and Fructosamine parameters in the adult Ethiopian population are different from the reference interval given by manufacturers.

5. Materials and Methods

5.1 Study Area

The study was conducted at Addis Ababa in selected four sub-cities. Addis Ababa is the Capital city of Ethiopia with an area of 530 km2 and a total population of 2.7 million(1.3 million males, 1.4 million females) according to the 2007 census report of the Central Statistical Agency of Ethiopia (56). It has 10 sub-cities. The health service coverage of Addis Ababa is 71%. Samples (blood, urine, and stool) were collected from 4 sub-cities (Arada, Kirkos, Akaki-Kality, and Yeka-sub city). Arada sub-city has an area of 9.9 sq km with a total population of 225,999 and 10 weredas whereas Kirkos sub city with an area of 14.62 sq km with a total population 235,441 and 11 weredas. Another study area was Akaki-Kality with 118.08 sq.km with a total population of 195,273, and with 11 weredas whereas Yeka sub city which found north east part of Addis Ababa with a total area of 85.98 sq.km with a total population of 368,418 and with 13 weredas. The samples were processed at the Addis Ababa, Department of Medical Laboratory Sciences main laboratory. The analysis was performed at the Ethiopian Public Health Institute (EPHI), National references laboratory for Clinical Chemistry, Addis Ababa. The reference laboratory is equipped with state-of-art analyzers. It is responsible for the provision of high-level diagnostic laboratory testing services for patients referred to the institute and specimens referred from all regional and federal health facilities. Moreover, the laboratory also participates in different research activities both biomedical and survey and surveillances.

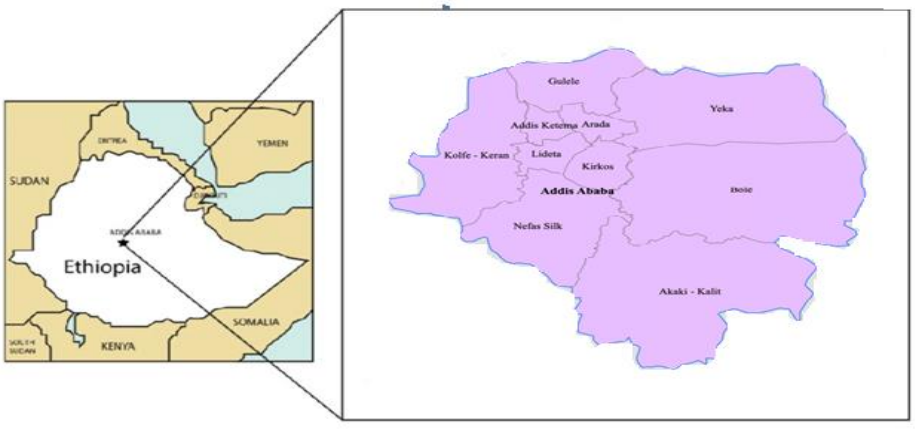

Figure 3. Administrative map of Addis Ababa city and its sub-cities

5.2 Study Design and Study Period

A cross-sectional study design was implemented from December 2019 to April 2020.

5.3 Population

5.3.1 Source population

- The source population for this study was people living in the city of Addis Ababa, Ethiopia with the age of 18 years and above.

5.3.2 Study Population

- The study population was people with 18 years and above that lives in Arada, Kirkos, Akaki, and Yeka sub-cities, Addis Ababa City, who fulfills the eligibility criteria.

5.4 Inclusion and exclusion criteria

5.4.1. Inclusion criteria

¾ Apparently healthy individuals aged 18 years and above who were voluntary to participate in the study and lived in the study area for at least 5 years.

5.4.2. Exclusion criteria

¾ Those experiencing an acute illness and having chronic diseases like diabetes mellitus, chronic renal insufficiency, heart disease, anemia, thyroid, liver diseases, and cancer of any type

¾ Individuals taking pharmacologically active substances: all prescription drugs, and have habit of smoking, alcohol consumption and obese

¾ Individuals having malaria, intestinal parasites and abnormal urine chemical tests and C-reactive protein

5.5 Sampling Techniques and Sample Size

5.5.1 Sampling Techniques

Probability Proportional to Size (PPS) sampling method was employed, where the size depends on the number of households of Woredas (former Kebeles) in a city. Accordingly, all the woredas in the town were considered /selected to be the participants of the study. Since Addis Ababa is a very large city, four sub-cities were selected based on PPS, namely Arada, Kirkos,

Akaki, and Yeka sub-cities; thus all woredas under the selected sub-city were included. To recruit 344 participants, the numbers of households were determined by dividing the total household in the selected towns (sub-cities for A.A) by the estimated number of individuals per household which is 4 for urban. Individuals in every K^{th} household were approached at their households through health extension workers. Once volunteering participants fulfilling the eligibility criteria are identified by the health extension workers, they were invited to go to nearby health facilities for interviews using a structured questionnaire and to facilitate biological sample collection.

5.5.2 Sample Size

CLSI recommends that to establish a reference interval, it is necessary to collect samples from a sufficient number of reference individuals to yield a minimum of 120 samples for analysis for each partition (e.g. sex, age range) with a power of 90% (5). In the current proposed study, for HbA1c and Fructosamine, sex partition is needed. For two partitions (gender-based) (2 x120) 240 samples were needed. According to previous studies in other African countries about 30% do not meet the eligibility criteria (57). In total, 344 individuals were enrolled (i.e, 30 % x 240=72 to be excluded during data analysis); thus giving a total minimum sample size of 344 needed from Addis Ababa city for this study.

5.6 Study Variables

5.6.1 Dependent Variables

- Reference interval of HbA1c and fructosamine

5.6.2 Independent Variables

- Sex
- Age

5.7 Method of data collection and analytical procedure.

Demographic data were collected as part of the National reference interval study using standardized questionnaires. Blood samples of about 8-10 ml from adults were collected in EDTA tubes for CBC and HbA1c, and serum separator tubes using a multisampling needle (method). To minimize diurnal variation and other factors blood samples were collected from fasting study participants at least for 8 hours.

Whole blood was analyzed on the same day of sample collection for CBC (separate study) and HbA1c. Specimens in serum separator tubes were allowed to clot for 30 minutes and centrifuged at 4500 rpm for 10 minutes. Serum separated from whole blood and transferred to two cryovials (each with 1 ml serum) and stored at -80^{O}C until analysis at National Reference Laboratory for Tuberculosis, EPHI.

5.8 Analytical methods

5.8.1 Hemoglobin A1c

HbA1c levels were measured using Cobas 6000 Clinical Chemistry analyzer (Roche Diagnostics GmbH, Mannheim, Germany). Cobas 6000 is a continuous and random-access analyzer with a consolidated test menu for routine clinical chemistry, specific proteins, drugs of abuse screening and therapeutic drug monitoring (TDM), and different measuring technologies.

The HbA1c determination was based on the turbidimetric inhibition immunoassay (TINIA) for hemolyzed whole blood with an inter-assay coefficient of variation (CV) of 1.3% and 1.0 for PreciControl HbA1c norm and PreciControl HbA1c path, respectively (58).

1. Sample and addition of R1 (buffer/antibody reagent): Glycohemoglobin (HbA1c) in the sample reacts with anti-HbA1c antibody to form soluble antigen-antibody complexes. Since the specific HbA1c antibody site is present only once on the HbA1c molecule, the formation of insoluble complexes does not take place.

2. Addition of R 3 (buffer/polyhapten reagent) and start of reaction: The polyhaptens react with excess anti-HbA1c antibodies to form an insoluble antibody-polyhapten complex which can be determined turbidimetrically **Table 1.**

Liberated hemoglobin in the hemolyzed sample is converted to a derivative having a characteristic absorption spectrum which is measured bichromatically during the pre-incubation phase (sample + R1) of the above immunological reaction. A separate Hb reagent is consequently not necessary.

The analyzer automatically calculates the analyte concentration (in % or mmol/mol) of each sample against calibrator/standards. The measuring range of HbA1c is 23-196 mmol/mol or 4.2-20.1% (defined by the lower detection limit and the maximum of the master curve).

The final result is expressed as % HbA1c and is calculated from the HbA1c/Hb ratio as follows:

Protocol 1 (mmol/mol HbA1c according. to IFCC):

$$HbA1c\ (mmol/mol) = (HbA1c/Hb) \times 1000$$

Protocol 2 (% HbA1c according to DCCT/NGSP)

Diabetes Control and Complications Trial (DCCT) and the National Glycohemoglobin Standardization Program):

$$HbA1c\ (\%) = (HbA1c/Hb) \times 91.5 + 2.15$$

Table 1. Product characteristics of HbA1c

Assay	HbA1c
Sample material	Whole blood
Assay volume	5µl
Measuring Range	4.2-20.1 % HbA1c (DCCT/NGSP) 23-196 mmol/mol (IFCC)
Traceability	DCCT/NGSP

5.8.2 Fructosamine

The formation of fructosamine is a two-step reaction, which is dependent on the glucose concentration. Levels of fructosamine was measured using colorimetric method Cobas 6000 clinical chemistry analyzer (Roche Diagnostics GmbH, Mannheim, Germany), using fructosamine reagent with an inter-assay coefficient of variation (CV) of 1.6% and 0.7 for Precinorm Fructosamine and Precipath Fructosamine, respectively (59).

Briefly, the analytical method for fructosamine (glycated protein) is based on the ability of ketoamines to reduce nitroblue tetrazolium in an alkaline medium. The rate of formation of formazan is directly proportional to the fructosamine concentration and is measured photometrically at 700/546 nm **Table 2.**

Table 2. Product characteristics of fructosamine

Assay	Fructosamine
Sample material	Serum/Plasma
Assay volume	6µl
Measuring Range	14-1000µl
Traceability	Fructose polylysine standard.

5.9 Quality Assurance

The good quality of the results was maintained by using standard operating procedures (SOPs) for analysis of all parameters. Laboratory analysis was done by an experienced Laboratory technologist.

In addition to this, two levels of quality control materials (PreciControl HbA1c norm and PreciControl HbA1c path for HbA1c, Precinorm Fructosamine, and Precipath Fructosamine for fructosamine) were measured every day before the actual analysis. Every working day only samples between 50- 100 were analyzed. Moreover, the National reference laboratory for Clinical Chemistry, EPHI is also participated in the external quality assurance program (EQA-One world Accuracy) and also the lab accredited in 2017 by the Ethiopia national accreditation office e (ENAO). Pretested questionnaire were used for data collection by health extension workers.

5.10 Data Management and Statistical Analysis

The Data was recorded in the Microsoft Office Excel 2013 and double checked for completeness, and transferred to SPSS (Statistical packages for social sciences) version 20. Before the actual analysis, the distribution normality checked by Kolmogorov-Smirnov test. Mann-Whitney test was done to see whether partition between males and females was needed or not. We used the non-parametric method to calculate the reference interval (the 2.5^{th} and 97.5^{th} percentiles for the lower reference intervals and upper reference intervals) with 90% confidence interval after reviewing the distribution of the data. Before the acthe actual analysis conducted

88 study particpinats excluded, 46(13.37%) with insufficent and missing, 30(8.72%) with CRP result, 12(3.49%) outliner according Tukey.

5.11 Ethical Consideration

Ethical Clearance was obtained from the Department of Medical Laboratory Technology, College of Health Sciences, Addis Ababa University, and appropriate consent was received from individuals participating during the study time. A consent form was prepared and detailed explanation of the objectives of the study, risks, and benefits, the confidentiality of the study explained to the study participants. During the study, any abnormal finding was communicated to the nearest health facility for follow up.

5.12 Dissemination

The study findings will be presented at the Department of Medical Laboratory Sciences of Addis Ababa University and copies will be kept in the library for future reference. The findings will also be communicated to EPHI, Addis Ababa Health Bureau and other stakeholders through presenting at scientific conferences. Manuscript will be submitted to local or international peer reviewed journals.

5.13 Operational definition

Apparently Healthy: Individuals with no clinical condition and negative for the screening tests like stool, urinalysis and C-reactive protein

Reference Interval: the interval between the lower 2.5th and upper 97.5th percentile limit

Reference individual: a person selected from reference population for testing on the basis of well-defined criteria.

Reference population: a group encompassing of all the reference individuals.

Reference sample group: an adequate number of individual's recruited from reference population to represent it.

6. Result

6.1 Demographic Characteristics

Out of the total 344 participants, 256 (121 males, 135 females,) of them fulfilled the eligibility criteria and were included to establish a reference interval for HbA1c and fructosamine **(Table 3).** About half of the study participants (both male and female) were 21-39 years of age and more than three-fourths of the study participants had < 25 kg/m2. About 65(54%) and 78(58%) of male and female study participants were government employees, and almost half of the study participants (both Male and Female) were from Amhara ethnicity. The majority of the study participants were college/diploma/degree, and single.

Table 3. Socio-demographic characteristics of study participants of Addis Ababa City, n=256

Variable	Male n (%)	Female n (%)
Sex	121(47.3)	135(52.7)
Age		
18-20	16(13.2)	5(3.7)
21-29	45(37.2)	43(31.9)
30-39	42(34.7)	51(37.8)
40 and above	18(14.9)	36(26.7)
BMI (kg/m^2)		
<25	92(76)	78(58.2)
≥25	29(24)	56(41.8)
Occupation		
Student	14(11.7)	2(1.5)
House wife	0(0)	28(20.7)
Government employee	65(54.2)	78(57.8)
Private employee	38(31.7)	23(17.0)
Others	3(2.5)	4(3.0)
Ethnicity		
Oromo	17(14.0)	23(17.5)
Amhara	58(47.9)	73(54.1)
Tigray	6(5.0)	4(3.0)
Gurage	4(3.3)	11(8.1)
Others	36(29.8)	24(17.8)
Educational Status		
Illiterate	1(0.8)	7(5.2)
Read and write	0(0)	6(4.4)
Primary(1-8)	21(17.5)	19(14.1)
Secondary(9-12)	24(20)	38(28.1)
College/Diploma/Degree and above	74(61.7)	65(48.1)
Marital Status		
Single	65(54.2)	52(38.5)
Married	45(37.5)	66(48.9)
Divorced	1(0.8)	8(5.9)
Widowed	2(1.7)	5(3.7)
Others	7(5.8)	4(3.0)

The mean ± SD of HbA1c and fructosamine were presented in Figure 3. The mean ±SD of fructosamine and HbA1c of study participants were 243.5μmol/L ±27.0 and 5.37%±0.41, respectively.

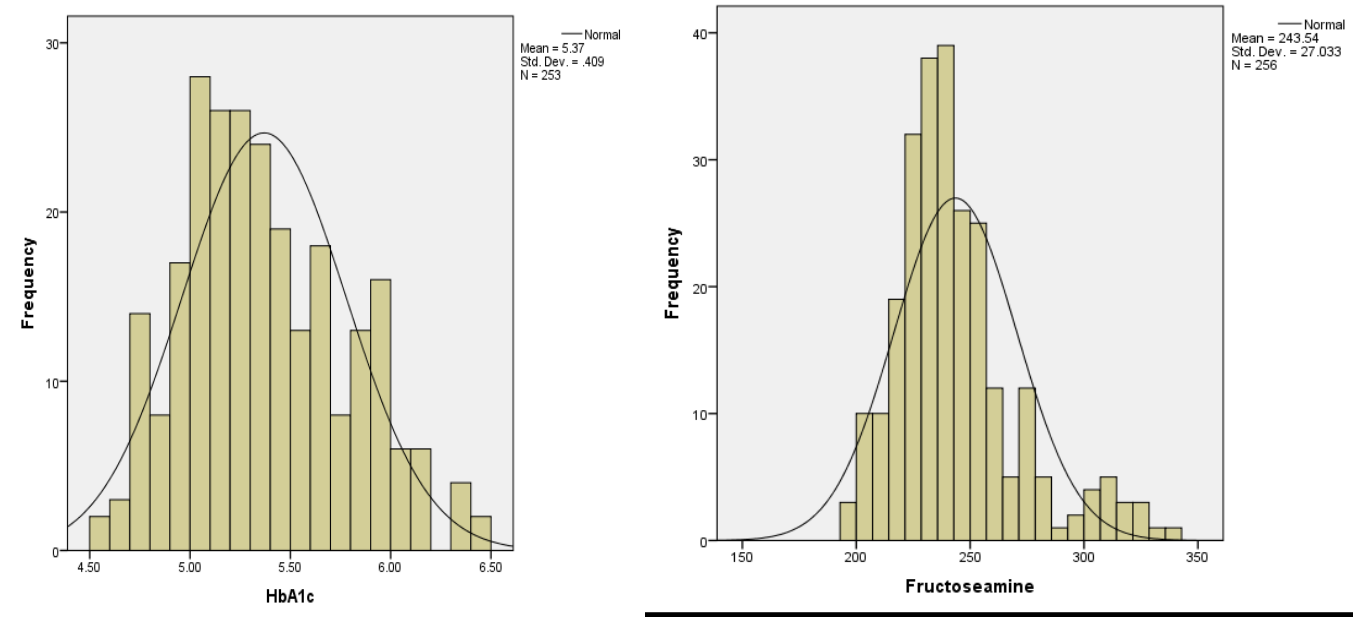

Figure 4. Histogram shows distribution of HbA1c and fructosamine of study participants Addis Ababa city, Ethiopia.

6.2 Hemoglobin A1c (HbA1c)

The minimum and maximum of HbA1c of the study participants were 4.53% and 6.45%, respectively, with 4.63% and 6.45% in males and 4.53% and 6.30% in females respectively. The overall distribution of HbA1c was not normally distributed *(P<0.0001).* The calculated 2.5th and 97.5th percentile of HbA1c for both gender were 4.70% - 6.27%, with 4.71% - 6.19% in male, and 4.67% - 6.36% in female **(Table 4).**

Table 4. The reference Interval of HbA1c (% and mmol/mol) among apparently healthy adults subject, Addis Ababa, Ethiopia (n=253) (male = 120, female = 133)

Analyte	Sex	Range	Median	2.5^{th} -97.5^{th} percentile	90% CI		*P value*
					lower reference limit	upper reference limit	
HbA1c (%)	Combined	4.53 – 6.45	5.30	4.70 – 6.27	4.63, 4.74	6.12, 6.37	
	Male	4.63 – 6.45	5.23	4.71 – 6.19	4.63, 4.75	6.12, 6.45	*0.07*
	Female	4.53 – 6.30	5.29	4.67 – 6.36	4.53, 4.74	6.01, 6.43	
HbA1c/mol)	Combined	26.0 – 47.0	34.0	28.0 – 44.7	27.0, 28.0	43.0, 46.0	
	Male	27.0 – 47.0	34.0	28.0 – 44.0	27.0, 28.0	43.0, 47.0	*0.07*
	Female	26.0 – 47.0	35.0	28.0 – 46.0	26.0, 28.0	42.0, 47.0	

The level of differences was tested for HbA1c within different groups by adjusting sex and age groups. HbA1c interval has shown statistical difference among different age group (p=0.0187) but the differences was not observed in male age groups, **Table 5,** The level of HbA1c among female study subjects has shown weak correlation with age(r=0.1803, p=0.0378) (**Figure 5**).

Table 5. Sex adjusted age specific reference interval of HbA1c (%), Addis Ababa, Ethiopia

	Age group (in years) specific central 95% ref value HbA1c (%)				$X^2(d.f)^*$, *P =value*
	18-20	**21-29**	**30-39**	**+40**	
Both Sex	4.63 - 6.55 n=21	4.45 - 6.01 n=89	4.50 - 6.03 n=90	4.63 - 6.27 n=53	*9.988(3), p=0.0187*
Male	4.67 - 6.50 n=16	4.49 - 6.05 n=45	4.46 - 5.98 n=40	4.35 - 6.40 n=19	*1.417(3), p=0.7016*
Female	3.87- 6.43 n=5	4.51- 6.09 n=44	4.51 - 6.11 n=50	4.77 - 6.40 n=34	*10.301(3). P=0.0162*
P=value	0.5087	0.3108	0.3441	0.1403	

**Kruskal-Wallis rank test*

*** Mann-Whitney two sample rank sum test*

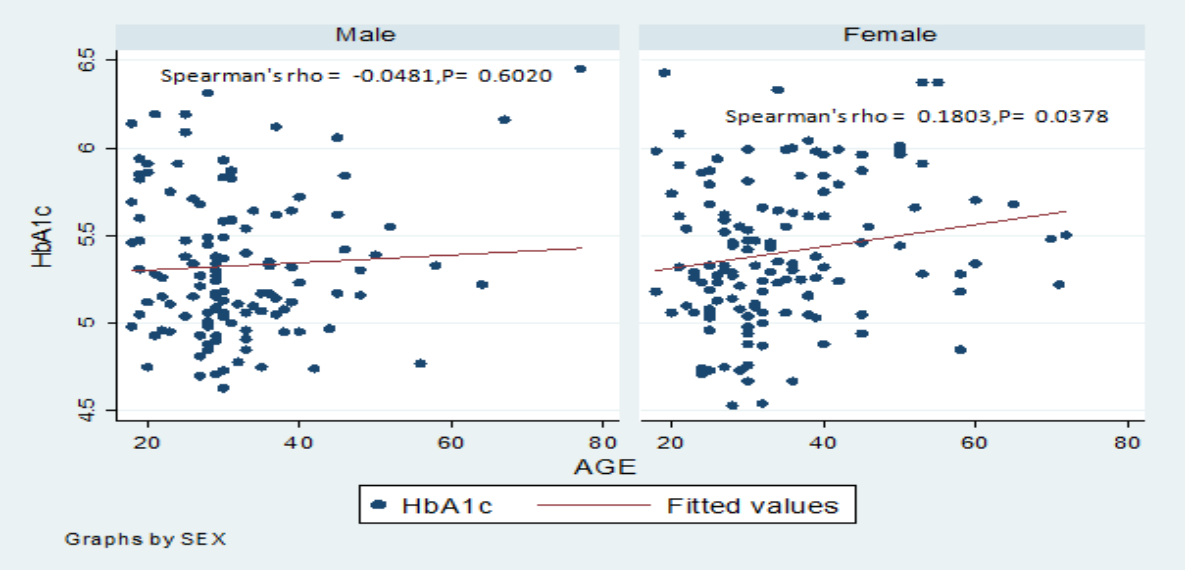

Figure 5. Regression fit of age versus HbA1c (%) of both males and females.

6.3 Fructosamine

The minimum and maximum of fructosamine of our study participants were 195.0 µmol/L and 337.0 µmol/L, respectively, with 195.0 µmol/L and 337.0 µmol/L in males and 197.0 µmol/L and 328.0µmol/L in females respectively. The overall distribution of fructosamine was not normally distributed. The calculated 2.5th and 97.5th percentile of fructosamine for both gender were 203.0 µmol/L and 321.0 µmol/L respectively with 203.0 µmol/L and 322.0 µmol/L in male, and 202.0 µmol/L and 312.8 µmol/L in female (**Table 6**).

Table 6. The reference Interval of fructosamine (µmol/L) among apparently healthy adults subject, Addis Ababa, Ethiopia (n=256) (male = 121, female = 135)

Analyte	Sex	Range	Median	2.5th 97.5th percentile	90% CI		*P value*
					lower reference limit	upper reference limit	
Fructosami	Combined	195.0 – 337.0	238.0	203.0 – 321.0	198.0, 207.0	308.0, 328.0	
	Male	195.0 – 337.0	238	203.0 – 322.0	195.0, 207.0	309.0, 337.0	*0.23*
	Female	197.0 – 328.0	233.5	202.6 – 312.8	197.0, 209.0	280.0, 328.0	

Sex adjusted age groups specific reference interval of fructosamine was tested for normality among different groups. There was no statistical different observed for age groups for fructosamine levels (**Table 7**).

Table 7. Sex adjusted age specific reference interval of fructosamine (µmol/L), Addis Ababa, Ethiopia

	Age (in years)group specific central 95% ref value fructosamine(µmol/L)				X^2(d.f)*$P=Value$
	18 – 20	**21 - 29**	**30 - 39**	**+ 40**	
Both Sex	197 -278 n=21	188 - 286 n=88	177 - 292 n=93	184 -310 n=54	*4.386(3), p value =0.2227*
Male	193 - 285 n=16	178 - 290 n=45	171 - 306 n=42	173 - 324 n=18	*4.225(3), p value=0.2382*
Female	204 -267 n=5	196 - 282 n=43	180 -282 n=51	184 - 307 n=36	*7.729(3), p value=0.0520*
P= value**	0.4570	0.3852	***0.0093***	0.6267	

**Kruskal-Wallis rank test*

*** Mann-Whitney two sample rank sum tes*

There was no correlation between fructosamine and age for males (r=0.1406, p=0.1241) and also between fructosamine and age in females (r=0.0703, p=0.4177) (**Figure 6**).

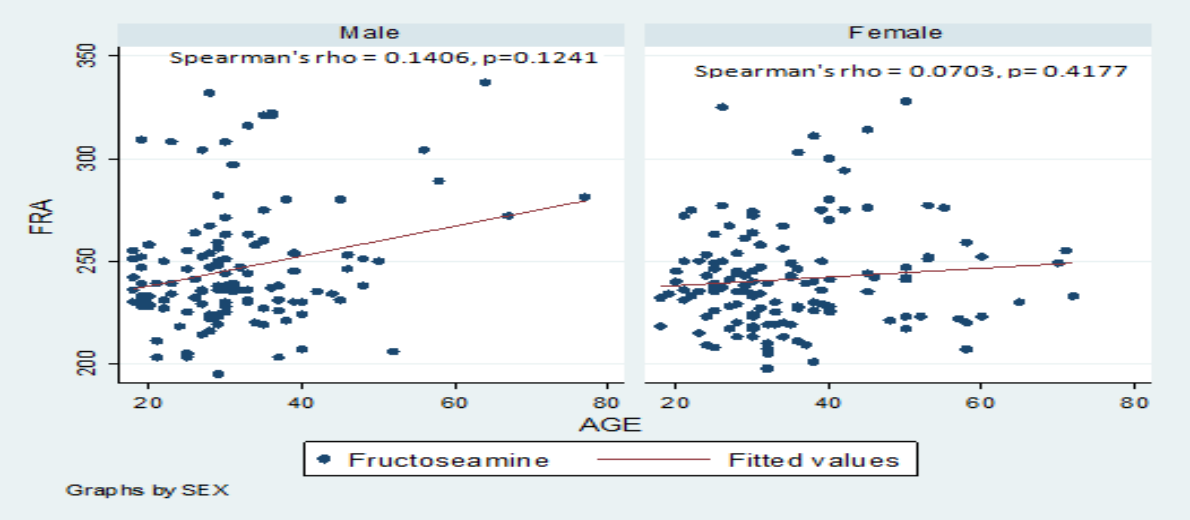

Figure 6. Regression fit of age versus fructosamine (µmol/L) (%) of both males and females.

7. Discussion

Most of the clinical laboratories in Ethiopia based on reference intervals established for other populations and studies indicated that variations observed between the western population and the African population (60–62). The current study aimed to established reference intervals for HbA1c and fructosamine in apparently healthy adults. The study utilized the Roche platform for analysis which is currently the predominantly available clinical chemistry analyzer and will improve the current practice in the country. In this study, the 2.5^{th} and 97.5^{th} percentile reference intervals were established for HbA1c and fructosamine, which are the commonly used parameters to diagnose, treatment, and monitoring of DM.

7.1 HbA1c

The reference interval established in this study for HbA1c was 4.70% to 6.27% (DCCT/NGSP), 28.0 mmol/mol to 44.7 mmol/mol (IFCC) for both gender with 4.71% (28.0 mmol/mol) to 6.19% (44.7mmol/mol) in males and 4.67% (28.0mmol/mol) to 6.36% (46.0mmol/mol) in females. The parameters are reported using different units; percentage according to the National Glycohemoglobin standardization Program and the Diabetes Control and Complication Trial criteria and in mmol/mol according to IFCC (34,63). In the present study, females had a higher value of HbA1c than males, this might be due to females had lower hemoglobin levels compared to males since HbA1c value is calculated from the ratio of glycated hemoglobin to hemoglobin value (10,64).

The result of this study is consistent with the result reported from Brazil with a large sample size (n=6230, male=3011, female=3219). In the Brazilian study (66), they established HbA1c reference interval from 4.5% to 6.1% for males and 4.4% to 6.1% for female study participants and no differences were found between males and females in this study.

Another study from Thailand using 144 subjects (99 males, 45 females) showed that the established reference interval for HbA1c was 4.79% to 6.15% and the authors concluded that HbA1c value is not affected by sex but affected by age group (67). Similar study also conducted in Thailand in 699 study participants and they found that 4.8% to 6.6% HbA1c value which is higher than the current study upper limit (68).

HbA1c assay was developed in 1978 and received globally to diagnose and monitor DM starting since 1980 (69). Nowadays, many methods are developed and available on the market to

measure HbA1c and almost all suppliers suggested different reference intervals on their packing insert (70). ADA and WHO recommended HbA1c value ≥ 6.5% as a diagnostic cut of value for diabetics, and ADA further recommended HbA1c value < 5.7% as normal, and value between 5.7% to 6.4% as pre-diabetes (71,72).

The current reference interval used by the national reference laboratory for clinical chemistry is 4.8 % -5.9 % (DCCT/NGSP) and 29 mmol/mol - 42 mmol/mol (IFCC) (73) and the established HbA1c value was comparable to the reference intervals given my manufacture with a slight difference in the upper limit. The possible explanation for the difference could be explained by the abnormalities of erythrocyte structure due to genetic differences of population (74), nutrition, and altitude (75) since the study area is above 2350 meters and the above cutoff value recommended by these two organizations may not apply to the Ethiopian adult population..

7.2 Fructosamine

Traditional biomarkers like glucose and HbA1c which are currently used for diagnostic and prognostic strategies in diabetes, may be biased due to the rapid changes of glucose hemostasis, red blood cell disorder, and renal disease (76,77). Fructosamine (the total glycated serum proteins) are alternate biomarkers of glycemia that have been accepted to offer additional information to HbA1c or to provide a reliable measure when HbA1c is observed not to be trustworthy (78).

Compared with glucose and HbA1c, fructosamine shows advantages that can accurately estimate short-term changes in glycemia that correspond to the half-life of albumin (79). When HbA1c values are less accurate in ongoing hemolytic anemia and in hematological diseases that change the survival lifetime of RBC, fructosamine may be a more alternative marker (80,81).

The current reference interval used by the laboratory is 205-285 umol/L and it was established by Roche three decades ago from 555 “apparently healthy” blood donors between 20 and 60 years of age (82). In the current study, we established a reference interval for fructosamine with a range from 203- 321umol/L and the currenet study differe in the upper limit when compared to established references interval by manufacturer.

There were different researchers who established reference intervals for the fructosamine parameters. The study conducted in Brazil indicated that the established fructosamine reference

interval 196–269 μmol/L and 186–248 μmol/L for males and females, respectively where males had higher fructosamine levels compared to females (p=0.006) (68). Another study conducted in China by Qiang Zhou and *et al.*, 2017 using 458 study participants (male=226, females=298) indicated that the reference intervals for both genders were 220-298μmol/L and only 23μmol/L absolute difference observed with our upper references limit (83).

In another study conducted in the USA using large sample size (n=1799), the 2.5^{th} and 97.5^{th} percentiles were 194.8 and 258.0μmol/L (84). Differences among studies may also emerge from the use of different kits. The data obtained from this study demonstrated that there was no significant gender difference in the fructosamine level and the small differences between both genders probably do not influence the interpretation of clinical results. Previous reports indicated from China Beijing (83), New Zealand (85) have also demonstrated similar results. However, this finding is in contrast with some previous findings where a significant difference in serum fructosamine level was revealed between men and women (55).

In the present study, to establish reference interval we used the Roche Cobas 6000 in which some reports used the same analyzer Roche Diagnostic kits and analyzers. The current finding is nearly similar to the previous finding by Chen et al. in China among 1497 healthy individuals which used the same kits and analyzer with our study. The authors reported that the reference range for Fructosamine was 216-290 μmol/L (53). However, the reference interval established for fructosamine in the current study was higher (36 μmol/L absolute difference) than Roche package insert value in upper cut-point interval value.

The relation between HbA1c and fructosamine with the age was evaluated. In the current study, age had weak positive association (r=0.1803, p=0.0378) with HbA1c among female participant. This result supported with other studies conducted USA by Dubowitz et al. (86), and Pani et al. (87) and a possible explanation for this could be alternation of glucose metabolism during aging (88) which result from increased adbdominal fat mass, mitochoderia dysfunction, and decaresed physical activity and increased oxidativer stress and inflammation during aging (89).

8. **Strength and limitation of the study**

8.1 Strength of the study

First, to the best of our knowelege, this is the first effort to establish a reference range for HbA1c and fructosamine levels in the Ethiopian population.

Second, the laboratory analysis was done at a national reference laboratory for clinical chemistry and this laboratory is equipped with state-of-art clinical chemistry analyzers, which implemented quality laboratory management systems and accredited since 2017 by the Ethiopian National Accreditation Office (ENAO).

Third, we used Roche HbA1c and fructosamine kits; nowadays these assays are the predominant assay in Ethiopia and maybe fairly generalized for health facilities in Addis Ababa using similar platform.

8.2 Limitation of the study

There are some limitations in this study.

- The study population was limited to Addis Ababa only, which might not apply to the rest of the regions.
- The study did not check the effect of serum albumin level which is known to affect fructosamine concentration; however, since the study participants are apparently healthy fulfilling the set exclusion criteria, it is less likely for the value to be influenced by abnormal albumin level.

9. Conclusion and Recommendation

9.1 Conclusion

The current study established reference intervals for fructosamine and HbA1c for the important demographic group. The established referenc interval was no gender difference both on HbA1c and fructosamine level whereas there was age group difference between on HbA1c level. The established reference interval for HbA1c is comparable with the reference interval recommended by the international organization, but the reference interval established for fructosamine (especially upper limit) is slightly higher than the current national reference laboratory report. The result of this study, is helpful for our population instead of using a reference interval that is developed from the western population with different socioeconomic and demographic characteristics.

9.2 Recommendation

- We recommend the current reference interval study will be used for medical care for diagnosis and monitoring of diabetes mellitus.
- We recommend also every laboratory should establish its own reference interval for its population for better diagnosis, treatment, and monitoring. This study will serve as a baseline data for further investigation using large sample size and different socio-demographic status.

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Annex I . English Version Questionnaire

Questionnaires to be filled by health professionals

Part I. General information

Code Number_____Region_____Zone_____

Woreda_____/ city /_sub city_____Kebele_____

Part II. Personal information

1. Age (in years)_____
2. Sex _____
3. Place of Birth_____
4. For how long (years) did you live in the birth place?_____
5. How long do you live in this specific area? (If different from the birth place)_____years

No.	Questions	Responses
	Part III. SOCIO-DEMOGRAPHIC INFORMATION	
6.	Educational status	1. Illiterate 2. Read and write 3. Primary (1-8) 4. Secondary (9-12) 5. College diploma/degree and above
7.	Occupation	1. Student 2. House wife 3. Government employee 4. Private employee 5. Farmer 6. Others (specify) _____
8.	Marital status	1. Single 2. Married 3. Divorced 4. Widowed 5. Not applicable (children)
9.	Religion	1. Orthodox Christian 2. Muslim 3. Protestant 4. Catholic 5. Others (Specify) _____
10.	Ethnicity	_____If mixed, specify _____
11.	Residence	1. Rural 2. Urban

	Questions 7-12 are additional questions to Students	
12.	Father's Age	_____
13.	Mother's Age	_____
14.	Father's Educational Level	1. Illiterate 2. Read and write 3. Primary (1-8) 4. Secondary (9-12) 5. College diploma/degree and above
15.	Mother's Educational Level	_____
16.	Father's Occupation	
17.	Mother's Occupation	
18.	Monthly income (in birr collected from salary, rent, and other income)	_____Birr
19.	Family Size (Number of People)	_____
20.	Source of water	1. Pipe 2. Spring water 3. Well water 4. River 5. Other sources (specify)
21.	Type of house	1. Mud 2. Cement 3. Wood 4. Bricks 5. others/specify_____
22.	Presence of or contact with Pet animals (e.g. Cat, Dog)	1. Yes 2. No
23.	Presence of domestic animals	1. Yes 2. No
	Part IV. Clinical information	
	Questions 24-28 for female participant who are pregnant specify	
24.	Gestation_____(weeks)	
25.	Parity _____	
26.	Iron supplementation:	1. Yes 2. No
27.	Folate supplementation	1. Yes 2. No
28.	Iron and folate combined supplementation	1. Yes 2. No
29.	Did you take any type of drug for any illness for the last three month?	1. Yes 2. No

30.	If yes to Q29, what type of drug? (more than one answer possible)	1. Anti-protozoa 2. Anti-helminthic 3. Anti-allergy 4. Birth control pills 5. Anti-bacterial 6. Anti-TB 7. Other (specify)
	History of common diseases	
31.	History of diabetes	1. Yes 2. No
32.	History of Hypertension	1. Yes 2. No
33.	History of Blood transfusion for the last 1 year	1. Yes 2. No
34.	Any history of blood transfusion	1. Yes 2. No
35.	History of Hospital Admission for the last 1 year	1. Yes 2. No
36.	History of Surgical procedure for the last three years?	1. Yes 2. No
37.	History of chronic gastritis	1. Yes 2. No
38.	History of Malaria for the last 6 month	1. Yes 2. No
39.	History of TB for the last two years	1. Yes 2. No
40.	History of Cancer	1. Yes 2. No
41.	History of Cardiac illness	1. Yes 2. No
42.	History of Bleeding disorders	1. Yes 2. No
43.	History of allergy	1. Yes 2. No
44.	History of Wheezing	1. Yes 2. No

Part V. Nutritional habit and your life style

How often do you eat the following food? (put a "√ " mark)							
No.	**Food type**	**A Once/day**	**B More than Once/ day**	**C 2-3 times/week**	**D Occasionally (e.g holidays, special ceremonies)**	**E Never**	**Remarks**
45.	Roots and Tuber (Potato, sweet potato, Enset, Cassava)						
46.	Legumes (Beans, peas, chicken pea, etc)						
47.	Cereals (Corn, Teff, Wheat, sorghum, etc)						
48.	Vegetables (Tomato,						

	cabbage, etc)						
49.	Fruits (Orange, banana, etc)						
50.	Meat (including poultry, fish, etc)						
51.	Milk and Milk products (Butter, yoghurt, cheese, etc)						
52.	Egg						
53.	Tea and/or coffee						
How frequent do you consume/use the following (put a √ mark)							
		Once/day (Regular)	More than once/day	2-3 times/week	Once a week	Occasionally (holiday, special ceremony)	Never
54.	Alcohol						
55.	*Khat*						
56.	Cigarettes						

	Part V. Life style/Habit Continued…	
57.	Do you have Fasting habit?	1. Yes 2. No
58.	If Yes, How is your fasting habit?	1. Eating vegetable food only 2. Complete abstinence from food then eating all kinds of food 3. Complete abstinence from food then eating vegetable food only
59.	Did you eat undercooked/raw meat?	1. Yes 2. No
60.	Do you have the habit of physical Exercise?	1. Yes 2. No
61.	If yes, how many times do you do the exercise per week?	
62.	Any sexual contact	1. Yes 2. No 3, Not applicable (children)
63.	If yes to Q45, condom use`	1. Yes 2. No
	Part VI. Anthropometric measurement	
64.	Height (in cm)	_____
65.	Weight (in kg)	_____
66.	MUAC	_____in cm (will be interpreted later)
67.	Blood pressure (mm Hg)	_____

❖ We thank you for your cooperation!

Annex II: Questionnaire Amharic version (ቃለ መጠይቅ)

ክፍል 1. አጠቃላይ መረጃ

ኮድ_____ክልል_____ዞን_____

ወረዳ_____ከተማ/ክፍለከተማ_____ቀበሌ _____

ክፍል 2. የግል መረጃ

እድሜ _____

ጾታ_____

የትውልድ ቦታ

በትውልድ ቦታዎ ለምን ያህል ጊዜ ኖረዋል?_____

አሁን ያሉበት ቦታ ለምን ያህል ጊዜ ኖረዋል? (ከትውልድ ቦታዎ የተለየ ከሆነ)_____ዓመት

ቁጥር.	ጥያቄ	ምላሽ
	ክፍል 3. ማህበራዊና ኢኮኖሚያዊ መረጃ	
24.	የትምህርት ደረጃ	6. ያልተማሩ 7. ማበብና መፃፍ 8. አንደኛ ደረጃ (1-8) 9. ሁለተኛ ደረጃ (9-12) 10. ኮሌጅ ዲፕሎማ/ዲግሪ እና ከዚያ በላይ
25.	ሥራ	7. ተማሪ 8. የቤት እመቤት 9. የመንግስት ሠራተኛ 10. የግል ተቀጣሪ 11. ገበሬ 12. ሌላ ካለ ይግለፁ _____
26.	የጋብቻ ሁኔታ	6. ያላገቡ 7. ያገቡ 8. የተፋቱ 9. ባል/ሚስት የሞተባቸው 10. አይመለከታቸውም (ህፃናት)
27.	ሃይማኖት	6. ኦርቶዶክስ ክርስቲያን 7. ሙስሊም 8. ፕሮቴስታንት 9. ካቶሊክ 10. ሌላ ካለ ይግለፁ _____
28.	ብሄረሰብ	_____ድብልቅ ከሆኑ ይግለፁ
29.	መኖሪያ ቦታ	2. ገጠር 2. ከተማ
	ጥያቄ 7-12 ለተማሪዎች ተጨማሪ ጥያቄዎች	
30.	የአባት እድሜ	_____
31.	የእናት እድሜ	_____
32.	የአባት የትምህርት ደረጃ	6. ያልተማሩ 7. ማንበብና መፃፍ 8. አንደኛ ደረጃ (1-8) 9. ሁለተኛ ደረጃ (9-12) 10. ኮሌጅ ዲፕሎማ/ዲግሪ እና ከዚያ በላይ

33.	የእናት የትምህርት ደረጃ (ከተ/ቁ 14 ይምረጡ)	_____
34.	የአባት ሥራ	1. ተማሪ 2. የቤት እመቤት 3. የመንግስት ሠራተኛ 4. የግል ተቀጣሪ 5. ገበሬ 6. ሌላ ካለ ይግለፁ _____
35.	የእናት ሥራ (ከተ/ቁ 16 ይምረጡ)	_____
36.	ወሃዊ ገቢ (በብር ከደሞዝ፣ ኪራይ፣ እና ሌሎች ገቢዎች)	_____ብር
37.	የቤተሰብ ብዛት	_____
38.	የውሃ ምንጭ	6. ቧንቧ 7. የምንጭ 8. የጉድጓድ 9. የወንዝ 10. ሌላ ካለ ይግለፁ _____
39.	የቤት አይነት	2. ጭቃ 2. ሲሚንቶ 3. እንጨት 4. ጡብ/ሸክላ 5. ሌላ ካለ ይግለፁ _____
40.	የቤት ውስጥ ለማዳ እንስሳ መኖር ወይም ንክኪ (ለምሳሌ ድመት፣ ውሻ)	2. አለ 2. የለም
41.	የቤት እንስሳት መኖር	2. አለ 2. የለም
	ክፍል 4. የጤና መረጃ	
	ከ 24-28 ያሉት ጥያቄዎች ለነፍሰጡር ሴቶች ብቻ ነው	
45.	ከፀነሱ ስንት ጊዜዎ ነው?	_____(ሳምንት)
46.	ለስንተኛ ጊዜ ነው የፀነሱት?	_____
47.	ተጨማሪ ብረት ንጥረነገር	2. አዎን 2. የለም
48.	ተጨማሪ ፎሌት ንጥረነገር	2. አዎን 2. የለም
49.	ተጨማሪ የብረት ንጥረነገር ና ፎሌት	2. አዎን 2. የለም
50.	ባፉት ሶስት ወራ ለማንኛውም ዓይነት ህመም	2. አዎን 2. የለም

	ማንኛውንም ዓይነት መድሃኒት ወስደኋል?	
51.	ለተራ ቁጥር 29 መልስዎ ወስጃለሁ ከሆነ የትኛውን ዓይነት መድሃኒት ነው ወሰዱት? (ከአንድ በላይ መልስ ይቻላል)	8. ፀረ-ፕሮቶዞአ 9. ፀረ-ሄልሚንትስ 10. ፀረ-አለርጂ 11. የወሊድ መከላከያ ኪኒን 12. ፀረ-ባክቴሪያ 13. ፀረ-ቲቢ 14. ሌላ ካለ ይግለፁ _____
	የሚከተሉት የህመም ዓይነቶች አሞዎት ያውቃል?	
52.	የስኳር ህመም?	2. አዎን 2. የለም
53.	የደም ግፊት ከፍ ማለት?	1. አዎን 2. የለም
54.	ባለፈው 1 ዓመት ደም ተሰጥቶዎ ያውቃል?	1. አዎን 2. የለም
55.	ማንኛውም ጊዜ ደም ተሰጥቶዎ ያውቃል?	1. አዎን 2. የለም
56.	ባለፈው 1 ዓመት ሆስፒታል ተኝተው ያውቃሉ?	1. አዎን 2. የለም
57.	ባለፉት 3 ዓመታት የቀዶ ህክምና ተደርጎልዎ ያውቃል?	1. አዎን 2. የለም
58.	የቆየ የጨጓራ ህመም አለብዎት?	1. አዎን 2. የለም
59.	ባፉት 6 ወራት የወባ ህመም አጋጥሞዎት ያውቃል?	1. አዎን 2. የለም
60.	ባለፉት 2 ዓመታት የቲቢ ህመም ኖሮዎት ያውቃል?	1. አዎን 2. የለም
61.	ካንሰር ህመም	1. አዎን 2. የለም
62.	የልብ ህመም	1. አዎን 2. የለም
63.	የመድማት ችግር/ህመም	1. አዎን 2. የለም
64.	አለርጂ (የሰውነት መቆጣት)	1. አዎን 2. የለም
65.	የመተንፈስ ችግር (ሲተነፍሱ ሲር ሲር የሚል ድምፅ)	1. አዎን 2. የለም

ክፍል 5. የአመጋገብ እና የህይወት ልምድዎ

የሚከተሉትን የምግብ ዓይነቶች ምን ያህል ጊዜ ይመገቢቸዋል ? (“√ “ ይህን ምልክት ያስቀምጡ)							
ተ/ቁ	የምግብ ዓይነት	1 በቀን አንድ ጊዜ	2 በቀን ከአንድ ጊዜ በላይ	3 በሳምንት ከ 2 እስከ 3 ጊዜ	4 አልፎ አልፎ (ለምሳሌ፣ ለበዓል፣ ልዩ ዝግጅቶች ሲኖሩ)	5 ተጠቅሜ አላውቅም	ማብራሪያ
45.	ሥራ ሥር (ድንች፤ ስኪር ድንች፣ እንሰት፣ ካሳቫ ወዘተ)						
46.	አባዝርት (Legumes፡ ባቄል፣ አተር፣ ሽንብራ ወዘተ)						
47.	ጥራጥሬ (በቆሎ፣ ጤፍ፣ ስንዴ፣ ማሽላ)						
48.	አትክልት (ቲማቲም፣ ጎመን፣ ወዘተ)						
49.	ፍራፍሬ (ብርትኳን፣ ሙዝ፣ ወዘተ)						
50.	ሥጋ (የዶሮ፣ የ አሳን ጨምሮ)						
51.	ወተትና የወተት ተዋፅዎ (እርጎ፣ ቅቤ፣ አይብ፣ ወዘተ)						
52.	እንቁላል						
53.	ሻይ እና/ወይም ቡና						
የሚከተሉትን ምን ያህል ይበላሉ/ይጠቀማሉ (√ ይህን ምልክት ያስቀምጡ)							
		በቀን አንድ ጊዜ (ሁልጊዜ)	በቀን ከ1 ጊዜ በላይ	በሳምንት ከ 2 እስከ 3 ጊዜ	በሳምንት 1 ቀን	አልፎ አልፎ (ለምሳሌ፣ ለበዓል፣ ልዩ ዝግጅቶች ሲኖሩ)	ተጠቅሜ አላውቅም
54.	አልኮል						
55.	ጫት						
56.	ሲጋራ						

	ከ ክፍል 5 የቀጠለ. የህይወት አመራርና ልምዶች	
57.	የመፅም ልምድ አለዎት?	2. አዎን 2. የለም
58.	መልስዎ አዎን ከሆነ፣ የመፅም ልምድዎ እንዴት ነው?	4. አትክልቶችን ብቻ መመገብ 5. በአጠቃላይ ከምግብ መታቀብ ከዚያም ያገኙትን መመገብ 6. በአጠቃላይ ከምግብ መታቀብ ከዚያም አትክልቶችን መመገብ
59.	በደንብ ያልበሰለ ወይም ጥሬ ሥጋ ይመገባሉ?	2. አዎን 2. የለም
60.	የሰውነት እንቅስቃሴ የማድረግ ልምድ አለዎት?	2. አዎን 2. የለም
61.	መልስዎ አለኝ ከሆነ በሳምንት ለምን ያህል ጊዜ ይንቀሳቀሳሉ?	
62.	የግብረ ሥጋ ግኑኝነት አድርገው ያውቃሉ	1. አዎን 2. የለም 3, አይመለከትም (ለህፃናት)
63.	ለ ተ/ቁ 66 መልስዎ አዎን ከሆነ፣ ኮንዶም ይጠቀማሉ?`	2. አዎን 2. የለም
	ክፍል 6. ክብደት፣ ቁመት፣ የክንድና የደም ግፊት ልኬት	
64.	ቁመት	_____ሴንቲ ሜትር
65.	ክብደት	_____ኪሎ ግራም
66.	የክንድ መሃለኛው ክፍል ዙሪያው (MUAC)	_____ሴንቲ ሜትር
67.	የደም ግፊት (በሚሊሜትር ሜርኩሪ)	_____(mm Hg)

❖ ስለትብብርዎ እናመሰግናለን!

ቃለ መጠይቅ የተደረገበት ቀን:_____

ቃለ መጠይቁን ያካሄደው ስም_____ ፊርማ_____

Annex III

Ethiopian Public Health Institute
National Reference Laboratory for Clinical Chemistry

Procedure for HbA1c

Compiler:	Signature:
Approver:	Signature:
Effective Date: 01, May 2018	Version No:2.0
Document Number: NRLCC/ALS/SOP5.5-062	Copy No:

REVISION AND AMENDMENT

Version Change History and Description of Amendment

Revision .No	Version. No	Page No	Description of Amendment	Amendment Date	Effective Date	Name & Signature of Reviewer	Name & Signature of approval

Purpose	This procedure provides instructions for performing HbA1c on the Cobas Intgera 400 Plus and COBAS 501 System
Abbreviations	RT= Room Temperature EDTA= Ethyl diamine tetra acetic acid QC= Quality Control HbA1c= Hemoglobin A1c NRLCC = National Reference Laboratory for Clinical Chemistry
Clinical Utility	Hemoglobin (Hb) consists of four protein subunits, each containing a heme moiety, and is the red-pigmented protein located in the erythrocytes. Its main function is the transport of oxygen and carbon dioxide in blood. Each Hb molecule is able to bind four oxygen molecules. Hb consists of a variety of subfractions and derivatives. Among this eterogeneous group of hemoglobins HbA1c is one of the glycated hemoglobins, a subfraction formed by the attachment of various sugars to the Hb molecule. HbA1c is formed in two steps by the non-enzymatic reaction of glucose with the N-terminal amino group of the ß-chain of normal adult Hb (HbA). The first step is reversible and yields labile HbA1c. This is rearranged to form stable HbA1c in a second reaction step. In the erythrocytes, the relative amount of HbA converted to stable HbA1c increases with the average concentration of glucose in the blood. The conversion to stable HbA1c is limited by the erythrocyte's life span of approximately 100 to 120 days. As a result, HbA1c reflects the average blood glucose level during the preceding 2 to 3 months. HbA1c is thus suitable to monitor long-term blood glucose control in individuals with diabetes mellitus. Glucose levels closer to the time of the assay have agreater influence on the HbA1c level.
Principle	This method uses TTABa) as the detergent in the hemolyzing reagent to eliminate interference from leukocytes (TTAB does not lyse leukocytes).Sample pretreatment to remove labile HbA1c is not necessary. All hemoglobin variants which are glycated at the ß-chain N-terminus and which have antibody-recognizable regions identical to that of HbA1c are determined by this assay. Consequently, the metabolic state of patients having uremia or the most frequent hemoglobinopathies (HbAS, HbAC, HbAE) can be determined using this assay.

Materials

Reagents	
1. Hba1c	Catalog No.05479207 190
2. Activator	Catalog 04 663 632 190
3. Deproteinizer	Catalog 20 763 071 122
4. Cleaner	Catalog 20 754 765 322
5. NaCl Diluent 9 %	Cat. No. 20756350 322

Reagents preparation: Ready for use
Reagents stability and storage:
Unopened at 2-8ºC up to the stated expiry date
On board in use at 10 to 15°C for 4 weeks
Note: Store reagent kit upright

Supplies
1. Activator bottle Catalog 04 745 086 190
2. Cleaner Cassette Catalog 20 764 337 322
3. Micro Cuvettes Catalog 21 043 862 001
4. Sample Cup micro 0.5ml Catalog 11 406 680 001
5. Diluent Catalog 03375790 190
5. Waste Container Catalog 21 044 850 001
Equipment
● Cobas Intgera 400 plus and COBAS 501 •Adaptor Cup Catalog 28 086 834 001 • Data Station • Cassette Mixer Catalog 28 085 714 001

Sample

Sample type	Amount equire	Transport and Storage	Stability
Whole blood	5µl	Transport and store serum depend on stability Only transport whole blood at RT or 2-8 °C	3 days at 15-25 °C 7 days at 2-8 °C 6 months at (-15)-(-25) °C

Sample retention: Specimens are discarded in accordance with NHIVRL Specimen retention policy. This refers to both Specimens in the Primary and Secondary Containers.

Special Safety Precautions Refer to the NHIVRL safety manual MAN5.2-001

Calibration

Calibrator	Level Stability	Frequency	Preparation (y/n)
C.f.a.s. HbA1c	•Unopened at 2-8ºC up to the stated expiry date • After opening 2 hours at 15‑25 °C or 28 days at 2‑8 °C	● With every new Cassette or lot changed ● After 29 days when using the same Cassette ● After preventive maintenance • when QC out of range	Yes

Note:

- Fill the *Cobas Intgera 400 plus and COBAS 501* Calibrator Log form when running a Calibrator
- Calibrators must be at room temperature before use

Quality Control

Control	Level	Stability	Frequency	Preparat on (y/n)
PreciControl HbA1c norm PreciControl HbA1c path	Level 1 Level 2	•Unopened at 2-8ºC up to the stated expiry date • for 7 days after opning	● Daily • after Calibration	Yes

.

Procedure Refer to Cobas integra 400 PLUS & COBAS 501 manual

Result Interpretation

Step	Action
1	This corresponds to a measuring range of 23-196 mmol/mol HbA1c (IFCC) and 4.2-20.1 % HbA1c (DCCT/NGSP) at a typical hemoglobin concentration of 8.2 mmol/L (13.2 g/dL). In rare cases of “>Test” flags which might occur with the use of the whole blood application, remix the whole blood sample and repeat the analysis with the same settings. It is recommended to switch the auto rerun function off.

Expected Values

Analyte	Reference Range		Analytical Range	Units
HbA1c	without diabetic	29-42 mmol/mol 4.8-5.9%	4.2-20.1 % HbA1c (DCCT/NGSP) 23-196 mmol/mol (IFCC)	mmol/mol %
	In a poorly controlled diabetic population	195mmol/mol 20%		

Limitations

Levodopa causes artificially high fructosamine results. Oxytetracycline causes artificially high fructosamine results

In hydremic states (pregnancy for instance) it may be favorable to relate fructosamine to protein using the following formula:

$$\text{Fructosamine}_{corr} = \frac{\text{measured fructosamine} \times 72}{\text{measured total protein (in g/L)}}$$

Dysproteinemic states may affect fructosamine values.

- In very rare cases, gammopathy, in particular type IgM (Waldenström's macroglobulinemia), may cause unreliable results

Performance Characterstics

Precision

Precision was determined using human samples and controls in accordance with the CLSI (Clinical and Laboratory Standards Institute) EP5 requirements with repeatability and intermediate precision (2 aliquots per run, 2 runs per day, 21 days). The following results were obtained (databased on DCCT/NGSP values):

	Mean %HbA1c	SD %	CV %
PreciControl HbA1c norm	5.3	0.07	1.3
PreciControl HbA1c path	9.9	0.11	1.1
Human sample 1	4.4	0.07	1.6
Human sample 2	5.6	0.09	1.6
Human sample 3	8.0	0.08	1.0
Human sample 4	10.6	0.11	1.1
Intermediate precision	Mean % HbA1c	SD	CV
PreciControl HbA1c norm	5.3	0.08	1.4
PreciControl HbA1c path	9.9	0.15	1.5
Human sample 1	4.4	0.09	1.9

Method comparison

Evaluation of method comparison data is according to NGSP certification criteria. The mean difference between the two methods and the 95 % confidence intervals of the differences in the range from 4-10 % (DCCT/NGSP) are given. 95 % of the differences between the values obtained for individual samples with both methods fall within the range defined by the lower and upper 95 % confidence intervals of the differences.

Sample size (n) = 82

Mean difference	0.07 % HbA1c
Lower 95 % confidence interval of Differences	-0.50 % HbA1c
Upper 95 % confidence interval of differences	0.65 % HbA1c

The sample concentrations were between 5.0 % and 9.9 % (DCCT/NGSP values).

Subsidiary Document	Document Unique ID	Document Name
	NRLCC/ALS/QPM/4.2/001	Quality Policy Manual

Reference

- Roche Cobas Integra 400 PLUS & COBAS 501Operator Manual
- Roche Cobas Integra 400 PLUS & COBAS 501 Package Inserts
- Tietz NW, editor. Text book of Clinical Chemistry.3rd ed. Philadelphia: WB Saunders,2001
- Lothar Thomas: Clinical Laboratory Diagnostics, use and assessment of Clinical Laboratory Results 1st ed.1998

Declaration

I, the undersigned laboratory personnel, certify that I am conducting every steps of the procedures incorporated in this SOP after a prior reading.

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Ethiopian Public Health Institute
National Reference Laboratory for Clinical Chemistry

Procedure for Fructosamine

Compiler:	Signature:
Approver:	Signature:
Effective Date: 01, May 2018	Version No:2.0
Document Number: NRLCC/ALS/SOP5.5-062	Copy No:

REVISION AND AMENDMENT

Version Change History and Description of Amendment

Revision .No	Version. No	Page No	Description of Amendment	Amendment Date	Effective Date	Name & Signature of Reviewer	Name & Signature of approval

Purpose

This procedure provides instructions for performing Fructosamine on the Cobas Intgera 400 Plus and COBAS 501 System

Abbreviations

RT= Room Temperature
EDTA= Ethyl diamine tetra acetic acid
QC= Quality Control
FRA= Fructosamine
NRLCC = National Reference Laboratory for Clinical Chemistry

Clinical Utility

Fructosamine represents non-enzymatic glycation attached to blood and tissue proteins. The formation of fructosamine is a two-step reaction, which is dependent on the glucose concentration. As a first step a Schiff Base is formed by the reversible coupling of glucose to protein which, in a second step, is transformed by non-reversible Amadori rearrangement to the corresponding ketoamine. This ketoamine is designated as fructosamine. The formation of fructosamine increases with the level of blood glucose. Metabolization occurs within 1 to 3 weeks, corresponding to the turnover of most serum proteins. The concentration of fructosamine thus reflects the average of the continuously varying blood glucose concentrations during this period, serving as a blood glucose memory. Fructosamine is therefore a rapid indicator of glycemia in the diagnosis and management of diabetes mellitus.

Principle

Colorimetric test by reaction with nitroblue tetrazolium. The colorimetric test for fructosamine (glycated protein) is based on the ability of ketoamines to reduce nitroblue tetrazolium in alkaline medium. The rate of formation of formazan is directly proportional to the fructosamine concentration and is measured photometrically.

Materials

Reagents	
1. Fructosamine	Catalog No. 04537939 190
2. Activator	Catalog 04 663 632 190
3. Deproteinizer	Catalog 20 763 071 122
4. Cleaner	Catalog 20 754 765 322
5. NaCl Diluent 9 %	Cat. No. 20756350 322

Reagents preparation: Ready for use
Reagents stability and storage:
Unopened at 2-8ºC up to the stated expiry date
On board in use at 10 to 15°C for 8 weeks
Note: Store reagent kit upright

Supplies
1. Activator bottle Catalog 04 745 086 190
2. Cleaner Cassette Catalog 20 764 337 322
3. Micro Cuvettes Catalog 21 043 862 001
4. Sample Cup micro 0.5ml Catalog 11 406 680 001
5. Diluent Catalog 03375790 190
5. Waste Container Catalog 21 044 850 001
Equipment
● Cobas Intgera 400 plus and COBAS 501 •Adaptor Cup Catalog 28 086 834 001 • Data Station • Cassette Mixer Catalog 28 085 714 001

Sample

Sample type	Amount required	Transport and Storage	Stability
Serum	2µl	Transport and store serum depend on stability Only transport serum at RT or 2-8 °C	3 days at 15-25 °C 2 weeks at 4-8 °C 2 months at -20 °C
Plasma	2µl	Transport and store plasma depend on stability Only transport plasma at RT or 2-8°C	3 days at 15-25 °C 2 weeks at 4-8 °C 2 months at -20 °C

Sample retention: Specimens are discarded in accordance with NHIVRL

Specimen retention policy. This refers to both Specimens in the Primary and Secondary Containers.

Special Safety Precautions Refer to the NHIVRL safety manual MAN5.2-001

Calibration

Calibrator	Level Stability	Frequency	Preparation (y/n)
Precimat Fructosamine	•Unopened at 2-8ºC up to the stated expiry date • After opening 2 hours at 15-25 °C or 28 days at 2-8 °C	• With every new Cassette or lot changed • After 7 days when using the same Cassette • After preventive maintenance • when QC out of range	Yes

Note:

- Fill the *Cobas Intgera 400 plus and COBAS 501* Calibrator Log form when running a Calibrator
- Calibrators must be at room temperature before use

Quality Control

Control	Level	Stability	Frequency	Preparation (y/n)
Precinorm Fructosamine Precipath Fructosamine	Level 1 Level 2	•Unopened at 2-8ºC up to the stated expiry date • for 7 days after reconstitute	• Daily • after Calibration	Yes

.

Procedure Refer to Cobas integra 400 PLUS & COBAS 501 manual

Result Interpretation

Step	Action
1	Determine samples having higher concentrations via the rerun function. Dilution of samples via the rerun function is a 1:2 dilution. Results from samples diluted using the rerun function are automatically multiplied by a factor of 2.

Expected Values

Analyte	Reference Range		Analytical Range	Units
Fructosamine	Adults without diabetic	205-285	14-1000	µmol/L
	In a poorly controlled diabetic population	228-563		

Limitations

Levodopa causes artificially high fructosamine results. Oxytetracycline causes artificially high fructosamine results

In hydremic states (pregnancy for instance) it may be favorable to relate fructosamine to protein using the following formula:

$$\text{Fructosamine}_{corr} = \frac{\text{measured fructosamine} \times 72}{\text{measured total protein (in g/L)}}$$

Dysproteinemic states may affect fructosamine values.

- In very rare cases, gammopathy, in particular type IgM (Waldenström's macroglobulinemia), may cause unreliable results

Performance Characterstics

Precision

Precision was determined using human samples and controls in an internal protocol with repeatability (n = 21) and intermediate precision (3 aliquots per run, 1 run per day, 21 days). The following results were obtained:

Repeatability	Mean µmol/L	SD µmol/L	CV %
Precinorm Fructosamine	262	4	1.6
Precipath Fructosamine	498	4	0.7
Human serum 1	262	2	0.9
Human serum 2	208	2	1.0
Intermediate precision	**Mean µmol/L**	**SD µmol/L**	**CV %**
Precinorm Fructosamine	262	4	1.5
Precipath Fructosamine	489	6	1.2
Human serum 3	266	4	1.5
Human serum 4	210	4	1.8

Method comparison

Fructosamine values for human serum and plasma samples obtained on a Roche/Hitachi cobas c 501 analyzer (y) were compared with those determined on Roche/Hitachi 917/MODULAR P analyzers (x), using the corresponding Roche/Hitachi reagent.

Sample size (n) = 231

Passing/Bablok	Linear regression
y = 0.968x + 15.0 µmol/L	y = 0.967x + 15.5 µmol/L
τ = 0.946	r = 0.998

The sample concentrations were between 166 and 836 µmol/L.

Subsidiary Document

Document Unique ID	Document Name
NRLCC/ALS/QPM/4.2/001	Quality Policy Manual

Reference

- Roche Cobas Integra 400 PLUS & COBAS 501Operator Manual
- Roche Cobas Integra 400 PLUS & COBAS 501 Package Inserts
- Tietz NW, editor. Text book of Clinical Chemistry.3rd ed. Philadelphia: WB Saunders,2001
- Lothar Thomas: Clinical Laboratory Diagnostics, use and assessment of Clinical Laboratory Results 1st ed.1998

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Declaration

I, the undersigned, declare that this M.Sc. thesis is my original work, has not been presented for a degree in this or any other university and that all sources of materials used for the thesis have been duly acknowledged.

M.Sc. candidate: **Tigist Getahun (B.Sc.)**

Signature: _____

Date of submission: _____

This thesis has been submitted with our approval as advisors.

Advisor: **Aster Tsegaye (MSc, PhD)**

Signature: _____

Date: _____

Place: Addis Ababa, Ethiopia.

Advisor: **Samuel Kinde (MSc, PhD candidate)**

Signature: _____

Date: _____

Place: Addis Ababa, Ethiopia.

Advisor: **Feyissa Challa (MSc, PhD candidate)**

Signature: _____

Date: _____

Place: Addis Ababa, Ethiopia.