THE ESTABLISHMENT OF NORMAL ADULT REFERENCE VALUES FOR A SELECTED PANEL OF LABORATORY ANALYTES IN SOUTH AFRICA

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A research proposal submitted in the fulfilment of the requirements for the Doctor of Philosophy (PhD): Biomedical Sciences

In the Faculty of Health and Wellness Science at the CAPE PENINSULA UNIVERSTIY OF TECHNOLOGY

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BELVILLE

FEBRUARY 2019

DECLARATION

I, *Francois Christiaan Smit*, declare that the contents of this thesis represent ***my own unaided work***, and that the thesis has not previously been submitted for academic examination towards any qualification. Furthermore, it represents my own opinions and not necessarily those of the Cape Peninsula University of Technology

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Signature Date

ACKNOWLEDGEMENTS

I hereby wish to give thanks and acknowledge:

- My Lord and Saviour, Jesus Christ, for giving me the strength, courage, and health during the good and bad times to complete this thesis. To Him all the Glory and Praise.
- My supervisor, Professor Tandi Matsha, for your continued support, encouragement and mentorship. It truly was an honour to have you as my supervisor.
- My co-supervisors, Prof. Rajiv Erasmus (Department of Chemical Pathology, University of Stellenbosch) and Prof. Kyoshi Ichihara (Yamaguchi University, Japan) for your mentorship and leadership during this project.
- Dr. Mariza Hoffmann, previously from the Department Chemical Pathology, University of Stellenbosch, who was my right hand during this project. Thank you for all the hard and long hours, even over weekends. You became one of my close friends and I admire you as one of my colleagues.
- Dr John Douglas, PathCare Chief Executive Officer, and the Pathcare Executive Committee for grating me permission to do this project.
- All the managers and laboratory staff from PathCare, Louis Leipoldt as well as PathCare Reference Laboratory for the processing of the samples. There are too many of you to mention individually.
- Ms. Janette Wassüng, former Quality Assurance Department (QAD) manager of PathCare Laboratories for all the technical support and advice. For me, you are one of the most dedicated and compassionate medical technologists who has a passion for the profession and I always try and inspire myself in my profession to be as dedicated and hard-working as you are.
- Mr. Arno Theron and all staff at PathCare QAD for the sample processing for freezing as well as all the hard work you did for compiling our final database.
- My family and friends for your encouragement, love and support which carried me through.
- The financial assistance of the Cape Peninsula University of Technology towards this research project is acknowledged. The opinions expressed in this thesis and the conclusions arrived at, are those of the author, and are not necessarily to be attributed to the Cape Peninsula University of Technology.

DEDICATION

Ek skryf hierdie toewyding in my moedertaal omdat ek trots Afrikaans is.

Soos mens ouer word, leer die lewe jou as mens baie lesse en maak jou as individu baie wyser. Een van daardie lesse is die waardering wat mens het vir die opofferings wat mens se ouers maak om jou as hulle kind ‘n beter lewe te kon gee.

En soms vergeet mens hierdie opofferings wat ouers vir hulle kinders maak en dit uit liefde. Dit is waarom ek baie graag hierdie tesis opdra aan my ouers:

- My pa die wyle Fasie Smit en
- My ma Brenda Smit

My pa is skielik oorlede in Oktober 2012 net toe ek met die projek begin het en het nooit die geleendheid gehad om my te sien gradueer met ‘n my Doktorsgraad nie.

My ma Brenda Smit het nogsteeds volgehou en die pad saam met my gestap en na 6 jaar kyk ek terug met dankbaarheid vir al haar hulp.

Daarom weet ek nie hoe ek dit in woorde kan beskryf hoeveel ek julle ondersteuning en opoffering kan voor dankie sê nie. Al die jare se ondersteuning en aanmoediging het vrugte afgepluk end it alles deur julle liefde. Dit is baie waar dat ouers enige iets vir hulle kinders se geluk sal doen, al moet hulle self opofferings maak.

Ek sal julle innig dankbaar wees en innig liefhê tot die dag van my dood.

Ook aan Handrè le Roux my lewensmaat: Dankie vir al jou ondersteuning, liefde en geduld gedurende hierdie 6 jaar. Dinge was maar soms baie op en af maar ons liefde vir mekaar het die toets van tyd weerstaan.

LIST OF ABBREVIATIONS, SYMBOLS, TERMS AND UNITS

%	Percentage
3N- ANOVA	Three-level-nested analysis of variance
° C	Degrees Celsius
µmolL	Micromol per Liter
g/dL	Grams per deciliter
g/L	Grams per liter
g/mL	Grams per milliliter
kg/m^2	Kilograms per meter squared
pg	Picograms
mmol/L	Millimols per Liter
Afr	African
Alb	Albumin
ALP	Alkaline Phosphatase
ALT	Alanine Aminotransferase
Amy	Amylase
ANOVA	Analysis of Variance
AST	Aspartate Aminotransferase
Baso	Basophils
BMI	Body Mass Index
Ca	Calcium
Cau	Caucasian (White ethic group)
CDL	Clinical Decision Limit
ChE	Cholinesterase
CI	Confidence Interval
CK	Creatine Kinase
Cl	Chloride
CLSI	Clinical & Laboratory Standards Institute
CPUT	Cape Peninsula University of Technology
Cre	Creatinine
CRM	Certified Reference Material
C-RIDL	Committee on Reference Intervals and Decision Limits

DIC	Disseminated Intravascular Coagulation
Eos	Eosinophils
FBC	Full Blood Count
Fe	Iron
Fer	Ferritin
FT3	Free Triiodothyronine
FT4	Free Thyroxine
GGT	Gamma-glutamyltransferase
Glu	Glucose
Hb	Haemoglobin
Hct	Haematocrit
hsC-RP	High Sensitivity C-Reactive Protein
HDL-C	High – Density Lipoprotein Cholesterol
Hypertension	Increased Blood Pressure
IFCC	The International Federation of Clinical Chemistry
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IP	Inorganic Phosphate
K	Potassium
LAVE	Latent Abnormal Values Exclusion
LDH	Lactate Dehydrogenase
LDL-C	Low – Density lipoprotein Cholesterol
LL	Lower Limit
Lymph	Lymphocyte
Mean	Average obtained from a group of observations
MCV	Mean Cell Volume
MCH	Mean Cell Haemoglobin
MCHC	Mean Cell Haemoglobin Concentration
Mono	Monocyte
Mortality	Rate of measurement of the number of deaths
Mix	Mixed Ancestry
Mg	Magnesium

MRA	Multiple Regression Analysis
Na	Sodium
Neu	Neutrophils
NAfr	Non-African
NP	Non-parametric
P	Parametric
Plt	Platelets
PSA	Prostate Specific Antigen
RBC	Red blood cell
RDW	Red Cell Distribution Width
RI	Reference Interval
RNP	Reference Measurement Procedure
RP	Partial Correlation Coefficient
RV	Reverence Value
QC	Quality Control
SD	Standard Deviation
SDR	Standard Deviation Ratio
SV	Sources of variation
TBili	Total Bilirubin
TC	Total Cholesterol
Tf	Transferrin
TG	Triglycerides
TP	Total Protein
UA	Uric Acid
Ul	Upper Limit
VLDL	Very Low – Density Lipoprotein
WHO	World Health Organisation
WCC	White Cell Count
ZAF/Af	African Population of South Africa
ZAF/NAf	Non-African Population of South Africa

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LITERATURE REVIEW

1. Introduction

The word: "*Normal*" was established in the English language in 1520 – 30's, derived from the Latin word: "normãlis" which means: "made according to the carpenter's square" (Collins English Dictionary, 2009). In medicine, normal can be defined as: "the ususal or expected state, form, amount or degree" (The American Heritage Stedman's Medical Dictionary, 2002). However, in the clinical laboratory set-up, the concept normal is found to be obsolete and impercise to fufill the modern demands of clinical pathology (Ashwood & Burtis, 2001). In 1965, an alternative concept named: reference values was proposed, with the establishment of the expert panel on Theory of Reference Values created in 1970 (Ashwood & Burtis, 2001). About 30 years ago, the Internation Federation of Clinical Chemisrty (IFCC), together with the Clinical Laboratory Standards Insitute (CLSI) issued a detailed set of standards and standards for each laboratory to follow when establishing reference intervals (IFCC Part 1 , 1986), (IFCC Part 2, 1987), (IFCC Part 3, 1988), (IFCC Part 4, 1991), (IFCC Part 5, 1987), (IFCC Part 6, 1987). The IFCC also recommended the use of the term reference values and related terms namely: reference individual, reference limit, reference interval and observed values (Ashwood & Burtis, 2001).

One of the most common decision support tools used for the interpretation of numerical pathology reports today are reference values (Barker & Jones, 2008). A reference value for most laboratories is defined by threshold values between which test results of a spesific percentage of apparently healthy individuals would fall, namely 95% (Barker & Jones, 2008). Presuming the underlying data of apparently healthy individuals follows a normal Gausian distribution, it is quite easy to estimate the 95% confidence interval of the upper – and lower reference limit values for a particular reference value (Ichihara & Boyd, 2010). Should it not be the case, several stastical parameters are availble to process the data obtained from reference value studies to create and impliment the new reverence interval (Ichihara & Boyd, 2010). Working reference values must include most population characteristics, derived form from the laboratory mean, resulting in patient values that will within these reference values will have statistically a lower probability of being affected, while those falling outside the reference values have a higher statistical probability of having disease. In this regard, it is evident that the clinical laboratory plays an important role not only in establishing these reference values, but also in the decision making process of patients (Barker & Jones, 2008).

Population characteristics all have a final bearing on reference value determination and should be included during the statistical analysis when computing working reference values for a population group (Barker & Jones, 2008). Some of these caracteristics or variables has been reported in this project namely: age, gender, ethnicity, anthropometric measurements (weight, height, and waist circumference), blood pressure, body mass index (BMI), eating habits, chronic disease, pregnancy, Hepatitis B (HBV) and C (HCV), and Human Immuno-Deficiency Virus (HIV).

The challenges of establishing reference values are well understood. Stratification by age and gender is the minimum pre-requisite, therefore for the age and gender were two of the many variables that were included for the purpose of this study. A reference range study for Homocysteine (Hcys) done in India on males and females showed a steady increase in mean values for Hcys over the age range of 20 – 29 years. This trend continued into the 50-year age range of the population; however, it was different for both males and females (Lahiri, Datta, & Das, 2014). A similar study showed a statistical difference between gender reference ranges in Uric Acid (UA); however, one of the limitations encountered was the non-conclusion of for the age groups 30 years and below as most of their participants was in the elderly age group (Das *et al.,* 2014).

In reference range studies ethnicity or ethnic groups would also play a major factor and was used as another variable for this study. One such study Nguyen and co-workers reported the plasma soluble (pro)renin receptor concentrations are dependent on ethnicity in healthy subjects (Nguyen *et al*., 2013). Chakraborty and Chakraborty proved that Vitamin C an E determination in a healthy Bengali population group was not only method dependant on reference range determination, but also dependant on ethnicity, racial genetic make-up, socio-demograpic layout of the pupolation and diet when compared to other reference ranges used for the same tests in the Western world (Chakraborty & Chakraborty, 2013). The theory of ehtnicity playing a role in reference range determination was also shown in a study done by Bailey and co-workers. They aimed in establishing the effects of various variables on a panel of biochemical markers in children from different ethnic groups, aged 2 days to 18 years of age as part of The Canadian Laboratory Initiative for Paediatric Reference Intervals (CALIPER) program. Their results showed a statistically significant difference in reference ranges in 50% (7 out of 14) biocemical markers of the cohort (Bailey *et al*., 2013).

Body mass index (BMI) measurement was performed on all participants of the project and reported in kg/m^2. BMI play an important role in reference range determination since it shows an statistical correlation with the measured analytes in most cases. Tamakoshi and co-workers did a study in 2002 to evaluate the effects of metabolic syndrome on C – reactive protein (C-RP) in men and found a stitistically significant postive correlation between C-RP and BMI (Tamakoshi *et al*., 2003). Iacobellis *et al.* showed that thyroid stimulating hormone (TSH) and BMI showed a statistical positive correlation in a thyroid hormone study performed on obese women with euthyroidism (normal thyroid function) (Iacobellis *et al.*, 2005).

An important set of variables investigated in this study was the effect of chronic disease on the determination of reference ranges. More spesifically participants knowlingly suffering from Human Immunodeficiency virus (HIV), Hepatitis B - (HBV) and C virus (HCV), as well as diabetes were excluded to participate in this project. It was shown in numerous studies that chronic dease does play an role and have a statistical effect on measuered analystes. Wei and co-workers showed that BMI, nephritis, kidney function and hypertenion had an influence on serum cystatin when evaluating glumelular filtration rate (GFR) (Wei *et al.* 2014). Another study showed that chronic disease in eldery men showed decreased concentrations and androgens and sex hormone – binding globulin (SHBG) and was inversely associated with central fat mass (CFM) (Frost *et al*., 2013).

Clinical Chemistry showed a great advancement of the years from the predominantly manual assays to automated clinical chemitry analysers for most methods. This, in itself, brought alng many challenges in itself. Futhermore, a spesific test may have more than one alanytical platform, making it even more important for laboratories to provide statistical, relevant and reliable reference values that is not only method and analyser spesific, but fulfill the demands of the patient population it serves

2. Protocol and set up of a multivariate study

Previous studies of this nature showed that proper planning steps must be in place before any attempt should be made in endevour in a project of this nature (Ozarda *et al*., 2013). This fact was reeinterated by the investigators as the project progressed to the more advanced

stages. Furthermore, adaptability and flexibility for such plans must be allowed as senario's and situations change during the course of such a project, as was the case with this project.

Not many reference range studies of this nature has been done in Africa, which includes South Africa as well. South Africa, as well as other African countries, merely adapted reference ranges from textbooks such as Tietz Fundamentals of Clinical Chemistry (Burtis & Ashwood, 2001). However, the validity of these ranges for the different population groups within South Africa was never established on a larger scale. Furthermore, studies such as these require input from all stakehoulders in the industry, capital, man power as well as participant willingness to participate in projects of these natures. The resulting information in the end will strive to globalise medical practise as a whole (Ozarda *et al*., 2013).

The CLSI, toghether with the International Fedration of Clinical Chemistry IFCC compiled a document which covers most of the aspects which can be used for setting up reference range studies. This document, CSLI/IFCC Document C28-A3, contains the majority of the information required by investigators when planning to do a multivariate referenge range study of this nature. It addresses issues such as: the selection of suitable participants, pre-analytical and analytical considerations, analysis of reference values obtained, transference, reference range validation, and medical decision limits as to name a few (CLSI, 2008).

Other regional studies proposed additional guidelines which originated from document CLSI/IFCC C28-A3 (Fuentes-Arderiu *et al.,* 2005), (Rustad *et al.*, 2004). However, all these documents did not cover all the aspects required for multi – centre reference range studies such as this one suffiently. Therefore, Orzada *et al.*, on behalf of the IFCC C-RIDL, drafted a common standard operating procedure and protocol to aid laboratories worldwide to assist them in planning and drafting a protocol of their own when setting up a reference range study of this nature (Ozarda *et al.,* 2013).

The countries involved in the international study conducted their own multicentre study by obtaining and deriving country – spesific reference intervals. Each country was responsible for recruiting appropriate healy participants, draw blood samples from these recruited participants and process all these samples in accordance with the SOP stated in the previous paragraph. Furthermore, each participating country should make use of a centralised analysing

laboratory to ensure the elimination of variations when analysing at different laboratories. Each country could use any measurement platform they choose. Measurement of standard reference material, or a panel of sera, was done by each participating laboratory to ensure reverence range tracebility to the reference measuring procedure of the standardized analytes. This ensured harmonisation of all participating countries' results for the final determination of reference ranges. The sources of variations seen in the country – spesific reference intervals was examined statistically and compared to the global study. Additional questionnaire items and analytes could be added according to the investigation and needs of each participating country (Ozarda *et al.,* 2013).

Due to the fact that no study of this nature was conducted in Africa before, the Africa Reference Range Group (ARRG) was created in June 2012, which became part of the muticenter international reference range group. South Africa was the first country in Africa to start with this project, followed by Kenya and Nigeria in 2015.

The investigators choose to analyse 42 biochemical – and endocrinological analytes, as well as a complete blood count (CBC) on each participant.

3. Statistical determination of reference intervals

When working with lage sets of databases with the end-product being reference intervals, one must carefully condsider which statistical method the investigator must use as well as the computation tehniques for every step of the process (Ichihara & Boyd, 2010). Furthermore, investigators must etsblish their sample size beforehand as to determine the reproducibility of the results and the potential sources of variation to interpret these results in the end.

Once the dataset has ben obtained, this dataset must be analysed, it characteristics identiefied and the optimal statistical technique must be used to see the distributon of the data. Once RI's have been determined using the desired statistical technique, the use of these reference interva;s by other laboratories may have to undergo further transformation (Ichihara & Boyd, 2010).

In the desing phase of any study of this magnitude, one of the first considerations the investigators must determine is the amount of participants the investigators are planning to recruit, in otherwords, determine the desired sample size. If one assumes that the distribution of a dataset follows a normal Gausian distribution, it is quite easy to determine and estimate the 95% confidence interval for both the lower limit of the dataset as well as the upper limmit of the dataset.

To ensure propper reference interval determination and statistical analysis, especially in a multivariate study such as this, at least 400 participants are required. Furthermore, if the study requires the determination of separate intervals for male and female sujects, the approximate amount of participants will be 800. To determine the reference intervals for a population of participants divided into different racial group, one needs to determine the reference intervals for different decades of ages, in other words: reference intervals for participants in their 20's, 30's, 40's 50'sand 60' (Ichihara & Boyd, 2010).

The documentation of sources of variation in a study of this nature is imperative. This is to ensure the correct interpretation of participant's results for the determination of reference intervals. Furthermore, this will assist the investigators in excluding any abnormal results amongst apparently healthy participants which may also be indicative of an underlying diease state (Ichihara & Boyd, 2010). This information will further assist the investigators to determine if partitoning of any given reference interval is required if any influence is observed on the results of these factors. Therefore, part of the study design is the collection of participant information with regards to general healt status, occupation, sleeping habbits, work- and excersise routine, eating habbits and suppliments as well as any medication and allergies by means of a comprehensive questionniare. The questionnaire used for the purposes of this study can be seen in **Appendix E**.

The analysis of these sources of variations are very important in reference interval determination. Several statistical mehods exsists to take into account these variations when doing reference interval determination. For example, one such simple test to see the impact of any variation factor on a dataset would be the Mann-Whitney U-test, otherwise known simply as the t-test. If you have three or more subgroups the use of a one way analysis of variation, simply known as analysis of variation (ANOVA). It is not however not the norm to use such

univariate statistical methods when performing reference interval determination, especially where a mirriad of factors can have an influnce on the final results (Ichihara & Boyd, 2010).

One must keep in mind that any study of this nature is one must always be aware that this is what is known as an observational study because many sources can have an influence on the end result, in this case reference intervals. That is why the impact of all the sources of variation should be kept into account when determining reference intervals. That is why there are many of these methods availible for investigators to use (Ichihara & Boyd, 2010).

3.1 Nested ANOVA

In statistics, a confounding variable (also confounding factor, a confound, or confounder) is an extraneous variable in a statistical model that correlates (directly or inversely) with both the dependent variable and the independent variable (Pearl, 2009), (Van der Weele & Shpitser, 2013). When performing univariate analysis, especially with reference range determination, one can overcome the problem of confounding variables by making use of nested ANOVA. This will allow a simultaneous comparison of two or more sources of variation. When using nested ANOVA, the impact or magnitude of each of these variables will be expressed in terms of the standard deviation (SD) or coefficient of variation (CV). Furthermore, categorical data for analysis is required when using nested ANOVA. Age would be considered a continuous variable and can be compared to for example gender, geography, eating habits or race (Ichihara & Boyd, 2010).

3.2 Multiple regression analysis (MRA)

In, regression analysis is a statistical process for estimating the relationships among variables. It includes many techniques for modeling and analyzing several variables, when the focus is on the relationship between a dependent variable and one or more independent variables or predictor. This model provides the investigator to determine the impact of all the variables simultaneously and automatically adjusts for confounding influences of other parameters by including them concurrently in the regression model (Ichihara & Boyd, 2010).

This can be done by predicting the impact of each parameter and expressing it as a regression coefficient. The only drawback is that this magnitude of each variation cannot be expressed as a SD or CV as with nested ANOVA. Furthermore, inclusion of too many variables

in the MRA model can hamper the reproducibility of the model. However, MRA can be used for the identification of potential parameters that can be included when performing nested ANOVA (Ichihara & Boyd, 2010).

3.3 Partitioning of reference intervals

Once contributing sources of variation has been determined using any of the mentioned methods mentioned above, for example gender or race, one must determine whether these sources of variation require separate reference intervals for different gender- and race groups. This process is referred to as partitioning and several proposed methods exists for partitioning (Ichihara & Boyd, 2010). For the global study, we made use of the Ichihara method.

3.3.1 Ichihara method

The use of the Ichihara method is dependant on a two- or three level nested ANOVA. It makes use of the infromation which is aquired form the SD obtained from each source of variation. Whe using this method, it will calculate the standard deviation ratio (SDR) of each variable, provided that the data supplied has been transfromed to yield a Gaussian distribution (Ichihara, *et al.*, 2008). This method was the one used by the investigtors of this study to compare the results obtained in the South African study to the results obtained in the global study described later. For completeness sake, some other methods are expained below to show the large amount fo availible methods that are at the dispoal to to investigators for the derevation of reference intervals.

3.3.2 Fraser method

For analytes with a Gaussian distribution, Fraser and co-workers have determined optimal, desirable and minimum bias limits. The principle is that the minimal standard will allow a flagging rate of about 5.7% at one end compared to the expected 2.5%. An alternative approach may be to set intervals to a number greater than the central 95% in some cases. In other words, this method is based on the degree or magnitude of biological variation, which is expressed as the standard deviation (SD) (Ichihara, *et al.*, 2008).

3.3.3 Harris-Boyd method

This method can be utlised when there are 2 subgroups whereby it evaluates the practical significance between the difference between these 2 subgroup's means by making adaptations or changes for differeing sample sizes (Harris & Boyd, 1990), (Harris & Boyd, 1995).

3.3.4 Lahti method

This last method is based on the percentages of reference values in each subgroup which is situated outside the lower limit and upper limit of the reference intervals derived without making use of partitioning and therefore focusses on the statistically unstable peripheral part of sample distribution (Lahti, 2004).

3.4 The exclusion of extreme values

Extreme value theory or extreme value analysis (EVA) is a branch of statistics dealing with the extreme deviations from the median of probability distributions. It seeks to assess, from a given ordered sample of a given random variable, the probability of events that are more extreme than any previously observed (Ichihara & Boyd, 2010).

One can make use of the univariate approach of multivariate approach. When considering the univariate approach, it is important to note that the presence of extreme values can only be judged by assuming the distribution pattern of the reference values in question. Normally, if the non-parametric method for reference interval determination is used, it would not be possible to examine the extreme values, however; there are some robust non-parametric methods that an investigator can use. The all still have their limitations Some of these methods include: the Turkey method, Dixon test and Smirnov method (Ichihara & Boyd, 2010).

When examining any value, it is not a good policy to see where the value falls in the distribution of data. This value must rather be examined together with information related to the value(s) concerned. This type of investigation would otherwise be known as a multivariate approach. The latent abnormal value exclusion (LAVE) method is an example of multivariate analysis. This method was first utilized by Ichihara and Kawai in 1996 to derive reference intervals for serum proteins in the Japanese population as well as other later studies in Japan (Ichihara, *et al.* , 2008); (Ichihara, *et al*., 2004). LAVE has been developed to exclude abnormal

results that was hidden within the reference inteervals. The pitfall to this method is that it does not judge what the abnormality of such a value might be in isolation. Instead, this method was designed to look at other concurrently meased test results. LAVE is a real-time approach for determination of multiple reference interval determination which can be done simultaneously in which no exclusion of values are made in the innitial computation of reference intervals (Ichihara & Boyd, 2010).

3.5 The calculation of reference intervals: parametric versus non-parametric.

3.5.1 Parametric method (P method)

Parametric statistics is a branch of statistics which assumes that sample data comes from a population that follows a probability distribution based on a fixed set of parameters. The parametric model relies on a fixed parameter set assumes more about a given population than non-parametric methods. When the assumptions are correct, parametric methods will produce more accurate and precise estimates than non-parametric methods, i.e. have more statistical power. As more is assumed when the assumptions are not correct, they have a greater chance of failing, and for this reason are not a robust statistical method. On the other hand, parametric formulae are often simpler to write down and faster to compute. For this reason, their simplicity can make up for their lack of robustness, especially if care is taken to examine diagnostic statistics. When looking at power transformation of data the most flexible method to achieve Gaussian transformation of a reference population is when the investigators makes use of the Box-Cox and was organically recommended by the IFCC expert committee on reference intervals 1987 (Solberg, 1991).

3.5.2 Non-parametric method (NP method)

Non-parametric statistics are statistics based on parameterized families of probability distributions. They include both descriptive and inferential statistics. The typical parameters are the mean, variance, as well as a few other parameters. Unlike parametric statistics, non-parametric statistics make no assumptions about the probability distributions of the variables being assessed. The difference between parametric models and non-parametric models is that the former has a fixed number of parameters, while the latter grows the number of parameters with the amount of training data. The non-parametric model does not have any parameters: parameters are determined by the training data, not the model. Non-parametric methods are widely used for studying populations that take on a ranked order. The use of non-parametric

methods may be necessary when data have a ranking but no clear numerical interpretation, such as when assessing preferences. In terms of levels of measurement, non-parametric methods result in "ordinal" data. As non-parametric methods make fewer assumptions, their applicability is much wider than the corresponding parametric methods. In particular, they may be applied in situations where less is known about the application in question. Also, due to the reliance on fewer assumptions, non-parametric methods are more robust (Bagdonavicius, Kruopis, & Nikulin, 2011).

Another justification for the use of non-parametric methods is simplicity. In certain cases, even when the use of parametric methods is justified, non-parametric methods may be easier to use. Due both to this simplicity and to their greater robustness, some statisticians are leaving less room for improper use and misunderstanding (Bagdonavicius, *et al.,* 2011). The wider applicability and increased robustness of non-parametric tests comes at a cost: in cases where a parametric test would be appropriate, non-parametric tests have less power. In other words, a larger sample size can be required to draw conclusions with the same degree of confidence (Bagdonavicius, Kruopis, & Nikulin, 2011).

4. Sample analysis and investigation of the South African study

The countries involved in the international study conducted their own multicentre study by obtaining and deriving country – spesific reference intervals, as explained in CLSI/IFCC Document C28-A3. The sources of variations seen in the country – spesific reference intervals was examined statistically and compared to the global study. Additional questionnaire items and analytes could be added according to the investigation and needs of each participating country. The ARGG investigators choose to analyse 42 biochemical – and endocrinological analytes, as well as a full blood count (FBC) on each participant. These are disucssed at lenght in the following chapters.

5. Research rationale and ojective of the South African study

This was the first project of its kind ever undertaken in South Africa. Although other RI studies were performed in South Africa, none of them to our knowledg was even compared statistically on a global scale with other countries. What makes this study even more effective is that it was performed with a standardised method compared to the other participating countries. The statistical method for the reference interval determination was also performed according to the standardised method proposed by the C-RIDL and IFCC.

The research questions for this study were: 1) How do the South African results of this study compare globally, 2) does variables such as age, gender, BMI and ethnicity play a role in RI determination 3) what other sources of variations must be considered for RI determination 4) were the same trends noted amongst different ethnic – and gender groups globally

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MANUSCRIPT ONE

Establishment of reference intervals of biochemical analytes for South African adults: A study conducted as a part of the IFCC global multicentre study on reference values. Part 1: Assessment of methods for derivation and comparison of reference intervals

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Abstract

Background

This study was conducted as a part of IFCC global study to establish reference intervals (RIs) of major laboratory tests for South African population in consideration of ethnic differences in values.

Methods

A total of 1245 healthy volunteers aged ≥18 years composed of 651 black African (Afr), 385 Caucasian (Cau), and 209 mixed ancestry (Mix) were recruited using the global study protocol. Fasting blood samples were collected from the participants. Serum specimens were measured for 40 chemistry and immunochemistry analytes, DXC. Before data analyses, 34 individuals with extreme values in nutritional and/or inflammatory markers were excluded together with 54 individuals suspected of latent HIV infection with high IgG and low albumin and/or high CRP. Sources of variation reference values (RVs) were investigated using the multiple regression analysis. Need for partitioning reference values by race, sex and age was judged by the standard deviation ratio (SDR). Latent abnormal values exclusion (LAVE) method was applied to cope with remaining abnormal values, before deriving RIs by both parametric and non-parametric methods.

Results

Partitioning of RIs by gender was required in 4 analytes: albumin, creatinine, HDL-cholesterol and the LAVE method led to prominent changes in RIs for 7 analytes: triglyceride, AST, ALT, GGT, CK, CRP and ferritin. The superiority of the parametric method (P) over the non-parametric method (NP) was also shown from the narrower confidence limits of the RIs. Prominent racial differences in RIs (SDR>0.4) were observed for urea, total cholesterol, LDL-C, ALT, amylase, CRP, immunoglobulin G and A, requiring partition of RVs into African and non-African (Cau+Mix), therefore emphasizing the need to partition by race. By comparing the results obtained in this study in the African population group and the results of the international study, the African population showed vast differences in RI's in certain analytes namely: Further partition by age was required for TC, LDL-C, and urea (for females). The LAVE method was effective in modifying the RIs for triglyceride, ALT, GGT, and CRP. Parametric methods led to invariably narrower confidence intervals of RI limits than the nonparametric method.

Conclusion

This study enabled us to evaluate rational methods for the determination of RIs. Recruitment of participants in South Africa posed to be challenging for the investigators but could be overcome with constant adjustment in recruitment strategies

Keywords

ethnic differences; gender differences; latent abnormal values exclusion methods; parametric method; nonparametric method; standard deviation ratio; nested ANOVA

Abbreviations

3N-ANOVA: three-level-nested analysis of variance
Afr: African
Alb: albumin
ALP: alkaline phosphatase
ALT: alanine aminotransferase
AMY: amylase
AST: aspartate aminotransferase
BMI: body mass index; Ca: calcium
C-RIDL: Committee on Reference Intervals and Decision Limits, IFCC
Cau: Caucasian
ChE: cholinesterase
CK: creatine kinase
CI: confidence interval
Cl: chloride
CLSI: Clinical and Laboratory Standards Institute
Cre: creatinine
CRP: high sensitivity C- reactive protein
Fe: Iron
FT3; free triiodothyronine
FT4: free thyroxine
GGT: gamma-glutamyltransferase
Glu: glucose
HDL-C: HDL-cholesterol
IP: inorganic phosphate
K: potassium
LAVE: latent abnormal values exclusion
LDH: lactate dehydrogenase
LDL-C: LDL-cholesterol
LL: lower limit
Mix: mixed ancestry
Mg: magnesium
MRA: multiple regression analysis
Na: sodium
NAfr: non-African
NP: non-parametric
P: parametric
PSA: prostate specific antigen
RI: reference interval
RMP: reference measurement procedure
rp: partial correlation coefficient
SDR: standard deviation ratio
SV: sources of variation
TBil: total bilirubin
TC: total cholesterol
Tf: transferrin
TG: triglycerides
TP: total protein
UA: uric acid
UL: upper limit

1. Introduction

Reference intervals (RIs) play an important role in medical decision making, especially for diagnosis and monitoring disease. Therefore, derivation of reliable RIs is one of the most important missions of clinical laboratory. RIs should be derived from healthy individuals based on the same population as patients to be served by the laboratory. Besides, each laboratory is required to verify the RIs regularly (Ozarda *et al.,* 2013). However, the selection and recruitment of a sufficient number of participants are very challenging and time-consuming. In some instances, laboratories utilize RIs supplied by in vitro diagnostic manufacturers, but these RIs may not be suitable for a given patient population (Borai*, et al*., 2016). Differences may be seen in the RIs depending on populations living under different environmental and dietary conditions (Johnson, *et al.,* 2004). To date there has been no study for establishing RIs for the South African population in consideration of race, age and gender.

Therefore, we joined the international multi-centre project led by the Committee on Reference Intervals and Decision Limits (C-RIDL) of the IFCC. Other collaborating countries include the USA, Turkey, Japan, the UK, China, India, Saudi Arabia, Argentina, Russia, the Philippines, Nepal, Pakistan, Kenya, Nigeria, and Bangladesh. We employed up-to-date analytical methods and the latest automated instruments for measurements for measurements of 3# commonly tested analytes. We expected that the project will provide us with a unique opportunity to compare South Africa's RIs with those from countries of widely different demographic profiles. The study also provided an opportunity to investigate the controversies over the need for secondary exclusion of individuals by use of the latent abnormal values exclusion (LAVE) method (Ichihara & Boyd, 2010), (Ichihara, 2014) and the use of parametric and nonparametric methods for derivation of RIs. The main goal of this study was however to derive country specific RIs for the adult South African population utilizing the common protocol proposed by the C-RIDL (Ozarda, *et al*., 2013).

2. Materials and Methods

The study design was adopted from the common study protocol (Ozarda, *et al.*, 2013) as in other countries and is briefly described below.

2.1 Ethical considerations

Ethical approval was obtained from the Faculty of Health and Wellness Sciences' Research and Ethical Committee of Cape Peninsula University of Technology, Reference Number: CPUT/NHREC: REC-230408-014 as well as the University of Stellenbosch's Health Research Ethics Committee 1 protocol number: S12/05/147. Written informed consent was obtained from each volunteer.

2.2 Study population and recruitment

Suitable participants were selected according to the criteria described below and were recruited from the healthy South African population. All participants had to be South African born citizens regardless of their ethnic origin. Those originally born in the former South West Africa (a province of South Africa in the early 1980's), now Namibia, and continued to live in South African were also allowed to participate. The study was conducted between October 2012 and February 2015 as a multi-centre involving three core institutions located in three different provinces in South Africa: PathCare Laboratories in Cape Town, Western Cape Province, which acted as the central laboratory, Water Sisulu University in Mthatha, Eastern Cape Province, and Wits University in Johannesburg, Gauteng Province. At each institution, samples were gathered from 3 to 4 local blood collection centres. Each core institution was responsible for blood sampling, serum separation, aliquoting, and freezing at −80°C., as described below. Each participant was handed out a study information leaflet, informing them of the purpose of the study as well as any risks involved when participating in such a study. Particpants were required to give informed consent to participate in this study by completing the last page of this study information leaflet. The information leaflet and consent form can be seen in **Appendix B** (English) and **Appendix C** (Afrikaans) respectively.

2.3. Inclusion and exclusion criteria

The volunteers had to be subjectively feeling well and regard themselves as healthy for participation. The target range of age was between 18 and 65 years. The volunteers over the age of 65 were also recruited, although their test results were not used for the derivation of RIs but for analyses on sources of variation of RVs. Ideally each participant is not under any medication, but we allowed up to 3 medications if they did not suffer from major illness described below. Since South Africa has high obesity rates (Cois & Day, 2016), volunteers with body mass index (BMI) of $\geq 30 kg/m^2$ was not included.

The participants were excluded if they suffered or had any of the following conditions: 1) diagnosed with diabetes and/or diabetes treatment; 2) reported history of chronic liver or kidney disease; 3) had results from their blood samples that clearly indicated a presence of a severe disease; 4) had been hospitalised or been seriously ill during the previous 4 weeks of participation; 5) donated blood in the previous 3 months; 6) known carrier state of hepatitis B virus (HBV), hepatitis C virus (HCV) or HIV positive; 7) pregnant or within one year after childbirth and 8) participated in another research study involving an investigational product in the past 12 weeks. The items for a health-status questionnaire were adapted from the one used in the previous Asian project (Ichihara, *et al*., 2013), but modified according to South African local needs as described later.

2.4. Participant demographics and anthropometric measurements

Anthropometric measurements: weight (Wt; kg), height (Ht; m), waist circumference (WC; cm), blood pressure was performed prior to sample collection and each participant had to be sitting down calmly for at least 20 minutes prior to sample collection. Body mass index (BMI) was calculated as Wt/Ht^2 (kg/m^2). A health status survey was conducted via a questionnaire adapted from the one used in the previous Asian project (Ichihara, *et al*., 2013) to obtain information on BMI, ABO blood type, alcohol consumption, smoking habits, exercise, recent episodes of infection or allergy, menstrual status. For this study, we added additional items to the questionnaire on the intake and frequency of oily fish per week as well as the duration of sun exposure per day and which parts of their bodies had the sun exposure as some Vitamin D (VitD) tests and skin pigment reading measurements were done on a small group of participants to determine their VitD status. For male participants, an additional question was asked on dysuria during the past 3 months as the test of PSA. For female participants a separate question was asked on the regularity and duration of their menstrual cycle and on hormone replacement therapy. The adapted questionnaire for the South African study can be seen in **Appendix E**.

2.5. Sampling and specimen processing

Participants were requested to avoid excessive physical exertion or exercise 3 days prior to the sampling. Furthermore, they were requested to avoid excessive eating and drinking the night before sampling, and to fast at least for 10 – 12 hours. From each volunteer, 31 mL of blood was drawn into three 9.0 mL SST III Vacutainer with clot activator and gel and one 4.0 mL K_2E EDTA sample for full blood count (FBC). However, analytical results of FBC are not

described in this report. All documentation and completed questionnaires were checked by the investigators by means of completing an African Reference Range Study checklist as can be seen in **Appendix A**. A PathCare Reference Range Pathology Request form accompanied each participant's samples to the analysing laboratory, labelled with a 9-digit barcoded laboratory requisition number as well as study number as identifier used for each participant. An example of this request form can be seen in **Appendix D**.

2.6. Transportation of specimens

Samples collected at each local site in the Cape Town region were transported within 2 to 4 hours after sampling was completed to PathCare Laboratories in Goodwood, Cape Town stored in a cooler at 2 – 8 ºC. The samples were centrifuged and separated immediately after collection and stored at – 80ºC for further analysis. This was because all specimens were analysed collectively in batches of 100 – 300 samples 1–2 times per These samples were then frozen and sent to PathCare on dry ice within 24 hours. After arrival, these samples were immediately stored at – 80 ^{o} C.

2.7. Target analytes and measurements

PathCare Laboratories in Cape Town (ISO 15189 accredited laboratory) acted as a central laboratory for receiving and analysing samples. Measured were 37 analytes listed in **Table 1**. They were comprised of standardised analytes (enzymes, major serum proteins, lipids and electrolytes) as well as non-standardised analytes (ferritin, thyroid function tests, vitamin D, PSA, etc.). The analytes depending on the assay principle of chemical reaction or immune-turbidimetry were measured using an automated Beckman Coulter DXC analyser for the specimens obtained between 2012 and 2013 or using an AU analyser for the specimens obtained in 2014, while those analytes depending on labelled immunoassays were measured by Beckman Coulter DxI analysers.

2.8. Statistical analysis

All data was captured on a Microsoft Excel spreadsheet and analysed using the same statistical methods described in the previous studies (Ichihara, *et al*., 2010), but are described in brief below. The graphical figures in presenting the results were all made by use of StatFlex Version 6 with flexible graphical capability (Artech Co., Ltd, Osaka, Japan).

2.8.1 Partitioning criteria and data regression based on ANOVA

By use of the 3-level nested ANOVA, the magnitude of variations of test results that was attributed to race, gender, and age was expressed as standard deviation ratio (SDR): between-ethnicity SDR (SDR_{rc}), between-gender SDR (SDR_{sex}) and between-age group SDR (SDR_{age}) after partitioning age at 30, 40, and 50 years (Ichihara & Boy, 2010). The two-level nested ANOVA was also perfomed to predict SDR_{rc} and SDR_{age} seprately for each sex. SDR≥0.40 was regarded as a guide for judging the need for partitioning RVs by a given factor (Ichihara & Boyd, 2010), (Ichihara, *et al*., 2014).

Regarding the analyses of racial differences and the computation of SDRrc, we performed in two ways: in three race groups of black African (Afr), Mixed Ancestry (Mix), and Caucasian (Cau) and in two race groups of Afr and Non-African (Non-Afr) by combining Mix and Cau groups.

2.8.2 Latent abnormal values exclusion (LAVE) method

In this project, it was inevitable that participants with latent diseases of common occurrence such as metabolic syndrome, inflammation, and anaemia were included in the sample population. Furthermore, there exist volunteers who failed to adhere to the basal conditions required for this study or who were not truthful in answering to the questionnaire. Therefore, to overcome these inevitable problems, the LAVE method was required for some analytes. LAVE is an iterative optimization methodmethod (Ichihara, *et al*., 1996, 2010 & Boy, 2010) for refining RIs by excluding those subjects who have abnormal results in a set of reference anlaytes (other than the one under derivation of the RI). As the reference test items, we set 11 analytes: Alb, UA, TG, HDL-C, LDL-C, AST, ALT, GGT, LDH, CK, and CRP. We allowed up to one abnormal value in them.

2.8.3 Derivation of RIs and the 90% confidene interval (CI) of the RI limits

The scheme for derivation of RIs and the 90% CI of the RI limits for this study was essenially the same as reported by Ichihara *et al*. (Ichihara, *et al*., 2016). RIs were determined using both the parametric (P)and non-parametric (NP) methods. For the P method, the RVs were transformed to a Gaussian distribution by making use of the modified Box-Cox power transformation equation. For the NP method, the RVs were first sorted and the 2.5 and 97.5 percentile points were determined. 90% CIs for P and NP methods were predicted by use of the bootstrap method with repeated resampling of 100 times. Therefore, the final RI limits,

lowe limit (LL) upper limit (UL) and the midpoint were smoothed by taking the averages of their resampled values.

2.9. Quality control

2.9.1. Mini-panel

For the purpose of this study dedicated QC monitoring was undertaken for standardized and non-standardized reagents by use of multiple specimens that were prepared suggested by the C-RIDL common protocol and standard operating procedure (SOP) (Ozarda, *et al.,* 2013). Accordingly, a mini-panel of sera from five healthy individuals (2 males and 3 females of different ethnic groups and age range) were prepared and measured over the period of collective measurements in order to closely monitor between-day variations of test results (Ichihara, 2014). The between-day and within-day CV SD of each analyte between both the DXC and AU analysers are shown in **Table 1.**

2.9.2. Panel of sera and assignment of traceable values for standardisation

Two panels of sera manufactured by the C-RIDL in 2011 and 2014, respectively, were obtained 1) to ensure standardization of RIs through recalibration based on assigned values set to the serum panels, 2) to align RVs of samples obtained between the two periods, and 3) to allow for comparison of RVs with those of the results from other countries. The scheme 2) and 3) depend on linear regression-based alignment of results between the laboratories (Ichihara, *et al*., 2013) The analytes with assigned values in the panels are reported in the previous reports (Ichihara, 2014).

3. Results

After sample processing, each pariticpant results were evaluated by the investigators and emailed or posted to them, depending on their choise of cummunication. For participants to understand the interpretation of their results, an African Reference Range Project Results Report was sent with which explained each test parameter as well as the imact on their health if abnormal. An example of such a report can be seen in **Appendix F**.

3.1 Characteristics of participants

The overall number of volunteers we recruited were 1433. However, there was imbalance in the age distribution with higher proportion in 18−29-year-old in both genders. Therefore, for data analyses, we first applied a random filter to delete 188 individuals to even out the sample size for each decade of age and sex. As another preliminary step for data validation, we scanned obvious extreme values analyte by analyte and found 1 individual with TG > 5.0 mmol/L, 1 with ALT and AST > 130 U/L, 2 with FT4>18 pmol/L, 1 with Alb<25 g/L, 28 with IgG> 30 g/L, and 1 with CRP> 60 mg/L. Therefore, we first deleted a total of 33 individuals with multiple extreme values in one or more analytes. Since we assumed the high IgG and low Alb is highly suggestive of latent HIV infection, we applied another step to exclude individuals with IgG> 20 g/L and (Alb< 30 g/L or CRP> 5.0 mg/L), which led to further reduction in data size of 69. Therefore, the final data size for use in the subsequent data analyses was 1143 (=1433 – 188 – 33 – 69).

The population characteristics of the participants are summarised in **Table 2** with regard to race, gender, age, BMI, WC, systolic blood pressure (SBP; mmHg), diastolic blood pressure (DBP; mmHg), and smoking habit (%). Among 1143 volunteers, 43% were males and 57% were females. The racial compositions were 551 (48%) Afr, 209 (18%) Mix and 383 (34%) Cau respectively. Volunteers with smoking habit was 10.3% overall with Mix showed the highest percentage (males: 16.9% and females 19.7%).

3.2 Ethnic-, gender-, and age-related changes in RIs

The magnitude of three major sources of variation: gender, race, and age were calculated for each analyte as SDRsex, SDRrc, and SDRage as shown in **Table 3**.

We evaluated SDRrc in two ways: one by dividing RVs by three racial groups (Afr, Mix, and Cau) and the other by two racial groups (Afr and NAfr[Cau+Mix]), with derivation of $SDRrc_3$ and $SDRrc_2$, respectively. For each method, we first applied 3N-ANOVA with gender, racial group, and age group (partitioned at 30, 40, and 50-year-old), then, applied 2N-ANOVA separately for each gender to derive gender-specific SDRrc and SDRage.

For graphical interpretation of the results in **Table 3**, we drew box-whisker charts after stratifying RVs by gender and race. The typical graphical profiles of gender- and race-related

changes of RVs are shown for 12 analytes in **Figure 1**. All 37 analytes are in **Appendix G (Supplementary Figure 1)**.

In general, there were not many differences between $SDRrc_2$ and $SDRrc_3$ except for TBil, LDL-C, AMY, and TC. However, because of a smaller data sizes of RVs in Mix group, in the final derivation of RIs, we adopted two race group option for determining race-specific RIs one for Afr and the other for NAfr.

Partitioning of RVs into 2 racial groups (Afr and Nafr) was found necessary for 11 analytes namely: TP, Urea, Cre, TBil, TC, LDL-C, ALT, AMY, ChE, IgA and IgG.

Five analytes required partition of RIs by gender ($SDR_{sex2} \geq 0.4$) namely: Alb, UA, Cre, CK and ferritin. Although HDL-C had SDR_{sex} of 0.37, the UL for both Afr and Non-Afr was significantly different between two sexes. Therefore, gender partitioning of RVs was also done for HDL-C.

Although SDR_{age} was computed by stratifying RVs by the following four age groups: 18−29; 30−39; 40−49, and 50− year old, because of too small number in each subgroup, we chose to partition RVs at age 45. Age related RIs were derived for 8 analytes namely: 1) males: PSA; 2) females: Urea, UA, ALP, ChE and Ferritin and 3) both genders: TC and LDL-C. Age-related changes will be discussed in more details in the Part 2, which dealt with sources of variation of RVs. Furthermore, partitioning by ethnicity of RIs was required for 10 analytes namely: TP, urea, TBili, TC, LDL-C, ALT, ChE, Amy, IgG and IgA.

3.3 Recalibration of derived RIs for standardization

As described earlier, this project measured both panel I and panel II on 2 separate occations during the course of the study. In order to perform proper recalibration or allignment of results, the precision of RI limits, LL and UL were recaulculated after calculating the RIs with or without LAVE.

Since the panel of sera have values assigned for confirming the status of standardization of our assays, we compared our test results with them as shown in **Appendix I and J** respectively.

The need for recalibration was judged by a ratio of bias for the LL (or UL), ΔLL (or ΔUL), to the SD comprising the RI, which corresponds to between-individual SD, as follows:

$$\Delta LL \text{ ratio} = | LL_{-} - LL_{+} | / (UL_{+} - LL_{+})/3.92$$
$$\Delta UL \text{ ratio} = | UL_{-} - UL_{+} | / (UL_{+} - LL_{+})/3.92$$

where LL_{+} and LL_{-} (or UL_{+} and UL_{-}) represent LL (or UL) with and without recalibration. We set the critical value for ΔLL (or ΔUL) ratio as 0.375 in analogy to the theory of allowable analytical bias in laboratory tests becasue the numerator of ΔLL (or ΔUL) is a bias or change caused by the recalibration. By this reasoning, five analytes, Cre, AST, ALT, LDH, and GGT, were judged as requiring recalibration of RIs based on the linear regression coefficients shown in **Figure 2**.

3.4 Reference intervals

3.4.1 Comparison among methods for derivation of the RIs

Two serum panels were produced by the C-RIDL in 2011 and in 2014, respectively composed of 80 sera in panel 1 and 100 sera in panel 2 from healthy individuals with nearly equal gender and age distribution. These 2 panels were analysed in this study to harmonise the RIs and compare them to the other countries who took part in the study. As can be seen in **Figure 2**, six of the analytes in the South African study required RI recalibration after comparing the values to the assigned values of the different panels namely: HDL when compared to the assigned value in panel I and Cre, AST, ALT GGT and LDH when compared to their assigned values from panel II.

3.4.2 Final list of RIs inconsideration of ethnic-, gender-, and age-related changes

The RIs for South Africa can be seen in **Appendix K (Supplementary Table 1)**. To simply these findings, **Table 4** shows the major analytes that require RIs separation for age, race and gender. Overall, eleven analytes required RI separation by race namely: TP, Urea, Cre, TBil, TC, LDL-C, ALT, AMY, ChE, IgG and IgA. Of these, four analytes required RIs separation by gender namely: Urea, Cre, ALT and ChE. Furthermore, three analytes required separation of RIs by age whereby 2 separate RIs for these analytes had to be generated for <45 years of age and ≥ 45 years of age respectively. These analytes are Urea, TC,and LDL-C.

Of all the analytes measured for this study, only the LAVE (+) RIs for ALT was adopted for this study.

4. Discussion

In this study, we successfully derived RIs for the South African population by comparing the values obtained from 3 racial groups. We proved that the P method is superior in the determination than the NP method. Furthermore, we also showed the need for LAVE in derivation of RIs.

4.1 Challenge in recruitment

HIV/AIDS in South Africa is a prominent health concern. South Africa has the largest antiretroviral treatment (ART) programme globally and these efforts have been largely financed from its own domestic resources. It is believed to have more people with HIV/AIDS than any other country in the world. The 2014 United Nations AIDS report estimated that 7 million adult South Africans had HIV/AIDS (19.2%) of South Africa's population of 55 million. The number of infected population is larger than in any other single country in the world (United Nations, 2014). Furthermore, 380 000 new HIV infections has been reported in South African in 2014 as well as 180 000 AIDS related deaths (United Nations, 2014). We clearly stated in the information sheet and the health-status questionnaire about the inclusion and exclusion criteria of this study which include questions like “Subjectively feeling healthy”, “not currently under medical care”, “not known carrier status of HIV, HBV or HCV”. However, we found that there were many subjects with very high IgG suspected of latent HIV or other chronic infections. Therefore, we had to apply secondary exclusion criteria of IgG $\geq$ 20 g/L, CRP $\geq$ 5mg/L, or Alb $<$ 30 g/L, which were determined by logistic regression analysis. After applying the criteria, 69 (47 males; 22 females) cases fell into the categories and were excluded from the analysis with reduction of total data size from 1433 to 1143. They were all Afr, none was from the other ethnic groups.

As additional problem of latent diseases, we diagnosed four cases of mild hyperthyroidism from high FT4, low FT3 and anti-thyroid antibodies. Thyroid disorders are common, with a lifetime risk of 12% of the population (Sigurd, 2016). However, because of

the low prevalence, their inclusion would not have made any difference to the derivation of RIs.

4.2 Ethnic differences in RIs

The differences in RIs between Caucasian and Mixed Ancestry population were generally smaller compared to those between African and other two. Besides, the number of volunteers in the Mixed were below 100. Therefore, the comparison and determination of RIs were the African polulation (AF) and non African (NAF) population by combining RVs of the Mixed Ancestry and Caucasion population.

Racial differences in reference values were noted in many analytes: TP, TBil, Urea, TC, LDL-C, IgG, IgA, ALT, AMY, ChE. The most conspicuos were ALT, LDL-C, and IgG. For ALT, there have been many reports from epidemological data based on population sampling scheme whereby very low ALT RIs were observed in African Americans which, in turn, makes them less susceptable to non-alcoholic fatty liver disease (Browning, *et al.* 2004); (Pan & Fallon, 2014); (Guerrero, *et al.,* 2009); (Ichihara, *et al.,* 2016). With this study, we confirmed the tendency of low ALT among African from apparently heatlhy individuals. The investigators thereby deduct from this finding that a possible genetic factor might contribute to lower ALT in the African population, regardles of their location globally.

For LDL-C, there was significant ethnic differences seen in RIs between Afr and non-Afr. In the non-Afr group the RI's were higher in both males and females in comparison with the Afr population. This tendency has also been shown in other reports (Sliwa, *et al.*, 2012); (Ellman, *et al.*, 2015). Therefore, we confirmed the same tendency of higher LDL-C in non-Afr compared to Afr.

For IgG, three Asian studies revealed a large regional differences in IgG among healthy individuals (Ichihara, *et al*., 2004); (Ichihara, *et al*., 2008); (Ichihara*, et al.*, 2013). The same finding was seen in the harmonised global study (Ichihara, *et al*., 2016) as well as in this study. Zemlin and co-workers hypothesized that serum protein electrophoresis patterns would be abnormal in untreated HIV subjects and correlate with markers of disease severity. (Zemlin *et al*.; 2015). This was a cross-sectional study whereby they performed serum protein electrophoresis on 72 HIV-positive subjects; clinically well and not on ART and 42 HIV-

negative controls recruited from the Voluntary Testing and Prevention clinic, Emavundleni in Crossroads, Cape Town, Western Cape, South Africa. One of their major findings was that there was a significant increase in the concentration of IgG in HIV positive cohort when compared to the negative cohort ($p < 0.001$). For our study, we therefore conclude that the increased IgG in the Afr population group was due to the latent HIV cases as described earlier.

For AMY, significantly higher RI's were seen in both males and females in the Afr racial group when compared the non-Afr group. Perry *et al.* showed that copy number of the salivary amylase gene (*AMY1*) is correlated positively with salivary amylase protein level and that individuals from populations with high-starch diets have, on average, more *AMY1* copies than those with traditionally low-starch diets (Perry*, et al.*, 2007). Love *et al.* conducted a study and reported all black Africans, urban and rural alike, made starchy foods, such as maize porridge, potatoes, rice and bread, an essential part of most meals as a result of traditional or habitual food consumption patterns (Love, *et al.*, 2001). We therefore deduct from these studies that AMY RIs in Afr will be higher than in the non-Afr population groups.

As for the other analytes, racial differences was observed in all of them in the global harmonised study (Ichihara, *et al.*, 2016).

4.3 Gender differences in RIs

This study has shown that partitioning of RIs by gender was indicated in 6 analytes namely: Alb, UA, Cre, HDL-C, CK and Ferr. The same findings were seen when compared to the global study either in a prominent – or moderate manner (Ichihara, *et al.*, 2016). Furthermore, it has been noted that females has more age-related changes in reference intervals, especially after menupause (Ichihara, *et al*., 2016). This, however, will be disussed in more detail in the part 2 of this report. Compared to the global study, our study findings showed no gender partitioning of RIs was required for Alt, GGT, IgM, Urea, TG, IP, AST, ALP or Tf. Furthermore, HDL-C and IgM shower an greater RI for females than in males. Gender related changes in these analytes was also seen in the global study, either in a prominent – or moderate manner (Ichihara, *et al*., 2016).

4.4 P-method versus NP-method in the derivation of RIs

As has been already discussed in other studies, we noted a problem of using nonparametric method in deriving RIs with wider UL and wider 90% CI of the UL almost invariable, indicating unnatural tailing of the distribution to the higher side. While they can be detected by the shape of the central portion by the parametric method and excluded by including a step of excluding values outside mean ± 3.5SD (outer 0.05% of values). Previous reports have shown that the P method based on the modified Box-Cox formula remains the method of choice over and above the NP method for the derivation of RIs in almost all situations (Ichihara, *et al.*, 2016). Excpetion to this rule of course would be in scenario's where the reference distribution would be truncated on the lower side due to the limited level of assay quantification (Ichihara, *et al.*, 2016). The use of the P-method versus NP-method for RIs determination for this study was clearly shown as can be seen in **Figure 3;** all analytes shown in **Supplimentary Figure 2 (Appendix H)**.

4.5 Need for LAVE methods

LAVE has several advantages namely: 1) truncation of reference distributions does not occur, because this method excludes those participants whose results are outside of the RI of any given analyte, other than the analyte for which the RI is being derived and 2) there is no effect at all on alaytes whose values are on rare occations just outside the normal RI of the analyte (Ichihara, *et al*., 2016). There is, however; an disadvantage of using LAVE because when applied it causes a reduction in the number of participants whose results are included in the final computation of the RIs.

During the process of defining RIs for this project, the investigators had to adopt a strict policy whereby the abnormal results of participants had to be excluded due to underlying disease. Many pathological conditions exist that can have an effect on each analytes (Borai, *et al.*, 2016) however, we presumed that if this prevalence is low amongst aparently healthy individuals, the abnormal results from such participants will not have a major influence on the determination of RIs. However, careful considerarion was required for those participants with latent but highly prevalent disorders such as metabolic syndrome and undiagnosed diabetes mellitus in the Mixed Ancestry population group in South Africa (Erasmus, *et al*., 2012) as well as latent HIV/AIDS cases which remained after the afore-mentioned secondary exclusion procedure. Therefore, the LAVE method was applied in our study on 77 analytes to exclude

those participants with abnormal results attributed to those conditions as the performance of LAVE done in previous studies of this nature were well characterized (Borai, *et al*., 2016), (Ozarda, *et al*., 2014), (Yamakado, *et al*., 2015) which is also reflected in **Figure 3;** all analytes shown in **Appendix H (Supplimentary Figure 2).**

5. Limitations and strenghts of the study

One of the most significant findings in this study was to determine which participants we had to exclude due to latent disease of common prevalence. By imposing cut off values C-RP, Alb and IgG as described above, this limitation was overcome with great success. One major limitation of the study was the recruitment of a small number of participants from the mixed ancestry population group. This population group had to be combined with he caucasian population group. This was then named non-AF as the results of these 2 population groups were similair when compared. This could mask the effect of latent disease in the mixed ancestry population group which could not identified due to the small number of participants.

Further investiagion into racial differences in RIs would be of great value when comparing South Africa's results with the other ongoing projects in Africa. Similar projects were conducted in Kenya and Nigeria with Ghana to commence with their project in due course.

6. Conclusion

As a part of global multi-centre collaborative project for derivation of RIs, this study is considered to be the largest and first study of this nature for the South African population to establish RIs for the majority of general biochemistry analytes under a standardized and harmonised protocol and furthermore, for those results to be compared to this standardised protocol. Partitioning of RIs by gender, ethnicity and age was necessary for many analytes however, the latter will be discussed in more detail in part 2 of this report. Partitioning of RIs by gender was required in 4 analytes: albumin, creatinine, HDL-cholesterol (C) and ferritin and partitioning by ethnicity for 10 analytes: total protein, urea, total bilirubin, total C, LDL-C, alanine aminotransferase, choline esterase, amylase, Immunoglobulin G and A. Regarding the

controversies over the optimal statistical methods for derivation of the RI, this study proved the superiority of the P over NP method and the need for application of LAVE method for analytes which are easily influenced by the presence of highly prevalent HIV/AIDS among apparently healthy individuals.

7. Conflict of interest

The authors state that there is no conflict of interests with regards to the publication of this article.

8. Acknowledgements

The authors would like to thank the following collaborators who contributed to the local study in South Africa:

1) Beckman Coulter South Africa for the technical advise and support throughout the project. Furthermore, for the sponsorship of the reagents, quality control material and calibrators for this project.
2) PathCare Laboratories and their CEO, Dr John Douglas, for the major contribution to this project. This included: a) sample preparation and storage prior to analysis at their headoffice at N1 City, Cape Town, b) the administration- and nursing staff of PathCare, Luois Leipoldt for assisting the investigators ant each clinic and taking the samples from the participants and c) the laboratory staff at PathCare Louis Leipoldt as well as PathCare Parl, N1 City, Cape Town, for the analysis of the prepared samples.
3) In particular we would like to acknowledge Arno Theron as well as his collegues at PathCare QA Department who prepared the samples after each clinic and coordinated the sample storage prior to analysis.

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Table 1: List of analytes with methods and between run SD and CV; comparison between the Beckman Coulter DXC,AU and DXI analysers shown respectively.

			Beckman Coulter DXC		Beckman Coulter AU	
Analyte	**Abbreviation**	**Method**	**Between Run SD**	**Between Run CV**	**Between Run SD**	**Between Run CV**
Sodium	Na	Ion selective electrode / diluted (indirect)	1.7	0.0	1,2	0,0
Potassium	K	Ion selective electrode / diluted (indirect)	0.3	0.1	0,2	0,1
Chloride	Cl	Ion selective electrode / diluted (indirect)	2.4	0.0	1,6	0,0
Urea	Urea	Urease	1.3	0.3	0,4	0,1
Creatinine	Cre	Modified kinetic Jaffè	11.7	0.2	13,0	0,2
Total Protein	TP	Buiret	4.7	0.1	2,8	0,0
Albumin	Alb	Bromocresol Green dye binding	1.8	0.0	1,6	0,0
Total Bilirubin	Tbil	Diazonium salt	2.4	0.3	4,4	0,5
GGT	GGT	Gamma-glutamyl-3-carboxy-4-nitroanilide	16.3	0.7	9,0	0,4
ALP	ALP	P-nitro-phenylphosphate hydrolysis	19.5	0.3	8,2	0,1
LD	LD	Lactate to Pyruvate	35.5	0.2	14,4	0,1
Calcium	Ca	Arsenazo III dye	0.1	0.0	0,1	0,0
Magnesium	Mg	Xylidyl blue	0.1	0.1	0,0	0,1
Phosphate	IP	Molybdate hydolysis	0.1	0.1	0,0	0,0
Glucose	GLU	Hexokinase	0.9	0.2	0.8	0.1
Lipase	Lip	1, 2-Diglyceride hydrolysis	18.5	0.4	13,2	0,5
Cholesterol	TC	Cholesterol oxidase	1.0	0.2	1,6	0,3
Triglycerides	Trig	Glycerol phosphate oxidase	0.6	0.4	0,5	0,4
HDL-C	HDL-C	Two phase selective accelerator detergent	0.5	0.4	0,5	0,3
LDL-C	LDL-C	Two phase selective accelerator detergent	0.8	0.3	1,1	0,4
Uric acid	UA	Modified Trinder reaction with Uricase	0.1	0.2	0,0	0,1
hsCRP	C-RP	Turbidimetry	3.4	0.8	2,0	0,8
Amylase	AMY	2-chloro-4-nitrophenyl-α-D-maltotrioside	20.2	0.3	21,4	0,4
IgA	IgA	Turbidimetry	0.5	0.3	1,3	0,5
IgG	IgG	Turbidimetry	4.5	0.4	1,5	0,2
IgM	IgM	Turbidimetry	0.3	0.3	0,5	0,5
ALT	ALT	NADH (without P-5′-P)	10.7	0.8	17,4	0,8
AST	AST	NADH (without P-5′-P)	4.5	0.2	8,9	0,4
Cholinesterase	Che	Butyrylthiocholine hydrolysis	1.2	0.2	1.2	0.2
CK	CK	Creatine phosphate dephosphorilysation	65.0	0.5	119,0	0,6
Iron	Fe	2, 4, 6-Tri-(2-pyridyl)-5-triazine chromogen	8.6	0.6	4,1	0,2
Transferrin	Tf	Turbidimetry	4.0	1.3	0,4	0,2

			Beckman Coulter DXI	
			Between Run SD	**Between run CV**
TG-Ab	TG-Ab	Two-site immune – enzymatic immunoassay	7.4	4.8
TPO-Ab	TPO-Ab	Two-site immune – enzymatic immunoassay	1.1	1.1
TSH	TSH	Two-site immune – enzymatic immunoassay	0.7	0.5
FT4	FT4	Two-site immune – enzymatic immunoassay	1.5	0.1
FT3	FT3	Two-site immune – enzymatic immunoassay	0.6	0.1
Ferritin	Fer	Turbidimetry	40.3	0.6
PSA	PSA	Two-site immune – enzymatic immunoassay	0.6	1.6

Table 2: Population characteristics of participants in South African study.

Race	Gender	N	Age					P	N	BMI	SDR	N	Waist - C	SDR	N	SBP	SDR	N	DBP	SDR	Smoke
			18 - 29	30 - 39	40 - 49	50 - 59	60 +														
	M	291	77	79	60	57	18		285	26.1 ± 5.8		276	90 ± 14.6		171	130 ± 17.5		171	83 ± 10.5		6.6 %
Afr								0.281			0.177			0.000			0.112			0.000	
	F	260	80	72	45	46	17		250	27.7 ± 5.9		246	89 ± 14.0		229	126 ± 19.5		228	82 ± 11.3		0.9 %
	M	74	24	17	15	14	4		72	25.7 ± 3.9		71	89 ± 10.7		71	124 ± 14.7		71	84 ± 10.8		16.9%
Mix								0.566			0.000			0.334			0.124			0.288	
	F	135	47	30	34	17	7		132	25.3 ± 4.6		130	83 ± 12.2		133	120 ± 18.3		133	80 ± 11.2		19.7%
	M	125	44	28	28	19	6		124	26.0 ± 3.6		123	89 ± 11.3		124	129 ± 16.7		124	81 ± 11.6		10.8%
Cau								0.317			0.292			**0.439**			**0.643**			0.245	
	F	258	90	49	46	46	27		256	24.3 ± 4.1		254	82 ± 11.5		254	115 ± 14.0		253	77 ± 10.1		9.0 %
Total		1143	362	275	228	199	79		1119	25.9 ± 5.2		1100	87 ± 13.3		982	123 ± 17.9		980	81 ± 11.1		10.3%

Table 3: The magnitude of three major sources of variation: gender (SDR_{sex}), race (SDR_{rc}), and age (SDR_{age}) by use of 3-level and 2-level nested ANOVA (3N-, 2N-ANOVA)

	3 level nested ANOVA			2 level nested ANOVA		
Analyte	**SDR_{sex}**	**SDR_{rc} (M, F)**	**SDR_{age} (M, F)**	**SDR_{sex}**	**SDR_{rc} (M, F)**	**SDR_{age} (M, F)**
TP	0.00	**0.52** (**0.49; 0.54**)	0.06 (0.04; 0.08)	0.00	**0.49** (**0.46**; **0.51**)	0.04 (0.00; 0.06)
Alb	**0.42**	0.16 (0.19; 0.11)	0.00 (0.32; 0.11)	**0.42**	0.20 (0.29; 0.14)	0.00 (0.33; 0.13)
BUN	0.00	**0.47** (**0.62;** 0.27)	0.25 (0.21; **0.45**)	0.00	**0.49** (**0.65**; 0.30)	0.24 (0.22; **0.45**)
UA	**0.87**	0.12 (0.21; 0.00)	0.24 (0.28; **0.40**)	**0.88**	0.09 (0.20; 0.00)	0.24 (0.26**; 0.40**)
Cre	**1.00**	0.26 (0.33; 0.17)	0.00 (0.10; 0.06)	**1.01**	0.34 (0.33; 0.40)	0.00 (0.14; 0.05)
TBil	0.00	**0.51** (**0.67**; 0.37)	0.00 (0.24; 0.15)	0.12	**0.50** (**0.65**; 0.39)	0.00 (0.22; 0.15)
Na	0.03	0.04 (0.00; 0.00)	0.00 (0.34; 0.25)	0.03	0.04 (0.00; 0.00)	0.00 (0.33; 0.26)
K	0.00	0.21 (0.24; 0.18)	0.00 (0.03; 0.08)	0.00	0.18 (0.22; 0.16)	0.00 (0.00; 0.02)
Cl	0.00	0.15 (0.18; 0.06)	0.00 (0.34; 0.12)	0.00	0.12 (0.15; 0.06)	0.00 (0.33; 0.10)
Ca	0.32	0.00 (0.00; 0.00)	0.24 (0.22; 0.26)	0.32	0.00 (0.00; 0.00)	0.24 (0.20; 0.27)
IP	0.09	0.00 (0.00; 0.00)	0.30 (**0.40**; 0.38)	0.10	0.00 (0.00; 0.00)	0.30 (**0.42**; 0.37)
Mg	0.00	0.05 (0.04; 0.00)	0.00 (0.16; 0.23)	0.00	0.10 (0.13; 0.00)	0.00 (0.15; 0.23)
TCho	0.00	**0.50** (**0.53**; **0.50**)	**0.47** (**0.51**; **0.52**)	0.00	**0.43** (**0.47**; **0.41**)	**0.48** (**0.53**; **0.52**)
TG	0.24	0.00 (0.00; 0.00)	**0.49** (**0.48; 0.50**)	0.25	0.00 (0.00; 0.00)	**0.51** (**0.48**; **0.53**)
HDL-C	**0.37**	0.27 (0.00; 0.35)	0.00 (0.14; 0.15)	**0.38**	0.31 (0.00; 0.38)	0.00 (0.14; 0.11)
LDL-C	0.00	**0.63** (**0.72**; **0.56**)	0.25 (**0.41**; **0.47**)	0.00	**0.55** (**0.67**; **0.47**)	0.26 (**0.42**; **0.47**)
Lip	0.00	0.00 (0.00; 0.00)	0.00 (0.23; 0.22)	0.00	0.10 (0.00; 0.09)	0.00 (0.22; 0.22)
ALT	0.09	**0.50** (**0.62**; 0.37)	0.10 (0.18; 0.26)	0.23	**0.47** (**0.58**; 0.36)	0.07 (0.18; 0.24)
AST	0.33	0.23 (0.21; 0.35)	0.00 (0.25; 0.26)	0.34	0.23 (0.21; 0.34)	0.00 (0.25; 0.24)
ALP	0.24	0.24 (0.18; 0.22)	0.00 (0.16; **0.40**)	0.26	0.22 (0.15; 0.17)	0.00 (0.17; 0.41)
AMY	0.00	**0.60** (**0.84**; **0.59**)	0.00 (0.00; 0.12)	0.00	**0.59** (**0.85**; **0.59**)	0.00 (0.00; 0.11)
LDH	0.00	0.13 (0.20; 0.00)	0.00 (0.14; 0.17)	0.00	0.14 (0.18; 0.00)	0.00 (0.12; 0.18)
CK	**0.55**	0.23 (0.00; 0.33)	0.00 (0.24; 0.00)	**0.56**	0.21 (0.00; 0.31)	0.00 (0.24; 0.00)
GGT	0.08	0.07 (0.27; 0.18)	0.00 (0.31; 0.37)	0.09	0.05 (0.24; 0.14)	0.00 (0.31; 0.38)
CRP	0.00	0.22 (0.23; 0.21)	0.21 (0.20; 0.27)	0.00	0.22 (0.20; 0.24)	0.21 (0.18; 0.28)
ChE	0.00	**0.40** (**0.57**; 0.00)	0.21 (0.20; **0.42**)	0.15	0.35 (**0.53**; 0.00)	0.21 (0.18; **0.43**)
Fe	0.27	0.19 (0.28; 0.34)	0.00 (0.04; 0.00)	0.30	0.24 (0.27; 0.42)	0.00 (0.00; 0.10)
Ferritin	**0.71**	0.00 (0.00; 0.00)	**0.46** (0.22; **0.57**)	**0.73**	0.00 (0.11; 0.00)	**0.47** (0.23; **0.58**)
Tf	0.32	0.00 (0.00; 0.00)	0.11 (0.00; 0.28)	0.32	0.00 (0.00; 0.00)	0.13 (0.00; 0.30)
IgA	0.00	**0.49** (0.37; **0.56**)	0.00 (0.25; 0.31)	0.00	**0.48** (**0.43**; **0.52**)	0.00 (0.23; 0.31)
IgG	0.00	**0.46** (**1.05**; **0.94**)	0.00 (0.00; 0.06)	0.00	**0.45** (**1.06**; **0.96**)	0.00 (0.07; 0.00)
IgM	0.37	0.00 (0.17; 0.00)	0.26 (0.16; 0.33)	0.37	0.00 (0.18; 0.00)	0.26 (0.17; 0.33)
FT3	0.30	0.28 (0.18; 0.32)	0.00 (0.14; 0.16)	0.32	0.28 (0.16; 0.33)	0.00 (0.15; 0.18)
FT4	0.02	0.18 (0.00; 0.23)	0.15 (0.20; 0.13)	0.06	0.18 (0.00; 0.25)	0.17 (0.21; 0.15)
TSH	0.00	0.00 (0.00; 0.00)	0.00 (0.13; 0.11)	0.00	0.10 (0.00; 0.14)	0.00 (0.17; 0.08)
Thyro	0.00	0.13 (0.16; 0.14)	0.00 (0.00; 0.00)	0.00	0.12 (0.14; 0.12)	0.00 (0.00; 0.09)
TgAb	0.00	0.05 (0.10; 0.00)	0.04 (0.00; 0.09)	0.00	0.09 (0.16; 0.00)	0.00 (0.00; 0.07)
PSA	------	----- (0.00; N/A)	----- (**0.47;** N/A)	-----	----- (0.00; N/A)	----- (**0.46;** N/A)

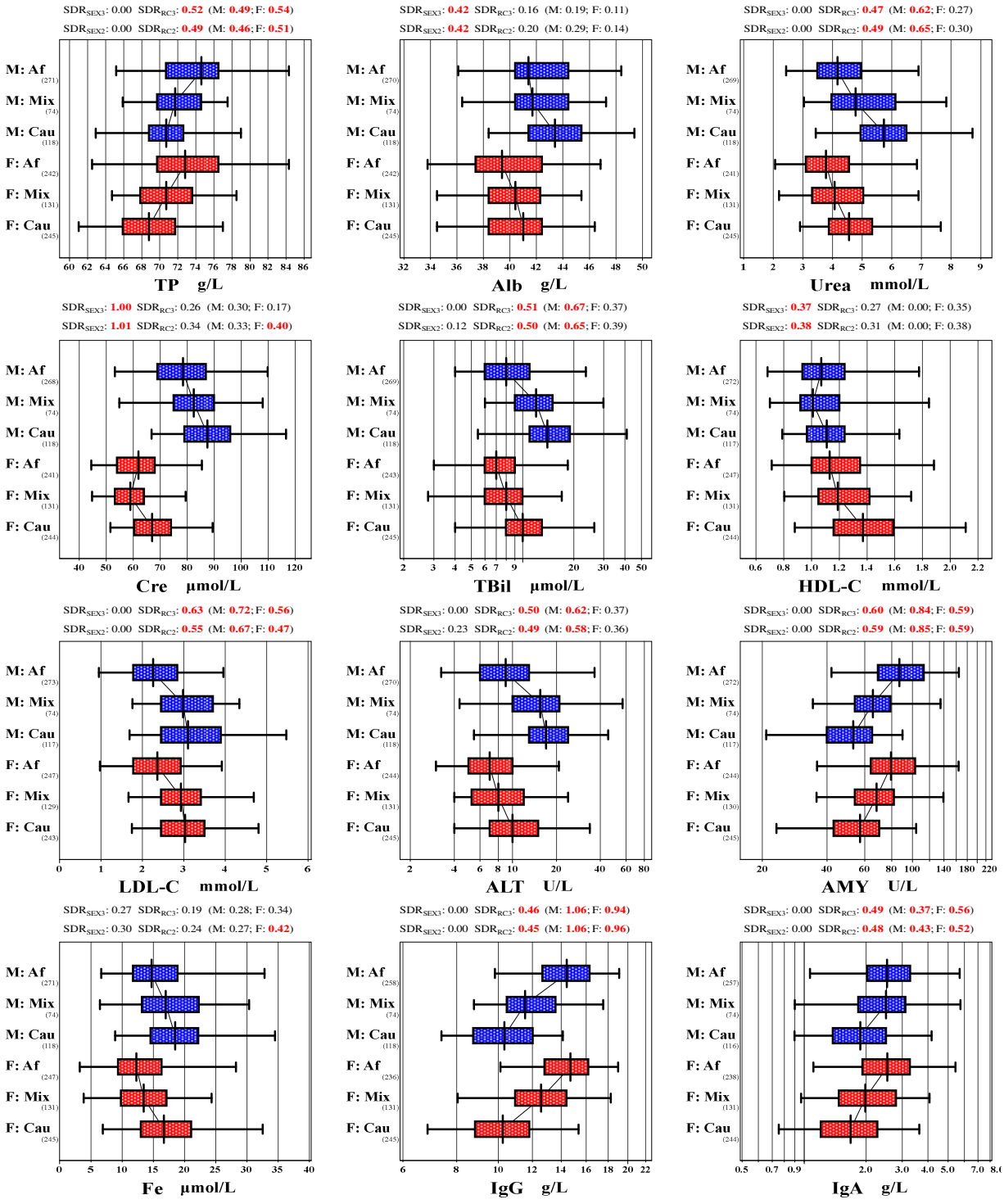

Figure 1: Box-whiskers chart of 12 analytes indicating partitioning of RIs by gender and race.

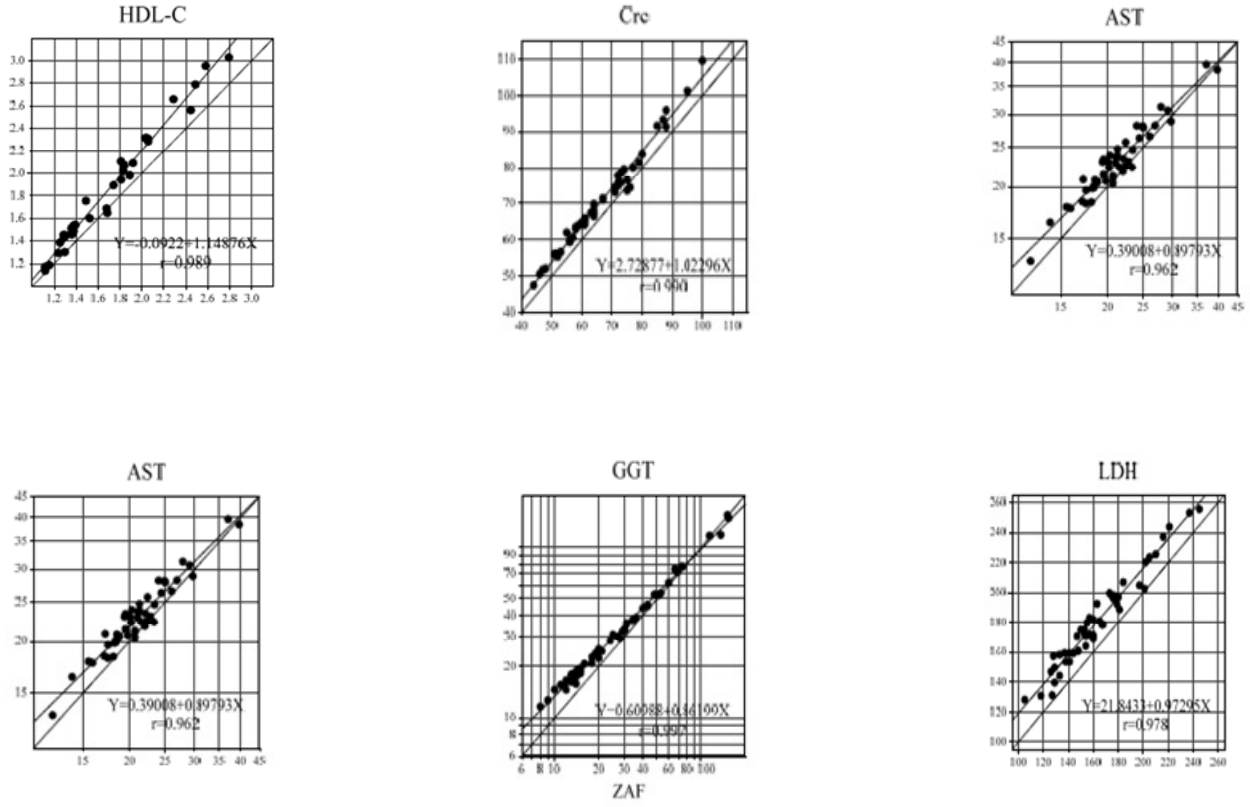

Figure 2: RIs requiring recalibration after comparison of HDL with assigned value of panel I and Cre, AST, ALT, GGT and LDH compared to assigned value of panel II

Table 4: Selected RIs partitioned by ethnicity, gender and age; Afr versus non – Afr (LAVE+ RIs highlighted in blue)

					AFRICAN								NON-AFRICAN							
Item	**Units**	**Method**	**Gender**	**Age**	**n**	**LL-L**	**LL-H**	**LL**	**Mean**	**UL**	**UL-L**	**UL-H**	**n**	**LL-L**	**LL-H**	**LL**	**Mean**	**UL**	**LL-L**	**LL-H**
TP	g/L	LAVE (-)	M + F	All	502	**63**	**66**	64	74	83	**83**	**85**	532	**62**	**63**	62	70	78	**77**	**79**
Urea	mmol/L	LAVE (-)	M	All	626	**2.38**	**2.69**	2.51	4.22	6.93	**6.59**	**7.58**	185	**3.06**	**3.45**	3.28	5.36	8.31	**7.90**	**8.92**
			F	< 45	185	**1.75**	**2.15**	3.28	5.36	8.31	**5.71**	**6.61**	165	**2.32**	**2.64**	1.95	3.6	6.14	**5.96**	**6.80**
				≥ 45	165	**2.29**	**2.97**	1.95	3.6	6.14	**7.30**	**9.04**	238	**2.33**	**3.06**	2.48	4.14	6.37	**7.55**	**8.68**
Cre	µmol/L	LAVE (-)	M	All	263	**55**	**62**	58	83	114	**110**	**120**	184	**62**	**74**	69	91	117	**113**	**122**
			F		184	**46**	**50**	69	91	117	**87**	**93**	238	**50**	**54**	48	65	89	**89**	**95**
TBil	µmol/L	LAVE (-)	M + F	All	499	**2.9**	**4.2**	3.5	7.8	19.1	**17.3**	**21.3**	524	**3.1**	**4.9**	4.3	10.5	24.1	**22.1**	**28.4**
TC	mmol/L	LAVE (-)	M + F	< 45	347	**2.42**	**2.72**	2.59	4.12	6.05	**5.87**	**6.31**	369	**3.27**	**3.56**	3.42	4.86	6.82	**6.58**	**7.20**
			M + F	≥ 45	164	**2.86**	**3.24**	3.04	4.76	7.39	**7.06**	**7.80**	173	**3.67**	**4.17**	3.90	5.73	7.81	**7.49**	**8.18**
LDL-C	mmol/L	LAVE (-)	M + F	< 45	348	**0.80**	**1.00**	0.9	2.2	3.67	**3.55**	**3.79**	371	**1.59**	**1.75**	1.65	2.83	4.47	**4.32**	**4.72**
			M + F	≥ 45	371	**1.15**	**1.41**	1.65	2.83	4.47	**4.41**	**5.05**	164	**1.80**	**2.17**	1.27	2.51	4.68	**5.02**	**5.65**
ALT	IU/L	LAVE (+)	M	All	211	**2.8**	**4.8**	3.8	10.3	31	**25.9**	**38.8**	155	**4.1**	**8.3**	6.7	19.9	53	**45.6**	**65.0**
			F		155	**2.4**	**3.1**	6.7	19.9	53	**18.4**	**23.7**	207	**3.1**	**4.4**	2.6	7.8	21.1	**28.1**	**34.3**
AMY	IU/L	LAVE (-)	M + F	All	502	**39**	**46**	41	84	161	**152**	**173**	526	**25**	**31**	28	59	111	**104**	**117**
ChE	kU/L	LAVE (-)	M	All	264	**4.5**	**5.1**	4.7	7.4	10.6	**10.2**	**11.0**	185	**5.5**	**6.3**	5.8	8.7	11.9	**11.4**	**12.4**
			F		185	**4.2**	**4.8**	4.5	7.1	10.1	**9.8**	**10.4**	240	**4.6**	**5.2**	4.9	7.4	10.6	**10.3**	**11.0**
IgG	g/L	LAVE (-)	M + F	All	483	**9.8**	**10.3**	10.1	14.5	19.6	**19.3**	**20.1**	531	**7.0**	**7.5**	7.3	10.9	16.9	**16.4**	**17.6**
IgA	g/L	LAVE (-)	M + F	All	483	**1.0**	**1.2**	1.1	2.5	5.3	**4.9**	**5.7**	528	**0.8**	**0.9**	0.8	1.8	4.2	**4.0**	**4.5**

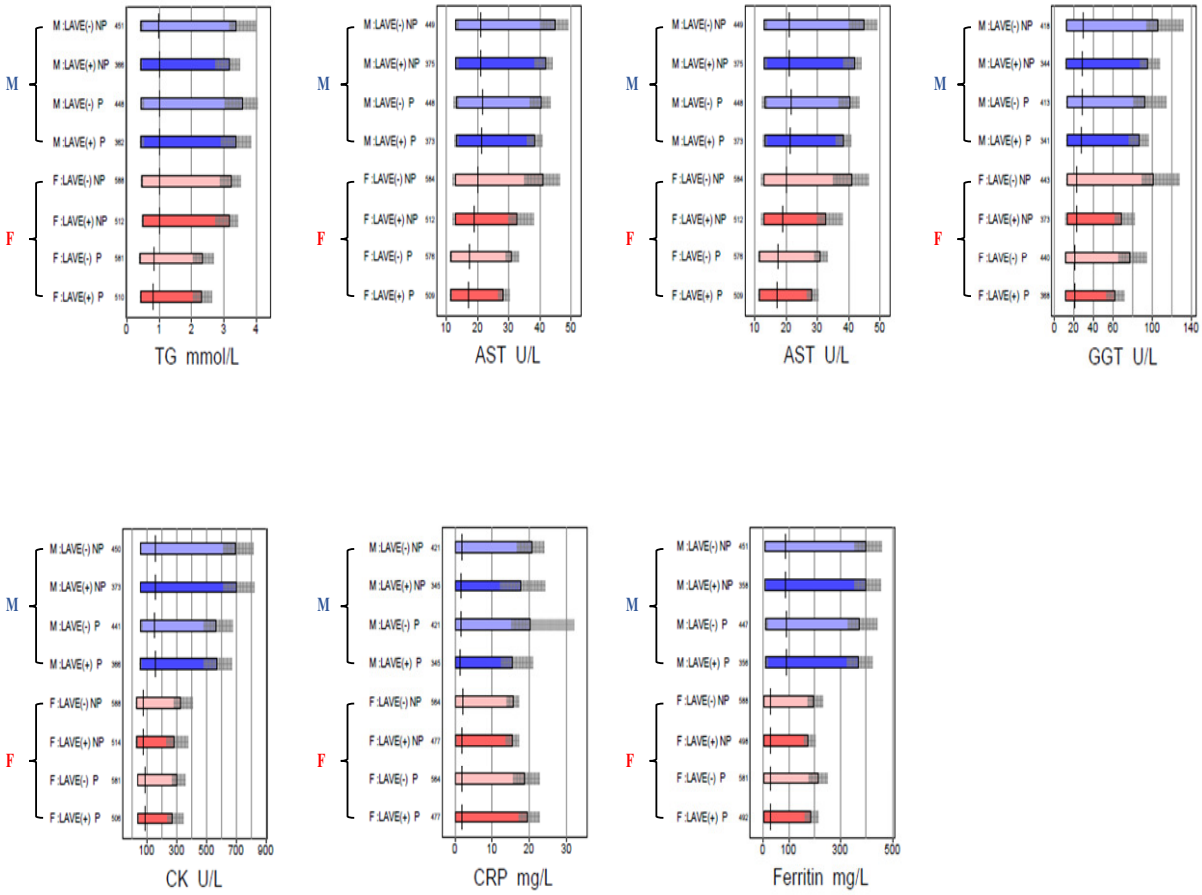

Figure 3: Analytes whereby LAVE (+) was applied in the derivation of RIs; P versus NP method.

MANUSCRIPT TWO

Exploration of sources of variation in the establishment of reference intervals of clinical chemistry analytes for the adult population in South Africa. Part 2: A study conducted as part of the IFCC global multicentre study on reference values.

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Abstract

Background

This study was conducted as a part of IFCC global study to establish reference intervals (RIs) of major laboratory tests for South African population in consideration of ethnic differences in values.

Methods

A total of 1245 healthy volunteers aged ≥18 years composed of 651 black African (Afr), 385 Caucasian (Cau), and 209 mixed ancestry (Mix) were recruited using the global study protocol. Fasting blood samples were collected from the participants. Serum specimens were measured for 40 chemistry and immunochemistry analytes, DXC. MRA was performed analyte by analyte for each gender and separated by ethnicity (Afr. Versus non-Afr.) to explore the importance of BMI and age. Any analyte with a $r_p \geq |0.20|$ was considered as significant.

Results

Age related changes was seen in 24 analytes namely: Alb, Urea, UA, Cre, TBil, Ca, IP, Mg, TC, TG, HDL-C, LDL-C, Lip, ALP, CK, GGT, ChE, Ferritin, Tf, C-RP, IgA, IgM, FT3 and PSA in males.

BMI related changes were observed in 12 analytes when comparing Afr with non-Afr ethnic groups namely: UA, TG, HDL-C, LDL-C, ALT, AST, ALP, LDH, CK, GGT, C-RP and Ferr.

Conclusion

This study shown that RI partitioning should be done for race and gender, other sources of variation must be considered such as age and BMI. As a matter of fact, in this study, age and BMI showed to have a major effect in the determination of RIs which has shown the importance of considering these variables.

Keywords

Reference values, Worldwide multicentre study, Body mass index (BMI), Multiple regression analysis, Age-related changes, BMI-related changes.

Non-standard abbreviations

Alb	Albumin
ALP	Alkaline Phosphatase
ALT	Alanine Aminotransferase
AMY	Amylase
AST	Aspartate Aminotransferase
BMI	Body Mass Index
Ca	Calcium
CDL	Clinical Decision Limit
CK	Creatine Kinase
CI	Confidence Interval
Cl	Chloride
CLSI	Clinical and Laboratory Standards Institute
Cre	Creatinine
CRM	Certified Reference Materials
CRP	C-reactive protein
Fe	Iron
GGT	Gamma-glutamyltransferase
Glu	Glucose
HDL-C	HDL-cholesterol
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IP	Inorganic Phosphate
K	Potassium
LAVE	Latent Abnormal Values Exclusion
LDH	Lactate Dehydrogenase
LDL-C	LDL-cholesterol
LL	Lower Limit

MRA	Multiple Regression Analysis
Mg	Magnesium
Na	Sodium
RI	Reference Interval
RV	Reference Value
SD	Standard Deviation
SDR	Standard Deviation Ratio
SV	Sources of Variation
TBil	Total Bilirubin
TC	Total Cholesterol
Tf	Transferrin
TG	Triglycerides
TP	Total Protein
UA	Uric Acid
UL	Upper Limit
ZAF/Af	African population of South Africa
ZAF/NAf	Non-African population of South Africa

1. Introduction

The global study in reference intervals not only enabled us to determine reference intervals (RIs) for South Africa, it also enabled the investigators to compare the South African RIs with the global study. Furthermore, it gave them the opportunity to investigate and clarify the sources of variations (SVs) of each laboratory test among healthy individuals.

Numerous studies have been conducted in East – and South - east Asia in the past to determine 1) the feasibility of establishing common reference intervals and 2) to determine if any difference in RIs exist when comparing results with variables such as age, race and gender (Ichihara *et al.,* 2008); (Ichihara *et al.,* 2013); (Ichihara, Ceriotti *et al.,* 2013).

With the coordinated gobal study on RIs coordinated the Committee on Reference Intervals and Decision Limits (C-RIDL), the International Federation of Clinical Chemistry (IFCC) and the studies mentioned above, South Africa conducted a similair study from 2012 – 2014 to establish 1) common reference intervals that could be used between different racial -, age – and gender groups within South Africa with a common protocol as used in the global study; 2) compare these results with the global study coorinated by the C-RIDL / IFCC and 3) to explore the sources of variations (SV) including age, gender, age and body mass index (BMI) on these results. This was achieved by making use of the common protocol that was described by the C-RIDL / IFCC whereby a detailed health questionnaire was distributed to all participants (Ozada *et al.,* 2013). Furthermore, by making use of this common protocol, it allowed us to allign the RIs based on the test results of a standardised reference serum panel I and panel II and allign the South African results in a well-controlled manner with the global results and by making use of different statistical methods (Ichihara, 2014).

In part 1 of the South African results, we focused one the standard deviation ratio of gender (SDR_{sex}) and race (SRD_{race}) as sources of variation on reference intervals. In this paper, we want to report on BMI and age as sources of variation on the South African RIs study by making use of multiple regression analysis (MRA).

2. Methods and materials

The study design for South Africa was adopted from the common study protocol as was used in other countries who formed part of the global study (Ozada *et al.,* 2013). Recruitment of participants and various statistical methods, sample labelling and completion of relevant paper work used for this project was well described in part 1 of the South African report. Sample analysis took place at an ICO 15189 acxredited laboratory (PathCare Laboratories, Cape Town).

2.1 Ethical considerations

Ethical approval was obtained from the Faculty of Health and Wellness Sciences' Research and Ethical Committee of Cape Peninsula University of Technology, Reference Number: CPUT/NHREC: REC-230408-014 as well as the University of Stellenbosch's Health Research Ethics Committee 1 protocol number: S12/05/147. Written informed consent was obtained from each volunteer.

2.2 Analysis of sources of variation (SV) by means of multiple regression analysis (MRA)

MRA was performed analyte by analyte for each gender and separated by ethnicity (Afr. Versus non-Afr.) to explore the importance of BMI and age. The results of the standardized partial regression coefficients (r_p) is seen in **Table 1,** whereby $r_p \geq |0.20|$ was considered as significant.

3. Results

3.1. Age related changes in test results

Looking at **Table 1**, age related changes was seen in 24 analytes namely: Alb, Urea, UA, Cre, TBil, Ca, IP, Mg, TC, TG, HDL-C, LDL-C, Lip, ALP, CK, GGT, ChE, Ferritin, Tf, C-RP, IgA, IgM, FT3 and PSA in males. This is illustrated in **Figure 1** (all analytes **supplementary figure 1**; **Appendix K).**

3.2. BMI related changes in test results

BMI related changes were observed in 12 analytes when comparing Afr with non-Afr ethnic groups namely: UA, TG, HDL-C, LDL-C, ALT, AST, ALP, LDH, CK, GGT, C-RP and Ferr. This is illustrated in **Figure 2.**

4. Discussion

A study of this nature for the derivation of RIs ensures that there is an opportunity to analyse potential biological SV's of RIs amongst healthy individuals. Furthermore, it enables us to compare these RI's to other similar studies done internationally, seeing that all these studies were performed utilising the same analysis scheme as we have done in this study.

What this study and other studies of this nature showed is that age and gender as SV's are strong factors to consider when partitioning RIs. However, looking at the global study as well as similar studies done globally in other countries, SVs including BMI and ethnicity also play a role when it comes to partitioning of RIs (Ichihara *et al.*, 2016). This might be due to the lack of knowledge to perform such statistical analysis (Ichihara *et al.*, 2016). Some authors also attribute this to insufficient sample size (Ichihara *et al.*, 2016). Furthermore, the Clinical and Laboratory Standards Institute (CLSI) guidelines (IFCC, 2010), which deals with the method used for the determination and analysis of major RIs have never included SVs analysis (Ceriotti *et al.*, 2010); (Rustad *et al.*, 2004).

Previous studies have shown that analysis of SVs cannot be performed by simple stratification of RVs. The need for multiple regression analysis (MRA) when looking at factors for the partitioning of RVs and the effect of gender as a SV on partitioning of RVs (Ichihara & Kawai, 1997). Furthermore, the theoretical aspect of such phenomena has been described previously (Ichihara & Boyd, 2010); (Ichihara, 2014) and has been discussed in depth in part one of this report. As a result of this, a series of papers on RI studies which included MRA for the exploration of SVs as an important part of the study has been published, which included the South African study described here and in part 1 (Borai *et*

al., 2016); (Ichihara *et al.*, 2007); (Ozada *et al.*, 2014); (Shimizu & Ichihara, 2015); (Xai, e*t al.*, 2016).

In the global study, BMI as an SV has shown obvious association with RVs, especially in those analytes representing the status of nutrition and inflammation (Ichihara *et al.*, 2016). The same changes were seen in the South African study. Furthermore, between race differences in RVs were seen in TG for females, ALT and AST for males and GGT for both genders, all of which is more prominently seen in Afr versus non-Afr.

In this study, gender-related changes in RIs was noted for a few analytes. Alb, Ca and CK showed a decrease in age more prominently in males than in females, Ureas showed a decrease in age more prominently in females than in males. Furthermore, Tf showed an increase in age in females and Ferritin an increase in age in females. It was noted that females overall have much more pronounced age-related changes in RIs than males, especially after menopause. Elderly females are affected by a range of clinical disorders including endocrine, cardiovascular, skeletal, urogenital tract and immunological systems, body mass, vasomotor tone, mood and sleep pattern. Reference intervals for many diagnostic biochemical tests for the menopause need to be used when interpreting results in clinical investigations for patient management (Honour, 2018). The same trend was seen in the international study (Ichihara *et al.*, 2016).

More peculiar results seen in this study was a high BMI r_p for T3 in non-Afr, a strong negative r_p of age for CK in non-Afr and a low r_p of age for LDH in both ethic groups.

5. Conclusion

As a part of global multi-center collaborative project for derivation of RIs, this study is considered to be the largest and first study of this nature for the South African population to establish RIs for the majority of general biochemistry analytes under a standardized and harmonised protocol and furthermore, for those results to be compared to this standardised protocol. Volunteers were presumed to be apparently healthy however, this study has identified those with latent disease. However; for RI studies such as these, careful consideration and

recruitment must be performed to ensure that all the participants are as healthy as possible. This can be achieved by proper screening procedures beforehand and by making use of a comprehensive questionnaire to try and eliminate those suspected of latent disease.

Not only has this study shown that RI partitioning should be done for race and gender, other sources of variation must be considered such as age and BMI. As a matter of fact, in this study, age and BMI showed to have a major effect in the determination of RIs which has shown the importance of considering these variables.

Regarding the peculiar trends seen in some of this study's results, comparison of other African countries namely Nigeria and Kenya, participating in this study would be of cardinal importance to see if similar trends occurred in these counties.

6. Conflict of interest

The authors state that there is no conflict of interests with regards to the publication of this article.

7. Acknowledgements

The authors would like to thank the following collaborators who contributed to the local study in South Africa:

1) Beckman Coulter South Africa for the technical advise and support throughout the project. Furthermore, for the sponsorship of the reagents, quality control material and calibrators for this project.
2) PathCare Laboratories and their CEO, Dr John Douglas, for the major contribution to this project. This included: a) sample preparation and storage prior to analysis at their headoffice at N1 City, Cape Town, b) the administration- and nursing staff of PathCare, Louis Leipoldt for assisting the investigators ant each clinic and taking the samples from the participants and c) the laboratory staff at PathCare Luois Leipoldt as well as PathCare Parl, N1 City, Cape Town, for the analysis of the prepared samples.

3) In particular we would like to acknowledge Arno Theron as well as his collegues at PathCare QA Department who prepared the samples after each clinic and coordinated the sample storage prior to analysis.

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Table 1: Association of age and BMI separated by gender and ethnicity on tests results by means of multiple regression analysis, whereby $r_p \geq |0.20|$ was considered as significant.

	Male								Female							
	African				Non-African				African				Non-African			
Item	n	R	Age	BMI	n	R	Age	BMI	n	R	Age	BMI	n	R	Age	BMI
TP	261	0,124	-0,126	0,056	189	0,103	-0,105	0,005	231	0,082	0,061	0,037	370	0,183	-0,156	0,165
Alb	262	0,277	-0,187	-0,164	189	0,323	-0,330	0,044	232	0,165	-0,075	-0,122	370	0,039	-0,031	-0,015
Urea	262	0,281	0,232	0,109	188	0,065	0,065	0,002	231	0,268	0,274	-0,017	370	0,364	0,379	-0,050
UA	266	0,321	0,248	0,149	188	0,231	-0,089	0,235	237	0,447	0,346	0,189	370	0,288	0,071	0,255
Cre	262	0,037	0,033	-0,026	189	0,222	-0,200	0,153	231	0,109	0,105	0,011	369	0,099	0,032	-0,106
TBil	264	0,186	-0,086	-0,144	189	0,213	-0,206	-0,024	233	0,121	0,010	-0,124	370	0,237	0,249	-0,131
Na	266	0,155	0,155	0,001	188	0,144	0,055	0,120	231	0,190	0,167	0,050	369	0,228	0,199	0,062
K	270	0,131	0,087	-0,121	189	0,158	0,099	0,103	236	0,057	0,037	-0,057	370	0,106	-0,083	0,101
Cl	270	0,172	0,153	0,049	188	0,180	0,116	0,113	236	0,131	-0,082	0,132	370	0,057	-0,006	0,059
Ca	270	0,203	-0,031	-0,193	189	0,320	**0,329**	0,058	237	0,171	0,055	-0,180	370	0,092	-0,038	-0,072
IP	264	0,169	-0,174	0,027	189	0,398	**0,358**	-0,111	235	0,191	-0,063	-0,160	369	0,268	**0,208**	-0,112
Mg	263	0,178	0,183	-0,031	189	0,122	0,116	-0,071	233	0,236	**0,247**	-0,043	370	0,205	0,190	0,036
TC	268	0,348	**0,259**	0,177	188	0,500	**0,484**	0,058	238	0,365	**0,323**	0,095	369	0,436	**0,449**	-0,042
TG	264	0,382	**0,273**	**0,207**	187	0,471	**0,353**	**0,242**	235	0,551	**0,533**	0,045	369	0,328	0,180	**0,219**
HDL-C	265	0,242	**0,212**	-0,182	188	0,149	0,107	-0,133	237	0,189	0,164	-0,164	369	0,334	**0,225**	**0,338**
LDL-C	266	0,294	0,130	**0,233**	188	0,459	**0,417**	0,119	237	0,281	**0,201**	0,139	366	0,448	**0,384**	0,130
Lip	265	0,252	**0,250**	0,008	188	0,113	0,111	-0,056	235	0,047	-0,023	0,049	368	0,206	0,192	-0,169
ALT	264	0,066	-0,030	0,067	189	0,498	0,050	**0,485**	235	0,158	0,094	0,100	370	0,311	0,128	**0,242**
AST	262	0,019	-0,006	-0,016	189	0,290	-0,128	**0,291**	234	0,184	0,177	-0,137	370	0,196	0,168	0,059
ALP	268	0,101	0,101	0,000	189	0,237	-0,009	**0,239**	237	0,237	**0,212**	0,058	369	0,455	**0,212**	**0,335**
AMY	265	0,202	0,114	-0,199	188	0,101	-0,002	-0,101	234	0,185	-0,109	-0,117	369	0,200	0,143	-0,198
LDH	268	0,166	0,069	0,135	189	0,204	-0,028	**0,208**	236	0,092	-0,041	0,097	369	0,339	0,102	**0,290**
CK	263	0,139	-0,132	-0,021	189	0,369	**0,358**	**0,203**	230	0,106	-0,087	0,098	369	0,148	-0,060	0,158
GGT	253	0,259	**0,218**	0,099	162	0,344	**0,229**	**0,216**	213	0,413	**0,406**	0,023	229	0,278	0,157	0,186
ChE	264	0,346	0,096	**0,310**	189	0,292	-0,004	**0,293**	236	0,395	**0,268**	**0,214**	369	0,383	0,186	**0,275**
Fe	264	0,135	-0,064	-0,103	189	0,121	-0,097	-0,054	237	0,110	0,116	-0,055	370	0,206	0,106	**0,218**
Ferritin	273	0,247	**0,253**	-0,031	189	0,257	0,191	0,134	238	0,504	**0,512**	-0,027	370	0,470	**0,462**	0,022
Tf	264	0,143	-0,117	0,115	189	0,115	0,012	0,111	234	0,221	**0,231**	0,039	368	0,267	**0,282**	0,058
CRP	251	0,425	0,093	**0,397**	172	0,307	0,160	**0,229**	231	0,469	**0,262**	**0,317**	338	0,392	-0,016	**0,397**
IgG	251	0,190	-0,042	0,197	189	0,085	0,084	-0,043	227	0,124	0,003	0,123	370	0,114	-0,114	0,082
IgA	250	0,159	0,159	0,001	186	0,250	**0,256**	-0,077	229	0,365	**0,351**	0,036	369	0,249	0,131	0,171
IgM	258	0,066	-0,065	-0,002	189	0,247	**0,222**	-0,069	230	0,295	**0,300**	0,016	369	0,230	**0,222**	-0,020
FT3	271	0,161	-0,165	0,059	189	0,352	**0,274**	**0,293**	236	0,186	-0,185	0,122	368	0,265	-0,168	**0,272**
FT4	271	0,069	-0,029	0,070	189	0,097	-0,045	0,098	236	0,082	-0,082	0,053	370	0,145	0,155	-0,058
TSH	253	0,143	0,034	0,130	138	0,072	0,061	0,031	203	0,144	0,110	0,066	275	0,104	0,058	-0,104
Thyro	239	0,128	0,086	0,076	165	0,121	0,032	0,112	199	0,048	-0,005	0,049	321	0,102	0,109	-0,048
TgAb	260	0,102	0,028	0,091	189	0,084	-0,075	-0,024	232	0,091	-0,096	0,032	366	0,064	-0,032	-0,046
PSA	228	0,334	**0,346**	-0,132	185	0,483	**0,495**	-0,080								

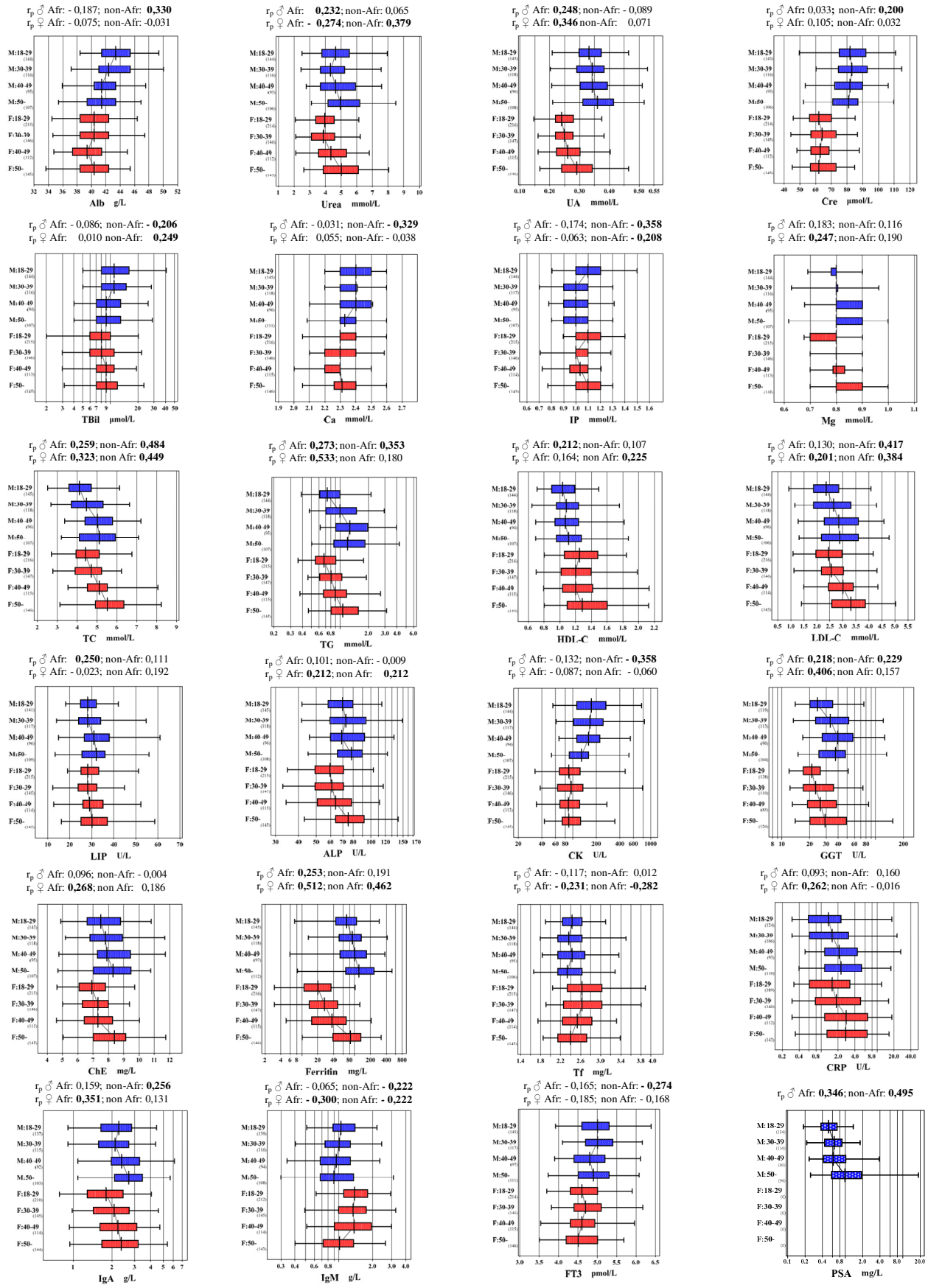

Figure 1: Age – related changes in 24 analytes; $r_p \geq |0.200|$ considered as significant

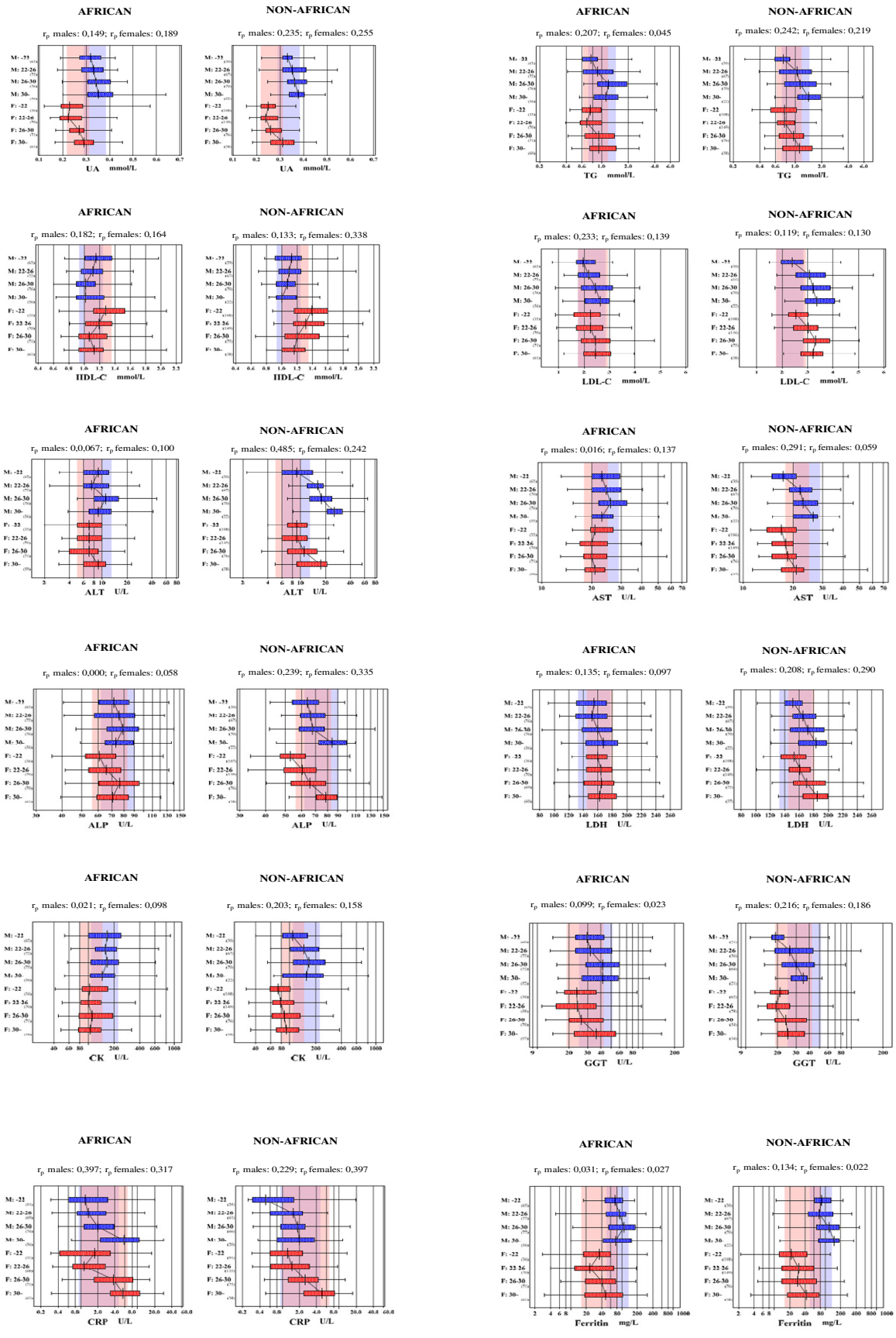

Figure 2: BMI related changeds of 12 analytes stratified according to gender and ethnicity

MANUSCRIPT THREE

White cell differential and platelet count reference ranges obtained from a healthy urban South African population residing in the Western Cape of South Africa.

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Short Title: Haematological reference ranges in the Western Cape, South Africa

Key Words: White cells, differential counts, Africans, Mixed Ancestry, Caucasian

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Disclosure summary

There is no conflict of interest to declare with respect to this manuscript. The results in this manuscript have not been previously published in whole or part.

Publication details: Pending acceptance in International Journal of Clinical Haematology

Abstract

Background

Research has suggested that compared to Europeans, individuals of African descent have lower white cell and neutrophil counts. These differences could lead to incorrect clinical decisions and therefore ethnic specific reference ranges are required. The Western Cape region of South Africa is uniquely diverse, comprising of Caucasian, Mixed Ancestry and those of African descent. The aim of this study was to compare the white cell, differential and platelet counts across the three major ethnic groups residing in this area and to propose appropriate RI's.

Methods

This study formed part of the project led by the Committee on Reference Intervals and Decision Limits (C-RIDL) and the strict guidelines laid out by the committee were followed. Full blood count and differential counts were performed on a Beckman Coulter ACT 5 diff AL analyser within 2-4 hours of collection and were reported as mean (standard deviation), 2.5th and 97.5th percentiles. Comparisons were analysed using SPSS v25 and Statistica v.13 and a p value of <0.05 was considered significant.

Results

Reference ranges for Caucasian and Mixed Ancestry individuals were similar while white cell ($p = 0.016$), monocyte ($p < 0.001$) and neutrophil ($p = 0.034$) RI's were significantly lower in African participants. There were however no clinically significant differences between the eosinophil, basophil, lymphocyte and platelet counts across the three population groups.

Conclusion

In conclusion, subjects of mixed ancestry in this region have similar reference intervals to those of European descent, while lower white cell and neutrophil counts in Africans have been confirmed.

1. Introduction

A reference interval is defined as the variation of a measurement in normal healthy individuals (Ichihara, *et al.*, 2008), (Ichihara, Itoh, *et al.*, 2008), (Ichihara, *et al.,* 2013). These intervals are important as they are used by health care professionals to interpret laboratory results and make important clinical decisions such as the cessation or the initiation of therapy. Establishing a representative reference interval (RI) has several challenges as many factors can influence the value being measured. These include sex, race, age, altitude, endemic diseases and the environment (Ichihara, *et al.*, 2008), (Ichihara, Itoh, *et al.*, 2008), (Ichihara, *et al.,* 2013). It is therefore well established that a good local reference interval (RIs) should take all the above factors into account (Aytekin & Emerk, 2008).

Most African countries have not generated local RIs and use ranges established in Europe, the United Kingdom (UK) or the United States of America (USA). Previous studies have however suggested that individuals of African descent have lower white cell, neutrophil and platelet counts in comparison to Caucasians (Bain, 1996). These results have been supported by local South African studies in which Africans were reported to have lower neutrophils counts and higher lymphocyte counts (Lawrie, *et al.,* 2009). These differences could potentially lead to misinterpretation, incorrect clinical decisions and the wastage of limited health care resources. Furthermore, it has also been documented that the lower white cell and neutrophil counts in Africans have resulted in the exclusion of these population groups from international clinical oncology trials which require a neutrophil count of $>1.5\ x10^9/l$ (Hsieh, *et al.,* 2010). It has therefore been suggested that ethnic-specific reference ranges should be established.

Some research groups from Africa and Southern Africa have attempted to generate normal ranges for haematological values (Buchanan, *et al.,* 2013), (Kueviako, *et al.,* 2010). However, these have largely concentrated on the black African populations and have often not followed the strict criteria proposed by the Clinical and Laboratory Standard Institutes (CLSI, 2011). The Western Cape province of South Africa has a unique and diverse population consisting of Black Africans, Caucasians and individuals of Mixed Ancestry and therefore a study examining the differences in the haematologic values of these groups is important. The aim of this project was to analyse and compare the white cell, platelet and differential count

results from a cohort of normal South Africans representing the three major population groups residing in the Western Cape and attempt to propose RIs for this region of Southern Africa.

2. Material and methods

This report forms part of the international multi-centre project led by the Committee on Reference Intervals and Decision Limits (C-RIDL) of the International Federation of Clinical Chemistry (IFCC). Other collaborating countries include the USA, Turkey, Japan, the UK, China, India, Saudi Arabia, Argentina, Russia, the Philippines, Nepal, Pakistan, Kenya, Nigeria, and Bangladesh. The study also provided an opportunity to investigate the controversies over the need for secondary exclusion of individuals by use of the latent abnormal values exclusion (LAVE) method (Ichihara, 2013) and the use of parametric (P) and nonparametric methods (NP) for derivation of RIs. The study design was adopted from the common study protocol proposed by C-RIDL (Ozarda, *et al.,* 2013) and is briefly described below.

2.1 Ethical considerations

Ethical approval was obtained from the Faculty of Health and Wellness Sciences' Research and Ethical Committee of the Cape Peninsula University of Technology, Reference Number: CPUT/NHREC: REC-230408-014 as well as the University of Stellenbosch Health Research Ethics Committee 1 protocol number: S12/05/147. Written informed consent was obtained from each volunteer.

2.2 Study population and recruitment

Suitable participants were selected according to the criteria described below and were recruited from the healthy South African population. All participants had to be South African born citizens regardless of their ethnic origin. Those originally born in Namibia, but live in South Africa were also allowed to participate. The study was conducted between October 2012 and February 2015. For the purpose of this study, blood was collected from African (Afr), Caucasian (Cau) and Mixed Ancestry (MA) males and females respectively.

2.3 Inclusion and exclusion criteria

All participants were required to fill in a health questionnaire which had been adapted from previous studies (Ichihara, *et al.,* 2013). This was used to obtain information on body mass index (BMI), ABO blood type, alcohol consumption, smoking habits, exercise, recent episodes of infection or allergy and menstrual status. All healthy individuals between the ages of 18 and 65 years were included while participants over the age of 65 were used to analyse sources of variation in the results. Ideally each participant should not be on any medication, however, according to international protocol, volunteers using up to 3 medications could be included provided they did not suffer from major illness as described below.

Participants were excluded if they suffered from any of the following conditions: 1) diabetes; 2) chronic liver or kidney disease; 3) had blood results which clearly indicated the presence of a severe disease; 4) had been hospitalised or been seriously ill during the previous 4 weeks; 5) donated blood in the previous 3 months; 6) were known carriers of hepatitis B virus (HBV), hepatitis C virus (HCV) or HIV 7) were pregnant or within one year after childbirth or 8) had participated in a clinical trial in the past 12 weeks. The items for the health-status questionnaire were adapted from the one used in the previous Asian project (Ichihara, *et al.,* 2013), but modified to suit the South African context.

2.4 Participant anthropometric measurements

All participants had the following anthropometric measurements performed prior to sample collection. These included weight (Wt; kg), height (Ht; m), waist circumference (WC; cm), and blood pressure (systolic blood pressure: SBP; diastolic blood pressure: DBP). All measurements were performed according to World Health Organisation (WHO) guidelines. The body mass index (BMI) was calculated as Wt/Ht^2 (kg/m^2) and those with a BMI of $>30kg/m^2$ were excluded.

2.5 Sample collection, transportation and analysis

Participants were requested to avoid excessive eating, alcohol consumption and excessive physical exertion for 3 days, prior to sampling. Furthermore, participants were requested to fast for 10 – 12 hours. Thirty-one mL of blood was drawn into three 9.0 mL SST III Vacutainer tubes with a clot activator and gel and one 4.0 mL K_2E EDTA sample for haematological analyses.

Full blood count (FBC) samples collected at each local site in the Cape Town region, were transported within 2 to 4 hours to an ISO 15189 accredited laboratory (PathCare Laboratories in Goodwood, Cape Town) and stored in a cooler at 2 –8 ºC. The following haematological parameters were analysed using a Beckman Coulter ACT 5 diff AL analyser: These included a white cell count, platelet count, and a full 5-part differential count.

The AC•T 5diff AL analyser is a fully-automated haematology analyser which generates a full blood count and White blood cell (WBC) five-part differential. This is determined simultaneously using Absorbance Cytochemistry, Volume (ACV) Technology and WBC/basophilic (BASO) methodology. The ACV technology is able to identify lymphocytes (LYMPH), monocytes (MONO), neutrophils (NEU), eosinophils (EOS), immature cells, and atypical lymphocytes while the WBC/BASO methodology makes use of differential lysis and impedance technology to generate a WBC count, basophil (BASO) percentage, and absolute count. The Coulter impedance principle is used to analyse the final red blood cell (RBC) and platelet (PLT) counts. (Beckman Coulter, 2017)

2.6 Statistical analysis

Statistical analysis was performed using SPSS v25 (IBM corp, Armonk, New York, USA) and Statistica v.13 (TIBCO Software Inc. (2017). The results were reported as mean (standard deviation), 2.5th and 97.5th percentiles and percentages. For comparison, the analysis of variation (ANOVA) was used and a p-value of <0.05 indicated statistical significance.

3. Results

3.1 Population characteristics

Blood was analysed from 711 participants of which 248 (34%) were male. Four hundred and twenty-seven of the participants were Caucasian (141 male), 65 were African (30 male) and 219 were of mixed ancestry (77 male). There were no significant differences in the anthropometric measurements, including fasting blood glucose, between the three ethnic groups, however the mean age of the African participants was significantly lower ($p = 0.014$) as seen in **Table 1**.

3.2 White cells, differential counts and platelets.

The white cell count and differential count parameters were similar between males and females, and although there was a gender difference between the platelet counts of Caucasians (p=0.04), this was not clinically significant.

Overall, Africans had lower white cell counts compared to Caucasian and Mixed Ancestry participants (p = 0.016). The monocyte percentage and absolute counts were significantly lower in both African males and females (p = <0.001), and although the percentage of lymphocytes was higher amongst Africans (p = 0.033) the absolute counts were similar. Absolute neutrophil counts were significantly lower in both male and female Africans, compared to Caucasian and Mixed Ancestry participants (p = 0.034) as seen **Table 2**.

3.3 Reference Intervals

The above results indicated that although Caucasian and Mixed Ancestry individuals have similar reference intervals, the results for white cells, neutrophils and monocytes were significantly lower in those of African descent and therefore separate reference ranges for these parameters should be generated. These ranges are reflected in **Table 2.**

There were however no clinically significant differences between the eosinophil, basophil, lymphocyte and platelet counts across the three population groups and therefore the following common reference ranges for these parameters are proposed as summarised in **Table 3**.

4. Discussion

The main aim of this project was to compare the platelet, white cell and differential reference intervals between the 3 main population groups residing in the Western Cape, South Africa. The results have demonstrated that although the reference ranges of Caucasian and Mixed Ancestry individuals are similar, the ranges for white cells, monocytes and neutrophils were significantly different in African participants.

Neutrophils are part of the innate immune system and reduced numbers can cause increased bacterial infections and septicaemia. Normal reference intervals for neutrophils in the USA are reported as 1.8 x 10^9 to 7.7 x 10^9/L (Kratz, *et al.,* 2004) but can vary depending on the region. An absolute count of less than 1.5 x10^9/L is considered as neutropaenic, with an increased risk of infection. Consequently, individuals who have neutropaenia are excluded from clinical trials which include drugs known to cause decreases in white cells and neutrophils (Hsieh, *et al.,* 2010).

Benign ethnic neutropaenia (BEN) is a term used to describe individuals of African descent who have low neutrophil counts but with no risk of infection (Hsieh, *et al.,* 2010). Originally, it was hypothesized that this phenomenon was as the result of increased neutrophils being marginated to the vascular endothelium. Recent research has however revealed that the reduced neutrophil count is probably due to the Duffy Null polymorphism (SNP rs2814778) frequently seen in Africans and which is known to protect against infection by *Plasmodium vivax* (Reich, *et al.,* 2009). The results of our study demonstrated significantly lower white cell and neutrophil counts in Africans, with neutrophil counts being as low as 1.05 x 10^9/l. These results support others who have reported similar reference ranges in healthy African individuals (Bain, 1996), (Lawrie, *et al.,* 2009), (Kueviako, *et al.,* 2010), (Zeh, *et al.,* 2011), (Taha, *et al.,* 2018). These reports have all proposed separate reference ranges for Africans, which is important, as they could influence clinical decision making, especially in the field of oncology and the initiation chemotherapy.

Furthermore, we have also demonstrated that the Mixed Ancestry population of the Western Cape share similar reference intervals to those of Caucasian descent. The Mixed Ancestry people of Cape Town are a unique population group comprising of the original indigenous Khoisan people (32-43%), Bantu speaking Africans (20-36%), Europeans (21-28%) and Asians (9-11%) (Statistics South Africa, 2011). According to the last census, this group make up 48% of the Western Cape population (Lim, *et al.,* 2015).

Although previous studies have reported similar findings in Hispanics who are also of mixed ancestry (Lim, *et al.,* 2015), studies in Southern Africa have usually combined this group with those of Caucasian descent (Lawrie *et al.*2009). This study therefore adds

valuable information for this region and could be significant for other mixed ancestry populations in Africa.

The results of this current study also support previous reports (Bain, 1996), (Lawrie, *et al.,* 2009) which observed monocyte counts as being lower in Black African participants. This however is in contrast to other African studies which have reported higher monocyte, eosinophil and basophil counts (Lugada, *et al.,* 2004). It has been hypothesized that this could be the result of elevated parasitic and inflammatory conditions which are common in these regions. Our study followed strict inclusion and exclusion criteria as recommended by the Committee on Reference Intervals and Decision Limits (C-RIDL) and therefore individuals with clinically significant pathologies were excluded. This therefore would explain the differing results.

As previously described (Bain, 1996), our study also observed higher platelet counts amongst Caucasian females compared to their male counterparts ($p = 0.04$). This gender difference was not observed in the Black African or Mixed Ancestry groups and as there were no clinically statistical differences across the different ethnic groups, the following reference interval for males (139.2-424.4 $x10^9$/l) and females (150.8-417.6 x 10^9/l), which are similar to international RIs, are proposed.

The major strength of this study, which has been different from previous ones in this region, is that the mixed ancestry participants were separated from those of Caucasian descent rather than grouping these two important population groups together. In addition, the strict guidelines of the Committee on Reference Intervals and Decision Limits (C-RIDL) were followed ensuring that only healthy individuals were included. This ensures that the results can be compared to other countries participating in the international multi centre project. A limitation of this research was however the lower number of African volunteers in comparison to Caucasians. Future research should aim to include higher numbers of African volunteers and strive to generate reference ranges for adolescents and those younger than 18 years.

Africa has many diverse population groups and it has been recommended that reference intervals be generated for each region on the continent. In this study, we have confirmed the lower white cell and neutrophil counts in the Xhosa speaking African population of the Western

Cape and have recommended a separate reference interval for this ethnic group. This is important as it would reduce the number of individuals falsely diagnosed with neutropaenia and who are consequently excluded from clinical trials and receiving chemotherapy. This article has also concluded that the reference intervals of Mixed Ancestry individuals residing in the Western Cape are similar to those of European descent.

The demographics of South Africa, Africa and the world are constantly changing and therefore Haematology laboratory professionals should be constantly aware of the influence sex and ethnicity has on the interpretation laboratory results.

5. Aknowledgements

The authors would like to thank the following collaborators who contributed to the local study in South Africa:

1) Beckman Coulter South Africa for the technical advise and support throughout the project and for the sponsorship of the reagents, quality control material and calibrators for this project.
2) PathCare Laboratories, Dr John Douglas, the administration- and nursing staff of PathCare, Louis Leipoldt Hospital for assisting the investigators at each clinic, the laboratory staff at PathCare Louis Leipoldt as well as PathCare Parl, N1 City.
3) Arno Theron and colleugues at PathCare Quality Assurance Department for particpant sample splitting, storage and distribution to the relevant departments within PathCare for analysis.

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Table 1: Participant characteristics

Parameter				**P value: ethnicity**
	African Mean (SD) range	Mixed ancestry Mean (SD) range	Caucasian Mean (SD) range	
Age (years)	27(9) 18-52	31(11) 18-56	31 (12) 19-59	P = 0.014
Waist circumference (cm)	83.2 (9.6) 65-106	84.3 (13.3) 65-115	86.2 (11.1) 67-105	P = 0.073
Body mass index (kg/m^2)	24.9 (3.9) 18.2-33.8	24.3 (3.7) 18.3-31.8	23.7 (3.2) 18.6-30.2	P = 0.152
Systolic Blood Pressure (mmHg)	115 (15) 93-161	119 (16) 93-156	117 (15) 95-148	P = 0.134
Diastolic Blood Pressure (mmHg)	77(11) 52-95	79 (11) 56-102	77 (10) 55-96	P = 0.100
Fasting Blood Glucose (mmol/L)	4.4 (0.4) 3.6-5.2	4.4 (0.5) 3.7-5.4	4.5 (0.4) 3.7-5.5	P = 0.079

SD: Standard Deviation; LL: Lower Limit; UL: Upper Limit

Table 2: White cells, platelets and differential count reference intervals

Parameter	Males			Females			P value: ethnicity
	African *Mean (SD)* *range*	*Mixed ancestry* *Mean (SD)* *range*	*Caucasian* *Mean (SD)* *range*	*African* *Mean (SD)* *range*	*Mixed Ancestry* *Mean (SD)* *range*	*Caucasian* *Mean (SD)* *range*	
White cells ($x10^9$/l)	5.32 (1.41) 3.0-9.1	5.8 (1.35) 3.7-9.39	6.23(1.9) 3.57-12.21	5.68 (1.68) 2.28-9.19	6.24 (1.91) 3.5-10.93	6.08 (1.7) 3.3-9.88	**P = 0.016**
Neutrophils %	54.3 (9.36) 32.95-73.66	55.7 (8.19) 37.89-73.58	55.95 (10.4) 30.69-78.1	54.02 (9.53) 32.95-73.66	55.97 (7.88) 40.04-70.8	55.25 (8.72) 36.78-72.08	P = 0.371
Neutrophils $x10^9$/l	2.95 (1.12) 1.1-5.49	3.29 (1.14) 1.51-6.35	3.59 (1.63) 1.5-8.49	3.13 (1.24) 1.05-6.11	3.56 (1.45) 1.58-7.49	3.42(1.26) 1.54-6.65	**P = 0.034**
Lymphocytes %	37.81 (8) 22.7-55	35.05 (7.38) 20.09-50.56	34.87 (9.67) 14.09-59.41	37.4 (8.55) 20.76-58.17	34.56 (6.93) 19.97-50.5	35.43 (7.94) 20.98-52.54	**P = 0.033**
Lymphocytes $x10^9$/l	1.95 (0.44) 1.09-3.26	1.97 (0.42) 1.27-3.11	2.07 (0.57) 1.14-3.6	2.07 (0.62) 0.98-3.59	2.09 (0.56) 1.19-3.38	2.09 (0.55) 1.23-3.41	P = 0.521
Monocytes%	4.23 (1.76) 1.8-8.6	5.56 (0.49) 2.55-8.81	5.55(1.7) 2.47-9.84	4.87 (2.05) 1.56-9.8	5.47 (1.96) 2.29-10.57	5.25 (1.71) 2.56-9.1	**P < 0.001**
Monocytes $x10^9$/l	0.22 (0.08) 0.06-0.39	0.32 (0.1) 0.17-0.64	0.34 (0.15) 0.15-0.79	0.27 (0.11) 0.06-0.49	0.33 (0.13) 0.14-0.69	0.32 (0.14) 0.11-0.67	**P < 0.001**
Eosinophils%	3.22 (2.44) 0.4-13.8	3.09 (1.59) 1.1-7.31	3.06 (1.95) 0.87-7.47	3.18 (2.31) 0.92-11.5	3.39 (2.11) 1.09-9.31	3.44 (2.52) 1.0-10.18	P = 0.986
Eosinophils x 10^9/l	0.17 (0.14) 0.02-0.67	0.18 (0.1) 0.05-0.43	0.19(0.11) 0.04-0.47	0.17 (0.12) 0.04-0.57	0.21 (0.13) 0.07-0.56	0.21 (0.18) 0.05-0.71	P = 0.337
Basophils%	0.44 (0.16) 0.2-0.7	0.56 (0.11) 0.4-0.76	0.57 (0.14) 0.4-0.8	0.52 (0.1) 0.4-0.69	0.61 (0.14) 0.4-0.8	0.63 (0.21) 0.3-1.02	NS
Basophils x 10^9/l	0.02 (0.01) 0.01-0.05	0.03 (0.01) 0.02-0.06	0.04(0.02) 0.02-0.08	0.03 (0.01) 0.01-0.06	0.04 (0.02) 0.02-0.09	0.04 (0.02) 0.01-0.09	NS
Platelets x 10^9/l	263.62 (71.47) 133.0-428.0	273.0 (64.73) 178.45-454.1	284.36 (68.79) 137.33-423.75	261.2 (53.16) 161.1-411.05	270.7 (66.23) 139.9-420.6	267.15 (65.9) 155.0-415.8	P = 0.296

SD: standard deviation, NS: not significant, Range: 25percentile to 97.5 percentile

Table 3: Reference intervals common to all ethnic groups.

Parameter	**Overall**		**Males**		**Females**	
	Mean (SD)	*Range*	*Mean (SD)*	*Range*	*Mean (SD)*	*Range*
Lymphocytes x10^9/l	2.06 (0.54)	1.18-3.37	2.02 (0.5)	1.18-3.4	2.09(0.56)	1.17-3.36
Eosinophils%	3.29(2.23)	1.00-8.75	3.10(1.94)	0.96-7.52	3.39(2.36)	1.00-9.32
Eosinophils x 10^9/l	0.2(0.14)	0.05-0.55	0.18(0.11)	0.05-0.49	0.20(0.16)	0.05-0.59
Basophils%	0.59(0.17)	0.30-0.90	0.54(0.14)	0.30-0.80	0.61(0.18)	0.30-1.00
Basophils x 10^9/l	0.04(0.18)	0.01-0.08	0.03(0.01)	0.01-0.07	0.04(0.02)	0.01-0.08
Platelets x 10^9/l	270.81(65.82)	150.38-418.88	277.06(68.16)	139.20-427.40	267.55(64.42)	150.80-417.60

Range: 25 percentile to 97.5 percentile; SD: standard deviation

MANUSCRIPT FOUR

Reference interval determination for glycated albumin in a South African population

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Running title: Glycated albumin and reference intervals

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Keywords: glycated albumin, glycated proteins, reference interval, diabetes mellitus, prediabetes

Abbreviations: (HbA1c) glycated hemoglobin; (GA%) glycated albumin; (BMI) body mass index; (OGTT) oral glucose tolerance test; (ADA) American Diabetes Association; (TSH) Thyroid stimulating hormone; (MDRD) Modification of Diet in Renal Disease; (GGT) γ-glutamyl transferase; (ALT) alanine aminotransferase**.**

Manuscript publication details: Under final review for publication in Annals of Clinical Biochemistry (preliminary accepted)

Abstract

Background

Glycated proteins, such as glycated haemoglobin (HbA1c) and glycated albumin (GA%), are increasingly being used for the assessment of glycaemic control and the diagnosis of diabetes mellitus. GA% is an intermediate marker of glycaemic control that is not influenced by factors that affect HbA1c levels and may be a better marker in these situations.

Methods

We measured GA% using an enzymatic method on stored serum samples of healthy individuals who were recruited in Cape Town, South Africa. An overall reference interval and specific reference intervals for age, sex, ethnicity and body mass index (BMI) were determined using non-parametric methods.

Results

The reference interval (2.5^{th} to 97.5^{th} percentile) for GA% of 663 healthy individuals (mean age, 34 years, 38.6% males) ranged from 10.7% to 15.2%. The median GA% in females was higher than in males ($p < 0.0001$). Females had a reference interval of 11.0% to 15.5%, whereas it was 10.6% to 14.4% in males. The median GA% for Caucasians and Mixed-Ancestry subjects were both 12.9%, whereas Black subjects had a higher median GA% of 13.3% ($p = 0.0025$). As an inverse relationship was observed between BMI and GA%, we determined reference intervals in obese, overweight and normal weight subjects. The GA% reference intervals for subjects with BMI <25 kg/m^2 was 11.2% to 15.3%, for BMI 25-30 kg/m^2 it was 10.5% to 14.9% and 10.0% to 14.6% for BMI >30 kg/m^2 ($p = 0.0001$).

Conclusions

The overall reference interval showed good correlation with reference intervals determined in other studies and we have for the first time determined these values in obese and non-obese healthy South Africans.

1. Introduction

Diabetes mellitus is a worldwide pandemic. According to recent estimations 424.9 million people between 20 and 79 years of age are living with diabetes and this number is expected to rise to 628.6 million by the year 2045 (IDF, 2017). This pandemic place an immense burden on economies and health care systems worldwide. Globally it is estimated that up to half of patients with diabetes remain undiagnosed (IDF, 2017). In Africa, approximately 77% all diabetes related deaths occurred in patients below 60 years of age (IDF, 2017). This high prevalence may be the consequence of diabetes complications due to late diagnosis and poor control (Hall, *et al.,* 2017).

Most diagnostic criteria for diabetes utilize glucose-based parameters, which are subject to wide intra-individual variability, are inconvenient for the patient as a fasting sample is required, and are also influenced by other factors such as stress, medication and concurrent illness (Sack, *et al.,* 2015). The oral glucose tolerance test (OGTT) is a cumbersome procedure and must be performed twice to confirm the diagnosis of diabetes. The OGTT has many prerequisites and is performed after a 10 to 16 hours fasting period and must be performed between 7am and 9am (Sacks, *et al.,* 2015). Ideally, the focus for diabetes diagnosis should shift to non-fasting parameters, including glycated proteins. Glycation is pathological non-enzymatic protein modification due to exposure to glucose and levels increase in diabetes (Taniguchi, *et al.,*2016). HbA1c is a glycated protein (haemoglobin) and was recommended by the American Diabetes Association (ADA) as one of the diagnostic criteria of diabetes in 2009 (Florkowski, 2013). Using HbA1c for the diagnosis and follow-up of diabetes has limitations in patients with conditions that affect red blood cell turnover, such as haemoglobinopathies, iron deficiency and haemolytic anaemias (English, *et al.,* 2015). HbA1c also differs in various racial groups and numerous studies have found that the recommended cut-off of 6.5% may not be useful in some populations (Hird, *et al,.*2016), (Zemlin, *et al.,* 2011), (Oueslati, *et al.,*2017), (Liu, *et al.,*2016), (Ding, *et al.,* 2018).

Fructosamine is another intermediate marker and refers to ketoamines formed during non-enzymatic glycation of serum proteins, including albumin, immunoglobulins and lipoproteins (Selvin, *et al.,* 2015). Ketoamines are stable products that form after the attachment of glucose derivatives to the free amine groups of all proteins (Dinu & Moţa, 2014).

Albumin is the most abundant serum protein and is more rapidly glycated than HbA1c, as glucose does not need to enter the intracellular space Roohk & Zaidi, 2008). Glycated albumin (GA%) is the main fructosamine, constituting 80% of total glycated serum proteins. It is more specific than fructosamine, as it is not influenced by other serum proteins which have variable half-lives (Freitas, Ehlert & Camargo 2017). GA% is the glycation product of albumin and an intermediate marker of glycemic control which allows for quick treatment follow-up in diabetics, as albumin has a half-life of 2-3 weeks (Lee, *et al.,*2011*).* Additionally, GA% is not influenced by many of the factors that affect HbA1c levels and may be an ideal marker of glycaemic control in these situations (Freitas, Ehlert & Camargo 2017).

Reference intervals for GA% have been determined in a few populations (Selvin *et al.,* 2018), (Testa, *et al.,* 2017), (Zhou & Shi, 2017), (Kohzuma, *et al.,* 2011), (Furusyo, *et al.,*2011), (Paroni, *et al.,* 2007), but to our knowledge, this is the first study determining GA% reference intervals in South Africa. It is recommended that each laboratory determine their own reference intervals, as geographical area and diet can influence the values (quantILab, 2016). The aim of this study was to determine reference intervals for GA% using an enzymatic method in a well-characterized healthy population.

2. Methods

2.1 Study population

The study subjects were identified from a database of the "Establishing Adult Reference Intervals for Selected Analytes in South Africa" study. The original database consists of 1436 subjects, but only subjects who were recruited in the Cape Town area (853 subjects) had fasting plasma glucose performed and were therefore included in this study. The protocol for the multi - centre reference interval study has been published elsewhere (Ozarda, *et al.,* 2013). Written informed consent was obtained from all subjects during the recruitment period. The Health Research Ethics Committee of Stellenbosch University, Cape Town (South Africa) approved this study (S12/05/147). This study was performed according to the Declaration of Helsinki.

Study subjects were excluded according to the exclusion criteria described elsewhere (Phatlhane, *et al.,* 2016). Additional exclusion criteria for the current study included missing

data for GA% (insufficient samples) and various conditions that could potentially affect albumin metabolism or levels: fasting plasma glucose > 126mg/dL (7 mmol/L) (excluding subjects with diabetes), thyroid stimulating hormone (TSH) < 0.37 mIU/L and >3.5 mIU/L (to exclude thyroid pathology), estimated glomerular filtration rate (eGFR) according to Modification of Diet in Renal Disease (MDRD) <60 mL/min/1.73m^2 (to exclude renal disease), bilirubin > 2.34 mg/dL (40 µmol/L), γ-glutamyl transferase (GGT) >121 U/L, alanine aminotransferase (ALT) > 100 U/L (to exclude liver disease) and subjects on medication that could affect albumin metabolism. There were no subjects with an albumin below 3,0 g/dL (30 g/L). Patients on oral contraceptive medication, oestrogen or thyroxine (with TSH within the reference interval) were not excluded according to the guidelines set by Ozarda *et. al*. in the multicentre reference interval study (Ozarda, *et al.,* 2013).

2.2 Biochemical analysis

GA% analysis was performed on stored serum samples that were collected between 2012 and 2015. The de-identified patient serum aliquots were immediately stored at -80 degrees Celsius after centrifugation. Nathan *et. al.* reported that GA% is stable for up to 23 years in serum samples stored at -80 degree Celsius (Nathan, *et al.,*2011). GA% analysis was performed with an enzymatic assay from Werfen (quantILab Glycated Albumin, Milan, Italy) on the Roche Cobas 6000 analyzer (Roche Diagnostics, Mannheim, Germany). GA% is expressed as a percentage of glycated albumin (g/L) to albumin (g/L). Therefore, glycated albumin and albumin is determined separately using different reagents for each analyte. Glycated albumin (g/L) is determined after elimination of endogenous glycated proteins by ketoamine oxidase (KAOD) (quantILab 2016). The steps of glycated albumin (g/L) determination are shown in the following reaction sequence (quantILab, 2016), where
KAOD = ketoamine oxidase, POD = peroxidase (quantILab, 2016):

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$$Glycated\ Amino\ Acids \xrightarrow{KAOD} Glucosone + Amino\ Acids + H_2O_2$$

$$Glycated\ Albumin \xrightarrow{Protease} Glycated\ Amino\ Acids$$

$$Glycated\ Amino\ Acids \xrightarrow{KAOD} Glucosone + Amino\ Acids + H_2O_2$$

$$H_2O_2 + 4 - AA + TODB \xrightarrow{POD} Bluish - purple\ dye + H_2O$$

Albumin was determined with the bromocresol purple method. The GA% calculation includes an inter-method arithmetic factor, which makes the results comparable to GA% results determined by high performance liquid chromatography (HPLC) (quantILab, 2016).

$$GA\% = 2.9 + 87.719 \left(\frac{Glycated\ Albumin\ (g/L)}{Albumin\ (g/L)} \right)$$

Multiple chemistry analytes including fasting plasma glucose, TSH, serum creatinine, and GGT, ALT and total bilirubin were routinely measured in an ISO 15189 pathology laboratory (PathCare Laboratories, Cape Town, South Africa).

2.3 Validation study

The enzymatic glycated albumin assay (Werfen) was validated for use on a Cobas 6000 analyzer (Roche) according to the Clinical Laboratory Standards Institute's (CLSI) guidelines (EP15-A3) (CLSI, 2014). The accuracy and precision were determined by analyzing high and low control samples that were provided by the assay manufacturer (Werfen).

2.4 Statistical analysis

Demographic and laboratory data were merged and analyzed using Stata 15 (College Station, Texas, USA) and NCCS 12 statistical software (Kaysville, Utah, USA). Non-parametric statistical methods were used to determine the 95% reference limits, with the lower limit defined as the 2.5^{th} percentile and the upper limit as the 97.5^{th} percentile (NCCLS, 2000). A bootstrapping method (3000 samples) was used to improve precision and accuracy of reference limits. This resampling procedure also served for predicting 95% confidence intervals (CI) for the limits of the reference interval. Our underlying assumption was that the 2.5% and 97.5% centiles contain 95% of the distribution of normal values of the reference population. Mann-Whitney U tests were used to analyze differences by gender and Kruskal Wallis for categories of age and body mass index (BMI). A two-sided p-value < 0.05 was considered statistically significant.

2.5 Sources of variation and multivariable analysis

The data were first analyzed to establish the partitioning requirements of the four covariates, namely gender, age, ethnicity and BMI. The data were then subjected to tests for

determining its suitability for parametric treatment by first transforming the GA% into normal using the Box-Cox transformation. Dummy variables of gender, age groups, ethnicity and BMI categories were created and included in the linear regression model. The relative significance of each independent variable was predicted from the significance level of the regression coefficient. Independent variables were considered of practical importance when its standardized Beta regression coefficient, (which correspond to the partial correlation coefficient ($|r_p|$)) was greater than 0.15. A standardized beta coefficient compares the strength of the effect of each individual independent variable to the dependent variable (GA%). The higher the absolute value of the beta coefficient, the stronger the effect. Standardized beta coefficients have standard deviations as their units coefficients and have the advantage of being comparable from one independent variable to the other because the unit of measurement has been eliminated.

3. Results

3.1 Method validation study

The within-assay CV for GA% was 2.2% at a target mean of 15.7% and 1.3% at a target mean of 37.4%. The within-laboratory CV was 2.3% at a target mean of 15.7% and 1.4% at a target mean of 37.4%. The total error at both control levels were below the desirable total allowable error of 6.09% that were determined by Montagnana *et. al.* (Montagnana, *et al.,* 2013).

The manufacturer's claims for within-assay CV range between 0.84 and 1.05% and the within-laboratory CV between 1.08 to 1.51%. We did not meet the desirable goals of Montagnana *et. al.* (1.03%) or the manufacturer's claims (1.08-1.51%) for the within-laboratory CV, but our CV's were in keeping with the CV's reported in a recent publication by Selvin *et al.* using the Lucica GA-L (Asahi Kasei Pharma) glycated albumin assay. They reported CV's of 2.1% at a mean GA% of 22.7% and 1.8% at a mean GA% of 56% (Selvin, *et al., 2018).*

3.2 Demographics and characteristics of subjects

The characteristics of the subjects are described in **Table 1**. Of the 663 adults included in the study, 427 (64.5%) were in the age category 18-38 years. The mean age of the study subjects was 33.9 years (SD=13.7). There were 407 (61.4%) females and 256 (38.6%) males. More than 50% of the subjects were Caucasian, 31.1% were Mixed-Ancestry and 11.6% were Africans. Only 12% of the participants had BMI's greater than 30 kg/m^2 and 53.5% were in the BMI category of <25 kg/m^2.

3.3 Reference intervals

The overall reference interval for 663 subjects (after exclusions) was 10.7% to 15.2%. Females had a slightly higher median GA% of 13.2% (95% CI 13.1-13.3) compared to 12.4% (95% CI 12.3-12.6) for males ($p < 0.0001$). The median GA% for Black Africans was 13.3% and is higher than the medians for Caucasians and Mixed-Ancestry subjects, which were 12.9% for both these groups ($p = 0.0025$). A statistically significant inverse correlation was demonstrated between GA% and BMI ($p = 0.0001$).

3.4 Sources of variation and multivariable analysis

Multiple linear regression was performed to explore the importance of gender, BMI, age and ethnicity as sources of variations as well as adjusting for confounding. Gender, BMI categories and ethnicity were significantly associated with the GA% in all the models as shown in the **Table 3**. Gender, BMI and ethnicity were considered of practical importance because their standardized regression coefficients (Beta) were greater than a cut-off of 0.15, implying a stronger effect on GA% ($p < 0.001$). From the magnitude of standardized beta coefficients, age-related changes were not associated with GA% and was considered of lesser importance.

4. Discussion

This is the first study on GA% reference interval determination conducted in an African population and included subjects from South Africa. We found that the overall reference intervals for this population were 10.7% (95% CI 10.5 - 10.9) to 15.2% (95% CI 15.0 - 15.3). Importantly we found that in this population the reference intervals were significantly higher in females ($p < 0.0001$) and differed according to BMI. The GA% reference intervals for

subjects with BMI <25 kg/m^2 was 11.2% to 15.3%, for BMI 25-30 kg/m^2 it was 10.5% to 14.9% and 10.0% to 14.6% for BMI >30 kg/m^2 (p = 0.0001). Furthermore, the GA% levels were significantly higher in Black Africans (13.3%) compared to Caucasians and Mixed-Ancestry subjects, both 12.9%, p=0.0025.

The reference interval for GA% according to the Werfen package insert is 9% to 16% and is based on a preliminary reference interval study performed by Testa *et. al.* in 2017. The reference interval determined in this study differs from those reported in several studies (Paroni, *et al.,*2007), (quantILab, 2016), (Ozarda, *et al.,* 2013), (Phatlhane, *et al.,* 2016), (Nathan, *et al.,* 2011), (CLSI, 2014) but is in keeping with a recent publication by Selvin *et. al.* in 2018 that demonstrated a reference interval of 10.7% to 15.1% in middle-aged adults (between 47 and 68 years, mean age 55.3 years) with an equal sex distribution and 15% Black Americans (Selvin, *et al.,* 2018). Important differences between the Selvin *et. al.* study and our study is that we included subjects of a wider age range (between 18 and 76 years, mean age 33.9 years) and also included subjects of Mixed-Ancestry (31.1%). The origin of Mixed-Ancestry individuals in South Africa comprises Khoisan, European, African and Indian origins (De Wit, *el al.,* 2010). The Mixed-Ancestry community of Cape Town was shown to have a high frequency of undiagnosed diabetes and the prevalence of diabetes and metabolic syndrome is increasing (Erasmus, *et al.,* 2012).

Another similarity between our study and that of Selvin *et. al*. is the gender-specific reference intervals whereby the GA% reference intervals were significantly higher in females. These differences could be attributed to the differences in obesity rates between males and females as observed in this study. However, the inverse relationship we observed between BMI and GA% disputes this hypothesis as it would have resulted in lower median GA% in females than in males if this difference was due to differences in BMI alone. The GA% and BMI relationship has also been reported in previous studies (Selvin, *et al.,* 2018), (Miyashita, *et al.,* 2007), (Koga, *et al.,* 2007), (Sumner, *et al.,*2016) and is thought to be due to an increase in inflammatory cytokines in overweight and obese non-diabetic subjects (Koga, *et al.,* 2007), (Koga, *et al.,* 2015). Further studies are necessary to determine whether a correction factor for BMI could be included in the GA% equation (Szkudlarek, *et al.,* 2016). Therefore, the difference observed between females and males is more likely to be due to a true gender-related difference rather than BMI.

A previous study by Wu and co-workers (Wu, *et al.,* 2016) demonstrated an association between age and GA%. In this study the researchers determined that there was a 0.31% increase in GA% for every 10-year increase in age. Age-specific reference intervals were determined in our population after dividing the group by terciles. In our study, there was no statistically significant difference between the age groups, but this may be due to the lower mean ± SD age of our study population (33.9 ± 13.7 years) compared to that of Wu and co-workers of 50.4 ± 12.6 years (Wu, *et al.,* 2016). Although our study comprised of individuals with an age range of 18 to 76 years, we could not compute reliable reference intervals in participants between 59 and 76 years as they comprised of only 38 participants (5.7%).

The ethnic-specific reference intervals demonstrated a slightly higher interval for Africans of 10.9% to 15.9%. This was also demonstrated in previous studies that included different ethnic groups. The Black Americans in the study by Selvin *et. al.* had a reference interval of 10.9% to 15.5% (Selvin, *et al.,* 2018). Another study that included White and Black Americans, demonstrated a mean GA% of 13.52% and 14.19% for these groups respectively (Kohzuma, *et al.,* 2011). The determination of reference intervals for subjects of Mixed-Ancestry is very relevant in the South African context, where 48.8% of the Western Cape province population is of Mixed Ancestry origin (Statistics South Africa, 2012). In addition, a previous study demonstrated that a Mixed Ancestry population in Bellville (Cape Town, South Africa) has a high prevalence of metabolic syndrome, which put them at high risk for the development of diabetes (Erasmus, *et al.,* 2012).

The strengths are that the study was conducted according to a well-written protocol for a worldwide multi - centre reference interval study and subjects were recruited specifically for a reference interval study. The subjects were healthy, without diabetes, and confirmed to be non-diabetic by means of fasting blood glucose measurement. The wide array of measurements performed during the previous reference interval study allowed exclusion of subjects with conditions that may have confounding effects on the results.

A limitation of this study is that there was not an equal distribution of subjects according to gender and ethnicity. The ethnic distribution of the sample was not representative of the ethnic distribution of the South African population at national or provincial (Western Cape) level according to the 2011 national census results, with only 11.6% of subjects being Africans

(Statistics South Africa, 2012). An additional limitation was that human immunodeficiency virus (HIV) and tuberculosis were not excluded in this population. The Western Cape is known to have a high prevalence of both and as albumin is a negative acute phase reactant, the albumin levels may be decreased (Poolman, *et al.,* 2017), (Zemlin, Burgess & Engelbrecht, 2009).

Future studies are needed to determine GA% reference intervals in other African populations. Also, GA% cut-offs in dysglycaemia and its usefulness as a screening tool for diabetes and prediabetes in this population should be determined.

5. Acknowledgements

Ms M Hoffman, for technical assistance with setting up the Roche® Cobas® 6000, performing the method validation study and analyzing study samples.

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Table 1: Characteristics of study subjects included in GA% reference interval determination study, *n =663*

Variable	n	**Mean (SD) or %**
Age (years)		33.9 (13.7)
18-38 years, %	427	64.5
39 -58 years, %	197	29.8
59 -76 years, %	38	5.7
Gender:		
Female, %	407	61.4
Male, %	256	38.6
Ethnicity:		
Africans, %	77	11.6
Caucasians, %	380	57.3
Mixed-Ancestry, %	206	31.1
Body mass index:		25.2 (4.2)
<25 kg/m^2, %	355	53.5
25-30 kg/m^2, %	228	34.4
>30 kg/m^2, %	80	12
eGFR (MDRD, mL/min/1.73m^2)	661	86.5 (7.5)

*SD-Standard Deviation

Table 2: Two-sided 95% Robust Reference Intervals for Glycated albumin (%)

	n	2.5th Percentile (95% CI)	97.5th Percentile (95% CI)	50th centile (95% CI)
Overall	663	10.7 (10.5 - 10.9)	15.2 (15.0 - 15.3)	12.9 (12.8 - 13.0)
Gender				**p < 0.0001****
Female	407	11.0 (10.7 - 11.2)	15.5 (15.3 - 15.7)	13.2 (13.1 - 13.3)
Male	256	10.6 (10.3 - 10.9)	14.4 (14.1 to 14.6)	12.4 (12.3 -12.6)
Age				**p = 0.4701**
18-38 yrs, %	427	10.8 (10.6 – 11.0)	15.1 (14.9 – 15.3)	12.9(12.8-13.0)
39-58 yrs, %	197	10.5 (10.1 – 10.9)	15.3 (14.9 – 15.7)	12.9(12.7-13.1)
59-76 yrs, %	38	10.8 (10.2 – 11.4)	15.5 (14.8 – 16.2)	13.1(12.8-13.7)
Ethnicity				**p = 0.0025****
Africans	77	10.9 (10.3 - 11.4)	15.9 (15.3 - 16.4)	13.3 (13.0 - 13.8)
Caucasians	380	10.7 (10.6 - 11.0)	15.0 (14.8 - 15.2)	12.9 (12.8 - 13.1)
Mixed Ancestry	206	10.6 (10.2 - 11.0)	15.2 (14.8 - 15.6)	12.9 (12.7 - 13.0)
BMI				**p = 0.0001****
<25 kg/m^2	355	11.2 (11.0 - 11.4)	15.3 (15.0 - 15.5)	13.2 (13.1 - 13.3)
25-30 kg/m^2	228	10.5 (10.0 - 10.9)	14.9 (14.5 - 14.3)	12.7 (12.4 - 12.9)
>30 kg/m^2	80	10.0 (9.7 - 10.4)	14.6 (14.0 - 15.3)	12.2 (11.9 -12.7)

CI- Confidence In□erv□l; BMI-Body M□ss Index; **S□□is□ic□lly signific□n□

Table 3: Association of Gender, BMI, Age and Ethnicity on test results evaluated by multiple regression model

Model	Variables	|Standardized Coefficients Beta|	p-value
Gender	**Gender**	0.30	< 0.001
Gender + BMI	**Gender**	0.27	< 0.001
	BMI Group		
	<25 kg/m^2	Reference	
	25-30 kg/m^2	0.19	< 0.001
	>30 kg/m^2	0.27	< 0.001
Gender + BMI + Age	**Gender**	0.27	
	BMI Group		
	<25 kg/m^2	Reference	
	25-30 kg/m^2	0,20	< 0.001
	>30 kg/m^2	0,29	< 0.001
	Age group		
	18-38 yrs	Reference	
	39-58 yrs	0,03	0,423
	59-76 yrs	0,07	0,068
Gender + BMI + Age + Ethnicity	**Gender**	0,26	< 0.001
	BMI Group		
	<25 kg/m^2	Reference	
	25-30 kg/m^2	0,20	< 0.001
	>30 kg/m^2	0,31	< 0.001
	Age group		
	18-38 yrs	Reference	
	39-58 yrs	0,06	0,126
	59-76 yrs	0,09	0,140
	Ethnicity		
	Africans	Reference	
	Caucasians	0,21	< 0.001
	Mixed Ancestry	0,27	< 0.001

MANUSCRIPT FIVE

The iron status of a healthy South African adult population

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Running title: Iron status in South African adults

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Keywords: Iron deficiency, anaemia, ferritin, iron status.

Manuscript publication details: Clinica Chima Acta 460 (2016). Pages 240 - 245

Abstract

Introduction

Iron deficiency is associated with significant morbidity and mortality, can present with or without haematological changes and is a major cause of microcytic anaemia. In South Africa and Africa in general, there is a paucity of studies on the iron status of healthy adult non pregnant females and males >18 years of age. The aim of the study was to determine the prevalence of iron deficiency in a healthy South African population.

Methods

A total of 651 healthy adults >18 years were included in the study. Blood samples were taken for the determination of iron status, haematological and inflammatory parameters. A ferritin level of < 30µg/L was used to define iron deficiency and these subjects were further divided into those with and without anaemia. Diet and menstrual history in females was further investigated.

Results

Overall, the prevalence of anaemia was 12.6% and iron deficiency was found in 78% of anaemic subjects. The prevalence of iron deficiency was 39.8% in all participants and females and Africans had a very high prevalence of 56.6% and 50.7% respectively. Significant ($p < 0.05$) differences were found in concentrations of ferritin, haemoglobin, iron, transferrin, transferrin saturation, MCV and MCH between the groups.

Conclusion

Anaemia is a minor health problem but a large proportion of subjects with iron deficiency do not present with anaemia. The prevalence of iron deficiency was high especially in females and African participants.

1. Introduction

Iron is an essential trace element that plays a crucial role in various metabolic functions. It is a structural component of haem-containing proteins like haemoglobin (Hb) and myoglobin which bind oxygen for delivery to the tissues. It is also involved in energy production, redox reactions and DNA synthesis. However, free iron is toxic and therefore it is tightly regulated by its binding and incorporation into proteins (Gantz, 2013).

A reduction in the total body iron is referred to as iron deficiency (ID) and it can occur as a result of reduced intake, decreased absorption or increased losses of iron (Bermejo & Garcia-Lopez, 2009), (Camaschella, 2015). ID progresses through three stages namely depleted iron stores, iron deficient erythropoiesis and finally iron deficiency anaemia (IDA) (Kiss, 2015). In iron depletion, the total body iron is decreased but erythropoiesis is unaffected whereas in iron deficient erythropoiesis, due to insufficient supply of iron to erythroid tissues, erythropoiesis is affected. However, the Hb is still within normal limits and thus referred to as iron deficiency without anaemia (IDWA). The last and severe stage of ID presents with anaemia (WHO, 2007). ID is the most common nutritional deficiency and the top ranking cause of anaemia in the world (Kassebaum, *et al.,* 2014). Children, pregnant and lactating women are considered at high risk because of the increased iron requirements. Non-pregnant women of reproductive age are also at risk of ID due to the loss of blood through menstruation (Camaschella, 2015).

In adults, anaemia is defined as an Hb concentration of below 13 g/dl in males and below 12 g/dl in non-pregnant females (Beutler & Waalen, 2006). Anaemia is easy to diagnose, however to confirm that the anaemia is a result of ID can prove to be difficult. The gold standard for the diagnosis of ID is a bone marrow aspirate (BMA) which is highly specific but invasive and expensive (Lopez, *et al.,* 2016). Serum ferritin is the most specific non-invasive biochemical test to diagnose ID, as it reflects total body iron stores. A serum ferritin level below 12µg/L is generally accepted as consistent with depleted iron stores (Ali, Luxton & Walker, 1978). However, Mast *et al.* found that the sensitivity and specificity of ferritin below 12µg/L compared to BMA was 25% and 98% respectively. The sensitivity increased to 92% and the specificity remained the same when a cut-off of 30µg/l was used (Mast, *et al.,* 1998). Importantly, this cut-off may only be used in the absence of inflammation as ferritin is an acute

phase protein and levels will be higher in the presence of inflammation (WHO, 2007), (Lopez, *et al.,* 2016). The WHO and Centres for Disease Control and Prevention (CDC) Joint Report recommends that ferritin, Hb, mean cell volume (MCV), soluble transferrin receptors and zinc protoporphyrin are the best indicators of iron status in all populations. The performance of other tests such as iron, transferrin and transferrin saturation are improved when used in conjunction with the above tests (Lopez, *et al.,* 2016).

Importantly, IDA is associated with significant morbidity and mortality as it can affect neurocognitive development and function, work productivity and even result in death (Stoltzfus, 2003). In 2000, Nojilana *et al*. estimated that 0.4% of all deaths in South Africa were a result of IDA (Nojilana, *et al.,* 2007). Various studies examined the prevalence of IDA in the South African population; however, these studies have been conducted mostly in children, pregnant women and the elderly population Nojilana, *et al.,* 2007), (Charlton, *et al.,*1997), (Mamabolo & Alberts, 2014), (Motadi, *et al.,* 2015). There is a paucity of studies on the iron status of healthy adult non pregnant females and males over 18 years of age (Shisana, *et al.,* 2013), (Wolmarans, *et al.,*2003), (Lawrie, Coetzee & Glencross, 2008). Thus, the aim of this study was to determine the prevalence of iron deficiency in a healthy population representative of South Africa.

2. Methods

2.1 Study population

From a database of the 'Establishing Adult Reference Intervals for Selected Analytes in South Africa' study consisting of 1433 subjects, 651 adults meeting the inclusion criteria were included in our study. The study involved the recruitment of healthy individuals aged 18 years and older from the general public as well as students, hospital and laboratory staff. The protocol and standard operating procedures used in the main study are published elsewhere (Ozarda, *et al.,*2013). All participants completed a questionnaire and written informed consent was obtained. The questionnaire included the following: (1) demographic details i.e. age, gender and ethnicity; (2) personal habits i.e. smoking and alcohol history; (3) physical activity and exercise; (4) dietary information; (5) current health status i.e. recent infections, medication intake including nutritional supplements and allergies; (6) menstrual history in females. The

study was approved by the Health Research Ethics Committee of Stellenbosch University, Cape Town, South Africa (S12/05/147).

We excluded the following individuals: those not born in South Africa; with recent results pointing to a severe disease; a recent illness or hospitalisation 2 weeks prior to recruitment; who donated blood up to 3 months prior to recruitment; known carriers of Hepatitis B, Hepatitis C or Human Immunodeficiency Virus (HIV); pregnant or within one year of childbirth; and those who participated in a research study involving an investigational drug in the past 12 weeks. Additional exclusion criteria included: missing data for ferritin, iron, transferrin, transferrin saturation, highly sensitive C-reactive protein (hsCRP), Hb, MCV and mean cell haemoglobin (MCH); and participants with hsCRP concentrations ≥10 mg/L and WCC >11 x 10^9.

2.2 Biochemical analysis

Blood samples were collected, following a 12–14 hour fast, after subjects were seated for 20 – 30 minutes. Biochemical parameters were analyzed at an ISO 15189 accredited laboratory (PathCare Reference Laboratory, Cape Town, South Africa).

The measurement of serum ferritin was performed on the Beckman Coulter Access immunoassay system (Beckman Coulter Inc. Brea, CA, USA). The assay is a paramagnetic particle, chemiluminescent immunoassay and the calibrator is traceable to the World Health Organisation (WHO) 3rd International standard for ferritin (IS 94/572). The assay has a linear range of 0.2–1500 µg/L. Transferrin, iron and hsCRP measurements were performed on the Beckman DXC automated analyser (Beckman Coulter Inc. Brea, CA, USA). Transferrin was measured in serum using an immunoturbidimetric method that utilises anti-human transferrin antibodies. The assay's linear range is 0.75–7.5 g/L. hs-CRP was determined in serum by an immunoturbidimetric assay utilising anti-CRP antibody coated latex particles with a linear range of 0.08–80mg/L. Serum iron was measured using a colorimetric assay that utilises 2,4,6-Tripyridyl-s-triazine (TPTZ) as a chromogen. TPTZ reacts with ferrous iron to form a blue coloured complex that is measured photometrically. The linear range of the assay is 2–179µmol/L. Transferrin saturation was calculated according to the formula: % saturation = [iron / (transferrin x 25.6)] x 100%.

The Beckman Coulter AC•T Diff5 haematology analyzer (Beckman Coulter Inc. Brea, CA, USA) was used for the determination of Hb, MCV, MCH and WCC using EDTA whole blood. This analyser is based on the Coulter principle of sizing and counting for quantitative determination of Hb, MCV, MCH and WCC.

2.3 Definitions & Statistical analysis

Serum ferritin was used to define iron deficiency. A cut-off level of 30µg/L for serum ferritin was chosen to distinguish between participants with ID (ferritin < 30µg/L) and those who are iron replete (IR) (ferritin > 30µg/L). For the diagnosis of anaemia, an Hb of < 13g/dl in males and < 12g/dl in females was used (WHO criteria). We used the laboratory reference ranges to define microcytic hypochromic anaemia as an Hb below cut-off for gender as well as an MCV <81fl (microcytosis) and MCH <28pg (hypochromia). Subjects with ID (ferritin < 30µg/L) were further subdivided into two groups namely IDA (Hb below cut-off for gender) and IDWA (Hb above cut-off for gender).

Data was analysed using Microsoft Excel® (Microsoft, Redmond, WA, USA) and SPSS® v20 statistical software (SPSS, Chicago, IL, USA). The Shapiro Wilk test was performed and non-parametric tests were applied as not all the data fitted a Gaussian distribution. Descriptive data is reported as median values and interquartile ranges (IQR). The Mann Whitney U test was used for comparisons between the groups. Statistically significant differences were indicated by $p < 0.05$.

3. Results

3.1 Study population characteristics

Of the 651 participants included, 410 (63%) were female and 241(37%) male. The majority of participants were of Caucasian origin (55.5%) followed by Mixed Ancestry (33.9%) and African (10.6%). The age of all participants ranged from 18 to 76 years with a median age of 30 (22-44) years.

3.2 Prevalence of anaemia

The prevalence of anaemia in all participants was 12.6%. Female participants had a much higher prevalence of anaemia (18.3%) compared to 2.9% in male participants. Anaemia was more prevalent (31.9%) in Africans compared to 17.2% and 6.1% in the Mixed Ancestry and Caucasians respectively. Microcytic hypochromic anaemia was present in only 22 (26.8%) participants of which 20(91%) were female. However, ID (ferritin < 30µg/L) was found in 64 (78%) of the participants with anaemia. The cause of anaemia was not determined in the 18 (22%) subjects in the IR group (**Table 1**).

3.3 Iron status of study participants

We found a prevalence of 39.8% for ID in this study population. African participants had the highest prevalence of ID (50.7%) compared to 43.4% and 35.5% in the Mixed Ancestry and Caucasian participants respectively (**Table 1**). **Table 2** shows the medians and IQRs of iron status and haematological parameters. There was a statistically significant ($p < 0.001$) difference in the concentrations of ferritin, transferrin, iron, transferrin saturation, Hb, MCV and MCH between the ID and IR groups (**Table 2**).

ID was more prevalent in female participants (56.6%) compared to males (11.2%). The median age in the ID group was 26 (21-36) years (**Figure 1**) and these women had a lower median ferritin concentration of 14 (8-21) µg/L (**Figure 2**) compared to the IR group with a median age and ferritin of 43 (28-54) years (**Figure 1**) and 61 (40-100) µg/L (**Figure 2**) respectively. In addition, the Hb in the ID group was lower than the IR group [12.5 (11.9-13) vs. 13.3 (12.8-13.8) g/dl] (**Figure 3**).

3.4 Prevalence of IDA

Overall, the prevalence of IDA in this population was 9.8% (**Table 1**). Almost all (97%) of the participants with IDA were female with an IDA prevalence of 15.1%. African participants had the highest prevalence of IDA (26.1%) compared to 14% and 4.2% in the Mixed Ancestry and Caucasians respectively. A statistically significant difference ($p < 0.001$) was observed when Africans were compared with Caucasians and Mixed Ancestry participants with Caucasians (**Table 1**).

The median ferritin and Hb concentrations were significantly ($p < 0.001$) lower in the IDA group compared to the IDWA group. A statistically significant difference ($p < 0.05$) was also observed between the IDA and IDWA groups for iron, transferrin, transferrin saturation, MCV and MCH concentrations (**Table 3**).

3.5 Diet and menstrual history in females

Most (34.1%) of the women in the ID group consumed meat 1-2 times a week whereas in the IR group the majority (39.9%) consumed meat 3-4 times a week. There were also twice as many women in the ID group who do not eat meat (5.6% vs. 2.8%) and fish (12.5% vs. 5.6%) compared to the IR group. There were more women (44.9%) that consumed vegetables every day in the IR group compared to the ID group (11.6%).

The questionnaire was used to determine if women had regular/ irregular cycles or in menopause as well as the use of hormonal therapy. More women with ID were still menstruating compared to those with normal iron stores. Most (53.9%) of the women with ID reported regular menstrual cycles, 14.7% had irregular cycles, 21.1% were on oral or depot contraceptives and 8.2% were menopausal. In the IR group 30.3% had regular cycles, 5.6% irregular cycles, 20.8% on contraceptives and 34.8% were menopausal.

4. Discussion

As data on iron status in South African adults is lacking, the purpose of this study was to determine the iron status of relatively healthy South African adults participating in the 'Establishing Adult Reference Intervals for Selected Analytes in South Africa' study. The prevalence of 12.6% for anaemia found in this population is considered a minor public health problem according to the WHO/CDC classification (WHO, 2007). This prevalence is much lower than the 17.5% found in the South African National Health and Nutrition Examination Survey (SANHNES) performed in 2012 (Shisana, *et al.,* 2013). Similar to the SANHANES study, we found a higher prevalence of anaemia in females compared to males. In the SANHANES study the adult population was defined as subjects older than 15 years, whereas in our study the participants were older than 18 years. Additionally, we followed a strict recruitment strategy to ensure a healthy population was included; however, the same does not

apply to the SANHANES study where all subjects, irrespective of their health status, were eligible for inclusion in the study.

There is a lack of reliable epidemiological data for ID because its prevalence is often predicted using the prevalence of anaemia (Lopez, *et al.,* 2016), (WHO, 2001). The WHO estimates that if the prevalence of IDA in the population evaluated is > 20% then ID is present in 50% of the population (WHO, 2001). In our study the prevalence of IDA in females was 15.1%, however ID was present in 56.6% of females. This demonstrates that using the WHO estimate would have resulted in an underestimation of the prevalence of ID in female participants. In a South African study of urban non-pregnant adult female health care workers, 10% were found to have possible IDA based on the presence of microcytic hypochromic anaemia (Lawrie, Coetzee & Glencross, 2008). Our population was also recruited from urban areas and consisted of students, health care workers as well healthy volunteers from the general population. If we had used a similar approach to Lawrie *et al.*, the estimated prevalence of IDA would be 3.4% in all participants and 4.9% in females. Interestingly, microcytic hypochromic anaemia was found in the same male participants with IDA based on our criteria. It is clear that the use of haematological parameters alone does not provide a reliable assessment of iron status in females. We, however, cannot draw any conclusions for men because of the very small number of male participants with IDA (0.8%) in our study.

Microcytic anaemia is not specific for ID and is also found in patients with anaemia of inflammation, thalassaemias and sideroblastic anaemia (De Loughery, 2014). Haematological parameters in combination with biochemical tests such as serum ferritin and transferrin saturation provide an accurate assessment of the iron status (Lopez, *et al.,* 2016), (Friedman, *et al.,*2015). In the SANHNES study, the prevalence of iron deficiency, as determined by ferritin <15µg/L, and IDA in women aged 16-35 years, was 15.3% and 9.7% respectively; however, there is no available data for the prevalence of ID in men (Shisana, *et al.,* 2013). Although it seems that our prevalence of ID and IDA is higher than reported in the SANHANES, we used a higher cut-off for ferritin (30µg/L) in our study. Secondly, we did not categorize participants into age groups and thus our prevalence is for female participants of all ages. Lastly, our exclusion criteria reduced the effect of inflammation on ferritin and therefore our findings are a more reliable indication of ID compared to SANHANES where subjects with chronic diseases and inflammation may have been misclassified.

Wolmarans *et al*. found that 27.4% had low iron status in a study conducted in non-pregnant adult South African women 18-55 years of age (Wolmarans, *et al.,*2003). This prevalence is lower than our study; however, some of the women may have been misclassified due to the presence of infection. C - RP was elevated in 16% of women and almost half of them reported a cold at the time of blood collection (Wolmarans, *et al.,*2003). Although we excluded participants with a recent illness and a CRP concentration ≥10 mg/L the possibility of misclassification of participants still exists.

An elevated serum ferritin is found in inflammatory states which includes subjects with mixed anaemia i.e. anaemia of chronic disease (ACD) and IDA; therefore, a higher cut-off should be used for diagnosing ID. Ferritin concentrations of up 100µg/L are considered diagnostic of ID in inflammatory states (Camaschella, 2015), (Friedman, *et al.,*2015). An alternative marker such as soluble transferrin receptors (sTfR) may be of clinical value in inflammatory states. The concentration of sTfR is increased in ID but it is not affected by inflammation (Asobayire, *et al.,*2001), (Braga, *et al.,*2014). However, elevated sTfR concentrations can be found in conditions with increased erythropoiesis such as haemolytic anaemia (Speeckaert, Speeckaert & Delanghe, 2010). Although sTfR is considered highly sensitive for ID, ferritin is considered to be more specific (Mast, *et al.,* 1998), (Braga, *et al.,* 2014). Current available ferritin assays are traceable to the high order WHO International Internal Standard and this can allow the use of the same cut-offs and comparisons between different studies. In contrast the lack of standardisation of sTfR assays has led to variable cut-offs and limits comparisons between studies (Braga, *et al.,*2014), (Speeckaert, Speeckaert & Delanghe, 2010). In addition, the lack of available routine automated assays and the cost of sTfR prevent its use in resource limited settings especially in Africa (Asobayire, *et al.,*2001). The expression of hepcidin, a peptide hormone that regulates cellular iron export, is increased in inflammation and decreased in ID (Andrews & Schmidt, 2007), (Goodnough, Nemeth & Ganz, 2010). The measurement of hepcidin may be helpful in inflammatory conditions; however, its use is limited by the lack of available routine and standardised assays (Camaschella, 2015). In our setting and throughout Africa, the cost of hepcidin assay would be prohibitive.

There are two forms of iron, haem and non-haem, which are obtained from the diet. Haem iron, which is well absorbed, is an excellent source of iron found in red meat, fish and

poultry whereas non-haem iron found in vegetables and iron fortified foods is inefficiently absorbed (Andrews & Schmidt, 2007). It is not surprising that ID was more prevalent in women who consumed less meat and fish. The women with ID were also much younger and the majority were still menstruating compared to those with IR stores. Iron losses are more in women of reproductive age because of menstruation and hence they require more iron than postmenopausal women and men.

Recent research has shown that there are inter-ethnic variations in the genes involved in iron metabolism which are associated with a low iron status and anaemia. However, these studies have been conducted mostly in European and Asian populations (Gichohi-Wainaima, Towers, *et al.,* 2015). To our knowledge there is only one study conducted in South Africa on African females that has explored the association between genetic variants from genome wide association studies and low iron status. This study found that there was no association between the previously described variants and low iron status in African females (Gichohi-Wainaina, *et al.,* 2015). African participants in our study had a higher prevalence of ID and IDA compared to other ethnic groups. We cannot explain if this is attributed to dietary and environmental factors or inter-ethnic genetic variations. This highlights the need for further genomic studies in the South African population to identify the inter-ethnic genetic variations associated with a low iron status.

Our study had some limitations. Due to the small number of African participants, we cannot draw any conclusions regarding the differences observed between the ethnic groups. We also did not determine if these differences are due to inter-ethnic variations of genes involved in iron homeostasis, which may have affected the results. Additionally, we did not compare our findings with BMA which is considered the gold standard for ID. We also did not measure sTfR which may have allowed us to include the participants with CRP concentrations > 10mg/L. Finally, we did not examine the association between ID and alcohol as well as BMI.

However, we had numerous strengths, the most important being the recruitment of healthy participants using very strict criteria. We also excluded subjects with possible infection and evaluated dietary and menstrual patterns. Also, to our knowledge, this is the first South African study looking at the iron status of healthy males.

5. Conclusion

We evaluated a healthy South African adult population and using a cut-off of ferritin < 30µg/L we found a 39.8% prevalence of ID. Although we found a higher prevalence than previous studies comparisons cannot be made because of differences in the population studied and study design. In addition, we examined an urban population and these findings may be different in a rural population. The use of haematological parameters as screening tests for ID is flawed and will result in a gross underestimation of the problem because the proportion of subjects with ID that have anaemia is rather small. In addition, IDA is the last and severe stage of ID and it contributes significantly to the burden of disease and mortality. ID was a bigger problem in females and this highlights the need for health care providers to increase awareness and in selected cases screen by measuring serum ferritin. This approach will allow for early detection of subjects with ID and may help to decrease the prevalence of IDA found in pregnant women. The importance of dietary iron cannot be ignored; we demonstrated that women with ID consumed less iron rich foods. ID is a preventable problem and, in our setting, a feasible intervention is to increase awareness amongst health care workers and provision of focused nutritional education at each health visit especially at primary health care level. There exists a need for further studies which are more representative of the South African population at large.

6. Funding / Acknowledgements

The study was funded by the National Health Laboratory Services and Medical Research Council. Beckman Coulter donated the reagents and the samples were analysed free of charge at PathCare Private Laboratories. These funding organisations did not play any role in the conception and design of the study or the writing of this article.

7. Conflict of interest: None

8. Authorship

All authors have made substantial contributions to the following: (1) the conception and design of the study, acquisition of data, analysis and interpretation of data, (2) drafting of the article and revising it critically for intellectual content, (3) final approval of submitted version.

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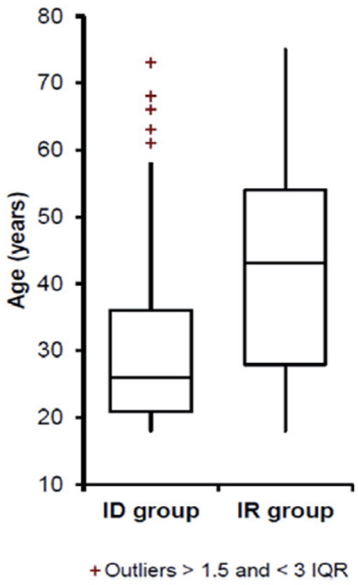

Figure 1: Box plot illustrating the age of female participants in the ID and IR groups ($p < 0.001$)

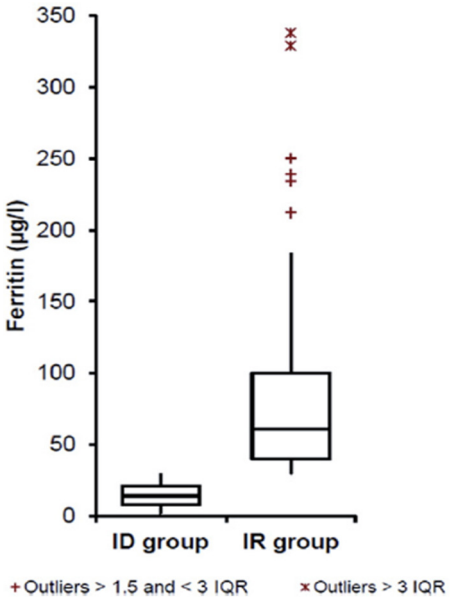

Figure 2: Box plot illustrating ferritin concentrations of female participants in the ID and IR groups ($p < 0.001$)

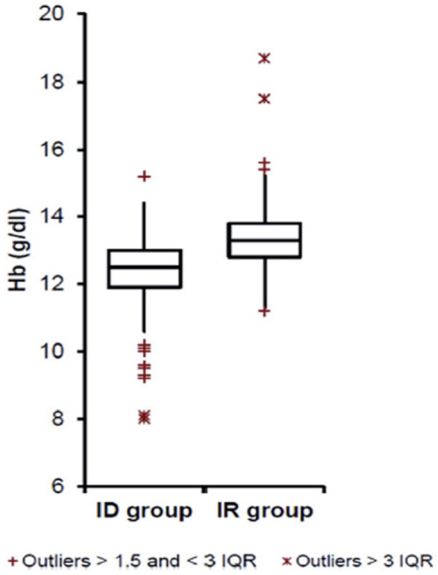

Figure 3: Box plot illustrating the Hb concentrations of female participants in the ID and IR groups ($p < 0.001$)

Table 1: Iron status in different ethnic groups

Variable	Total (n = 651)		African (n = 69)		Mixed ancestry (n = 221)		Caucasian (n = 361)		p value
	Anaemia	No anaemia	Anaemia	No anaemia	Anaemia	No anaemia	Anaemia	No anaemia	
Iron deficiency, n(%)	64(9.8)	195(30)	18(26.1)[a]	17(24.6)	31(14)[b]	65(29.4)	15(4.2)[c]	113(31.3)	< 0.0001[ac] 0.0001[bc]
Iron replete, n(%)	18(2.8)	374(57.4)	4(5.8)[a]	30(43.5)	7(3.2)[b]	118(53.4)	7(1.9)[c]	226(62.6)	NS[bc] 0.0385[ac]
All participants, n(%)	82(12.6)	569(87.4)	22(31.9)[a]	47(68.1)	38(17.2)[b]	183(82.8)	22(6.1)[c]	339(93.9)	< 0.0001[bc] 0.0001[ac]

[ac], significant difference between African and Caucasian; [bc], difference between Mixed Ancestry and Caucasian; NS no significant difference

Table 2: Iron status and haematological parameter medians (IQR)

Parameter	Iron deficient (n = 259)	Iron replete (n = 392)	Abnormal range	p value
Ferritin (µg/l)	14(8-21)	83(50-131)	<30	< 0.001
Transferrin (g/l)	2.9(2.7-3.3)	2.5(2.2-2.8)	>3.6	< 0.001
Iron (µmol/l)	13.7(9.7-18.4)	18(14.6-22.4)	<9 (F) <12 (M)	< 0.001
Transferrin saturation (%)	19(13-26)	28(22-36)	<20	< 0.001
Hb (g/dl)	12.6(12-13.2)	13.3(12.8-13.8)	<12 (F) <13 (M)	< 0.001
MCV (fl)	88(84-91)	91(88-94)	<81	< 0.001
MCH (pg)	30(28-31)	31(30-32)	<28	< 0.001

IQR = Interquartile range

Table 3: Parameter medians (IQR) of participants with iron deficiency

Parameter	IDA (n = 64)	IDWA (n = 195)	Abnormal range	p value
Ferritin (µg/l)	8(4-14)	17(11-23)	<30	< 0.001
Transferrin (g/l)	3.2(2.9-3.5)	2.9(2.7-3.1)	>3.6	< 0.001
Iron (µmol/l)	8.8(5.6-13.2)	15.4(11.3-19.1)	<9 (F) <12 (M)	< 0.001
Transferrin saturation (%)	12(7-19)	20(15-27)	<20	0.007
Hb (g/dl)	11.4(10.9-11.6)	12.9(12.4-13.5)	<12 (F) <13 (M)	< 0.001
MCV (fl)	83(79-87)	89(87-92)	<81	< 0.001
MCH (pg)	27(26-29)	30(29-31)	<28	< 0.001

IQR = Interquartile range; IDA = Iron deficiency anaemia; IDWA = Iron deficiency without anaemia

MANUSCRIPT SIX

Comparison of LDL – Cholesterol (LDL-c) estimate using the Friedewald Formula and the newly proposed De Cordova formula with a directly measured LDL – Cholesterol in a healthy South African population.

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Short tile: Friedewald versus De Cordova LDL-c formula

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DECLARATIONS:
Competing Interests: None

Funding: National Health Laboratory Services/Medical Research Council funding

Ethical approval: The ethics committee of Stellenbosch University approved this study (REC number: S12/05/147)

Manuscript publication details: Annals of Clinical Biochemistry 51(6), November 2014. Pages: 672 - 679

Abstract

Background

The accurate determination of low-density lipoprotein cholesterol (LDL-c) is pertinent in clinical practice. Most laboratories employ the Friedewald formula, for convenient estimation of LDL-c, despite its shortfalls. Different formulae have been proposed for use, for more accurate but convenient estimation of LDL-c. Here, we compare a new formula recently proposed by de Cordova *et al*, with that of Friedewald and LDL-c determined by a homogeneous assay. We also assess its performance at very low TG levels against the modified Friedewald formula recommended by Ahmadi *et al.*

Methods

A database of 587 adults from the "Establishing Reference Intervals for Selected Analytes in South Africa" study was utilized. Fasting samples were assayed for lipids. LDL-c was determined by the Daiichi method. Performance of the Friedewald and the de Cordova formulae were compared. This was exclusively repeated at very low TG levels (<1.13mmol/l), this time, including the Ahmadi formula.

Results

The Friedewald formula and the de Cordova formula both had high correlations with the direct LDL-c (r=0.98 and r=0.97 respectively), although the latter showed an inconsistent bias at different LDL-c levels. The two formulae had a higher correlation (r=0.98) than the Ahmadi formula (r=0.92) at very low TG levels.

Conclusion

The Friedewald formula showed better agreement with the direct LDL-c than the de Cordova formula, at various LDL-c levels, in our population. It also outperformed the Ahmadi formula at very low TG levels. We therefore advise that it remains the formula of choice for LDL-c estimation in South Africa.

1. Introduction

Low density lipoprotein cholesterol (LDL-c) is a lipoprotein historically classified based on its hydrated density as determined by ultracentrifugation. Its association with an increased risk for cardiovascular disease has been confirmed by epidemiologic studies, clinical trials, genome wide association studies and meta-analyses in humans (Cohen, *et al.,* 2006), (Kizer, *et al.,* 2010), (Angelakopoulou, *et al.,* 2012).

LDL-c-lowering therapy has also been shown to reduce the risk for coronary artery disease (HPSCG, 2002), (SSSG, 1994), (Goldberg, *et al.,* 1998). Due to these reasons, the expert panel for the detection, assessment and management of elevated blood cholesterol in adults- the National Cholesterol Education Program (NCEP) Adult Treatment Panel (ATP) III, continues to focus on LDL cholesterol as the primary target in the diagnosis of dyslipidaemias and in therapy with lipid-lowering agents (NECP, 2001). NCEP guidelines also require that clinical laboratories estimate LDL-c using methodologies with a total analytical error of < 12% and an imprecision of <4% (Bachorik & Ross, 1995).

Traditionally, the reference method for the determination of LDL-c is by beta-quantitation following ultra-centrifugation. This method is however, time-consuming, labour-intensive, expensive, requires highly skilled technicians and different types of equipment which make reproducibility of the procedure difficult. Hence, its application is limited to specialized lipid laboratories (Nauck, Warnick & Rifai., 2002). In 1972, William Friedewald developed a new method for the estimation of LDL-c, which eliminates the need for ultracentrifugation and is therefore cost-effective and convenient. This method was developed using a sample size of 448 individuals made up of patients with either type II or type IV familial hyperlipoproteinaemia, and their normal relatives. The range of LDL-c in the study was from 0.72mmol/L to 21.72mmol/L. The Friedewald formula estimates LDL-c from total cholesterol (TC), high density lipoprotein cholesterol (HDL-c) and triglycerides (TG), thus: LDL-c = TC-HDL-c – TG/2.2 (Friedewald, Levy & Fredrickson, 1972).

Due to the three parameters involved in the estimation of the Friedewald formula, it is difficult to meet the NCEP criteria for total error and imprecision using this method, as it will involve the imprecision due to the individual parameters. In addition to this shortfall, the

Friedewald formula estimates LDL-c inaccurately in non-fasting samples, at TG levels greater than 4.5 mmol/L (Friedewald, Levy & Fredrickson, 1972) and also at very low TG levels (<1.13mmol/L) (Ahmadi & Boroumand, 2008) in certain disease conditions such as diabetes mellitus, hepatopathies and nephropathies (Matas, *et al.,* 1994), (Nauk, *et al.,* 1996).

In the past two decades, direct methods for measurement of LDL-c have been developed (Sugiuchi, *et al.,* 1998), (Miki, 1999), (Okada, *et al.,* 1998), (Rifai, *et al.,* 1998). These methods are expensive for most clinical laboratories. Hence, despite its numerous shortfalls, the Friedewald formula remains widely applied by routine laboratories worldwide. More recently, several authors have derived various formulae in specific populations to circumvent the drawbacks of the Friedewald formula (Ahmadi & Boroumand, 2008), (Anandaraja, *et al*., 2005), (Chen, *et al.,* 2010), (Hattori, *et al.,* 1998), (Teerakanchana, *et al.,* 2007). These authors have recommended the verification of their formulae in populations different from those of their study. De Cordova *et al.* very recently published a new and simple formula from a large Brazilian database of 10,664 individuals; with LDL-c values ranging from 0.62 to 10.94 mmol/L. This new formula outperformed the Friedewald formula and other previously published formulae in their study population. The formula estimates LDL-c thus: LDL-c = 0.75 (TC – HDL-c). It also does not require a fasting specimen as it is independent of TG (De Cordova & De Cordova, 2013).

Here, we compare the recently published de Cordova formula, the Friedewald formula and a directly measured LDL-c in a healthy South African population. We also verified its performance at very low TG levels (< 1.13 mmol/L), against the modified Friedewald formula proposed by Ahmadi *et al*, which estimates LDL-c viz: LDL-c = TC/1.19 + TG/0.81 – HDL-c/1.1 -0.98 (Ahmadi & Boroumand, 2008).

2. Materials and methods

2.1 Study Population

Our study utilised the database of the 'Establishing Adult Reference Intervals for Selected Analytes in South Africa', a study which was approved by the Health Research Ethics Committee of Stellenbosch University, Cape Town, South Africa. Healthy individuals, aged 18 – 65 years, were recruited from students, laboratory and hospital staff and the general public. The study criteria included males and females of South African Origin, aged 18 - 65 years of age, who were feeling subjectively well, who were not taking any medications except the contraceptive-pill or estrogens or thyroxin if the subject was on a maintenance dose. We excluded individuals who were not born in South Africa, obese individuals (body mass index ≥ 30 kg/m^2), heavy alcohol consumers (ethanol ≥ 70g/day), heavy smokers (tobacco >20 cigarettes/day), individuals taking regular medication for a chronic disease or recently (≤14 days) recovered from acute illness, injury, or surgery requiring hospitalization. Individuals with known carrier states of Hepatitis B Virus, Hepatitis C Virus, or Human Immunodeficiency Virus, those pregnant or within one year after childbirth and those with incomplete questionnaires or unsigned consent forms were also excluded from the study.

Blood samples were collected after a 12-14 hour fast, into serum separator tubes for determination of lipids TC, HDL-c, LDL-c, and TG. Blood was collected after subjects were seated for 20 to 30 minutes and centrifuged within 4 hours of collection. The serum was frozen at – 80 ^{o}C until analysis. Specimen analysis was carried out within six months of collection, at a single ISO 15189 accredited laboratory (PathCare Laboratories, Goodwood, Cape Town) was used for sample analysis.

All assays were performed on the Beckman DXC automated analyser using reagents from Beckman Coulter (Brea, CA). The LDL-c method is a 2- phase homogeneous method based on the Daiichi method (Nakamura, *et al.,* 1975). It depends on a unique detergent which solubilizes only the non-LDL lipoprotein particles, with the consumption of the products by an enzymatic reaction with cholesterol esterase and cholesterol oxidase, to yield a colourless product. In the second phase, a reagent containing N,N'- bis- (4- sulphobutyl)-m-toluidine disodium(DSBmT), buffer (pH 6.3), and a detergent which solubilizes the remaining LDL particles. A chromogenic coupler allows for colour formation and this is measured

spectrophotometrically. The method had an imprecision of less than 2%. The total cholesterol measurement was performed by a colorimetric 3-step enzymatic reaction. The CV was less than 3%. HDL-c assay was based on a 2- phase homogenous, colorimetric, enzymatic reaction, whose CV was less than 3%. The triglyceride procedure had a CV of less than 2%. It is based on a sequence of three coupled enzymatic reactions which yield a product measured colourimetrically (Buccolo & David, 1973).

2.2 Data analysis

Data was extracted from Microsoft Excel and analysed using Statistica version 11.0 software. Statsoft Inc. (2012). Pearson's correlation analyses of the LDL-c as determined by the Friedewald formula and the de Cordova formula, were performed against the directly measured LDL-c. These were repeated in different groups according to race; gender; and gender with race. Correlation analysis was performed for very low TG values (<1.13mmol/L) using the de Cordova formula and the modified Friedewald formula proposed by Ahmadi *et al.*, (LDL-c = TC/1.19 + TG/0.81 – HDL/1.1 – 0.98), from here on referred to as 'the Ahmadi formula' (Ahmadi & Boroumand, 2008). The Bland-Altman plot was employed to assess the agreement between the de Cordova formula and the direct LDL-c, and also the agreement between the Friedewald formula and the direct LDL-c. This was repeated at very low TG levels, also employing the Ahmadi formula. The range of agreement was defined as mean bias ± 2SD.

3. Results

A total of 587 subjects were recruited. Of these, 389 were females and 189 were males. There were 307 Caucasians (102 males), and 204 of Mixed Ancestry (60 males). Individuals of African ancestry constituted only 13% (49 females and 27 males). The range of direct LDL-c values was from 0.70 to 7.00 mmol/L. Total cholesterol levels ranged from 2.30 to 9.10mmol/L and HDL-c levels from 0.39 to 2.40 mmol/L, while TG levels ranged from 0.12 to 6.32 mmol/L. Two subjects had TG values > 4.5mmol/L. Very low (<1.13mmol/L) TG levels were seen in 81 % (473) of the subjects. **Table 1** shows the mean (SD) and the range of the LDL-c values as estimated by the direct assay, the Friedewald formula and the de Cordova formula.

Pearson's correlation analyses demonstrated a very high correlation between the de Cordova formula and the direct LDL-c ($r = 0.97$, $p < 0.001$). This was slightly lower than the correlation observed between the Friedewald formula and the direct LDL-c ($r = 0.98$, $p < 0.001$). These are shown in **Figure 1**.

At very low TG levels, the de Cordova formula and the Friedewald formula showed equally higher correlation ($r = 0.98$, $p < 0.001$) with the direct LDL-c than the modified Friedewald formula proposed by Ahmadi *et al*., for very low TG levels; which showed a correlation of $r = 0.92$, $p < 0.001$ as seen in **Figure 2.**

Various group analyses were carried out according to races (African, Mixed Ancestry and Caucasian), gender (males and females) and gender and race (African females/African males; Mixed Ancestry females/Mixed Ancestry males; and Caucasian females/Caucasian males), correlating the Friedewald formula and the de Cordova formula with the direct LDL-c in each of these groups.

Among male subjects, there was a slightly higher level of correlation with the Friedewald formula ($r = 0.98$, $p < 0.001$) compared to the de Cordova formula ($r = 0.97$, $p < 0.001$). This marginally higher difference was also seen among the female subjects with the Friedewald formula: $r = 0.98$, $p < 0.001$, and the de Cordova formula $r = 0.97$, $p < 0.001$.

In group analyses according to ethnicity, the Friedewald formula had a slightly higher correlation with the direct LDL-c ($r = 0.98$, $p < 0.001$) than the de Cordova formula ($r = 0.97$, $p < 0.001$) among Caucasian participants. A lower correlation was observed in both formulae, in Mixed Ancestry participants, where the Friedewald formula once again, had a slightly higher r value (0.97) than the de Cordova formula ($r = 0.96$, $p < 0.001$). Equal degrees of correlation were observed between each of the two formulae (de Cordova and Friedewald) and the direct LDL-c ($r = 0.98$, $p < 0.001$) among African participants

Sub-group analyses of the two formulae with the direct LDL-c, according to gender, together with race showed the highest correlations, using the Friedewald formula, in Afican males and Caucasian males ($r = 0.99$, $p < 0.001$). The lowest correlation with the direct LDL-c was found Mixed Ancestry males, using the de Cordova formula ($r = 0.94$, $p < 0.001$). Equal

degrees of correlation were found between each of the 2 formulae (de Cordova and Friedewald) and the direct LDL-c, among African females (r = 0.98) and Caucasian females (r = 0.97). The Friedewald formula however had a slightly higher correlation with the direct LDL-c than the correlation between the de Cordova formula and the direct LDL-c in the sub-groups of African males (r = 0.99 versus r = 0.97), Caucasian males (r = 0.99 versus r = 0.96) and Mixed Ancestry males (r = 0.96 versus r = 0.94).

Figure 3 shows a Bland-Altman plot of the difference of the direct LDL-c and the LDL-c derived from the de Cordova formula, against the mean of the two LDL-c values; this had a mean bias of 0.18 ± 0.50 mmol/L. A positive bias is also noted at lower LDL-c values, and a negative bias at higher LDL-c values. A similar plot of the difference between the direct LDL-c and the LDL-c derived by the Friedewald formula, against the mean of the two LDL-c values gave a mean bias of – 0.3 ± 0.36mmol/L.

At very low TG values, the Bland-Altman plot of the direct LDL-c and the LDL-c derived from the de Cordova formula gave a mean bias of 0.19 ± 0.44mmol/L, with a negative bias at high LDL-c values and a positive bias at low LDL-c values. A similar plot using the Friedewald formula gave a mean bias of -0.33 ±0.30mmol/L, while that using the Ahmadi formula gave a mean bias of 0.18 ± 0.65mmol/L. The three plots are shown in **Figure 4**.

4. Discussion

LDL-c is pivotal in the development of artherosclerosis and coronary artery disease. Its levels are used as targets in the diagnosis and management of dyslipidaemias. The Friedewald formula was developed over 40 years ago, as a convenient way to estimate LDL-c (Friedewald, Levy & Fredrickson, 1972). Several authors have demonstrated the important limitations of the Friedewald formula, its poor performance in various conditions and also its inability to meet the National Cholesterol Education Program (NCEP) requirement for imprecision for LDL-c determination. Other authors have proposed new formulae to circumvent these limitations of the Friedewald formula and recommended verification of these formulae in different populations before adoption (Ahmadi, *et al.,* 2008), (Ahmadi & Boroumand, 2008), (Anandaraja, *et al*., 2005), (Chen, *et al.,* 2010), (Hattori, *et al.,* 1998), (Teerakanchana, *et al.,*

2007). De Cordova *et al.* recently published a simple, presumably, more accurate formula for the estimation of LDL-c (De Cordova & De Cordova., 2013). We compared this newly published formula and the Friedewald formula, against LDL-c determined by a homogeneous method, in a healthy South African population of different ethnic origins.

The two formulae were generally, highly correlated with the direct LDL-c. However, as high correlation coefficients do not imply agreement of methods, we examined the degree of agreement between each of the two formulae and the direct LDL-c, using the Bland-Altman difference plot. The Bland-Altman plot suggests a lesser agreement exists between the de Cordova formula and the directly measured LDL-c. Using the de Cordova formula, an LDL-c will be underestimated by 0.18mmol/L, but this value can vary from -0.32 to 0.68mmol/L. This shows a level of discrepancy of up to 0.50 mmol/L for any value of LDL-c. In particular, the higher the LDL-c, the more the negative bias and the lower the LDL-c level, the more the positive bias, further making this large discrepancy unpredictable. The Friedewald formula, though showing an over-estimation of LDL-c by 0.3mmol/L, demonstrates a better agreement with the direct LDL-c as the degree of variation is from -0.66 to 0.07mmol/L. This variation of up to 0.36 mmol/L is narrower than the 0.50mmol/L observed with the de Cordova formula. In addition, there is a uniform distribution of points with the Bland-Altman plot for the Friedewald formula; hence the bias observed with the Friedewald formula is uniform at the various levels of LDL-c and therefore, more predictable (**Figure 3**).

At very low TG values, the Friedewald formula still outperformed the de Cordova and the Ahmadi formula. Though the de Cordova formula showed a lower mean bias than the Ahmadi formula, the presence of the inconsistent bias at different levels of LDL-c (positive bias at low LDL-c levels and a negative bias at high LDL-c levels) observed with the de Cordova formula, makes it less reliable for estimating LDL-c at these TG levels (**Figure 4**). This was not in agreement with the study by de Cordova *et al*, who observed a superior performance of the de Cordova formula to the Friedewald formula at low TG levels (De Cordova & De Cordova, 2013).

Therefore, these results imply theoretically, that there is a higher likelihood for a patient in a particular risk group classified according to the NCEP- ATP III classification for the prevention and management of adults at risk of coronary artery disease, to be erroneously

classified by the de Cordova formula, compared to the Friedewald formula. It also implies that this incorrect classification may be less predictable due to the presence of differing bias at various LDL-c levels. This has implications in the management of patients, as the estimated LDL-c values can easily vary unpredictably with the de Cordova formula, around decision limits and thus lead to unnecessary institution of therapy or withholding therapy in a patient requiring treatment.

Our finding of an overestimation of LDL-c by the Friedewald formula is in agreement with those of Cordova *et al. (*Cordova, *et al.,* 2004 and other authors (Ahmadi, *et al.,* 2008), (Anandaraja, *et al.,* 2005) but different from those of Vujovic *et al.* who evaluated different formulae for LDL-c estimation in a Serbian population and found that Friedewald formula significantly underestimates directly measured LDL-c in all TC, TG and LDL-c ranges (Vujovic, *et al.*, 2010). Tighe *et al.* also evaluated healthy individuals with TG levels < 4.52mmol/L and found that the negative bias in the Friedewald formula occurs frequently at low TG concentrations (Tighe, *et al.,* 2006).

Our study has a number of limitations. The first and major one is that it was carried out among healthy adults whose racial origins are representative of the South African population. However, the large Brazilian cohort which de Cordova *et al.* had studied may have included individuals with various medical conditions. In our study, there were only 2 values of TG greater than 4.5mmol/L; hence our comparison of the performance of the two formulae does not include extremely high TG levels in contrast to the de Cordova study. The use of a non-reference method for the determination of LDL-c may be a second source of limitation to our findings. However, the study by de Cordova *et al.* employed the Wako method, which is also a non-reference method that meets the NCEP standard for precision in the determination of LDL-c. Our method is a homogeneous LDL-c method based on the Daiichi method; it also meets the NCEP standard for precision. These differing findings from the two studies may be due to differing methods for the determination of LDL-c and HDL-c and the differences in the study population.

In conclusion, in a healthy South African population, our data has shown a slightly greater, more uniform bias but better agreement of the Friedewald formula with a directly measured LDL-c based on the Daiichi method when compared to the newly published formula

by de Cordova *et al.* Though there is a need to verify these findings in a larger South African cohort, including subjects with extremely elevated TG levels and other conditions in which the Friedewald formula has previously been found to be inaccurate, we advise that the Friedewald formula remains the formula of choice to calculate LDL-c in South Africa.

5. Acknowledgements

The authors would like to acknowledge PathCare Laboratories, South Africa, for the lipid analysis, Beckman Coulter for the reagents utilized for the study and Prof M Kidd for the statistical analysis.

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Table 1: Mean (SD) values and ranges of LDL-c as determined by the direct LDL-c assay and the Friedewald and de Cordova formulae.

LDL-c	**Mean(SD) (mmol/L)**	**Range (mmol/L)**
Direct LDL-c	2.79 (0.87)	0.70 – 7.00
De Cordova	2.61 (0.72)	0.85 – 5.72
Friedewald* LDL-c	3.09 (0.86)	0.82 – 7.24

*Includes 2 subjects who had TG values greater than 4.5mmol/L.

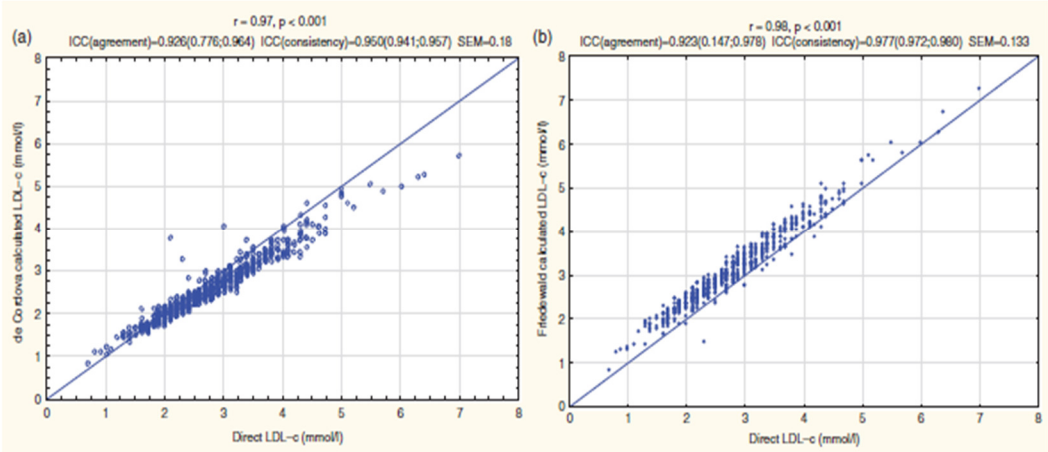

Figure 1: Correlation of LDL-c calculated by the De Cordova formula with the direct LDL-c (a) and correlation ofLDL-c calculated by the Friedewald formula with the direct LDL-c (b).

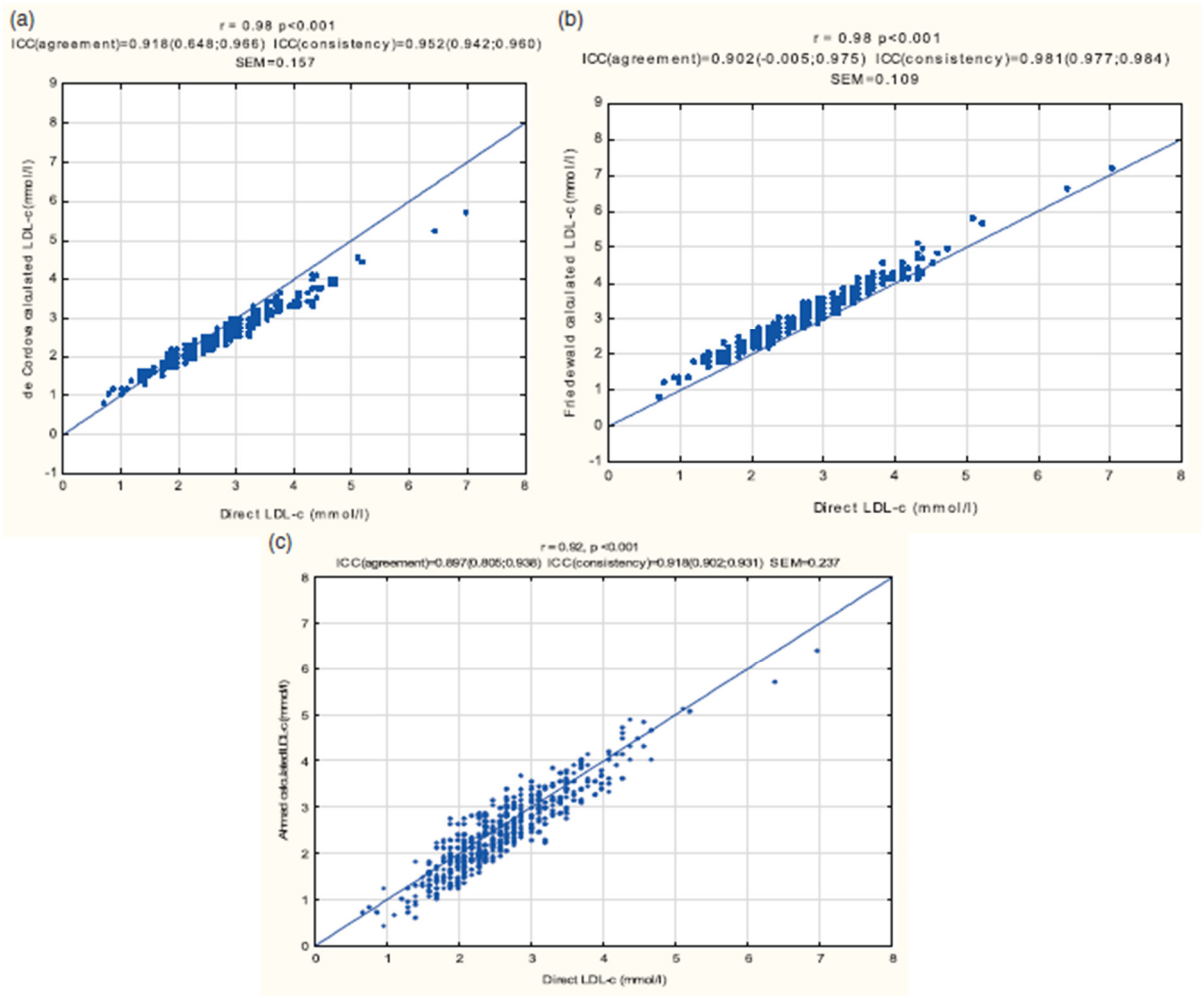

Figure 2: Correlation of LDL-c calculated by the de Cordova formula (a), the Friedewald formula (b) and the Ahmadi formula (c), with the direct LDL-c at very low TG levels.

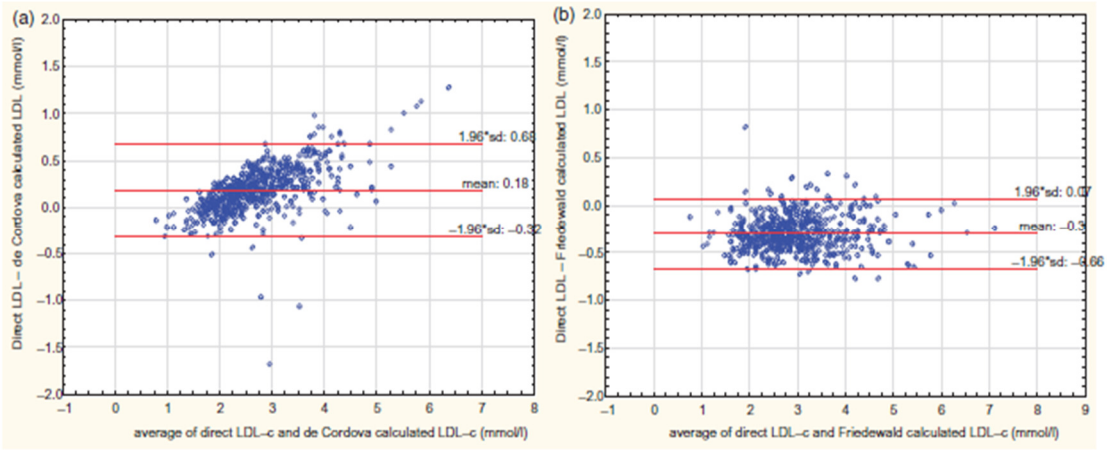

Figure 3: Bland-Altman plots of direct LDL-c and de Cordova calculated LDL-c (a), and direct LDL-c and Friedewald calculated LDL-c (b).

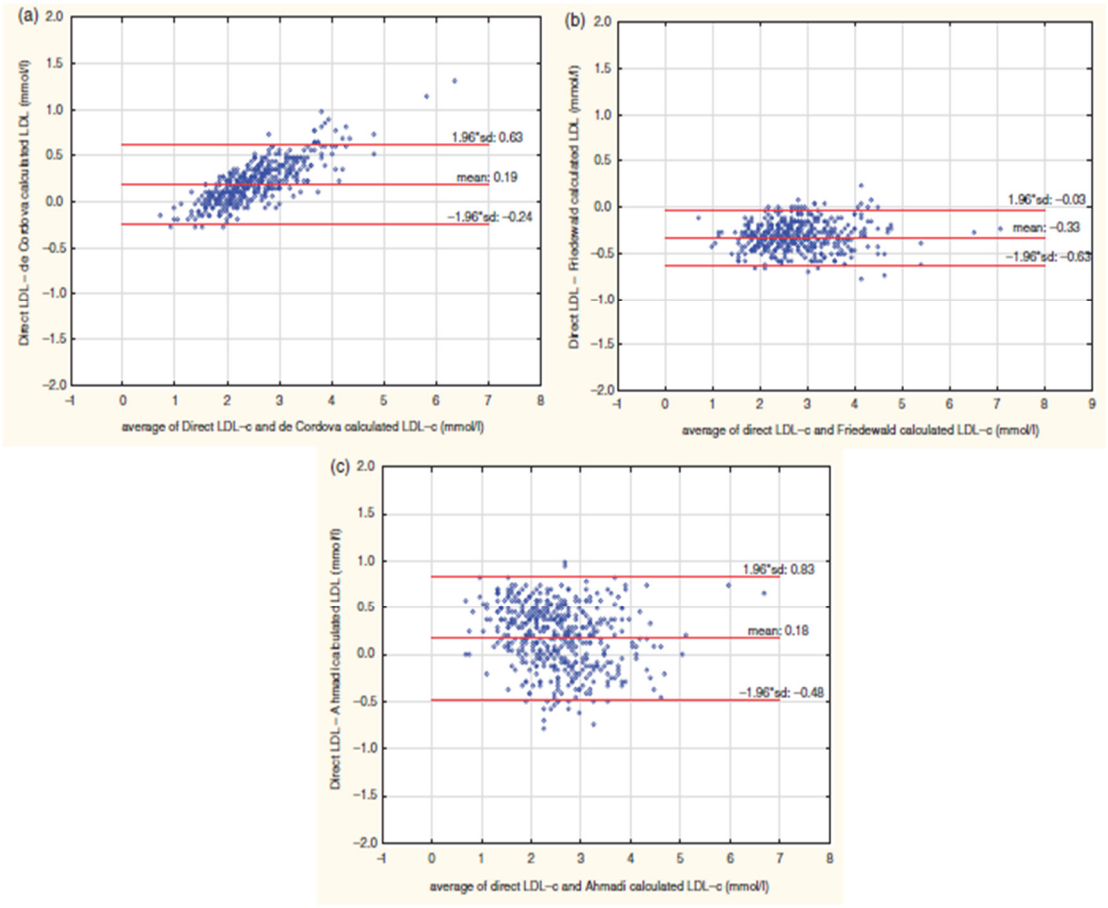

Figure 4: Bland-Altman plots of direct LDL-c and LDL-c estimated by the de Cordova formula (a), Friedewald formula (b) and Ahmadi formula (c) at very low TG levels (<1.13 mmol/L).

CONCLUSION

In this study, we successfully derived RIs for the South African population by comparing the values obtained from 3 racial groups. We proved that the P method is superior in the determination than the NP method. Furthermore, this was also the case with other research projects in Africa (Omuse, *et al.,* 2018) and globally (Borai, *et al*., 2016), (Ichihara, *et al.,* 2013), (Ichihara, *et al.,* 2016), (Ozarda, *et al.,* 2014) who participated in the global project and who made use of the same standardised recruitment – and analysis protocol suggested by Ozarda *et al.* (Ozarda, *et al.,*2013).

The need for LAVE in deriviation of RIs was also well demotrated as described earlier. LAVE has several advantages namely: 1) truncation of reference distributions does not occur, because this method excludes those participants whose results are outside of the RI of any given analyte, other than the analyte for which the RI is being derived and 2) there is no effect at all on alaytes whose values are on rare occations just outside the normal RI of the analyte (Ichihara, *et al*., 2016). There is, however; an disadvantage of using LAVE because when applied it causes a reduction in the number of participants whose results are included in the final computation of the RIs.

We looked at a number of sources of variation in the derivation of South African RIs namely: ethnicity, gender, age, and BMI. Differences in reference values were noted between Afr and non-Afr participants in many analytes as described earlier. Furthermore, our results compared well to the global harmonised study where differences in RIs was observed in all the participating countries Ichihara, *et al.,* 2013).

Partitioning of RIs by gender was required in 4 analytes: albumin, creatinine, HDL-cholesterol and the LAVE method led to prominent changes in RIs for 7 analytes: triglyceride, AST, ALT, GGT, CK, CRP and ferritin. The superiority of the parametric method (P) over the non-parametric method (NP) was also shown from the narrower confidence limits of the RIs. Prominent racial differences in RIs were observed for urea, total cholesterol, LDL-C, ALT, amylase, CRP, immunoglobulin G and A, requiring partition of RVs into African and non-African (Cau+Mix), therefore emphasizing the need to partition by race. By comparing the results obtained in this study in the Afr population group and the results of the international study, the Afr population showed vast differences in RI's in certain analytes namely: Further partition by age (< 45 and > 45 years of age) was required for TC, LDL-C, and urea (for

females). The LAVE method was effective in modifying the RIs for triglyceride, ALT, GGT, and CRP. Parametric methods led to invariably narrower confidence intervals of RI limits than the nonparametric method.

Age related changes was seen in 24 analytes namely: Alb, Urea, UA, Cre, TBil, Ca, IP, Mg, TC, TG, HDL-C, LDL-C, Lip, ALP, CK, GGT, ChE, Ferritin, Tf, C-RP, IgA, IgM, FT3 and PSA in males.

BMI related changes were observed in 12 analytes when comparing Afr with non-Afr ethnic groups namely: UA, TG, HDL-C, LDL-C, ALT, AST, ALP, LDH, CK, GGT, C-RP and Ferr.

Looking at haematological parameters, RIs for Caucasian and Mixed Ancestry individuals were similar while white cells, monocyte and neutrophil RIs were significantly lower in African participants. There were however no clinically significant differences between the eosinophil, basophil, lymphocyte and platelet counts across the three population groups.

The RIs for GA% of 663 healthy individuals (mean age, 34 years, 38.6% males) ranged from 10.7% to 15.2%. The median GA% in females were higher than in males. Females had a reference interval of 11.0% to 15.5%, whereas it was 10.6% to 14.4% in males. The median GA% for Cau and MA subjects were both 12.9%, whereas Afr subjects had a higher median GA% of 13.3%. As an inverse relationship was observed between BMI and GA%, we determined reference intervals in obese, overweight and normal weight subjects. The GA% reference intervals for subjects with BMI <25 kg/m^2 was 11.2% to 15.3%, for BMI 25-30 kg/m^2 it was 10.5% to 14.9% and 10.0% to 14.6% for BMI >30 kg/m^2. The overall reference interval showed good correlation with reference intervals determined in other studies and we have for the first time determined these values in obese and non-obese healthy South Africans.

Our iron study evaluation showed that the prevalence of anaemia was 12.6% and iron deficiency was found in 78% of anaemic subjects. The prevalence of iron deficiency was 39.8% in all participants and females and Africans had a very high prevalence of 56.6% and 50.7% respectively. Significant differences were found in concentrations of ferritin, haemoglobin, iron, transferrin, transferrin saturation, MCV and MCH between the groups. Anaemia is a

minor health problem but a large proportion of subjects with iron deficiency do not present with anaemia. The prevalence of iron deficiency was high especially in females and Afr participants.

We also investigated the determination of LDL – d by comparing the Friedewald formula and the de Cordova formula. The Friedewald formula and the de Cordova formula both had high correlations with the direct LDL-c , although the latter showed an inconsistent bias at different LDL-c levels. The two formulae had a higher correlation than the Ahmadi formula at very low TG levels. However, the Friedewald formula showed better agreement with the direct LDL- c than the de Cordova formula, at various LDL-c levels, in our population. It also outperformed the Ahmadi formula at very low TG levels. We therefore advise that it remains the formula of choice for LDL-c estimation in South Africa.

This project had quite a few strenghts. As a part of global multi-centre collaborative project for derivation of RIs, this study is considered to be the largest and first study of this nature for the South African population to establish RIs for the majority of general biochemistry - and haematological analytes under a standardized and harmonised protocol (Ozarda, *et al.,*2013) and furthermore, for those results to be compared to this standardised protocol. The subjects were healthy, without diabetes, and confirmed to be non-diabetic by means of fasting blood glucose measurement. The wide array of measurements performed during the previous reference interval study allowed exclusion of subjects with conditions that may have confounding effects on the results. Furthermore, we have also demonstrated that the MA population of the Western Cape share similar reference intervals to those of Cau descent. One of the most significant findings in this study was to determine which participants we had to exclude due to latent disease of common prevalence as described in detail earlier.

The project also had limitations. One major limitation of the study was the recruitment of a small number of participants from the mixed ancestry population group. This population group had to be combined with he caucasian population group in some instances. This was then named non-Afr as the results of these 2 population groups were similair when compared. This could mask the effect of latent disease in the mixed ancestry population group which could not identified due to the small number of participants. Lower number of Afr volunteers was also recruited in comparison to Cau. There was not an equal distribution of subjects according

to gender and ethnicity. The ethnic distribution of the sample was not representative of the ethnic distribution of the South African population at national or provincial level as was described earlier.

Further investiagion into racial differences in RIs would be of great value when comparing South Africa's results with the other ongoing projects in Africa. Africa has many diverse population groups and it has been recommended that RIs be generated for each region on the continent. The demographics of South Africa, Africa and the world are constantly changing and therefore laboratory professionals should be constantly aware of the influence gender, age, BMI and ethnicity has on the interpretation of laboratory results.

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APPENDIX DOCUMENTS

APPENDIX A: AFRICA REFERENCE RANGE STUDY CHECKLIST

BARCODE NUMBER

PLEASE NOTE: A COMPLETED COPY OF THIS CHECKLIST MUST ACCOMPANY ALL PARTICIPANT DOCUMENTATION.

CHECKLIST (Please tick each block with a √ to verify that the item was checked and completed)

1) Verify the pathology request form is correctly marked off for male or female participant. ☐

2) Verify that the correct test profile was marked off on the request form (male or female). ☐

3) Verify that each participant has fully completed their questionnaire. ☐

5) Verify that the participant AND phlebotomist has signed and dated the informed consent form. Ensure that the form is correctly labelled with the correct participant code as well as laboratory requisition number. ☐

6) Verify that all the documentation has been correctly labelled with the participant's study code as well as the correct laboratory requisition number (barcode label). ☐

7) Verify that the following blood collection tubes was collected from each participant:

- SST (yellow top) X 3 ☐
- EDTA (purple top) x 1 ☐
- Fluoride (grey top) x 1 ☐
- Lithium Heparin (green top) x 1 ☐

8) Verify that the collection tubes are correctly labelled with the same participant study code number as well as the correct laboratory requisition number (barcode) AND that it corresponds to the completed documentation. ☐

Name of the person who completed this check list (print): _____

Signature of the person who completed this checklist: _____

Date: _____

APPENDIX B

PARTICIPANT INFORMATION LEAFLET AND CONSENT FORM

ESTABLISHING ADULT REFERENCE VALUES FOR SELECTED ANALYTES

REFERENCE NUBER: S12/05/147

INVESTIGATOR: Francois Christiaan Smit

CO-INVESTIGATORS: J Wassung, Dr Mariza Hoffman, Profs. Rajiv Erasmus, Tandi Matsha and Kyoshi Ichihara (Japan)

ADDRESS: Department Biomedical Sciences, Cape Peninsula University of Technology, Bellville, Cape Town
Department of Chemical Pathology, NHLS, 9th floor, Tygerberg Hospital, Cape Town

CONTACT NUMBERS:
Francois Smit – 083 483 6678
Dr Mariza Hoffmann - (021) 938 4165
Professor RT Erasmus – (021) 938 4107

You are being invited to take part in a research project. Please take some time to read the information presented here, which will explain the details of this project. Please ask the study staff or doctor any questions about any part of this project that you do not fully understand. It is very important that you are fully satisfied that you clearly understand what this research entails and how you could be involved. Also, your participation is **entirely voluntary** and you are free to decline to participate. If you say no, this will not affect you negatively in any way whatsoever. You are also free to withdraw from the study at any point, even if you do agree to take part. This study has been approved by the Health Research Ethics Committee at Stellenbosch University and will be conducted according to the ethical guidelines and principles of the international Declaration of Helsinki, South African Guidelines for Good Clinical Practice and the Medical Research Council (MRC) Ethical Guidelines for Research.

What is this research study all about?

The department of Chemical Pathology (NHLS, Tygerberg Hospital), (to be replaced by the names of other participating institutions) is in the process of reviewing "normal values" for selected blood test. Doctors will use these normal values to compare against values of patients in order to assess the health of an individual e.g. comparing the blood sugar level of a diabetic patient with that of normal individuals.

A total number of ± 2000 healthy individuals, aged 18 – 80, will be asked to take part in the study. Candidates will be recruited from Tygerberg Hospital staff, Tygerberg community and students/staff from the University of Stellenbosch. The project is planned for 2012-2015.

You will be asked about your health status and your race. It is extremely important that you answer these questions honestly and to the best of your ability. The question on your race is important, as we want to see if we have to determine different normal values for different racial groups (this has been shown to be of value in various overseas countries). We will be unable to analyse your test results appropriately if this question is not answered.

All healthy (male and female) individuals will be considered for the study. Individuals with the following conditions will not be included in the study and samples will not be analysed:

- Under regular medications for illnesses (e.g. high blood sugar, high blood pressure, allergies and depression)?
- Underweight or overweight individuals
- Pregnant females or within 1 year after child birth
- Alcohol: more than 1400ml beer OR 580ml wine OR 175ml whiskey/brandy etc
- Individuals who had an operation or had to be admitted to hospital for an illness in the past 2
- Smoking more than 20 cigarettes per day

Why have you been invited to participate?

Since you are not known with any of the above conditions or diseases, you are seen as a "healthy individual" and we expect that all your test results will be "normal". Therefore, we will be able to use your test results to calculate "normal values".

What will your responsibilities be?

In order for this project to be a success, healthy individuals are expected to complete a questionnaire and allow for height, weight and blood pressure measurement. A small amount of blood (approximately 2 tablespoons) will be collected from selected individuals. This will have no adverse effects on the health of individuals.

Will you benefit from taking part in this research?

This study would be beneficial to the participants in that results of an individual will be made available, with comments provided by the Department of Chemical Pathology regarding the observed results. If results are severely abnormal, a referral letter will be written to your local clinic or general practitioner. There will be no direct financial benefits to individuals involved in the study. The establishment of normal values for our local population will help doctors to treat patients who are sick.

Are there any risks involved in your taking part in this research?

Apart from discomfort at the site of where bloods are drawn, there are no risks involved.

If you do not agree to take part, what alternatives do you have?

Your participation in this study is completely voluntary and you can withdraw at any stage of the study. Your participation will not influence any treatment you are currently on. You can withdraw from the study at any stage, in which event your stored samples will be discarded.

Who will have access to your data?

Your information will not be made public. Only the principal and co-investigators in the study will have access to your result. In addition, once we have drawn your blood, your samples and information will be identified with a number only. If the results of the study are published, your identity will not be made known.

What will happen in the unlikely event of some form of injury occurring as a direct result of your taking part in this research study?

There are no real risks that can result in any serious form of injury. A qualified medical doctor or nurse will draw a small amount of blood from the participants.

Is there anything else that you should know or do?

You can contact the Committee for Human Research at 021-938 9207 if you have any concerns or complaints that have not been adequately addressed by your study doctor.

Declaration by participant

By signing below, I ……..……. agree to take part in a research study entitled:

THE ESTABLISHMENT OF NORMAL ADULT REFERENCE VALUES FOR A SELECTED PANEL OF LABORATORY ANALYTES IN SOUTH AFRICA

I declare that:

- I have read or had read to me this information and consent form and it is written in a language with which I am fluent and comfortable.
- I have had a chance to ask questions and all my questions have been adequately answered.
- I understand that taking part in this study is voluntary and I have not been pressurized to take part.
- I may choose to leave the study at any time and will not be penalized or prejudiced in any way.
- I may be asked to leave the study before it has finished, if the study doctor or researcher feels it is in my best interests, or if I do not follow the study plan, as agreed to.

Signed at (*place*) .....….....…….. on (*date*) ……...…….. 20.....

Signature of participant……

Signature of witness……

Declaration by investigator

I *(name)* ……..…… declare that:

- I explained the information in this document to ……..
- I encouraged him/her to ask questions and took adequate time to answer them.
- I am satisfied that he/she adequately understands all aspects of the research, as discussed above
- I did/did not use a translator. (*If a translator is used then the translator must sign the declaration below.*

Signed at (*place*) .....….....…….. on (*date*) ……...…….. 20.....

Signature of investigator……

Signature of witness……

Declaration by translator (if required)

I *(name)* ……..…… declare that:

- I assisted the investigator (*name*) ……to explain the information in this document to (*name of participant*) ……..…….. using the language medium of Afrikaans/Xhosa.
- We encouraged him/her to ask questions and took adequate time to answer them.
- I conveyed a factually correct version of what was related to me.
- I am satisfied that the participant fully understands the content of this informed consent document and has had all his/her question satisfactorily answered.

Signed at (*place*) .....….....…….. on (*date*) ……...…….. 20.....

Signature of translator……..

Signature of witness……

APPENDIX C

DEELNEMERINLIGTINGSBLAD EN -TOESTEMMINGSVORM

"THE ESTABLISHMENT OF NORMAL ADULT REFERENCE VALUES FOR A SELECTED PANEL OF LABORATORY ANALYTES IN SOUTH AFRICA"

VERWYSINGSNOMMER: S12/05/147

HOOFNAVORSER: Francois Christiaan Smit

MEDENAVORSERS: J Wassung, Dr. Mariza Hoffmann, Profs. Rajiv Erasmus, Tandi Matsha en Kyoshi Ichihara (Japan)

ADRES: Departiment Biomediese Wettenskappe, Cape Peninsula Unirsiteit van Tegnologie, Bellville, Kaapstad
Departement van Chemiese Patologie, NHLS, 9de Vloer, Tygerberg Hospitaal, Kaapstad

KONTAKNOMMERS:
Francois Smit- 083 483 6678
Dr M Hoffmann – 021 938 4165
Prof RT Erasmus – 021 938 4107

U word genooi om deel te neem aan 'n navorsingsprojek. Lees asseblief hierdie inligtingsblad op u tyd deur aangesien die detail van die navorsingsprojek daarin verduidelik word. Indien daar enige deel van die navorsingsprojek is wat u nie ten volle verstaan nie, is u welkom om die navorsingspersoneel of dokter daaroor uit te vra. Dit is baie belangrik dat u ten volle moet verstaan wat die navorsingsprojek behels en hoe u daarby betrokke kan wees. U deelname is ook **volkome vrywillig** en dit staan u vry om deelname te weier. U sal op geen wyse hoegenaamd negatief beïnvloed word indien u sou weier om deel te neem nie. U mag ook te eniger tyd aan die navorsingsprojek onttrek, selfs al het u ingestem om deel te neem.

Hierdie navorsingsprojek is deur die Gesondheidsnavorsingsetiekkomitee (GNEK) van die Universiteit Stellenbosch **goedgekeur en sal uitgevoer word volgens die etiese riglyne en beginsels van die Internasionale Verklaring van Helsinki en die Etiese Riglyne vir Navorsing van die Mediese Navorsingsraad (MNR).**

Wat behels hierdie navorsingsprojek?

Die Departement van Chemiese Patologie (NHLS, Tygerberg Hospitaal) is besig om die "normaal waardes" wat by laboratorium toetse gerapporteer word, te hersien. Dokters sal hierdie "normaal waardes" gebruik om met pasiente se waardes te vergelyk om sodoende siektes te kan optel.

Daar sal in totaal 2000 mense gevra word om aan die studie deelneem, wie tussen die ourderdom van 18 en 80 jaar moet wees. Personeel en studente van Tygerberg Hospitaal, asook gesonde mense in die gemeenskap, sal gevra word om deel te neem. Die projek word beplan vir 2012 – 2015.

U sal uitgevra word oor u algemene gesondheid en u ras. Dit is besonder belangrik dat u hierdie vrae so eerlik en volledig antwoord as moonlik. U ras is van groot belang, aangesien ons wil bepaal of verskillende normaalwaardes vir verskillende rasse in ons land nodig is (verskeie oorsese studies het aangetoon dat dit baie belangrik is). Dit sal nie moontlik wees om u toetse ordentlik te kan interpreteer as die inligting nie verskaf word nie.

Alle gesonde individue sal gevra word om aan die studie deel te neem, en individue met die volgende, sal nie in die studie ingesluit word nie:

- Onder behandeling vir ‘n siekte, bv hoe bloeddruk, suikersiekte, allergie of depressie
- Ondergewig of oorgewig
- Swanger, of ‘n vrou wie in die afgelope jaar ‘n baba gehad het
- Alkohol gebruik van meer as 1400ml bier per dag, of 580 ml wyn per dag, of 175ml sterk drank per dag (whiskey of brandewyn)
- Rook meer as 20 sigarette per dag

Waarom is u genooi om deel te neem?

Aangesien u nie met een van die bogenoemde toestande/gewoontes bekend is nie, word u gesien as ‘n “gesonde persoon” en ons werwag dat al u toets uitslae “normaal” sal wees. Om die rede sal ons in staat wees om u toets uitslae te kan gebruik om “normaal waardes” vir toetse saam te stel

Wat sal u verantwoordelikhede wees?

Vir die studie om ‘n sukses te wees, sal u verwag word om a lys vrae te beandwoord en dan toe tel laat dat die studie personeel u weeg, meet en u bloeddruk bepaal. Daarna sal ‘n klein bietjie bloed (ongeveer 2 eetlepels vol) van u getrek word. Dit sal vir u geen gevaar inhou nie.

Sal u voordeel trek deur deel te neem aan hierdie navorsingsprojek?

Die studie sal u baat, want ‘n klomp toetse sal op u bloed uitgevoer word, en u sal die uitslae verniet kry. As abnormale uitslae gevind word, sal u deur middel van ‘n brief na u kliniek/huisdokter verwys word. U sal nie betaal word om and die studie deel te neem nie. Die opstel van normaal waardwes vir ons plaaslike bevolking sal dokters help om siek pasiente te diagnoseer en te behandel.

Is daar enige risiko's verbonde aan u deelname aan hierdie navorsingsprojek?

U sal geringe ongemak ervaar terwyl u bloed getrek word. Verder is daar geen risiko’s verbonde aan die studie.

Watter alternatiewe is daar indien u nie instem om deel te neem nie?

U deelname aan die studie is totaal vrywillig en u kan op enige stadium van die studie onttrek. U deelname/ ontrekking van die studie sal nie u heidige behandeling beinvloed nie. As u van die studie onttrek, sal die bloed wat by u getrek is, weggegooi word.

Wie sal toegang hê tot u mediese rekords?

U informasie sal nie bekend gemaak word nie. Slegs die hoof navorser en medenavorsers in die studie sal toegang he tot u informasie en toetsuitslae. Sodra u bloed getrek is, sal u slegs deur middel van ‘n studiekode identifiseer kan word. As die uitslae van die studie gepubliseer gaan word, sal u identiteit nie bekend gemaak word nie.

Wat sal gebeur in die onwaarskynlike geval van ’n besering wat mag voorkom as gevolg van u deelname aan hierdie navorsingsprojek?

Daar is geen risiko verbonde aan die deelname aan die studie nie. ‘n Gekwalifiseerde dokter/suster/verpleegster sal bloed trek by deelnemers.

Verklaring van deelnemer

Met die ondertekening van hierdie dokument onderneem ek, …….....……...……..……., om deel te neem aan ’n navorsingsprojek met die titel:

“THE ESTABLISHMENT OF NORMAL ADULT REFERENCE VALUES FOR A SELECTED PANEL OF LABORATORY ANALYTES IN SOUTH AFRICA”

Ek verklaar dat:

- Ek hierdie inligtings- en toestemmingsvorm gelees het of aan my laat voorlees het en dat dit in ’n taal geskryf is waarin ek vaardig en gemaklik mee is.
- Ek geleentheid gehad het om vrae te stel en dat al my vrae bevredigend beantwoord is.
- Ek verstaan dat deelname aan hierdie navorsingsprojek **vrywillig** is en dat daar geen druk op my geplaas is om deel te neem nie.
- Ek te eniger tyd aan die navorsingsprojek mag onttrek en dat ek nie op enige wyse daardeur benadeel sal word nie.
- Ek gevra mag word om van die navorsingsprojek te onttrek voordat dit afgehandel is indien die studiedokter of navorser van oordeel is dat dit in my beste belang is, of indien ek nie die ooreengekome navorsingsplan volg nie.

Geteken te *(plek*) .....……. op *(datum*) ……...…….. 20.....

Handtekening van deelnemer ……

Handtekening van getuie ……

Verklaring van navorser

Ek *(naam)* ……...…… verklaar dat:

- Ek die inligting in hierdie dokument verduidelik het aan …….....……..
- Ek hom/haar aangemoedig het om vrae te vra en voldoende tyd gebruik het om dit te beantwoord.
- Ek tevrede is dat hy/sy al die aspekte van die navorsingsprojek soos hierbo bespreek, voldoende verstaan.
- Ek ’n tolk gebruik het/nie ’n tolk gebruik het nie. (*Indien ’n tolk gebruik is, moet die tolk die onderstaande verklaring teken.)*

Geteken te *(plek*) .....……. op *(datum*) ……...…….. 20.....

Handtekening van deelnemer ……

Handtekening van getuie ……

Verklaring van tolk (indien van toepassing)

Ek *(naam)* ……...…… verklaar dat:

- Ek die navorser *(naam*) …….....……. bygestaan het om die inligting in hierdie dokument in Afrikaans/Xhosa aan……..... te verduidelik.
- Ons hom/haar aangemoedig het om vrae te vra en voldoende tyd gebruik het om dit te beantwoord.
- Ek ’n feitelik korrekte weergawe oorgedra het van wat aan my vertel is.
- Ek tevrede is dat die deelnemer die inhoud van hierdie dokument ten volle verstaan en dat al sy/haar vrae bevredigend beantwoord is.

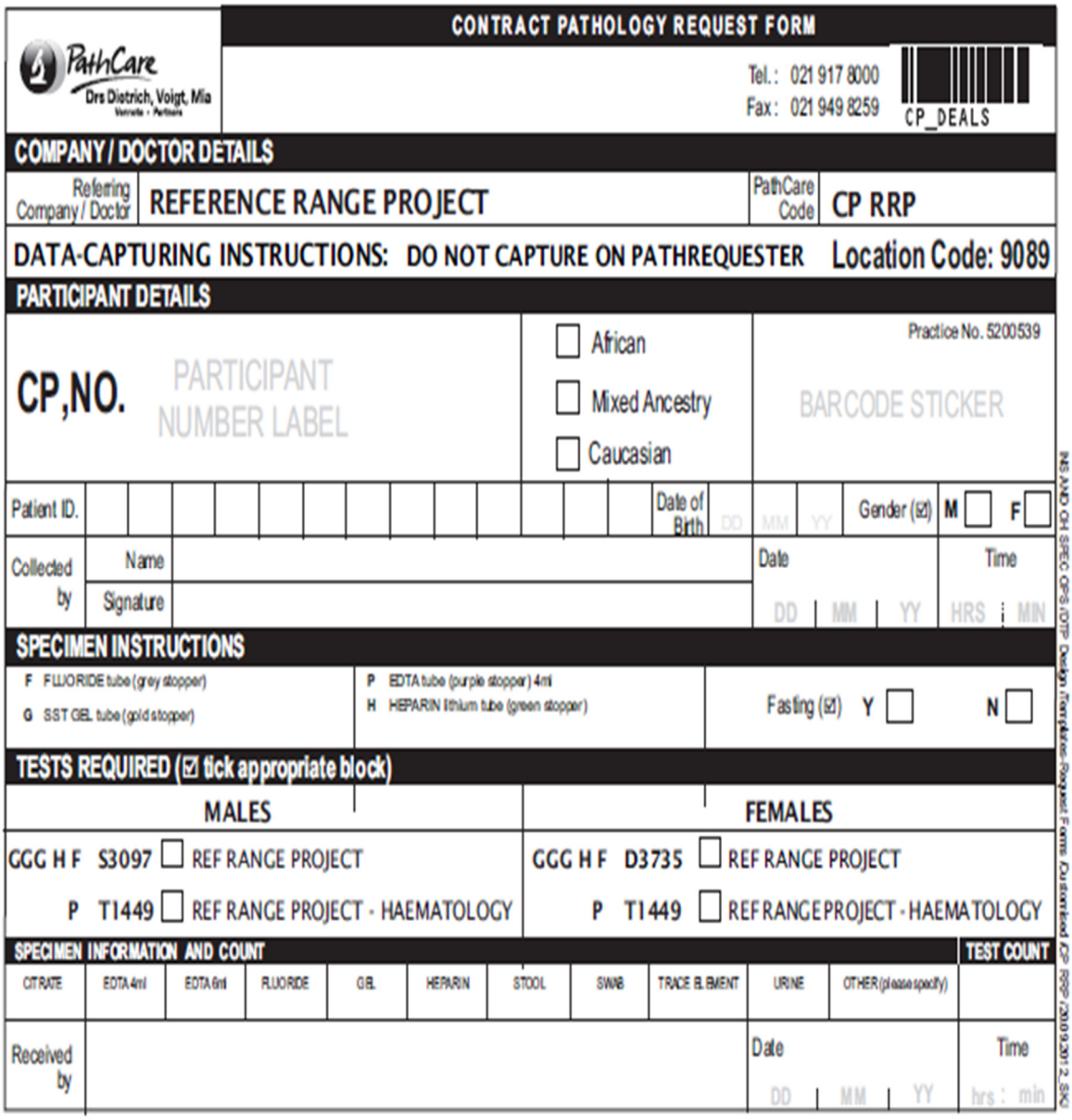
Geteken te *(plek*) .....……. op *(datum*) ……...…….. 20.....

Handtekening van deelnemer ……

Handtekening van getuie ……

APPENDIX D

REFERENCE RANGE STUDY PATHCARE PATHOLOGY REQUEST FORM



PathCare
Drs Dietrich, Voigt, Mia
Vennote + Partners

CONTRACT PATHOLOGY REQUEST FORM

Tel.: 021 917 8000
Fax: 021 949 8259

CP_DEALS

COMPANY / DOCTOR DETAILS

Referring Company / Doctor	REFERENCE RANGE PROJECT	PathCare Code	CP RRP

DATA-CAPTURING INSTRUCTIONS: DO NOT CAPTURE ON PATHREQUESTER **Location Code: 9089**

PARTICIPANT DETAILS

CP,NO. PARTICIPANT NUMBER LABEL

☐ African
☐ Mixed Ancestry
☐ Caucasian

Practice No. 5200539

BARCODE STICKER

Patient ID. | Date of Birth DD MM YY | Gender (☑) M ☐ F ☐

Collected by: Name | Signature | Date DD MM YY | Time HRS : MIN

SPECIMEN INSTRUCTIONS

F FLUORIDE tube (grey stopper)
G SST GEL tube (gold stopper)
P EDTA tube (purple stopper) 4ml
H HEPARIN lithium tube (green stopper)

Fasting (☑) Y ☐ N ☐

TESTS REQUIRED (☑ tick appropriate block)

MALES	FEMALES
GGG H F S3097 ☐ REF RANGE PROJECT	GGG H F D3735 ☐ REF RANGE PROJECT
P T1449 ☐ REF RANGE PROJECT - HAEMATOLOGY	P T1449 ☐ REF RANGE PROJECT - HAEMATOLOGY

SPECIMEN INFORMATION AND COUNT

CITRATE	EDTA 4ml	EDTA 6ml	FLUORIDE	GEL	HEPARIN	STOOL	SWAB	TRACE ELEMENT	URINE	OTHER (please specify)	TEST COUNT

Received by | Date DD MM YY | Time hrs : min

INS AND OH SPEC OPS IDTP Design Templates Request Forms Customised CP RRP 20093012.SK

APPENDIX E: PARTICIPANT QUESTIONNAIRE

INTERNATIONAL REFERENCE RANGE STUDY QUESTIONAIRE

Study number ☐ Laboratory number ☐

Date of sampling ☐ Year/month/Day Time of sampling ☐ hour : minutes

CONTACT DETAILS OF PARTICIPANT

Name and surname: _____
ID number: _____
Postal address: _____
_____
Contact numbers: Cell: _____ Home/Office: _____ Fax: _____
E-mail address: _____

INCLUSION CRITERIA

1. Do you consider yourself healthy? ☐ NO ☐ YES
2. Are you at least 18 years of age? ☐ NO ☐ YES

EXCLUSION CRITERIA

1. Are you obese (BMI > 30 kg/m2) ☐ NO ☐ YES
2. Are you a heavy drinker (> 5 glasses of wine/5 beers/5 shots per day)? ☐ NO ☐ YES
3. Are you a heavy smoker (> 20 cigarettes/day)? ☐ NO ☐ YES
4. Do you have/had chronic disease (including diabetes, hypertension, depression etc) requiring regular medication? ☐ NO ☐ YES
5. Do you have results from a blood test that point to a severe disease? ☐ NO ☐ YES
6. Have you been hospitalized or seriously ill in the past 2 weeks? ☐ NO ☐ YES
7. Have you given blood as a donor in the previous 3 months? ☐ NO ☐ YES
8. Are you a known carrier of Hepatitis B/C or HIV? ☐ NO ☐ YES
9. Are you pregnant or within one year after childbirth? ☐ NO ☐ YES
10. Have you participated in a research study involving an investigational drug in the past 12 weeks? ☐ NO ☐ YES

DEMOGRAPHICS

Are you fasting ? ☐ NO ☐ YES
Last food intake: ☐ (AM / PM)
Time since last meal ☐ (hours)

Age: ☐ years

Gender: ☐ Male ☐ Female

Ethnicity: ☐ Caucasian ☐ African ☐ Mixed ☐ Other (specify) _____
Descent of origin: _____

Occupation: ☐

Measurements: Height ☐ cm Skin pigment reading ☐
Weight ☐ kg Waist circumference ☐ cm

Blood pressure: ☐ mmHg

ABO blood type: ☐ A ☐ B ☐ AB ☐ O ☐ not known

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PERSONAL HABITS

Do you smoke? ☐ NO ☐ YES

If yes, how many per day for how many years? ☐ nr/day ☐ total nr of years

Do you drink alcohol? ☐ NO ☐ YES

If yes, how often? ☐ Rarely ☐ 1x/week ☐ 2 - 3 x/w ☐ 4 - x/w ☐ every day

If yes, how much do you drink in the typical week.

Beer	☐	litres
Cider	☐	litres
Wine	☐	glasses (6 glasses/bottle)
Spirits	☐	bottles

* Calculation by the investigator:
Amount of alcohol consumption in gram of ethanol per day and week

☐ grams per day

☐ grams per week

PHYSICAL ACTIVITIES AND EXERCISE

Daily activities

Time spent standing/walking	☐	hours/day
Time spent sitting	☐	hours/day
Time spent sleeping	☐	hours/day
Time spent outdoors	☐	hours/day

Physical activity at work

Does your work involve vigorous activity? ☐ NO ☐ YES

If yes, how much time do you spend on the activity (e.g. digging)? ☐ hours ☐ min/typical day

Does your work involve moderate intensity activity (e.g. walking)? ☐ NO ☐ YES

If yes, how much time do you spend on this activity? ☐ hours ☐ min/typical day

Physical activity (sports, fitness, recreation) on a regular basis (at least once per week for > 1 year)

Do you do any vigorous exercise regularly (e.g. running, aerobics)? ☐ NO ☐ YES

If yes, what type? ☐

If yes, frequency and duration ☐ days/wk ☐ hrs + min on a typical day

Do you do any moderate-intensity exercise regularly (e.g. brisk walking)? ☐ NO ☐ YES

If yes, what type? ☐

If yes, frequency and duration ☐ days/wk ☐ hrs + min on a typical day

MEAL PATTERN (frequency and amount per week)

Meat	☐ None	☐ 1 - 2 x/W	☐ 3 - 4 x/W	☐ 5 - 6 x/W	☐ every day
Fish	☐ None	☐ 1 - 2 x/W	☐ 3 - 4 x/W	☐ 5 - 6 x/W	☐ every day
Vegetables	☐ None	☐ 1 - 2 x/W	☐ 3 - 4 x/W	☐ 5 - 6 x/W	☐ every day
Beans	☐ None	☐ 1 - 2 x/W	☐ 3 - 4 x/W	☐ 5 - 6 x/W	☐ every day
Milk/diary	☐ None	☐ 1 - 2 x/W	☐ 3 - 4 x/W	☐ 5 - 6 x/W	☐ every day
Fruit	☐ None	☐ 1 - 2 x/W	☐ 3 - 4 x/W	☐ 5 - 6 x/W	☐ every day
Sweets	☐ None	☐ 1 - 2 x/W	☐ 3 - 4 x/W	☐ 5 - 6 x/W	☐ every day
Fried/sauteed	☐ None	☐ 1 - 2 x/W	☐ 3 - 4 x/W	☐ 5 - 6 x/W	☐ every day
Salty foods	☐ None	☐ 1 - 2 x/W	☐ 3 - 4 x/W	☐ 5 - 6 x/W	☐ every day
Hot/spicy food	☐ None	☐ 1 - 2 x/W	☐ 3 - 4 x/W	☐ 5 - 6 x/W	☐ every day

CURRENT HEALTH STATUS

Have you suffered from any of the following infections which required treatment in the past year?

Bacterial infection (e.g. pharingitis, pneumonia)	☐ No	☐ once/y	☐ twice/y	☐ three/more	
Viral infection (e.g. influenza, viral gastroenteritis)	☐ No	☐ once/y	☐ twice/y	☐ three/more	
Parasite infection	☐ No	☐ treated < past 2 yrs	☐ currently treated	☐ untreated	

Do you have air pollution in your living environment?

Polution	☐ None	☐ Mild	☐ Moderate	☐ Fairly	☐ Heavy
Source	☐ Car	☐ Factory	☐ Both	☐ Other_____	
Time spent outside in air polution	☐ hrs/day				

Are you taking any nutritional supplements regularly? ☐ NO ☐ YES

If yes, specify type _____

Are you taking any medication regularly? ☐ NO ☐ YES

If yes, specify ALL DRUGS _____

Have you had any of the following allergic disorders in recent years?

☐ Allergic rhinitis ☐ Bronchial asthma
☐ Food allergy ☐ Other (Specify):_____
☐ Atopic dermatitis

Have you had chest pain in the past year for which you seeked medical attention or was hospitalized? ☐ NO ☐ YES

VITAMIN D STATUS

Have you eaten olly fish (e.g. pilchards, sardines, snoek, mackerel, salmon, induding canned, fresh or smoked) in the last week? ☐ NO ☐ YES

If yes, how many times? ☐ times/week

On an avarage day, how much time do you spend outdoors between 10 am and 4 pm?

☐ < 15 min	☐ 15 min	☐ 30min	☐ 1 hr	☐ 2 hrs	
☐ 3 hrs	☐ 4 hrs	☐ 5 hrs	☐ 6 hrs		

When you are outdoors, what parts of your body are exposed and NOT covered?

☐ Face ☐ Arms ☐ Hands ☐ Legs ☐ Torso

WOMEN

When was the first day of your last period? ☐ yyyy/mm/dd

How is your menstrual cycle?

☐ regular ☐ irregular ☐ menopause
☐ under hormone therapy or taking contraceptive pills

If "regular", what is the averge lengh of the cycle? ☐ days

Do you suffer from hypermenorrhoea (heavy/lasting > 7 days) or hypomenorrhoea (estremely light)?

☐ hypermenorrhoea ☐ hypomenorrhoea

MEN

Have you had any urinary problems in the past 3 months (frequent urge to urinate, burning or painful urination or difficulty urinating)? ☐ NO ☐ YES

THANK YOU FOR YOUR COOPERATION

YOUR INFORMATION WILL BE USED SOLELY FOR THIS STUDY

APPENDIX F:
ARICA REFERENCE RANGE PROJECT RESULTS REPORT FOR PARTICIPANTS

Dear Participant

Please read the general information on tests performed on your blood samples with your results attached.

You need to know that 5% of all healthy people have abnormal results. Medical and mathematical statistics guarantee that. By definition, a "normal" test result range is the range that includes 95% of all healthy adults. So it's possible that you are in the 5% of normal healthy adults who have an abnormal high or low result.

An elevated urea level, with a normal creatinine, does not indicate renal impairment. It can be due to mild dehydration, a high protein diet, and can also occasionally be seen with gastro-intestinal bleeds (peptic ulcers).

Your LDL-cholesterol (bad type of cholesterol) is above the target – lifestyle changes are advised, followed by repeat measurement. At this stage, your overall cardiovascular risk (risk of cardiovascular disease calculated by the Framingham Risk score based on age, blood pressure, cholesterol, smoking history and presence or absence of diabetes) is moderately (10 year risk 8,7%), and therefore cholesterol treatment might be indicated in the future

The likely cause of your elevated CK level (muscle enzyme) is exercise, as you indicated that you walk quite regularly.

KIDNEY FUNCTION:

Sodium, potassium, chloride (electrolytes) PLUS urea and creatinine

The main functions of the kidneys are to excrete waste products (e.g. urea and creatinine), to maintain fluid volume and composition (sodium, potassium and chloride), and hormone synthesis. The tests most commonly used to assess kidney function are urea and creatinine, of which the concentrations will rise with kidney injury.

BONE HEALTH

Calcium, magnesium and phosphate

Calcium:

Calcium is the most abundant mineral in the human body, and has many important functions. Its most important function is to serve as a structural element (teeth and bone), but it also plays a very important role in the neuromuscular system (muscle contraction). In addition, it is needed for specific enzymes to work, and plays a role in intracellular signaling. The level of calcium in the blood is controlled mostly by vitamin D and parathyroid hormone (produced by the parathyroid glands).

High levels of calcium can be seen in malignant diseases, with overactive parathyroid glands, in people with an overactive thyroid, with the intake of certain drugs (Vitamin D intoxication, Thiazide diuretics, Lithium therapy), with immobilization, and as a result of certain diseases (tuberculosis and sarcoidosis).

Low levels of calcium can be seen in cases with low parathyroid hormone levels or in vitamin D deficiency, renal failure or pancreatitis.

Magnesium:

Magnesium is the fourth most abundant cation found in the body, and is distributed mostly in the bone, muscle and soft tissue. It plays a very important role as cofactor for enzymes, and together with calcium affects membrane excitability.

A high level of magnesium is uncommon and is mostly seen when magnesium treatment is given for severe hypertension in pregnancy (pre-eclampsia).

A low level of magnesium is more common, and seen in cases of malabsorption, malnutrition, alcoholism, liver disease, drug toxicity and renal disorders.

Phosphate:

Phosphates' function and concentration is closely linked to that of calcium.

High levels of phosphate can be seen in renal impairment, with hypoparathyroidism, excessive phosphate intake, vitamin D intoxication and in catabolic states.

Low levels of phosphate can be seen during the recovery phase of diabetic ketoacidosis, with parenteral nutrition low in phosphate, with a low dietary intake or with malabsorption, vomiting, vitamin D deficiency, alcohol withdrawal and with diuretic therapy (just to name a few).

LIPOGRAM

Total cholesterol, triglycerides, HDL-cholesterol (good cholesterol) and LDL-cholesterol (bad cholesterol).

Elevated levels of cholesterol are causally related to atherosclerosis, the process responsible for the majority of cardiovascular diseases. When evaluating the results of a fasting lipogram, it can be used in conjunction with other risk factors (e.g. obesity, hypertension, smoking and a family history of cardiovascular disease) to estimate the risk of cardiovascular disease.

Since all participants in the study were seen as "healthy" (low risk), the target for LDL cholesterol is < 3 mmol/L. If an LDL of > 3 mmol/L is found, lifestyle intervention with or without lipid lowering therapy needs to be considered.

Ultrasensitive CRP

Inflammation of the arteries is a risk factor for cardiovascular disease. It has been linked to an increased risk of heart disease, heart attacks, sudden death, stroke, and peripheral arterial disease. High Sensitivity (also called Ultra-sensitive) C-reactive protein is a protein found in the blood and what we call a "marker" for inflammation. One should aim for ultra-sensitive CRP of < 1 mg/L.

Immunoglobulins (IgG, IgA and IgM)

An immunoglobulin test measures the level of certain immunoglobulins, or antibodies, in the blood. Antibodies are proteins made by the immune system to fight antigens, such as bacteria, viruses, and toxins. The body makes different immunoglobulins to combat different antigens.

Immunoglobulin A (IgA): Is found in high concentrations in the mucous membranes, particularly those lining the respiratory passages and gastrointestinal tract, as well as in saliva and tears.

Immunoglobulin G (IgG): The most abundant type of antibody. Is found in all body fluids and protects against bacterial and viral infections.

Immunoglobulin M (IgM): Is found mainly in the blood and lymph fluid and is the first to be made by the body to fight a new infection.

IgA, IgG, and IgM are frequently measured simultaneously. Evaluated together, they can give doctors important information about immune system functioning, especially relating to infection or autoimmune disease. Certain cancers (e.g. myeloma) are also associated with an elevation of a specific type of immunoglobulin.

LIVER FUNCTION TEST

Total protein, albumin, bilirubin, alkaline phosphatase, gamma GT, ALT and AST

The liver is a vital organ and plays a very important role in sugar, fat and protein metabolism. In addition, it plays an important role in the synthesis of hormones, metabolism of drugs, storage of vitamins and iron, and excretion of waste products.

When evaluating liver function tests, total protein and albumin tells one about the synthesis function of the liver, and bilirubin about the excretion function of the liver. The liver enzymes tell you mostly about the integrity of the cells in the liver. When ALP and GGT are elevated together, it points towards the obstruction of bile ducts (as seen with e.g. gallstones). When ALT and AST are both elevated, it usually means that the liver cells are damaged (for example in hepatitis).

These enzymes are however not liver-specific. ALP is also found in bone and can be elevated

in bone disease. AST is also found in red blood cells and muscle cells and can be elevated with red blood cell or muscle damage. Excessive alcohol use and certain drugs can cause an increase in GGT.

CREATININE KINASE (CK)

Creatine kinase (CK) is an enzyme that is present in heart muscle, skeletal muscle, and the brain. CK values can be increased after muscle injury or damage, brain injury, a heart attack, electric shock and convulsions. High values are also seen in patients with uncontrolled hypothyroidism.

LACTATE DEHYDROGENASE (LDH)

LDH is widely distributed. Tissue breakdown releases LDH, and therefore LDH can be measured as a surrogate for tissue breakdown, e.g. haemolysis (breakdown of red blood cells). Other disorders indicated by elevated LDH include cancer, meningitis, encephalitis, acute pancreatitis, and HIV.

TROPONON I

For more than 15 years troponin has been known as a reliable marker of cardiac muscle tissue injury. It is considered to be more sensitive and significantly more specific in the diagnosis of myocardial infarction. Troponin can also be elevated in other non-cardiac conditions, e.g. pulmonary embolism, polymyocitis and dermatomyocitis, snake bite, endurance racing and carbon monoxide poisoning (to name a few).

CHOLINESTERASE

Cholinesterase is a family of enzymes that initiates the breakdown of the neurotransmitteracetylcholine. It is most commonly measured in suspected organophosphate exposure, and in cases of suspected scolineapnoee (taking long to wake up after anesthetics).

Low cholinesterase levels can be seen in organophosphate poisoning/exposure (used for spraying crops). It can also be measured before an operation, if it is suspected that one has a genetic defect causing lower enzyme activity, which can cause a specific drug (scoline) to be metabolized slower. This can result in a delayed recovery from anesthetics.

Other factors that can affect cholinesterase values include weight, hormonal status, and the use of oral contraceptives.

PANCREATIC FUNCTION

Serum amylase and lipase

Serum amylase is found in the salivary glands and the pancreas. Plasma amylase activity is usually significantly increased in patients with acute pancreatitis. However, there are many other causes for a raised amylase (perforated duodenal ulcer, intestinal obstruction, acute abdominal disorders, acute kidney injury, diabetic ketoacidosis, ruptured fallopian tube, salivary gland disorders, anorexia nervosa and morphine administration). For this reason, amylase is performed in conjunction with lipase, where pancreatitis is likely when both enzymes are significantly increased.

Low serum amylase can indicate diffuse pancreatic dysfunction, but in many cases the clinical relevance of a low serum amylase remains poorly understood.

FASTING PLASMA GLUCOSE

Fasting plasma glucose can be used to diagnose diabetes or prediabetes. It is more convenient than performing a glucose tolerance test. People with a fasting glucose level of 5,6 to 6,9 mmol/L have impaired fasting glucose (IFG), or prediabetes. A level of 7 mmol/L or above, confirmed by repeating the test on another day, means a person has diabetes.

IRON STUDIES

Iron, transferrin, transferrin saturation and ferritin

Iron concentrations vary significantly during the day. For this reason, iron is usually evaluated

in conjunction with its carrier protein (transferrin), to calculate the transferrin saturation.

Low transferrin saturation is usually seen in iron deficiency and chronic disease, where high transferrin saturation is usually seen with iron therapy and iron overload.

Ferritin is an acute phase protein produced by the liver and gives an idea of the body's iron stores. Low serum ferritin is seen in iron deficiency, where high ferritin levels can be seen in acute infection, iron overload, liver disease and some malignancies.

THYROID FUNCTION TESTS

T4, T3 and TSH and thyroid auto-antibodies (thyroglobulin antibodies and thyroid peroxidase antibodies)

The thyroid gland secretes thyroid hormone (T4 and T3). Thyroxin (T4) synthesis and release is stimulated by the pituitary hormone, TSH. Thyroid hormones are essential for normal growth and development and have many effects on metabolic processes. The most obvious overall effect is to stimulate the basal metabolic rate.

An increase in thyroid hormone production (hyperthyroidism) can be seen in Grave's disease, toxic multi-nodular goiter, solitary toxic adenoma, thyroiditis, intake of iodine-containing drugs, excessive eltroxin intake, during early pregnancy due to very high HCG values, and in rare cases in the presence of a pituitary tumor.

Low thyroid hormone levels (hypothyroidism) can be seen in Hashimoto's thyroiditis, post thyroid surgery or after receiving radioactive iodine, congenital causes, and due to pituitary or hypothalamic disease.

The measurement of thyroid antibodies can help to establish the cause of hyper- and hypothyroidism, as positive values indicate an underlying auto-immune disease (Hashimoto's thyroiditis, or Grave's disease).

VITAMIN D (NOT MEASURED ON ALL STUDY PARTICIPANTS)

Vitamin D is one of the fat-soluble vitamins. It can be taken in the diet (e.g. fish, eggs, cod liver or supplements), but can also be manufactured in the body when sun exposure is adequate. The major biologic function of vitamin D is to maintain normal blood levels of calcium and phosphorus. Vitamin D aids in the absorption of calcium, helping to form and maintain strong bones. It is used, alone or in combination with calcium, to increase bone mineral density and decrease fractures. Recently, research also suggests that vitamin D may provide protection from osteoporosis, hypertension (high blood pressure), cancer, and several autoimmune diseases.

PROSTATE SPECIFIC ANTIGEN (PSA)

Prostate specific antigen (PSA) is produced at high concentrations by normal and malignant prostatic epithelium. It is mainly secreted into seminal fluid, where it digests the gel forming after ejaculation. Only minor amounts of PSA leak out into circulation from the normal prostate, but the release of PSA is increased in prostatic disease. Thus PSA is a sensitive serum marker for prostate cancer but its specificity is limited by a high frequency of falsely elevated values in men with benign prostatic hyperplasia (non-cancerous enlargement of the prostate). PSA measurement should always be accompanied by a digital rectal examination, as normal PSA concentrations do not exclude the possibility of prostate cancer.

PLEASE NOTE THAT:

- **LABORATORY TEST SHOULD ALWAYS BE INTERPRETED TOGETHER WITH THE CLINICAL PICTURE**
- **ALL ABNORMAL TEST RESULTS SHOULD BE CONFIRMED AND IF PERSISTANTLY ABNORMAL, SHOULD BE INVESTIGATED**

UNFORTUNATELY, REPEAT TESTING AND REFERRAL IS BEYOND THE SCOPE OF THIS PROJECT

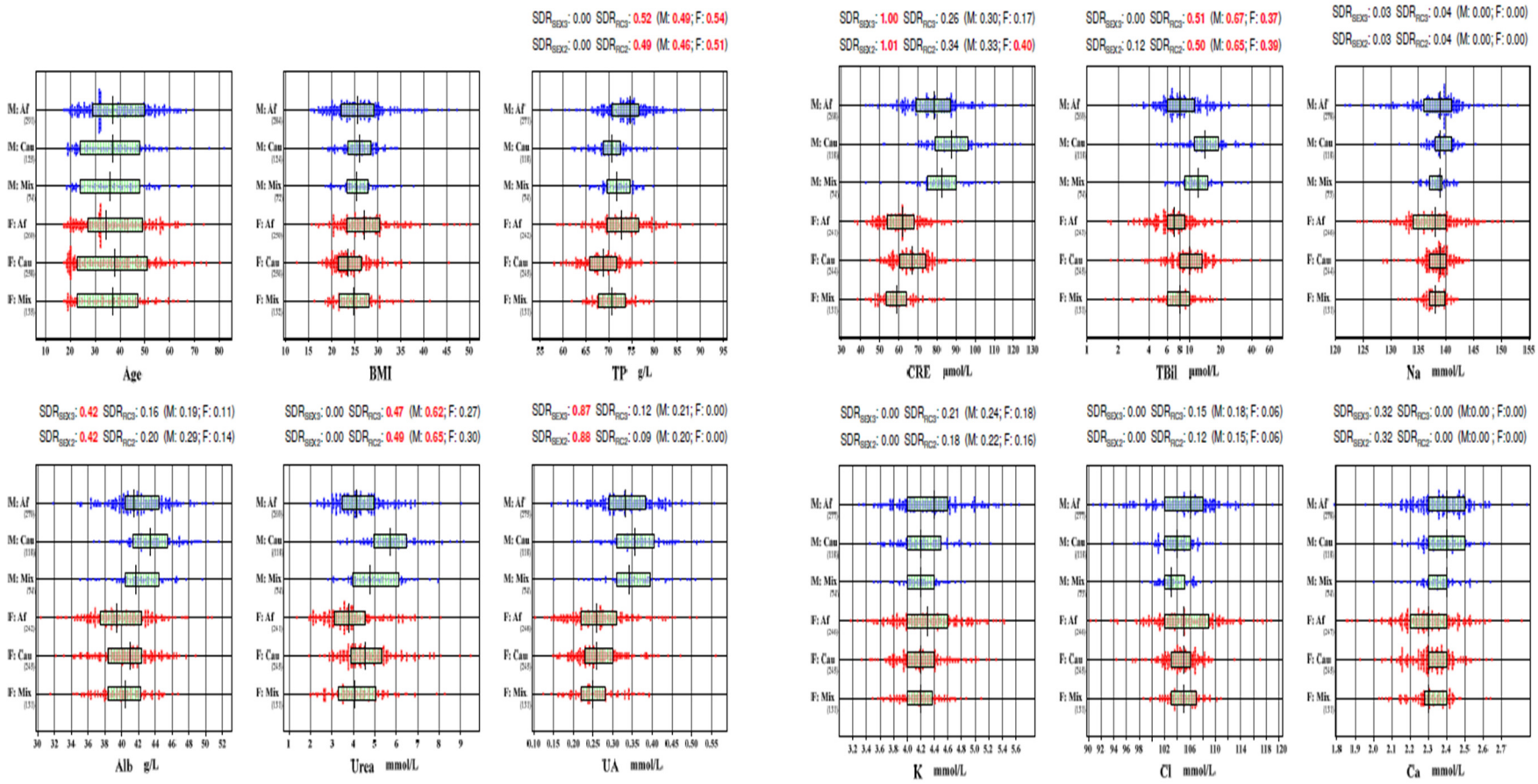
Thank you very much for participating in this very important study.

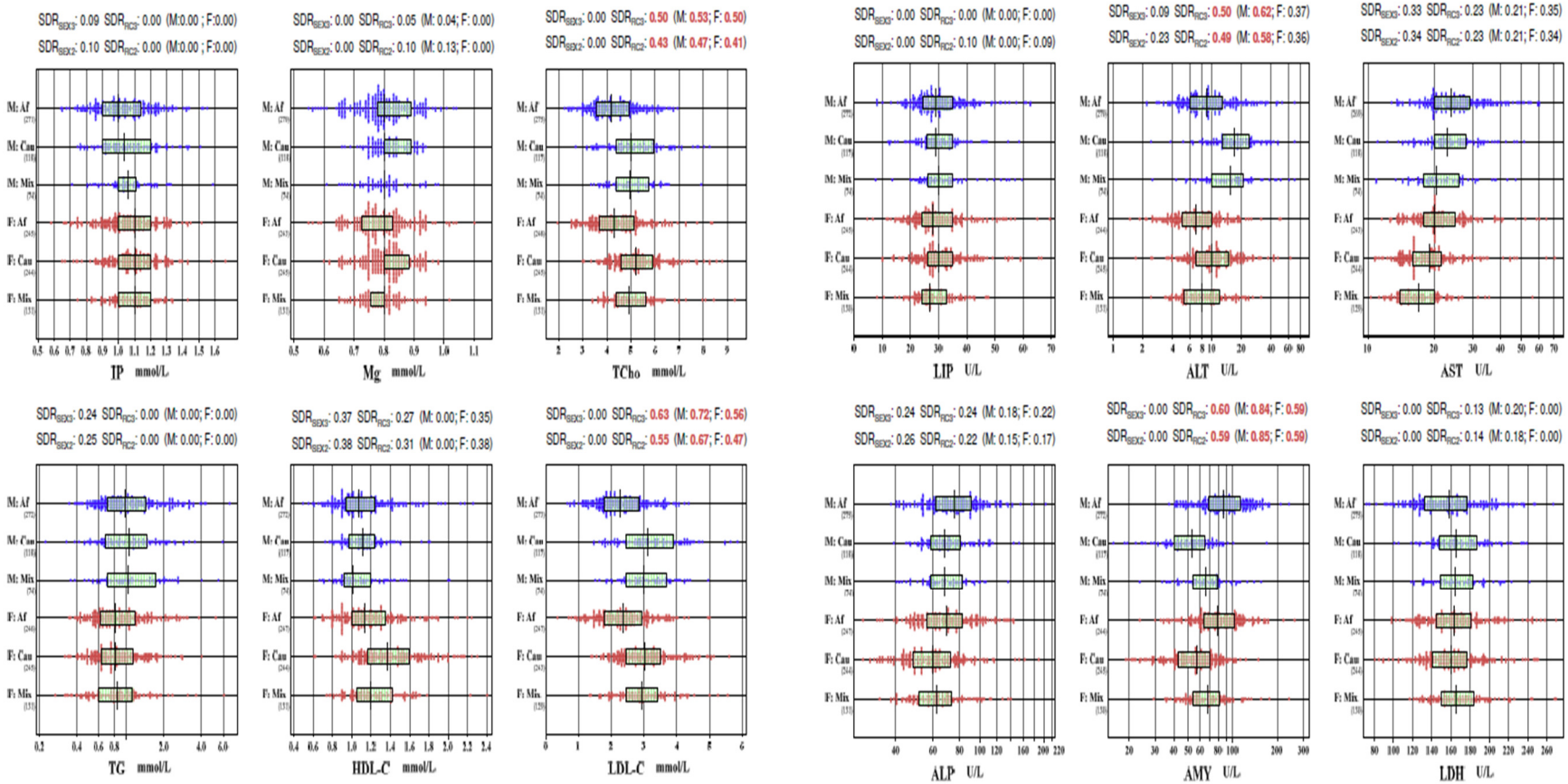
Regards

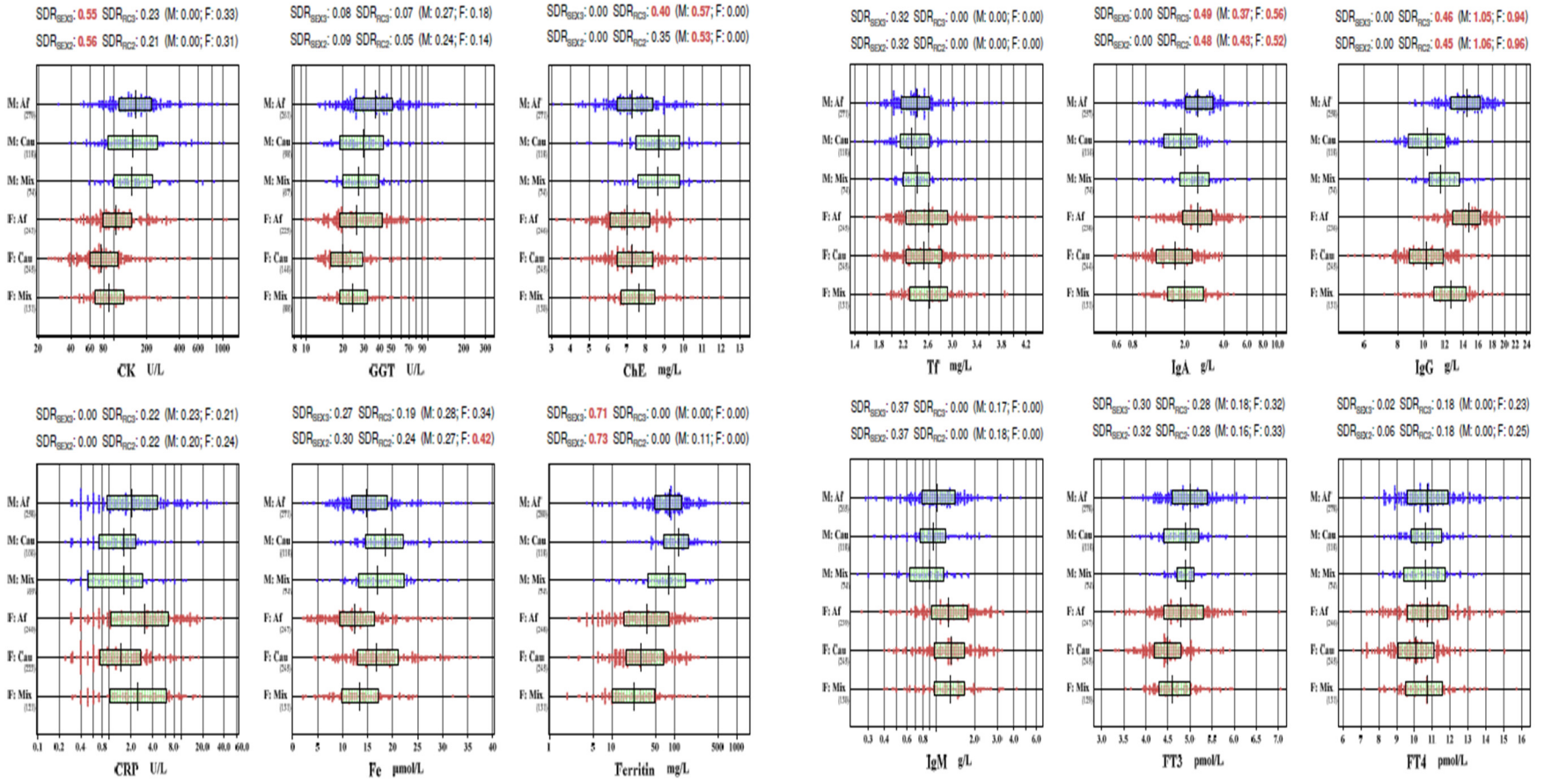
Dr. M Hoffmann

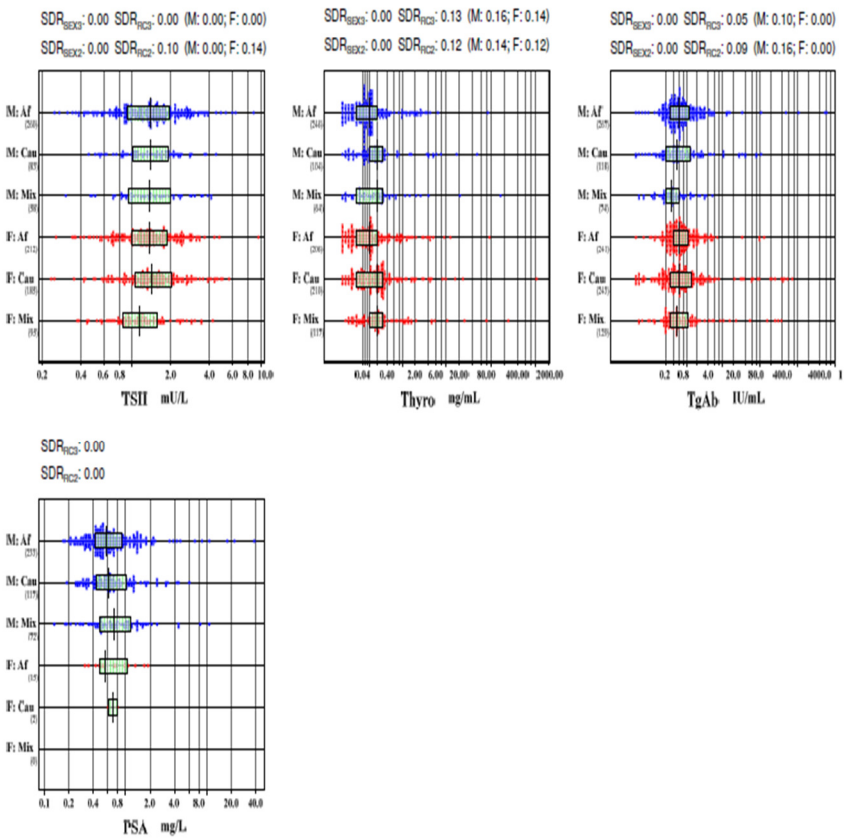
APPENDIX G

Supplementary Figure 1 (Manuscript One): Ethnic - and gender related changes for all analytes



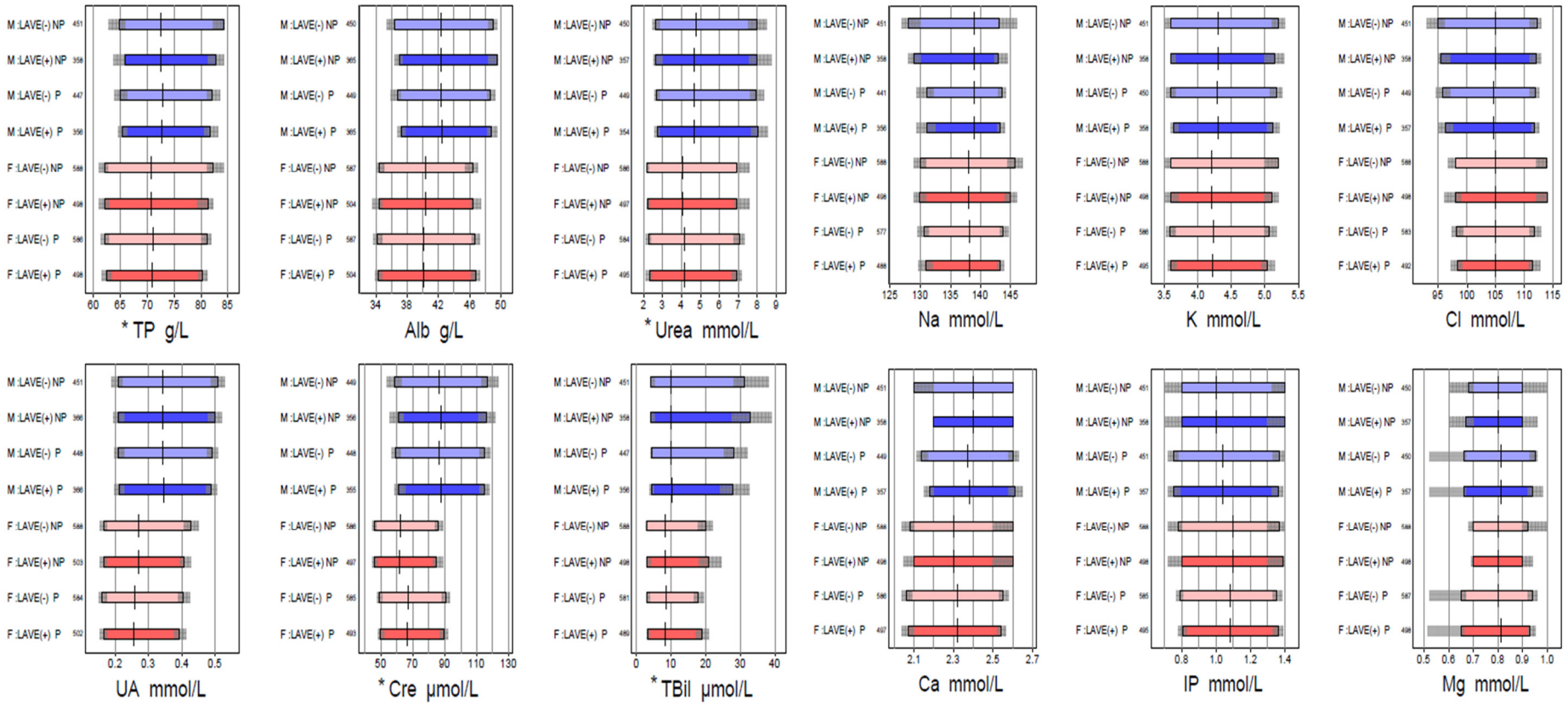

SDR_SEX3: 0.09 SDR_RC3: 0.00 (M:0.00 ; F:0.00)
SDR_SEX2: 0.10 SDR_RC2: 0.00 (M:0.00 ; F:0.00)
IP mmol/L
SDR_SEX3: 0.00 SDR_RC3: 0.05 (M: 0.04; F: 0.00)
SDR_SEX2: 0.00 SDR_RC2: 0.10 (M: 0.13; F: 0.00)
Mg mmol/L
SDR_SEX3: 0.00 SDR_RC3: 0.50 (M: 0.53; F: 0.50)
SDR_SEX2: 0.00 SDR_RC2: 0.43 (M: 0.47; F: 0.41)
TCho mmol/L
SDR_SEX3: 0.00 SDR_RC3: 0.00 (M: 0.00; F: 0.00)
SDR_SEX2: 0.00 SDR_RC2: 0.10 (M: 0.00; F: 0.09)
LIP U/L
SDR_SEX3: 0.09 SDR_RC3: 0.50 (M: 0.62; F: 0.37)
SDR_SEX2: 0.23 SDR_RC2: 0.49 (M: 0.58; F: 0.36)
ALT U/L
SDR_SEX3: 0.33 SDR_RC3: 0.23 (M: 0.21; F: 0.35)
SDR_SEX2: 0.34 SDR_RC2: 0.23 (M: 0.21; F: 0.34)
AST U/L
SDR_SEX3: 0.24 SDR_RC3: 0.00 (M: 0.00; F: 0.00)
SDR_SEX2: 0.25 SDR_RC2: 0.00 (M: 0.00; F: 0.00)
TG mmol/L
SDR_SEX3: 0.37 SDR_RC3: 0.27 (M: 0.00; F: 0.35)
SDR_SEX2: 0.38 SDR_RC2: 0.31 (M: 0.00; F: 0.38)
HDL-C mmol/L
SDR_SEX3: 0.00 SDR_RC3: 0.63 (M: 0.72; F: 0.56)
SDR_SEX2: 0.00 SDR_RC2: 0.55 (M: 0.67; F: 0.47)
LDL-C mmol/L
SDR_SEX3: 0.24 SDR_RC3: 0.24 (M: 0.18; F: 0.22)
SDR_SEX2: 0.26 SDR_RC2: 0.22 (M: 0.15; F: 0.17)
ALP U/L
SDR_SEX3: 0.00 SDR_RC3: 0.60 (M: 0.84; F: 0.59)
SDR_SEX2: 0.00 SDR_RC2: 0.59 (M: 0.85; F: 0.59)
AMY U/L
SDR_SEX3: 0.00 SDR_RC3: 0.13 (M: 0.20; F: 0.00)
SDR_SEX2: 0.00 SDR_RC2: 0.14 (M: 0.18; F: 0.00)
LDH U/L
M: Af
M: Cau
M: Mix
F: Af
F: Cau
F: Mix

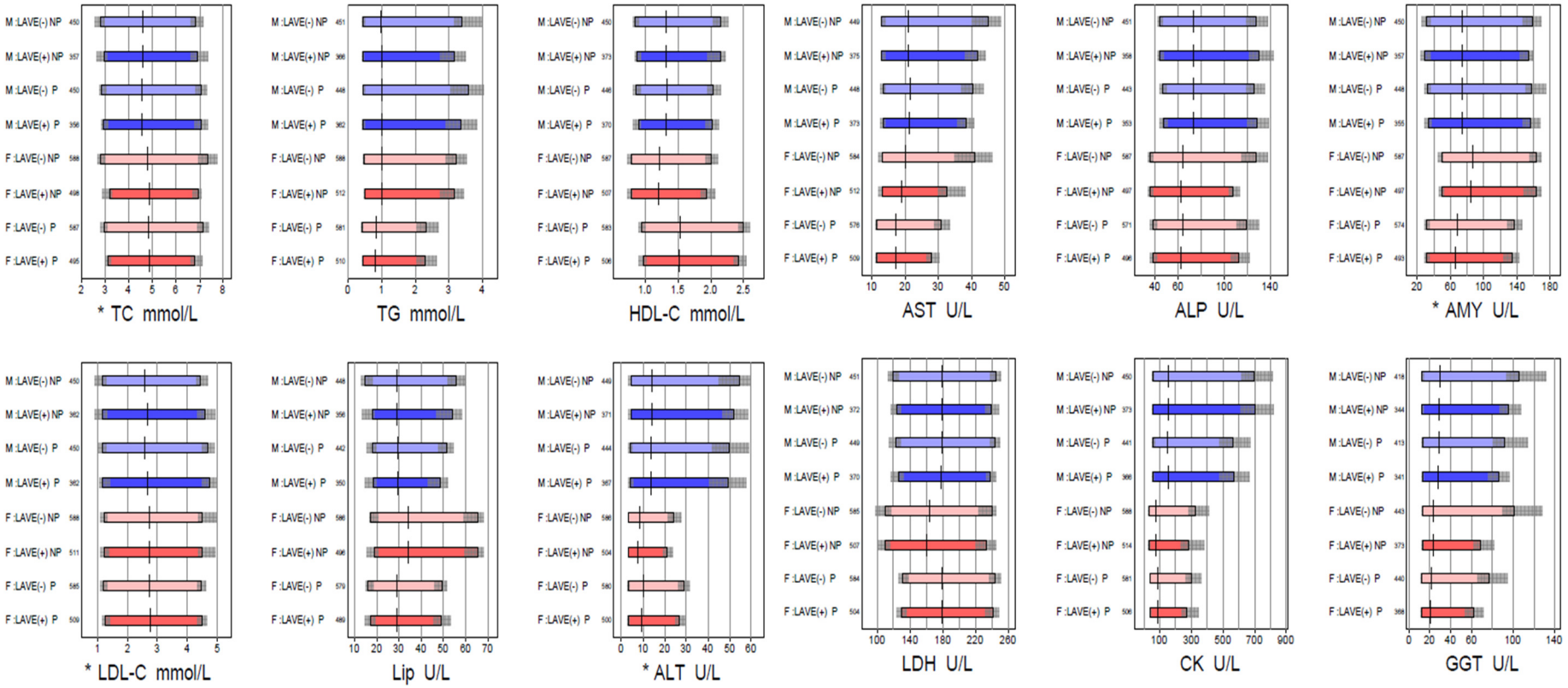

SDR_SEX3: 0.55 SDR_RC3: 0.23 (M: 0.00; F: 0.33)
SDR_SEX2: 0.56 SDR_RC2: 0.21 (M: 0.00; F: 0.31)
M: Af
M: Cau
M: Mix
F: Af
F: Cau
F: Mix
CK U/L
SDR_SEX3: 0.08 SDR_RC3: 0.07 (M: 0.27; F: 0.18)
SDR_SEX2: 0.09 SDR_RC2: 0.05 (M: 0.24; F: 0.14)
GGT U/L
SDR_SEX3: 0.00 SDR_RC3: 0.40 (M: 0.57; F: 0.00)
SDR_SEX2: 0.00 SDR_RC2: 0.35 (M: 0.53; F: 0.00)
ChE mg/L
SDR_SEX3: 0.32 SDR_RC3: 0.00 (M: 0.00; F: 0.00)
SDR_SEX2: 0.32 SDR_RC2: 0.00 (M: 0.00; F: 0.00)
Tf mg/L
SDR_SEX3: 0.00 SDR_RC3: 0.49 (M: 0.37; F: 0.56)
SDR_SEX2: 0.00 SDR_RC2: 0.48 (M: 0.43; F: 0.52)
IgA g/L
SDR_SEX3: 0.00 SDR_RC3: 0.46 (M: 1.05; F: 0.94)
SDR_SEX2: 0.00 SDR_RC2: 0.45 (M: 1.06; F: 0.96)
IgG g/L
SDR_SEX3: 0.00 SDR_RC3: 0.22 (M: 0.23; F: 0.21)
SDR_SEX2: 0.00 SDR_RC2: 0.22 (M: 0.20; F: 0.24)
CRP U/L
SDR_SEX3: 0.27 SDR_RC3: 0.19 (M: 0.28; F: 0.34)
SDR_SEX2: 0.30 SDR_RC2: 0.24 (M: 0.27; F: 0.42)
Fe μmol/L
SDR_SEX3: 0.71 SDR_RC3: 0.00 (M: 0.00; F: 0.00)
SDR_SEX2: 0.73 SDR_RC2: 0.00 (M: 0.11; F: 0.00)
Ferritin mg/L
SDR_SEX3: 0.37 SDR_RC3: 0.00 (M: 0.17; F: 0.00)
SDR_SEX2: 0.37 SDR_RC2: 0.00 (M: 0.18; F: 0.00)
IgM g/L
SDR_SEX3: 0.30 SDR_RC3: 0.28 (M: 0.18; F: 0.32)
SDR_SEX2: 0.32 SDR_RC2: 0.28 (M: 0.16; F: 0.33)
FT3 pmol/L
SDR_SEX3: 0.02 SDR_RC3: 0.18 (M: 0.00; F: 0.23)
SDR_SEX2: 0.06 SDR_RC2: 0.18 (M: 0.00; F: 0.25)
FT4 pmol/L

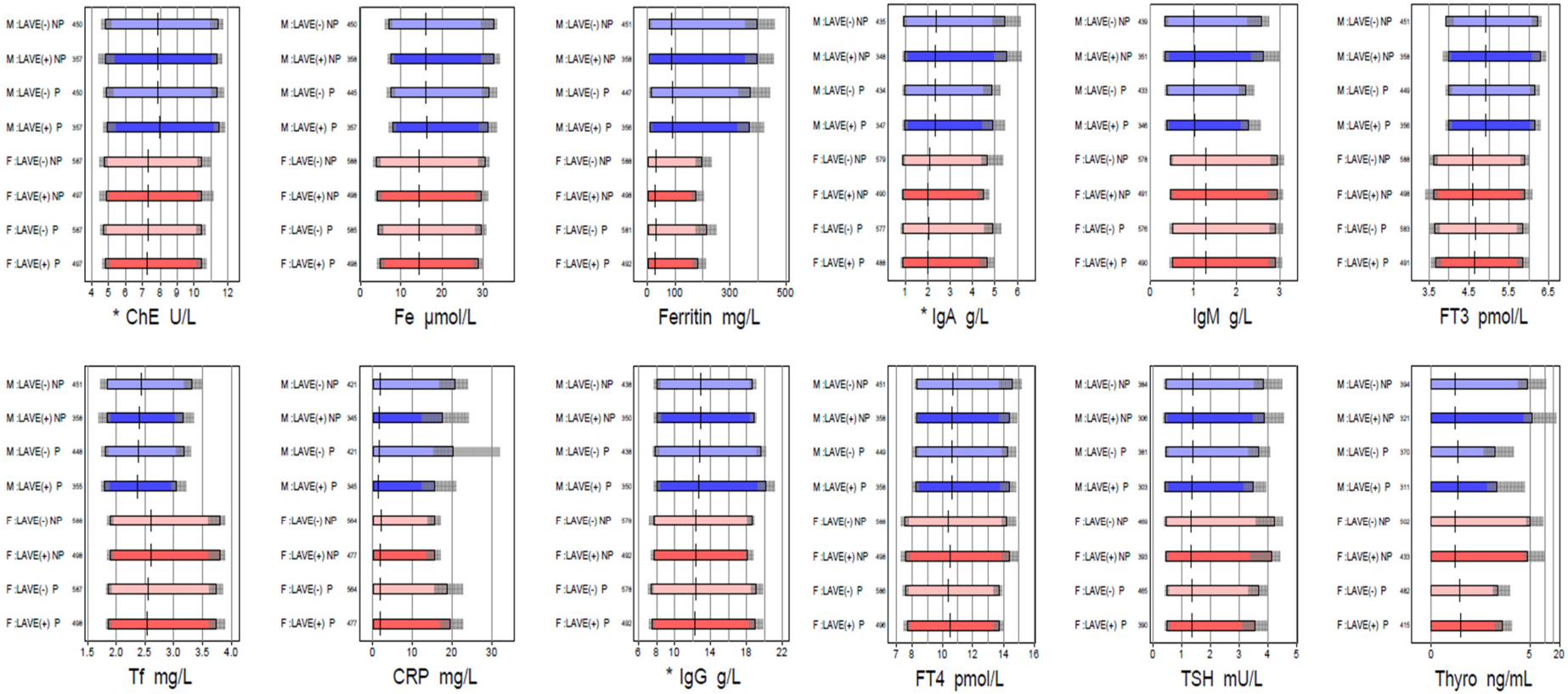

SDR_SEX3: 0.00 SDR_RC3: 0.00 (M: 0.00; F: 0.00)
SDR_SEX2: 0.00 SDR_RC2: 0.10 (M: 0.00; F: 0.14)
M: Af
M: Cau
M: Mix
F: Af
F: Cau
F: Mix
0.2 0.4 0.6 0.8 2.0 4.0 6.0 8.0 10.0
TSH mU/L
SDR_SEX3: 0.00 SDR_RC3: 0.13 (M: 0.16; F: 0.14)
SDR_SEX2: 0.00 SDR_RC2: 0.12 (M: 0.14; F: 0.12)
0.04 0.40 2.00 6.00 20.00 80.00 400.00 2000.00
Thyro ng/mL
SDR_SEX3: 0.00 SDR_RC3: 0.05 (M: 0.10; F: 0.00)
SDR_SEX2: 0.00 SDR_RC2: 0.09 (M: 0.16; F: 0.00)
0.2 0.8 4.0 20.0 80.0 400.0 4000.0
TgAb IU/mL
SDR_RC3: 0.00
SDR_RC2: 0.00
0.1 0.2 0.4 0.8 2.0 4.0 8.0 20.0 40.0
PSA mg/L

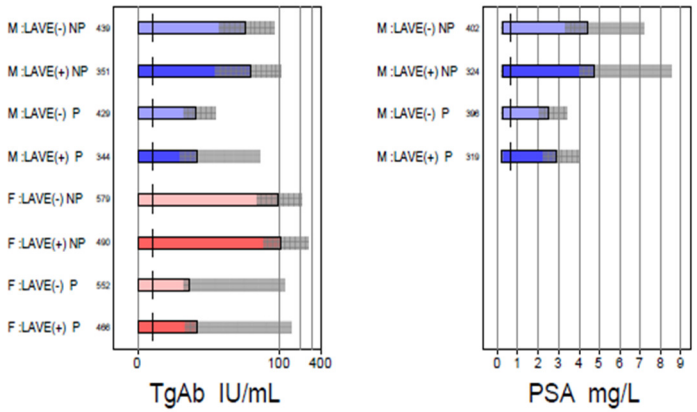
APPENDIX H

Supplementary Figure 2 (Manuscript One): Comparison of Reference Intervals and their 90% CIs amongst 4 derivation methods namely: P-method versus NP-method and LAVE(+) versus LAVE (-) all analytes.



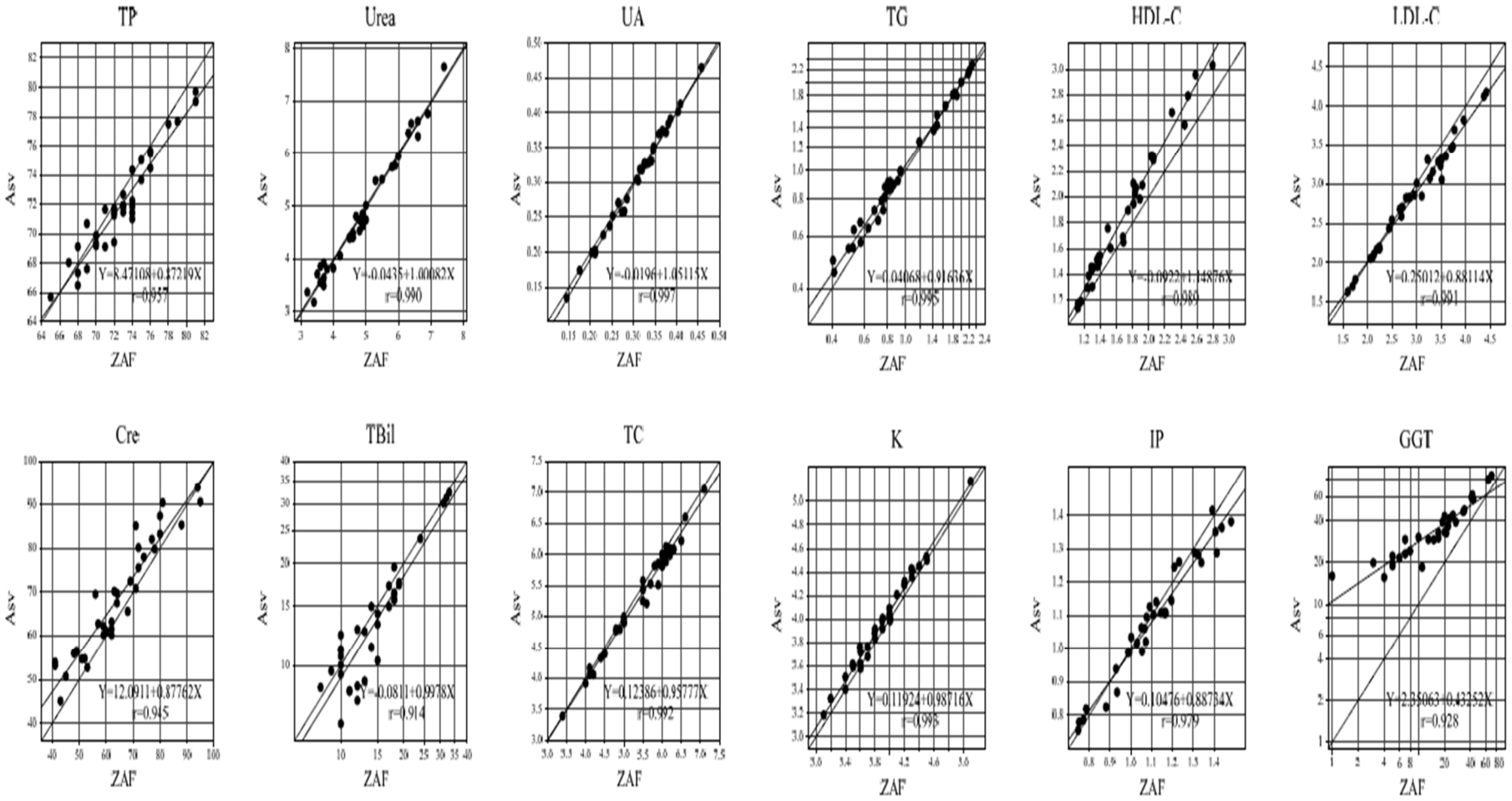

M :LAVE(-) NP
M :LAVE(+) NP
M :LAVE(-) P
M :LAVE(+) P
F :LAVE(-) NP
F :LAVE(+) NP
F :LAVE(-) P
F :LAVE(+) P
* TC mmol/L
TG mmol/L
HDL-C mmol/L
AST U/L
ALP U/L
* AMY U/L
* LDL-C mmol/L
Lip U/L
* ALT U/L
LDH U/L
CK U/L
GGT U/L

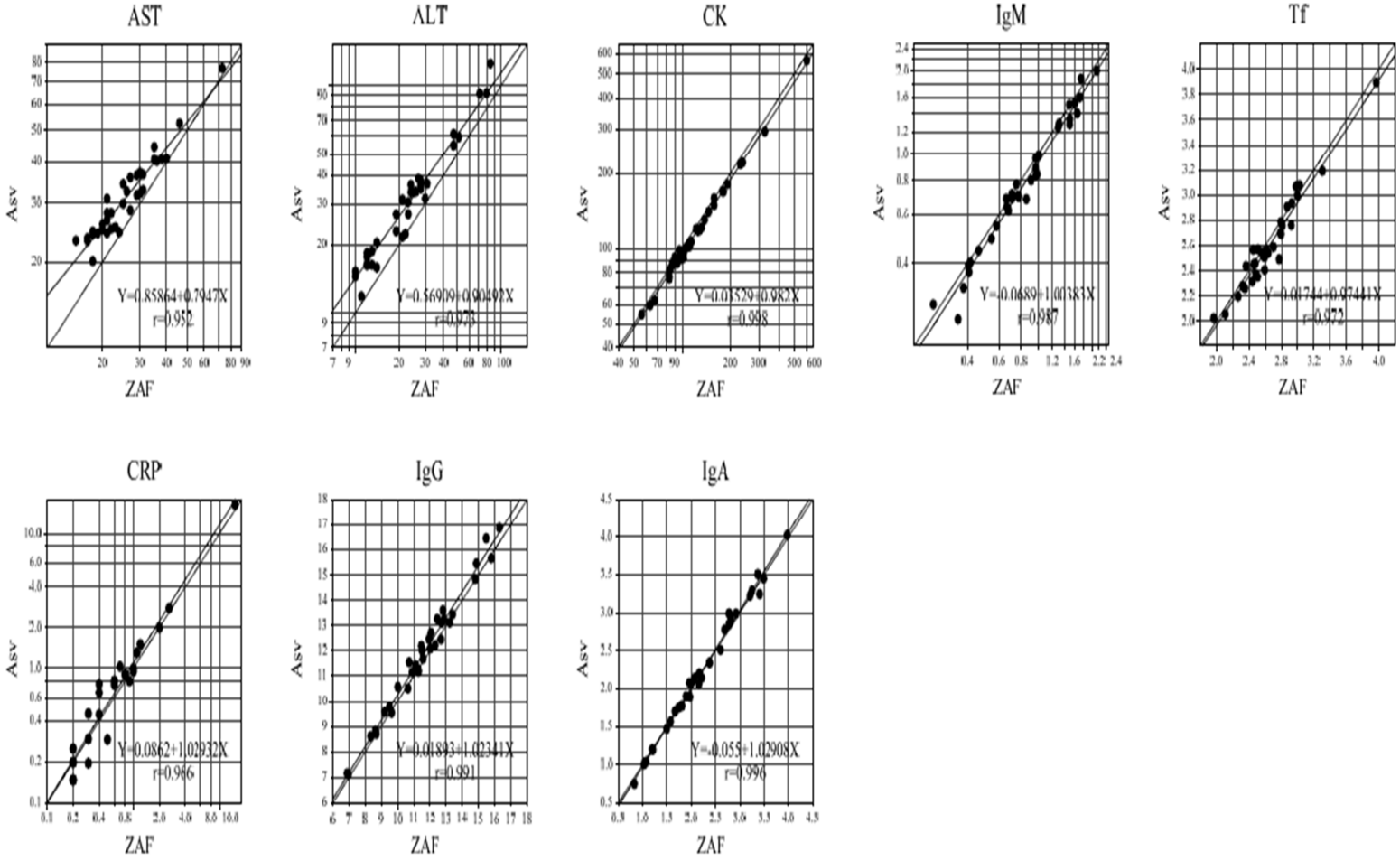

M :LAVE(-) NP
M :LAVE(+) NP
M :LAVE(-) P
M :LAVE(+) P
F :LAVE(-) NP
F :LAVE(+) NP
F :LAVE(-) P
F :LAVE(+) P
* ChE U/L
Fe µmol/L
Ferritin mg/L
* IgA g/L
IgM g/L
FT3 pmol/L
Tf mg/L
CRP mg/L
* IgG g/L
FT4 pmol/L
TSH mU/L
Thyro ng/mL


M :LAVE(-) NP 439
M :LAVE(+) NP 351
M :LAVE(-) P 429
M :LAVE(+) P 344
F :LAVE(-) NP 579
F :LAVE(+) NP 490
F :LAVE(-) P 552
F :LAVE(+) P 466
0
100
400
TgAb IU/mL
M :LAVE(-) NP 402
M :LAVE(+) NP 324
M :LAVE(-) P 396
M :LAVE(+) P 319
0 1 2 3 4 5 6 7 8 9
PSA mg/L

APPENDIX I:

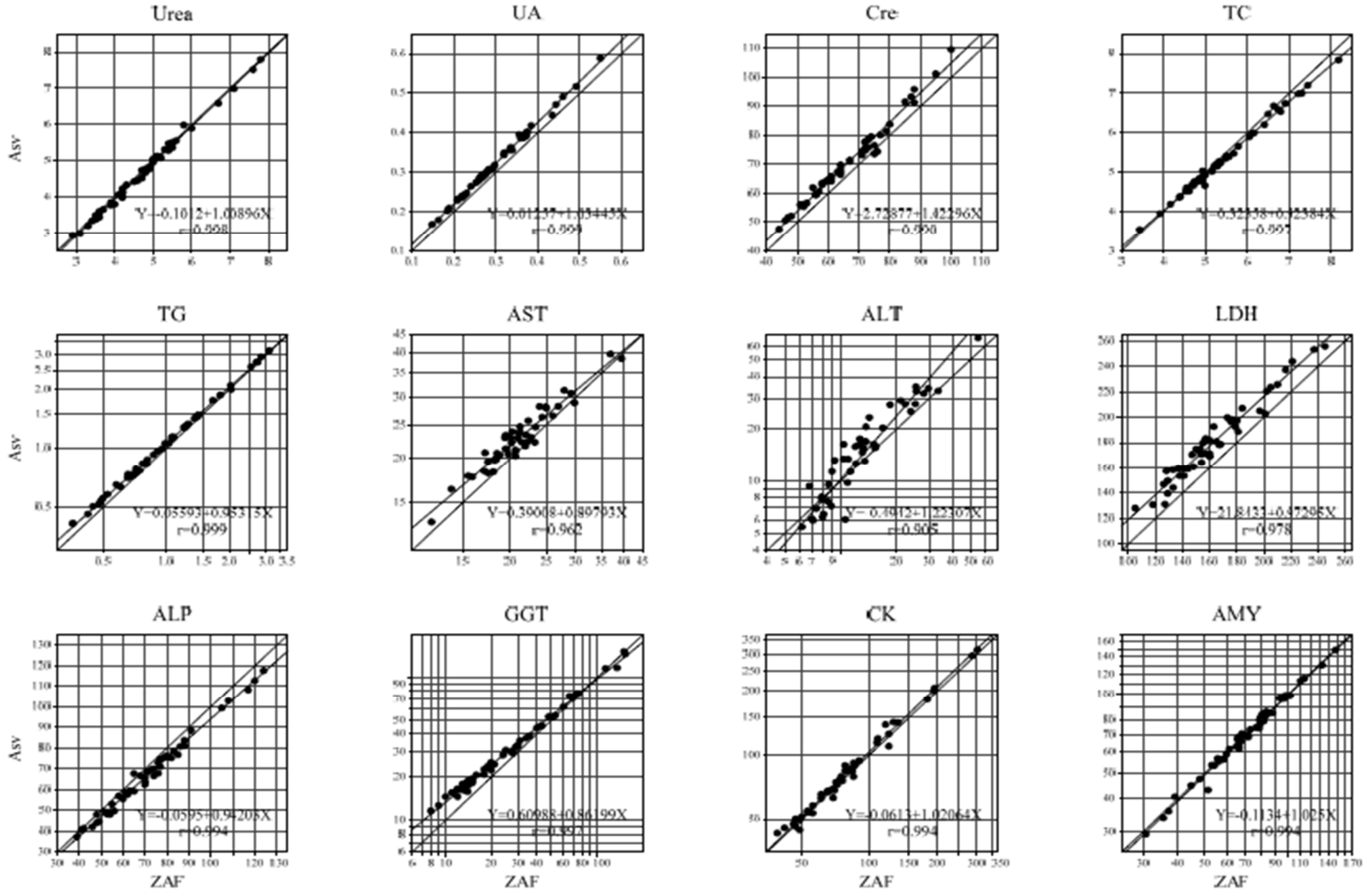
Supplementary Figure 3A (Manuscript One): Comparison of test results with panel of sera I




AST
Asv
ZAF
Y=0.85864+0.7947X
r=0.952
ALT
Asv
ZAF
Y=0.56909+0.90492X
r=0.973
CK
Asv
ZAF
Y=0.03529+0.982X
r=0.998
IgM
Asv
ZAF
Y=-0.0689+1.00383X
r=0.987
Tf
Asv
ZAF
Y=-0.0174+0.97441X
r=0.975
CRP
Asv
ZAF
Y=0.0862+1.02932X
r=0.966
IgG
Asv
ZAF
Y=0.01893+1.02341X
r=0.991
IgA
Asv
ZAF
Y=-0.055+1.02908X
r=0.996

APPENDIX J

Supplementary Figure 3B (Manuscript One): Comparison of test results with panel of sera II



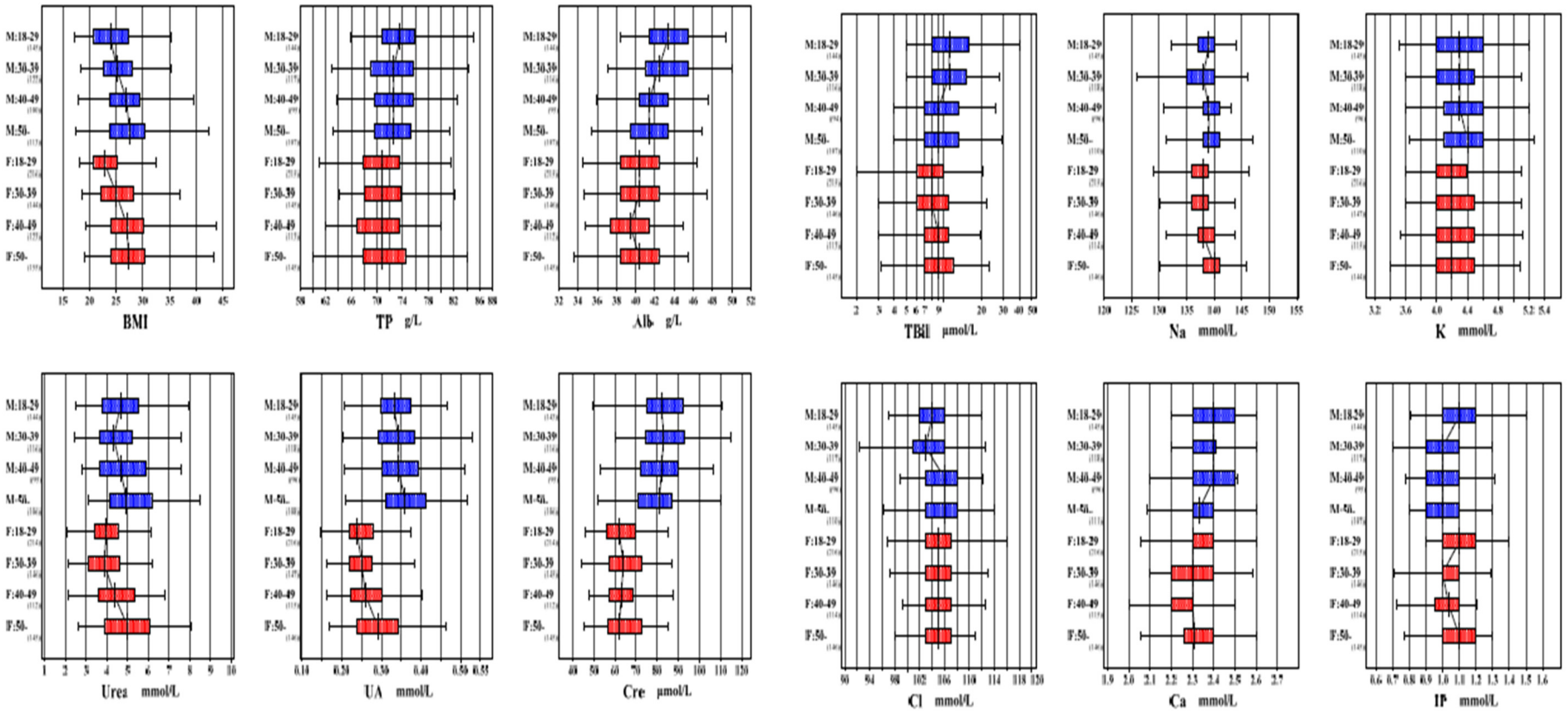
APPENDIX K

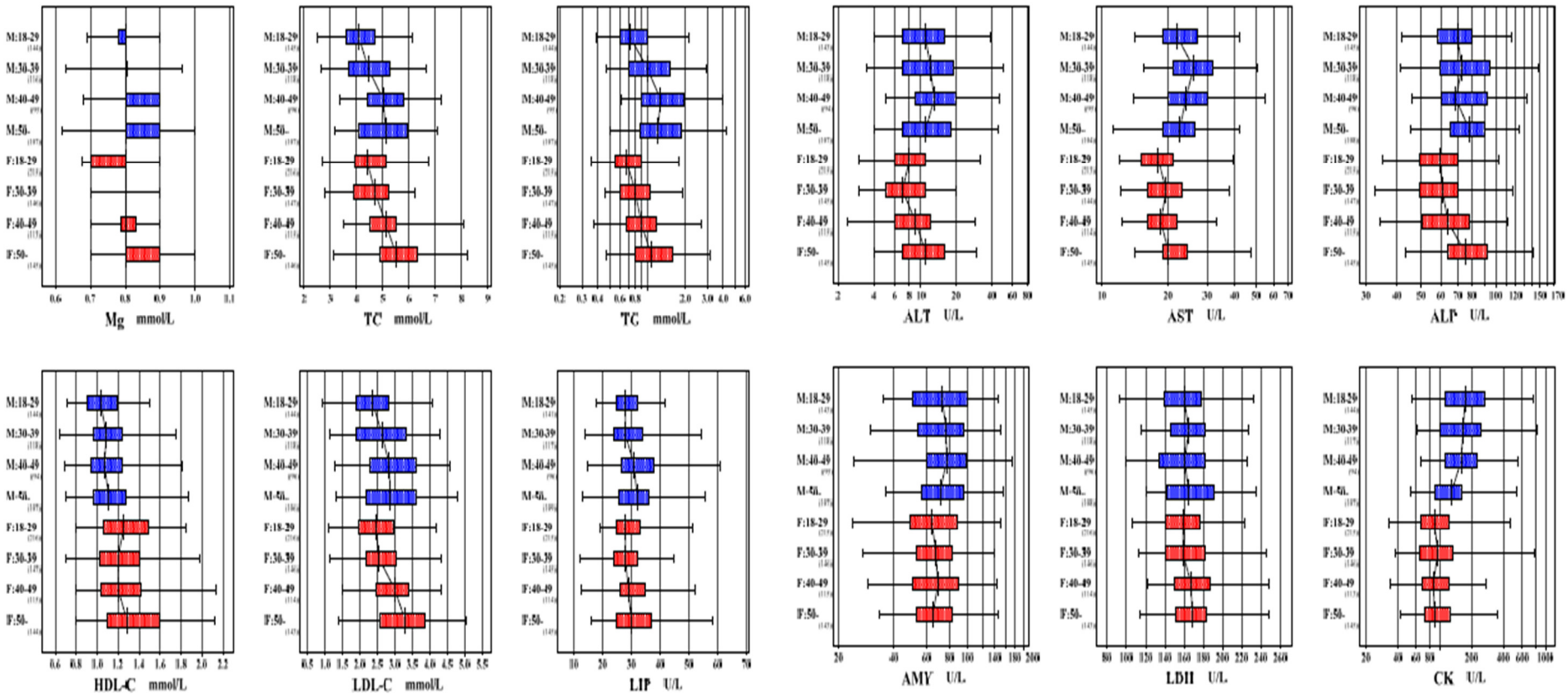
Supplementary Table 1 (Manuscript One): South African derived RI's all analytes all races partitioned by age and gender

Item	Sex	Method	Age	N	LL-L	LL-H	LL	Me	UL	UL-L	UL-H
TP	M+F	LAVE(–)	All	1034	62	64	**63**	**72**	**82**	81	82
Alb	**M**	LAVE(–)	All	449	36	37	**37**	**42**	**49**	48	49
	F		All	587	34	35	**34**	**40**	**47**	46	47
Urea	**M**	LAVE(–)	All	449	2.56	2.79	**2.66**	**4.66**	**7.93**	7.63	8.32
	F		<45	402	2.01	2.29	**2.16**	**3.92**	**6.29**	6.01	6.57
	F		≥45	196	2.39	2.78	**2.55**	**4.72**	**8.15**	7.70	8.74
UA	**M**	LAVE(–)	All	448	196	226	**208**	**344**	**489**	474	508
	F		<45	405	145	167	**156**	**249**	**373**	357	391
	F		≥45	195	169	195	**176**	**279**	**466**	441	497
Cre	**M**	LAVE(–)	All	448	53	58	**55**	**82**	**110**	107	113
	F		All	585	44	47	**45**	**63**	**86**	84	88
TBil	M+F	LAVE(–)	All	1017	3.1	4.1	**3.8**	**9.0**	**22.2**	20.7	24.5
Na	M+F	LAVE(–)	All	1017	130	131	**131**	**139**	**144**	143	144
K	M+F	LAVE(–)	All	1036	3.6	3.6	**3.6**	**4.3**	**5.1**	5.1	5.2
Cl	M+F	LAVE(–)	All	1029	96	98	**97**	**105**	**112**	111	112
Ca	M+F	LAVE(–)	All	1035	2.07	2.12	**2.10**	**2.34**	**2.58**	2.57	2.60
IP	M+F	LAVE(–)	All	1037	0.75	0.79	**0.76**	**1.07**	**1.36**	1.34	1.39
Mg	M+F	LAVE(–)	All	1037	0.52	0.67	**0.65**	**0.81**	**0.94**	0.92	0.96
TC	M+F	LAVE(–)	<45	716	2.70	2.92	**2.81**	**4.51**	**6.53**	6.34	6.84
	M+F		≥45	337	3.02	3.34	**3.17**	**5.30**	**7.66**	7.42	7.95
TG	M+F	LAVE(+)	<45	621	0.38	0.43	**0.41**	**0.80**	**2.42**	2.13	2.67
	M+F		≥45	265	0.48	0.57	**0.50**	**1.06**	**2.90**	2.53	3.40
HDL-C	**M**	LAVE(–)	All	446	0.67	0.76	0.71	1.08	1.64	1.56	1.73
	F		All	583	0.74	0.82	0.78	1.24	1.99	1.93	2.07
LDL-C	M+F	LAVE(–)	<45	719	1.02	1.18	**1.11**	**2.54**	**4.20**	4.04	4.36
	M+F		≥45	337	1.24	1.50	**1.40**	**3.04**	**5.12**	4.88	5.34
AST	**M**	LAVE(+)	All	373	14	15	**14**	**23**	**42**	40	45
	F		All	509	12	13	**12**	**19**	**31**	29	33
ALT	**M**	LAVE(+)	All	367	2.9	4.7	**3.8**	**11.8**	**40.8**	33.6	47.4
	F		All	500	2.7	3.4	**2.8**	**8.2**	**22.3**	20.7	24.5
LDH	M+F	LAVE(–)	All	1031	103	113	**109**	**162**	**229**	223	235
ALP	**M**	LAVE(–)	All	443	44	49	**47**	**73**	**126**	119	135
	F		<45	392	35	40	**37**	**60**	**110**	101	121
	F		≥45	191	37	49	**45**	**74**	**135**	123	155
GGT	**M**	LAVE(+)	All	341	14	15	**14**	**32**	**99**	87	111
	F		<45	253	11	14	**13**	**22**	**66**	56	79
	F		≥45	125	13	14	**14**	**27**	**90**	73	110
CK	**M**	LAVE(+)	All	366	52	65	**58**	**156**	**569**	481	668
	F		All	506	35	44	**39**	**89**	**271**	240	341
LIP	M+F	LAVE(–)	All	1017	15	19	**17**	**29**	**50**	47	52
ChE	**M**	LAVE(–)	All	450	4.65	5.23	**4.88**	**7.91**	**11.4**	11.0	11.8
	F		<45	404	4.39	4.85	**4.62**	**7.09**	**9.6**	9.3	9.9
	F		≥45	196	4.52	5.41	**4.86**	**7.98**	**11.3**	10.9	11.9
CRP	M+F	LAVE(+)	All	822	0.29	0.34	**0.32**	**1.79**	**17.76**	15.02	21.14
Fe	M+F	LAVE(–)	All	1032	4.7	6.3	**5.6**	**15.2**	**30.2**	29.4	31.6
Ferritin	**M**	LAVE(+)	All	447	9.2	19.0	**13.1**	**90**	**374**	331	443
	F		<45	345	2.8	4.5	**3.1**	**23**	**112**	90	134
	F		≥45	149	5.3	9.9	**7.0**	**65**	**247**	209	286
Tf	M+F	LAVE(–)	All	1036	1.82	1.90	**1.85**	**2.48**	**3.53**	3.45	3.63
IgA	M+F	LAVE(–)	All	1013	0.9	0.9	**0.9**	**2.2**	**4.8**	4.6	5.1
IgG	M+F	LAVE(–)	All	1016	7.3	7.8	**7.6**	**12.5**	**19.3**	19.0	19.8
IgM	**M**	LAVE(–)	All	433	0.35	0.42	**0.39**	**1.00**	**2.21**	2.06	2.41
	F		All	576	0.45	0.54	**0.50**	**1.28**	**2.89**	2.77	3.08
FT3	M+F	LAVE(–)	All	1032	3.68	3.85	**3.76**	**4.77**	**6.03**	5.95	6.17
FT4	M+F	LAVE(–)	All	1035	7.7	8.0	**7.8**	**10.5**	**13.9**	13.6	14.2
TSH	M+F	LAVE(–)	All	847	0.41	0.50	**0.46**	**1.36**	**3.63**	3.40	3.94
Thyro	M+F	LAVE(–)	All	858	0.00	0.00	**0.00**	**0.13**	**1.11**	0.81	2.23
TgAb	M+F	LAVE(–)	All	960	0.00	0.04	**0.01**	**0.62**	**4.70**	3.29	336
PSA	**M**	LAVE(–)	<45	287	0.20	0.24	**0.22**	**0.57**	**1.71**	1.51	1.96
	M		≥45	119	0.14	0.29	**0.21**	**0.93**	**9.02**	5.14	15.06

APPENDIX L

Supplementary Figure 1 (Manuscript Two): Age related changes in all analytes partitioned by gender

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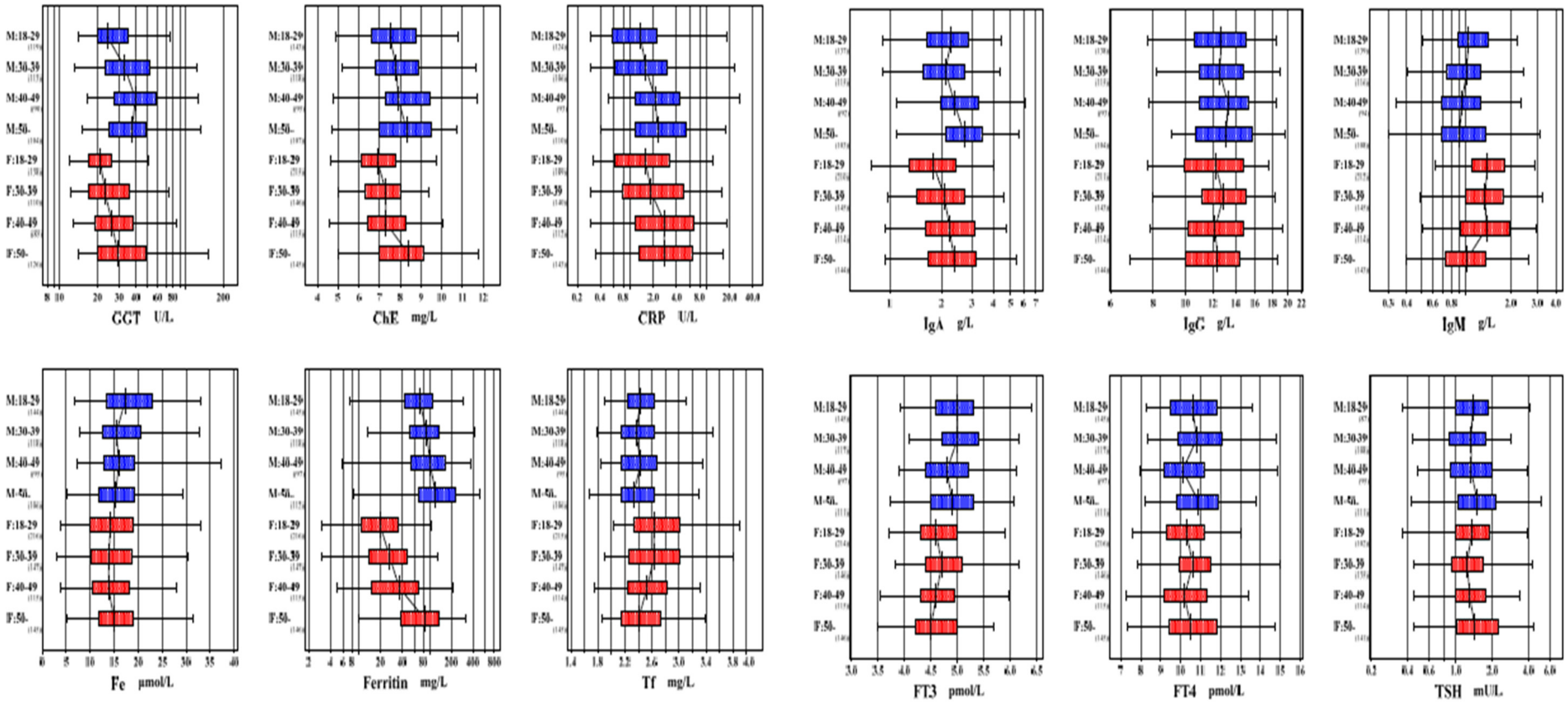
M:30-39
M:40-49
M:50-
F:18-29
F:30-39
F:40-49
F:50-
Mg mmol/L
TC mmol/L
TG mmol/L
ALT U/L
AST U/L
ALP U/L
HDL-C mmol/L
LDL-C mmol/L
LIP U/L
AMY U/L
LDH U/L
CK U/L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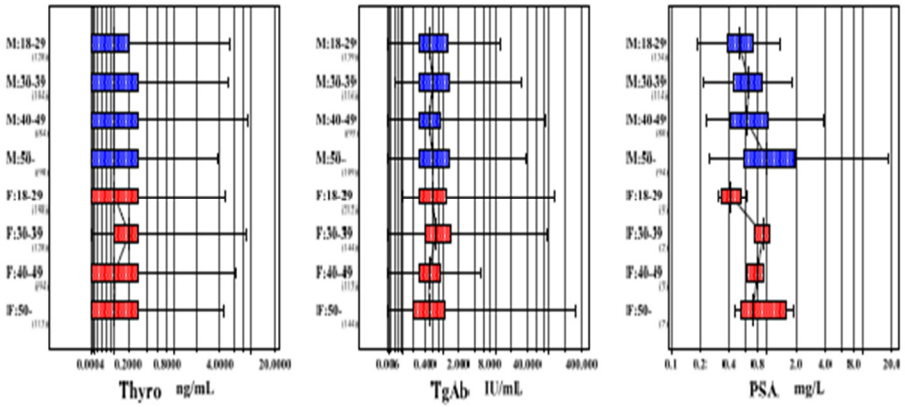
M:30-39
M:40-49
M:50-
F:18-29
F:30-39
F:40-49
F:50-
GGT U/L
ChE mg/L
CRP U/L
IgA g/L
IgG g/L
IgM g/L
Fe μmol/L
Ferritin mg/L
Tf mg/L
FT3 pmol/L
FT4 pmol/L
TSH mUL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M:40-49
M:50-
F:18-29
F:30-39
F:40-49
F:50-
0.0004 0.2000 0.8000 4.0000 20.0000
Thyro ng/mL
0.006 0.400 2.000 8.000 40.000 400.000
TgAb IU/mL
0.1 0.2 0.4 0.8 2.0 4.0 8.0 20.0
PSA mg/L

APPENDIX M

Supplementary Table 1 (Manuscript three): Population characteristics of participants partitioned by gender and ethnicity

	AFRICAN												MIXED ANCESTRY												CAUCASIAN											
	OVERALL				MALE				FEMALE				OVERALL				MALE				FEMALE				OVERALL				MALE				FEMALE			
	± SD	LL	Mean	UL	± SD	LL	Mean	UL	± SD	LL	Mean	UL	± SD	LL	Mean	UL	± SD	LL	Mean	UL	± SD	LL	Mean	UL	± SD	LL	Mean	UL	± SD	LL	Mean	UL	± SD	LL	Mean	UL
Age (years)	8.9	18.0	26.9	52.0	10.4	18.0	29.6	54.0	6.9	18.0	24.9	41.0	11.5	18.0	31.5	56.0	12.3	19.0	34.3	56.0	10.8	18.0	30.0	57.0	12.3	19.0	30.6	59.0	11.0	19.0	29.2	52.0	12.9	18.0	31.2	60.0
Waiste Circumference (cm)	9.6	65.0	83.2	106.0	8.1	64.0	79.8	94.0	9.9	71.0	85.7	106.0	13.3	65.0	84.3	115.0	12.1	66.0	85.8	106.0	13.9	62.0	83.6	118.0	11.1	67.0	86.2	105.0	9.3	67.0	85.7	105.0	11.9	66.5	86.4	105.0
Body Mass Index (kg/m^2)	3.9	18.2	24.9	33.8	4.6	17.8	25.0	34.3	3.4	19.6	24.8	31.7	3.7	18.3	24.3	31.8	3.6	19.2	25.2	33.0	3.7	18.0	23.8	31.0	3.2	18.6	23.7	30.2	3.2	19.5	24.8	30.2	3.0	18.2	23.1	30.1
Systolic Blood Pressure (mmHg)	14.9	93.0	114.8	161.0	16.2	96.0	123.0	169.0	10.2	93.0	108.6	124.0	16.0	93.0	118.9	156.0	14.8	98.0	126.6	156.0	15.1	90.0	115.1	156.0	15.0	95.0	117.1	148.0	15.5	98.0	127.0	166.0	12.1	93.0	112.4	142.0
Diastolic Blood Pressure (mmHg)	10.6	52.0	76.9	95.0	10.8	50.0	80.7	109.0	9.6	60.0	73.9	92.0	11.3	56.0	78.7	102.0	11.0	64.0	83.2	106.0	10.9	56.0	76.5	98.0	10.2	55.0	76.5	96.0	11.5	57.0	79.1	101.0	9.3	55.0	75.3	93.0
Fasting Blood Glucose (mmol/L)	0.4	3.6	4.4	5.2	0.4	3.8	4.4	5.5	0.4	3.5	4.4	5.2	0.5	3.7	4.4	5.4	0.6	3.2	4.4	5.5	0.4	3.8	4.4	5.2	0.4	3.7	4.5	5.5	0.5	3.7	4.5	5.6	0.4	3.7	4.5	5.5

Clinica Chimica Acta 467 (2017) 83-97

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A global multicenter study on reference values: 2. Exploration of sources of variation across the countries

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a r t i c l e i n f o

Article history:
Received 31 December 2015
Received in revised form 8 September 2016
Accepted 20 September 2016
Available online 22 September 2016

Keywords:
Reference values
Worldwide multicenter study
Body mass index (BMI)
Multiple regression analysis
Age-related changes
Ethnicity-related changes
BMI-related changes
Smoking-related changes
Alcohol-related changes

a b s t r a c t

Objectives: The intent of this study, based on a global multicenter study of reference values (RVs) for serum analytes was to explore biological sources of variation (SVs) of the RVs among 12 countries around the world.
Methods: As described in the first part of this paper, RVs of 50 major serum analytes from 13,396 healthy individuals living in 12 countries were obtained. Analyzed in this study were 23 clinical chemistry analytes and 8 analytes measured by immunoturbidimetry. Multiple regression analysis was performed for each gender, country by country, analyte by analyte, by setting four major SVs (age, BMI, and levels of drinking and smoking) as a fixed set of explanatory variables. For analytes with skewed distributions, log-transformation was applied. The association of each source of variation with RVs was expressed as the partial correlation coefficient (r_p).
Results: Obvious gender and age-related changes in the RVs were observed in many analytes, almost consistently between countries. Compilation of age-related variations of RVs after adjusting for between-country differences revealed peculiar patterns specific to each analyte. Judged from the r_p, BMI related changes were observed for many nutritional and inflammatory markers in almost all countries. However, the slope of linear regression of BMI vs. RV differed greatly among countries for some analytes. Alcohol and smoking-related changes were observed less conspicuously in a limited number of analytes.
Conclusion: The features of sex, age, alcohol, and smoking-related changes in RVs of the analytes were largely comparable worldwide. The finding of differences in BMI-related changes among countries in some analytes is quite relevant to understanding ethnic differences in susceptibility to nutritionally related diseases.
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http://dx.doi.org/10.1016/j.cca.2016.09.015
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Non-standard abbreviations

Alb	albumin
ALP	alkaline phosphatase
ALT	alanine aminotransferase
AMY	amylase
ARG	Argentina
AST	aspartate aminotransferase
BMI	body mass index
Ca	calcium
CDL	clinical decision limit
CK	creatine kinase
CI	confidence interval
Cl	chloride
CLSI	Clinical and Laboratory Standards Institute
C3	complement component 3
C4	complement component 4
CHN	China
Cre	creatinine
CRM	certified reference materials
CRP	C-reactive protein
CV (b)	CV of the regression slope b
DL	drugs for dyslipidemia; decision limit
DMS	data management system
ETOH	alcohol consumption
Fe	iron
GGT	gamma-glutamyltransferase
Glu	glucose
GBR	Great Britain
GH	growth hormone
HDL-C	HDL-cholesterol
HBV	hepatitis B virus
HCV	hepatitis C virus
HT	drugs for hypertension
IgA	immunoglobulin A
IgG	immunoglobulin G
IgM	immunoglobulin M
IND	India
IP	inorganic phosphate
JPN	Japan
K	potassium
LAVE	latent abnormal values exclusion
LDH	lactate dehydrogenase
LDL-C	LDL-cholesterol
LL	lower limit
MRA	multiple regression analysis
Me	median
Mg	magnesium
Na	sodium
PAK	Pakistan
PHL	Philippines
RI	reference interval
RMP	reference measurement procedure
RUS	Russia
RV	reference value
r_p	partial correlation coefficient
SAU	Saudi Arabia
SD	standard deviation
SDR	standard deviation ratio
Sk	skewness
Smk	smoking cigarettes
SV	sources of variation
TBil	total bilirubin
TC	total cholesterol
Tf	transferrin
TG	triglycerides
TP	total protein
TUR	Turkey
UA	uric acid
UL	upper limit
ZAF	South Africa
ZAF/Af	African of South Africa
ZAF/NAf	Non-African of South Africa

1. Introduction

The current reference interval (RI) study not only allows determination of RIs but also provides us with an invaluable opportunity to explore and clarify sources of variation (SVs) of each laboratory tests among healthy individuals. In 2004, a multicenter study was conducted in the East and Southeast Asian countries to assess the feasibility of establishing common reference intervals. That study unexpectedly uncovered regional differences in test values for a variety of analytes, especially for inflammatory or nutritional markers, such as IgG, C3, and CRP [1]. To confirm these finding, a large scale study to derive common RIs was conducted in 2009 involving 3500 healthy volunteers recruited by 67 laboratories from 7 countries in East and South-east Asia. By the use of a collective measurement scheme, 72 analytes (25 chemistries and 47 immunoassays) were measured for each specimen [2,3]. The study clearly revealed regional differences in one third of the analytes as determined by use of the criterion of an SD ratio (SDR: between-factor SD divided by between-individual SD) N 0.3, which corresponds to the allowable analytical bias based on between-individual SD [4,5]. Notable examples were HDL-C, IgG, C3, CRP, PTH, and folate.

With this background, the current global multicenter study on reference values (RVs) coordinated by the C-RIDL/IFCC was carefully planned with two major objectives: (1) to establish optimal methodologies for derivation of RIs through analysis of real-world datasets gathered from studies around the world, and (2) to explore their SVs including age, gender, BMI, drinking, smoking, geographical regionality and ethnicity. The use of a common protocol with a detailed health-status questionnaire [6], and the scheme of aligning RVs based on the test results of a standard reference serum panel measured in common allowed us to perform the analysis in a well-controlled manner [7]. In the first part of this report on the global study, various methodological issues related to derivation of the RIs were addressed and thoughts on each issue were provided as a consensus among the collaborators [8]. In this second part of the paper, SVs of RVs found in the 12 countries were systematically evaluated, using the same dataset as analyzed in the first part. The main focus of the analyses were as follows:

(1) to evaluate the importance of age, BMI, and levels of alcohol ingestion and smoking as a major SVs of RVs in the various countries (ethnic groups) by use of multiple regression analysis (MRA),
(2) to confirm ethnicity related differences in BMI-related changes in RVs, which were noted for some analytes (ALT, TG, HDL-C, and CRP) in the preliminary analysis [5],
(3) to make a BMI adjusted comparison of RVs among the countries, and
(4) to delineate gender- and age-related profiles of RVs from a large number of datasets compiled from the 12 countries.

2. Methods

2.1. Source data

Included in this interim analysis are the results from 12 countries: China (CHN), Japan (JPN), Philippines (PHL), India (IND), Pakistan (PAK), Saudi Arabia (SAU), Turkey (TUR), Russia (RUS), UK (GBR), South Africa (ZAF), USA, and Argentina (ARG). The demographic profiles of each country's study are as reported in the part 1 of this paper [8]. In brief, the total numbers of subjects were 13,396 (male 6347; female 7049). No ethnicity related distinctions of individuals were made for any countries except South Africa, where RVs were partitioned as Africans and non-Africans. Although the number of individuals above 65 years of age varied greatly, the distributions of ages under 65 were well balanced. Distributions of BMI differed greatly among the countries as shown in Fig. 1. As shown in Table 1 of part 1 [8], the proportions of those who smoked cigarettes were quite comparable among the countries. In contrast, the proportions of individuals who drank alcohol occasionally or regularly differed greatly between countries, partly for religious reasons. In this study of SVs, unlike the study for deriving the

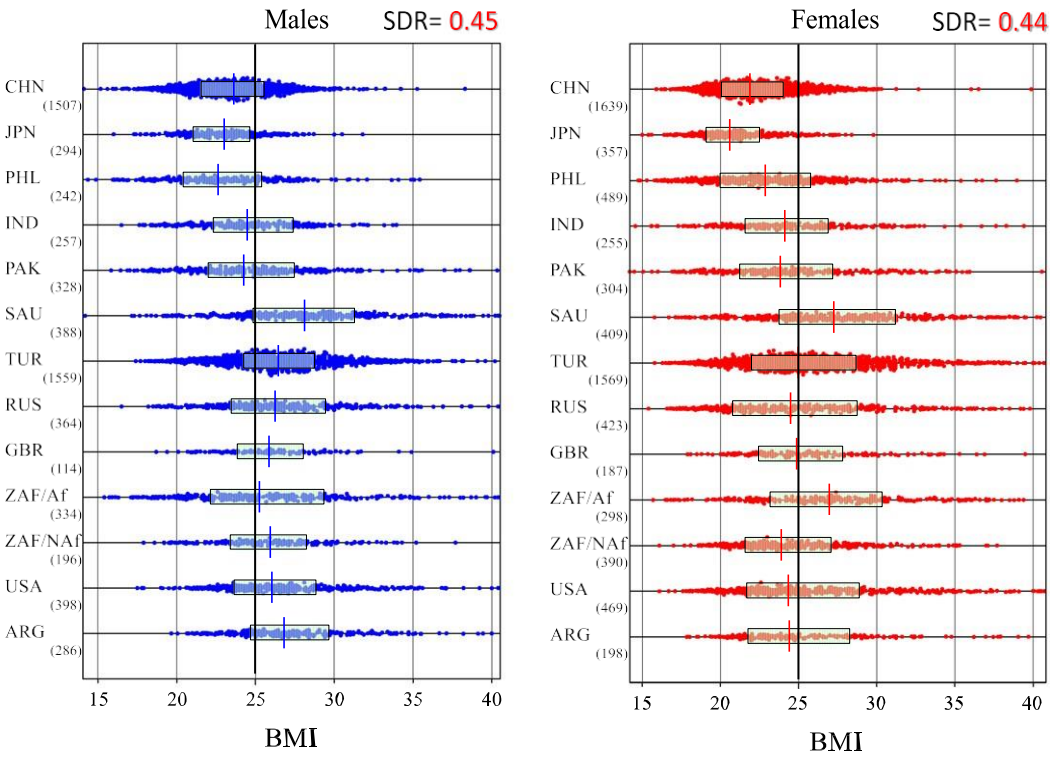

Fig. 1. Comparison of BMI among the ethnic groups in males and females. The distributions of BMI for males and females were compared among ethnic groups. The horizontal box in each scattergram represents the central 50% range and the vertical line in the center denotes the median point. The long vertical line was drawn at BMI = 25 kg/m^2 as a guide. As judged from the SDR of around 0.45 for both genders, there are large between-group differences in BMI among the ethnic groups.

RIs, RVs from individuals receiving medications were not excluded from the analysis, because their numbers were relatively small [see Table 1 of [8] and no difference was observed in the preliminary multiple regression analysis (MRA) with or without the exclusion of those individuals, although we noted a non-negligible effect of oral contraceptive in the derivation of Alb, TG, and ALP [8] .

Among 50 target analytes [6], RVs of the following 31 analytes were chosen for analyses in this study: TP, Alb, UN, UA, Cre, TBil, Glu, TC, TG, HDL-C, LDL-C, K, Ca, IP, Mg, Fe, LD, GGT, AMY, AST, ALT, ALP, CK, CRP, IgG, IgA, IgM, C3, C4, Tf, and TTR. RVs of Na and Cl were excluded because of the difficulty in harmonizing the values for these analyses.

2.2. Analysis of sources of variation by multiple regression analysis

MRA was performed, country by country, analyte by analyte, separately for each gender. The value of each analyte was set as an object variable and a fixed set of SVs were included as explanatory variables [of age, BMI, and levels of alcohol consumption (ETOH) and smoking cigarettes (Smk). ETOH was ranked into five grades by ethanol in g per day: 0: none; 1:≤12.5 g; 2:12.5 þ to ≤25 g; 3:25 þ to ≤50 g; 4:50 g þ ethanol/day. Smk was ranked into 3 grades by the average number of cigarettes per day: 0:none; 1:≤20; 2:N20/day. The South African dataset was again analyzed in two parts by ethnicity. Regarding ETOH, it was excluded from the analysis for Saudi Arabia and Pakistan (where drinking alcohol is prohibited for religious reasons), and for Smk, it was not included in the analysis of females in India, Pakistan, and African of South African because of a very low rate of smoking [also see Table 1 in [8].

Each of 25 analytes was set one by one as an objective variable. Values for analytes which showed skewed distributions of values were logarithmically (log) transformed before MRA. These include TBil, Cre, TG, AST, ALT, LDH, GGT, ALP, CK, AMY, and CRP. The outlying points outside mean ± 4 × SD of the RV distribution were excluded before MRA (if transformed, mean and SD were computed in the log scale).

The association of each SV with the object variable was expressed as a standardized partial regression coefficient (r_p) which corresponds to the partial correlation coefficient, with values between −1.0 and 1.0. Because of the generally large data sizes, statistical testing of r_p is not suitable; therefore, we arbitrarily regarded 0.15 ≤ | r_p | þ 0.25 as slight, 0.25 ≤ | r_p | þ 0.35 as moderate, and 0.35 ≤ | r_p | as obvious associations.

2.3. Association of age and BMI with the RVs for each country

Linear-relationships of the RVs with age or BMI were analyzed country by country using the least-square linear regression in order to determine whether or not the relationships were constant among the countries. For age, the analysis was selectively performed in males for RVs of Alb which had a prominent age-related change, and in females for RVs of IP, ALP, and LDH, which showed prominent increase after menopause. For BMI, regression analysis was performed separately for each gender using RVs of UA, TG, HDL-C, ALT, GGT, and CRP with the highest associations with BMI. Again, in the analysis of RVs of ALT, GGT, and CRP, the RVs were transformed logarithmically to minimize the skewness in the distributions.

2.4. BMI adjusted comparison of the RVs among the countries

With conspicuous differences in BMI among the countries (Fig. 1) and our observation of ethnicity related differences in the effect of BMI on RVs, BMI adjusted analysis of between-country differences was performed. The adjustment was made, country by country, by use of the following formulas, assuming BMI of the i-th individual (BMI_i) was shifted to BMI_0 (=25 kg/m^2):

$b = \Delta y_i / \Delta BMI_i$ ⋯ definition of the slope

$\Delta y_i = b \times (BMI_i - BMI_0)$

$Y_i = y_i - \Delta y_i$

where b is the slope of the least squares regression line y=a+b× BMI. BMI_i and y_i represent the BMI and test results of the *i*-th individual. Y_i corresponds to y_i adjusted for the difference of BMI from BMI_0 (ΔBMI_i). By the definition of the slope b, the change of the y_i (Δy_i) by shifting from BMI_i to BMI_0 can be computed by the second formula above. Then, the adjusted value Y_i can be simply computed by the third formula.

Likewise, when y_i is logarithmically transformed, the adjusted Y_i by shifting BMIi to BMI_0 can be computed based on the least square linear regression line $\log y = a + b \times BMI$ using the following formulas (Fig. 2A).

$$b = \Delta \log y / \Delta BMI$$

$$\Delta \log y_i = b \times (BMI_i - BMI_0)$$

$$\log Y_i = \log y_i - \Delta \log y_i$$

$$Y_i = e^{\log y_i - \Delta \log y_i}$$

2.5. *Sex and age related profile of the RVs adjusted for between-country differences*

All the test results were recalibrated to the assigned values if available, and otherwise aligned to the values for China, which had the largest number of RVs. Then, in consideration of between country differences observed between the countries as reported in part one of this report [8], the aligned test results for each analyte were adjusted country by country so that the median of country g (Me_g) was adjusted to the median of China (Me_0). Therefore, the *i*-th value of country g, x_{gi} was adjusted to X_{gi} by use of the following formula.

$$X_{gi} = x_{gi} - (Me_g - Me_0)$$

In case of analyte whose values were log transformed, the following formula were used to adjust for the difference D_g in the medians on the log-scale (Fig. 2B).

$$D_g = \log Me_g - \log Me_0$$

$$\log X_{gi} = \log x_{gi} - D_g$$

$$X_{gi} = e^{\log x_{gi} - D_g}$$

3. Results

3.1. *Comparison of partial correlation coefficients for age and BMI among the countries*

The lists of partial correlation coefficients (r_p) obtained country by country, analyte by analyte, for each gender were organized for each SV into four tables (Suppl. Table 1). Test items in each table were sorted by the descending order of their average r_p ($\overline{r_p}$) for the countries examined. The analytes with $|\overline{r_p}| \geq 0.10$ for either of the sexes for the analysis of age and BMI (or $|\overline{r_p}| \geq 0.07$ for ETOH or Smk) were chosen and their distributions of r_p are shown in Fig. 3.

Age as a SV in males showed moderately positive associations with TC and Glu; slight associations with LDL-C, urea, CRP, and TG; moderately negative (neg) association with Alb; and slightly negative associations with IgM and TP. In females, age showed associations with urea and TC; moderate associations with LDL-C, Glu, and LDH; slight associations with TG, ALP, Tf (neg), IgM (neg) and 6 other analytes. It is notable that LDH and ALP in females showed a relatively wide among-country difference in the degree of age-association. It is also notable that degree of age-associations is generally more prominent in females, especially for urea, Alb, LDH, and ALP.

BMI as a SV in males showed a prominent association with C3; moderate association with ALT, CRP, UA, GGT, and TG; slight association with HDL-C (neg) and 4 other analytes. In females, BMI showed prominent association with C3 and CRP, moderate association with UA and HDL-C (neg), and slight association with TG, GGT, AMY (neg), and four other analytes. It is notable that the associations of ALP and AMY with BMI are more prominent in females.

Smk as a SV had only a week associations with TG, GGT, TBil (neg), IgG (neg), and AMY (neg) in both genders. ETOH as a SV also had a week associations only with GGT, HDL-C, UA and IgG (neg).

3.2. *Consistency of age-related changes among ethnic groups*

The partial correlation coefficient r_p computed by the MRA showed closeness of the linear association between values of a given SV (x) and RVs (y). However, r_p does not necessarily mean that the slope of the linear association is steeper in proportion to r_p. Therefore, regarding age as a SV, its relationship with RVs and the slope was evaluated by least-square linear regression analysis. Chosen for the analysis were Alb of males, and ALP for females. A two-dimensional scattergram was drawn for each ethnic group using the same scale for x- and y-axes. The graphs are displayed in the descending order of the slopes in Fig. 4. Regarding Alb, there is apparent consistency in the linear relationship among the 10 ethnic groups. There is some fluctuation of the

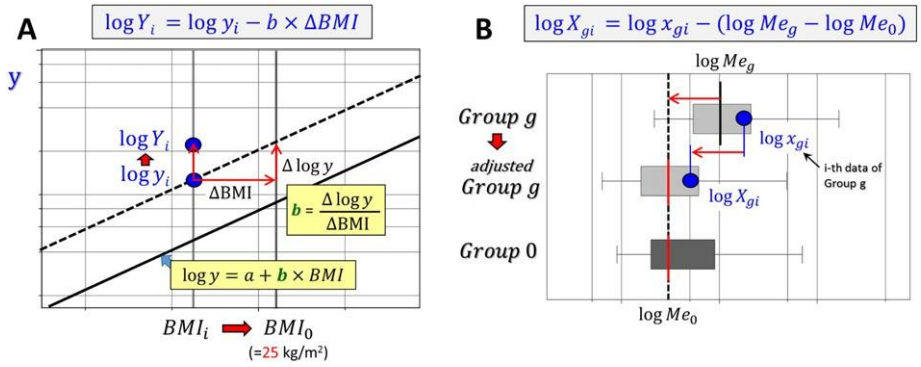

Fig. 2. Adjustment of test values on logarithmically transformed scale. The left panel (A) illustrates a formula to adjust a test value y of the *i*-th individual for a change of BMI from BMI_i to BMI_0 (=25 kg/m^2) by use of a least-square linear regression equation. The right panel (B) illustrates how to shift the *i*-th test results x_{gi} of Group g by a difference of medians between Group g and Group 0 in logarithmic scale.

slopes, which range from −0.04 to −0.133 (a 3.3-fold spread), but the difference can be reduced to 2.0-fold by excluding the groups on both ends with small sample sizes. It is notable that the slopes (b), as a whole, are not consistent with the correlation coefficients (r).

For ALP in females, a prominent elevation after menopause (around 50 years) was apparent in almost all 13 ethnic groups. The magnitude of between-group differences was expressed in SDR (computed by one-way ANOVA). The fluctuations in SDR were small, implying there are

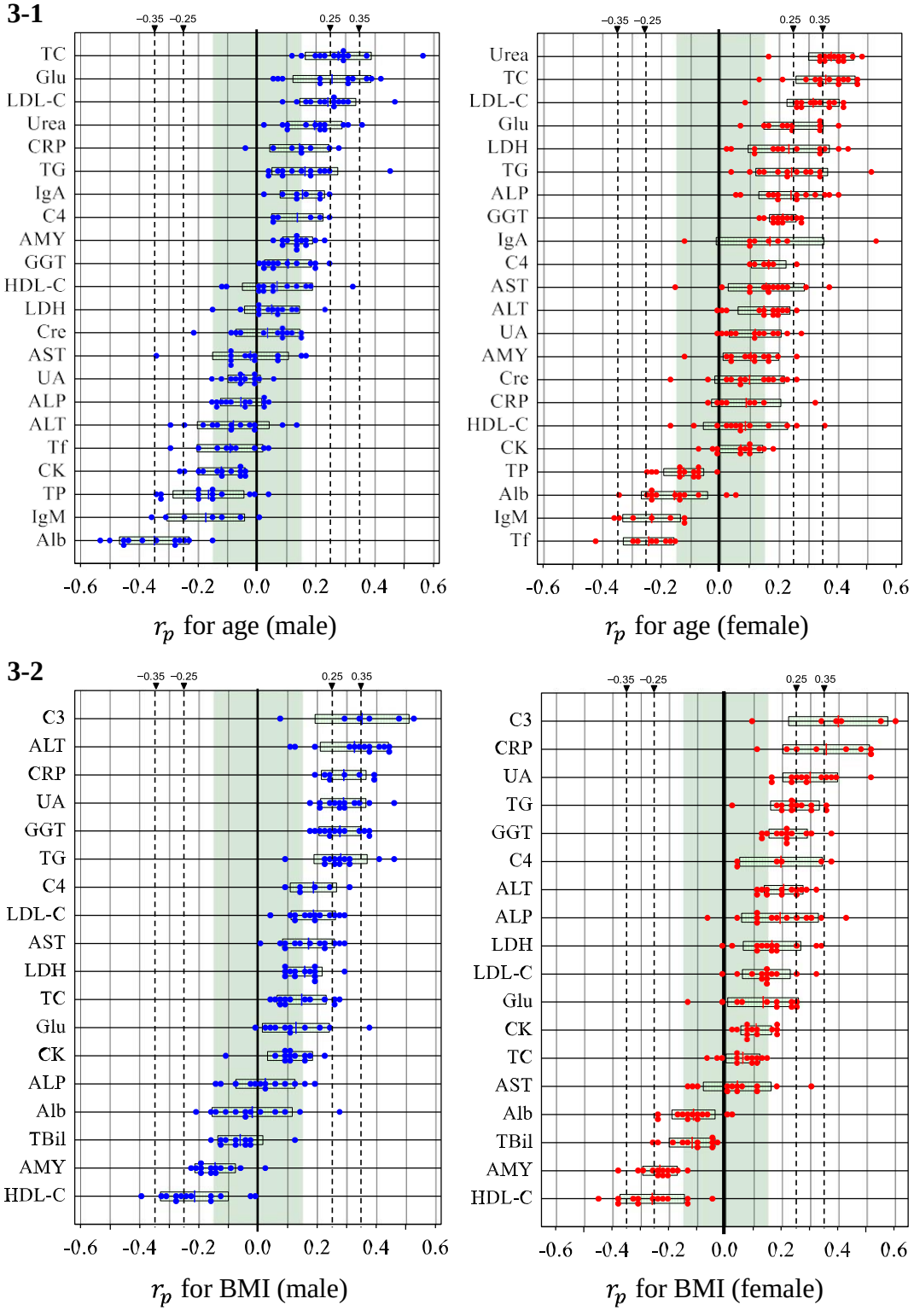

Fig. 3. Comparison of MRA results across the countries in males and females. Multiple regression analysis (MRA) was performed, country by country, for each analyte by setting age, BMI, and levels of alcohol consumption (ETOH) and smoking (Smk) as a fixed set of explanatory variables. The analysis was done separately for each gender. The association of each source of variation (SV) with test results of a given analyte was expressed as a partial regression coefficient (r_p) with values between −1.0 and 1.0. The results of r_p from all the countries were gathered, SV by SV, for each gender. The distributions of r_p for each analyte are shown in descending order of average r_p of all the countries. Because of the large data size, $0.15 \le |\overline{r_p}| < 0.25$ was regarded as slight, $0.25 \le |\overline{r_p}| < 0.35$ as moderate, $0.35 \le |\overline{r_p}|$ as prominent association. Fig. 3-1: Distribution of r_p for age. Fig. 3-2: Distribution of r_p for BMI in males and females. Fig. 3-3: Distribution of r_p for habit of smoking (Smk) and drinking alcohol (ETOH).

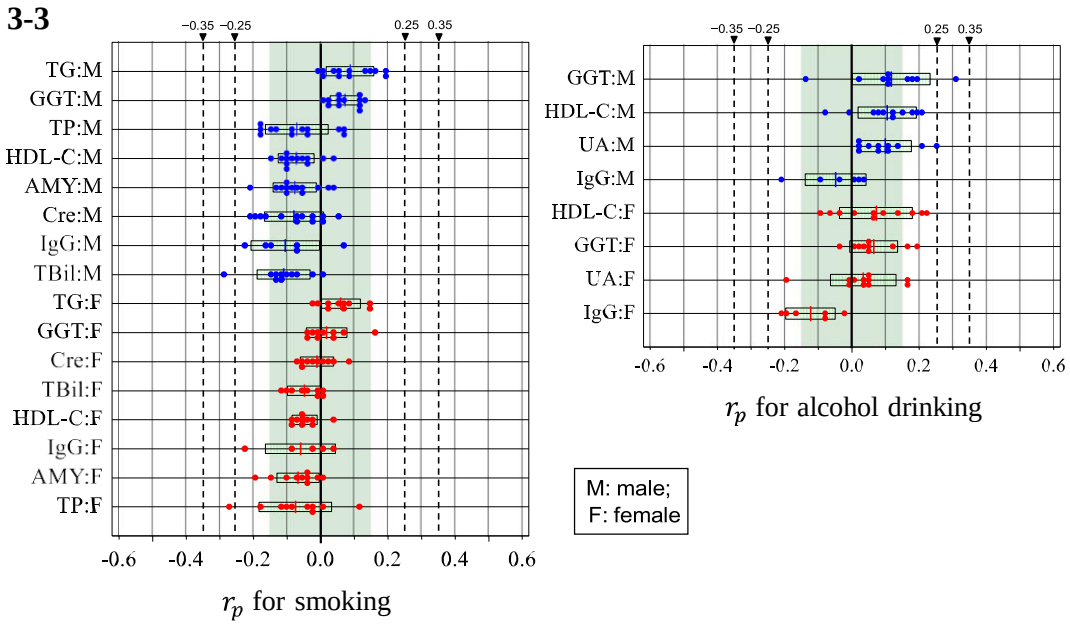

Fig. 3 (*continued*).

no major differences in age-related changes in female ALP among groups. The same analyses performed for urea, TC, and LDL-C gave similar results (data not shown).

3.3. Consistency of BMI-related changes among the countries

For BMI as a SV, the same analyses were performed to compare r_p with the slopes of the linear regressions between BMI and test results. Chosen for analysis were UA, TG, HDL-C, ALT, GGT, and CRP which showed obvious associations with BMI. Typical results are shown in Fig. 5 for ALT (Fig. 5-1), HDL-C (Fig. 5-2), and CRP (Fig. 5-3) for males. Regarding log-transformed ALT, there are conspicuous differences in slopes of the least-square regression line for 13 ethnic groups. The slopes differed greatly, ranging from 0.003 to 0.091, a factor of 30 fold (6.9 fold after excluding one group on each end). For HDL-C, the slopes also differed greatly among the ethnic groups, ranging from −0.0004 to −0.0050 (a factor of 12.5-fold; reduced to 3.1-fold after removing two groups from the ends). In contrast, the slopes of log-transformed CRP with BMI ranged from 0.061 to 0.114 (the factor of 1.9 fold; the same after exclusion). It is of note that the slope (b) is as a whole not related to the correlation coefficient (r), which is a parameter representing the closeness of the relationship. The results of the same analyses for UA, ALT, and GGT are shown in Suppl. Fig. 1. In all, there are quite conspicuous ethnic differences in BMI-related changes for the test results in each of these analytes except CRP.

3.4. BMI-adjusted comparison of RVs among ethnic groups

The results of the above analyses point to the need for BMI adjusted comparison of RVs among the countries because there exist large between-country differences in BMI as shown in Fig. 1. In Fig. 6, two examples are shown for between-country comparison of RVs for log-transformed male TG and for female HDL-C. Adjusted comparisons for other analytes affected by BMI are shown in Suppl. Fig. 2. In terms of SDR, the changes are small with only slight shifts of RVs for each ethnic group. The interpretations of adjusted differences in RVs among the ethnic groups are described in the Discussion.

3.5. Gender- and age-related profiles of RVs after application of the LAVE method and adjustment for between-ethnic groups differences.

Fig. 7 shows the effects of applying the LAVE method and adjustment for between-ethnic group differences on the gender- and age-related profiles of RVs. Gender and age-related scattergrams of RVs for TG and ALP were drawn in three ways. Plotted in Graph A were RVs of all ethnic groups which were made traceable to the reference measurement procedures (RMPs) based on the assigned values in the panel: i.e., all the RVs of each ethnic group were aligned to the assigned values of the serum panel. In Graph B, RVs made traceable to RMPs were plotted only when test results of other related analytes were within respective RIs. In Graph C, RVs of each ethnic groups after applying the LAVE method were aligned to those of China (CHN) by adjusting for the difference of its median from that of China. The horizontal broken lines are shown as a guide for ease of comparison. The final adjusted gender- and age-profiles are shown in Suppl. Fig. 3 for major analytes.

4. Discussion

This primary study for derivation of RIs provides us with a good opportunity to analyze potential biological SVs of RVs among healthy individuals. Conventionally, gender and age are the only SVs considered because they are often strong enough to require partitioning RVs. Not much attention has been paid to other biological SVs such as BMI, alcohol consumption, cigarette smoking, exercise, food preferences, menstrual cycle, ABO blood groups, etc. This situation may be attributable to an insufficient sample size or lack of knowledge or facility for performing the analyses. In fact, no description is given in the Clinical and Laboratory Standards Institute (CLSI) guideline [9] regarding the method to be used for the analysis, and major RI studies in the past have not included SV analysis [10,11]. Reliable analysis of SVs cannot be done by simple stratification of the RVs. The need for multiple linear regression analysis (MRA) for the purpose was first described by Ichihara and Kawai when they looked into factors for partitioning RVs and encountered problems of interaction and confounding phenomena of gender on RVs [12]. The theoretical aspect of these phenomena was described elsewhere [4,5]. After the first report, a series of papers on

RI studies which included MRA for exploration of SVs as an integral part of the study were published [1,13-17]. The analyses targeting the same SVs as in this study (BMI, smoking, and alcohol consumption) are available in [1,12-15,17]. Additionally, the association of ABO blood group on RVs was evaluated in the RI study for isozymes of ALP, LDH, and AMY [16] and menstrual cycle related changes were reported on 85 major laboratory tests [18] as a secondary analysis of the 2009 Asian study on RVs [2,3].

The effects of additional biological SVs, such as ethnicity and the geographical effect on RVs are of interest, but these studies can be achieved only by building international collaborations. Therefore, there are very few investigations reported so far which were conducted as primary studies for deriving RIs [1,2,10,11,19]. On the other hand, there have been a large number of publications comparing laboratory tests results among multiple ethnic groups. These were, however, conducted as a part of large epidemiological studies aiming at uncovering risk factors for common disorders like the metabolic syndrome, cardiovascular diseases, diabetes, hypertension, and steatohepatitis. The target populations were those that had an increased likelihood of contracting the highly prevalent diseases, and thus, the sampling schemes used were "random population based sampling". Therefore, unlike the current reference interval study, they were not based on the concept of

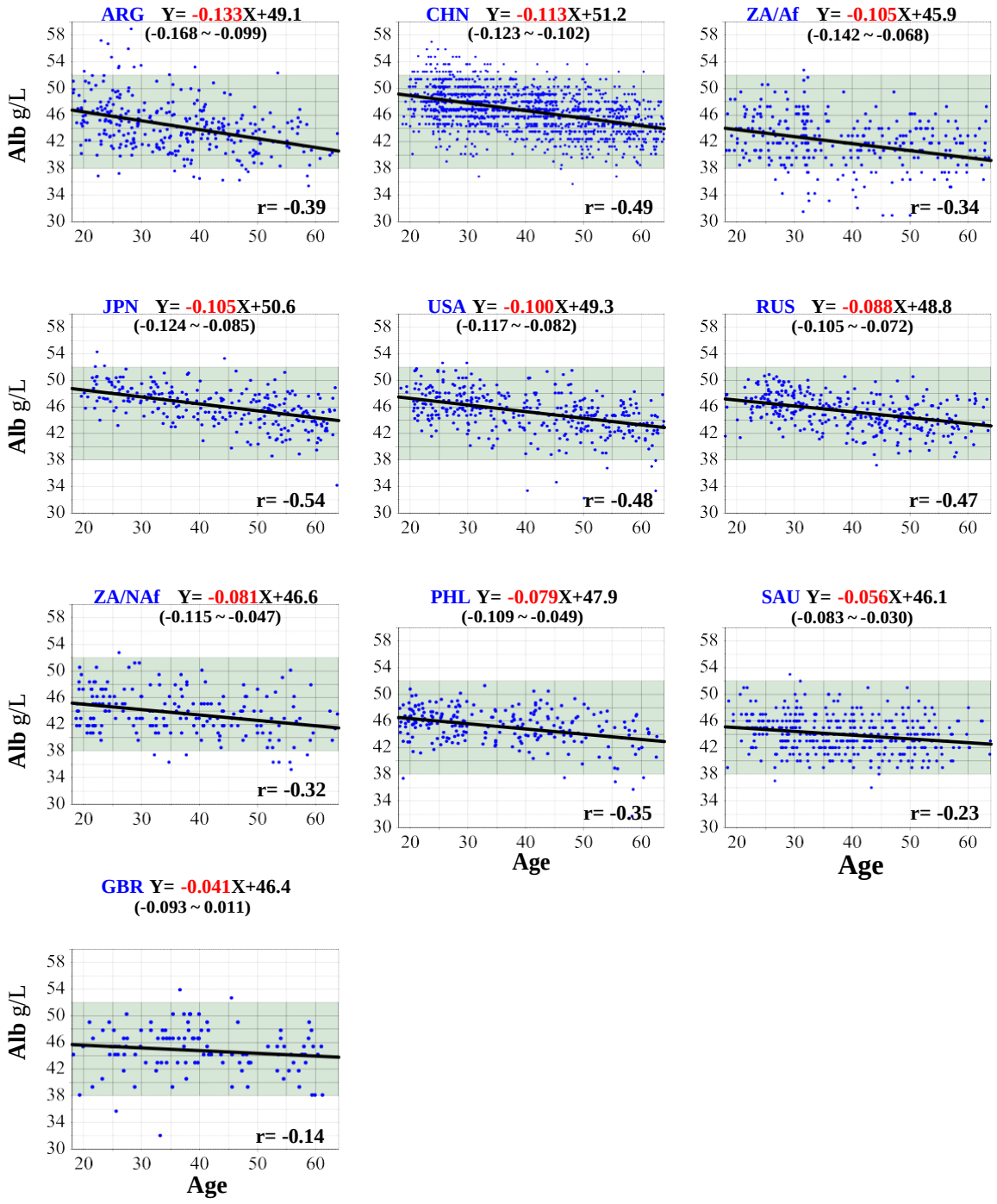

Fig. 4. Comparison of age-related change in RVs among ethnic groups. In panel 4-1, the relationship between age and RVs of male Alb is shown for each ethnic group. The least-squares regression line was computed and its equation is shown on top of each graph to allow comparison of slopes among the ethnic groups. Shown below each slope in red letter is its 95% CI. The simple correlation coefficient (r) is shown at the right bottom. In panel 4-2, the relationships between age and RVs of female ALP were plotted. The significance of age related changes was

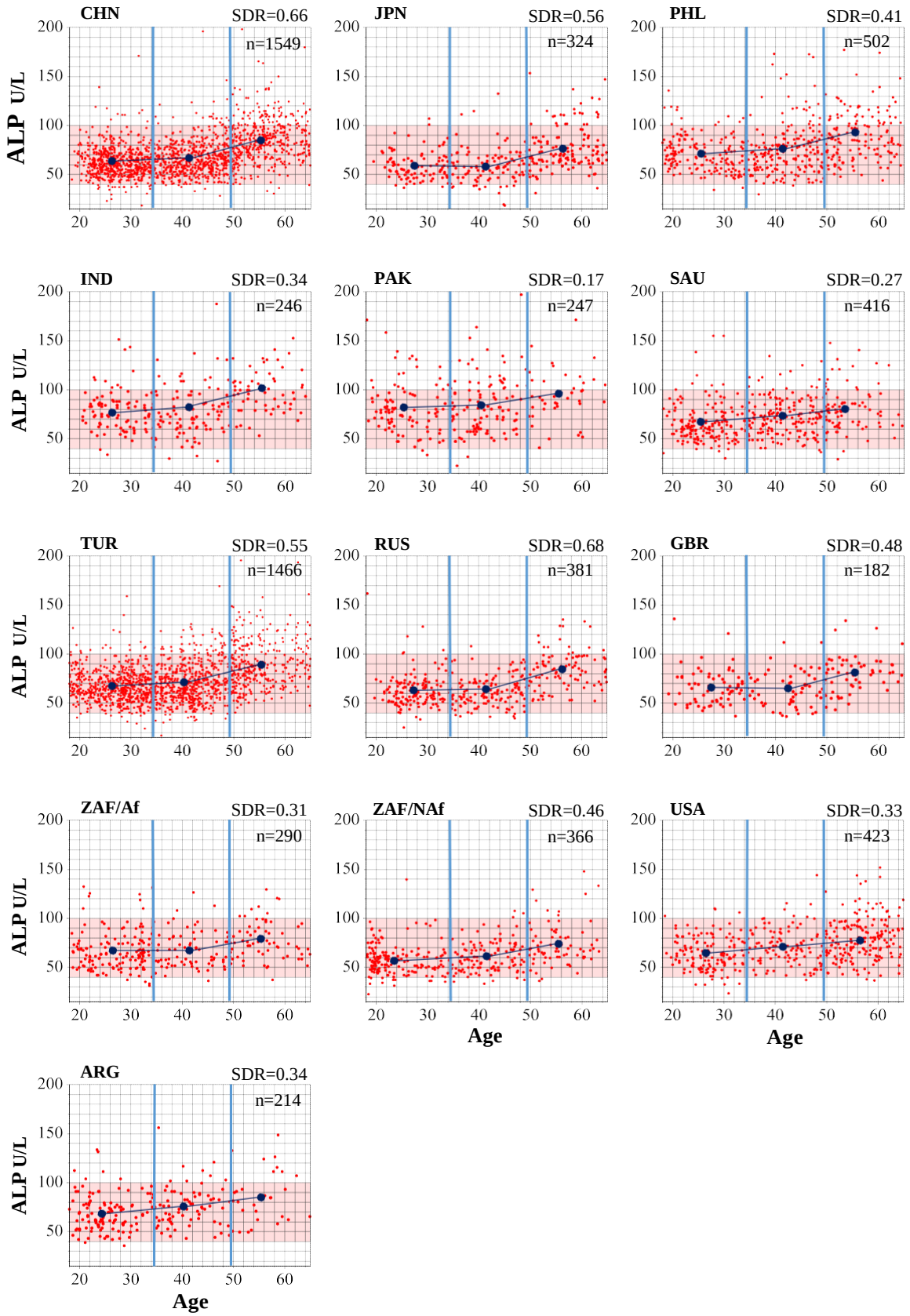

Fig. 4 (*continued*).

"healthiness" but included individuals who might have the latent diseases targeted by the studies. Examples include a large scale multinational study like the INTERHEART study which involved 52 countries around the world and aimed at elucidation of risk factors such as serum lipids for myocardial infarction [20-22]; or nationwide studies in the United States with balanced sampling from multiple ethnic

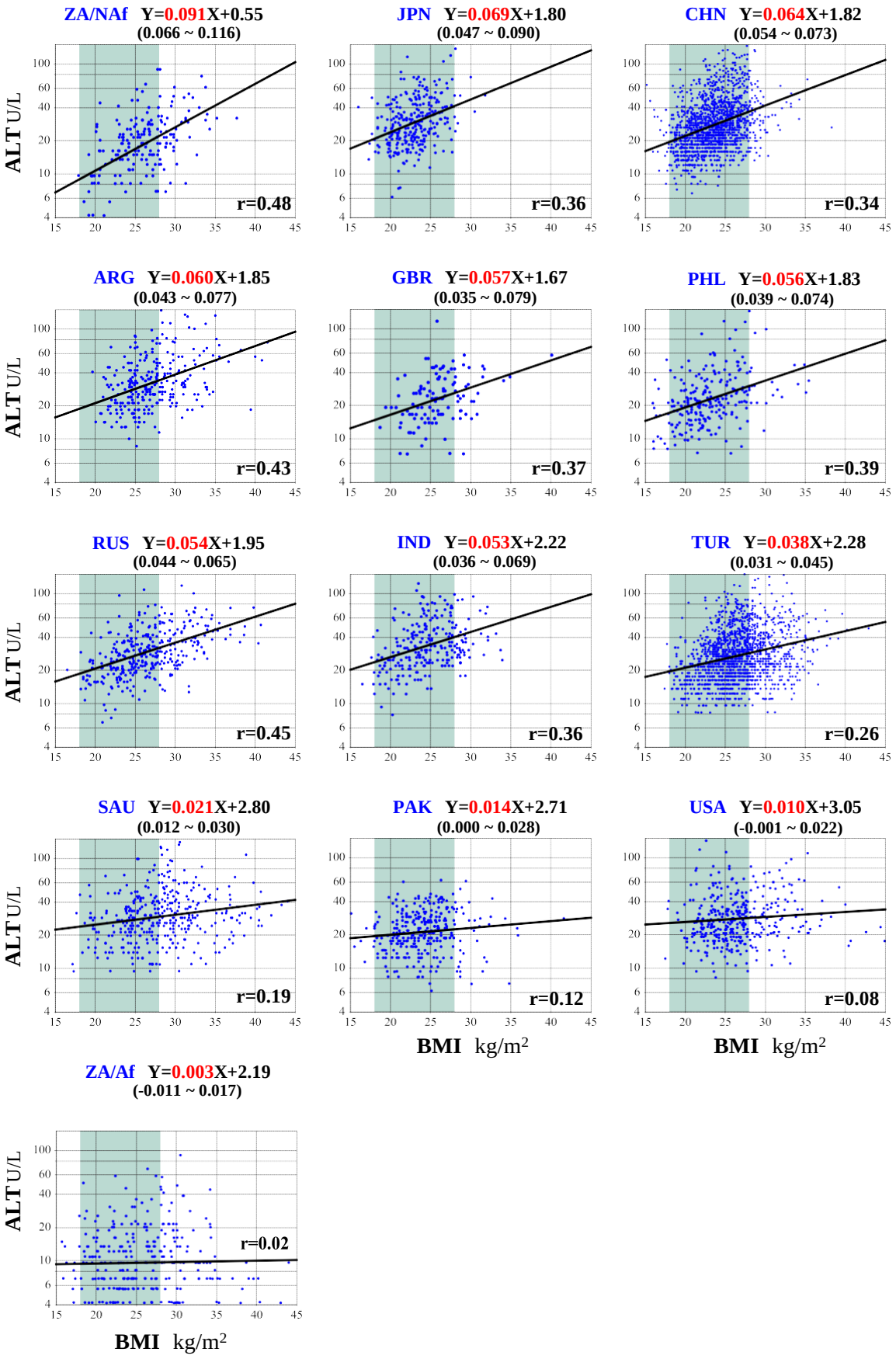

Fig. 5. Comparison of BMI-related changes in RVs among ethnic groups. In panel 5-1, the relationship between BMI and RVs of ALT in males (in logarithmic scale) is shown for each ethnic group. The least-square regression line was computed and its equation is shown on top of each graph to allow comparison of slopes among the ethnic groups. Shown below each slope in red letters is its 95% CI. The simple regression coefficient (r) is shown at the right bottom. The same analyses were done for HDL-C and CRP as shown in panels 5-2, and 5-3, respectively. Other male results for UA, TG, and GGT, and female results for all six analytes are available in Suppl. Fig. 1.

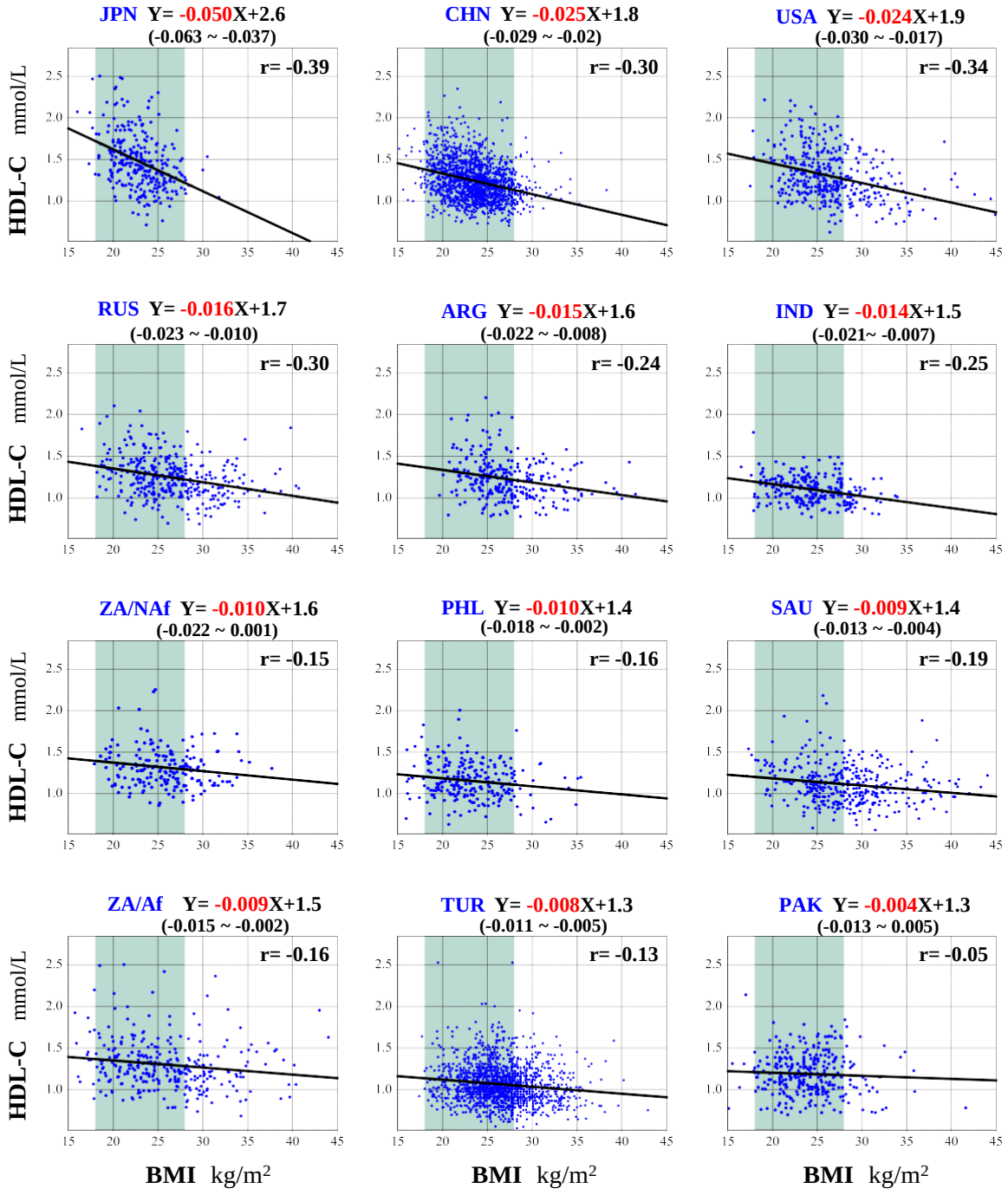

Fig. 5 (*continued*).

groups such as the National Health and Nutrition Examination Survey (NHANES) [23,24] and the Women's Health Study [25], or in Canada the Study of Health Assessment and Risk in Ethnic groups (SHARE) [26]. These studies targeted the analysis of multiple serum risk factors for common diseases. Numerous epidemiological studies for elucidation of health-related risk factors were also done in local multiracial communities of Framingham [27], Dallas [28], London [29], Manchester [30], and Newcastle [31]. These studies are cited below in the discussion of ethnicity-related changes in RVs.

On the other hand, in this global project on RVs, we adopted a "convenience sampling" scheme, targeting those who were easily accessible from hospitals and clinical laboratories. Therefore, epidemiologists may criticize that the reference individuals do not represent minor social groups with special occupations, economic conditions, or ethnicity. However, the study on RVs needs to focus on the status of "healthiness" in volunteers who fulfill pre-defined conditions, and, in this particular project, who do not possess multiple abnormal results in major laboratory test results. Therefore, presumptive test results from minor subgroups would have negligible effect on data analysis due to their small size or would form a cluster of abnormal results but be excluded by application of the LAVE method. From this perspective, our international collaborative study is the first comprehensible one which uncovered ethnic differences in "health-associated" RVs in a global scale.

As for representation of ethnic groups within each country, the sampling scheme is far from ideal for multiracial countries like the US and the UK with coverage of just three cities (Rochester, MN; Salt Lake

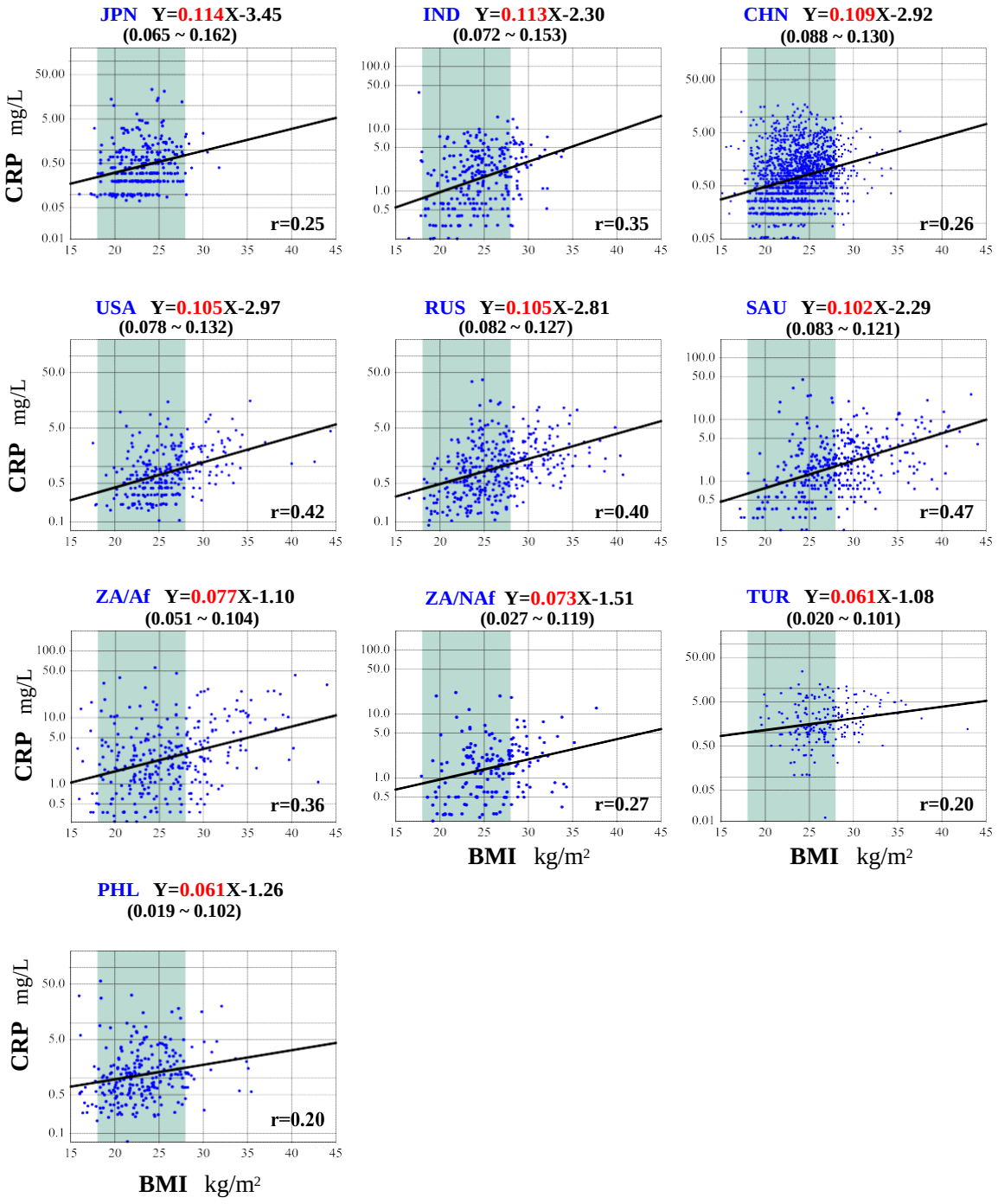

Fig. 5 (*continued*).

City, UT; Davis, CA) in the US and one (Leeds) in the UK. We need to accept that the RVs just represent the Caucasian population, which constitutes a vast majority. On the other hand, in South Africa, the sampling was separately conducted in three ethnic groups: African, Caucasian, and mixed origin (Chinese, Indian, etc.). Due to a lack of sufficient data for the third group and similarity of the last two, the data analyses for South Africa were done in two ethnic groups: African and Non-African as representative of the country.

For other countries, ethnic compositions are regarded as representative of each country because of the following reasons. In Turkey and China, the sampling was carried out nationwide and no regional differences were noted as a whole [15,17]. In Saudi Arabia, Japan, and Russia, the sampling was done in geographically distinct major cities but all subjects were from a single ethnic group. In the Philippines, Pakistan, India, and Argentina, only individual cities were covered but the population sampled was representative of each country.

Among the multitude of biological SVs evaluated in this study gender, age and BMI are the three most important ones. Age-related changes in RVs proved to be a universal phenomenon regardless of the countries as shown in Fig. 3-1. With the availability of a large number of RVs from 12 countries, it became possible to compile all of them

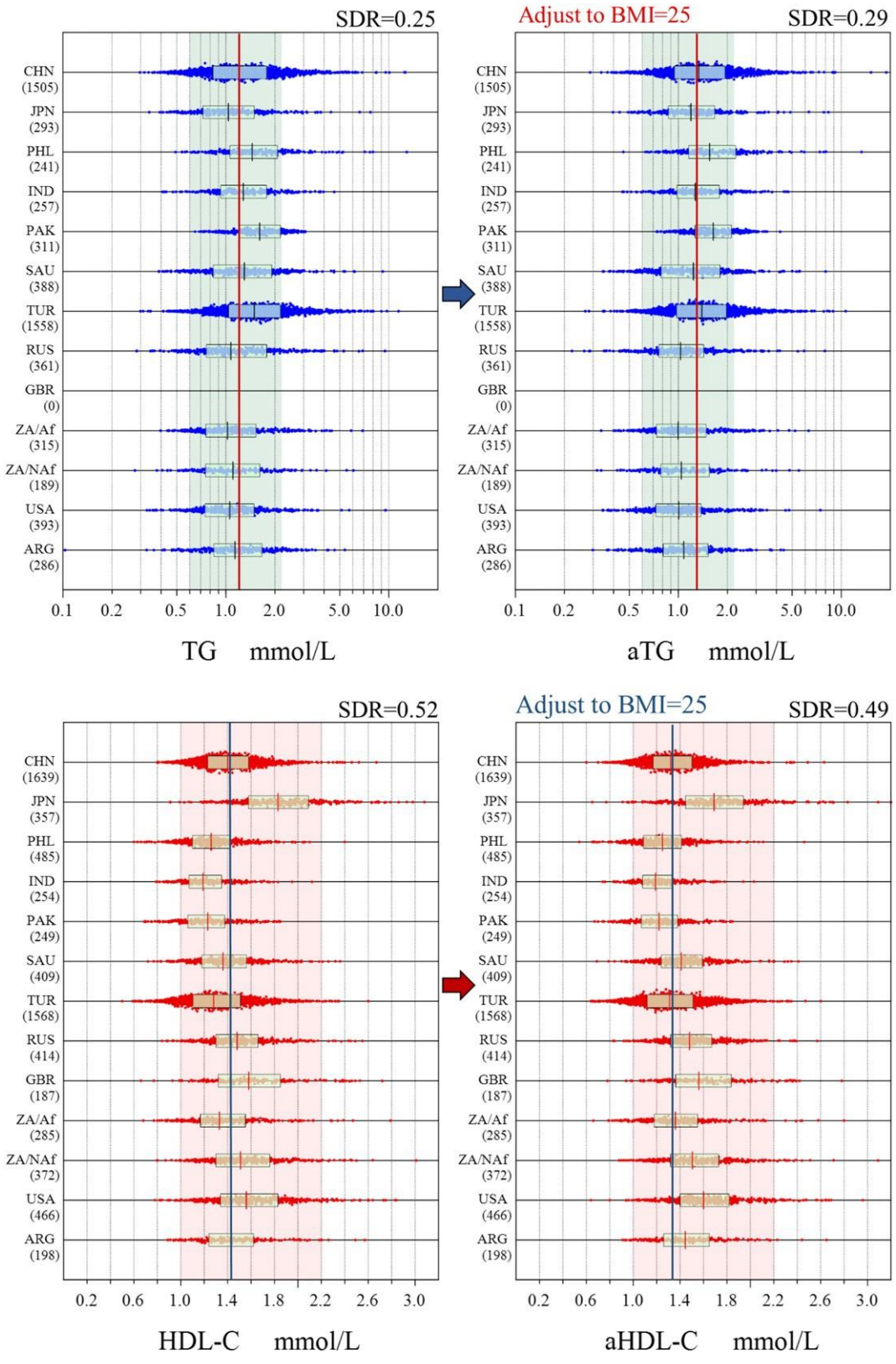

Fig. 6. BMI-adjusted comparison among the ethnic groups. Distributions of RVs for male TG (the top panel) and female HDL-C (the bottom panel) after alignment to the assigned values were compared by scattergrams among the ethnic groups before (left) and after (right) adjustment for the difference in each individual's BMI from the reference BMI of 25 kg/m^2. The details of the computation for the adjustment are described in the Methods section.

and delineate gender and age-specific profiles by three steps: (1) alignment of values either to assigned values or to China, (2) removal of potentially abnormal values by applying the LAVE method, and (3) adjusting RVs to those of China based on the difference in medians between China and the country concerned as shown in Suppl. Fig. 3. The figures are of great relevance in interpreting the RVs appropriately in consideration of gender and age. From the figures, it is apparent that almost all analytes have specific profiles of its own.

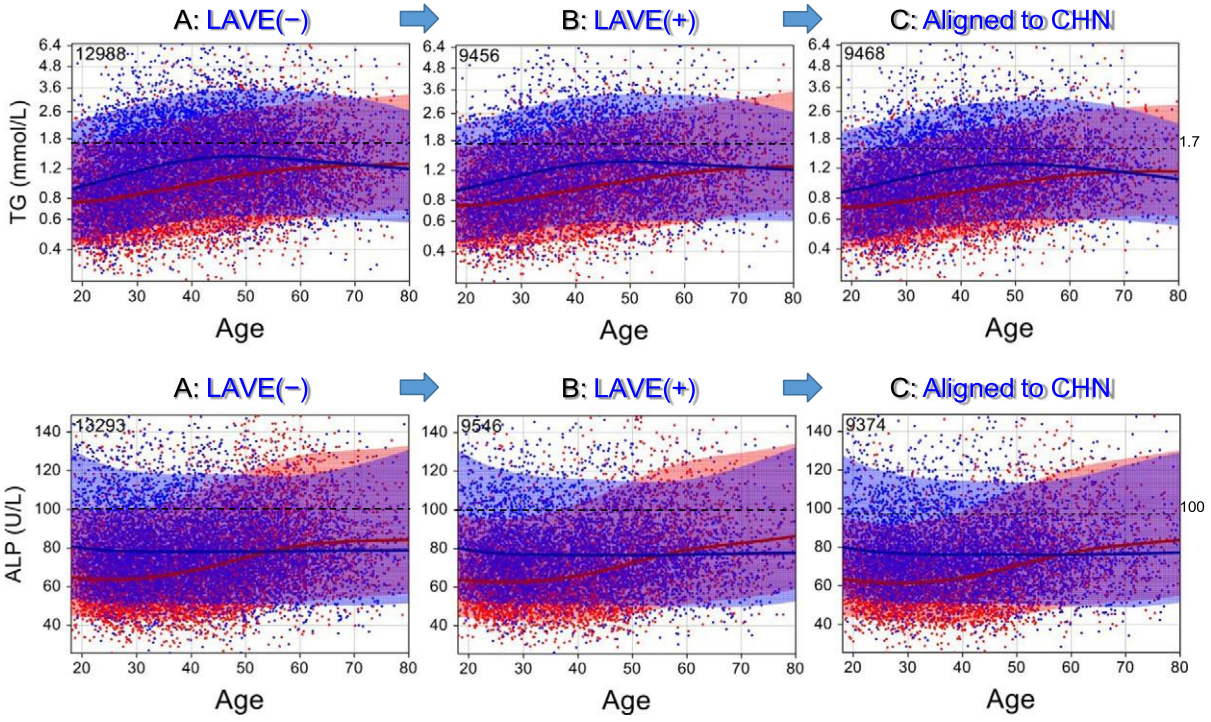

Fig. 7. Effect of the LAVE method and adjustment for between-country differences on the gender- and age-related profiles of RVs. Gender and age-related scattergrams of RVs for TG and ALP were drawn in three ways. Plotted in Graph A (blue: males; red: females) are the RVs for all the ethnic groups which were made traceable to the RMPs based on the assigned values in the panel ; i.e., all the RVs of each ethnic group were aligned to the assigned values of the serum panel. In Graph B, RVs made traceable to RMPs were plotted only when test results of other related analytes were within the respective RIs. In Graph C, RVs of each ethnic groups after applying the LAVE method were aligned to those of China (CHN) by adjusting for the difference of the medians from that of China. The horizontal broken lines are shown as a guide for ease of comparison.

The majority of the analytes show gender-differences in RVs. The most prominent ones were observed for UA, Cre, HDL-C, ALT, GGT, CK, and IgM, whereas moderate degrees of gender-differences were observed in Alb, urea, TG, IP, Fe, AST, ALP, C3, and Tf. It is notable that females generally have much more pronounced age-related changes in RVs than males, especially after menopause as indicated by generally higher partial correlation coefficients for age (Suppl. Fig. 4-1). This is especially true for urea, UA, IP, AST, ALT, ALP, LDH, GGT, CK, IgM, and Tf (all $P < 0.0001$ by paired t-test). In contrast, Alb showed more prominent reduction with age in males ($P < 0.0001$) than in females.

The third important biological SV is BMI. It showed obvious association with RVs for many analytes, especially those representing the statuses of nutrition and inflammation as shown in Suppl. Fig. 4-2. It is of note that BMI-related changes in RVs of ALP and AMY were more prominent in females ($P < 0.001$ and $P < 0.005$, respectively). This is attributed to an easily noticeable linear relationship of BMI with RVs of liver ALP (ALP2) and pancreatic AMY only in females as reported by Shimizu and Ichihara [16]. Contrastingly, the associations of BMI with ALT and GGT ($P < 0.001$), and AST ($P < 0.01$) were more prominent in males ($P < 0.001$). No gender difference in BMI related changes was observed for HDL-C and UA.

The most impressive findings of this study regarding biological sources of variations are between ethnic differences in BMI-related changes in RVs as shown in Fig. 5 and in Suppl. Fig. 1. These figures show different slopes for the regression lines between BMI (x) and RVs (y) among the countries−e.g.,: for ALT, HDL-C, TG, and UA. The lines were very steep for some countries (implying a slight change in BMI results in a large change in test values). In sharp contrast, the lines were very flat for other countries (no change of values by the change of BMI). As an exceptional case, CRP showed fairly consistent relationships with BMI among the countries.

However, it should be noted that these ethnic differences in the slope of the BMI-RV relationship cannot be predicted from r_p computed by MRA, because r_p just indicates how tight the linear relationship between BMI and RVs is and thus does not always indicate the steepness of the linear relationship.

This fact clearly indicates that comparisons of between-country differences of the RVs requires country by country adjustment according to the BMI-RV relationship. However, in a practical sense, the BMI adjusted comparison shown in Suppl. Fig. 3 indicates that the adjustment resulted in almost negligible changes in SDR for between-ethnic group differences.

With this confirmation, we can now turn to discuss the results shown in Fig. 3 and Suppl. Fig. 1-1 and 1-2 of the first part of the report of this study [8]. Judging from those figures, the most important findings are the lack of practically important degrees of between-ethnic group differences (SDR < 0.3) in males for UA, TC, TG, IP, Fe, AST, LDH, ALP, GGT, IgA, IgM, and Tf, and in females for TG, IP, LDH, ALP, IgM, and Tf. This information points to the feasibility of sharing RVs around the world for those commonly tested analytes, although extreme environmental, geographical or dietary factors might have to be considered in some situations. In any case, since this is an interim report of the worldwide campaign, we plan to derive globally applicable common RIs after results from five other countries become available in 2016.

On the other hand, we identified obvious between ethnic group difference (SDR ≥ 0.4) in males for TP, Alb urea, TBil, HDL-C, Mg, CRP, IgG, C3, Insulin, Vit. B12, and folate, and in females for TP, Alb, Cre, TBil, HDL-C, Mg, ALT, GGT, CRP, IgG, C3, C4, PTH, Vit. B12, and folate. Among those analytes, the underlined ones have been already documented in the 2009 East and Southeast Asian multicenter study for derivation of common RIs [2,3]. These findings were confirmed by our findings in the global study.

For CRP, there have been many epidemiological reports on between ethnic differences in serum CRP because it has been implicated as a risk factor for cardiovascular diseases [24,25,32–36]. In most reports, South Asians from India, Bangladesh, Pakistan, and African American have higher levels of CRP even after adjustment for BMI [33,35]. In contrast,

CRP is low in Chinese and Japanese living in the USA [25,32]. Socioeconomic status is implicated as an additional factor for the elevated CRP in the multiracial community [24]. In the 2009 Asian study, healthy volunteers from Vietnam, Malaysia, and Indonesia had significantly higher values, and Japanese and Koreans had much lower values [2]. Since IgG, C3, and C4 were also higher in South Asian, the elevated CRP was attributed to increased activity of infectious agents in the area near the equator. In this study, we confirmed higher CRP levels in African and South African populations in contrast to the non-African population in both genders. Higher CRP was also observed in Pakistan, India, and Turkey, while lower CRP was observed in China and Japan. With regard to the slope of BMI vs CRP, it is very impressive to note that between-ethnic group differences in the slope were very small compared with those observed for other analytes considered to be nutritional risk factors: UA, ALT, TG, HDL-C, and GGT. Therefore, elevated CRP can be interpreted without adjustment for BMI.

Regarding ALT, the Asian study showed no ethnic difference, but this study revealed very low ALT levels in South African populations, which led to higher SDR for between ethnic group differences in ALT. However, removal of the African group lowered the SDR. It has been already well documented that African Americans have very low ALT and thus are less susceptible to non-alcoholic fatty liver disease [28,36–38]. In fact, no association was found between BMI and ALT in this study for the African group in South Africa (Fig. 5).

The same phenomenon was observed for LDL-C: The Asian study showed no ethnic difference but this study showed very low LDL-C in the African group in South Africa. Removal of the African group again led to SDR b 0.3 in both genders.

For HDL-C, a strong ethnic difference was confirmed in this study with tendency of very high level (especially among women) in Japan and China, but very low levels were recorded in volunteers from India, Pakistan, Turkey and South Africa (African population). The same trend of low HDL-C has been documented in South Asians [26,30,31].

5. Conclusion

Analyses of between-ethnic group differences in laboratory tests have been heretofore performed primarily as a part of epidemiological studies for prevention of common diseases. However, volunteers for these studies were recruited by use of population based sampling without attention being paid to the status of "healthiness". Therefore, a substantial number of individuals with latent diseases was included. On the other hand, in reference interval studies, it is mandatory to choose individuals who are as healthy as possible. In this regard, the current study provided a great opportunity to elucidate "health" related RVs and allowed us an opportunity to compare them between ethnic groups as well as between gender, age, BMI, and other SVs.

Regarding gender and age-related changes in RVs, we have elucidated them from a large number of healthy subjects after adjusting for between-country differences and removal of individuals with latent diseases by use of the LAVE method. It became obvious that each analyte has its own specific gender and age-related profiles which may be more important in clinical practice than fixed RIs derived without consideration of gender or age.

BMI was confirmed to have close associations with RVs of many analytes as has been previously reported in many epidemiological studies. However, the most impressive finding of this study was the between-ethnic group differences in the steepness of relationships between BMI and RVs for UA, HDL-C, ALT, and GGT, which are important in order to understand why some ethnic groups have susceptibility to nutritionally related diseases.

Regarding the peculiar trends of RVs representing African population in South Africa, we need to confirm the findings by integrating data from Kenya and Nigeria which will become available in 2016.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.cca.2016.09.015.

Research funding

A Scientific Research Fund (No. 24256003: 2012-2014) provided by the Japan Society for the Promotion of Science (JSPS) was used to partially support local studies (to cover breakfast after sampling for volunteers, personnel fee for recruitment and sampling, consumables such as sampling equipment) in India, Saudi Arabia, Philippines, US, and Argentina.

Conflict of interest statement

The authors state that there are no conflicts of interest with regard to publication of this article.

Acknowledgements

We are grateful to the collaborators who coordinated the local study, especially,

Dr. Ling Qiu (Peking Union Medical College, Beijing, China), Prof. Rajiv Erasmus (University of Stellenbosch and National Health Laboratory Services, Tygerberg, South Africa), Dr. Anwar Borai (King Saud bin Abdulaziz University for Health Sciences, Jeddah, Saudi Arabia), Dr. Svetlana Evgina (Beckman Coulter LLC, Moscow, Russia), Dr. Irina Skibo (Helix Medical Laboratories, St. Petersburg), Dr. Tester Ashavaid (P. D. Hinduja National Hospital and Medical Research Center, Mumbai, India), Prof. Dilshad Khan (National University of Medical Sciences, Rawalpindi, Pakistan), Prof. Laura Schreier (Pharmacy and Biochemistry, University of Buenos Aires, Argentina), Reynan Roll (West Visayas State University Medical Center, Iloilo, Philippines), Ms. Ashley Bunker (ARUP Laboratories, Salt Lake City, UT, USA), Mr. Reo Kawano (Clinical Research Center, Yamaguchi University Hospital, Ube, Japan), Dr. David Armbruster (Abbott Laboratories, Abbott Park, IL, USA), and Kazuo Mori (Beckman Coulter Japan, Tokyo, Japan). Finally, we are grateful to Professor Andrew M. Johnson for his detailed scientific editing of this manuscript.

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