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DO HAEMOGLOBIN VARIANTS CAUSE MISLEADING RESULTS OF INDICATORS OF GLYCEMIC CONTROL IN DIABETIC PATIENTS

Final Report

September 2001

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Summary

Surveys carried out by the Ministry of Health and Quality of Life and Collaboration Centres of the World Health Organization in the years 1987,1992 and 1998 had shown that there is rising prevalence of Type 2 diabetes in the Island nation of Mauritius and Rodrigues. One in five Mauritian adults is likely to be affected. Of significance , the rate of poorly controlled diabetic patients was high , being around 40 % of the diagnosed cases.Fasting plasma glucose has been the sole indicator used to assess glycemic control in the patients. Glycated haemoglobin has been known to be a better indicator as reported in various studies elsewhere. However, it was important to assess the suitability of this test and to determine the appropriate cut off value to help in the management of treatment of diabetic patients in Mauritius.

A cohort of diagnosed cases of diabetic was studied over the period of 1997 to 2001. Fasting plasma glucose, serum fructosamine and glycated haemoglobin were assayed at various intervals to compare glycemic status in those patients. Haemoglobin variants were screened in representative samples of normal adults and diabetic patients to determine the prevalence in the population. Laboratory tests were also carried to determine the effect of any interference on those indicators. As a substudy , a group of patients were given intensive intervention with an education module and the indicators were compared for any significant change.

Results of the study have shown that :

(a) The prevalence of haemoglobin variants is 1.2% in the normal adult population of Mauritius and Rodrigues; the majority being HbS (1.1%) and the other type detected was HbC (0.1%) ; Very low prevalence was detected in the cohort studied.
(b) The best indicator for glycemic control is HbA1C with a cut off value of 7.5%. An appropriate cut off value when using fasting plasma glucose is 9.0 mmol/L.
(c) The poorly controlled diabetic patients comprise of a majority of insulin requiring cases of whom a significant number could be Type 1 cases.
(d) Intensive intervention with well defined education component has a beneficial effect on glycemic status ,which can be assessed by HbA1c as indicator.
(e) The interference by haemoglobin variants on the three indicators studied was not significant.

It is therefore recommended that:

(a) HbA1C be used as routine indicator for glycemic control in diabetic patients. This will also necessitate standardized methodology with appropriate quality control. There must be a well defined protocol for clinicians to use this service in the most cost effective way.
(b) Education be made an integral component of intervention for diabetic patients .This will require adequate monitoring and evaluation. HbA1C will be the indicator of choice.
(c) Further studies be carried to :
    - Determine the proportion of known Type 2 diabetic patients who resemble Type 1 and require insulin treatment;
    - Detect onset of complications in long standing poorly controlled cases; Assays of microalbuminuria, insulin response , and Cpeptide concentration will be appropriate.
    - Initiate suitable intervention programme for diagnosed cases of diabetes, with emphasis on education . Monitoring and evaluation will involve HbA1C as main indicator.
    - Develop a laboratory method to determine HbA1C or equivalent indicator which can be adapted in the Mauritian context as an appropriate , cost effective and cost efficient procedure to be used in the decentralized services of diabetes care in Mauritius.

September 9, 2001.

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The Research Team comprised of the following officers of the Ministry of Health and Quality of Life:

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INTRODUCTION

The adult population of the Island of Mauritius has one the highest prevalence of Type 2 Diabetes [1,2]. Mauritius is a multiracial island, the main groups being Asian Indian Hindus, Asian Indian Muslims, Creoles and a small number of Chinese. The first study for prevalence of NCDs carried in 1987 showed an overall crude prevalence of diabetes of 12.8% with 17.9% showing impaired glucose tolerance, which is a risk category for both Type 2 diabetes and ischaemic heart disease (IHD) [3].Major risk could be attributed to overweight, central adiposity and physical inactivity [4,5]. The crude prevalence of hypertension was 16.1% in males and 13.8 % in females [6] with Asian Indian Muslims lowest and Creoles highest. A follow-up survey was carried in 1992 [8].The prevalence of Type 2 diabetes and of IGT was not changed significantly, but there was more awareness in the community and the proportion of unknown cases had fallen. On the other hand the incidence of new diabetes cases in the five years was high and proportion of poorly controlled diabetic patients had increased to 42%. The proportion of obese or overweight individuals had increased also : from 26 % to 36 . The last survey carried in Mauritius was in 1998 [10]. Results are alarming. The prevalence of diabetes in the adult Mauritian population increased by 36 % since 1987, with about 37% poorly controlled. Diagnosed cases of hypertension has risen by 20% and dyslipidemia, an important risk factor of heart disease, has reached the 50% since 1992.

In the island of Rodrigues from 1992 to 1999 , there has been notable rise in diabetes (31%) , with one in every sixth person having Type 2 diabetes. Obesity is high and rate of hypertension is 48% , which may be one of the highest reported anywhere in the world [11]. Despite a five year(1987-1992) national programme aimed principally at changing of lifestyle, the rate of diabetes did not change significantly (1987: 12.8 % and 1992: 13.1%)[3,4]. In fact, the rate of poor glycemic control on the diabetics increased from 30% in 1987 to 42 % in 1992 [4].Fasting plasma glucose levels were used to indicate glycemic control in those studies.

It is now widely accepted that glycohaemoglobin (a complex of glucose and haemoglobin,HbA1c) is a valuable indicator for long term diabetic control [5].

Various methods to determine HbA1c are now available and the majority are based on either affinity chromatography, high pressure liquid chromatography, ion exchange chromatography, electrophoretic, or immunochemical methods [6].

Spurious elevation or decrease in results obtained in assays currently used have been reported when the patients have variations in their haemoglobin molecules [7].Mutation in the B-chain of the haemoglobin is responsible for such variation and the most commonly encountered Hb variants are HbS, HbC, and HbE [8]. The significance of the interference of these variants in the HbA1C results is important as it has a direct effect on the treatment regimen of the diabetic.

Routine methods for glycated hemoglobin assay can be divided into two major categories, based on how the glycated and non-glycated components are separated. Early studies employed ion-exchange chromatography using large columns to separate HbA1a.HbA1b and HbA1c from HbA1o.These have been replaced by much shorter mini-columns for the measurement of HbA1 or HbA1c [9]. In a well documented study, Schellekens at al [10] found an optimal separation between $HbA1_{a+1b}$ and HbA1c using 0.036M sodium phosphate buffer (pH 6.78).Other studies on elution buffers were carried out [11] and it was observed that a very critical equilibrium molecular weight be obtained between molarity and pH of the first eluting buffer and the temperature at which experiments are performed.

Much work on HbA1C is done on commercial minicolumns, in which generally EDTA blood lysed with proper lysing agent and thereafter HbA1 eluted from pre-equilibrated minicolumns with phosphate buffers at low molarities and pH 6.7. Further studies by Abrahams et al [12] and Rosenthal [13] showed that column methods are temperature dependent and very controlled conditions are necessary for obtaining acceptable results. It was also observed that ion exchange procedures in batches resulted in poor performance [14]

Another method commonly used is electrophoresis. Glycation at the N terminal of HbA changes with isoelectric point of the molecule by only 0.01 at pH unit. Conventional electrophoresis is therefore not suitable for the assay of Hba1c of HbA1 but methods based on electro endosmosis have been extensively used. Electrophoretic methods are insensitive to variations in buffer pH ionic strength.

In colorimetric methods, thiobarbituric acid is most commonly used and has a good correlation with minicolumn methods. These methods can be automated as well[15] .Fluorimetric method based on periodate oxidation has also been described [16] In the recent immunoassay techniques, the basic principle used is inhibition of HbA1c-specific antibody agglutination. A synthetic polymer containing an HbA1c-like glycopeptide causes agglutination of the antigen-antibody complex, which is then inhibited by HaA1c in the blood sample to be tested. This inhibition is then measured by a photometer.[17] .That human hemoglobins are heterogenous has been reported by several investigators .Schroeder at al [18] isolated minor fractions from adult red cell haemolysates. These minor fractions elute before the main component HbAo and are therefore called fast hemoglobins. At least three different fractions HbA1a ,HbA1b and HbA1c were identified. Huisman et al [19] reported an increase in the fast moving Hb in diabetic patients. This finding of diabetic Hb components was also reported by Rahbar [20] Structural studies later established that diabetic hemoglobin was identical to HbA1c[21].

Human adult red cell contain more than 90 % of its hemoglobin as nonglycated HbA and two minor hemoglobin fractions as HbA2 and HbF. The fast hemoglobin have shown to have amino acids which are identical to that of HbA but with the addition of a glucose molecule complexed to them.HbA1c is major fraction of the fast Hb, which accounts for 3-6% of total hemoglobin in man.

Although interefering substances such as gross lipemia, gross haemolysis, carbamylation products in uremic patients and acytelation products in alcoholics known to affect column methods but not the immunoassay, the latter is reckoned to be interfered by hemoglobin variants. Blood from normal adults contains up to 0.5% of fetal hemoglobin (HbF). HbF is significantly increased in patients suffering from thallassemia. It is also increased to a lesser extent during pregnancy and early infancy. HbF can interfere to a variable degree with many glycated Hb methods. Glycation of hemoglobin is a consequence of prolonged exposure to hyperglycemia, which is a primary cause of diabetic complications[22] .Hyperglycemia accelerates formation of non-enzymatic advanced glycosylation end products (AGE) on tissue macromolecule. It is a critical part of the evolution of diabetic complications, particularly as they are chemically irreversible and thus accumulate with time.The degree of accumulation for example has been shown to correlate with severity of retinopathy [23]. Formation of advanced gycosylated products begins

with the formation of the more familiar early glycosylation products,when glucose attaches to amino groups to form unstable Schiff base adducts.Levels of the labile base increase rapidly,and equilibrium is reached after several hours.ambient glucose concentration over that brief period determines the steady state level of Schiff base adducts. Once formed, Schiff base adducts of glucose and protein amino groups undergo a slow chemical rearrangement over a period of weeks to form a nonstable (but still chemically reversible) sugar protein adduct, the Amadori product [24] . Equilibrium of Amadori glycosylation products is reached over a period of approximately 28 days. Thus even on the very long-lived protein, the total amount of Amadori product is only proportional to the integrated glucose concentration of preceding four weeks. After the relatively brief period of time necessary to attain equilibrium, measured levels of Amadori products reach a constant steady state value which does not increase at a function of time beyond that point.

In countries with large population with abnormal hemoglobin results from diabetic patients are made difficult by the shortened half life of red cell, increase in HbF, and sometimes even the absence of HbA1.

In Mauritius no in depth study has been made to evaluate the prevalence of abnormal hemoglobin in adults and more so in the diabetic population. A study on school age children of age ranging 12-18 demonstrated that abnormal Hb was HbS in Creoles(2.2%) whereas in the Indian the authors found HbE (0.81%) and HbD (0.97%). [25] . The main aims of the present study are therefore to (a) document on the prevalence of hemoglobin variants in the adult population and particularly in the diabetics and (b) to determine any interference of possible variants on the methodology of measuring glycated hemoglobin. The initial task consists therefore to identify a group of diabetics and carry out their baseline investigations . Factors which could affect the percentage of glycated hemoglobin are to identified and the detection of variants carried out on the diabetics under study. An indication of the variation if any in the level of glycated Hb due to those factors can be obtained. Eventually, measurement of variants in a sample of adults representative of the adult Mauritian population can be carried out to determine the predominant types of variants in the population. Thereafter, the variants in the diabetic population can be compared and their effect independent of clinical factors on the methodology evaluated.

2. METHODOLOGY

2.1 *Questionnaire*

Two types of questionnaire were designed. The first one was to obtain demographic information of the subject to include anthropometric measurements and medical history and the second one was to have the subject attitude to common dietary knowledge concerned with diabetes.

After the design of the questionnaire, a pilot study was carried out to test it. Personnel were identified to be interviewers and were given a short training on how to approach people, how to read out the questions, what to say at the start and how to end the interview. They were also given guidance on how to behave or act and what to say when the respondent is unwilling to take part or does not wish to continue the interview. The interviewer was also told that as far as possible the interview should be private and confidential.

The pilot studies were carried out over short time and with a few respondents. Any difficulty revealed was noted and either the questionnaire was modified or appropriate interview setting identified through these pilot studies. It was also possible to evaluate the length or time of interview, and the number of interviews possible per day or per week. Indications also were obtained on certain cases where respondents became tired or unattentive due to the type of lengthy questions. From such results, it was possible to modify the structure of the questionnaire and in some cases a further training was given to interviewers. The final result of some of these questionnaires is shown in the annex.

2.2 *Survey Procedures and Measurement Techniques.*

An appropriate site was selected at the Diabetic Clinic of the Victoria Hospital. For the initial survey and for the study at the institution, participants were called in a fasting state .

2.2.1 *Operational Procedure*

2.2.1.1 Registration

On arrival, the subject was greeted, name taken and a card bearing a serial number was given. The name was then ticked off on the original name list. A short interview was carried out to obtain information on past medical history, family history and relevant sections of the questionnaire were appropriately filled in. This exercise allowed subject to have a short rest before the blood pressure was measured.

2.2.1.2 Blood pressure

A quiet area was chosen where the subject was allowed to rest for at least ten minutes in the seated position. Nursing staff were trained in measuring blood pressure. Standard calibrated mercury sphygmomanometers were used. The subject was asked to remove garments to expose the right arm which was rested comfortably on a table, with the elbow level with the heart, and the upper arm at an angle of about forty degrees to the trunk. Adult size cuffs were used. The cuff was applied firmly with the artery marker positioned correctly over the brachial artery.

The lower edge of the cuff was 2-3 cm above the cubital fossa, to allow space for the bell of the stethoscope. The pulse obliteration pressure was established by palpating the radial pulse with the fingers

on the left hand while inflating the cuff with the other. The cuff was inflated to about 30 mm Hg above that level. The stethoscope bell was then placed lightly over the position of the brachial artery. The cuff pressure was released at a steady state of about 2-3 mm per heart beat. Systolic pressure is the level where the first sounds identifiable as pulse are heard. Diastolic pressure was taken as that level where the sounds ceased (5th phase). The cuff was then deflated completely and measurements recorded. A second measurement was then carried out. If the readings of the two measurements differed by more than 10%, a third measurement was taken and recorded. The average measurement was noted.

2.2.1.3 Height and weight

The subject was in light clothing and without shoes when measurements were taken for height and weight. Height was recorded to the nearest centimetre, rounding up if midway, using an appropriate stadiometer. Calibration of the equipments was checked daily.

2.2.1.4 Waist and hip circumference

Whenever these measurements were taken, the subject was first requested to stand relaxed, breathing quietly and normally. One layer of light clothing was accepted. A dress-maker's measuring tape was applied horizontally. Waist girth was measured as being that level yielding the minimum circumference between the umbilicus and xiphoid process. Hip girth was recorded as the maximum circumference around the buttocks posteriorly and indicated anteriorly by the symphysis pubis. Measures were made to the nearest 0.5 cm and were repeated following both initial recordings. When a variation of greater than 2 cm occurred between duplicate readings, a third measurement was taken and recorded alongside the second. The two most consistent readings were noted for analyses.

2.3 *Blood Collection*

Venous or capillary blood was collected in the fasting state

The subject was in the seated position and the specimen was taken from the cubital fossa of the chosen arm by the standard technique. The venous blood was collected in tubes on which the subject number and name were recorded. Sterile conditions were observed and prolonged stasis using the tourniquet was avoided.

2.4 *Biochemistry*

The biochemical analyses were performed either using semiautomated techniques or by automated chemistry analysers. All apparatus and equipment were calibrated regularly and almost always before use. Stringent quality control systems were implemented to ensure reliable results. The procedure and interpretation of quality control are described in a later section. An outline is now given of the principle and method of assay of some common biochemical investigations carried out in this study.

2.4.1 *Specimen Handling*

Biochemical analyses were performed either in serum or on plasma. Blood collection was practised with a vacuum system such as Vacuutainer System [Becton-Dickingson, U K]. Serum was obtained from blood collected in plain tubes. Plasma was obtained upon centrifugation of blood collected in tubes containing specific anticoagulants/preservatives. In some cases, glucose assays were performed in plasma from blood collected in lithium heparin or in fluoride oxalate. Lipid analysis including apoproteins was performed on serum.

Glycated haemoglobin (HbA1C) was performed on whole blood collected in tubes containing potassium EDTA as anticoagulant.

2.4.2 *Determination of glucose*

A glucose oxidase was an enzymatic -colorimetric assay was used]. The method as per Trinders is hereby described as an example [26].

The principle of the test is as follows:

(a) Glucose ----------> gluconic acid+hydrogen peroxide

glucose oxidase

(b) phenol + amino-4-antipyrine +hydrogen peroxide ----------> quinoneimine + water

peroxidase (chromogen)

In the presence of the first enzyme glucose oxidase, glucose is converted to gluconic acid and hydrogen peroxide. The latter in presence of the second enzyme peroxidase reacts with phenol and amino-antipyrine to form a chromogen, whose colour is measured under defined conditions.

The main components of the reagent system are:

Reagent 1 (buffer)	Phosphate buffer	150 mmol/L
	Phenol	10 mm0l/L
Reagent 2 (Enzymes)	amino-antipyrine	0.4 mm0l/L
	Peroxidase	300 U/L
	Glucose oxidase	10000U/L

Reagents were prepared fresh before use, although at times prepared working reagent was kept at -4° C for not more than four weeks. In each batch of analyses, a standard solution of known concentration of glucose and at least two quality control specimens were assayed with the samples.

The procedure for analysis was as follows:

Semi-Automated system:

Deionized water (as reagent blank or standard solution working reagent (1.0 ml) was added to ten microlitres of sample and contents mixed. After incubation for 20 minutes at 20-25°C, the colour absorbance was read at a wavelength of 404 nanometres on a calibrated colorimeter. The concentration of glucose in the samples was deduced from the calibration curve or from the working standard solution.

2.4.3 *Determination of total cholesterol*

An enzymatic-colorimetric method was used. One such method is hereby described[27]. In the presence of the enzyme cholesterol esterase, cholesterol ester in the sample is converted to free cholesterol and fatty acids. The cholesterol is then oxidised by a second enzyme. Hydrogen peroxide formed during that reaction reacts with phenol and aminoantipyrine to form the chromogen whose colour is measured photometrically.

(a) Cholesterol ester ----------> cholesterol+fatty acids + Cholesterol esterase

(b) Cholesterol ----------> cholest-4-en-3one +H_2O_2
Chol. oxidase

(c) H_2O_2 + phenol + 4 aminoantipyrine --------> quinoneimine
Peroxidase +4H_2O

The concentrations of the main components of the reagent system are indicated below.

Reagent 1 Buffer	Phosphate buffer	0.1 mmol/L
	Phenol	15 mmol/L
	sodium cholate	3.74 mmol/L
	Surfactant	
Reagent 2 Enzymes	4-aminoantipyrine	0.5 mmol/L
	Peroxidase	> 1000 µ/L
	Cholesterol oxidase	> 200 µ/L
	Cholesterol esterase	> 125 µ/L

Reagent 2 was in powder form and was mixed with contents of bottle (buffer reagent) to prepare the working reagent. The latter was usually stored at 2-8°C for not more than four weeks.

In the semiautomated system, 1.0 ml of the working reagent was mixed with ten microlitres of sample, standard solution, or distilled water as blank, in respective tubes. After an incubation period of 10 minutes at 20-25°C, the final colour obtained was measured photometrically at a wavelength of 500 nm. The concentration of total cholesterol in the sample was calculated from the known value of the standard solution or from a calibration curve. Quality control samples were always set to assess the accuracy and reproducibility. In automated system, the programme for total cholesterol was appropriately set .

2.4.4 *Determination of High-Density Lipoprotein Cholesterol(HDL-C)*

The assay consisted first of all in separating the high density lipoproteins and then estimating the cholesterol bound to those fractions. The separation step was accomplished by a precipitation technique using phosphotungstic acid in the presence of magnesium ions. Chylomicrons and very low density lipoproteins (VLDL) and low density lipoproteins (LDL) are precipitated under these conditions. After centrifugation, the supernatant contains the high density lipoproteins (HDL). An aliquot of that supernatent was subsequently used to determine the cholesterol concentration as described above.[28]

In the first stage, serum (150 µL) was pipetted into a labelled tube followed by 150µL of a solution of sodium chloride (9 g/L). Then 30 µL of precipitating reagent (phosphotungstic acid, 40 g/L and MgCl2.6H2O, 100 g/L; pH 6.2) were added and mixed. The contents were allowed to stand at room temperature for 10 minutes, after which tubes were centrifuged at 5000 rpm for 15 mins. Aliquots of supernatant were processed as above for cholesterol measurement with a modified programme.

2.4.5 *Determination of Triglycerides*

The principle of assay of triglycerides is based on a sequence of coupled enzymatic reactions:

(a) Triglycerides --------> glycerol+ fatty acids

Lipase

(b) Glycerol + ATP ------> glycerol-3-phosphate +ADP

glycerokinase

(c) Glycerol-3-Phosphate ----------->dihydroxy acetone phosphate +hydrogen peroxide

[Enzyme:glycerol-3-phosphate oxidase]

(d)Hydroghen peroxide +parachlorophenol +aminoantipyryne--- ------->quinoeimine(chromogen) +water

[Enzyme:peroxidase]

The triglycerides are converted to dihydroxyacetone phosphate with eventual formation of hydrogen peroxide. The latter is then used to produce the chromogen whose colour is estimated photometrically.[29] The standard solution used is that of glycerol (equivalent to 2.29 mmol/L or 200 mg/dL). The concentrations of the various components of the reagent system are as below

Reagent 2 (buffer)	Trisbuffer pH 7.6	50 mmol/L
	Parachlorophenol	5.4 mmol/L
	Magnesium	2 mmol/L
Reagent 3 (enzymes)	Aminoantipyrine	0.4 mmol/L
	Lipase	10000 u/L
	Glycerokinase	200 U/L
	Glycero-3-phos-oxidase	2000 u/L
	Peroxidase	200 u/L
	ATP	0.8 mmol/L

Similar to above reagents, the triglyceride working reagent was prepared by mixing contents of the two reagent bottles. The working reagent was stored at 2-8°C for not more than four weeks. In the semiautomated system, ten microlitres of sample, standard solution or reagent blank (distilled water) were mixed with one ml of working reagent and contents incubated for 10 minutes at 25°C.

The absorbance of the final colour obtained was read at 500 nm. The concentration of triglycerides was read from a calibration curve or calculated from the known value of the standard. Quality control samples were assayed along side the test samples. The appropriate programme was set for the automated system.

2.4.6 *Determination of Apolipoproteins*

Determination Of apoproteins was carried out using Behring Kits (Behring Diagnostics,France).The principle is based on an immunoturbidimetric reaction. The specific antibodies of the reagent formed immune complexes with the proteins contained in the human serum sample. The turbidity generated was measured photometrically.

Reagents consisted of antisera to human serum proteins which were supplied diluted with a reaction buffer.antibodies reacting unspecifically were removed by carrier-bound protein fractions (solid phase immunoadsorption).The antisera were adjusted with the reaction buffer to ensure optimum suitability for use on the analytical system.addition of sodium azide eliminated microbial contaminants.

Determination of Uric acid:

Uric acid present in sample was determined according to :

uric acid +2H2O + O2 ------→allantoin +CO2 +H2O2

H2O2 + 3,5 dichloro 2 hydroxybezene sulfonic acid + 4 amino antipyrine ---------→ quinoeinimine +HCL +2H2O.

Standard and sample (20uL) were mixed with 1.0 mL of working reagent and incubated for 5 min at 37 C. absorbance was read at 520 nm.

2.4.7 *Determination of Urea*

Urea is hydrolysed in the presence of water and urease to produce ammonia and carbon dioxide. The ammonia produced in the first reaction combines with alpha oxoglutarate and NADH in the presence of glutamate dehydrogenase to yield glutamate and NAD.Ten microlitres of sample, standard ,or control solution are mixed with 1.0 mL of working reagent and absorbance read at 0,1,2,and 3 minute interval.

2.4.8 *Determination of Hemoglobin A1C*

It is a quantitative immunological determination determination of percent glycohemoglobin (HBA1C) in whole blood or hemolysate used to monitor long term blood glucose control in individuals with diabetes mellitus. HBA1c and Total Hb are determined from haemolysate, prepared manually or on-board from the automated system from whole blood.HBA1C is measured from haemolysate from a latex enhanced turbidimetric immunoassay .Total Hb is measured from the same haemolysate by colorimetric cyanide free alkaline haematin method.Both tests are performed at 550 nm from haemolysed blood in which Hb is proteolically degraded.

The final HBA1C test result is determined from the HBA1C/Hb ratio, including a conversion formula to match a HPLC reference method.

2.4.9 *Identification of human haemoglobin variants*

2.4.9.1 Manual Procedure

Haemoglobins are separated by electrophoresis in an alkaline medium, the separation being based on the complex interaction of the haemoglobin molecule with the support medium and the gel buffer.

Freshly collected EDTA anticoagulated whole blood were collected and stored at 2-8 C for not more than one week.200 UL of well mixed whole blood was mixed with 1000 uL of 0.9% NaCL solution, centrifuged at

3000 rpm for 5 min. Supernatant (1000 mL) is removed and discardded and a further 1000 uL of the NaCl solution to the tube.Steps repeated twice. The contents are treated as packed cells

The gel surface is blotted at the application point.The sample application template is aligned with the + sign at the edge of the gel. A blotter is placed on top of the template and finger rubbed accross the slits to ensure good contact. Sample (3uL) is applied to each slit and allowed to absorb for 5 min. Buffer is poured in the electrophoretic chamber. Following sample absorption , the template is lightly blotted and after 30 seconds both blotter and template are removed. The gel is then positioned in the chamber agarose side up aligning the positive and negative sides with the corresponding positions on the chamber. The gel is electrophoresed at 150 volts for 30 minutes. At the end of electyrophoresis , the plates are dried completely at 60-70 C and placed afterwards in the stain solution for 10 minutes. Destaining is carried out by rinsing the plate with two consecutive washes of destaining solution .Plate is allowed to remain in the wash for 1 minute or until the background is clear. The plate is dried at 60-70 C and is evaluated for the presence and absence of particular bands of interest.

2.4.9.2 Automated system: Helena Rep (Helena France)

Reagent kit Cat no 0326-0384 was used for the purpose. Blood sample (collected in either EDTA tube or in Heparin tube) was centrifuged, plasma removed and cells washed on 0.9% saline thrice. Washed cells (10 uL) were then mixed with 300 uL of lysing reagent , vortexed briefly and allowed to stand at room temperature for at least 5 minutes. Control solutions (AFSC,AFSA2, and ASA2) were appropriately diluted with the lysing reagent.These haemolysates (75 uL) were applied to sample cups and the sample tray was prepared and adjusted onto the automated system as per instructions of the Cat 0326-0384.After electrophoretic run, the agarose gel were stained and destained as per method and bands identified.

2.5 *Quality Control*

2.5.1 *Control of pre-analytical variables*

An important part of the activities of the present study was carried out outside the laboratory, at the site of survey the diabetic clinic of the Victoria Hospital. It is obvious that the many steps preceding specimen analysis are prone to 'error' or variation. This is here referred to as pre-analytical variation. To obtain reliable results, pre-analytical variation should be a minimum. Monitoring and controlling of this variation is therefore essential, which requires a coordinated effort of each member of the team. Training was given to the personnel involved in the 'pre-analytical activities' on pertinent aspects of control and standardization of techniques. Some of these are now outlined.

2.5.1.1 Identification of subject

Correct identification of subject, questionnaire and specimen was a major concern. It was inevitable to have handwritten labels, which are highly prone to errors. 'Double-checking' was therefore necessary in each case. Thus, the subject on arrival would receive a card bearing a specific serial number, alongside the subject's names. At each station (bleeding station, blood pressure station or at the height, weight measurement station) the names and the serial number was spelt out aloud. Finally a tick was placed on a 'register' book when identification was correct at each point and on the labels of the specimen tubes.

2.5.1.2 Turnaround Time

Delays in procedure were minimal, otherwise response rate would be low, which had to be avoided. Besides, delays are detrimental to the analytes in the specimen. Long standing specimens undergo various changes in the analyte concentration, such as a decrease of glucose due to glycolysis [30]. Plasma or serum required for analysis had to be separated as soon as possible but within the two-hour collection period[31].

2.5.1.3 Subject Preparation

Test results are affected by various physiological factors, some of which are controllable. Serum cholesterol and triglycerides are said to increase by six per cent with change of posture from lying to standing[32] . Clear instructions were also given to subjects on whether they should be fasting or be on any special diet. On day of blood collection, details were asked from the subject and subject status before blood collection was indicated in `remark' column of the register book.

2.5.1.4 Specimen Collection

Standardized technique of blood collection was used. Prolonged application of tourniquet, for example, was avoided. An increase of up to five per cent in serum total cholesterol is known to occur with various occlusion prolonging from one to three minutes [33] . Haemolysis is another factor to be prevented. That was done by use of an evacuated blood collection system and by avoiding any undue physical shock to blood contained in the tubes. Correct containers and appropriate anticoagulants and preservatives were always used.

2.5.1.4 Specimen Transport

After collection, specimen was instantly transported to site of analysis. Specific means of transport was made available for that purpose. Stability of specimen was controlled by

(a) instantly placing the specimen containers in cold racks, placed inside cool box containing ice blocks,

(b) transporting these boxes immediately to the laboratory and within the first two hours after collection. This procedure was adopted to cause less than ten per cent of the `real' analyte concentration as defined for a `stable' specimen [34-35]

2.5.1.5 Specimen Separation and Aliquoting

Specimen separation and aliquot was the final important procedure before analysis was performed. That was usually carried out in the laboratory though in some cases, plasma and serum were separated and aliquoted at the survey site. Two important aspects were considered: the equipment (centrifuges) and the personnel. The latter were given training on procedures of specimen separation and aliquoting. Proper and regular maintenance of the centrifuges was essential. For quality control purpose, the timer of the equipment, the speed and temperature were monitored[36] and a record kept of daily performance.

2.5.2 Control of Analytical Variation

The next important aspect on the study was the determination of analyte concentration in specimen collected from participants. Valid data obtained were essential to draw conclusions and make decisions at different levels of the present work. The analytical performance, therefore, had to be judged for its validity and that involved two important concepts, precision and accuracy.

(a) Analytical Precision

Analytical precision refers to "agreement between replicate measurements"[37]. In a replicate experiment the same material is analysed several times (at least twenty times). The variability obtained when analysis was done within the same run or series was referred to as "within-run precision". When the sample material was analysed in different runs but on same day, the variability was referred to as "within-day precision". "Between-day precision" refers to the variability obtained when sample material was analysed repeatedly on different days. It has been recommended to used the term "imprecision" to denote "the standard deviation or coefficient of variation of results of a set of replicate measurements" [*Buttner et al, 1976*]. That was also described as the 'random error', i.e analytical error which can either be positive or negative and whose direction and exact magnitude cannot be predicted. [38].

(b) Analytical Accuracy

The next important concept in validating an analytical performance is accuracy or systematic error. It is a measure of "agreement between best estimate of a quantity and its true value" [*Buttner et al, 1976*]. The term 'inaccuracy' has also been recommended to mean "the numerical difference between the mean of a set of replicate measurements and the true value." *[Buttner J et al, 1976]*. It also refers to systematic error, "analytical error that is always in one direction" [*Westgard J V, 1978]*. Two types of systematic error are defined. The constant systematic error is always in the same direction and of the same magnitude even as the concentration of analyte changes *[Westgard, J O, 1978]*. Proportional systematic error is always in one direction and its magnitude is a percentage of the concentration of the analyte being measured *[Westgard J O, 1978]*. In this study, quality goals for analytical performance had to be set. The precision goal was stated as an allowable standard deviation. The accuracy goal was stated as an allowable systematic error (or allowable bias).

3.0 RESULTS AND DISCUSSION

3. 1 *Selection of patients*

Patients who have been tested *at least* on both March 97 and Sept 97 have been included in the database for comparison purpose. The number of persons' results analysed are given in brackets:

(a) for those patients tested only on March 97 and Sept 97 (number= 117).

(b) for those patients tested on March 97,Sept 97 and March 98 (number=63).

(c) 75 patients out of the 117 persons mentioned above were tested in 2001; 40 of the 75 persons were on insulin and the rest on tablets (daonil). A total of 40 patients were tested an all the four occasions (number=40).

3.2 *Statistical results*

Since this exercise is a case study rather than a survey, only simple results are given.

Proportions of poorly controlled DM have been calculated . We cannot use the means for comparison purpose directly on these data because the readings are on different scales. Various cut off points have been used for the different tests in order to assess the comparability of these tests. Medians were used to compare the overall results of the 40 persons across the four different tests as they are not affected by extreme values. Means have been calculated in order to compare the same tests across different dates. The coefficients of variation ({standard deviation /mean} x100%) have been used to compare the variability within the results of each test.

Table A: Proportions of poorly controlled DM Number of patients : 117

Test	Cut off Value	Date 1.3.97	Date 1.9.97	Cut off value	Date 1.3.97	Date 1.9.97
GLUP	>=11.1	56.4%	75.2%	>=9.0	78.6%	86.3%
HBA1C	>=7.5	87.2%	88.9%	>=7.0	90.6%	95.6%
FRUCT	>=285	85.5%	89.7%			
HBA1C	>=8.0	76.1%	86.3%			

Table B: Proportions of poorly controlled DM Number of patients : 63

Test	Cut off Value	March 97	Sept 97	March 98	Cut off value	March 97	Sept 97	Marcl
GluP	>=11.1	60.3%	81.0%	58.7%	>=9.0	81.0%	88.9%	74.6%
HbA1C	>=7.5	95.2%	100.0%	77.8%	>=7.0	100%	100%	92.1%
Fruct	>=285	92.1%	100.0%	95.2%				
HbA1C	>=8.0	92.1%	100.0%	73.0%				

Table C: Proportions of poorly controlled DM *Number of patients : 40*

Test	Cut off Value	March 97	Sept 97	March 98	2001
HbA1C	>=7.0	100.0%	100.0%	90.0%	100.0%
HbA1C	>=7.5	95.5%	100.0%	75.0%	92.5%
HbA1C	>=8.0	92.5%	100.0%	75.0%	85.0%

Table D : Medians of the HbA1c results *Number of patients : 40*

Test	March 97	Sept 97	March 98	2001
HbA1C	9.65	10.75	10.05	10.10

Table E : Mean, Standard Deviation (SD) , Median, and Coefficient of Variation (CV%) have been calculated on the results 117 patients tested on Mar97. Number of patients = 117

Test	Samples taken on March97			
	Mean	SD	Median	CV%
GluP	12.8	5.3	12.1	41.6
HbA1C	9.5	2.0	9.4	21.2
Fruct	389	105	388	26.9

Looking at Tables A,B, and C, and comparing the proportions of poorly controlled DM across the different dates, an increase in the overall results is noted from March 97 to Sept 97 whereas a decrease is noted from Sept 97 to March 98. Although the decrease may be explained by the intervention programme (education) , the increase noted between the first two periods should be interpreted with caution. Referring back to the 63 patients and taking into the HbA1C values only (cut off= 7.5%) it is noted that the results of only 49 (77.8%) of the 63 have increased. It is also noted with concern that the results of the 40 patients in 2001 are higher than those in March98. However, looking at Table D, we notice that the medians of the HbA1c results for these 40 patients across the four different dates have varied within a small range (9.65 – 10.75). This could be explained by the fact that these patients are either on insulin or are taking tablets. These drugs are nevertheless helping to maintain the level of glucose at a *stable* level.

Comparing the coefficient of variations given at Table E , it is clear that HbA1C is much better than fasting plasma glucose [GluP] (21.2 % compared to 41.6%), i.e the % CV of Glup is twice as high as that of HbA1C. The %CV of fructosamine (26.9%) is slightly higher than of HbA1C. HbA1C is thus a better indicator for average blood glucose over longer periods. Glucose values vary too much and can thus generate many false positives and false negatives. Referring to the 117 patients and comparing GluP (cut off >=9.0) and HbA1C (>=7.5) for samples taken on March 97, in 18 cases (15.4%) the results are positive

with HbA1C but negative with GluP- a problem for the patient. On the other hand, in 8 cases (6.8%) the results are negative with HbA1C but positive with GluP- a problem for the health service.

It would have been of interest to study a sample measured in March 98 but without any education intervention. Nevertheless, from the different results of the various tests done in March 1998, it seems that at least part of the decrease in the proportion of poorly controlled DM could be assigned to the effect of health education programme. However , from the overall results , we may conclude that any drug regime is more effective when it is accompanied by a health education programme. We recommend that the intervention through health education to be done repeatedly and that all DM patients be given the facility of keeping the records of their daily level of blood glucose so as to better monitor the different doses of the drug intact.

Using the HbA1c results of the 75 persons tested in 2001, we observe that the Mean of the 40 patients on insulin is 10.26 whereas that of the 35 patients taking tablets is 9.86. The difference, although slight, could be explained by the fact that those on insulin are more serious cases (Type I ???) and that if they would not have been on insulin, the results would have been worse. We therefore further recommend further that all DM patients be closely follow up so as to determine at an earlier stage whether they must start an insulin regime.

Using the results from Table A and arbitrarily setting the Fruct Test as a standard, the most appropriate cut-off values for the HbA1c test is " >=7.5 ". Similarly, it can be said that the better cut-off value for the Glup test is " >=9.0 ". However, given the intrinsic complexity of human body and the principles on which the different tests are based, any attempt in establishing cut-off values by comparing results of the different tests is too hazardous. The level of correlation between the results of Glup and Hba1c is low . Looking at individual results of the two tests, we have seen that many patients who are negative with Glup at cut-off value ">=9.0" are positive with HbA1c at ">=7.5" ,and vice-versa.

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