On a Person by Person Basis, Glucose and HbA1c Results from a VAMS Dried Blood Sampling Device were not Clinically Acceptable Substitutes for Formal Venous Blood Samples.

A Closing Out Report
prepared by
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Based Upon the Original Laboratory Work of

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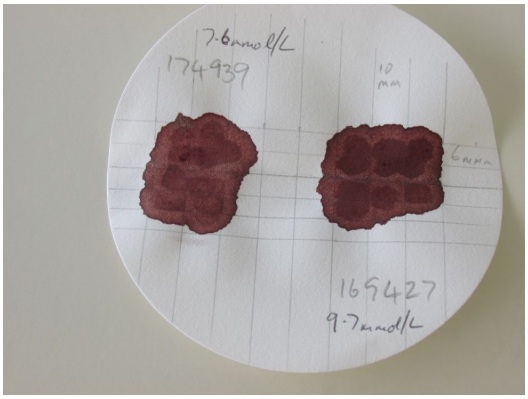
Introduction

The invention of the so called VAMS devices such as the Neoteryx Mitra sampler offered the prospect of making home collection of specimens by diabetic patients a more feasible option. However, before any such plan could be proposed it was important to evaluate these devices against the current handheld glucometer and formal venous blood samples analyzed in an accredited pathology laboratory. The results of two projects that ran in sequence are reported here. The first study evaluated the Mitra Neoteryx 10uL device from the point of view of glucose performance and the second study involved the same devices but from the point of view of haemoglobin A1c (HbA1c) performance. Both studies involved paired measurements of these analytes in two separate groups of 20 volunteers.

There is a long history of the use of dried blood samples in clinical biochemistry (1) and various levels of success have been reported across a very wide range of analytes (2,3). The availability of the VAMS devices offered a solution to the inherent ‘indeterminate volume of sample collected’ problem in dried blood sample techniques. Collection of a blood droplet onto filter paper has been the approach that has been successful primarily because the medium – filter paper – is manufactured to high tolerances of thickness and the common office hole punch device facilitates the sampling of the dried blood spot with a high degree of reproducibility. Nevertheless there are problems even in that approach because a large dried spot is visibly different from the centre to the edge. As the droplet soaks in red blood cells appear to concentrate at the centre and at the perimeter the blood appears paler and presumably ‘red cell’ depleted, Figure 1(left). Use of a more open weave medium than the usual Whatman Number 1 type of filter paper such as ‘facial tissue’ does provide a more homogeneous dried blood spot, Figure 1(right), but the thickness of such tissues is not as controlled as in analytical grade filter papers. In this study similar problems with inhomogeneous soaking of the blood droplet into the Mitra Samplers were encountered to such an extent that the variability and bias in the glucose and HbA1c results rendered them as unreliable surrogates for formal venous samples.

FIGURE 1 : Examples of Typical Dried Blood Spots on Filter Paper (left) and Facial Tissue Paper (right)



Literature Reviews
Please refer to the original theses submitted by Samarakkody and Shah respectively.

Materials

Glucose study :

- Mitra (RUO) 10µL Microsampling 4 pack device clam shell (Neoteryx, LLC Torrance, CA, USA)

FIGURE 2(a) : Neoteryx Mitra 10uL Dried Blood Sampler in Clam Shell Packaging.

Figure 2(b) : Neoteryx 10uL Dried Blood Samplers in the Opened Clam Shell Packaging.

Figure 2(c) : Neoteryx Mitra 10uL Dried Blood Sampler After Being Loaded with Finger Prick Blood Samples.

- Unistik 3 Extra safety lancets (Owen Mumford: Woodstock, Oxford, UK)
- Thermo Scientific Heraeus Multifuge 3SR+ Centrifuge (ThermoFisher Scientific, Germany)
- Glucose 201 RT glucometer strips (HemoCue AB: Ängelholm, Sweden)
- Alcowipe ProMed skin cleansing swabs (Promedica Pty Ltd. Briemar Nominees, Australia)
- Vacuette® 2 mL Grey top vacutainer tubes containing sodium fluoride (NaF)/ tri--potassium ethylenediaminetetraacetic acid (K3 EDTA) designed for blood glucose collection (Greiner Bio-one GmbH. Frickenhausen,Germany)
- Socorex Acura 825 adjustable pipettes (Wheaton Science Products, Millville, NJ)
- SSI free standing Ribbed Screw Top tubes 0.5 ml and 2.0 ml. (Scientific Specialties Incorporated, California 95242, U.S.A)

HbA1c study :

- Socorex positive displacement glass pipette: This pipette was used to sample 10µL venous blood for pre-treatment. At a setting of 10µL, we confirmed that it delivered 10.03µL with a CV% of 6.6%.
- Socorex Acura 825 adjustable pipettes (Wheaton Science Products, Millville, NJ)
- Vacuette® 2 mL Purple top vacutainer tubes containing di-potassium ethylenediaminetetraacetic acid (K2 EDTA) designed for blood haematology collection (Greiner Bio-one GmbH. Frickenhausen,Germany)

Reagents

Glucose study :

- Infinity™ Glucose Hexokinase Liquid Stable Reagent. (Cat No. 1520-200A, Thermo Fisher Scientific, USA)
- Trichloroacetic acid (TCA). (AnalaR, BDH laboratory Supplies, Poole, England)
- Triethanolamine (TEA) (Sigma-Aldriche Chemie GmbH, Switzerland)
- D-Glucose (Aldrich Chemical Company Inc. Milwaukee, USA)

HbA1c study :

- We adapted the program supplied by the Randox (Randox Laboratories Ltd.,UK) for the HbA1c measurement to suit our sample sizes and the requirements of KoneLab.

- Randox Total Hb and HbA1c kit (Catalogue number HA 8043).There were two reagents for the Randox Total Hb assay. Reagent R3 was the Haemoglobin denaturant reagent. That reagent contained a protease enzyme that lysed red blood cells. The R1 reagent for Total Hb which converted all haemoglobin derivatives into haematin in an alkaline solution of a non-ionic detergent as described by Wolf et al. (1984). This resulted in a green solution that was measured at 600 nm.

Follow up studies :

- Sysmex Haemoglobin reagent which contained the more effective levels of SLS at 1.7 g/L.

Apparatus

Glucose study :

- Hemocue® Glucose 201 glucometer.(HemoCue AB: Ängelholm, Sweden)

Glucose study and HbA1c study :

- A KoneLab 20XT (Thermo Electron Corporation, Finland) selective chemistry analyzer for in vitro diagnostic purposes. It was user programmable for a wide range of chemistries.

Figure 3 : THe Kone Lab XT20 Clinical Chemistry Analyzer.

- Horizontal Roller mixer.

HbA1c study :

- Sysmex XS 1000i (Sysmex Corporation, Japan) instruments was used to measure Total Haemoglobin (Hb). The Sysmex XS series of analyzers used the Sysmex's cyanide-free Sodium Laurel Sulphate (SLS) method for analyzing haemoglobin in a dedicated channel. Measurement results were reported in g/dL units. This reagent was found not to fully extract the Hb and HbA1c from the Mitra tips and was subsequently replaced with the Sysmex Haemoglobin reagent which contained more effective levels of SLS at 1.7 g/L.
- BioRad D-10 HbA1c analyzer.

Follow up studies :

- Microtitre Plate reader : TECAN infinite 200 Pro, (Tecan Austria GmBh., Grodig, Austria.
- Kleenex facial tissues, (Kimberley-Clark Australia Pty Ltd, NSW, Australia.)
- Cotton buds (Johnson and Johnson (Philippines), Paranaque City, Phillipines))
- Plastic heparinised 75mm haematocrit tubes, (Plasticrit, Drummond Scientific Co., Broomall, PA, USA.)
- Point of Care HbA1c analyzer : Siemens DCA Systems, (Siemens AG, Muenchen, Germany)
- Dried blood sampling kit : HemaXis DB Evaluation kit, (DBS System SA, Gland, Switzerland.)
- Filter Paper : Whatman #1 : GE Healthcare UK Ltd., Buckinghamshire, UK.

METHODS

Participants' samples.

- Blood sampling sites used in the glucose study and the HbA1c study :

Venous blood was collected from the participants preferred arm. Antecubital area was used to collect venepuncture blood. This area was wiped with a skin cleansing swab AlcoWipe containing 70% isopropyl alcohol and was thoroughly dried. A tourniquet was applied to the preferred arm and was released before the needle was withdrawn from the vein.

Two by 2ml samples of venous blood were collected into two grey top (fluoride/EDTA) Vacuette tubes during the glucose study.

During the HbA1c study the venous sample was collected into a purple top (EDTA) Vacuette tubes. Two Mitra samplers were pre labelled with participant's ID before proceeding. Using a transfer pipette, one large drop of venous blood from the participant's purple top tube was placed onto a petri dish, and the corresponding participant's Mitra tip was touched against the droplet at 45-degree angle and left there until completely soaked with blood. They were then placed back into the participant's clam shell case as recommended by Neoteryx and left at room temperature to dry for 24 hours.

- Finger prick capillary blood collection used in the glucose study and the HbA1c study :

The Unistik 3 Extra device was used to make the finger pricks for the capillary blood samples. This device contains a single use needle spring loaded 21G lancet with an incision depth of 2.0 mm. Immediately after sampling the needle retracts and the device can be disposed of safely.

According to the participant preference two of the fingers, usually the middle and the fourth fingers were selected for the three finger prick sites. Two sites were used to collect blood onto the Mitra samplers and the other was used for the glucometer strip. The finger prick sites were wiped with skin cleansing AlcoWipe swabs and were dried thoroughly. To get the best sample, the hand was warmed first. The lancet was pressed firmly and perpendicular against the finger tip at a position approximately 2mm below the level of the fingernail bed and the release button was pressed. The initial blood flow was usually slow so it was important to wait a few seconds to allow a reasonably sized blood droplet to form (~ 15µL) on the surface to avoid under sampling. Each Mitra tip was held at a 45° angle to the surface of the blood droplet, care being taken not to touch the skin of fingers. The Mitra tips were held in this position until the whole of the tips were a uniform red in colour. The tip samplers were then transferred to the clam shells and were left to air dry for 24 hours.

During the glucose study a Glucose 201 RT glucometer strip was held to the third blood drop and when fully filled, the surface was wiped and was read using the Hemocue® Glucose glucometer.

Assay methods.

The assay methods reported here were arrived at after various experiments to determine which procedure for each of the analytes would provide the best agreement with the results for those sample types with corresponding formal venous whole blood samples when analyzed by the conventional pathology laboratory techniques. These were then designated as the ‘best laboratory practice for the whole blood and dried blood samples’ for the purposes of the studies reported here.

- **Glucose Study :**

Plasma samples.

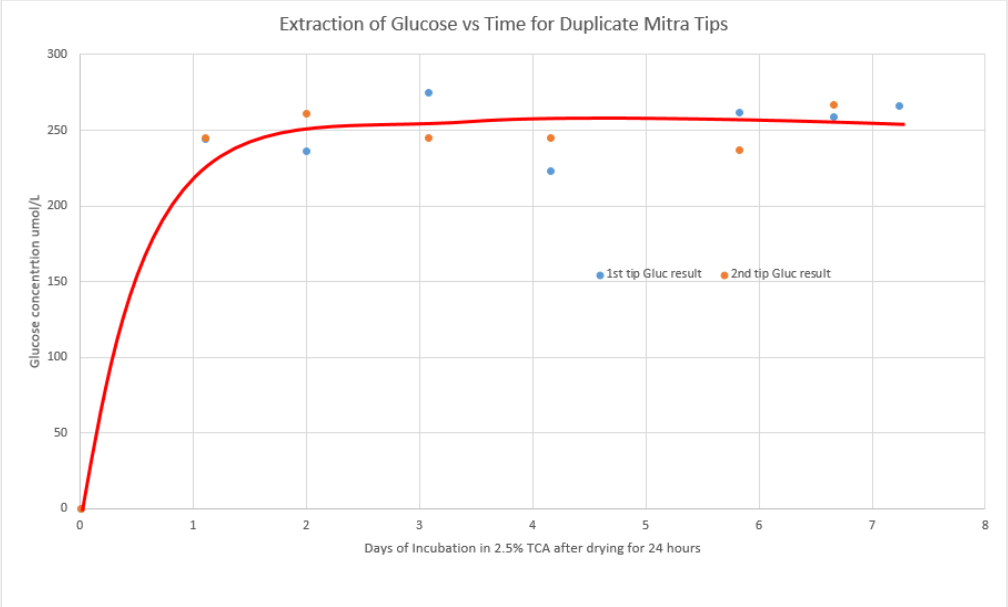
The Vacuette tubes were inverted and the blood was mixed well with the anticoagulant powder in the tube as soon as they were collected. One of the Vacuette tubes was centrifuged at 3000rpm for 15 minutes at room temperature using a Thermo Scientific Heraeus Multifuge 3SR+ Centrifuge. The plasma was pipetted out using a 1000µL micro pipette, into 2ml storage tubes. These were then used to measure the glucose concentration using InfinityTM Glucose Hexokinase Liquid Stable Reagent on the KONELAB 20XT analyzer.

Mitra samples.

Blood in the other Vacuette tube was mixed gently and using a pipette one drop of venous blood was taken onto a sterilized petri dish. Two new Mitra tips were used to draw up this venous blood samples. These were also left to air dry for 24 hours. There remaining blood containing Vacuette tube was frozen at - 20°C in the freezer for future use.

After 24 hours of drying, the dry absorptive tips of the Mitra samplers were removed by hand using a sterile paper, from the sampler cone and were placed into small 2mL tubes. Exactly 400 µL of 2.5% Trichloroacetic acid (TCA) was then added as per the recommendation of Burrin and Price in 1984 and were capped. TCA is a protein precipitant, which ensured that haemoglobin and all other blood proteins remained trapped in the matrix of the Mitra sampler tip while water soluble components such as glucose dissolved in the aqueous phase. It has been shown that with TCA all of the glucose can be eluted in 20 minutes from dried blood spots on filter paper but we observed that the optimum incubation time for the tips in 2.5% TCA was 48 hours, see FIGURE 4. This time was used for all the tips as the incubation time.

Figure 4 : Time course of glucose extraction from a dried blood sample on a Mitra 10uL sampler.



After two days in the TCA, the extractant was transferred using a 1000 uL micro pipette into another tube ready for measurement of glucose concentrations using the modified Infinity™ Glucose Hexokinase Liquid Stable Reagent. We observed that the buffering capacity of the unmodified Infinity Glucose reagent was insufficient for the TCA extracts and that additional Triethanolamine (TEA), which is a strong buffer had to be added. The hexokinase enzymatic method used by Burrin and Price in 1984 for the filter paper glucose determination was done by the use of Boehringer Kit no 263826. The concentration of TEA that was in that buffer solution was 200 mmol/L. The concentration of TEA in the buffer of InfinityTM Glucose Hexokinase Liquid Stable Reagent was 20mmol/L.. Additional TEA to match the previously published concentration was achieved by adding 10 ml of 0.1mol/L aqueous solution of of TEA to 50mL of the InfinityTM Glucose Hexokinase Liquid Stable Reagent. This modified reagent was used to measure the glucose concentrations in the TCA extracts of finger prick and venous Mitra tips.

The extracts were stored at 4 °C in the fridge after measurements and appeared to be stable indefinitely.

Standard glucose solutions for the venous plasma samples

A standard glucose solution of 19.53 mmol/L concentration was made by dissolving

0.720g of D-glucose in 200mL of distilled water. A series of working standards containing 4.88, 9.77, 14.65, 19.53 mmol/L were made by dilutions with distilled water.

Standard glucose solutions for the Mitra tip extraction calibration curve

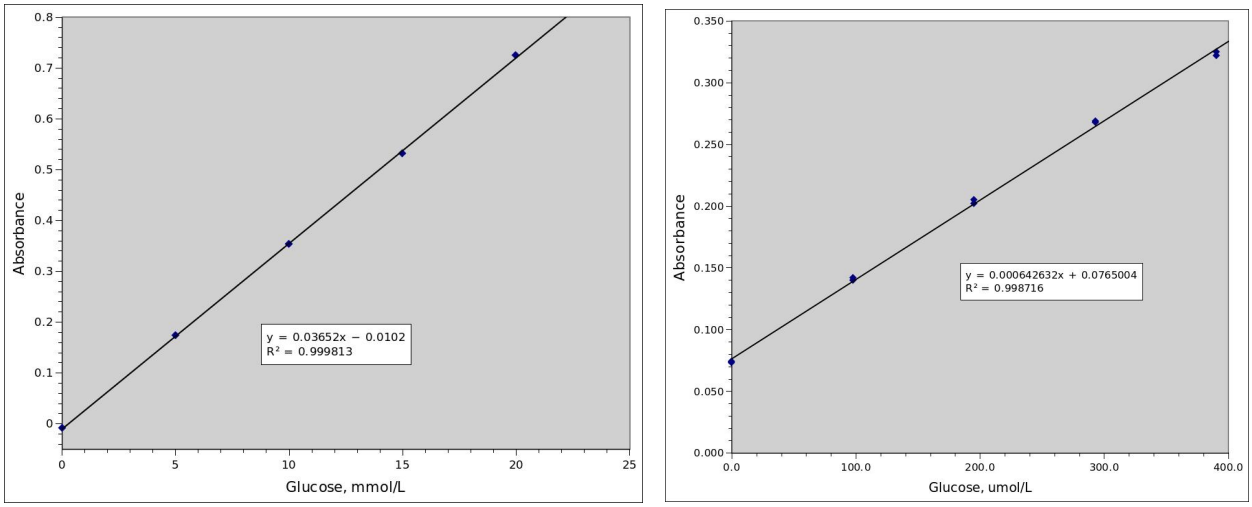
The standard solutions for this calibration were made by adding 980 µL of 2.5% TCA solution to 20 µL of each of the plasma glucose standards, a 1 in 50 dilution. The standard series was 0.0, 97.6, 195.3, 292.9, and 390.6 µmol/L.. These values were arrived at from a consideration that theoretically the Mitra sampler tip contained dried solutes derived from 10uL of blood sample which had effectively been dissolved into 400uL of TCA at the extraction step; a 1 in 40 dilution.

Secondary glucose calibrants for the Mitra tip extracts

Venous blood samples from seventeen patients who attended an ISO15189 accredited hospital pathology laboratory were used as secondary calibrants for the Mitra tip extracts.. The total volume of blood was divided into two parts where one part was centrifuged to separate the plasma. This plasma was used to measure the glucose concentration on a Cobas 3000 mainframe analyzer in the accredited pathology laboratory. The other part of blood was mixed well and a drop of blood was taken out using a pipette onto a sterilised petri dish. Two new Mitra tips were used to draw up this blood droplet. These tips were left to air dry for 24 hours.

After drying for 48 hours, the absorptive tips were removed from the sampler cones by hand using a sterile paper and were placed in 2 mL storage tubes containing 400µL of 2.5% TCA. After 2 days of incubation in TCA the extractant was transferred into another tube using a micro pipette and was measured on KONELAB 20XT for glucose using the modified version of Infinite TM Glucose Hexokinase Liquid Stable Reagent.
These Mitra tip glucose concentrations (in µmol/L) were plotted against the plasma glucose concentrations (in mmol/L) values of the corresponding plasma samples measured on KONELAB 20XT. In that way a Secondary Standards calibration curve was generated for use with the study Mitra tip extracts.

FIGURE 5 : Typical Calibrations Curves for the Glucose Assays : Plasma Glucoses on the Left and Mitra Tip Glucoses on the Right



Typical Calibration Data for the Plasma Glucose Assay :

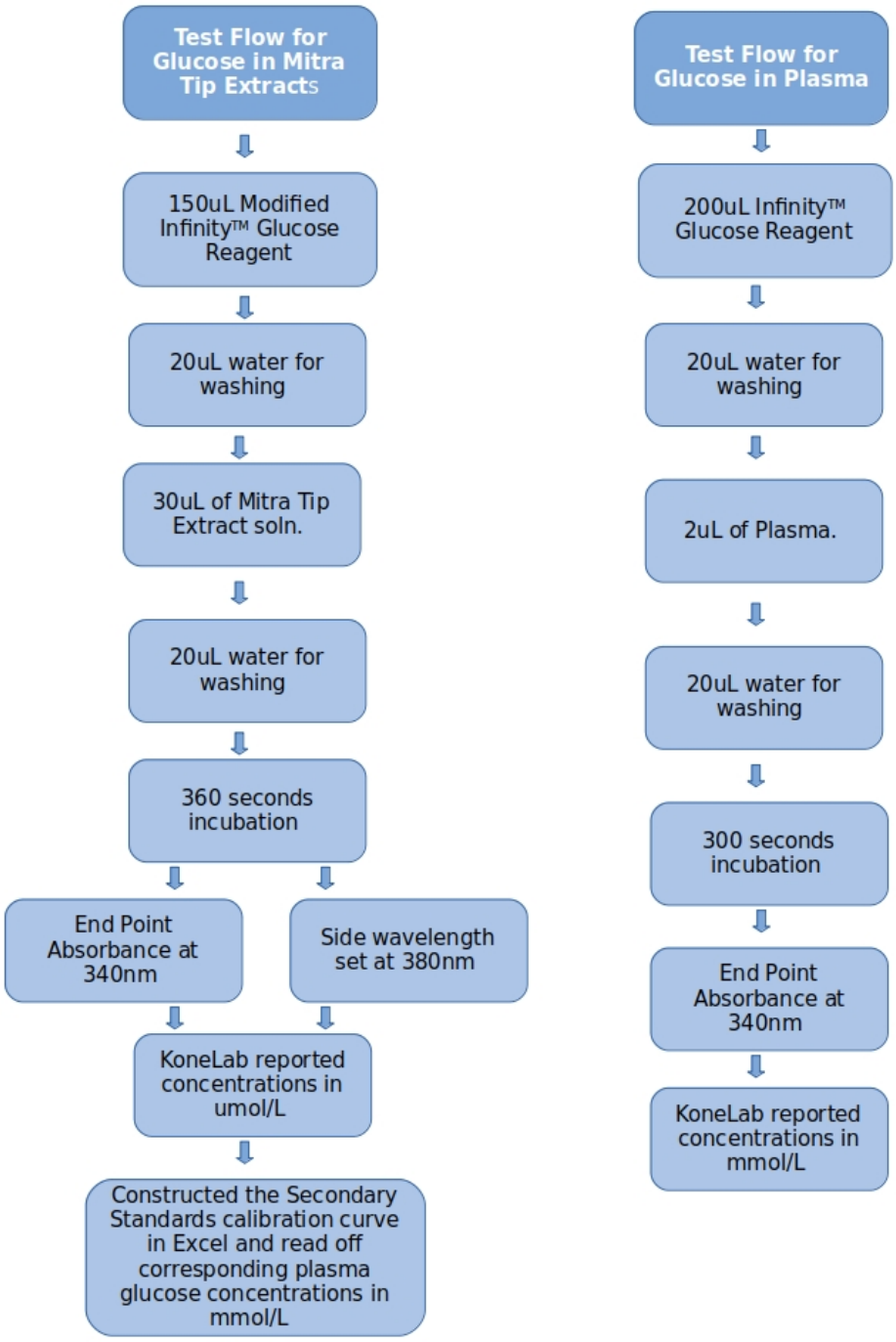
Glucose, mmol/L	Absorbance
0	−0.009
5	0.174
10	0.353
15	0.532
20	0.725

Typical Calibration Data for the Mitra Tips Glucose Assay :

Glucose, umol/L	Absorbance
0.0	0.074
0.0	0.073
97.7	0.140
97.7	0.142
195.3	0.202
195.3	0.205
292.9	0.269
292.9	0.268
390.6	0.325
390.6	0.322

Calibration curve for the Mitra Tips based upon the secondary standards has not been shown because this varies according to what samples the analyst chooses to use as the secondary standards.

Figure 6 : Test flow programmed into the Kone Lab XT20 for Mitra Tip Extract Glucose (left) and Plasma Glucose (rigtht)



- **HbA1c Study :**

There were two Randox reagents provided in the kit for the HbA1c method : HbA1c R1 and HbA1c R2. The HbA1c R1 reagent was a suspension of latex particles coated with an HbA1c specific mouse monoclonal antibody. When HbA1c in the samples bound to this antibody it prevented agglutination. The HbA1c R2 was the Agglutinator reagent which on addition post incubation agglutinated any latex particles that had not bound to the endogenous sample HbA1c. Consequently, as the concentration of HbA1c increased then the turbidity of the mixture decreased in a complex non-linear style.

Extraction of Hb and HbA1c from the dried blood on the Mitra samplers:

Using tissue paper each dried Mitra tip was manually removed and placed into their corresponding labelled extraction tubes.

- For the HbA1c extraction 400uL of Randox R3 reagent was added.
- For the Hb extraction 800uL of the Sysmex SLS was added.

All tubes of tips in their extraction solvent were left at room temperature for 48 hours. Then, using transfer pipettes, all extraction solvent was transferred into the corresponding pre-labelled clean tube for that participant, ready to be placed onto the analyzer at a later time. Intermediate storage was at 4**°** C.

Measurement of Total Hb in venous blood using the Sysmex XS 1000i:

Venous blood total Hb was measured on the haematology analyzer Sysmex XS 1000i immediately after blood collection. There was no pre-treatment step for venous blood analyzed for the Hb on the Sysmex. The procedure was followed was as per the manufacturer’s instructions.

Pre-treatment of venous blood sample prior to analysis on the KoneLab analyzer:

The first step of the procedures for Total Hb and HbA1c on KoneLab involved sample pretreatment of the venous blood with the Randox denaturant reagent. This involved the addition of 10µL venous blood using a Socorex positive displacement glass pipette to 800µL of SLS in an SSI tube. This was allowed to stand at room temperature for 5 minutes before transferring onto the KoneLab.

Test flow programmed into Konelab for Total Hb and HbA1c:

For Total Hb on the KoneLab the calibration type was set to ‘Linear’. The full test flow parameters are shown on the left-hand side of Figure 6.

For HbA1c on the KoneLab the calibration type was set to ‘Linear’ because the ‘Spline’ type option was not available on the instrument. No note was taken of the calculated HbA1c concentrations on the KoneLab printout. Instead the raw data pairs – standard concentration : KoneLab measured absorbance - were later typed into MyCurveFit. and the unknown sample concentrations calculated there off the four parameters logistic fit to the calibration data pairs; see Figure 7.The full test flow parameters for Hba1c are shown on the right-hand side of Figure 8.

FIGURE 7 : Typical Calibration Curve for the HbA1c Randox Reagent Based Assay

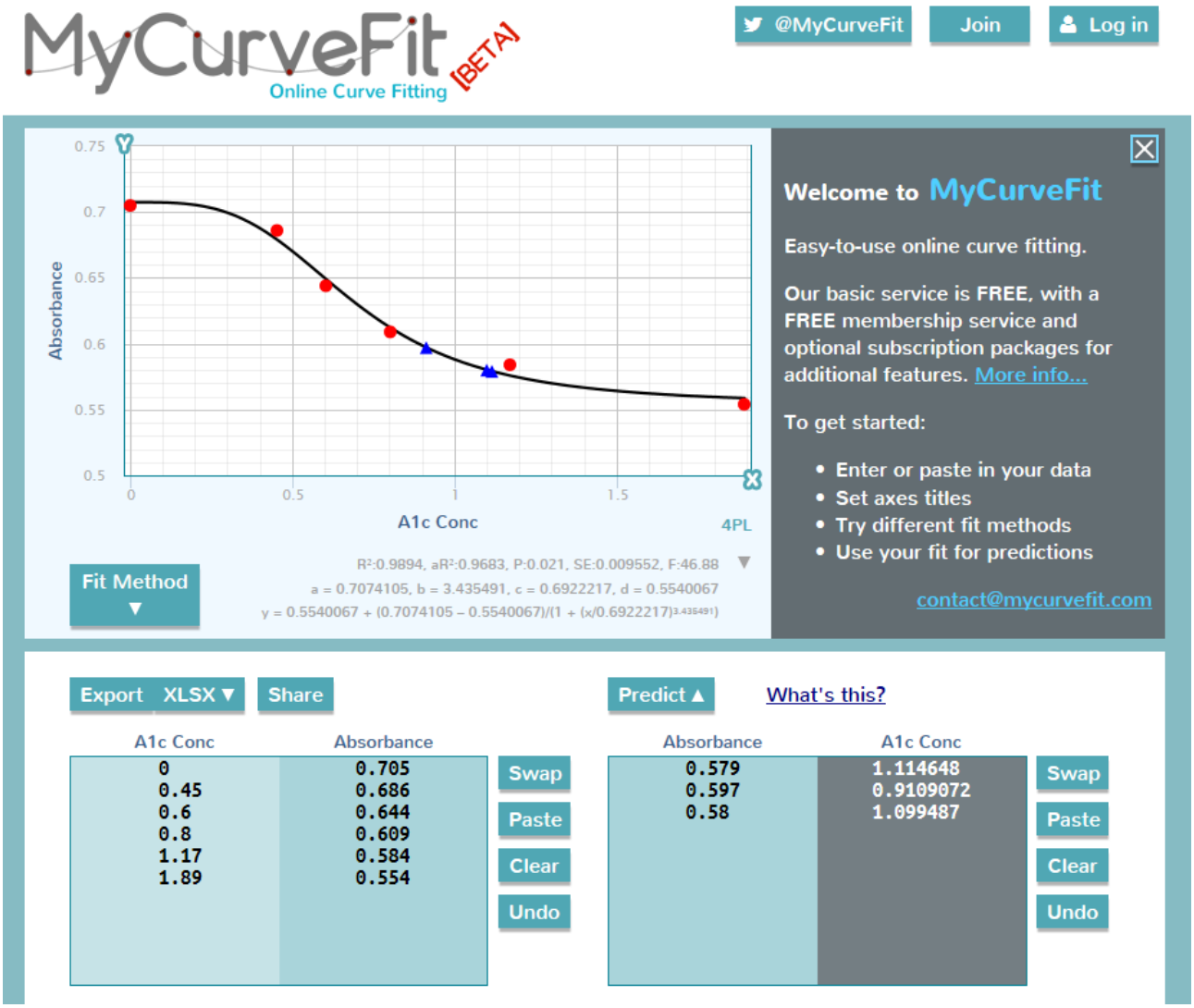
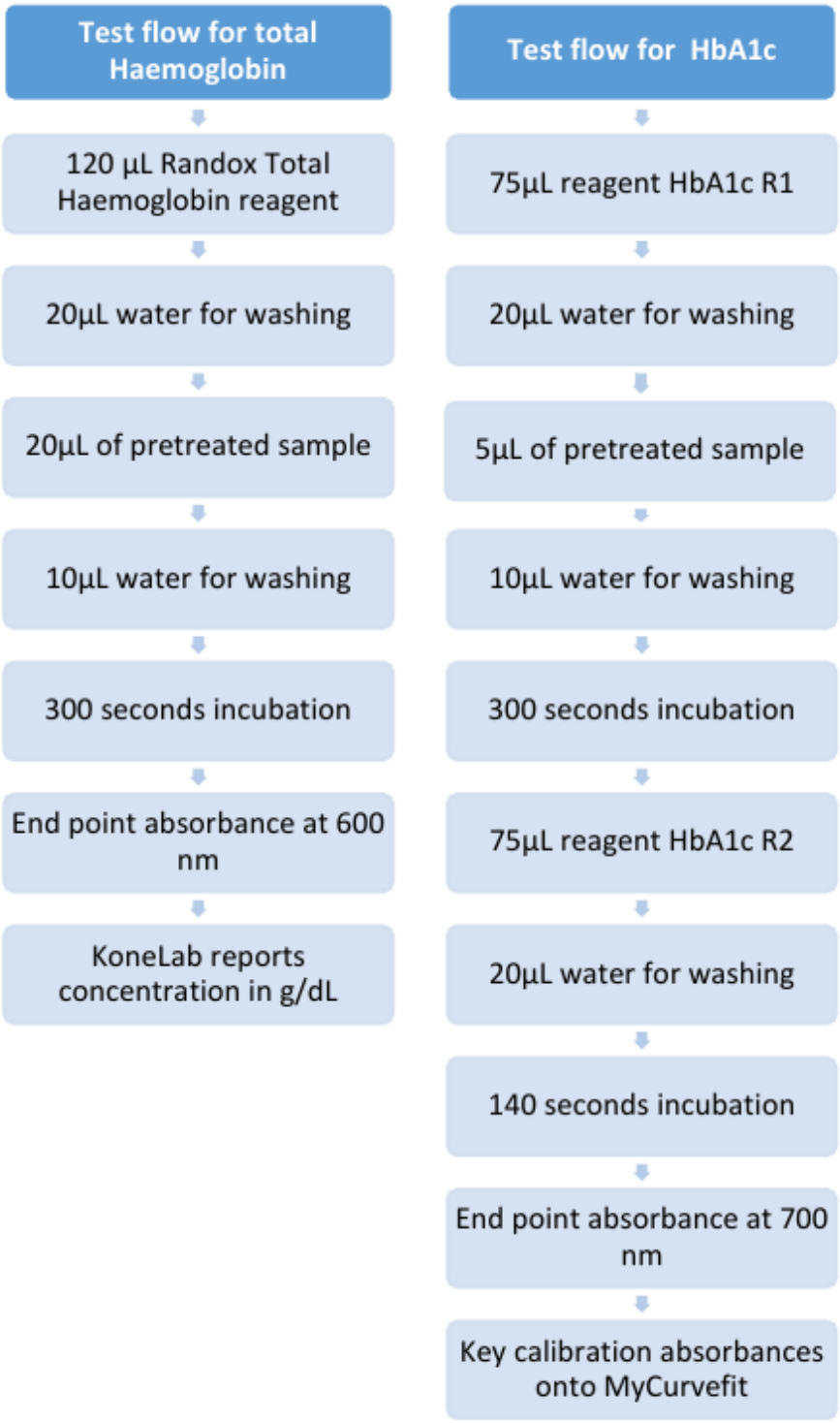


Figure 8 : Test flow programmed into the Kone Lab XT20 for Whole Blood Total Haemoglobin (left) and Haemoglobin A1c (right).



Statistical Analyses including Statistical and Clinical Significance Criteria :

Linear regression was used for all the glucose calibration curves. This was done by the KONELAB 20XT instrument for the plasma glucose measurements. For the Mitra tip samplers we used Excel Data Analysis Add-in.

For the HbA1c study the calibration data were fitted to a four parameter logistic using the online calculator at www.mycurvefit.com.

Assessments of the final 'best laboratory practice for the whole blood and dried blood samples' were made using the Paired Student's t-test.

For all statistical tests we considered a 'p' value of ≤ 0.05 confirmed statistically significant differences or effects.

◆ Clinical Significance Criteria For the Glucose Study.

The Royal College of Pathologists of Australia have defined the allowable limits of performance for glucose and these were used to evaluate the data analysis.

- +/- 0.4 mmol/L up to 5.0 mmol/L glucose
- +/- 8% for glucose levels greater than 5.0 mmol/L

We chose to use the diabetic clinical decision limit of 7.00 mmol/L (fasting glucose) as the point of which we would decide the acceptability of the data set. That is 7.00mmol/L +/- 0.56 mmol/L.

◆ Clinical Significance Criteria For the HbA1c Study.

The differences between our sample population means were also assessed against independently defined 'clinically significant change' criteria for Total Hb and % HbA1c.

The clinically significant change values for Total Hb and HbA1c were calculated at the 95% confidence level. The World Health Organisation (WHO) has recommended HbA1c of 5.7 – 6.4% as the high-risk range and 6.5% as the cut point for diagnosing diabetes. We, therefore, calculated that a clinically significant change in % HbA1c would be equal to (6.5% minus 5.7%) = 0.8%. In other words, if a clinician observed a patient's HbA1c had increased by 0.8% between consultations then the clinician could be justified in suspecting that the patient's glucose metabolism/control was "deteriorating". Conversely, if it fell by 0.8% then the patient's glucose metabolism/control could be considered as "improving".

For Total Hb, we took the recommendation of ± 7% as being clinically significant based upon the recommendation made on the ClinLab Navigator website. (http://www.clinlabnavigator.com/test-significant-change.html).

◆ Statistical Analyses Applied to all Data Sets.

- Box and Whisker Plots : These plots have been presented in the 'minimum to maximum' style.

- Dot Plots : These were generated using Gnumeric Spreadsheet 1.12.32 running on (Linux) Knoppix Version 8.1.

- Bland and Altman Plots : Full regression analyses of these plots were made with the following interpretative criteria :

a) **Statistically Significant Slope** : Interpreted according to the 'p' value of the regression analysis being ≤ 0.05. If statistically significant then this was taken as objective evidence of a systematic bias.

b) **Statistically Significant Intercept** : Interpreted according to the 'p' value of the regression analysis being ≤ 0.05. If statistically significant then this was taken as objective evidence of a fixed bias.

c) **Clinically Significant Bias** : The linear regression equation was solved for one or more values along the x-axis. The value chosen along the x-axis was selected according to the clinical decision limits generally accepted for the analyte under consideration.

- 'Subjective' Significance Criterion for both studies.

We defined the following subjective significance criterion :

The results of the dried blood samples must agree to within +/- 10% of the value obtained from the formal venous whole blood samples.

Our rationale was that in these days of near patient testing being regularly utilised in the clinical environment alongside formal 'main lab' testing it is essential that all methodologies within a single clinical environment should agree to within +/- 10%. Under such a condition we believed that regardless of the source of the data, the clinical staff would give equal credence to them when they came to using them to make their diagnostic and therapeutic decisions.

RESULTS AND DISCUSSION

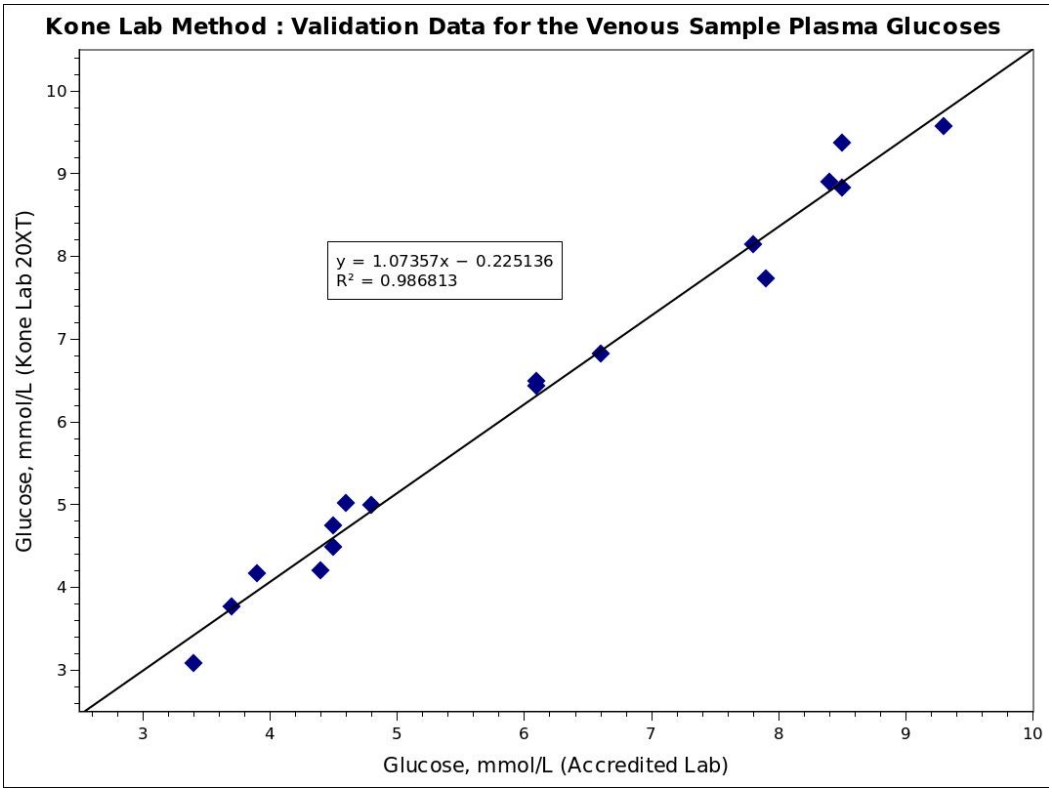
Of all the various possible permutations of calibrating the Mitra sampler tips analyses of the data showed that in house 'secondary standards' provided the closest agreements to the corresponding formal venous sample results. Only those results are presented here for evaluation and discussion. Then the data from the 'in vivo' studies are presented and discussed.

Validation Data for the Plasma Glucose Analyses on the Kone Lab XT20

Table 1. Measurements of glucose in the plasma derived from the venous blood samples collected from the accredited pathology laboratory compared to our KONELAB 20XT analysis of the same plasma samples.

Sample name:	Plasma Glucose Measurements by the accredited pathology laboratory, mmol/L	Plasma Glucose Measurements by KONELAB 20XT, mmol/L
A	6.1	6.43
B	4.8	4.99
C	6.6	6.82
D	7.9	7.73
E	8.5	8.83
F	4.5	4.49
G	3.4	3.08
H	7.8	8.15
I	4.4	4.20
J	3.7	3.76
K	3.9	4.17
L	8.4	8.90
M	8.5	9.37
N	9.3	9.57
O	6.1	6.49
P	4.5	4.75
Q	4.6	5.02
Mean	6.06	6.28
Standard deviation	1.99	2.15
SEM	0.12	0.13
Correlation Coefficient	0.9934	[redacted]
Slope	1.074	
Intercept	-0.2251	
Std Error of the Estimate of x from y	0.24	
Paired Student's t	t = -3.17 p = 0.01	

Figure 9 : Validation Plot for Venous Sample Glucose Measurements on the Kone Lab XT20



These data demonstrated

- That when the Accredited Laboratory measured a value of 5.0 mmol/L then the KoneLab would have reported a value of 5.1 mmol/L, which was well within the +/- 0.4 mmol/L limits set by the RCPA and was only 2% higher than the Accredited Laboratory measurement so it also satisfied our “Subjective” Significant Criterion of +/- 10%.
- That when the Accredited Laboratory measured a value of 7.0 mmol/L then the KoneLab would have reported a value of 7.3 mmol/L which was 4% higher than the Accredited Laboratory’s result, less than the +/- 8% limit set by the RCPA and less than our “Subjective” Significant Criterion of +/- 10%.

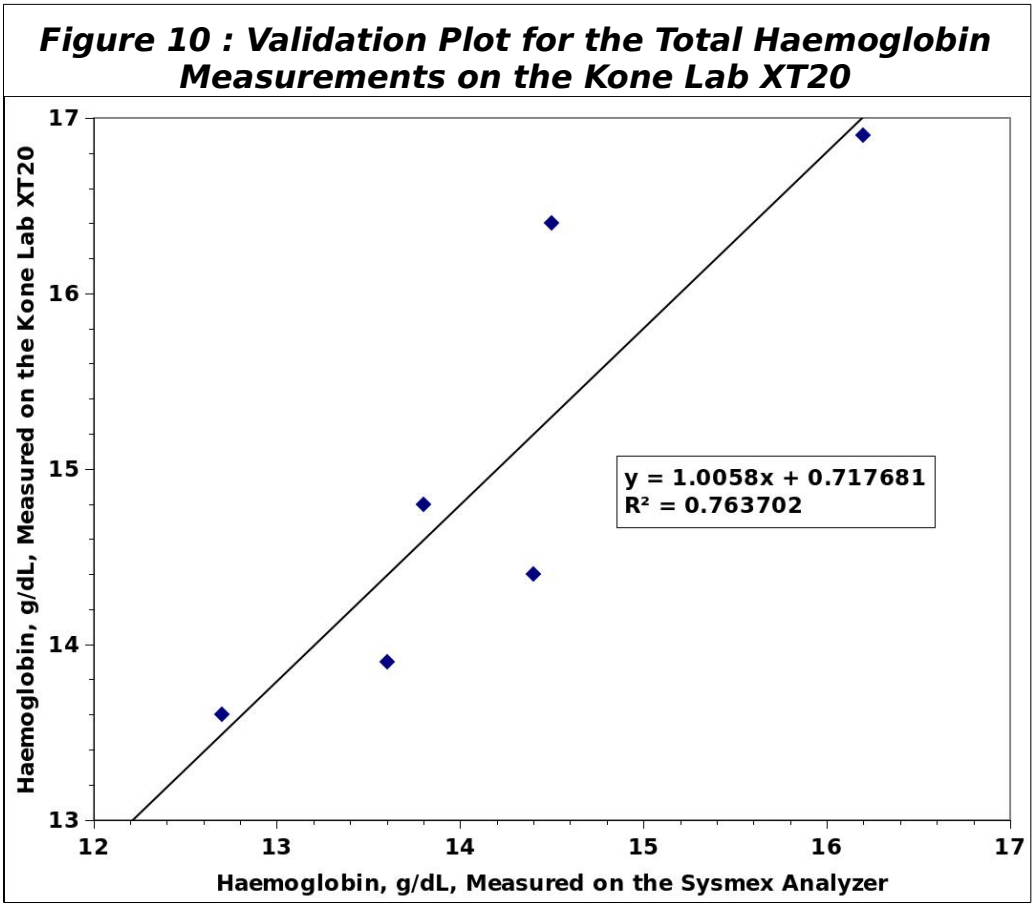
For these reasons we were satisfied with the performance of the plasma glucose assay on the KoneLab XT20.

Validation Data for the Whole Blood Total Haemoglobin Analyses on the Kone Lab XT20

Table 2. Measurements of Total Haemoglobin in the venous whole blood samples analyzed on the Sysmex Haematology Analyzer compared to the KONELAB 20XT analyses of the same whole blood samples.

Sample Name	Sysmex Hb g/dL	Venous blood on Kone Lab Hb g/dl
114577	12.7	13.6
118204	13.8	14.8
114961	14.4	14.4
119498	14.5	16.4
117729	13.6	13.9
117727	16.2	16.9
Mean	14.20	15.00
SD	1.17	1.35
SEM	0.48	0.55
Correlation Coefficient	0.8739	
Slope	1.0058	
Intercept	0.7177	
Standard Error of Estimate of x from y	0.64	
Paired Student's t	t = -2.98 p = 0.03	

Figure 10 : Validation Plot for the Total Haemoglobin Measurements on the Kone Lab XT20



The normal range for haemoglobin in men is 13 – 18 g/dL and 12 – 16 g/dL in women (www.mytransfusion.com.au/reasons-transfusion/anaemia) which give a composite males and females range of 12 – 18 g/dL. These validation data showed that when the Sysmex Analyzer read 12 g/dL then the Kone Lab method would have reported a value of 12.8 g/dL. This was 7% higher than the Sysmex but was acceptable since it matched the tolerable tolerance limit of +/- 75 set by the ClinLab Navigator website that has been referred to previously. It also fell inside our “Subjective” Significant Criterion of +/- 10%.

The agreement was better when the same calculations were made at the 18 g/dL level. The KoneLab would have reported a value of 18.8 g/dL which was 4.6% higher than the Sysmex result. This was inside the +/- 7% criteria set by the ClinLab Navigator website and was well inside our “Subjective” Significant Criterion of +/- 10%.

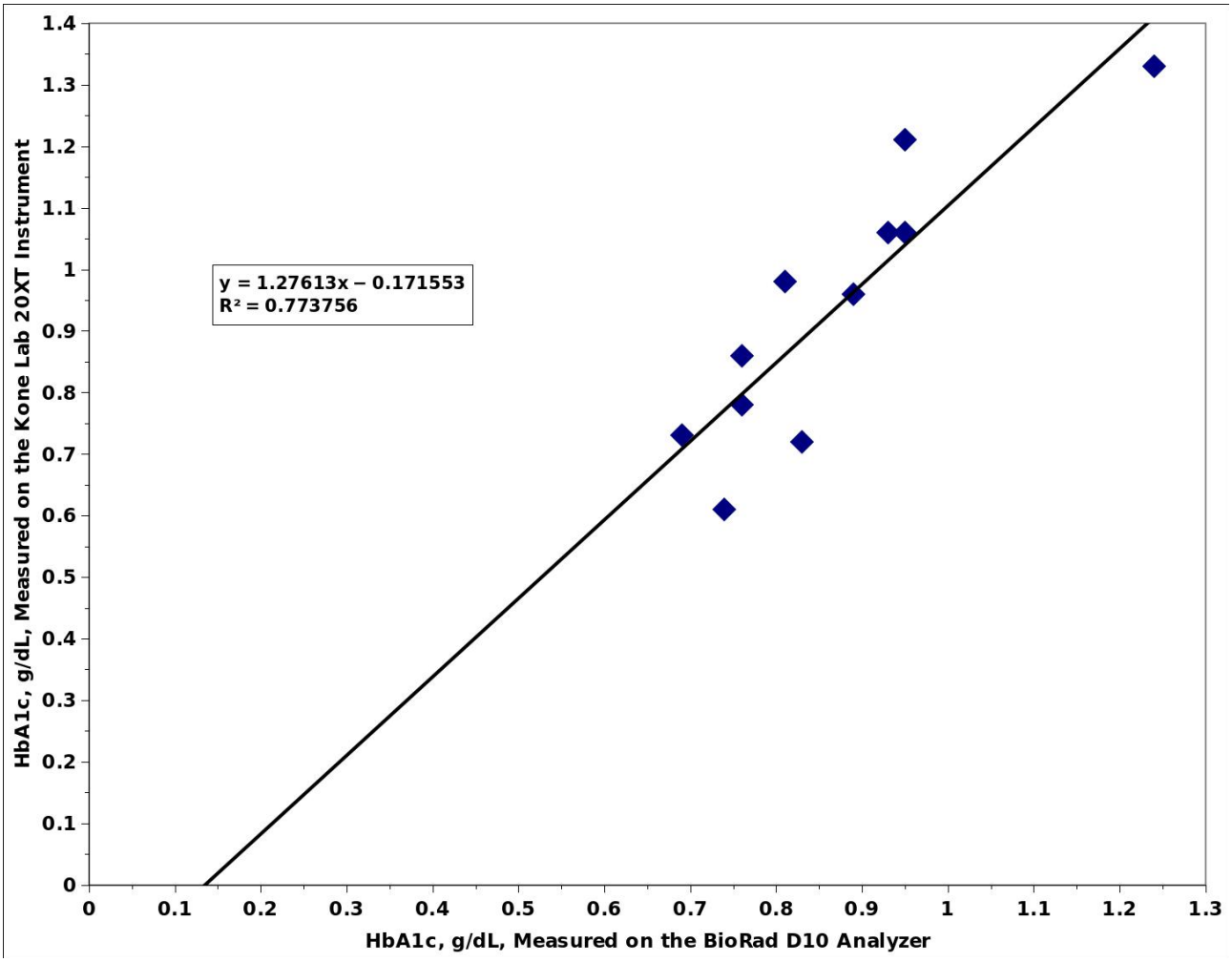
For these reasons we were satisfied with the performance of the Total Haemoglobin assay on the KoneLab XT20.

Validation Data for the Whole Blood HbA1c Analyses on the Kone Lab XT20

Table 3. Measurements of Haemoglobin A1c in the venous whole blood samples analyzed on the BioRad D10 Analyzer compared to the KONELAB 20XT analyses of the same whole blood samples.

Sample Name	PathologyLab BioRad D10 A1c, g/dL	KoneLab A1c, g/dL
96080	0.81	0.98
101501	0.74	0.61
101528	1.24	1.33
96772	0.83	0.72
96749	0.69	0.73
96579	0.93	1.06
103525	0.76	0.78
112112	0.89	0.96
98400	0.95	1.06
94932	0.95	1.21
108895	0.76	0.86
Mean	0.87	0.94
SD	0.152	0.221
SEM	0.046	0.067
Correlation Coefficient	0.8796	
Slope	1.276	
Intercept	−0.172	
Standard Error of Estimate of x from y	0.093	
Paired Student’s t	t = -2.00 p = 0.07	

Figure 11 : Validation Plot for the Haemoglobin A1c Measurements on the Kone Lab XT20



Given that the composite reference range for Total Haemoglobin is 12 – 18 g/dL, then the corresponding HbA1c concentrations at the WHO 'diabetes diagnostic level' of 6.5% would be 0.78 g/dL and 1.17 g/dL respectively. At these two concetrations the KoneLab XT20 would have reported values of 0.82 g/dL and 1.32 g/dL which when calculated as a percentages of the Total Haemoglobin concentrations would have been 6.8% and 7.3% HbA1c. Those results amounted to errors of only 0.3% HbA1c and 0.8% HbA1c respectively. The former was well inside the clinically acceptable limit of +/- 0.8% HbA1c and the latter was just on that limit. We have noted earlier in this report that the Randox Method for HbA1c did not perform well in our opinion at levels above 1g HbA1c/dL.

◆ Glucose Study : Results and discussion

TABLE 4: Results and Statistical Analyses of the Data from the Glucose Study.

Participant ID	Venous blood glucose mmol/L	1st Finger prick Mitra Tip, mmol/L	2nd Finger prick Mitra Tip, mmol/L
JS	5.13	3.13	2.88
SS	5.37	3.04	2.77
SG	5.61	5.10	5.27
WN	9.75	7.81	7.86
KJL	5.17	4.68	5.23
LC	6.02	4.52	5.48
SRD	4.74	4.06	4.17
JP	4.40	5.27	4.55
JT	5.09	4.09	4.33
PC	4.00	3.65	3.87
SF	5.37	5.74	5.83
CT	4.62	4.05	4.90
SuSa	5.12	4.16	3.74
APP	5.35	4.11	4.01
SGJ	4.96	3.77	3.94
JB	6.06	5.71	5.54
DL	4.77	4.55	5.05
SM	6.47	7.45	7.45
SB	5.47	6.19	6.50
BS	5.96	6.85	6.73
Mean	**5.50**	**5.211**	
Standard deviation	**1.170**	**1.355**	
CV %	**21%**	**27%**	
N	**20**	**40**	
Median	**5.26**	**4.62**	
Paired Student's t	**3.30**		
Significance, p, (Two tail test).	**0.002**		
Statistically significant	**Yes**		
Clinically significant by RCPA criteria.	**No** (+5.3%)	(Difference between Means) ÷ Venous Mean X 100	

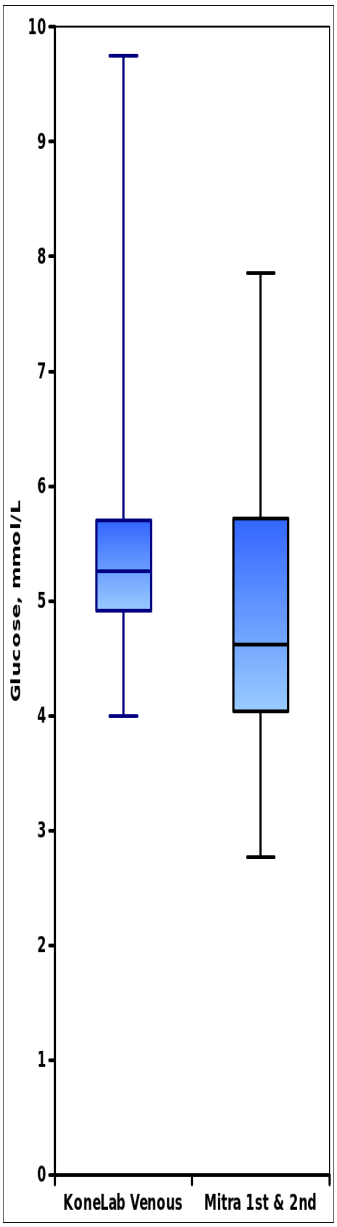


Figure 12 : Bland and Altman Plot of the Venous Glucose Results and the Mitra Sampler Glucose Results.

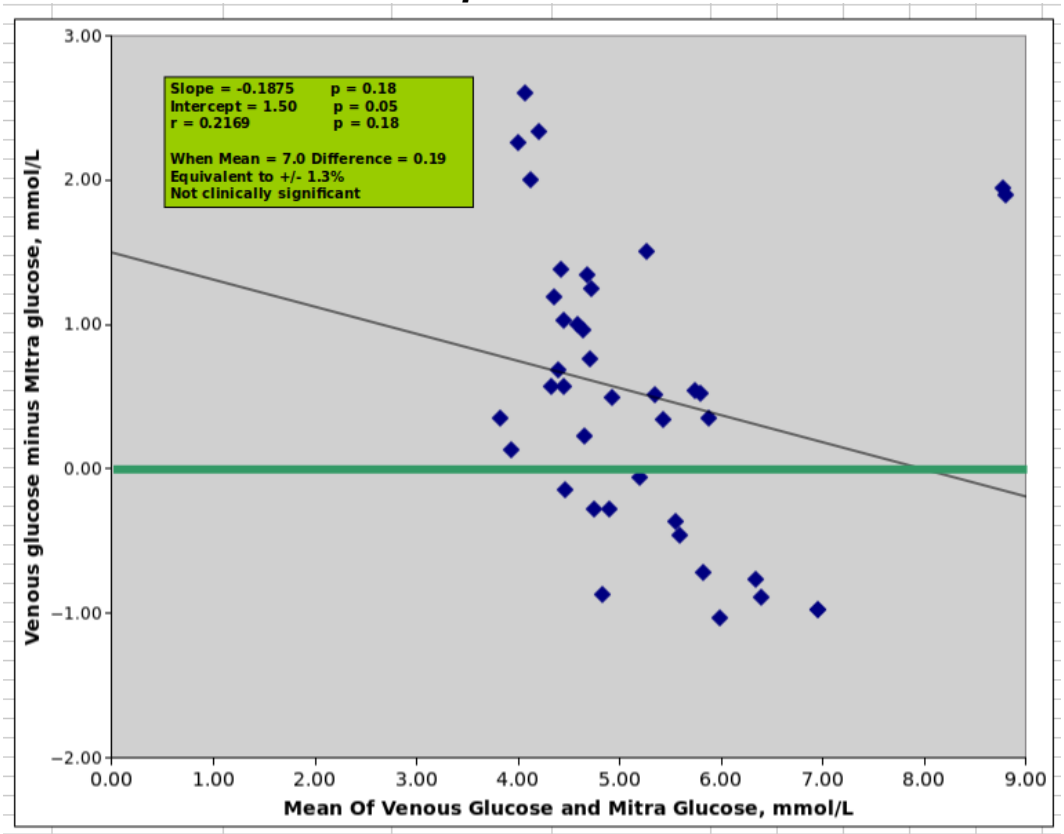


Figure 13

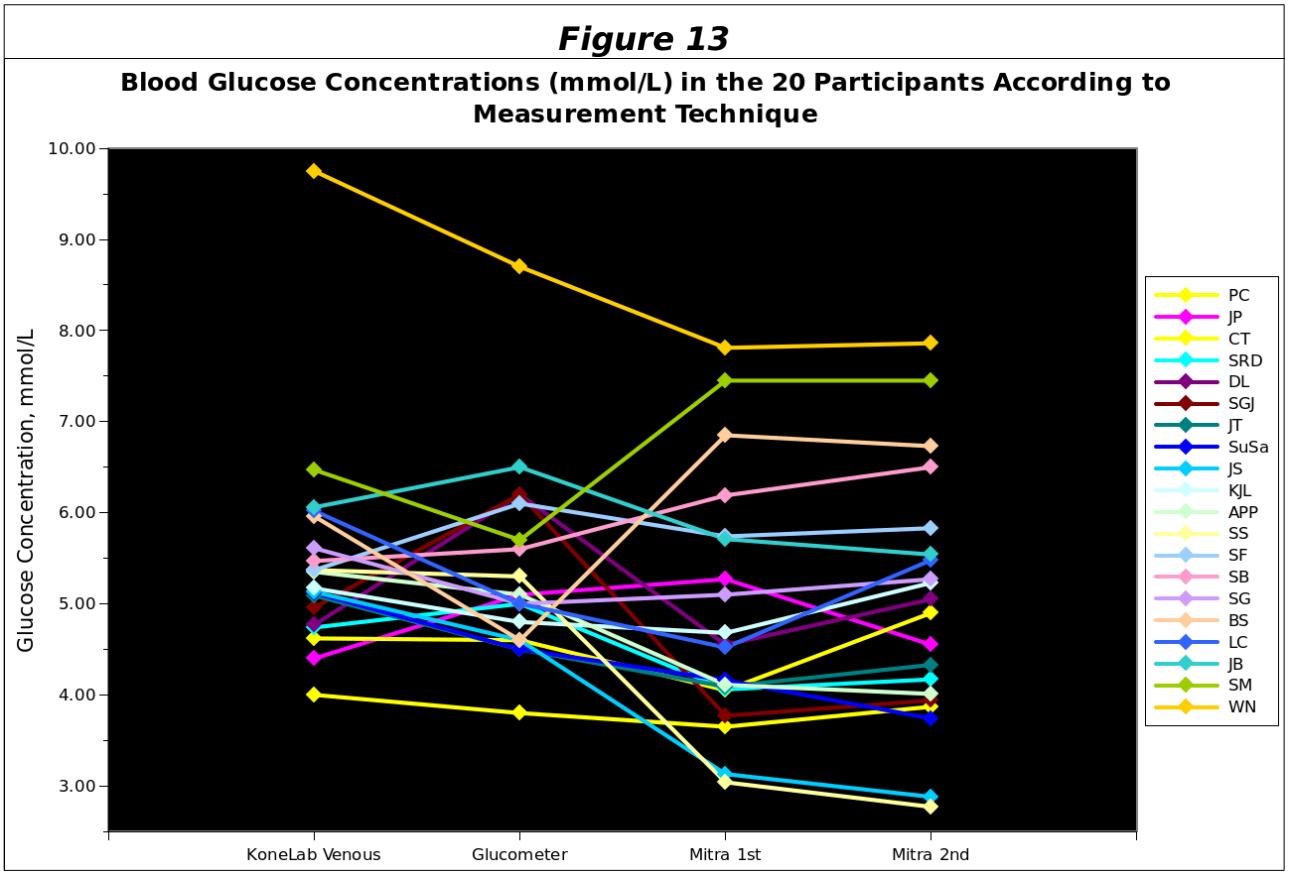
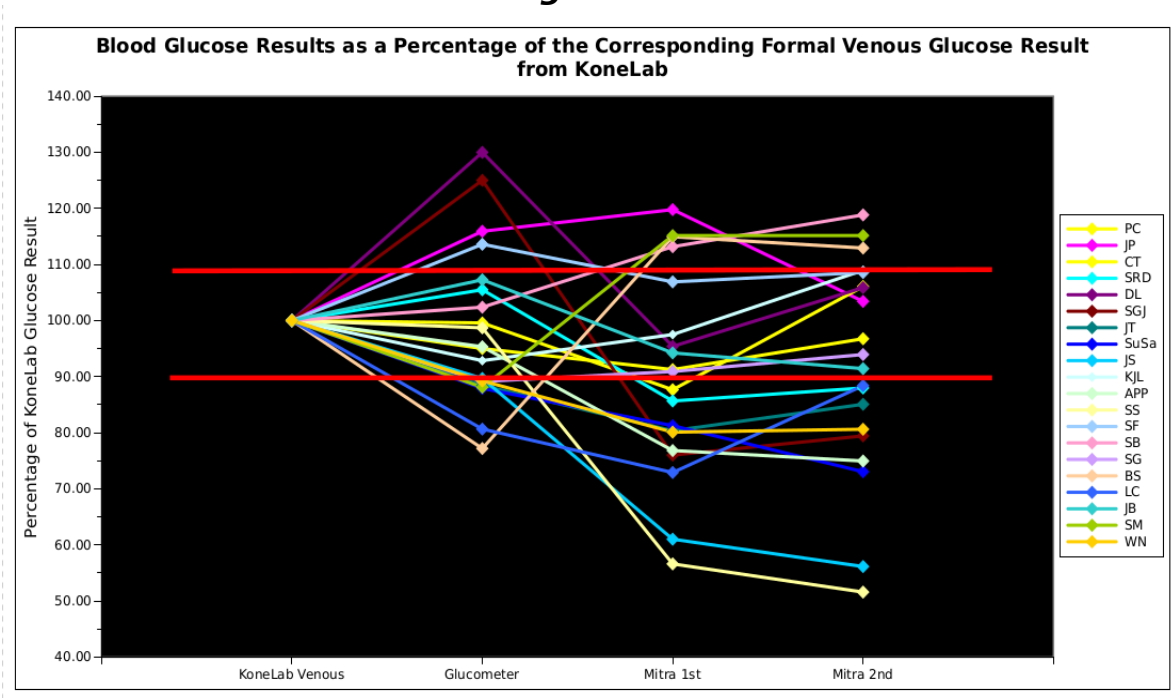


Figure 14



When the data were regarded as populations (Table 4 and Figure 12) then there were significant statistical differences. But when these differences were assessed against the RCPA criteria then these differences were not regarded as clinically significant.

However Figure 13 and particularly Figure 14 illustrated that on a person to person basis 8 of the 20 readings by the glucometer and 14 of the 40 readings from the Mitra Samplers fell outside of our +/- 10% “Subjective” Significant Criterion. These scores were too high to justify using either techniques as substitutes for formal venous sample analyses.

◆ HbA1c Study : Total Haemoglobin Results and Discussion

Table 5 : Results and Statistical Analyses of the Total Haemoglobin Data from the HbA1c Study.

Participant ID	Venous Blood THb, g/dL	Mitra1 THb, g/dL	Mitra2 THb, g/dL
SS	12.55	10	9.1
CJX	13.25	14.2	13.1
TH	13.95	12.7	12.2
CB	13.15	11.2	12.5
DS	11.95	10.5	12.6
IT	11.95	12	9.9
YM	12.25	11.7	11.6
LM	14.05	15.1	12.5
JD	13.45	12.2	11.6
GZ	12.15	11.2	10.2
JHX	13.15	11.9	12.8
JS	13.35	11.3	13.5
MB	15.75	16	15.1
YC	15.35	16	16.5
SM	14.55	15	13.2
PM	16.15	14.4	13.4
AS	14.35	14	13.3
JB	16.95	17	17.8
EZ	13.65	14.6	13.1
WCC	15.05	17.7	16.6
Mean	**13.85**	**13.23**	
Standard Deviation	**1.44**	**2.21**	
CV %	**10.36**	**16.67**	
N	**20**	**40**	
Median	**13.55**	**12.95**	
Paired Student's t	**2.98**		
Significance, p, (Two tail test)	**0.00**		
Statistically significant	**Yes**		
Clinically significant	**No** (Diff = 0.62 g/dL or 4.5%)	(Difference between Means) ÷ Venous Mean X 100	

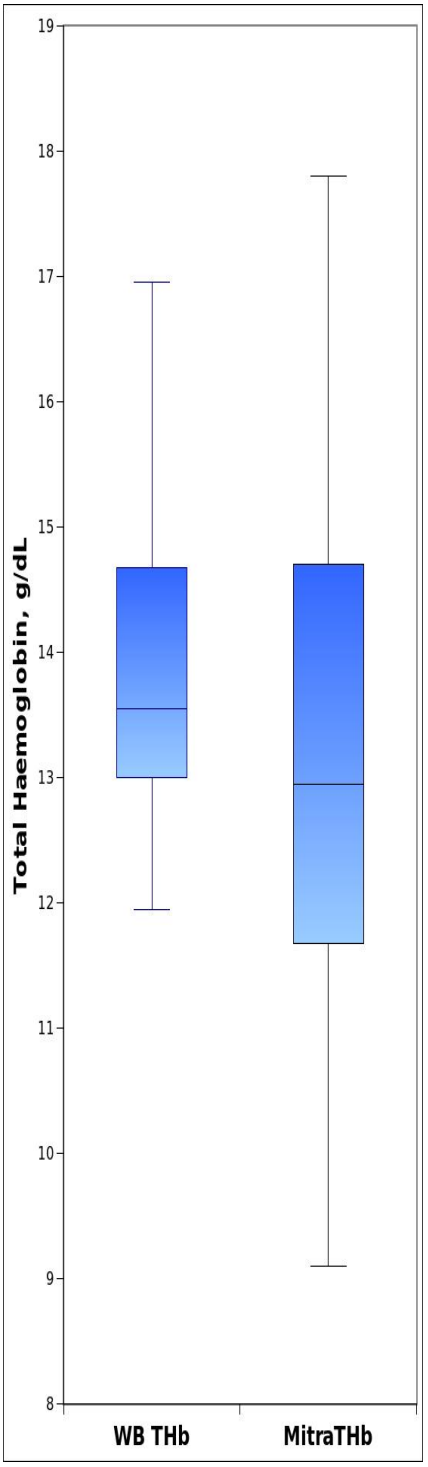


Figure 15 : Bland and Altman Plot of the Whole Blood Total Haemoglobin Results and the Mitra Sampler Total Haemoglobin Results.

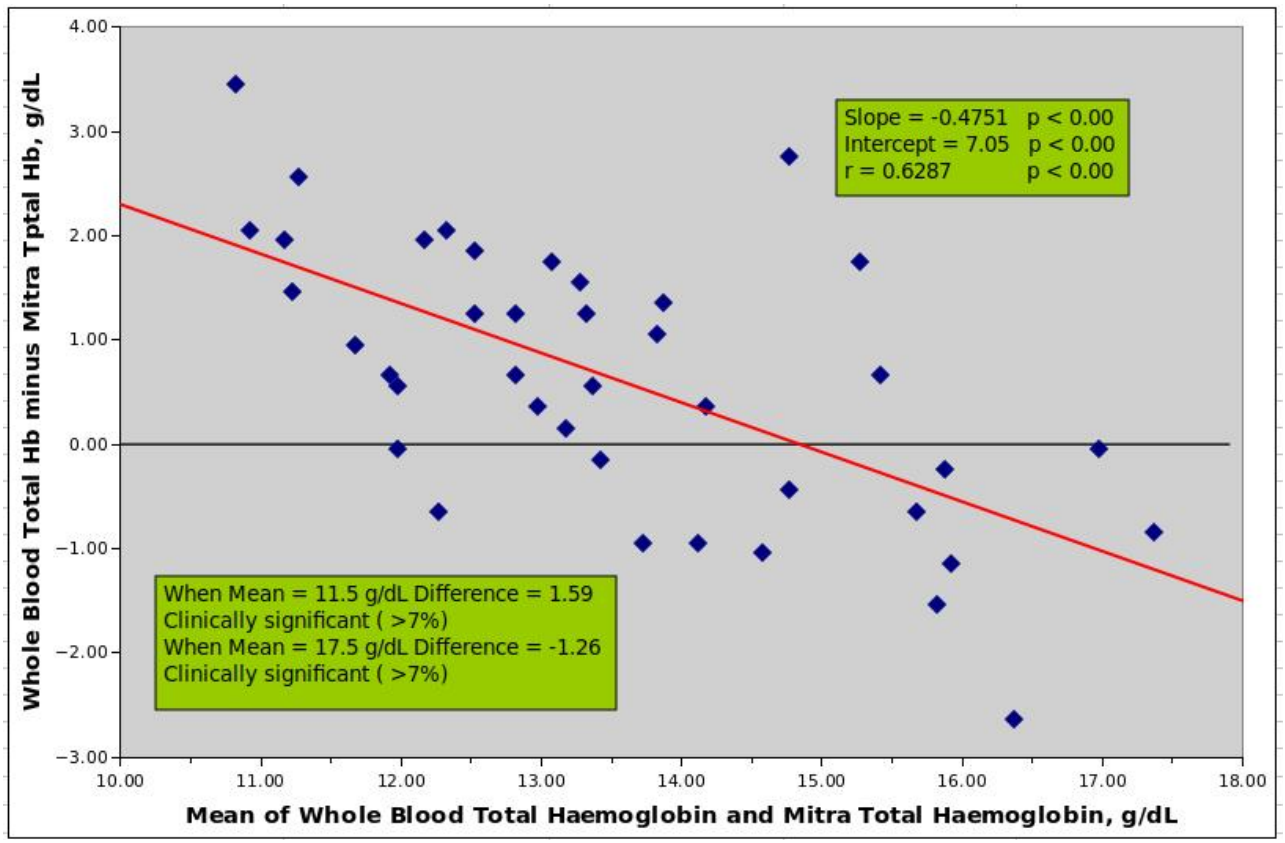


Figure 16

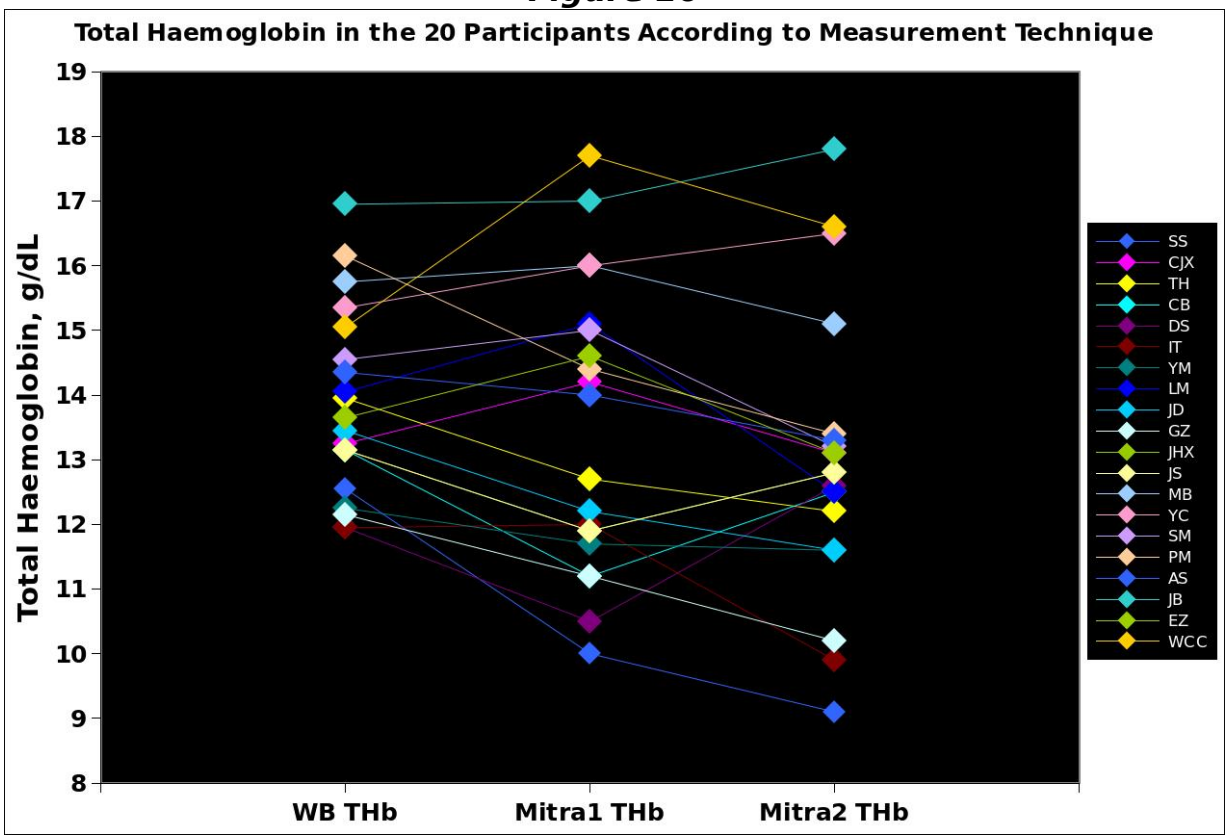
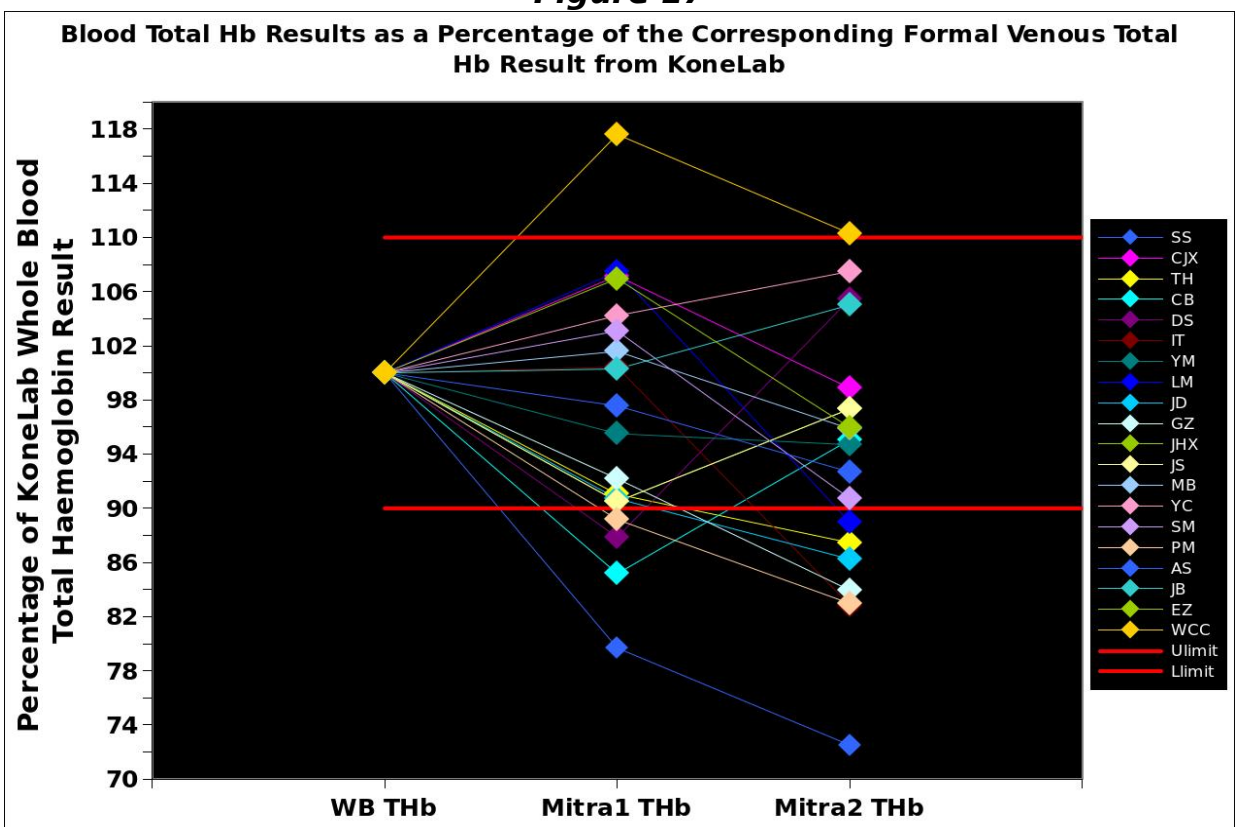


Figure 17



When the haemoglobin data were regarded as populations (Table 5 and Figure 15) then there were significant statistical differences. But when these differences in Table 5 were assessed against the RCPA criteria then these differences were not regarded as clinically significant on the simple difference between the two population means; (13.85 g/dL and 13.23 g/dL). In contrast the Bland and Altman Plot in Figure 15 demonstrated statistically significant fixed and systematic biases which were also determined to be clinically significant. Figure 17 illustrated that on a person to person basis 24 of the 40 readings from the Mitra Samplers fell outside of our +/- 10% “Subjective” Significant Criterion. These scores were too high to justify using the Mitra Samplers as substitutes for formal venous sample analyses.

◆ HbA1c Study : Percent HbA1c Results and Discussion

Table 6: Results and Statistical Analyses of the Percent HbA1c Data from the HbA1c Study Study.

Participant ID	Venous blood, % HbA1c	1st Finger prick Mitra Tip, % HbA1c	2nd Finger prick Mitra Tip, % HbA1c
SS	6.4	4.1	4.2
CJX	6.9	5.1	5
TH	6.9	5	4.9
CB	6.5	3.6	4
DS	6.9	5.3	5.1
IT	6.5	4.7	4.8
YM	5.7	4.3	4
LM	6.6	3.8	4
JD	6.9	6.2	6.1
GZ	6.6	4.9	4.1
JHX	5.9	4.3	4.1
JS	7.2	4.9	5.2
MB	8.7	8.1	8.3
YC	6.5	4.2	5.5
SM	6.2	6.1	4.7
PM	8.4	8.5	8.7
AS	7.1	6.1	6.5
JB	6.3	4.5	5.4
EZ	8.9	9.1	8.5
WCC	6.3	4.9	5.4
Mean	**6.90**	**5.40**	
Standard deviation	**0.86**	**1.51**	
CV %	**12.51**	**27.88**	
N	**20**	**40**	
Median	**6.60**	**4.95**	
Paired Student's t	**5.35**		
Significance, p, (Two tail test).	**< 0.00**		
Statistically significant	**Yes**		
Clinically significant by our WHO based criterion of +/- 0.8% HbA1c.	**Yes** (Diff = 1.5%)	(Difference between Means) ÷ Venous Mean X 100	

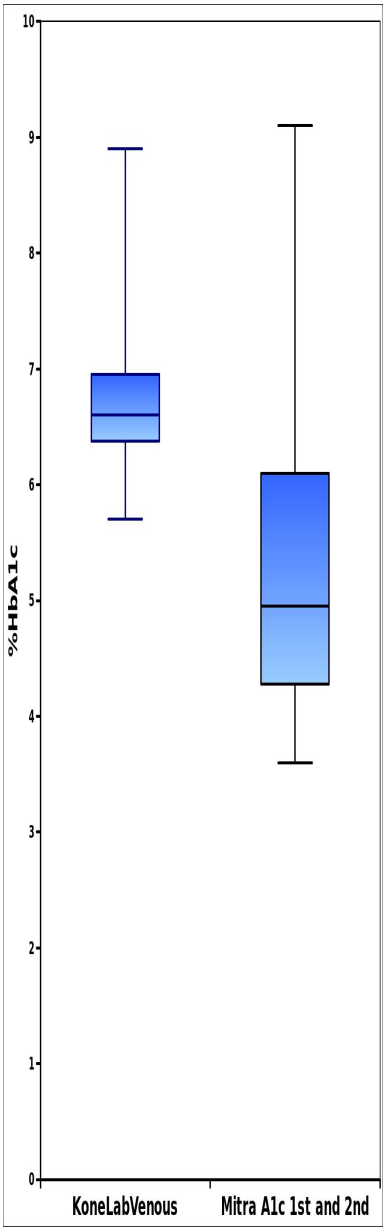


FIGURE 18: Bland and Altman Plot of the Venous %HbA1c Results and the Mitra Sampler %HbA1c Results.

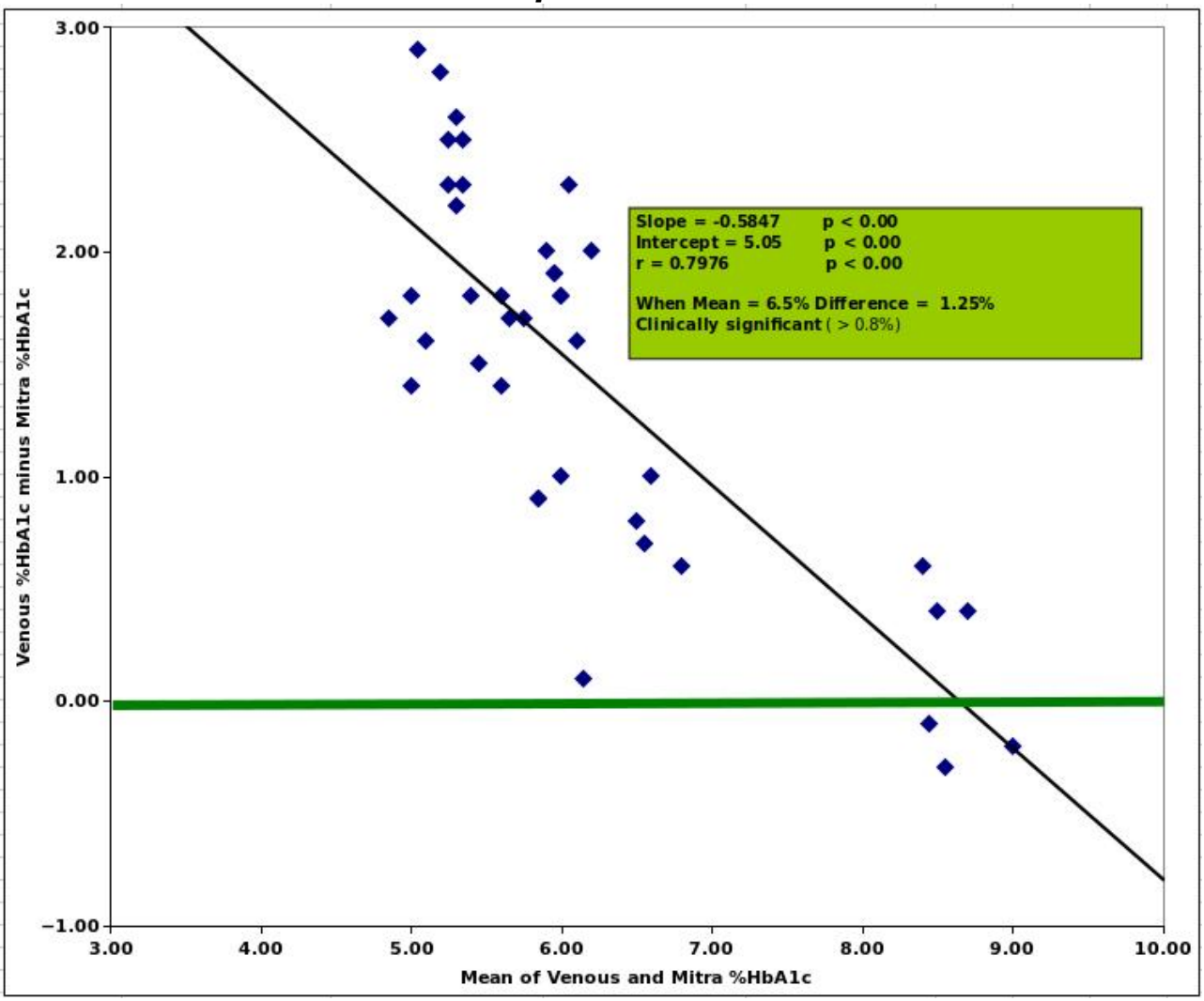


Figure 19.

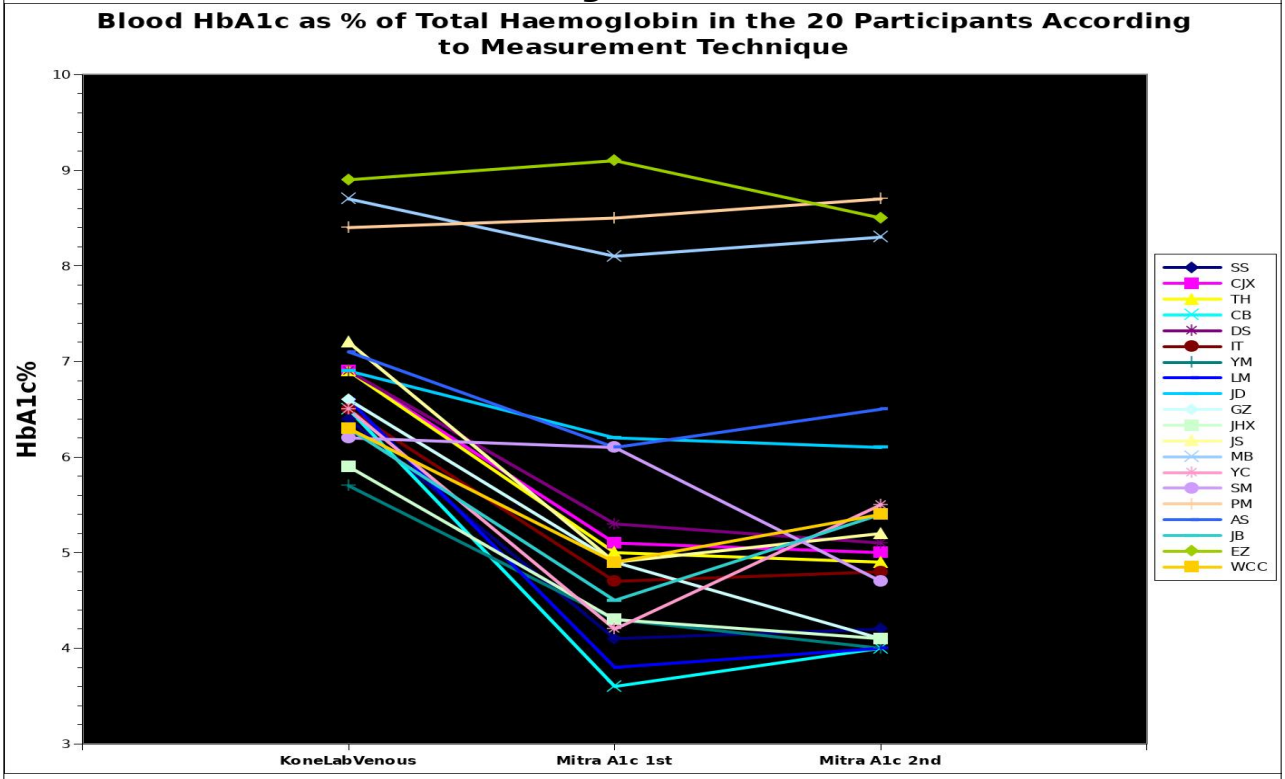
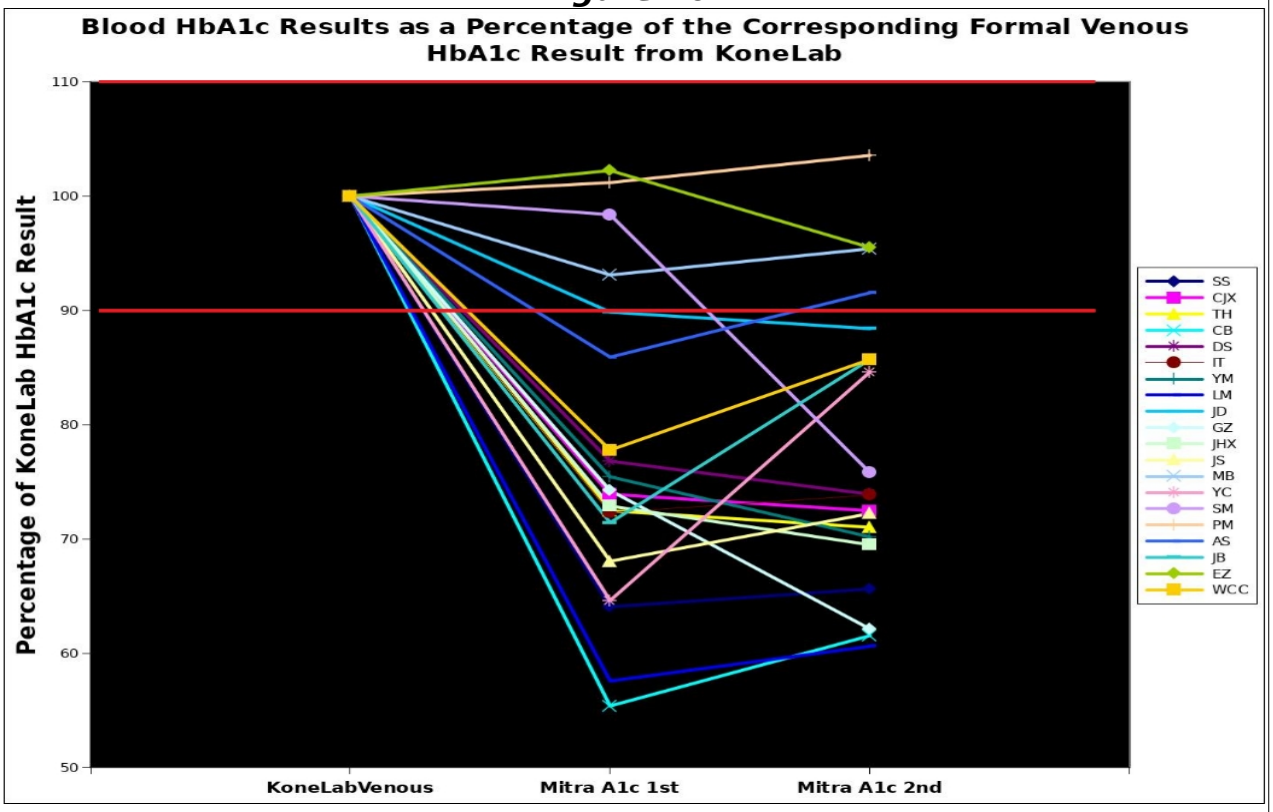


Figure 20.



When the HbA1c data were regarded as populations (Table 6 and Figure 18) then there were significant statistical and clinically significant differences. The Bland and Altman Plot in Figure 18 demonstrated statistically significant fixed and systematic biases which were also determined to be clinically significant. Figure 20 illustrated that on a person to person basis 32 of the 40 readings from the Mitra Samplers fell outside of our +/- 10% "Subjective" Significant Criterion. These scores were too high to justify using the Mitra Samplers as substitutes for formal venous sample analyses.

FOLLOW UP WORK

1. Optimisation of the HbA1c assay.

This work was inconclusive due to the lack of sufficient in date reagents to do the work.

The issue that still remains is that the the sensitivity and precision of the Randox Kit needs to be improved at the diagnostic 'window' concentrations of between 0.78 g/dL and 1.17 g/dL HbA1c. This will involve careful experiments where the ratio of latex to sample are varied so as to move the ODs for these concentrations up the calibration curve, see Figure 21 where the responses at the lower concentration are higher so that those 'window' concentrations are associated with a steeper part of the curve. Figure 22 plots the typical calibration curve used in the study (blue line) and the desirable/ideal form of the calibration curve (red line) in an optimised assay.

Figure 7 (repeated) : Typical Calibration Curve for the Randox Reagent Based HbA1c Assays.

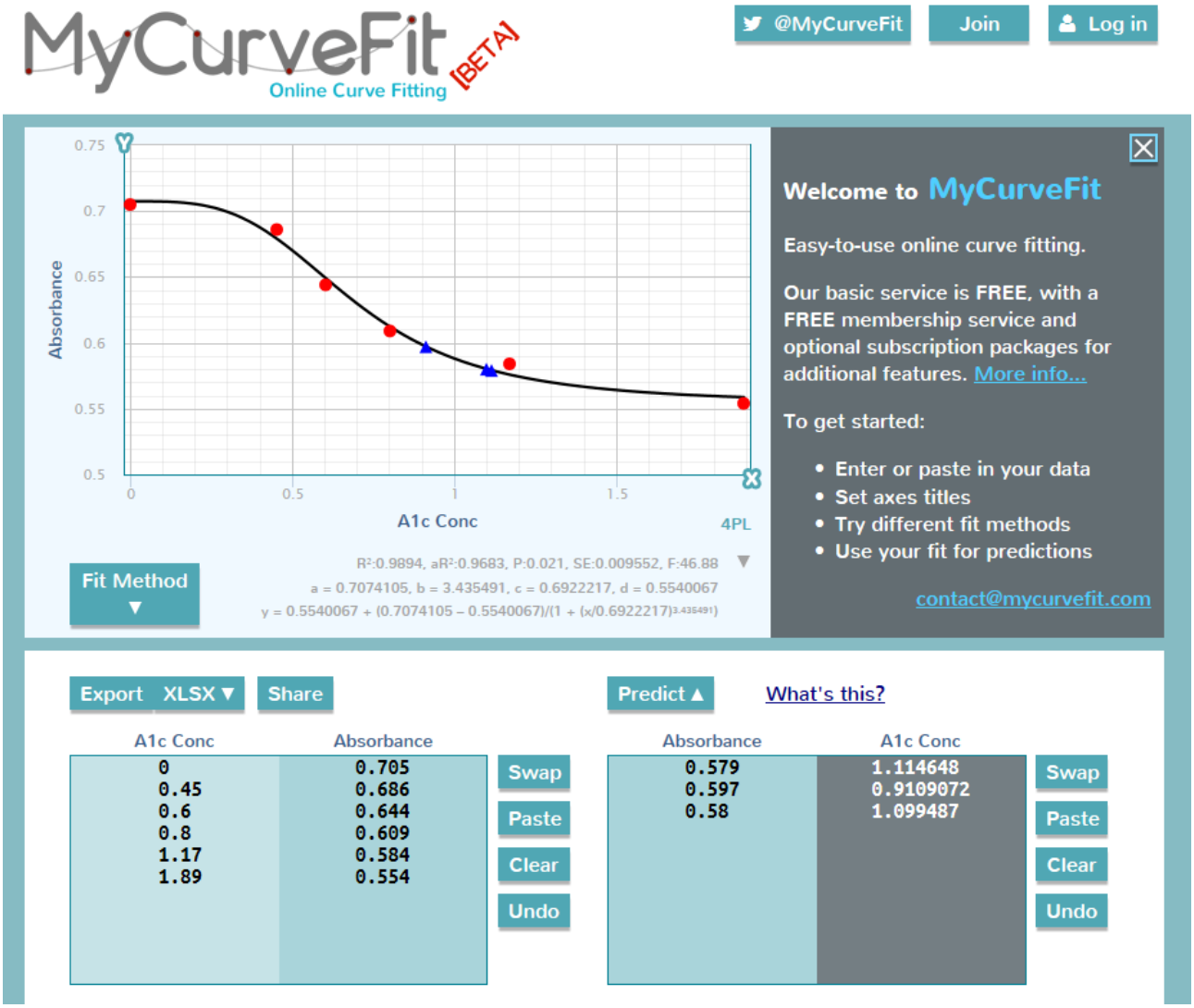


FIGURE 21 : Ideal Form of the Calibration Curve for the HbA1c Assay that Has Improved Sensitivity and Precision in the diagnostic 'window' between 0.78 g/dL and 1.17 g/dL HbA1c.

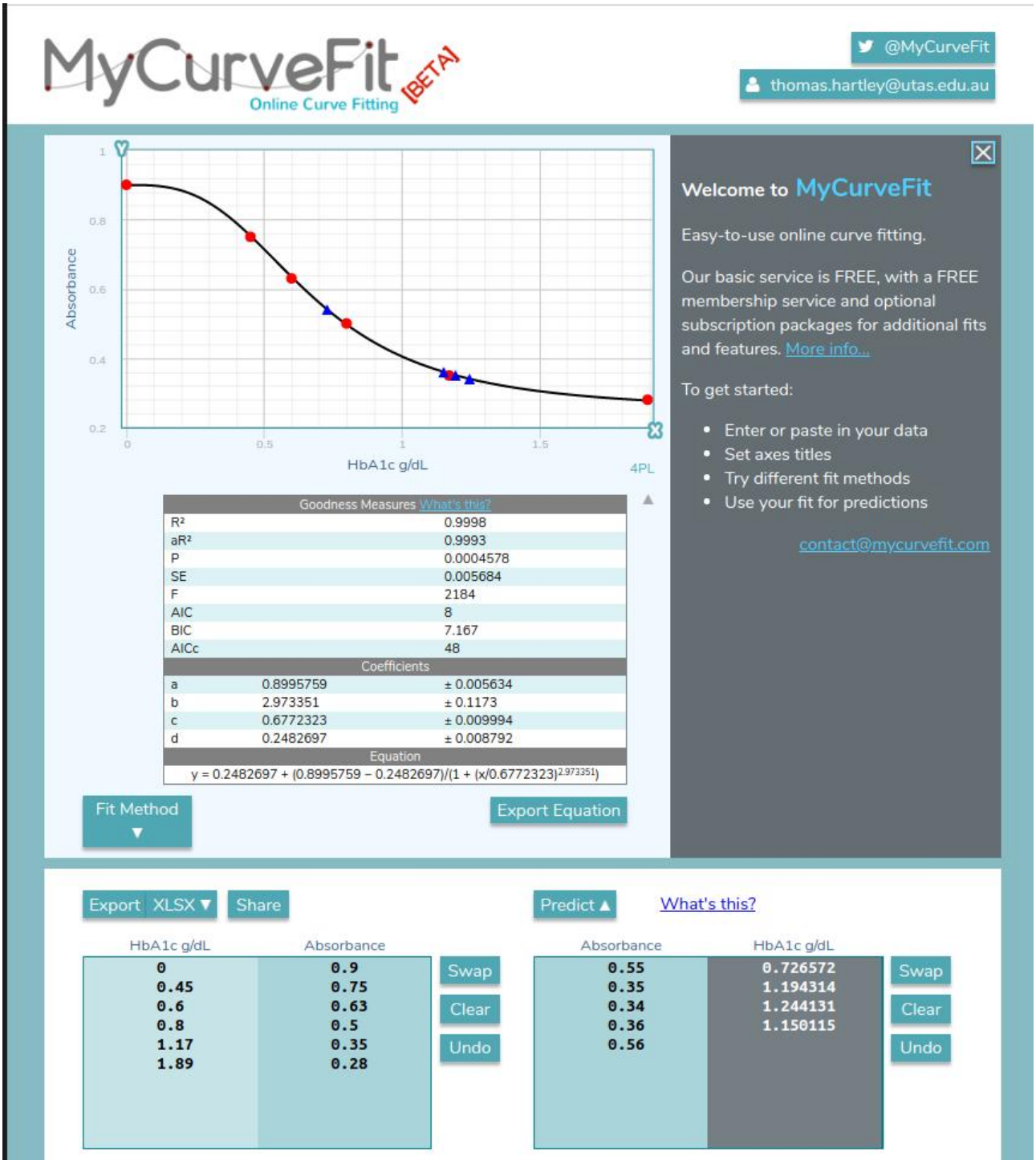
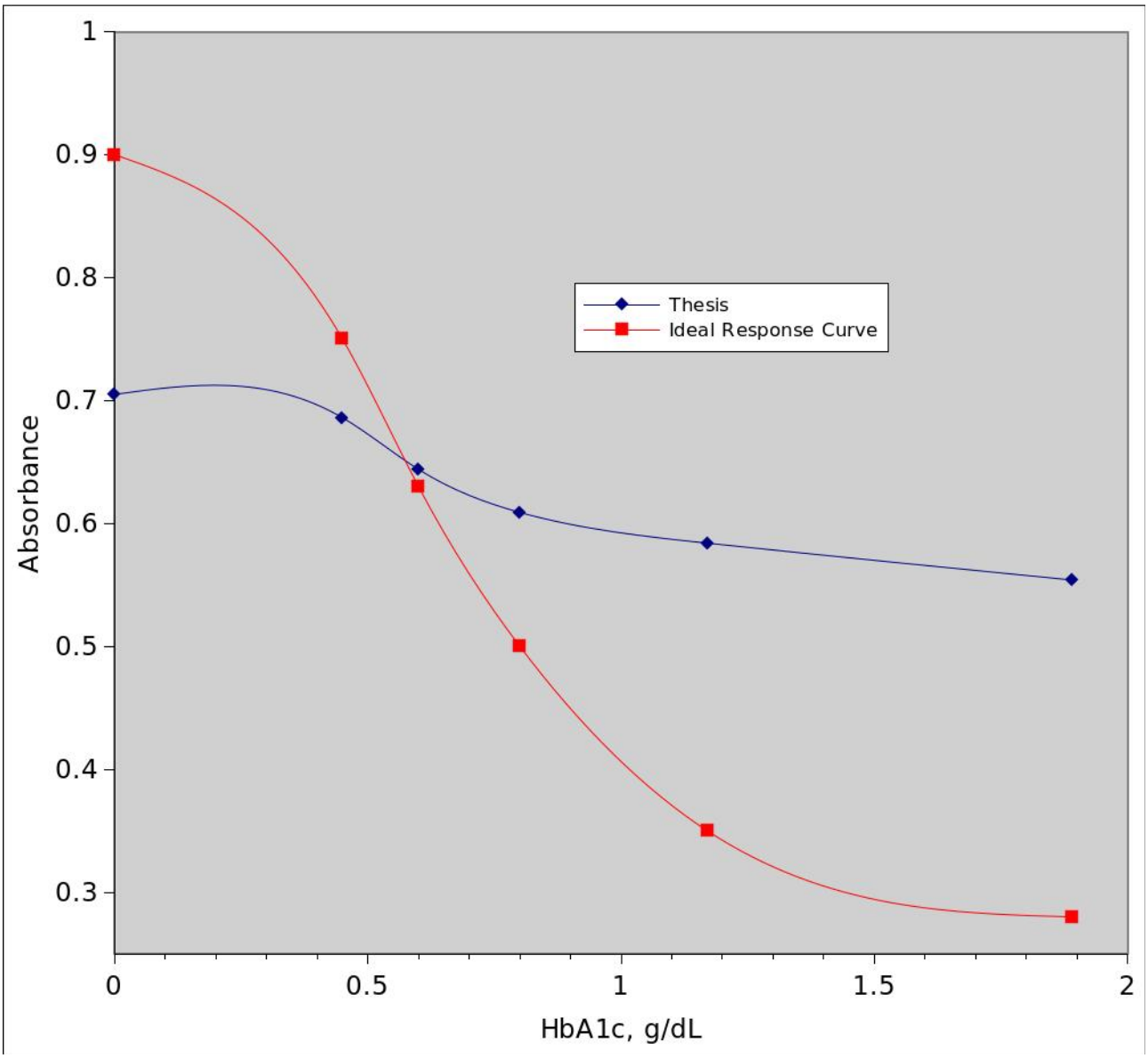


FIGURE 22
Ideal Response Curve for the HbA1c Assay (red) Compared to the Observed Curve (blue)

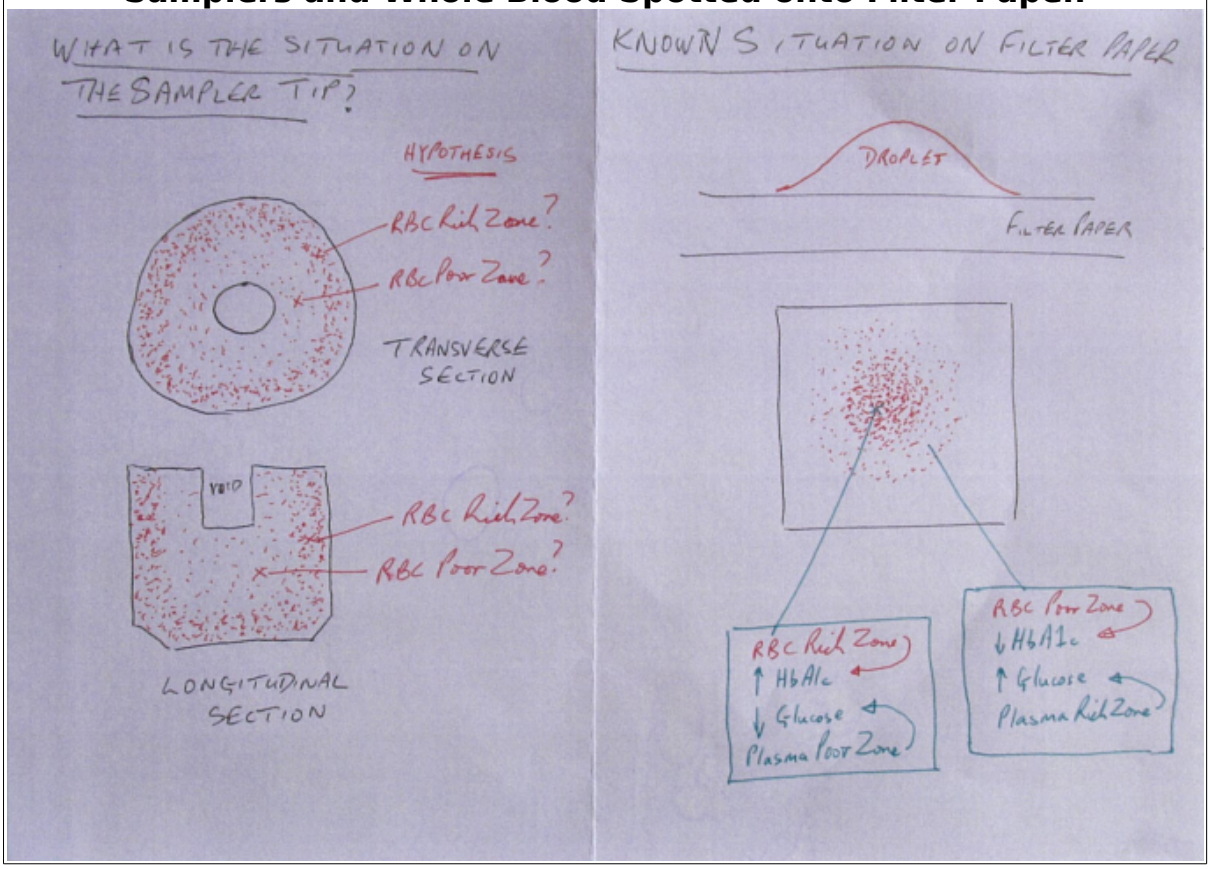


2. Investigation of the Volumetric Accuracy of the Mitra Samplers.

Mention was made in the Introduction about the inhomogeneous nature of blood spots on filter paper and it was therefore decided that a closer examination of how the blood soaked onto the Mitra Samplers was warranted. The suspicion was that the red blood cells would clog the micro pores of the tip at the surface and cause a diminished uptake of red blood cells while still allowing the passage of blood plasma into the central volume of the tip. The results of the HbA1c study supported this theory because the Mitra Sampler data were always lower (with one exception) than the corresponding formal venous samples eg see Figure 20. Theoretically the use of secondary standards in this assay should have compensated for this but it was later realised that the ‘grey top’ tubes used as the HbA1c secondary standards actually

contained HAEMOLYSED blood. (Fluoride/EDTA tubes are notorious for haemolysing the specimen within 24 hours. In addition the specimens in question had been deep frozen at -20ºC which will have also ensured that they were haemolysed. Haemolysed blood would have soaked into the Mitra Sampler tips more effectively than whole blood if our theory here is correct.) Our thinking on this problem is illustrated in the sketches in Figure 23.

FIGURE 23 : Hypothetical Distribution of Whole Blood in Mitra Samplers and Whole Blood Spotted onto Filter Paper.



Results of Fixing, Staining and Sectioning 'Loaded' Mitra Samplers Collected from Two Normal Individuals

Equipment :

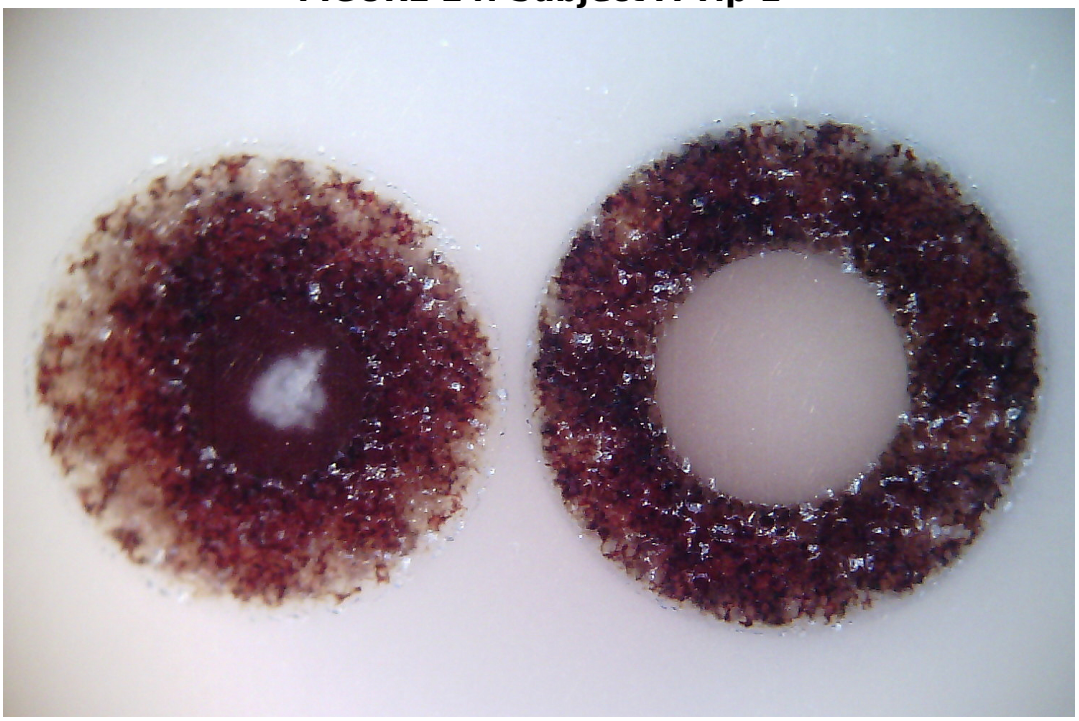
10 uL Mitra Samplers
Histology Embedding station
Binocular Microscope X20 magnification (X2 objective, X10 eyepiece)
Mobile phone camera (Huawei Y600)
Unistik 3 Extra spring loaded lancets, 21G needle, 2.0 mm depth)

Procedure : Two subjects loaded two Mitra Samplers from the one finger prick made with the spring loaded lancet device. These were then left dry for several days before processing. All four samplers loaded with blood easily apart from Tip 2 from Subject A which took two 'touch ons' to load adequately.

Tip 1 from Subject A was sectioned transversely at two levels. One level close to the end of the tip (Figure 24, left image) and at second level approximately half way down the long axis of the sampler (Figure 24, right image). These sections were fixed in formalin and

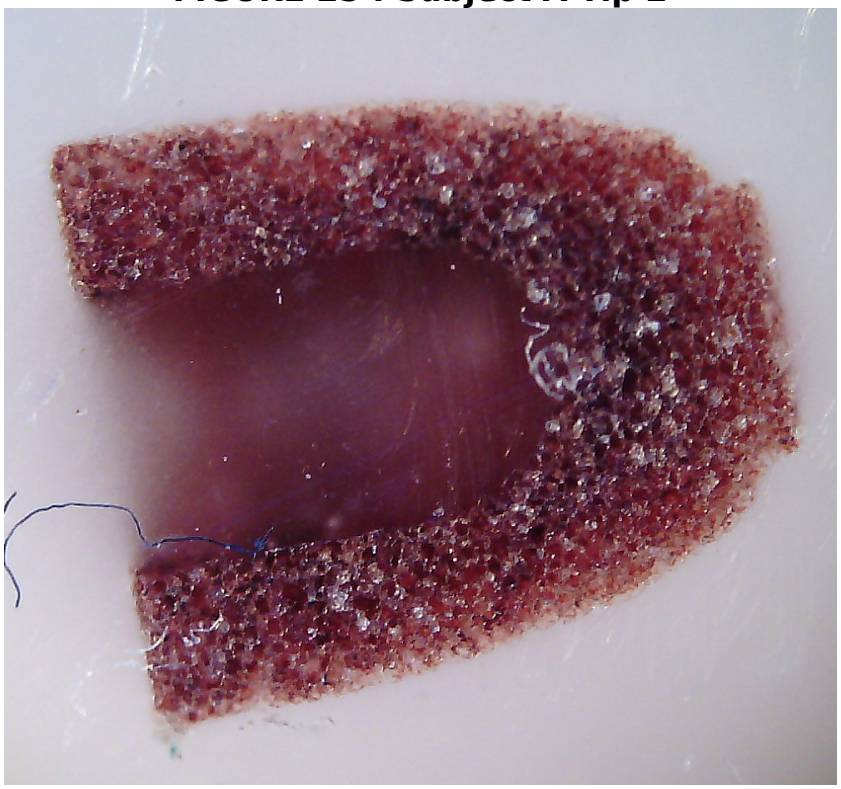
embedded in paraffin wax as per standard pathology laboratory procedures for small tissue specimens.

FIGURE 24: Subject A Tip 1



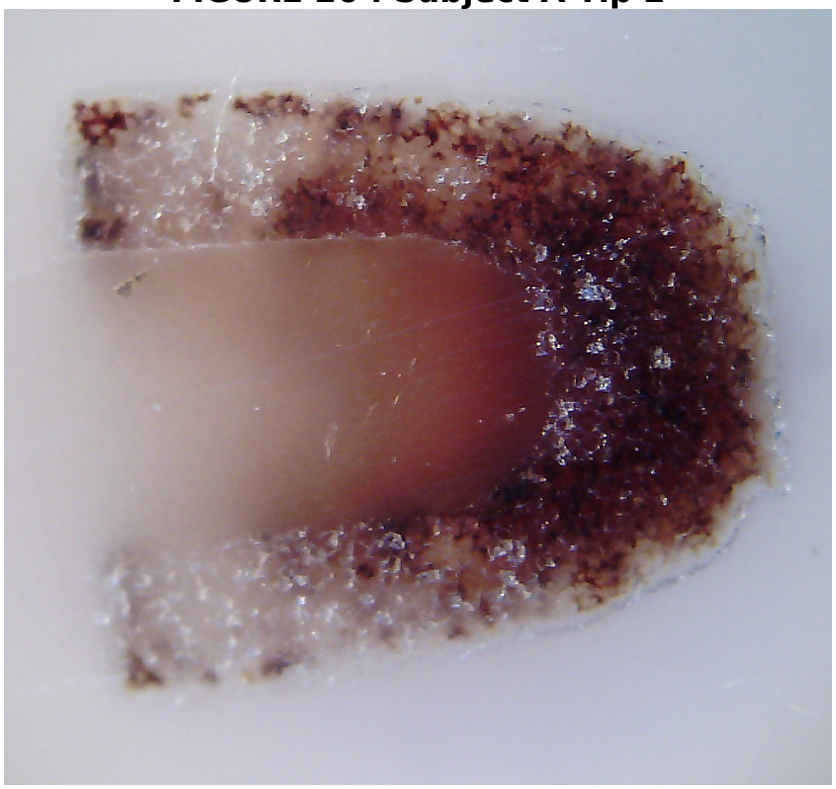
Tip 2 from Subject A was sectioned longitudinally. One of these sections was embedded in paraffin wax without any form of fixation (Figure 25)

FIGURE 25 : Subject A Tip 2



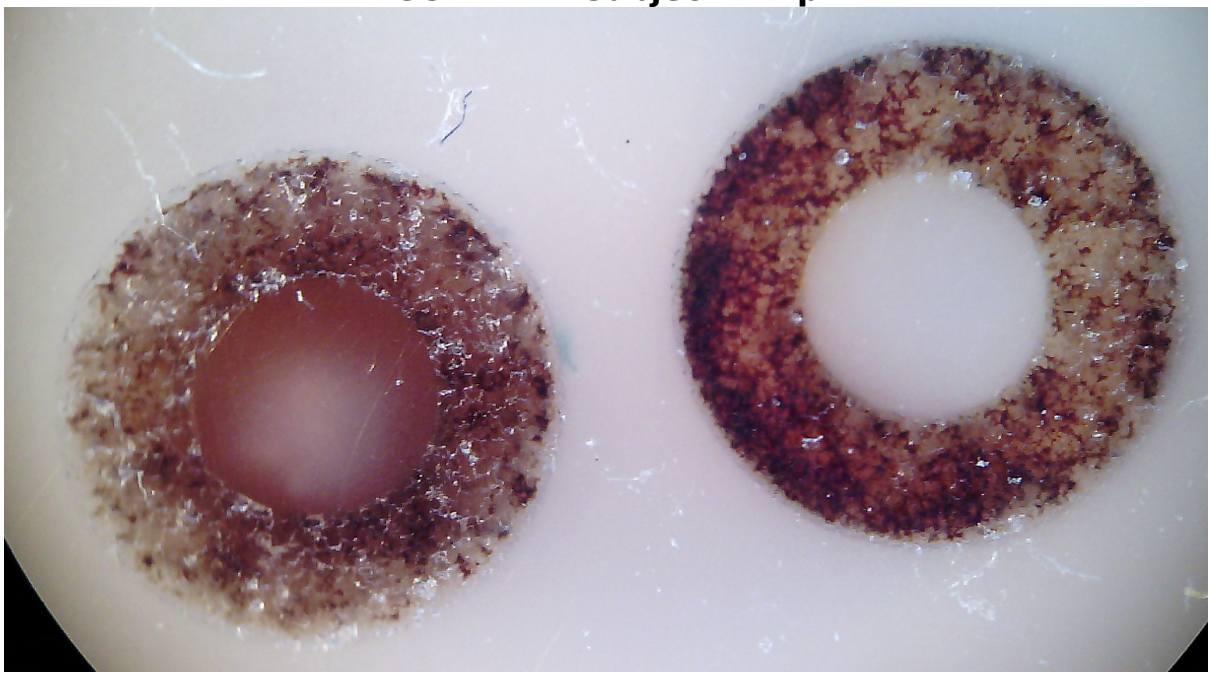
The other half was fixed with formalin before being embedded in paraffin wax (Figure 26)

FIGURE 26 : Subject A Tip 2



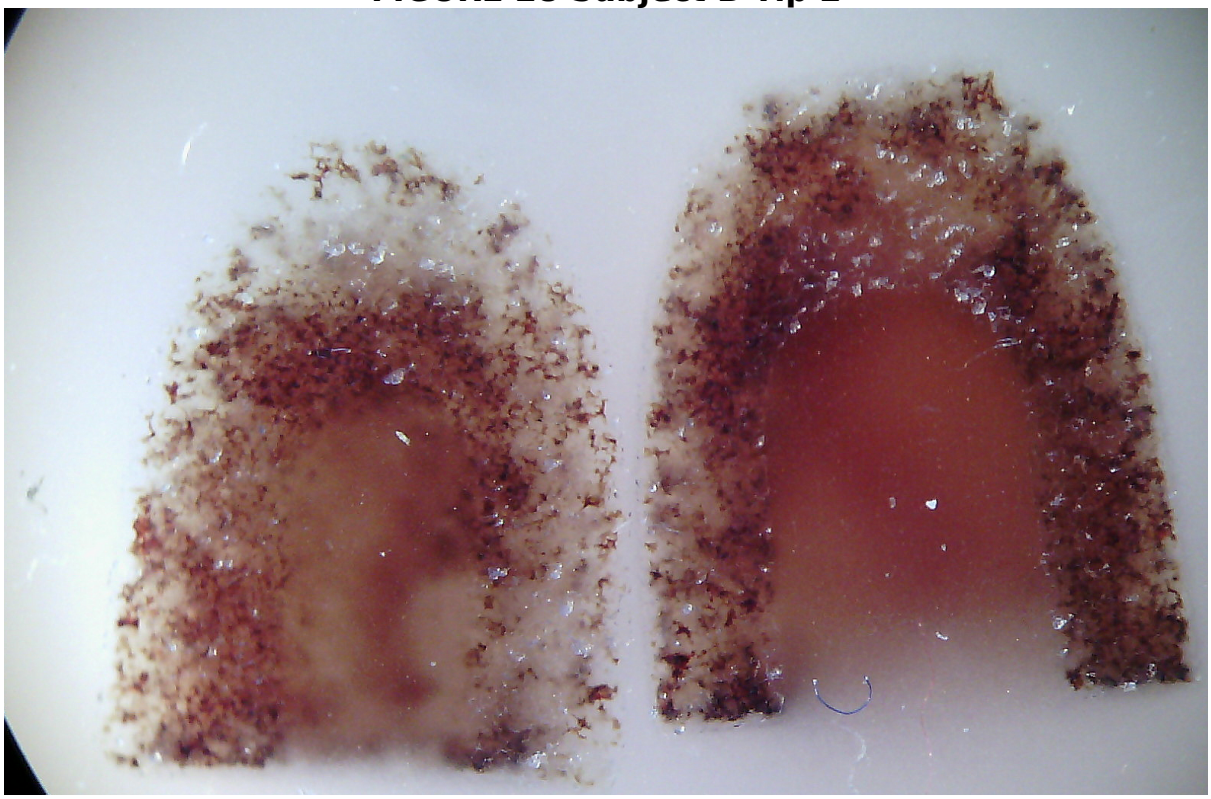
Tip 1 from Subject B was sectioned transversely at two levels. One level close to the end of the tip (Figure 27, left image) and at second level approximately half way down the long axis of the sampler (Figure 27, right image). These sections were fixed in formalin and embedded in paraffin wax as per standard pathology laboratory procedures for small tissue specimens.

FIGURE 27 : Subject B Tip 1



Tip 2 from Subject B was sectioned longitudinally. Both of these sections were fixed with formalin and embedded in paraffin wax as per standard pathology laboratory procedures for small tissue specimens (Fig 28).

FIGURE 28 Subject B Tip 2



Interpretation : Although the sample collectors were satisfied at the time that the samplers were adequately soaked with blood (apart for Subject A's second sampler which required two 'touch ons') the various tips showed some variations under microscopic examination. Subject A's Sampler Tip A showed quite light colouration around the circumference at the tip end (Figure 24, left image) and patches of light and heavy colouration in the middle region of the tip. Even the unfixed sampler tip (Figure 25) appeared to be uniformly soaked with blood but there was an impression that the outer layer was less blood stained that the interior and its other half, shown in Figure 26 definitely confirmed that blood penetration into the tip closest to the tip holder was quite sparse.

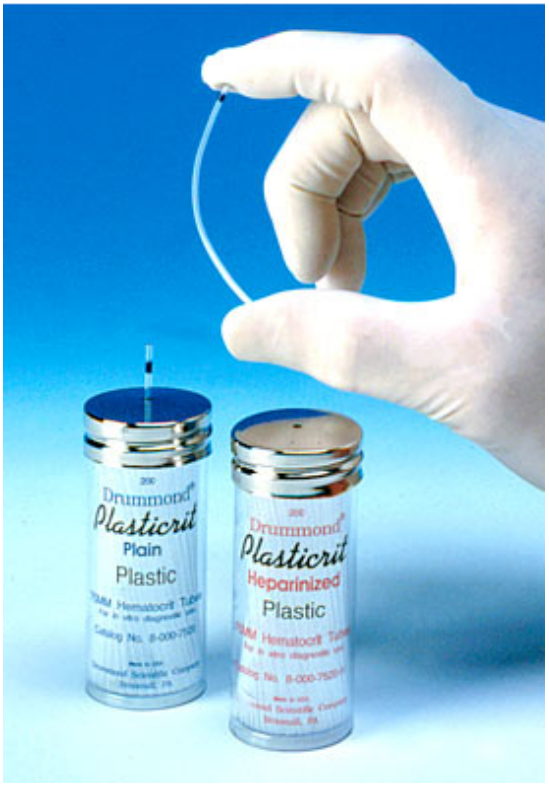
The tips from Subject B who bled more easily from the puncture showed similar variations in blood penetration into the tips. Figure 27 left image (Tip 1) showed uneven colouration with some quite light colouration around parts of the circumference. Figure 27 right image showed that penetration and colouration was greater in the left semicircle that the right. Figure 28 (Tip 2) also showed quite dramatically the blood uptake into the two halves, when viewed longitudinally, had been quite different.

Conclusions :

The work of Samarakkody on the validation of these samplers for glucose monitoring had demonstrated that they tended to read lower than the corresponding conventional venous samples. The follow up work by Shah demonstrated that they tended to give lower HbA1c results than the corresponding conventional venous samples. This lead to the hypothesis that perhaps the Mitra samplers tended to 'under sample' the red blood cells in the finger prick droplet when it came to HbA1c. In addition this outer layer of sequestered red blood cells may have impeded the full extraction of the glucose from the core of the tip and this could explain why the glucose concentrations when measured using the Mitra Samplers also tended to be lower than the formal venous samples. **The sectioning studies have confirmed visible sample inhomogeneity** but there is not enough detail for them to prove the hypothetical consequences on the glucose and HbA1c results. Suffice it to say that there is sufficient evidence to bring into question the claim that these are reproducible 10uL blood droplet samplers..

The next area for improvement is the accurate and reproducible volumetric collection of blood. To this end we would suggest that the plastic microhaematocrit tubes manufactured by Drummond Scientific (cat. No. 8-000-7520-H, marketed in Australia by Thermo Scientific) Fig 27 be investigated as possible finger prick droplet samplers. Being plastic they would be suitable for placing into the 'home self monitoring' environment compared to the conventional glass capillaries usually used for microhaematocrit collection and testing. The plan would be for the user to mark where the blood flowed up to before dabbing the tube onto an absorbant tissue or similar where it would dry. That way we would know the exact volume of blood that had been collected.

FIGURE 29 : Plastic Heparinised Microhaematocrit Tubes as Candidate Volumetric Samplers of Blood from Finger Prick Droplets.



These tubes have an approximate internal diameter of 1mm which would mean that 10uL of blood would fill a 12.7 mm length of a tube. It could be possible to get a workshop to cut exactly 12.7 mm lengths of these tubes and mount them in a suitable holder. This is not a new idea – the Siemens DCA Haemoglobin analyzer uses a micro capillary to draw up exactly 1 uL of blood from a patient's specimen tube, see Figure 30. These samplers are intended for use by laboratory technicians and not the patients. However it would not be difficult to make a patient safe version based upon the same concept. The advantage would be that the patient would be advised to completely fill the 12.7 mm length of capillary tube before they dabbed it onto the filter or tissue paper supplied as a 'patient kit'.

FIGURE 30 : The 1uL Whole Blood Sampler used in the Siemens DCA HbA1c Analyzer.

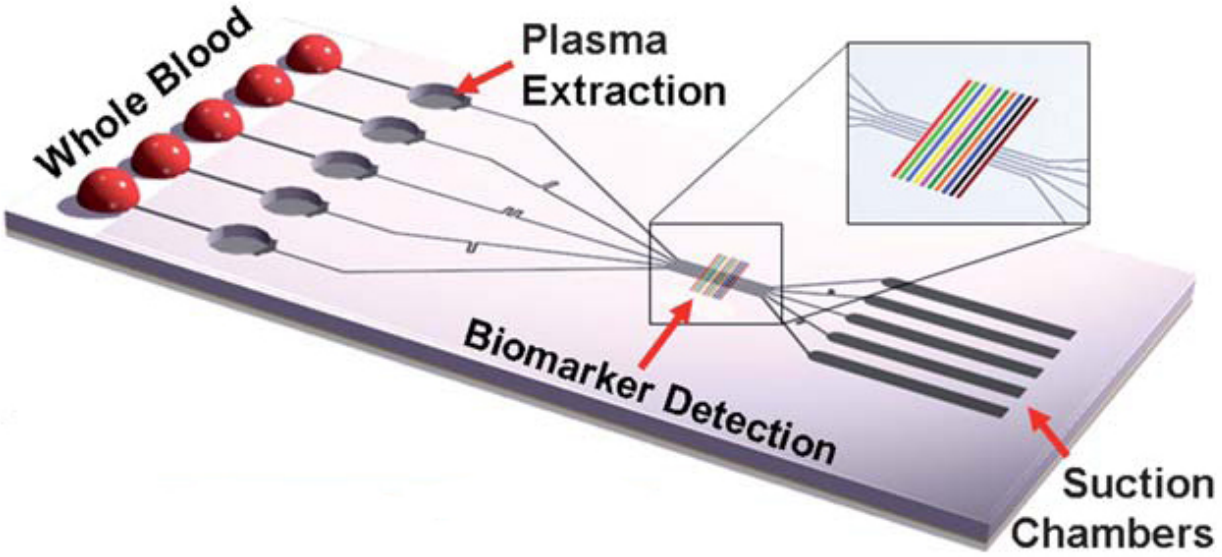
As part of this follow up work some trade samples of a whole blood droplet sampling system manufactured by DBS Systems SA in Switzerland was given short evaluation. This device is shown in Figure 31 after it had been used as directed by the manufacturers. The principal of operation is that there is a S-shaped capillary channel which when it is touched onto a finger prick blood droplet draws up exactly 10uL of blood. Then when the device is closed the blood is drawn out of the sample capillary because the distal end touches a pad of filter paper. The device has room for four samples alongside each other. In our evaluation we found that the ergonomics of positioning the device onto the blood droplet was poor. We also observed that if the device came out of contact with the blood droplet during sampling then there was a high risk of the thread of blood going into the capillary would break. Remaking contact with the the blood droplet did not guarantee that the sampler would continue to work or that the break in the sample thread would be bridged by the incoming blood. In other words there was a high risk of under sampling. Similarly if there was not a distinct convex meniscus of blood at the distal end then there was a high risk that the filter paper would not draw the blood out of the capillary. Overall we concluded that these samplers would not be a success in the routine clinical environment or home monitoring environment.

FIGURE 31 : The Hemaxis Finger Prick Blood Droplet 10uL Sampler Manufactured by DBS Systems.

CLOSING COMMENT

The attraction for successful home based sample collection and even home based analytical devices that are even more sophisticated than the glucometers and coagulometers continues. The invention of progressively more complex microfluidic devices may eventually yield devices that are truly equivalent to formal venous blood sampling but it is the view of this author that, until the dual challenges of obtaining accurate and reproducible volumetric sampling and really good blood flows from a lancet puncture, are solved then these devices are not going to replace the formal venous sample.

FIGURE 30 : https://www.microfluidicfuture.com/blog/simbas-everything-the-blood-touches-is-our-kingdom



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Declaration of conflicting interests

None of the researchers had any financial or other relationships with the providers of any items of instrumentation, reagent kits or blood sampling devices used in these studies.

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Ethical approval

Approval for this study was obtained from the Tasmania Health and Medical Human Research Ethics Committee. (Ethics Ref: H0014943.).
On arrival at the study centre, participants were given a questionnaire, an information sheet and an informed consent form. During the glucose study the participants were given their blood glucometer readings.

Guarantor

Not applicable.

Contributorship

JS and SS were enrolled in an MSc by course work and dissertation at the University of Tasmania. They performed all the participant recruitment and bench work associated with the HbA1c and glucose studies respectively. Their dissertations were used as the principal data sources for this paper.

TH, KA and JB were the academic supervisors.

TH provided supervision of the laboratory work and advised with statistical data handling. TH performed the follow up studies.

KA supervised of the ethical applications, participant recruitment, sample collections from participants and preparation of the dissertations.

AT performed the fixing, embedding and sectioning services during the follow up studies.

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Available online : http://www.medlabstats.com/dried-blood-samplers/dbs-utas.pdf