To determine the validity of the Sebia HbA1c assay for identifying variant haemoglobin disorders.

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Abstract

The purpose of this research is to determine if the Sebia HbA1c programme can be used to accurately identify patients with a variant haemoglobin chain. While this method is not currently validated as a diagnostic test for haemoglobin variants, it is a validated diagnostic test for diagnosing diabetes through the measurement of HbA1c. HbA1c testing is already part of the prenatal screening service to assess the risk of gestational diabetes in the Waikato and Bay of Plenty regions in New Zealand. However, as it is not a diagnostic test for variant haemoglobin detection, when an abnormal peak is detected, it is up to the clinician to order further tests to accurately identify the abnormal haemoglobin chain, resulting in increased costs and turnaround times for results. The only current diagnostic test is the Sebia Haemoglobin E method.

Data used in this study were from routine requests for haemoglobinopathy identification using the Sebia Haemoglobin E programme. These samples were run in parallel with the Sebia HbA1c programme to determine the difference between migration patterns and quantitation of haemoglobin peaks between both methods.

The results of this study demonstrate a good correlation between the two methods with a correlation coefficient of at least 0.9, however the HbA1c method shows a negative bias when compared to the reference method: the Haemoglobin E method. Despite this, laboratories could use the HbA1c technique as a presumptive method for identifying abnormal haemoglobin chains, provided adjustments to the reference intervals were made.

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Attestation of authorship

"I hereby declare that this submission is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person (except where explicitly defined in the acknowledgements), nor material which to a substantial extent has been submitted for the award of any other degree or diploma of a university or other institution of higher learning."

Kate Louise Mclaughlin

28/08/19

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Ethics approval

Full ethics approval was granted by the AUTEC committee on the 3rd April 2019. Application number is 19/42 and ethics approval has been granted until 3rd April 2022. Full ethics approval was also granted by the Waikato District Health Board and the Waikato District Health Board Research committee.

1. Background/Literature Review

Haemoglobinopathies are a term used to describe disorders of haemoglobin. These conditions were originally limited to the tropics and subtropics, however due to migration and diversification of populations, they are now the most common autosomal disorder globally. Recent data suggests that approximately 7% of the world's population are carriers of inherited haemoglobin disorders (Modell and Darlison, 2008). Furthermore, approximately 300,000-400,000 babies are born each year with severe forms of haemoglobinopathies, requiring medical intervention to improve the quality of life (Weatherall, 2010). If left untreated these conditions may result in death within the first few years of life, thus the effect of these conditions on global healthcare has a serious impact on identification and treatment of such conditions (Angastiniotis, 2013).

While most countries around the world offer prenatal screening for haemoglobinopathies, in New Zealand there is no official prenatal screening for Haemoglobinopathies and their associated disorders. Prenatal screening is designed to provide an accurate result as early in the gestation as possible. This is reliant on the prior identification of clinical conditions which can be attributed to the biological parents of an unborn child (Vrettou, Kakouru, Mamas and Trager-Synodios, 2018).

Haemoglobin is a quaternary structure comprising four subunits, with each subunit having one polypeptide chain and one heme group. The polypeptide chain differs in the maturity of the haemoglobin structure. Adult haemoglobin is made up of two alpha and two beta chains, while fetal haemoglobin is comprised of two alpha and two gamma chains. Fetal haemoglobin has a higher affinity for oxygen, which is critical in maintaining fetal health in utero (Marengo-Rowe, 2006).

Each heme group comprises a ferrous iron which binds reversibly to oxygen, which is deposited in oxygen lacking tissues around the body via the bloodstream. The binding of oxygen is dependent on the partial pressure of oxygen, therefore as oxygenation occurs, the coupling of another oxygen molecule to the heme structure is made easier (Giardina, Messana, Scatena and Castagnola, 1995). With a partial pressure of 100 mm Hg, the

haemoglobin molecule is completely saturated with oxygen, however with environmental changes the partial pressure of oxygen changes, altering the saturation state (Pitman, 2011).

There are several factors which determine the suitability of the oxygen transport around the human body. The oxygenation availability of the lungs, the distribution and pressure of blood flow, the haemoglobin concentration (the carrying capacity of the blood) and the oxygen affinity in tissues and peripheral capillaries. The success of the above factors is reliant on normal haemoglobin structures and normal environmental challenges in order to deliver oxygen effectively around the body (Marengo- Rowe, 2006). However, with Thalassemias and variant haemoglobin disorders, there are additional challenges the body encounters to successfully deliver oxygen throughout the body.

Disorders of haemoglobin can be divided into two categories; those in which there is a defect in the production of one of the globin subunits, resulting in a noticeable reduction or total absence, and those in which there is a structural defect in the one of the globin chains (Forget and Bunn, 2003 and Huisman, Carver and Baysal, 1997). A haemoglobin production defect is termed a thalassemia and a structural defect is termed a variant haemoglobin. Such defects occur from mutations, including deletion, duplication, inversions, insertions and other variations of genetic material. Some mutations may be harmless, however if the reading frame of the genetic material is altered, the end amino acid sequence may be modified (Bain, Wild, Stephens and Phelan, 2011).

If the modified gene causes a conformational change in the haemoglobin structure, the ability of the molecule to bind oxygen is reduced (Huisman, Carver and Efremov, 1996). All haemoglobinopathies are inherited recessively, with one copy inherited paternally and one inherited maternally (Weatherall et al, 2006).

While many haemoglobin disorders may go unnoticed, particularly if the defect is localised to one globin chain only (heterozygous variants), patients who have two copies of the defect (homozygous variants) are at greater risk health disorders which may require medical intervention. Furthermore, if a patient is found to have a haemoglobin disorder and partner screening is not performed, then any children arising from this partnership have a 25% chance of having a child with a homozygous phenotype (Trent, 2006).

Figure 1 below demonstrates the inheritance pattern of an affected gene, if both parents are carriers.

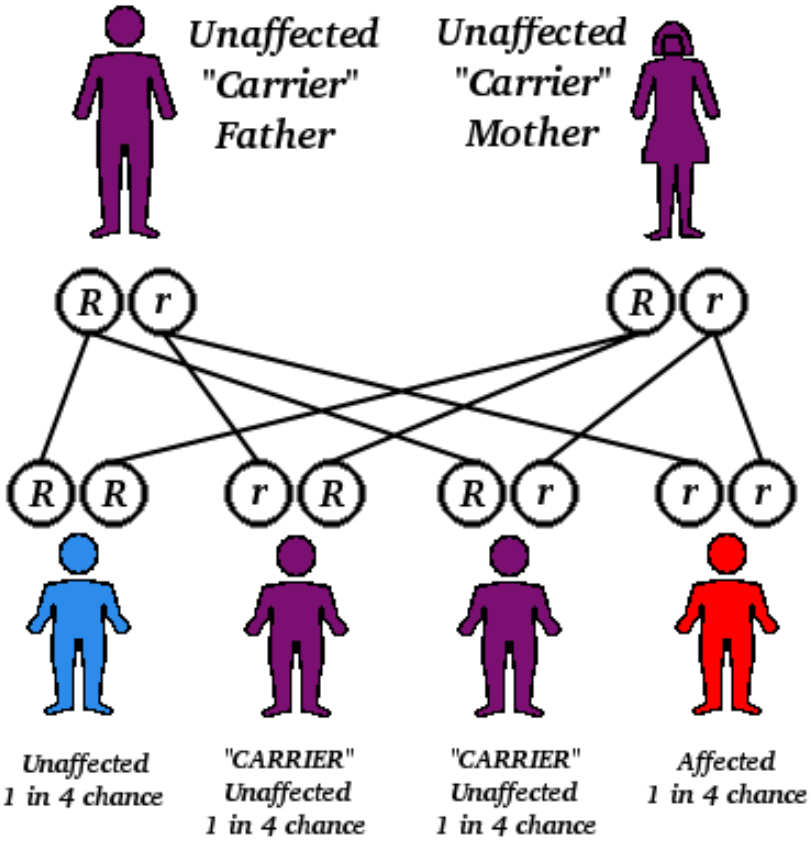

Figure 1 sourced from: https://www.researchgate.net/figure/inheritance-pattern-of-Thalassemia-as-an-autosomal-recessive-fashion_fig1_321289738

Haemoglobinopathies can have a significant impact on patient health and mental state and can result in a significant burden on global healthcare due to the treatment requirements. A study performed in 2018 by Hisam et al, described the emotional response from patients diagnosed with a haemoglobinopathy and the caregivers responsible for patient care. Their study found that half of the patients and their caregivers had apparent levels of stress above the threshold in which they deemed significant. Furthermore, it was found that some patient's families were placed under a significant financial burden due to the healthcare costs associated with haemoglobinopathies. Although this study was geographically limited to one area, similar findings could be assumed on a global scale.

The true monetary cost of haemoglobinopathies is difficult to quantify accurately. However several studies have been published, suggesting the financial impact of a patient with sickle

cell disease (HbS) has an estimated cost of $460, 151 USD over the patient's lifetime (Kauf, Coates, Huazhi, Mody-Patel and Hartzema, 2009), Similar studies suggest the financial implication of beta thalassemia to be approximately $148,899 USD over a thirty year period (Riewpaiboon, Nuchprayoon, Torcharus, Idaratna, Thacorncharoesap and Ubol, 2010). With advancing healthcare and technology to detect and diagnose clinical diseases such as haemoglobinopathies, more patients are exceeding the original life expectancy and are seeking additional healthcare to maintain their quality of life (Weatherall, 2010).

Treatment options include regular blood transfusions, iron chelation therapy and in some cases a bone marrow transplant may be required. These treatment options may be limited in terms of geographical location, financial stability and the ability of organizations to correctly match bone marrow donors with available resources (Rund, 2016).

1.1 Thalassemias

Thalassemias are a group of haemoglobin disorders that result from a variant gene in the globin chain which makes up the haemoglobin molecule (Public Health England, 2018). Thalassemias are categorised as alpha, beta, delta, gamma or a combination of beta/delta/gamma depending on which globin chain is affected (Weatherall and Clegg, 2001). The nomenclature directly correlates with the globin chain affected.

Alpha and beta globin genes are made up of seven clusters, located on two separate chromosomes. Alpha genes are located near the centre of chromosome 16 and beta globin genes located on the short arm of chromosome 11. Delta and gamma genes are classified as beta type genes and are also located on chromosome 11 (Das and Sharma, 2016). Children inherit two copies of the alpha gene from each biological parent and one each of beta, delta and gamma genes (Weatherall D, Akinyanju O, Fucharoen S, Olivieri N, and Musgrove P, 2006).

A reduced production rate of one globin chain can have significant pathological effects on the body. The results can be attributed to ineffective erythropoiesis (red blood cell production), through damage to red cell precursors or haemolytic anaemia due to mature red cell damage (Bain, 2001).

If haemoglobin production is normal, the effects of the thalassemia may go unnoticed until the patient is exposed to conditions where there is a greater need for tissue

oxygenation. However, any of the two outcomes can put the patient at a greater risk of anemia, requiring blood transfusions and increased healthcare needs to maintain adequate health.

1.2 Clinical Significance of Thalassemias

1.2.1 Beta thalassemias

Beta thalassemias can be classified as thalassemia major, thalassemia intermedia and thalassemia minor, depending on the phenotypic inheritance from each parent. Allele inheritance can be normal (β), reduced functionality (β^+) or completely non-functional (β^0) (Genetics Home Reference, 2019). Patients may present with anaemia, a decrease in red blood cell production, with varying severity based on environmental challenges.

- Thalassemia Major-β^0/ β^0

Results from the inheritance of two affected genes (one from each parent), termed homozygous. Patients present with severe anaemia and hepatosplenomegaly due to increased red cell turnover and ineffective red blood cell production, requiring regular blood transfusions (Ali and Wong, 1984). Iron chelation therapy is required to maintain serum ferritin levels to within normal limits to prevent secondary complications such as liver dysfunction, thyroid issues and diabetes. Bone disfiguration, particularly the maxilla and zygoma result from ineffective and excessive red blood cell production, becomes apparent if the patient is left untreated (Bain, 2001). Symptoms usually present in the first two years of life and require regular medical intervention through the utility of blood transfusions to maintain a normal quality of life (Bain et al, 2011).

- Thalassemia Intermedia- β^+/ β^0

A milder form of anaemia, typically presenting later in life. Patients inherit one affected gene and one normal gene and differ from thalassemia major patients in which blood transfusions are not dependent for survival (Bain, 2001). However, blood transfusions are required in events which they are exposed to low oxygen environments or nutrient poor diets (Ali and Wong, 1984). Clinical symptoms are typically similar to thalassemia major; however they are often less severe (Bain, 2001).

- Thalassemia minor- β^+/β

Affected patients are usually asymptomatic and may present with normal or slightly decreased haemoglobin levels. Anaemia presents in a mild form but can deteriorate in cases of nutritional deficiencies such as vitamin B12, folic acid and iron (Choudry, 2017).

1.2.2 Alpha Thalassemias

Alpha thalassemias can be divided into four categories, depending on the number of functioning alpha genes.

- Silent carrier alpha thalassemias (-α/αα)

Affected patients have three functioning alpha genes, which has little effect on haemoglobin production resulting in normal haemoglobin levels or mild anaemia.

- α Thalssemia trait- (--/αα) / (-α/-α)

Affected patients have one set of functioning alpha genes inherited from one parent or one absent and one functioning gene from each parent. Consequences result in mild forms of anaemia. Both silent carrier and alpha thalassemia trait patients are often asymptomatic and require little or no treatment (Galanello, 2013).

- Haemoglobin H disease/compound alpha thalassemia (--/-α)

Patients present with mild to moderate forms of haemolytic anaemia. Secondary complications include hepatosplenomegaly with some dependence on blood transfusions and folic acid supplementation (Krakas et al, 2015).

- Haemoglobin Bart's disease/homozygous alpha thalassemia (--/--)

Homozygous alpha thalassemia results from the inheritance of no functioning alpha genes. This condition is characterised by hydrops fetalis, where there is excess fluid build-up in the body prior to birth. Haemoglobin production is severely compromised which results in severe anaemia, enlarged liver and spleen and growth abnormalities. As a result, many babies are stillborn or die shortly after birth. Survival is dependent on early identification and inter-uterine blood transfusions (Singer, 2009 and Kohne, 2011).

1.2.3 Delta thalassemias- $\delta^{+}/\delta^{-}/\delta^{0}$

Delta thalassemias result from the partial deletion of the delta genes on chromosome 11. This condition has no clinical significance and diagnosis of homozygous forms are relatively simple, as there is complete loss of the A_2 peak. Heterozygous forms can be more difficult to diagnose as there are numerous contributors to a reduced A_2 peak percentage (Bain, Wild, Stephens and Phelan, 2010). While delta thalassemias have no clinical significance alone, they can co-exist in conjunction with beta thalassemia, complicating the identification and ultimate diagnosis (Mansoori, Ashad, Rashid and Karim, 2016).

1.3 Variant haemoglobins

Mutations which alter the end amino acid sequence result in several variations of normal adult haemoglobin. Currently over 700 variant haemoglobin structures have been identified, the most well documented and clinically significant variants being F, D, S, E and C haemoglobin (Weatherall and Clegg, 2001). A large proportion of variant haemoglobins arise from an amino acid substitution in one of the four polypeptide chains (Hempe, Granger and Craver, 1997). These substitutions commonly arise from mutations in the DNA, which alters the end translation of mRNA to form proteins. The clinical significance of the condition depends on the site and type of amino acid change (Schneider, 1978).

While many variant haemoglobins can be termed “clinically silent” in which they have no effect on laboratory parameters, such as the complete blood count, it is still important to correctly identify the globin chain affected as these conditions can progress to a clinically significant event. The ability to go unnoticed or remain silent is attributed to the fact that oxygen is still supplied to tissues, however the conformational change may result in a decreased affinity for oxygen, which in times of stress or higher demand for oxygen, may result in anaemia (Bain, 2001). There is also the possibility for variant haemoglobins to interact with other variant chains or beta-thalassemias, which is why the detection and identification of variants is so important (Bain, 2001).

Many variant haemoglobins have arisen as a result of selection pressures and as a protective mechanism against pathogens. Sickle cell disease is one example of this, where the red cell assumes a crescent shape, instead of the normal biconcave disk. This change results in a

shortened red cell life span (20-30 days) and the potential of the sickle cells to obstruct blood vessels, resulting in tissue hypoxia (Bunn, 1997 and Serjeant, 1992). Altering the conformation of the haemoglobin prevents parasites such as malaria from entering the red cell, however, it also prevents oxygenation and can cause a range of problems which can limit the quality of life and life expectancy of the patient (Public Health England, 2018).

1.4 Clinical significance of variant haemoglobins

Variant haemoglobin disorders can be subdivided into heterozygous, homozygous or compound heterozygous variants.

1.4.1 Heterozygous variants

Result from the inheritance of one affected gene and one normal gene (one from each parent). The presence of one normal gene allows a proportion of haemoglobin to function normally, often resulting in minimal or no clinical symptoms.

1.4.2 Homozygous variants

Result from the inheritance of two affected genes (one from each parent). Affected patients have no normal adult haemoglobin A, instead have two copies of either haemoglobin S, haemoglobin D, haemoglobin E or haemoglobin C. Clinical symptoms include anaemia with microcytosis and hypochromic cells, which if severe, require blood transfusions to maintain a normal quality of life (Warghade, Britto, Haryan, Dalvi, Bendre, Chheda, Matkar, Salunkhe, Chanekar, and Shah, 2018).

1.4.3 Compound heterozygous variants

Compound heterozygotes have inherited two mutant alleles. This can result in the patient having two variant haemoglobin chains, such as haemoglobin S and C or a variant haemoglobin combined with a thalassemia. Concurrent alpha thalassemias can be difficult to diagnose based on phenotypic findings, as they can result in subtle differences in both the haematology parameters and the variant haemoglobin percentage. (Bain, Wild, Phelan and Stephens, 2011).

1.5 Prevalence of haemoglobinopathies

The World Health Organisation (WHO) estimates that haemoglobinopathies are prevalent in 71% of countries, potentially affecting 89% of births. More specifically, it is suggested that 5.2% of individuals and over 7% of pregnant women carry a haemoglobin variant that is of major clinical significance (Modell and Darlison, 2008). The exact number of patients affected by haemoglobinopathies is difficult to determine. This is due to the lack of accurate health registers, which can be complicated in countries with lower socio-economic status. In the European WHO region only 6 out of the 34 member countries have a national register for thalassemia and sickle cell disease (Angastiniotis M, 2013).

Inherited haemoglobin disorders were originally limited to the tropic and sub-tropic regions of the globe However, due to migration and ethnically diverse populations, the effects of these disorders can be seen worldwide (Livingston, 1967 and Weatherall and Clegg, 2001). Table one below provides a historical estimate of the prevalence of haemoglobinopathies by regions classified by the World Health Organisation. This provides an estimate of the proportion of births per year, which may be affected by a haemoglobinopathy, sorted by geographic location.

Table 1.

The estimated prevalence's of carriers of haemoglobin gene variants and affected conceptions.

	Demography2003				% of the populationcarrying			Affected conceptions(per 1000)			Affected births (% of under-5 mortality)
WHO region	Population (millions)	CrudeBirthrate	Annual births (1000s)	Under-5 mortality rate	Significant variant[a]	α^+ thalassaemia[b]	Any variant[c]	Sickle-cell disorders[d]	Thalassaemias[e]	Total	
African	586	39.0	22 895	168	18.2	41.2	44.4	10.68	0.07	10.74	6.4
American	853	19.5	16 609	27	3.0	4.8	7.5	0.49	0.06	0.54	2.0
Eastern Mediterranean	573	29.3	16 798	108	4.4	19.0	21.7	0.84	0.70	1.54	1.4
European	879	11.9	10 459	25	1.1	2.3	3.3	0.07	0.13	0.20	0.8
South-east Asian	1 564	24.4	38 139	83	6.6	44.6	45.5	0.68	0.66	1.34	1.6
Western Pacific	1 761	13.6	23 914	38	3.2	10.3	13.2	0.00	0.76	0.76	2.0
World	6 217	20.7	128 814	81	5.2	20.7	24.0	2.28	0.46	2.73	3.4

[a] Significant variants include Hb S, Hb C, Hb E, Hb D etc. β thalassaemia, α^0 thalassaemia.[b] α^+ thalassaemia includes heterozygous and homozygous α^+ thalassaemia.[c] Allows for (1) coincidence of α and β variants, and (2) harmless combinations of β variants.[d] Sickle-cell disorders include SS, SC, S/β thalassaemia.[e] Thalassaemias include homozygous β thalassaemia, haemoglobin E/β thalassaemia, homozygous α^0 thalassaemia, α^0/ α^+ thalassaemia (haemoglobin H disease).

Table 1 sourced from: https://www.who.int/bulletin/volumes/86/6/06-036673-table-T1.html

While there is no specific data available for the prevalence of haemoglobinopathies in New Zealand, net migration levels for 2018 were estimated at 51,200 (+/- 800) (Stats NZ, 2019). Based on the statistics suggested above, with 5.2% of individuals affected by haemoglobinopathies worldwide, it could be assumed that up to 2,662 affected individuals are migrating to New Zealand annually.

While screening for the presence of haemoglobinopathies cannot necessarily prevent the progression of these conditions, it provides vital information that is necessary to improve the quality of life of affected individuals and reduce the global financial and sociological burden these conditions pose.

1.6 Haemoglobinopathy testing in New Zealand

While no specific prenatal screening programme exists in New Zealand, current testing protocols by most New Zealand laboratories involve two stages; a primary and secondary investigation.

- Primary investigations:
  - Perform complete blood count (CBC) and serum ferritin
  - Perform a haemaglobinopathy screen
- Secondary investigations:
  - Perform partner or family studies
  - Perform DNA analysis for final confirmation.

The above investigations are normally directed by a haematologist and secondary investigations are only performed to isolate the exact gene change or to provide more clarity if genetic counselling is offered. DNA analysis can also be suggested when a definitive diagnosis through electrophoresis is unable to be achieved. The haemoglobinopathy screen is a testing panel comprising several qualitative tests that may be useful when interpreting the result. This always requires an electrophoretic screen of proteins. (Neal, 2018)

1.7 HbA1c testing in New Zealand

HbA1c is currently used as a screening and diagnostic test for Diabetes Mellitus in New Zealand, as recommended by the New Zealand Society for the Study of Diabetes. This has replaced older diagnostic tests such as fasting plasma glucose or a glucose tolerance test. HbA1c testing has many advantages over the older diagnostic tests, which are more desirable for patients to comply with testing and for practitioners to interpret results (Bonora and Tuomilehto, 2011). HbA1c provides a more accurate representation of the amount of glucose in the blood, in comparison to fasting plasma glucose, as it is based on the coupling of glucose to the red cell which has an average life span of three months (Nathan, Turgeon and Regan, 2007). There is reduced variability between samples, as HbA1c is not affected by exercise and fasting status of the patient at the time of collection (Bonora and Tuomilehto, 2011). The sample is stable, which reduces the risk of pre-analytical errors and variation.

Several laboratory methods have been validated for the diagnostic use of HbA1c in New Zealand. Techniques include; capillary electrophoresis, high pressure liquid chromatography, spectrophotometric and immunoassay (Van Delft, Lenters, Bakker-Verweij, de Korte, Baylan, Harteveld and Giordano, 2009). Each method has strengths and weaknesses over the other, but spectrophotometric and immunoassay techniques are disadvantaged in that they cannot discriminate or identify variant haemoglobins.

1.8 Aims and Objectives

The purpose of this research is to validate the Sebia HbA1c programme to identify variant haemoglobin chains found through routine diabetic screening. In order to achieve this, the principal objectives are as follows; (1) to determine if the peak positions between the Sebia HbA1c and haemoglobin E programmes are comparable, (2) to determine if there is enough evidence to support the use of the Sebia HbA1c programme as an identification test for haemoglobin variants, (3) to determine the gene frequency of haemoglobin variants throughout the Waikato/Bay of Plenty regions.

To achieve this, two methodologies will be compared- the Sebia haemoglobin E method and the Sebia HbA1c method. The Haemoglobin E method is the only diagnostic test currently in use at Pathlab Waikato for the identification and confirmation of variant haemoglobin chains. Both methods utilize protein separation by charge, allowing individual blood proteins to be isolated and visualized. The haemoglobin E method allows normal blood proteins to be visualized with haemoglobin A_2 migrating first and haemoglobin A migrating last. The HbA1c method produces a similar migration pattern, with haemoglobin A_2 migrating first, followed by haemoglobin A and then the HbA1c fraction. The most common haemoglobin abnormalities which are mentioned above, F, D, S and E, migrate between haemoglobin A and haemoglobin A_2 on both techniques.

The research questions are as follows:

1. What is the agreement of the peak position of variant haemoglobin chains identified through HbA1c analysis compared to the peak positions which have been validated through the Sebia haemoglobin E programme? How does the peak percentage compare between the two techniques?

2. Is there enough confidence in the data to support the use of the HbA1c programme to identify haemoglobin variants without further phenotypic investigations? Could we use HbA1c as a first line screening test to exclude haemoglobin variants?
3. What is the gene frequency of haemoglobin variants though the Waikato/Bay of Plenty regions?

2. Methodology

Samples were analysed using the Sebia Capillarys 2 and Capillarys 3 Tera instrumentation. Both systems are a fully automated system which utilizes the principle of protein separation by charge. With this instrumentation, charged proteins are separated by their electrophoretic mobility through an alkaline buffer, through an electrical charged field. This buffer has a specific pH of 9.4, which influences the electrolyte pH and electroosmotic flow of the solution. Migration of separated proteins is influenced by the size and charge of the protein and will result in a distinct pattern when an electrical voltage is applied across the capillary (Fritsch and Krause, 2003). Protein separation occurs through capillary tubes comprised of silica, which have an internal diameter of 25 um.

The following illustration demonstrates the workflow utlised to obtain the results used in this study.

Collection of blood samples for routine thalassemia screening and testing using the Sebia Haemoglobin E method.

Retesting the same samples using the Sebia HbA1c method.

Data analysis- including obtaining the electrophoretogram of each sample to determine the presence of any abnormal haemoglobin through zones Z8 through to ZA_2 (see figure two below). Each zone Z(F), Z(D), Z(S) and Z(E) shows where abnormal haemoglobin proteins run.

Data analysis continued- each abnormal haemoglobin identified will be compared through both methods, with the abnormal haemoglobin percentage and location being graphed to allow the correlation coefficient to be determined. Finally, a Bland-Altman plot will be generated for each of the five abnormal haemoglobin chains (F, D, S, E and A_2) to

determine the average difference between the abnormal haemoglobin identified by both methods. This will determine if the differences in abnormal haemoglobin concentration are statistically significant.

2.1.1 Sebia Haemoglobin E programme

Whole blood samples are aspirated and mixed with a hemolysate solution, allowing the contents of the red cell to become free in solution, before being mixed with the alkaline buffer. The sample is then presented to the capillary tube, where aspiration and subsequent migration is controlled by peltier effect. A voltage is applied across the capillary tube, resulting in protein separation from anode to cathode. A detector is located towards the cathode and measures proteins at 415nm as they pass through the capillary tube. Separation of hemoglobin fractions and their migration pattern is recorded by the Phoresis software on the x axis from position 0 through to 300. The migration position is validated against the standardized position of HbA and HbA2. The remaining zones in the x axis are divided into 15 regions and the identification of any potential variant hemoglobin's is dependent on which region they migrate in. This separation into 15 zones allows for the differentiation of the most common variants HbS, HbD, HbC and HbE with a quantifiable peak % of abnormal hemoglobin in comparison to normal HbA haemoglobin.

Haemoglobin electrophoresis is a commonly used and well recognized technique in most medical laboratories for the qualitative detection of haemoglobinopathies.

2.1.2 Limitations of the Haemoglobin E programme

The haemoglobin E programme is a versatile technique to provide insight into proteins found in the red blood cell. Due to the genetic changes discussed above and the subsequent change in the amino acid sequence, the protein's net electrical charge is modified. When mixed with the alkaline buffer, this allows for distinctions between the main groups of variant haemoglobins that are of interest (HbF, HbD, HbS, HbE). Currently more than 1400 different types of variants have been described, which makes it impossible to individually determine using this or any other qualitative technique. Despite this, many lesser known variants exhibit similar electrophoretic mobility to the groups of interest, allowing variants to be grouped in zones (Bain, 2001).

2.1.3 Sebia HbA1c Programme

Haemoglobin glycation is a non-enzymatic reaction between the glucose molecule located within the erythrocyte (red blood cell) and the N-termino amino group of the haemoglobin s chains. This reaction is continually taking place during the life of the red blood cell. The rate of glycation is dependent on the level of blood glycaemia and is used as an indicator of diabetic control.

The principle of this test is similar to the haemoglobin E programme, in which whole blood samples are mixed with a lysate solution, freeing the contents, before being mixed with an alkaline buffer. The aspiration and migration apply the same principles as above, however the separation pattern is different. When an alkaine buffer is used, separation of blood proteins occurs in the following order; A2/C, E, S, D, F, A0, other Hb and then A1c.

2.1.4 Limitations of HbA1c testing

As the measurement of HbA1c is based on the coupling of glucose to the red cell over a three-month period, there are several factors which may interfere with this result. Any condition which alter the life cycle of the red span; for example, significant blood loss, anaemia and medications can falsely decrease the HbA1c level. The consequence of any haemoglobinopathy can lead to anaemia of varying severities, which can falsely elevate HbA1c results (Sinha, Mishra, Singh and Gupta 2012).

As variant haemoglobin disorders have a variation in the globin chain synthesis, the production of normal haemoglobin (HbA) is impaired or inhibited. Patients who are homozygous for a variant haemoglobin do not have any normal Haemoglobin A. Glucose will still couple to the variant haemoglobin, however the testing platform may not necessarily be able to discriminate between variant and normal haemoglobin, producing a false result. This is a significant disadvantage to immunoassay or spectrophotometric methods, as they have no ability to detect any haemoglobinopathies, they can still produce an analytically correct HbA1c result. However, depending on the expression of the affected haemoglobin chain, for example a patient who is homozygous for haemoglobin E, the result produced will be HbE1c as opposed to HbA1c.

2.2 Variant Haemoglobin peak detection

The Phoresis software captures data observed by the detector located at the cathode end of the capillary tubes. After reading at 415nm, percentages of relative haemoglobin is determined along an x axis. Figure 2 depicts a normal haemoglobin distribution, with a haemoglobin A percentage between 96.7 and 97.8 and an A2 percentage between 2.2 and 3.2, as validated by Sebia. The peak on the left hand side of figure 2 represents haemoglobin A and the peak on the right is haemoglobin A_2, both of which are normal proteins.

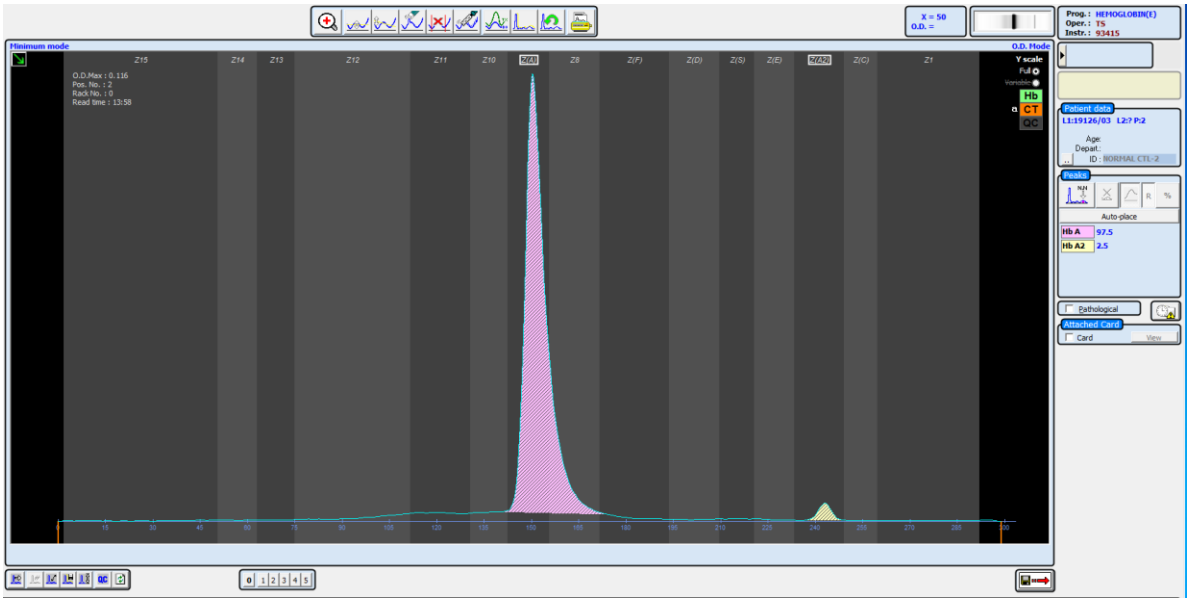

Figure 2: An example of a normal haemoglobin electrophoretogram.

The zoning along the X-axis allows the user to view the prospective variant haemoglobin peaks which run in each specific zone. These are built into the Phoresis software, which when the user hovers over each zone, a pop up window appears with the names of each variant haemoglobin peak known to run within the zone location (Sebia, 2013). An example of this can be viewed in figure three below. Figure three also indicates the location of an abnormal haemoglobin protein, migrating in conjunction with the normal proteins; haemoglobin A and A_2 as seen in figure two above.

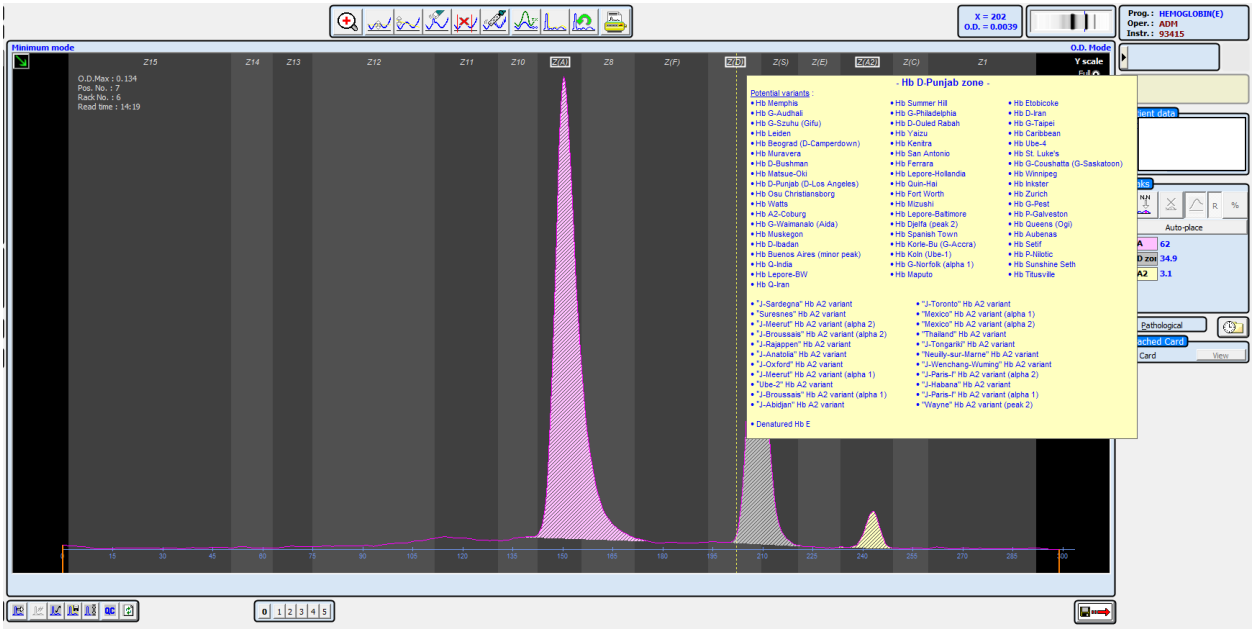

Figure 3: Elution of Haemoglobin D and identification of peaks within the haemoglobin D zone (Sebia, 2019)

2.3 Sample size and requirements

All data analysed in this study has been retrospective. Samples were analysed and reported as per normal laboratory protocol. There were no conditions associated with data selection and all samples which were tested for a thalassemia or haemoglobinopathy at Pathlab Waikato over the past three years has been included. Approximately 660 samples have been included in this study over the specified time frame.

2.3.1 Methods

Retrospective data analysis has been performed at Pathlab Waikato for the past three years on samples sent for routine thalassemia testing. When abnormal peaks, indicating a haemoglobinopathy might be present, standard laboratory protocol is to confirm the finding using an alternative method. The HbA1c programme, while not validated for haemoglobinopathy testing, is it able to detect the presence of suspected haemoglobinopathies. Therefore, this method can ultimately confirm the findings identified using the haemoglobin E programme.

2.3.2 Sample collection and storage

Patient blood was collected into vacutainer tubes containing K_2EDTA as per standard laboratory protocol. Samples were refrigerated at 4ºC until Haemoglobin E and HbA1c testing was performed. Samples were analyzed weekly, with both Haemoglobin E and HbA1c analysis occurring on the same day. Samples are stored at 4ºC for seven days and then discarded as per normal laboratory protocol.

2.4 Quality Assurance

HbA1c

Royal College of Pathologists of Australia Quality Assurance programme (RCPA) samples are analysed monthly to determine correct HbA1c quantitation by the instruments at Pathlab Waikato. Two levels of internal quality control material are run daily to ensure each capillary is measuring correctly.

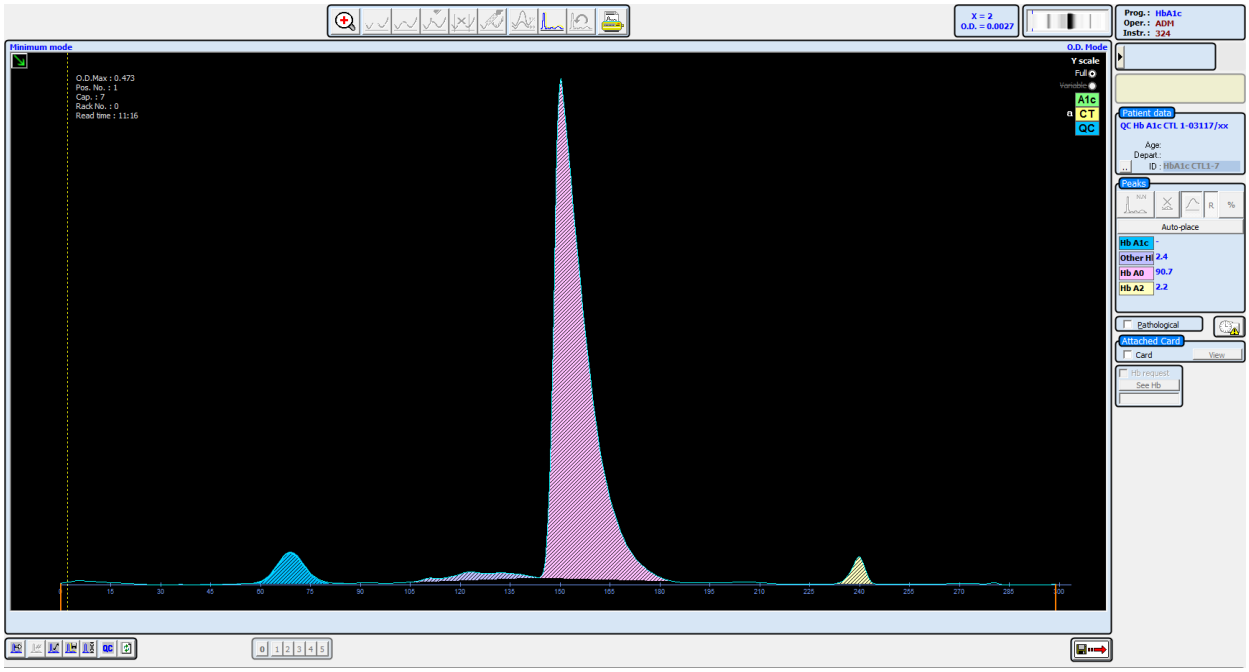

Figure 4: Internal quality control level 1 (Sebia, 2019)

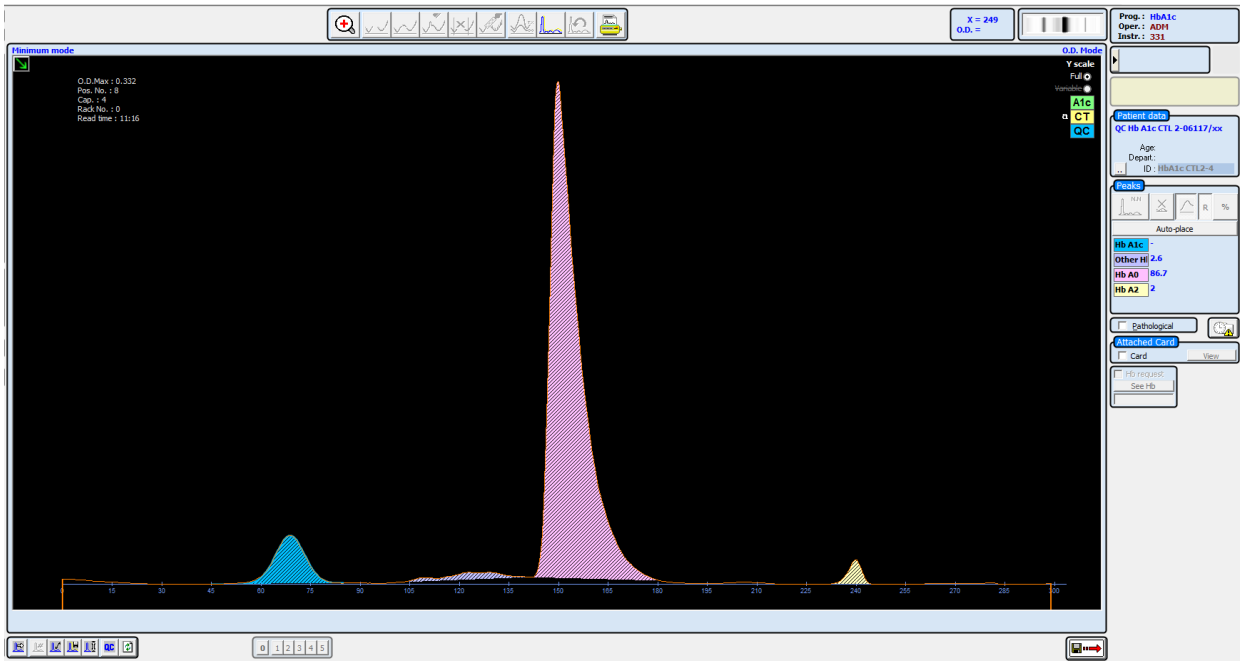

Figure 5: Internal quality control level 2 (Sebia, 2019)

Haemoglobin E

NEQAS quality assurance samples are analysed every three months. Before each batch of patient samples were run, a normal A_2 control was analysed to ensure the correct migration centering of the HbA peak was correct for each of the eight capillaries.

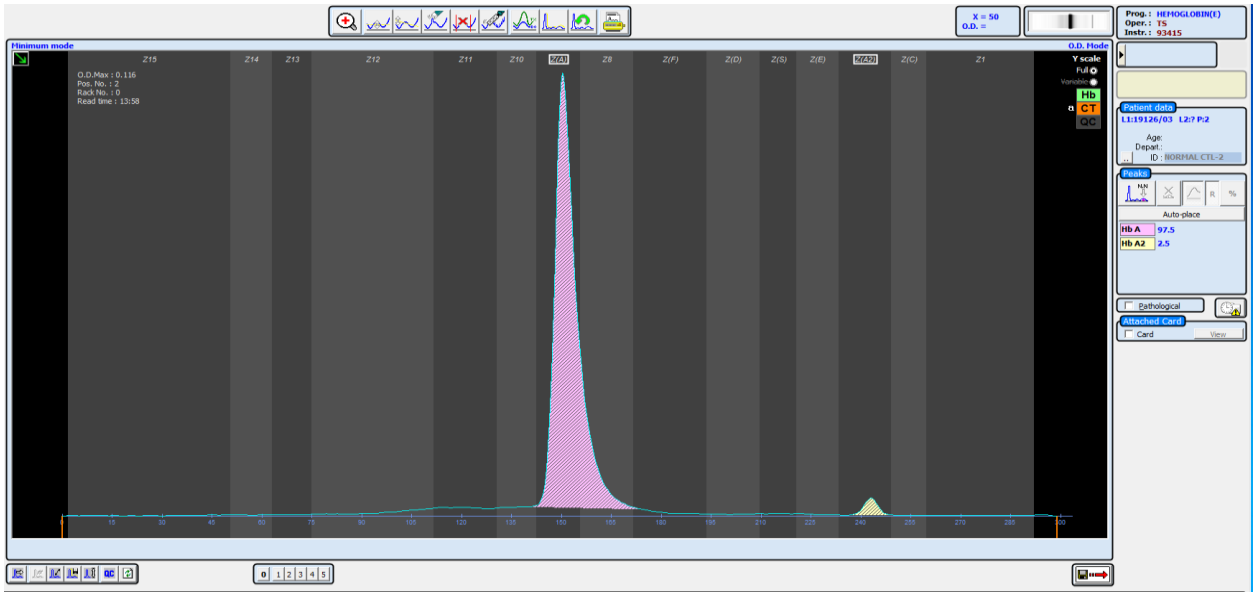

Figure 6: Internal normal A_2 haemoglobin control (Sebia, 2019)

2.5 Ethics

This study was given full ethics approval by the AUT Ethics committee on 3rd April 2019.

Since patient samples were used in this study, the key ethical points to consider were ensuring patient confidentiality and that the samples were being used in an appropriate manner. All the testing was performed at Pathlab Waikato, which is part of the Waikato District Health Board. Within this district health board there are policies and guidelines in place to comply with the obligations under the relevant legislations including the Human Tissue Act 2008, Treaty of Waitangi Act 1975 and the Health and Disability Commission 1996.

The samples in this study have already been screened for both diabetes and the presence of variant haemoglobin chains and appropriate follow up has already been suggested/undertaken.

Submission to the Health and Disability Commission is not required as this is not an intervention study and is principally for the purposes of an educational degree.

All data had been de-identified prior to study commencement.

2.6. Thalassemia and HbA1c testing:

Patient samples were presented to the capillarys 2 and capillarys 3 tera instruments for testing. Both instruments were set up as per the manufacturer's recommendations, with appropriate control material run and validated prior to patient sampling. As these instruments are a partially automated system, once the sample rack was presented for analysis, sample dilution, migration and protein detection were performed by the instrument.

Electrophoretograms were viewed by the operator and peak positions identified using the Phoresis software version 8.6.3 (Sebia, 2019).

2.7 Results analysis and statistical evaluation:

As the data used in this study has been performed retrospectively, data was acquired through the information technology (IT) department at Pathlab Waikato. A request was made to correlate all patients who had a thalassemia screen in the past three years, with the

following parameters included; complete blood count, mean cell haemoglobin, mean cell volume and ferritin levels. Data was de-identified by the IT department prior to receiving the data for review.

Using the Phoresis software, the peak positions and percentage for suspected F, D, S, E and A2 haemoglobinopathies were identified using the axis scale and peak qualifications within the software. The peak positions and percentages were compared for both the HbA1c and haemoglobin E programme.

3. Results analysis

3.1 Clinical Samples

A total of 660 samples were used for this study. Table two shows the breakdown of samples into the respective haemoglobin groups, which were identified from data obtained using the Sebia haemoglobin E method (the reference method).

Table 2. *Sample number sorted by haemoglobin group involvement*.

Samples identified using the Sebia haemoglobin E method (n=660)	**Haemoglobin variant group**
530	A_2
55	F
36	S
30	E
9	D

Table 3. *Mean peak x axis location of each abnormal haemoglobin detected with both haemoglobin E and HbA1c techniques and statistical output.*

Haemoglobin variant group	Mean peak location (x axis)		Standard deviation		Coefficient of variation	
	Hb technique	A1c technique	Hb technique	A1c technique	Hb technique	A1c technique
F	186	178	5.8	7.1	3.1	4.0
D	207	200	1.2	0.3	0.6	0.3
S	214	212	1.0	1.2	0.49	0.51
E	228	226	0.62	0.18	0.28	0.08
A_2	243	240	0	0	0	0

There was no difference between the haemoglobin A_2 peak location for both methods, as this location is required by both methods to correctly centre each electrophoretogram. The peak locations appear to run consistently throughout each method, with minor variation in

the locations of variant haemoglobin. The standard deviation (SD) and coefficient of variation (CV) demonstrate the variation of the peak location from the mean value. Both the SD and CV for all haemoglobin variant groups demonstrate little variation from the mean value, as indicated by the low values. However, haemoglobin F exhibited greater variation, which can be attributed to the migration pattern in relation to haemoglobin A. This is discussed further in section 3.2.2 below.

3.2 Agreement of HbA1c and Haemoglobin E testing- Correlation of haemoglobin peak percentages between the two techniques.

3.2.1 Haemoglobin A_2

Figure 7 shows the correlation between the peak percentage of haemoglobin A_2 measured by the haemoglobin E programme, when compared to the HbA1c technique. All data points migrated exactly as per the manufacturer's instructions. The correlation coefficient is 0.97, suggesting a strong positive correlation between the haemoglobin A_2 values obtained from each method.

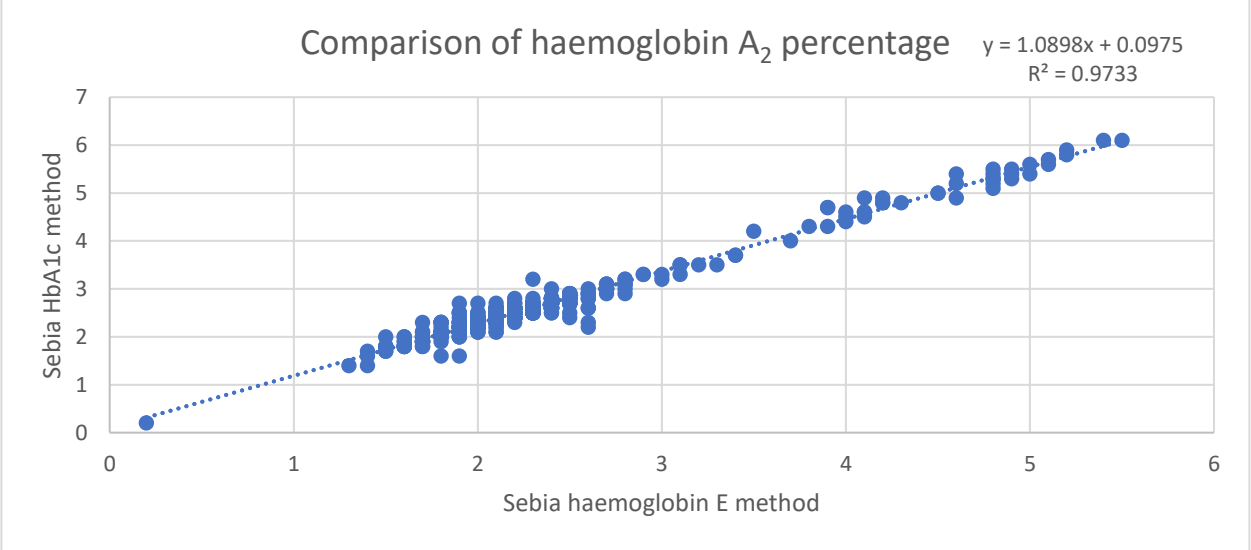

Figure 7: Percentage difference between haemoglobin A_2 peaks between both methodologies.

Figure 8 below shows a Bland Altman plot which describes the differences between the two methods used in this study. The results show a consistent negative bias between the two sets of data of approximately 0.48%. The results indicate that the haemoglobin A_2 percentages obtained by the HbA1c method are on average 0.48% lower than the standard method.

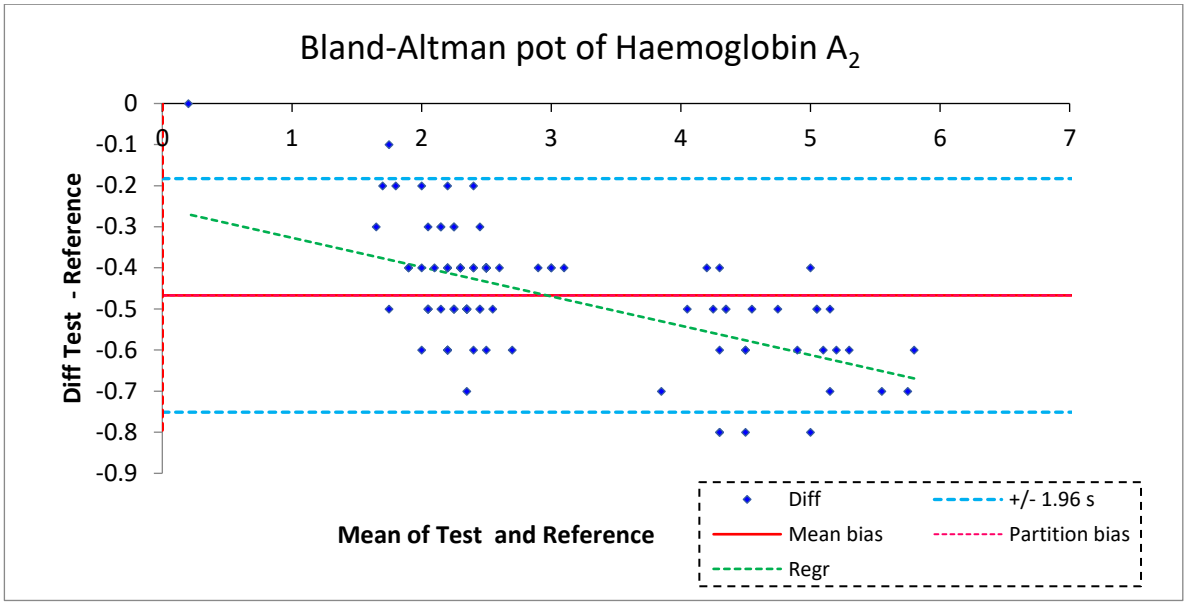

Figure 8: Bland-Altman plot representing the difference of haemoglobin A_2 between the two methods.

3.2.2 Haemoglobin F

Figure 9 shows the correlation of A_2 peaks between instruments with the presence of a Haemoglobin F peak.

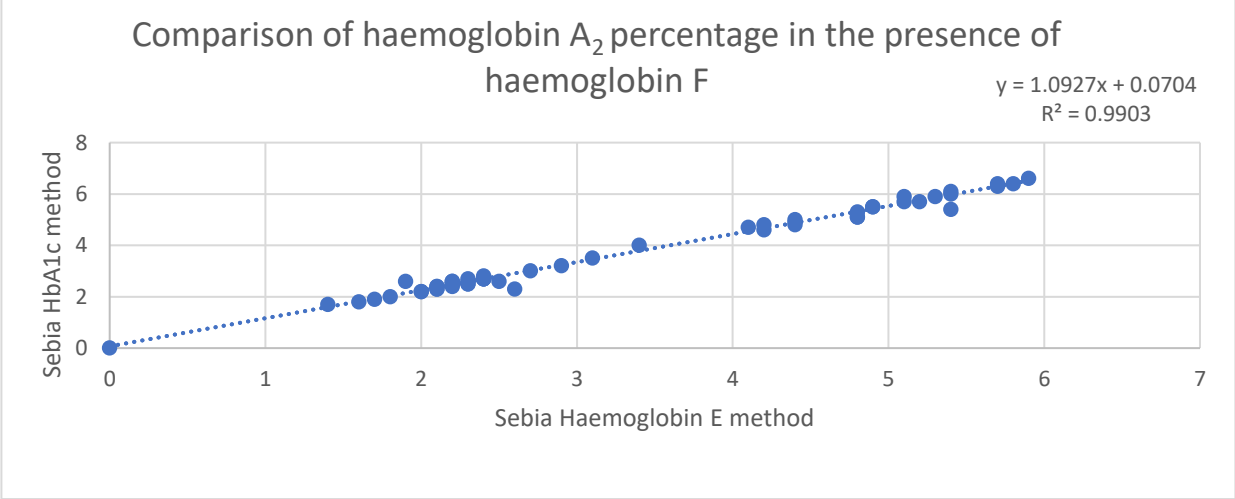

Figure 9: Difference of haemoglobin A_2 peak percentages.

Figure 10 shows the correlation of the peak percentages of Haemoglobin F between the two techniques. While the correlation is still acceptable, being at least 0.9, as the haemoglobin F

peak percentage increases, the percentage difference between the two methods increases. Haemoglobin F migrates closely to the A0 peak on the HbA1c technique. As this technique was only designed for HbA1c determination, the resolution between the A0 and haemoglobin F peak has not been extended.

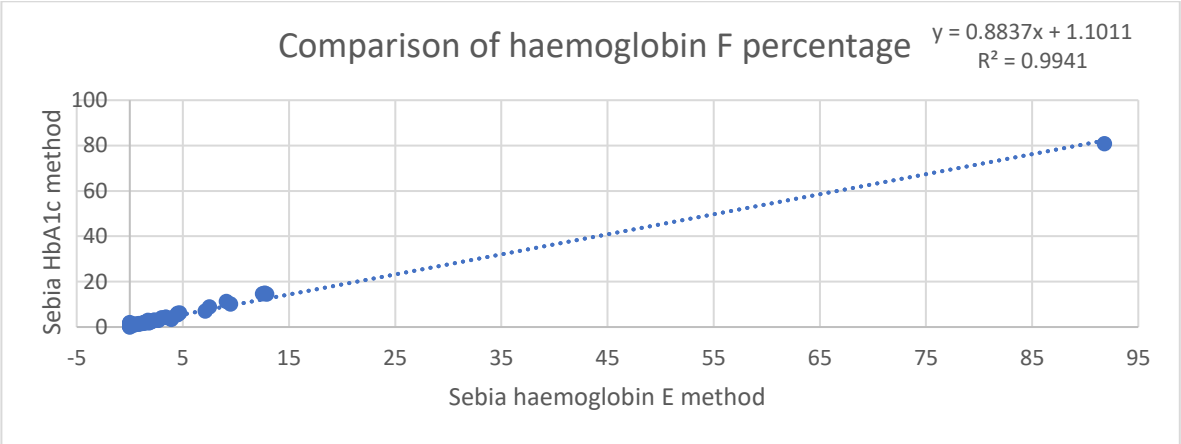

Figure 10: Comparison of HbF peak percentage between the two instruments.

The Bland-Altman plot below demonstrates the average difference between the peak positions of both methods. The results show a negative bias with an average difference of approximately 0.9% against the HbA1c programme. The haemoglobin A_2 peak was also reviewed and when Haemoglobin F is present in the sample, the average difference between the haemoglobin A_2 peaks were 0.4%. The P value of all results was less than 0.001, suggesting there is a significant difference between the two methods throughout all decision points.

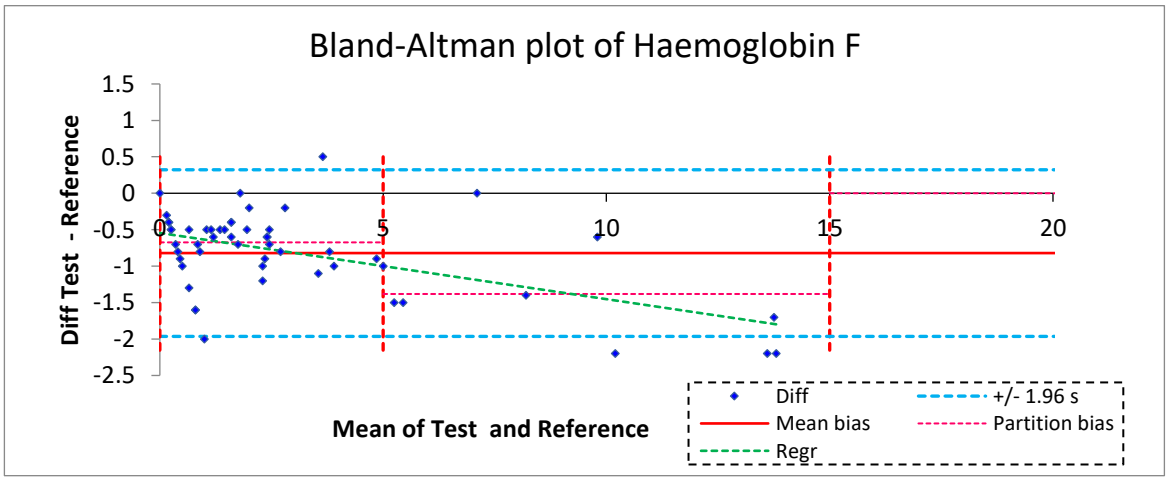

Figure 11: Bland-Altman plot representing the difference between haemoglobin F between the two techniques.

3.2.3 Haemoglobin D

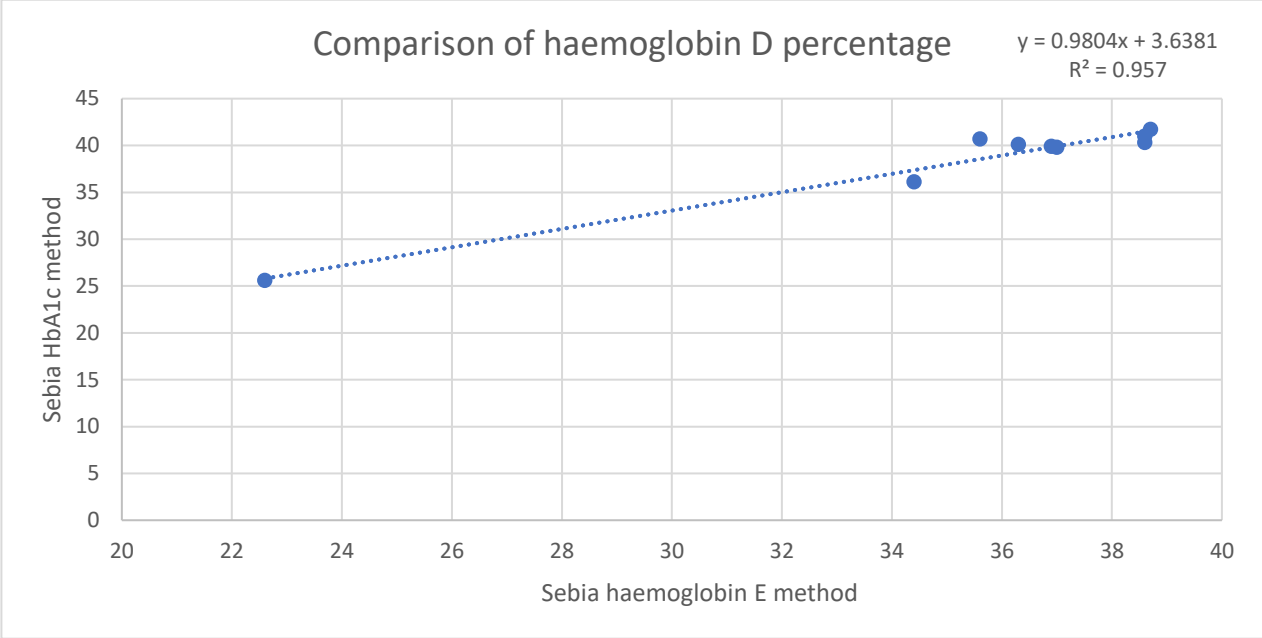

Figure 12: Comparison of the Haemoglobin D peak percentage

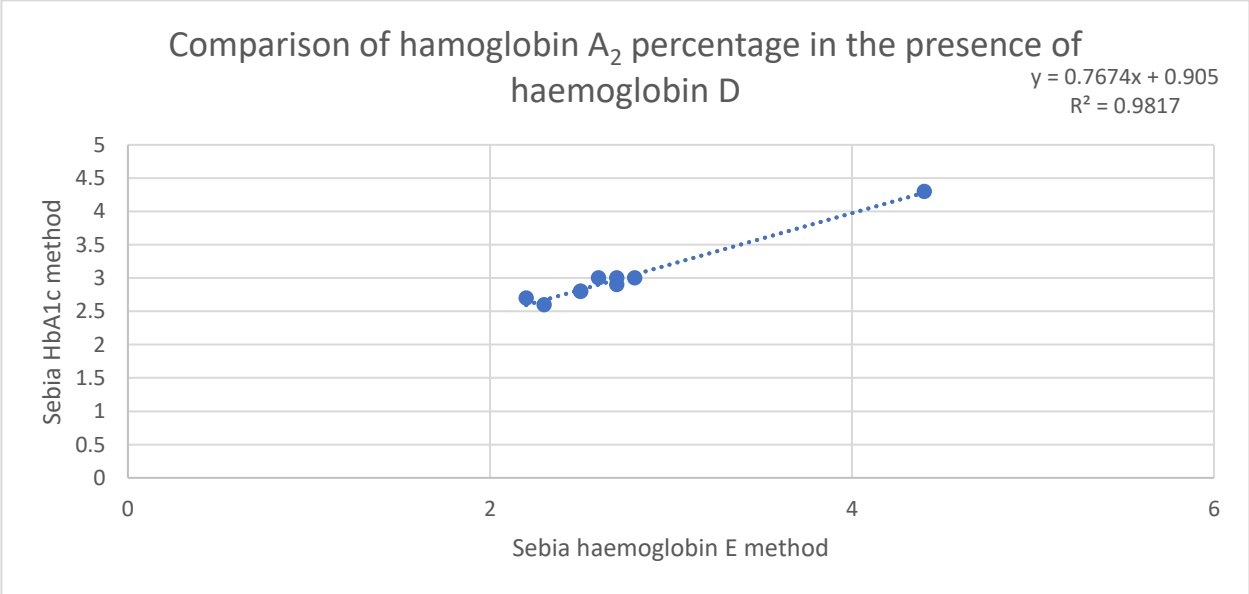

Figure 13: Difference between the haemoglobin A_2 peak percentage with the presence of haemoglobin D

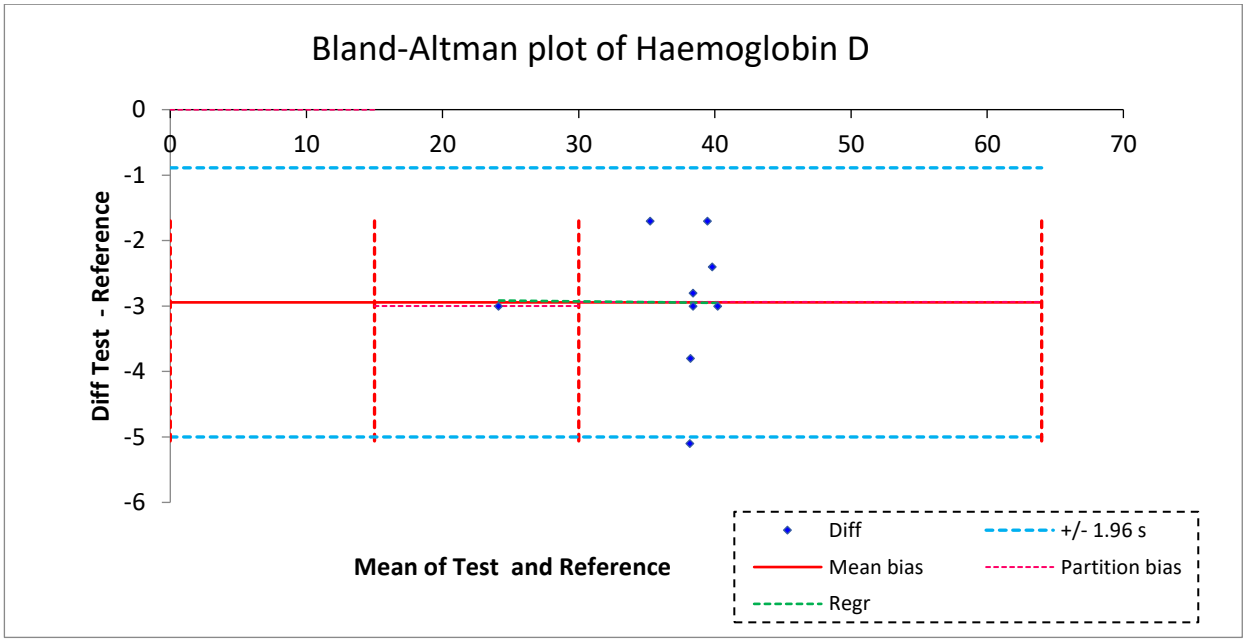

Figure 14: Bland-Altman plot of haemoglobin D showing the difference in haemoglobin peak percentage between both methodologies

Haemoglobin D, despite being limited in sample numbers, showed excellent correlation between the two techniques with a correlation coefficient of 0.957 and 0.9817 between the haemoglobin D and haemoglobin A_2 peaks respectively (figure 12 and 13). The Bland-Altman plot displayed a negative bias against the HbA1c technique, with the peaks obtained using HbA1c to be approximately 2.9% different. Due to the limited sample numbers, there were too few observations to determine the statistical significance of the difference between the two methods.

3.2.4 Haemoglobin S

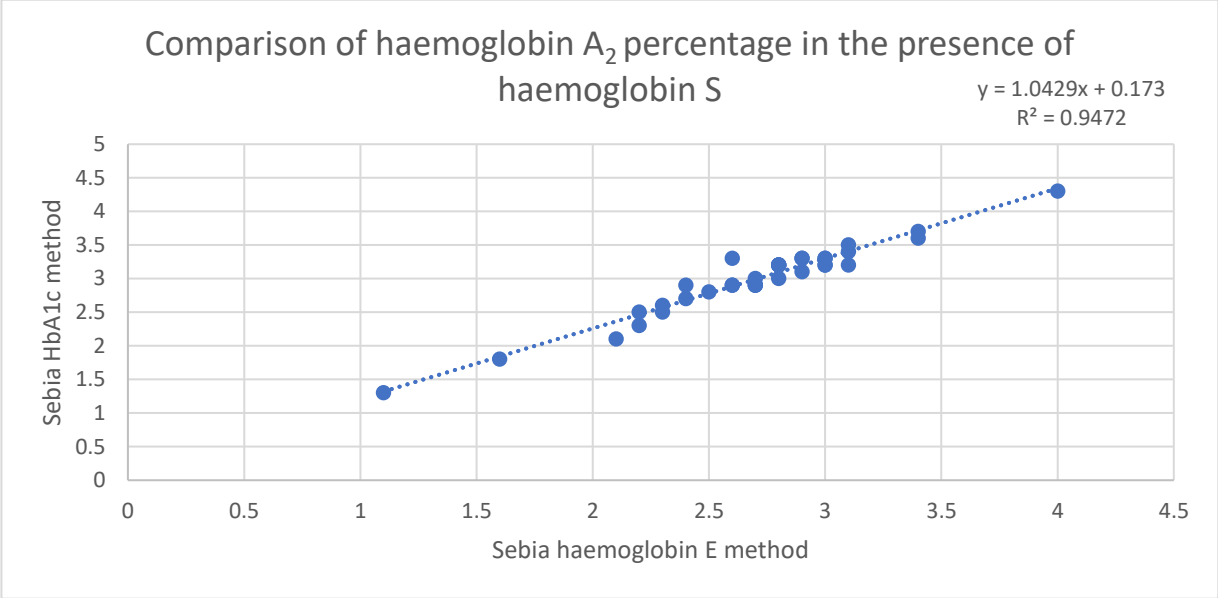

Figure 15. Difference between haemoglobin A_2 peak percentages in the presence of haemoglobin S.

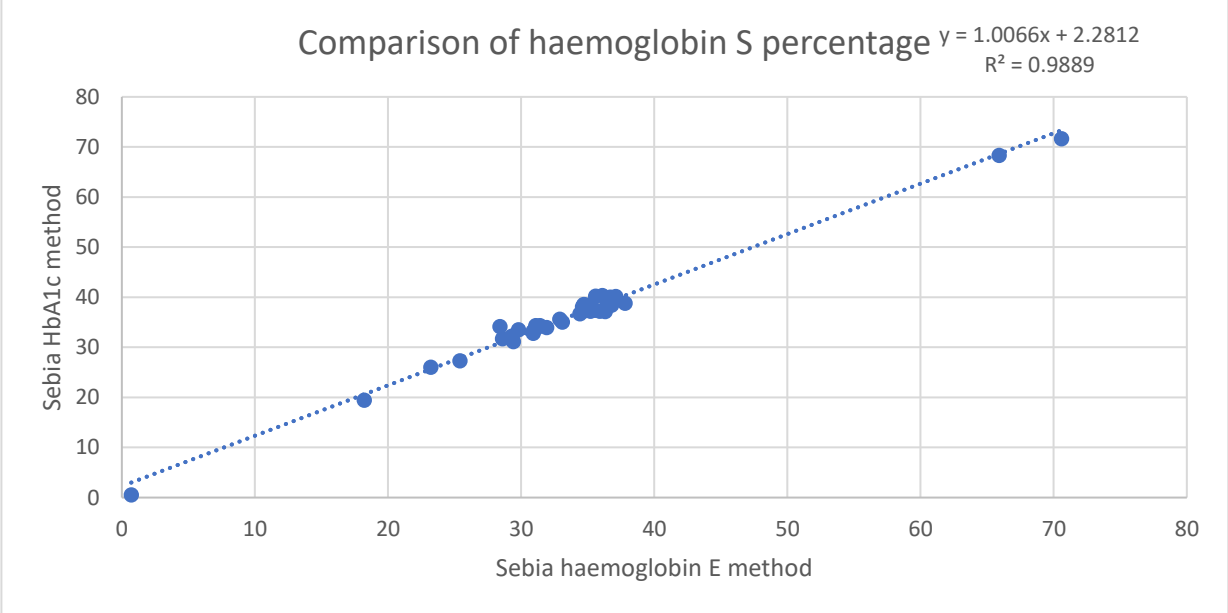

Figure 16. Difference between the peak percentages of haemoglobin S between the two methods.

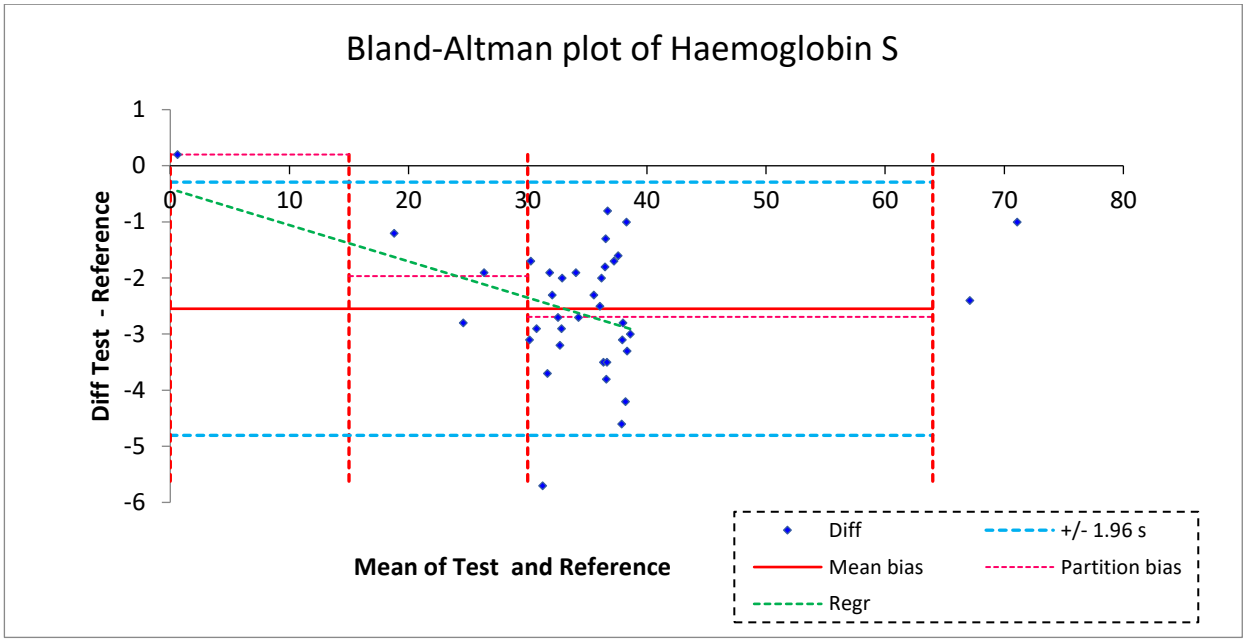

Figure 17: Bland-Altman plot representing the difference between the peak percentages of haemoglobin S.

Haemoglobin S demonstrated excellent correlation with both the peak percentage and haemoglobin A_2 percentage as seen in figures 15 and 16. The results of the Bland-Altman plot also indicate a negative bias against the HbA1c technique, with an average difference of 2.6% between the haemoglobin S peaks and 0.22% difference between the haemoglobin A_2 peak quantitation. The partitioning of results shows a p value of 0.051 for results obtained between 15 to 30%, indicating there is not a significant difference between the two methods. However, for results obtained above 30%, the p value drops below 0.001, suggesting results are more varied between the two methods as the peak percentage increases.

3.2.5 Haemoglobin E

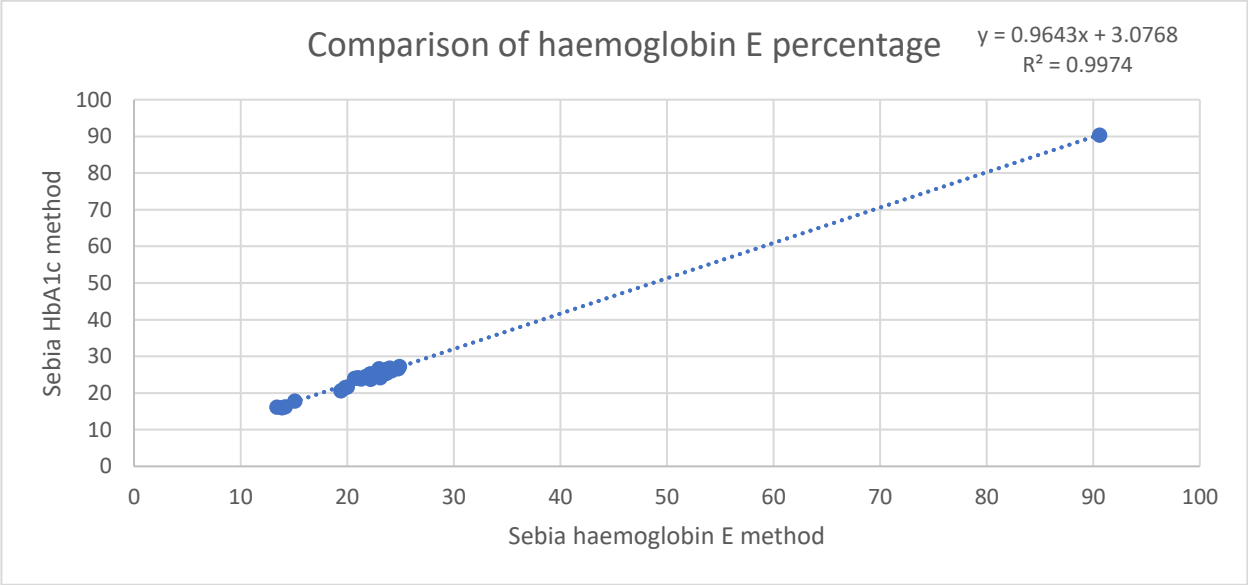

Figure 18. Difference in haemoglobin E peak percentages between the two methods

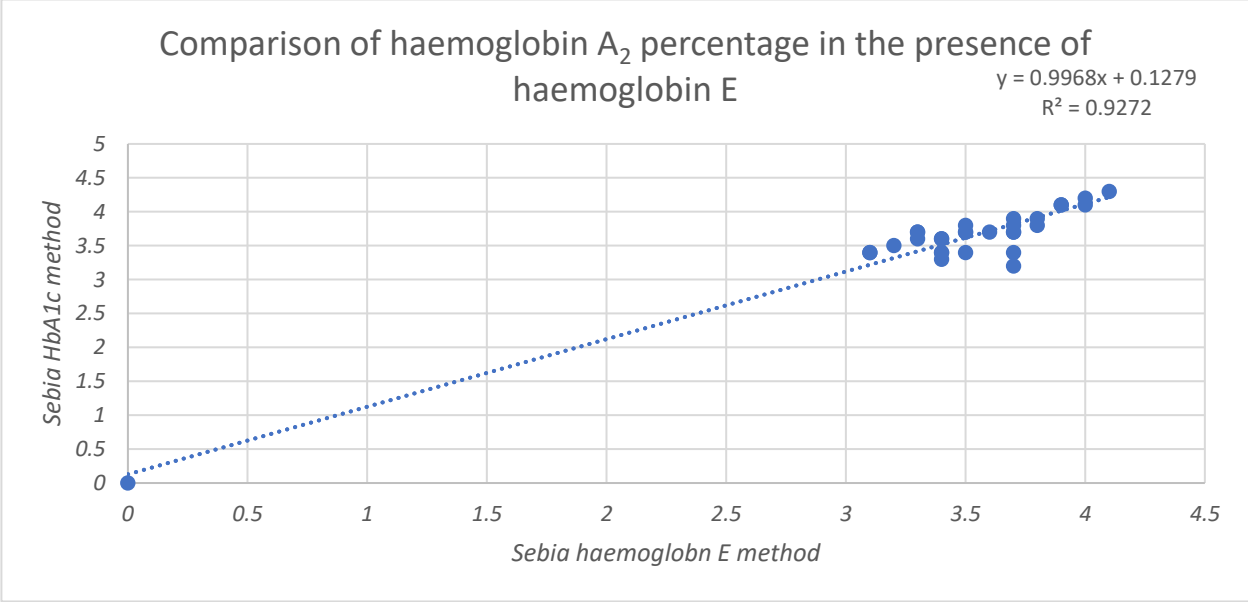

Figure 19. Difference in haemoglobin A_2 peak percentages between the two methods.

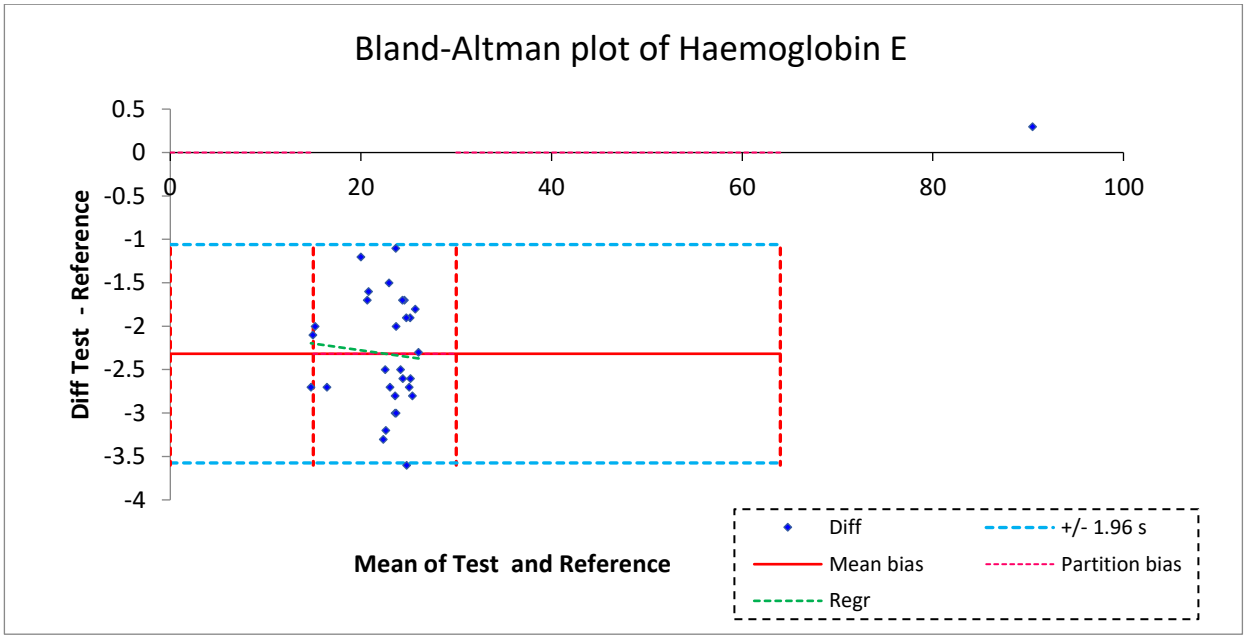

Figure 20: Bland-Altman plot describing the difference between haemoglobin E between the two techniques.

Haemoglobin E has also demonstrated excellent correlation between the two methodologies, with a correlation coefficient of 0.9974 and 0.9272 between the haemoglobin E and haemoglobin A_2 peak percentages (figures 18 and 19). The Bland-Altman plot is consistent with the findings discussed above, in which there is a negative bias against the HbA1c programme with an average difference of 2.4% between the haemoglobin E peaks. The difference between the haemoglobin A_2 peaks was 0.15%. The results observed demonstrate the difference between the two methods is statistically significant (p=0.001).

As there are no current studies evaluating the differences in peak positions between the Sebia HbA1c and haemoglobin E techniques, it is difficult to truly estimate the significance of the above findings. Despite this, the results obtained for all of the haemoglobin peaks of interest are all consistent with a negative bias against the HbA1c technique, suggesting the diagnostic cut off levels would need to be amended to reflect this. The presence of excellent correlation coefficients between both methods suggest good accuracy and precision of the HbA1c technique for identifying variant haemoglobin peaks.

3.3 Migration positions of normal haemoglobin samples

3.3.1 Haemoglobin E programme

Throughout all the result interpretation, the migration positions of normal haemoglobin A and A_2 were consistent throughout the sample population. HbA migrates at 150 and A_2 at 243, reproducibility that has been obtained by the calibration of each capillary by the validation of the normal A_2 control run prior to patient analysis. With each patient sample, HbA and HbA_2 is normalized to 150 and 243 correspondingly, with a peak quantitation visualized through the software. Centering the two peaks provides an internal reference to which each patient sample is compared and normalized against.

Migration positions of abnormal haemoglobin samples

With heterozygous samples, both the HbA and HbA_2 normalised to 150 and 243 as per the analysis of normal samples, with the abnormal peak migrating between the HbA and HbA_2 zones. The table below shows the migration zones of common haemoglobin peaks of interest which have been discussed above.

Table 4.

Migration zones of haemoglobin peaks of interest through the reference method- the Sebia haemoglobin E programme.

Haemoglobin F	171-193
Haemoglobin D	204-209
Haemoglobin S	210-220
Haemoglobin E	221-233

Homozygous samples, which typically have at least 90% variant haemoglobin and little or no haemoglobin A or A_2 are unable to migrate correctly as the peaks do not correlate to the internal reference curve. The Phoresis software uses the most recent reference data to position the peak in the approximate location as stated above. Although these samples migrate in the approximate positions of the suspected peaks, they cannot be validated or guaranteed. Therefore, the manufacturer recommends mixing the sample with a normal patient, which allows for the addition of haemoglobin A and A_2 to correctly center the chromatogram and allow the suspected peak to be visualized correctly (Sebia, 2013).

3.3.2 HbA1c programme

Migration positions of normal haemoglobin samples

The migration positions of the A0 and HbA_2 were standard throughout patient sample analysis. While the haemoglobin E programme standardizes peak positions using a standard sample, the HbA1c programme requires two levels of calibrators. The calibrators validate the HbA1c peak quantitation and an internal reference normalizes the A0 and HbA_2 to 150 and 240. Calibrators combined with the use of two levels of control material ensure each capillary is measuring correctly prior to patient sample analysis.

Migration positions of abnormal haemoglobin samples

Heterozygous patients who have a variant peak percentage of less than 41%, have an A0 and HbA_2 which runs in the standard position described above. The five common peaks of interest; Hb F, Hb D, Hb S and Hb E migrate between the A0 and HbA_2. Table 2 below depicts the migration positions of each peak.

Table 5.

Migration zones of haemoglobin peaks of interest through the comparison method- the HbA1c programme.

Haemoglobin F	181-185
Haemoglobin D	199-201
Haemoglobin S	212-214
Haemoglobin E	225-226

Homozygous samples migrate in a similar style to the haemoglobin E programme as described above. The absence of haemoglobin A and A_2 prevent the sample from standardizing against the internal reference. The software will place the peak according to the most recent positioning of haemoglobin A and A_2.

3.4 Peak percentages between haemoglobin E and HbA1c techniques

Table 6 shows the average difference in peak percentages between the Haemoglobin (E) and HbA1c techniques as obtained through the Bland-Altman plots. The results of the haemoglobin A_2 peak have minor differences, but there is a more noticeable difference between the abnormal haemoglobin peaks. This is not unexpected as despite the technology utilized by each methodology, ultimately they have been designed to identify different proteins in blood.

Table 6. *Average difference in haemoglobin peaks between the reference method- Sebia haemoglobin E and Sebia HbA1c method.*

	Abnormal haemoglobin Peak	Haemoglobin A_2 Peak
Haemoglobin F	0.61	0.39
Haemoglobin D	2.9	0.26
Haemoglobin S	2.5	0.24
Haemoglobin E	2.2	0.12
Haemoglobin A_2	-	0.31

4. Discussion

The purpose of this research is to determine if the Sebia HbA1c technique could be used accurately as a screening test to identify patients who have a variant haemoglobin or thalassemia. As previously discussed, the only technique which has been validated for identifying these abnormal haemoglobin techniques is the Sebia Haemoglobin E program. The identification of these abnormal haemoglobin are reliant on the primary requestor asking for a confirmatory test, often after it has been identified by other laboratory tests such as HbA1c. This results in increased turn around time for result release and increased costs to the laboratory and the overall healthcare system.

While both of the two techniques mentioned in this research will only give a phenotypic result and any uncertain or inconclusive results will require gene testing to confirm, they both provide an accurate representation of the five most common abnormal haemoglobin groups. This information can be used to estimate the burden that an abnormal haemoglobin peak may have on a patient, including healthcare, financial and social burden.

4.1 Detection of abnormal haemoglobin peaks

4.1.1 Haemoglobin E vs HbA1c peak position

Objective one was to determine the agreement between the peak positions generated from two methods; HbA1c and haemoglobin (E). While HbA1c has never been validated as a diagnostic test for detecting haemoglobinopathies, the methodology it uses, capillary electrophoresis, allows for enough separation of proteins to visualize abnormal haemoglobin peaks. As previously discussed, the HbA1c programme does have limitations, particularly when dealing with homozygous haemoglobinopathies. Despite this, the HbA1c programme has good agreement with the haemoglobin E programme, when looking at the five most common haemoglobinopathies. The results for the five most common haemoglobin variants demonstrated excellent correlation between the two techniques. There was a correlation coefficient of at least 0.9 between both the abnormal haemoglobin peak and the A_2 haemoglobin peak as demonstrated in the graphs above. As both techniques only allow for phenotypic identification of peaks, any complete confirmation would need to be done by gene testing. Despite this, the agreement of Haemoglobin E to HbA1c techniques suggests that there is enough confidence to allow for the presumptive

identification of abnormal haemoglobin techniques, providing there is appropriate clinical follow-up with confirmatory testing.

Lot to lot variation between reagent, control and calibrators can pose significant challenges for laboratories to produce consistent results (Thompson and Chesher, 2018). Since the data used in this study has been retrospectively collected, both techniques have been through several lot number changes in both reagent and calibration/control material. Despite this, the agreement between the peaks generated throughout both techniques has been consistent, indicating that the lot to lot variability is not significant in this study.

The results show that the Sebia HbA1c method can detect abnormal haemoglobin proteins, which are consistent with the findings obtained through the reference method; the Sebia haemoglobin E method. However, the abnormal haemoglobin peak location and percentage differ between the two techniques. Despite this, if the laboratory makes appropriate adjustments to the reference intervals and is aware of the differences of abnormal haemoglobin location through the electrophoretograms, then the Sebia HbA1c method could be used as a screening tool for identifying the five most common variant haemoglobin groups.

4.1.2 Haemoglobin E vs HbA1c peak percentage

Objective one further explores the significance of the peak percentages generated between the HbA1c and Haemoglobin E methodologies. The results in table 4 above show the average difference between the haemoglobin peak percentages between the two techniques.

Haemoglobin A_2 peak percentage

The average haemoglobin A_2 peak difference between the methodologies was 0.31%. While this number appears to be insignificant, when it is applied to the normal reference values suggested by Sebia (2.2-3.2%) it can move the result outside the normal reference values. If the operator has not corrected for this change, it can result in the misinterpretation of a suspected result. For example, a haemoglobin A_2 result of 2.3 by the haemoglobin E programme would be at the lower end of the reference interval, but if we apply the average difference, we would expect to see a result of 1.9 by HbA1c which is now outside the normal reference interval.

A Bland and Altman plot was performed using the first 100 data points of the haemoglobin A_2 data. The results of this indicate a negative bias of approximately 0.48% against the HbA1c technique, which can be accounted for by modifying the reference interval used by the laboratory. This is consistent with previous studies performed comparing the Sebia Haemoglobin E method and high pressure liquid chromatography (HPLC). A study completed by Keren et al, compared 297 haemoglobinopathy samples demonstrated a mean haemoglobin A_2 peak of 2.8% ± 0.8% by the Sebia Haemoglobin E technique and a mean haemoglobin A_2 peak of 2.3% ± 0.8% by HPLC (Keren, Hedstrom, Gulbranson, Ou and Bak, 2008).

A recent study performed by Eliosa Urrechaga (2012), investigated the correlation between the haemoglobin A_2 peaks between the Haemoglobin (E) and HbA1c techniques on the Capillarys 2 Flex piercing analyser. Results achieved in this study demonstrated excellent correlation between techniques and a correlation coefficient of 0.9766. The results demonstrated above in my comparison are consistent with the findings of this study, supporting the detection of Haemoglobin A_2 between the two techniques.

Variant Haemoglobin peak percentage

There was a more noticeable difference between the remaining four haemoglobin groups, however the largest difference was 2.9%. The results demonstrate a negative bias, indicating that the results produced by the HbA1c programme are on average 3% lower than the haemoglobin E programme. This difference in peak percentages could result in misdiagnosis if the laboratory has not made allowances for the differences in peak percentage, particularly if a heterozygous variant was co-migrating with an alpha thalassemia. However, as the proportion of abnormal haemoglobin should not be used alone to indicate disease severity, careful correlation with laboratory parameters such as full blood count and iron studies is essential to make an informed diagnosis.

4.2 Significance of the peak positions generated through HbA1c

The presumptive identification of abnormal haemoglobin peaks allows laboratory staff to make recommendations to clinical staff in a timely manner. The presence of a haemoglobinopathy does not necessarily indicate disease status. Other laboratory parameters such as complete blood count and iron studies will determine the extent of

anaemia (if any) and will contribute to the extent of abnormal haemoglobin involvement. HbA1c is a commonly requested test, used for the screening, diagnosis and monitoring of diabetic status (El Agouza, Abu Shahala and Sirdah, 2002). The Ministry of Health recommends screening patients every three to five years, depending on a patient's diabetic risk (BPAC guidelines, 2012). As this is a relatively frequently requested test and is often requested in conjunction with a complete blood count, the capability to detect and identify abnormal haemoglobin peaks allows the laboratory to suggest a likely diagnosis of the finding in a timely manner. Although any confirmation would require gene testing, if the appropriate complementary tests are undertaken at the same time, the clinical significance of the abnormal peak can be extrapolated.

4.3 Incorporation of HbA1c into routine prenatal screening programmes

Objective two was to determine if there is enough confidence in the data obtained to use the Sebia HbA1c method as a first line screening test for identifying haemoglobinopathies. The Ministry of Health recommends the use of HbA1c as a screening test during the first antenatal screen, a practice which has been adopted by all New Zealand laboratories. The initial antenatal screen is commonly performed at 12 weeks gestation and assesses the diabetic and infectious status of the mother (Ministry of Health, 2019). This screen also includes the complete blood count, which as discussed above, can be used in conjunction with any abnormal peak detected to make a probable identification and likely significance of any haemoglobinopathy. The data obtained from this study demonstrates excellent correlation and agreement between the two methods. However, the difference between the peak percentages would need to be corrected by using a modified reference range and key operators made aware of the changes prior to result review. The table below provides an adjusted haemoglobin A_2 reference interval which could be utilized by laboratories, based on the findings of this study. However, the suggested reference intervals below are based on a heterozygous phenotype of the affected haemoglobin chain. Care must be taken interpreting results which are suggestive of a concomitant alpha or beta thalassemia, as both the peak percentage and haemoglobin A_2 will change.

Table 7. *Diagnostic cutoff limits for haemoglobin A_2 as provided by Sebia and the modified cutoffs as suggested by this research*

Haemoglobin chain	Current diagnostic cutoff	Modified diagnostic cutoff
Haemoglobin A_2	2.2-3.2%	1.82-2.82%

The National Health Service in Britain has produced documentation to aid laboratory staff in diagnosing suspected haemoglobinopathies. The algorithm below provides guidelines for diagnostic cut-off levels of variant haemoglobin percentages.

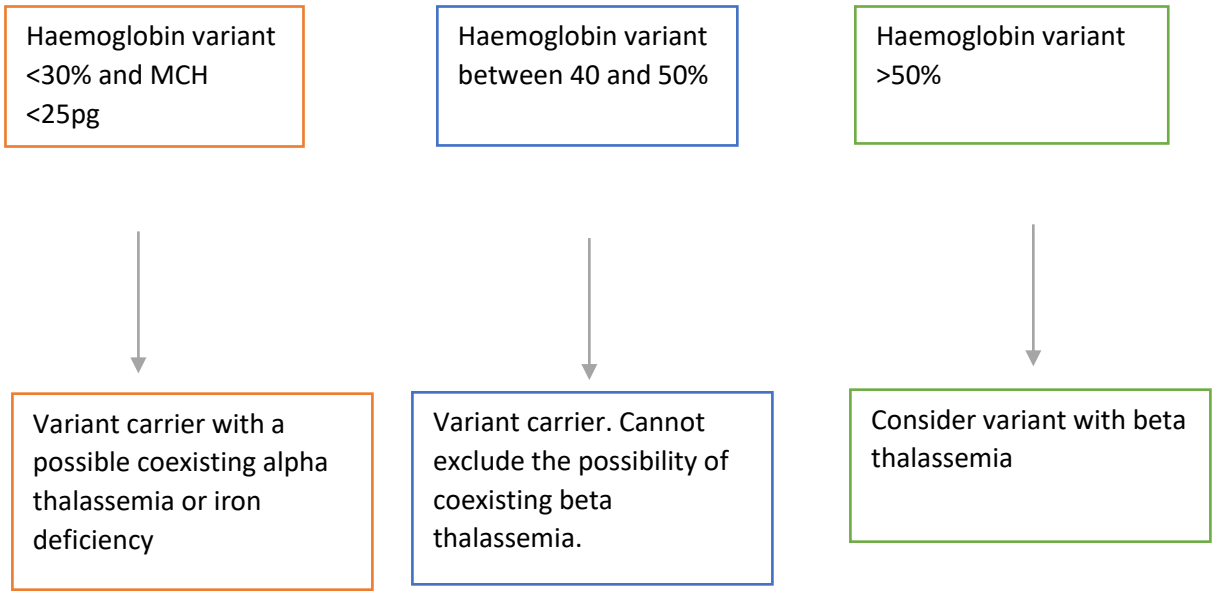

Figure 21: Interpretation guidelines for variant haemoglobin detection. Adapted from NHS sickle cell and thalassemia screening programme, 2017.

Based on the results described above, this algorithm would need to be adjusted to correct for the negative bias obtained by the HbA1c technique. The algorithm below shows modified acceptance guidelines which could be used by laboratories to interpret results obtained through HbA1c.

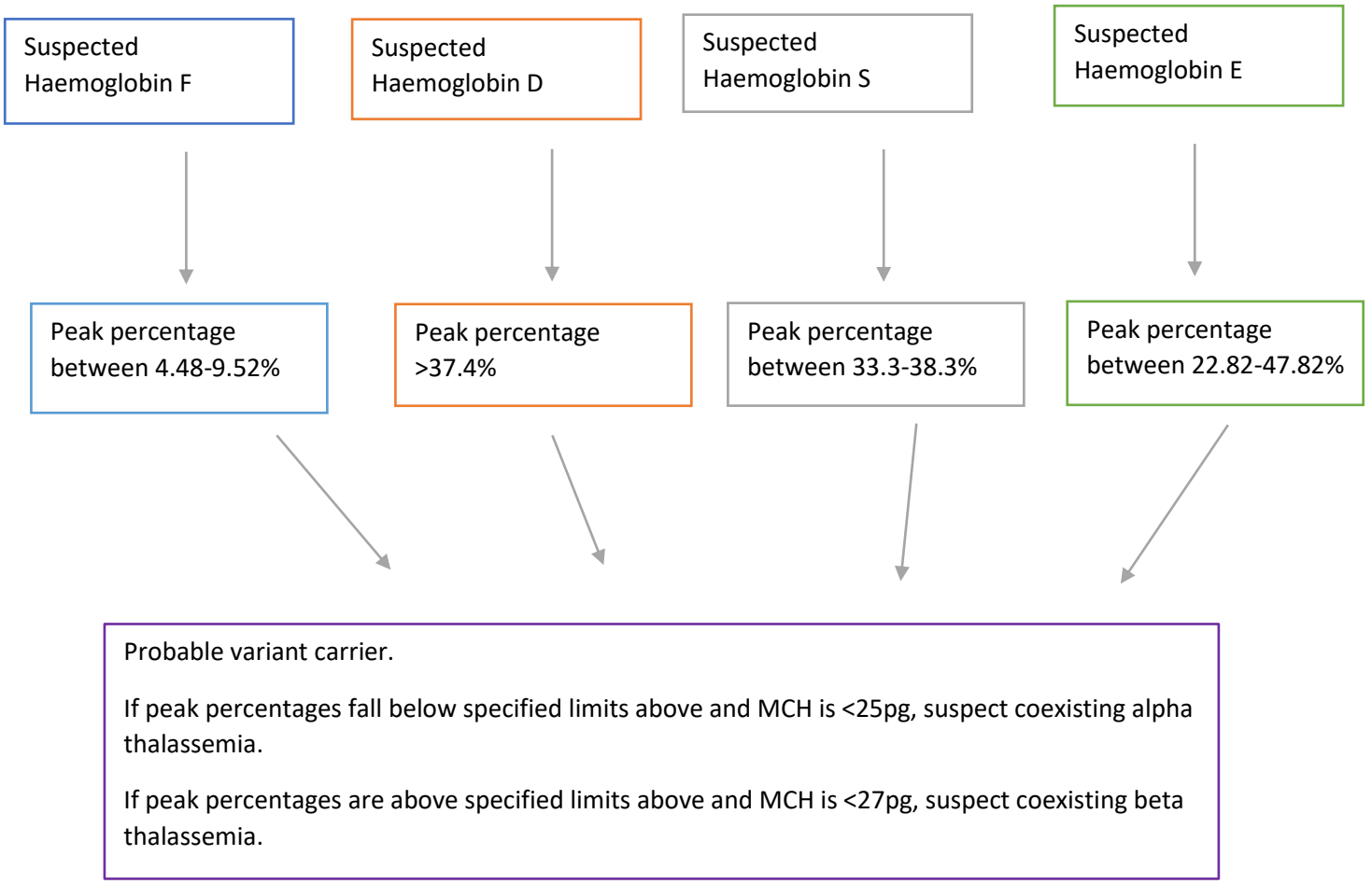

Figure 22: Suggested guidelines to aid laboratories in the interpretation of haemoglobinopathies.

However, this can only be used to screen patients if the laboratory utilizes technology, such as the Capillarys 3 to perform HbA1c analysis. As discussed previously, methodologies such as immunoassay and spectrophotometry cannot discriminate between variant haemoglobin chains and will assume all haemoglobin present in the sample is normal. HPLC techniques are also limited as they are unable to quantify haemoglobin A_2 in the presence of haemoglobin E due to close elution zones (Keren et al, 2008). Access to such technology can be limited by geographical location and budget allocation from district

health boards. To overcome this, laboratories could apply a risk-based model based on ethnicity and the outcome of the complete blood count to determine the likelihood of the child being affected by a haemoglobinopathy. If the biological partner of the child is of the same ethnicity, partner screening can be recommended at this stage to determine the inheritance risk to the child. Any of these results would be considered suggestive, however if a significant abnormality was detected, gene testing could be referred to determine the exact abnormality present. This could potentially reduce the turn-around time for result release, particularly if the mother is deemed high risk of having a haemoglobinopathy, and the pregnancy has already commenced. The key to any successful screening programme is informed consent. Patients must have enough information to understand the implications of prospective test results and have access to counselling services should this be required (Medical Sciences Council, 2011). Consent forms should be utilized to protect the laboratory particularly when surrogate services or adoption are employed or in cases of infidelity.

However, any screening based on ethnicity must be approached with care. Integrated and varied societies allow for genetically diverse population pools and therefore offspring. While certain ethnicities are predisposed to haemoglobinopathies, mixed populations may reduce the likelihood of identifying clinical risk due to genetic dilution. The idea of a screening model such as the one described above, is not to discriminate, but to educate prospective parents about the risk of haemoglobinopathies and the financial and social implications outlined above.

With the global population increasing at approximately 1% per year and historic data suggesting that 7% of the world's population is affected by a haemoglobinopathy, education is the most important factor to reduce the effects of these conditions. Despite the development of new technologies, the rate of population growth and the continued effects of haemoglobinopathies present burdens which are immeasurable to both healthcare and socioeconomic status of those affected.

4.4 Gene frequency of Haemoglobinopathies in the Waikato/Bay of Plenty regions

Objective three was to outline the gene frequency in the sample population, throughout the Waikato/Bay of Plenty regions. From a total sample population of 731 patient samples, the gene frequency has been extrapolated into the table below.

Table 8. *Estimated gene frequency of haemoglobin variants throughout the Waikato/Bay of plenty regions.*

Haemoglobin chain affected	Percentage of patients affected
A_2	0.173%
F	0.062%
D	0.014%
S	0.051%
E	0.041%

The involvement of the haemoglobin A_2 chain was calculated by identifying A_2 percentages outside the reference interval suggested by Sebia, from the Haemoglobin E programme (2.2-3.2%). This percentage includes patients with beta thalassemia of varying severity (increased haemoglobin A_2 percentage) and alpha thalassemia (decreased haemoglobin A_2). The estimated population of this region is 726,179 people (Stats NZ, 2019). The above data suggests that approximately 0.34% of people in the Waikato and Bay of Plenty regions are affected by a hemoglobinopathy. Statistic New Zealand estimates the latest population numbers as 4,957,400 people as at 31st March 2019. This includes approximately 58,020 births and 148,476 migrants arriving annually. We can suggest, based on the findings of this study, that approximately 16,855 people in New Zealand are currently affected by a haemoglobinopathy. This is based on the expected gene frequency obtained from the data table above.

Although this number may seem small, the consequences of haemoglobinopathies can be significant in terms of financial and emotional burden. In 2005, Statistics New Zealand has estimated that by the year 2021, the Asian population in New Zealand will increase by 390,000 people or 145% over historical figures determined in 2001 (Ewing, 2005). Figure 23 below shows a historic representation of the distribution of thalassemias globally. This

demonstrates a significant prevalence of thalassemias, however the proportion of variant haemoglobin disorders is also increased throughout these areas (Modell and Darlison, 2008). With the increase in genetically and culturally diverse population pools within New Zealand, the number of affected individuals will continue to increase. Education and effective screening techniques remain our best tools to attempt to reduce the prevalence of haemoglobinopathies within New Zealand.

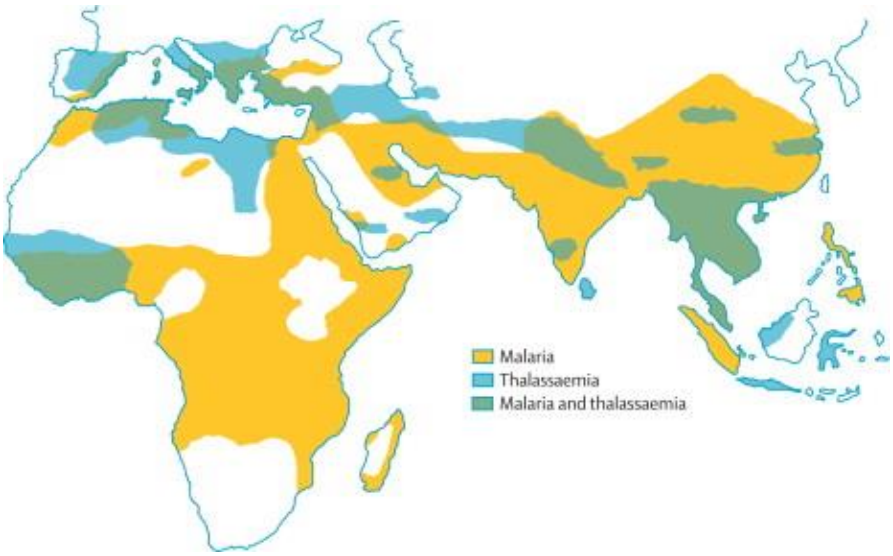

Figure 23: Global distribution of thalassemia in 2006. (Weatherall et al, 2006)

5.Conclusion

The purpose of this study was to determine if the Sebia HbA1c technique could be used to accurately identify haemoglobinopathies without the need for further investigations. Despite the good result correlation between the reference method, Sebia haemoglobin E technique and the HbA1c method, the results obtained through HbA1c demonstrated a consistent negative bias. This indicates that an alternative reference range would need to be utilized by the laboratory to accurately report patient results. Regardless of this, the HbA1c technique could be used to make a presumptive identification of a haemoglobinopathy, provided the correct complementary tests were requested at the same time. Laboratory tests such as complete blood count and iron studies can be used to estimate the severity of a suspected haemoglobinopathy and to provide insight into the clinical significance of any finding.

As HbA1c testing has been incorporated into all prenatal screening panels in New Zealand, the ability to detect and determine the likely clinical significance of an abnormal peak can be utilized by laboratories employing the Sebia HbA1c methodology. This screening test can provide valuable information to guide and educate patients about the genetic effects of hemoglobinopathies and the consequences they may pose to the next generation. Although the data obtained is limited to capillary electrophoresis methodology, the results could be extrapolated to be used as a risk profile to selectively screen populations which have an elevated prevalence of haemoglobinopathies.

The gene frequency of patients throughout the Waikato and Bay of Plenty regions, although are currently low in numbers, can pose a significant financial burden to healthcare within New Zealand. With integration and mixed societies and family structures, the need for screening against haemoglobinopathies is becoming more apparent.

5.1 Study limitations and future directions

With any research project, the results are often limited due to financial and time constraints. As this study has been undertaken as part of a university Master's degree, the sample number included in this study has been restricted due to time limitations. The results of this study were also limited by the gene frequency throughout the Waikato and Bay of Plenty regions. This results in a selection bias that represents a small proportion of the New

Zealand population, therefore the conclusions extrapolated from the data may exhibit a bias.

Future studies should be performed with a larger sample population from several geographical locations to provide a better estimation of the gene frequency of haemoglobinopathies within New Zealand. As both techniques examined during this research are looking at phenotypes only, it would be helpful to confirm the findings with gene testing.

This project was undertaken in a working medical laboratory, it is a representation of the processes and methods undertaken by one laboratory and one type of methodology. For improved statistical conclusions, it could be prudent to include results from laboratories using similar technologies and processes.

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Appendix A: Ethics approval

a) AUT ethics committee approval letter

3 April 2019

Fabrice Merien

Faculty of Health and Environmental Sciences

Dear Fabrice

Re Ethics Application: **19/42 To determine the validity of the Sebia HbA1c assay for identifying variant haemoglobin chains, when compared to the Sebia Haemoglobin E programme**

Thank you for providing evidence as requested, which satisfies the points raised by the Auckland University of Technology Ethics Committee (AUTEC).
Your ethics application has been approved for three years until 3 April 2022.

Standard Conditions of Approval

1. A progress report is due annually on the anniversary of the approval date, using form EA2, which is available online through http://www.aut.ac.nz/research/researchethics.
2. A final report is due at the expiration of the approval period, or, upon completion of project, using form EA3, which is available online through http://www.aut.ac.nz/research/researchethics.
3. Any amendments to the project must be approved by AUTEC prior to being implemented. Amendments can be requested using the EA2 form: http://www.aut.ac.nz/research/researchethics.
4. Any serious or unexpected adverse events must be reported to AUTEC Secretariat as a matter of priority.
5. Any unforeseen events that might affect continued ethical acceptability of the project should also be reported to the AUTEC Secretariat as a matter of priority.

Please quote the application number and title on all future correspondence related to this project.

AUTEC grants ethical approval only. If you require management approval for access for your research from another institution or organisation then you are responsible for obtaining it. You are reminded that it is your responsibility to ensure that the spelling and grammar of documents being provided to participants or external organisations is of a high standard.

For any enquiries, please contact ethics@aut.ac.nz

Yours sincerely,

Kate O'Connor
Executive Manager
Auckland University of Technology Ethics Committee

b) Waikato District Health Board research office approval letter

Research <research@waikatodhb.health.nz>

28/03/2019 11:59 AM

Thank you for this information Kate.

Given the information you have provided to me below and in the attached form, I agree that you require AUT ethics only for this project (and do not require Health & Disability Ethics Committee (HDEC) review). I have conferred with the Relationship Manager within Waikato DHB who covers Pathlab, and she is also happy with the research proposed.

The research is being undertaken within the Waikato DHB region, but not at the Waikato DHB hospitals, so it falls outside of my usual locality process. Therefore we do not require that you register the study with us; nor that you complete our Māori Consultation process.

Waikato DHB has a goal of eliminating health inequity. So the main points of interest for a retrospective study are that you are collecting ethnicity and that samples are treated with, and disposed of (if appropriate) in a culturally sensitive way. If you are collecting ethnicity of the samples, it may become clear if there are any inequities.

Kate, if you need formal Māori Consultation for the study for the ethics committee, please complete the attached two forms, and we can do that for you. But I am comfortable with the information you have provided.

Regards

Sarah

Sarah Brodnax | Coordinator – Governance | Quality & Patient Safety | Waikato DHB

13 Ohaupo Road | p 07 839 8899 ext 23589 | e sarah.brodnax@waikatodhb.health.nz

Waikato DHB vision: *Healthy People. Excellent Care*

Our values: *People at heart - Te iwi ngakaunui* | Give and earn respect - Whakamana | Listen to me talk to me - Whakarongo | Fair play - Mauri Pai | Growing the good – Whakapakari | Stronger together – Kotahitanga

Appendix B: Research outputs

a) Haemoglobin A_2- ***peak position through each method, zone classification and peak percentage between methodologies.***

Sample number	HbA1c peak position	Hb (E) peak position	Phoresis Zone	HbA1c Peak %	Hb (E) peak %
1	240	243	A2	2.2	2.6
2	240	243	A2	2.1	2.3
3	240	243	A2	2	2.3
4	240	243	A2	1.9	2.3
5	240	243	A2	2.3	2.5
6	240	243	A2	2.3	2.7
7	240	243	A2	2.1	2.5
8	240	243	A2	0.2	0.2
9	240	243	A2	1.6	1.8
10	240	243	A2	2.3	2.7
11	240	243	A2	1.7	1.8
12	240	243	A2	1.5	2
13	240	243	A2	4.6	5.4
14	240	243	A2	4.1	4.9
15	240	243	A2	3.9	4.7
16	240	243	A2	3.9	4.7
17	240	243	A2	1.9	2.5
18	240	243	A2	2.1	2.5
19	240	243	A2	1.5	1.8
20	240	243	A2	4.8	5.5
21	240	243	A2	1.9	2.1
22	240	243	A2	2	2.4
23	240	243	A2	5.2	5.9
24	240	243	A2	2	2.7
25	240	243	A2	3.5	4.2
26	240	243	A2	1.7	1.9
27	240	243	A2	2.1	2.4
28	240	243	A2	2.1	2.4
29	240	243	A2	5.4	6.1
30	240	243	A2	4.6	5.2
31	240	243	A2	4.8	5.4
32	240	243	A2	1.9	2.5
33	240	243	A2	2.1	2.6
34	240	243	A2	2	2.3
35	240	243	A2	2.3	2.7
36	240	243	A2	1.9	2.5
37	240	243	A2	2.1	2.7
38	240	243	A2	1.9	2.5
39	240	243	A2	2.4	3
40	240	243	A2	1.7	2.3

41	240	243	A2	4.2	4.8
42	240	243	A2	4.2	4.8
43	240	243	A2	4.9	5.5
44	240	243	A2	5.5	6.1
45	240	243	A2	2.2	2.8
46	240	243	A2	4	4.6
47	240	243	A2	5	5.6
48	240	243	A2	4.8	5.3
49	240	243	A2	4.8	5.3
50	240	243	A2	3.8	4.3
51	240	243	A2	2.1	2.6
52	240	243	A2	2.2	2.7
53	240	243	A2	1.9	2.4
54	240	243	A2	2.1	2.6
55	240	243	A2	4.5	5
56	240	243	A2	2.3	2.7
57	240	243	A2	1.9	2.2
58	240	243	A2	2.1	2.6
59	240	243	A2	2	2.5
60	240	243	A2	4.1	4.6
61	240	243	A2	4	4.5
62	240	243	A2	4.1	4.6
63	240	243	A2	2	2.5
64	240	243	A2	4.3	4.8
65	240	243	A2	2.3	2.8
66	240	243	A2	2.2	2.7
67	240	243	A2	2.1	2.6
68	240	243	A2	4.9	5.4
69	240	243	A2	2	2.5
70	240	243	A2	2.1	2.6
71	240	243	A2	2.1	2.6
72	240	243	A2	1.8	2.3
73	240	243	A2	1.8	2.3
74	240	243	A2	1.8	2.3
75	240	243	A2	1.8	2.3
76	240	243	A2	2.3	2.7
77	240	243	A2	2.3	2.7
78	240	243	A2	4.1	4.5
79	240	243	A2	2.3	2.7
80	240	243	A2	2.3	2.6
81	240	243	A2	2.3	2.7
82	240	243	A2	4	4.4
83	240	243	A2	2.3	2.7
84	240	243	A2	2.3	2.7
85	240	243	A2	2.8	3.2
86	240	243	A2	2.8	3.2
87	240	243	A2	4.8	5.2

88	240	243	A2	2.3	2.7
89	240	243	A2	2.3	2.7
90	240	243	A2	1.7	2.1
91	240	243	A2	1.7	2.1
92	240	243	A2	1.8	2.2
93	240	243	A2	2.2	2.6
94	240	243	A2	2.1	2.5
95	240	243	A2	2.4	2.8
96	240	243	A2	2	2.4
97	240	243	A2	2	2.4
98	240	243	A2	2.9	3.3
99	240	243	A2	2.7	3.1
100	240	243	A2	2.1	2.5
101	240	243	A2	2.2	2.6
102	240	243	A2	3.9	4.3
103	240	243	A2	2	2.4
104	240	243	A2	3.1	3.5
105	240	243	A2	2.1	2.5
106	240	243	A2	2.1	2.5
107	240	243	A2	2.2	2.6
108	240	243	A2	2.2	2.6
109	240	243	A2	2	2.4
110	240	243	A2	2	2.4
111	240	243	A2	2.2	2.5
112	240	243	A2	2.1	2.4
113	240	243	A2	2.5	2.9
114	240	243	A2	2.4	2.8
115	240	243	A2	1.6	2
116	240	243	A2	2.2	2.6
117	240	243	A2	2.1	2.5
118	240	243	A2	1.9	2.3
119	240	243	A2	2	2.4
120	240	243	A2	1.9	2.3
121	240	243	A2	2.2	2.6
122	240	243	A2	2.2	2.6
123	240	243	A2	2.5	2.9
124	240	243	A2	2.2	2.6
125	240	243	A2	2	2.4
126	240	243	A2	2.1	2.5
127	240	243	A2	1.9	2.3
128	240	243	A2	1.9	2.3
129	240	243	A2	2	2.4
130	240	243	A2	2.4	2.8
131	240	243	A2	2	2.4
132	240	243	A2	2.1	2.5
133	240	243	A2	2.2	2.6
134	240	243	A2	2	2.4

135	240	243	A2	2	2.4
136	240	243	A2	2.7	3.1
137	240	243	A2	2.4	2.8
138	240	243	A2	2.1	2.5
139	240	243	A2	2.2	2.6
140	240	243	A2	2.4	2.8
141	240	243	A2	2.4	2.8
142	240	243	A2	2.1	2.5
143	240	243	A2	1.6	2
144	240	243	A2	2.5	2.9
145	240	243	A2	2.2	2.6
146	240	243	A2	2.1	2.5
147	240	243	A2	2	2.4
148	240	243	A2	2.9	3.3
149	240	243	A2	2.4	2.8
150	240	243	A2	2.5	2.9
151	240	243	A2	2.6	3
152	240	243	A2	2.2	2.6
153	240	243	A2	3.1	3.5
154	240	243	A2	2.4	2.8
155	240	243	A2	4.9	5.3
156	240	243	A2	4.6	4.9
157	240	243	A2	2.4	2.7
158	240	243	A2	1.9	2.2
159	240	243	A2	2.4	2.7
160	240	243	A2	2.4	2.7
161	240	243	A2	2.4	2.7
162	240	243	A2	2.3	2.6
163	240	243	A2	2.4	2.7
164	240	243	A2	2.4	2.7
165	240	243	A2	2.3	2.6
166	240	243	A2	2.3	2.6
167	240	243	A2	2.3	2.6
168	240	243	A2	2.4	2.7
169	240	243	A2	1.9	2.2
170	240	243	A2	2.3	2.6
171	240	243	A2	2.3	2.6
172	240	243	A2	1.9	2.2
173	240	243	A2	2.3	2.6
174	240	243	A2	2.3	2.7
175	240	243	A2	2.4	2.7
176	240	243	A2	2.4	2.7
177	240	243	A2	1.9	2.2
178	240	243	A2	2.3	2.6
179	240	243	A2	2.3	2.6
180	240	243	A2	2.4	2.7
181	240	243	A2	2.3	2.6

182	240	243	A2	1.9	2.2
183	240	243	A2	2.3	2.6
184	240	243	A2	2.3	2.6
185	240	243	A2	2.3	2.6
186	240	243	A2	2.4	2.7
187	240	243	A2	2.3	2.6
188	240	243	A2	2.3	2.6
189	240	243	A2	2.8	3.1
190	240	243	A2	2.4	2.7
191	240	243	A2	2.4	2.7
192	240	243	A2	2.3	2.6
193	240	243	A2	2.3	2.6
194	240	243	A2	1.9	2.2
195	240	243	A2	1.9	2.2
196	240	243	A2	1.9	2.2
197	240	243	A2	2.4	2.7
198	240	243	A2	2.4	2.7
199	240	243	A2	2.3	2.6
200	240	243	A2	2.3	2.6
201	240	243	A2	2.3	2.6
202	240	243	A2	2.4	2.7
203	240	243	A2	3.4	3.7
204	240	243	A2	2.3	2.6
205	240	243	A2	2.3	2.6
206	240	243	A2	1.9	2.2
207	240	243	A2	2.3	2.6
208	240	243	A2	3.4	3.7
209	240	243	A2	2.8	3.1
210	240	243	A2	2.3	2.6
211	240	243	A2	2.3	2.6
212	240	243	A2	2.8	3.1
213	240	243	A2	1.9	2.2
214	240	243	A2	1.9	2.2
215	240	243	A2	2.4	2.7
216	240	243	A2	2.3	2.6
217	240	243	A2	2.3	2.6
218	240	243	A2	2.4	2.7
219	240	243	A2	2.3	2.6
220	240	243	A2	1.7	2
221	240	243	A2	1.7	2
222	240	243	A2	1.8	2.1
223	240	243	A2	1.8	2.1
224	240	243	A2	1.7	2
225	240	243	A2	1.5	1.8
226	240	243	A2	1.7	2
227	240	243	A2	1.5	1.8
228	240	243	A2	1.8	2.1

229	240	243	A2	1.8	2.1
230	240	243	A2	1.8	2.1
231	240	243	A2	1.7	2
232	240	243	A2	1.8	2.1
233	240	243	A2	1.8	2.1
234	240	243	A2	1.8	2.1
235	240	243	A2	1.4	1.7
236	240	243	A2	2.3	2.6
237	240	243	A2	2.1	2.4
238	240	243	A2	2.3	2.6
239	240	243	A2	2.2	2.5
240	240	243	A2	2	2.3
241	240	243	A2	2.1	2.4
242	240	243	A2	2.1	2.4
243	240	243	A2	2.1	2.4
244	240	243	A2	3.7	4
245	240	243	A2	2.1	2.4
246	240	243	A2	2	2.3
247	240	243	A2	4.8	5.1
248	240	243	A2	2.2	2.5
249	240	243	A2	2	2.3
250	240	243	A2	2	2.3
251	240	243	A2	2.1	2.4
252	240	243	A2	2.5	2.8
253	240	243	A2	2.1	2.4
254	240	243	A2	2.1	2.4
255	240	243	A2	2	2.3
256	240	243	A2	2.1	2.4
257	240	243	A2	2.2	2.5
258	240	243	A2	2.2	2.5
259	240	243	A2	2.1	2.4
260	240	243	A2	2.1	2.4
261	240	243	A2	2	2.3
262	240	243	A2	2	2.3
263	240	243	A2	2.5	2.8
264	240	243	A2	2	2.3
265	240	243	A2	2.1	2.4
266	240	243	A2	2.2	2.5
267	240	243	A2	2.1	2.4
268	240	243	A2	2.1	2.4
269	240	243	A2	3.2	3.5
270	240	243	A2	2.2	2.5
271	240	243	A2	3	3.3
272	240	243	A2	2.2	2.5
273	240	243	A2	2	2.3
274	240	243	A2	2.2	2.5
275	240	243	A2	2.1	2.4

276	240	243	A2	2.1	2.4
277	240	243	A2	2.2	2.5
278	240	243	A2	2	2.3
279	240	243	A2	2.5	2.8
280	240	243	A2	2	2.3
281	240	243	A2	2.5	2.8
282	240	243	A2	2.2	2.5
283	240	243	A2	2.2	2.5
284	240	243	A2	2.1	2.4
285	240	243	A2	2.1	2.4
286	240	243	A2	2.1	2.4
287	240	243	A2	2	2.3
288	240	243	A2	2.2	2.5
290	240	243	A2	2.2	2.5
291	240	243	A2	2	2.3
292	240	243	A2	2.5	2.8
293	240	243	A2	2.6	2.9
294	240	243	A2	2.1	2.4
295	240	243	A2	2.1	2.4
296	240	243	A2	2.2	2.5
297	240	243	A2	2.2	2.5
298	240	243	A2	2.2	2.5
299	240	243	A2	2.5	2.8
300	240	243	A2	2.1	2.4
301	240	243	A2	2	2.3
302	240	243	A2	2	2.3
303	240	243	A2	2	2.3
304	240	243	A2	2.2	2.5
305	240	243	A2	2.2	2.5
306	240	243	A2	2.2	2.5
307	240	243	A2	2.1	2.4
308	240	243	A2	2.5	2.8
309	240	243	A2	2.1	2.4
310	240	243	A2	2	2.3
311	240	243	A2	2.2	2.5
312	240	243	A2	2.7	3
313	240	243	A2	2.2	2.5
314	240	243	A2	2.5	2.8
315	240	243	A2	2.2	2.5
316	240	243	A2	2.1	2.4
317	240	243	A2	2.5	2.8
318	240	243	A2	2.2	2.5
319	240	243	A2	2.2	2.5
320	240	243	A2	2	2.3
321	240	243	A2	2.7	3
322	240	243	A2	1.6	1.9
323	240	243	A2	2.2	2.5

324	240	243	A2	2.1	2.4
325	240	243	A2	2.7	3
326	240	243	A2	2.1	2.4
327	240	243	A2	2.6	2.9
328	240	243	A2	2.5	2.8
329	240	243	A2	2.6	2.9
330	240	243	A2	2	2.3
331	240	243	A2	2.5	2.8
332	240	243	A2	1.6	1.9
333	240	243	A2	2	2.3
334	240	243	A2	2	2.3
335	240	243	A2	2.2	2.5
336	240	243	A2	2.7	3
337	240	243	A2	2.1	2.4
338	240	243	A2	2.2	2.5
339	240	243	A2	2.5	2.8
340	240	243	A2	2.2	2.5
341	240	243	A2	2.1	2.4
342	240	243	A2	2.2	2.5
343	240	243	A2	2.7	3
344	240	243	A2	2	2.3
345	240	243	A2	2.7	3
346	240	243	A2	2	2.3
347	240	243	A2	2.1	2.4
348	240	243	A2	2.5	2.8
349	240	243	A2	2.1	2.4
350	240	243	A2	2.5	2.8
351	240	243	A2	2	2.3
352	240	243	A2	2.2	2.5
353	240	243	A2	2.2	2.5
354	240	243	A2	1.9	2.1
355	240	243	A2	2.3	2.5
356	240	243	A2	2.3	2.5
357	240	243	A2	2.3	2.5
358	240	243	A2	2.4	2.6
359	240	243	A2	2	2.2
360	240	243	A2	1.9	2.1
361	240	243	A2	3	3.2
362	240	243	A2	2.3	2.5
363	240	243	A2	1.9	2.1
364	240	243	A2	2.4	2.6
365	240	243	A2	2	2.2
366	240	243	A2	2.5	2.7
367	240	243	A2	2	2.2
368	240	243	A2	2.3	2.5
369	240	243	A2	2.3	2.5
370	240	243	A2	1.9	2.1

371	240	243	A2	1.9	2.1
372	240	243	A2	2.5	2.7
373	240	243	A2	2	2.2
374	240	243	A2	2.5	2.7
375	240	243	A2	2.3	2.5
376	240	243	A2	1.9	2.1
377	240	243	A2	2.4	2.6
378	240	243	A2	2.5	2.7
379	240	243	A2	2.4	2.6
380	240	243	A2	2.3	2.5
381	240	243	A2	2	2.2
382	240	243	A2	2.4	2.6
383	240	243	A2	1.9	2.1
384	240	243	A2	2.3	2.7
385	240	243	A2	2.3	2.7
386	240	243	A2	2.3	2.6
387	240	243	A2	1.9	2.1
388	240	243	A2	2.4	2.6
389	240	243	A2	2	2.2
390	240	243	A2	3.3	3.5
391	240	243	A2	2.3	2.5
392	240	243	A2	1.9	2.1
393	240	243	A2	2.4	2.6
394	240	243	A2	2	2.2
395	240	243	A2	2.4	2.6
396	240	243	A2	2	2.2
397	240	243	A2	2.5	2.7
398	240	243	A2	2.5	2.7
399	240	243	A2	2.4	2.6
400	240	243	A2	2.3	2.5
401	240	243	A2	1.9	2.1
402	240	243	A2	2.5	2.7
403	240	243	A2	1.9	2.1
404	240	243	A2	2.8	3
405	240	243	A2	2.4	2.6
406	240	243	A2	2.4	2.6
407	240	243	A2	2.4	2.6
408	240	243	A2	2.3	2.5
409	240	243	A2	2	2.2
410	240	243	A2	2.3	2.5
411	240	243	A2	2.5	2.7
412	240	243	A2	2	2.2
413	240	243	A2	2	2.2
414	240	243	A2	2.3	2.5
415	240	243	A2	1.4	1.6
416	240	243	A2	2.3	2.5
417	240	243	A2	1.9	2.1

418	240	243	A2	2.5	2.7
419	240	243	A2	2	2.2
420	240	243	A2	1.9	2.1
421	240	243	A2	2.3	2.5
422	240	243	A2	1.9	2.1
423	240	243	A2	2.3	2.5
424	240	243	A2	2.2	2.4
425	240	243	A2	2.1	2.3
426	240	243	A2	1.7	1.9
427	240	243	A2	1.7	1.9
428	240	243	A2	1.5	1.7
429	240	243	A2	1.6	1.8
430	240	243	A2	1.7	1.9
431	240	243	A2	1.7	1.9
432	240	243	A2	1.8	2
433	240	243	A2	1.8	2
434	240	243	A2	1.7	1.9
435	240	243	A2	1.8	2
436	240	243	A2	1.6	1.8
437	240	243	A2	1.6	1.8
438	240	243	A2	1.8	2
439	240	243	A2	1.6	1.8
440	240	243	A2	1.7	1.9
441	240	243	A2	1.8	2
442	240	243	A2	1.7	1.9
443	240	243	A2	1.8	2
444	240	243	A2	1.6	1.8
445	240	243	A2	1.6	1.8
446	240	243	A2	1.5	1.7
447	240	243	A2	1.8	2
448	240	243	A2	2.6	2.8
449	240	243	A2	2.1	2.3
450	240	243	A2	2.1	2.3
451	240	243	A2	2.1	2.3
452	240	243	A2	2.2	2.4
453	240	243	A2	2.2	2.4
454	240	243	A2	2.1	2.3
455	240	243	A2	3.1	3.3
456	240	243	A2	2.1	2.3
457	240	243	A2	2.2	2.4
458	240	243	A2	2.2	2.4
459	240	243	A2	2.1	2.3
460	240	243	A2	2.2	2.4
461	240	243	A2	2.1	2.3
462	240	243	A2	2.2	2.4
463	240	243	A2	2.2	2.4
464	240	243	A2	2.2	2.4

465	240	243	A2	2.2	2.4
466	240	243	A2	2.1	2.3
467	240	243	A2	2.1	2.3
468	240	243	A2	2.1	2.4
469	240	243	A2	2.1	2.4
470	240	243	A2	2.2	2.4
471	240	243	A2	2.1	2.3
471	240	243	A2	2.1	2.3
473	240	243	A2	2.1	2.3
474	240	243	A2	2.1	2.3
475	240	243	A2	2.2	2.4
476	240	243	A2	2.1	2.3
477	240	243	A2	2.1	2.3
478	240	243	A2	2.1	2.3
479	240	243	A2	2.2	2.4
480	240	243	A2	2.1	2.3
481	240	243	A2	2.7	2.9
482	240	243	A2	2.1	2.3
483	240	243	A2	2.1	2.3
484	240	243	A2	2.2	2.4
485	240	243	A2	2.6	2.8
486	240	243	A2	2.1	2.3
487	240	243	A2	2.1	2.3
488	240	243	A2	2.1	2.3
489	240	243	A2	2.2	2.4
490	240	243	A2	2	2.1
491	240	243	A2	1.9	2
492	240	243	A2	2.8	2.9
493	240	243	A2	2	2.1
494	240	243	A2	1.7	1.8
495	240	243	A2	1.9	2
496	240	243	A2	2.3	2.6
497	240	243	A2	2.5	2.8
498	240	243	A2	2.1	2.2
499	240	243	A2	2	2.1
500	240	243	A2	1.7	1.8
501	240	243	A2	2.4	2.5
502	240	243	A2	1.9	2
503	240	243	A2	2.1	2.2
504	240	243	A2	2.4	2.5
505	240	243	A2	1.3	1.4
506	240	243	A2	1.8	1.9
507	240	243	A2	2.2	2.3
508	240	243	A2	2.1	2.1
509	240	243	A2	2.6	2.6
510	240	243	A2	2.5	2.8
511	240	243	A2	2.4	2.8

512	240	243	A2	2.6	2.6
513	240	243	A2	2.1	2.1
514	240	243	A2	2.5	2.5
515	240	243	A2	1.4	1.4
516	240	243	A2	2.5	2.4
517	240	243	A2	1.8	1.6
518	240	243	A2	2.5	2.9
519	240	243	A2	1.9	1.6
520	240	243	A2	2.6	2.3
521	240	243	A2	2.6	2.2
522	240	243	A2	2.2	2.4
523	240	243	A2	2.5	2.9
524	240	243	A2	2.5	2.8
525	240	243	A2	4.5	5
526	240	243	A2	4.2	4.9
527	240	243	A2	5.1	5.7
528	240	243	A2	5	5.4
529	240	243	A2	5.2	5.8
530	240	243	A2	5.1	5.6

b) Haemoglobin F- ***peak position through each method, zone classification and peak percentage between methodologies.***

Sample number	HbA1c peak position	HbA1c A2 peak position	Hb (E) peak position	Hb (E) A2 Peak Position	Phoresis Zone	A1c F Peak %	HbA1c A2 peak %	Hb (E) HbF peak %	Hb (E) A2 Peak %
531	181	240	188	243	F/A2	0.5	2.4	1.2	2.8
532	181	240	188	243	F/A2	0	2.9	1.3	3.2
533	182	240	190	243	F/A2	0	2.1	0.3	2.4
534	180	240	187	243	F/A2	3.4	2	4.4	2.2
535	179	240	187	243	F/A2	1.1	4.4	1.6	4.8
536	180	240	187	243	F/A2	1.4	4.8	2.1	5.3
537	178	240	188	243	F/A2	3.9	2.2	3.4	2.5
538	178	240	188	243	F/A2	0	2.2	2	2.6
539	180	240	188	243	F/A2	4.5	1.6	6	1.8
540	151	240	160	243	F/A2	0	2.2	0.4	2.6
541	180	240	187	243	F/A2	2.1	2.2	2.7	2.5
542	180	240	186	243	F/A2	7.1	2	7.1	2.2
543	180	240	188	243	F/A2	0.8	5.1	1.3	5.9
544	182	240	189	243	F/A2	0	3.1	1	3.5
545	180	240	185	243	F/A2	9.1	1.4	11.3	1.7
546	181	240	187	243	F/A2	0	5.7	0.9	6.4
547	182	240	188	243	F/A2	0	2.7	1.6	3
548	181	240	187	243	F/A2	1.8	4.9	2.8	5.5
549	180	240	187	243	F/A2	3.4	2.1	4.2	2.3
550	180	240	188	243	F/A2	0.5	2.1	1.3	2.4
551	179	240	187	243	F/A2	1.9	5.4	2.8	6.1

552	180	240	174	243	F/A2	9.5	1.9	10.1	2.6
553	180	240	186	243	F/A2	3	5.7	4.1	6.3
554	180	240	187	243	F/A2	2.1	2.1	2.8	2.3
555	179	240	187	243	F/A2	2.2	5.8	2.7	6.4
556	177	240	187	243	F/A2	4.5	5.2	5.5	5.7
557	180	240	187	243	F/A2	1.8	4.8	1.8	5.1
558	183	240	190	243	F/A2	0	4.1	0.5	4.7
559	180	240	187	243	F/A2	2.7	2.3	2.9	2.5
560	180	240	187	243	F/A2	2.3	2.2	3.1	2.4
561	177	240	186	243	F/A2	7.5	2.3	8.9	2.5
562	180	240	187	243	F/A2	0.9	4.4	1.5	4.9
563	182	240	189	243	F/A2	0	2.4	0.8	2.7
564	180	240	184	243	F/A2	12.9	1.8	14.6	2
565	181	240	188	243	F/A2	0	2.6	0	2.3
566	180	240	187	243	F/A2	1.4	5.1	1.8	5.7
567	180	240	186	243	F/A2	4.7	4.2	6.2	4.8
568	178	240	185	243	F/A2	12.5	1.7	14.7	1.9
569	185	240	188	243	F/A2	0.9	2.3	1.4	2.7
570	182	240	187	243	F/A2	1.3	5.9	1.9	6.6
571	178	240	187	243	F/A2	4.4	2.4	5.3	2.7
572	168	240	181	243	F	91.8	0	80.9	0
573	181	240	187	243	F/A2	0	4.4	0.5	5
574	183	240	190	243	F/A2	0	2.3	0.4	2.6
575	183	240	189	243	F/A2	0.4	2.4	0.9	2.7
576	180	240	187	243	F/A2	1.7	5.3	2.9	5.9
577	180	240	186	243	F/A2	1.9	4.9	2.1	5.5
578	182	240	188	243	F/A2	0	3.4	1.6	4
579	182	240	188	243	F/A2	1.7	2.4	2.2	2.7
580	182	240	189	243	F/A2	0	4.8	0.7	5.1
581	181	240	187	243	F/A2	2.1	4.2	2.7	4.6
582	175	240	184	243	F/A2	12.7	2.5	14.9	2.6
583	183	240	188	243	F/A2	1.2	5.4	1.7	6
584	184	240	188	243	F/A2	1.4	5.4	1.8	5.4

c) Haemoglobin D- ***peak position through each method, zone classification and peak percentage between methodologies.***

Sample number	HbA1c peak position	HbA1c A2 Position	Hb (E) Position	Hb (E) A2 Position	Phoresis Zone	A1c Peak %	HbA1c A2 peak %	Hb (E) Peak %	Hb (E) A2 Peak %
585	200	240	208	243	D/A2	22.6	4.4	25.6	4.3
586	200	240	207	243	D/A2	38.6	2.8	41	3
587	200	240	204	243	D/A2	38.6	2.7	40.3	3
588	201	240	207	243	D/A2	37	2.6	39.8	3
590	200	240	208	243	D/A2	35.6	2.2	40.7	2.7
591	201	240	207	243	D/A2	36.9	2.5	39.9	2.8
592	200	240	207	243	D/A2	38.7	2.5	41.7	2.8
593	201	240	208	243	D/A2	34.4	2.7	36.1	2.9

594	201	240	207	243	D/A2	36.3	2.3	40.1	2.6

d) Haemoglobin S- ***peak position through each method, zone classification and peak percentage between methodologies.***

Sample number	A1c peak position	A1c A2 peak position	Hb S Position	Hb A2 Position	Hb Zone	A1c S Peak %	A1c A2 peak %
595	212	240	214	243	S/A2	36.1	2.4
596	212	240	216	243	S/A2	0.7	1.1
597	212	240	215	243	S/A2	30.9	3.4
598	212	240	214	243	S/A2	29.8	2.8
599	218	240	212	243	S/A2	65.9	2.1
600	212	240	214	243	S/A2	31.2	3
601	212	240	212	243	S/A2	70.6	2.2
602	210	240	214	243	S/A2	23.2	4
603	212	240	214	243	S/A2	35.6	2.4
604	212	240	214	243	S/A2	36.4	3.1
605	212	240	215	243	S/A2	36.3	3.1
606	212	240	214	243	S/A2	31.4	2.6
607	212	240	214	243	S/A2	36.4	2.6
608	212	240	214	243	S/A2	36.7	2.8
609	212	240	214	243	S/A2	29.3	2.8
610	212	240	214	243	S/A2	37.8	2.7
611	212	240	214	243	S/A2	37.1	2.9
612	212	240	214	243	S/A2	28.4	2.6
613	212	240	214	243	S/A2	30.9	3.4
614	212	240	214	243	S/A2	36.8	2.3
615	212	240	214	243	S/A2	35.9	3
616	212	240	214	243	S/A2	33.1	2.7
617	212	240	214	243	S/A2	34.4	2.5
618	212	240	214	243	S/A2	34.6	2.3
619	212	240	214	243	S/A2	32.9	2.8
620	212	240	214	243	S/A2	36.6	2.2
621	212	240	214	243	S/A2	25.4	2.8
622	213	240	214	243	S/A2	29.4	3
623	213	240	214	243	S/Q2	31.9	3
624	212	240	214	243	S/A2	31.1	3.1
625	212	240	214	243	S/A2	34.7	1.6
626	212	240	214	243	S/A2	34.8	2.9
627	212	240	214	243	S/A2	35.6	2.7
628	212	240	215	243	S/A2	28.6	2.8
629	212	240	214	243	S/A2	34.9	2.7
630	212	240	214	243	S/A2	35.2	2.9

e) Hamoglobin E- ***peak position through each method, zone classification and peak percentage between methodologies.***

Sample number	A1c peak position	A1c A2 peak position	Hb Peak position	Hb A2 Position	Hb Zone	A1c E Peak %	A1c A2 peak %	Hb E Peak %	Hb A2 Peak %
631	226	240	228	243	E/A2	23	3.7	26.6	3.7
632	226	240	227	243	E/A2	21.7	3.4	24.4	3.3
633	226	240	229	243	E/A2	19.4	3.7	20.6	3.2
634	226	240	228	243	E/A2	14.2	3.1	16.2	3.4
635	226	240	227	243	E/A2	22.9	3.3	25.4	3.7
636	226	240	228	243	E/A2	22.2	3.5	25	3.4
637	226	240	228	243	E/A2	21	3.4	24.2	3.6
638	226	240	228	243	E/A2	24.9	3.4	27.2	3.4
639	226	240	227	243	E/A2	23.1	3.4	25.7	3.4
640	225	240	227	243	E/A2	22.1	3.7	25.1	3.8
641	226	240	227	243	E/A2	24.2	3.6	26.1	3.7
641	226	240	228	243	E/A2	13.4	4	16.1	4.2
643	226	240	228	243	E/A2	23.8	3.7	25.7	3.7
644	226	240	227	243	E/A2	24	3.1	26.8	3.4
645	226	240	228	243	E/A2	23.7	3.9	25.4	4.1
646	226	240	227	243	E/A2	22.7	4.1	24.7	4.3
647	226	240	228	243	E/A2	15.1	3.7	17.8	3.4
648	226	240	228	243	E/A2	23.7	3.5	26.4	3.7
649	226	240	228	243	E/A2	23.1	3.5	24.2	3.7
650	226	240	227	243	E/A2	21.3	3.3	23.8	3.7
651	226	240	227	243	E/A2	23.9	3.9	26.5	4.1
652	226	240	227	243	E/A2	20.7	3.5	24	3.8
653	226	240	227	243	E/A2	20	3.2	21.6	3.5
654	226	240	228	243	E/A2	13.9	3.8	16	3.8
655	226	240	227	243	E/A2	22.2	4	25.2	4.1
656	226	240	227	243	E/A2	22.2	3.8	23.7	3.9
657	226	240	228	243	E/A2	19.8	3.7	21.5	3.9
658	226	240	229	243	E/A2	90.6	0	90.3	0
659	226	240	227	243	E/A2	24.8	3.4	26.6	3.6
660	226	240	227	243	E/A2	23.5	3.3	25.2	3.6