Fibrinogen glycation and glycaemic control in type 2 diabetic subjects

LT Göttsche BDietetics

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Supervisor: Dr M Pieters
Co-supervisor: Dr DG van Zyl

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ABSTRACT

Type 2 diabetes mellitus is a worldwide pandemic that causes both micro- and macrovascular complications. Although the causal relationship between chronic hyperglycaemia and microvascular disease have been established, the relationship between chronic hyperglycaemia and macrovascular disease (including cardiovascular disease (CVD)) is not yet well defined. A possible causal mechanism may be related to the glycation of haemostatic proteins such as fibrinogen. Hyperglycaemia causes non-enzymatic glycation of proteins through direct binding of carbohydrates (glucose and to a minor extent fructose) to proteins. The results of this study indicated that uncontrolled African type 2 diabetic subjects had a significantly higher level of fibrinogen glycation than non-diabetic subjects and that achievement of glycaemic control indeed resulted in a significant decrease in fibrinogen glycation. Glycated fibrinogen also correlates with and compares well with HbA1c in monitoring glycaemic control. By correlating end fibrinogen glycation levels with the average fasting capillary glucose of different 4-day time-intervals (fibrinogen has a half-life of 4 days) the study indicated that fibrinogen could be used as a short-term indicator of glycaemic control. Because fibrinogen is involved in vascular disease itself, glycated fibrinogen may be a better long-term predictor of CVD than current markers of glycaemic control. It may also aid in the elucidation of the relationship between hyperglycaemia and CVD. The results of this study showed that fibrinogen glycation is indeed sensitive to fluctuations in glycaemic control.

ABSTRAK

Tipe 2 diabetes mellitus is 'n wêreldwye pandemie wat beide mikro- en makro-vaskulêre komplikasies veroorsaak. Alhoewel die oorsaak-verwantskap tussen chroniese hiperglisemie en mikro-vaskulêre siektes goed gedefinieer is, is die verwantskap tussen chroniese hiperglisemie en makro-vaskulêre komplikasies (insluitend kardio-vaskulêre siektes (KVS)) nog nie goed gedefinieer nie. 'n Moontlike meganisme mag verwant wees aan die glikosilering van haemostatiese poteïne soos fibrinogeen. Hiperglisemie veroorsaak nie-ensiematiese glikosilering van proteïne deur direkte binding van koolhidrate (glukose en tot 'n mindere mate fruktose) aan proteïne. Die resultate van hierdie studie dui aan dat ongekontroleerde Afrika tipe 2 diabetiese proefpersone 'n betekenisvolle hoër vlak van fibrinogeen glikosilering as nie-diabetiese proefpersone het en dat die bereiking van glisemiese kontrole wel 'n betekenisvolle verlaging in fibrinogeen glikosilering veroorsaak. Geglikosileerde fibrinogeen korreleer en vergelyk ook goed met HbA1c in die monitor van glisemiese kontrole. Deur end fibrinogeen waardes met die gemiddelde vastende kappilêre glukose van verskillende 4-dag tydsintervalle (fibrinogeen het 'n half-leeftyd van 4 dae) te korreleer, het die studie aangedui dat fibrinogeen ook as 'n kort-termyn indikator van glisemiese kontrole gebruik kan word. Omdat fibrinogeen self betrokke is by vaskulêre siektes, mag geglikosileerde fibrinogeen 'n beter lang-termyn voorspeller van KVS wees as die huidige merkers van glisemiese kontrole. Dit mag ook help om die verwantskap tussen hiperglisemie en KVS te verduidelik. Die resultate van hierdie studie het aangedui dat fibrinogeen glikosilering wel sensitief is vir veranderinge in glisemiese kontrole.

ABBREVIATIONS

CVD: Cardio-vascular disease

AGE: Advanced glycated end product

ROC: Receiver Operator Characteristic

HbA1c: Glycated haemoglobin

WHO: World Health Organisation

SADA: South African Diabetes Association

NIDDM: Non-insulin-dependant diabetes mellitus

IGT: Impaired glucose tolerance

IFG: Impaired fasting glucose

DKA: Diabetic ketoacidosis

HHS: Hyperosmolar hyperglycaemia state

ADA: American Diabetes Association

PKC: Protein kinase C

3-DG: 3-Deoxyglucosone

MGO: Methylglyoxal

CML: Carboxymethyl lysine

DOLD: Deoxyglucasone-lysine dimmer

GOLG: Glyoxal-lysine dimmer

MOLD: Methyl glyoxal lysine dimmer

VCAM-1: Vascular cell adehesion molecule-1

IL: Interleukin

TNF: Tumor necrosis factor

LDL: Low-density lipoprotein

AACE: American Association of Clinical Endocrinologists

HDL: High-density lipoprotein

VLDL: Very low-density lipoprotein

CNBr: Cyanogen-bromide

PAI: Plasminogen activator inhibitor

BMI: Body mass index

OGTT: Oral glucose tolerance test

SGM: Self-glucose monitoring

FBG: Fasting blood glucose

ELISA: Enzyme-linked immunosorbent assay

IM: Intra-muscular

HOMA: Homeostasis model assessment

CV: Coefficient volumes

MW: Molecular weight

HCl: Hydrochloride

NaCl: Sodium chloride

Na_2CO_3: Sodium carbonate

NaOH: Sodium hydroxide

$NaHCO_3$: Sodium bicarbonate

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CHAPTER 1: INTRODUCTION

1.1. Background and motivation

Type 2 diabetes mellitus is a worldwide pandemic that causes both micro- and macrovascular complications. Data from the Diabetes Control and Complications Trial (DCCT) have established the casual relationship between hyperglycaemia and micro-vascular diseases (The Diabetes Control and Complications Trial Research Group, 1993; The Diabetes Control and Complications Trial/ Epidemiology of Diabetes Interventions and Complications Research Group, 2000). The casual relationship between hyperglycaemia and macrovascular disease is however not as clearly understood although the bulk of evidence, confirm a relationship between glycaemic control and the extent of macro-vascular complications (Boyne & Saudek, 1999; Haffner, 1999).

Hyperglycaemia may cause atherosclerosis by several mechanisms. One of these is non-enzymatic glycation of plasma proteins. This may result in structural changes that may in turn affect the function of these proteins.

Fibrinogen has been shown to be an independent risk marker for cardiovascular disease (CVD) (Stec *et al.*, 2000; Danesh *et al.*, 2005) and may be related to the CVD increase seen amongst diabetic subjects. Diabetic patients have been shown to have high fibrinogen levels and that fibrinogen undergoes non-enzymatic glycation in the presence of uncontrolled blood glucose levels (Lütjens *et al.*, 1985). This glycation of fibrinogen may alter its structure and thus also its function. The study's focus is to help explain the role of glycated fibrinogen in the relationship between type 2 diabetes, hyperglycaemia and macro-vascular disease.

1.2 Aims and objectives

The study had two main aims of which the first was to determine whether controlling blood glucose levels in outpatient type 2 diabetic subjects would affect fibrinogen glycation. The other was to determine how well glycated fibrinogen compares to glycated haemoglobin (HbA1c) in its relation to glycaemic control.

The study included twenty uncontrolled type 2 black African diabetic subjects and twenty non-diabetic subjects (age and body mass index matched) as a reference group. During the first phase of the intervention period the diabetic subjects were seen by a diabetes educator. They were taught how to do self-glucose monitoring, how to co-ordinate insulin-use with meals, how to manage hypoglycaemia as well as glucagon administration. Baseline samples were taken at the end of Phase 1. During the second phase, the diabetic subjects were controlled with insulin, while the use of metformin was continued. Once glycaemic control was achieved, subjects

remained treated for 8 days (Phase 3) after which end samples were drawn. Blood samples of the non-diabetic subjects were drawn within one week of their matched diabetic subject's blood sampling. Both groups were outpatients because such a study would reflect real life situations.

1.3 Structure of dissertation

The dissertation consists of five chapters, including this introduction chapter.

The literature chapter provides some background information to the prevalence, pathogenesis and complications of type 2 diabetes mellitus. Markers of glycaemic control that are currently used are discussed. The relationship between fibrinogen and CVD are discussed where the characteristics of fibrinogen and the hypercoagulable state of diabetes and CVD are conferred. However, only the causes for the hypercoagulable state related to the topic of the dissertation are discussed which include elevated plasma fibrinogen, endothelial dysfunction and glycation of haemostatic proteins. How to determine glycated fibrinogen are explained, while some evidence for the use of fibrinogen as a marker of glycaemic control is provided.

Subject recruitment and characteristics are explained in the methodology chapter. The methodology chapter also deals with the study design, how blood sampling was done as well as how the analytic procedures and statistics were done.

The results of the study are discussed in chapter 4. This includes the SDS-PAGE analysis, baseline characteristics, baseline to end changes (deltas) and Receiver Operator Characteristic (ROC) curves. End glycated fibrinogen values were correlated with fasting capillary values at different time-intervals and these results are also given. The discussion on these results follows in chapter 5 where recommendations for further research are also given.

An article on the study was accepted for publication in Thrombosis Research (see Annexure A).

CHAPTER 2: LITERATURE SURVEY

2.1 Introduction

Type 2 diabetes is a widespread disease with a significant impact on the world's population. Individuals with diabetes need to monitor their blood glucose levels in order to achieve optimum glycaemic control. Poor glycaemic control may cause micro- and macrovascular complications. Although the casual relationship between chronic hyperglycaemia and microvascular disease have been established, the relationship between chronic hyperglycaemia and macrovascular disease (including CVD) is not yet well defined. A possible casual mechanism may be related to the glycation of haemostatic proteins such as fibrinogen.

Currently glycaemic control is measured by using the fructosamine assay or HbA1c. Since fibrinogen plays a role in CVD and is known to be elevated amongst diabetic subjects, it may be useful to study the effect of glycaemic control on glycated fibrinogen. It may provide more information between the relationship between hyperglycaemia and macrovascular disease.

Furthermore, HbA1c and fructosamine have the limitation that it measures glycaemic control as an average of a relatively long time period. The fructosamine assay and HbA1c measures glycaemic control for a 14 and120 day period respectively. Since fibrinogen has a half-life of four days, glycated fibrinogen could possibly be used as an indicator of glycaemic control over a shorter period of time.

In this chapter the prevalence, pathogenesis and complications of type 2 diabetes will be discussed. The currently used markers of glycaemic control are addressed, while evidence for the possible use of fibrinogen as a marker is provided. Characteristics of fibrinogen, its importance in diabetes and CVD and how to determine glycated fibrinogen is discussed.

2.2 Type 2 Diabetes

2.2.1 Prevalence

The World Health Organisation (WHO) estimates that there are currently 150 million cases of Type 2 diabetes worldwide. This is expected to double by 2025 (Visser *et al.*, 2003; WHO, 2003). The 2003 prevalence of diabetes in South Africa was 2 million and this is increasing at a rate of approximately 11% per annum (Novo Nordisk (pty) Ltd., 2003). The South African Diabetes Association (SADA) estimates that there are up to date, 3 million individuals in South Africa with type 2 diabetes, half of which have not yet been diagnosed (Marais, 2005).

2.2.2 Pathogenesis

Type 2 Diabetes Mellitus was until recently better known as non-insulin-dependant diabetes mellitus (NIDDM) or adult-onset diabetes mellitus. It is more commonly, but not exclusively, found in older individuals and certain ethnic groups (Parillo & Riccardi, 2004). The major pathogenic factor of type 2 diabetes is insulin resistance. Where insulin resistance is present, the demand for insulin production and secretion to maintain normoglycaemia is increased. Type 2 diabetes develops when the ability of the beta cells to secrete insulin cannot adequately compensate for the degree of insulin resistance. Thus, a predominant insulin resistance occurs with relative insulin deficiency. Type 2 diabetes may also be due to a predominant secretory defect in conjunction with insulin resistance (Lipkin, 1999; The expert committee on the diagnosis and classification of diabetes mellitus, 2002).

Insulin resistance is generally defined as a reduction in the ability of insulin to regulate glucose metabolism properly. A more complex definition is the inability of insulin to stimulate peripheral glucose disposal, reduce hepatic glucose production, suppress lipolysis, increase adipose tissue lipoprotein lipase, stimulate sodium reabsorption, alter vascular tone and act as a growth factor (Lipkin, 1999).

Key factors related to the development of insulin resistance are obesity and/or central adiposity (Augustin *et al.*, 2002; Bonoro *et al.*, 2002; Bavenholm *et al.*, 2003; Lawlor, 2003; Greenfield & Chambell, 2004). High intake of refined carbohydrates increases insulin secretion on demand, resulting in hyperinsulinaemia, which may down-regulate insulin receptors and eventually reduce insulin efficiency, thereby resulting in insulin resistance (Augustin *et al.*, 2002). Insulin resistance is also associated with diminished physical activity because insulin-stimulated glucose uptake is limited (Augustin *et al.*, 2002; Bavenholm *et al.*, 2003).

Aging is another important factor in the development of insulin resistance, probably because body weight tends to increase over the age of 55 years, with a concomitant decrease in lean body mass. There is also a decrease in growth hormone action in the elderly shown by a decrease insulin-growth factor 1 levels. Growth hormone deficiency is associated with increased fat mass, decreased muscle mass and subsequently decreased insulin sensitivity (Bloomgarden, 2002).

Hyperglycaemia, dyslipidaemia and hypertension can cause endothelial dysfunction, which may result in insulin resistance, by virtue of an impaired delivery of glucose and insulin to target tissues (Bonoro *et al.*, 2002; Caballero, 2003). Low birth weight, low offspring birth weight, short adult leg length and childhood social class were found to be independently associated with adult insulin resistance. One explanation for the association between low birth weight and

the risk of type 2 diabetes is that poor intrauterine nutrition leads to small birth size and foetal programming that result in changes in glucose metabolism, which might lead to insulin resistance and eventually type 2 diabetes (Lawlor, 2003).

Another theory, also known as the foetal insulin hypothesis, suggests that genetic polymorphisms that lead to increased insulin resistance and insulin-mediated impaired foetal growth underlie the association between birth weight and type 2 diabetes. The theory are supported by the finding that offspring birth weight is associated with increased risk of parental (in both mothers and fathers) insulin resistance and diabetes (Lawlor, 2003). The mechanism behind the association between offspring birth weight has not been significantly proven (Bloomgarden, 2002).

An explanation for the interaction between birth weight and adult body mass index (BMI) can be found in the "thrifty phenotype hypothesis". This hypothesis suggests that the foetus adapts to a poor intrauterine milieu by optimising the use of a reduced nutrient supply to ensure survival, but favouring the development of certain organs over that of others. This leads to persistent alterations in growth and function of developing tissues. An example hereof is an increased peripheral muscle resistance to the action of insulin in the developing foetus so that glucose is preferentially diverted to essential organs, such as the brain. Later in life, the adult has an increased risk for insulin resistance when he/she becomes obese, since he/she was "programmed" that glucose should preferentially be diverted to other organs. (Lawlor, 2003; Simmons, 2006).

Adult leg length is an indicator of childhood nutrition. Interruption of growth, due to nutrition deprivation, at any stage in an individual's life course, leads to relatively short legs and long torso. This is especially so when the interruption occurred in early childhood. The association between leg length, childhood social class and the risk of diabetes, appear to be mediated through insulin resistance (Lawlor, 2003).

Although insulin resistance is a precursor for type 2 Diabetes, individuals does not necessarily have diabetes when they are insulin resistant. There are different diagnostic cut-off values to determine in which category an individual falls. Table 2.1 below, adapted from World Health Organisation (1999), shows the plasma and blood values for the diagnosis of diabetes mellitus, impaired glucose tolerance (IGT) and impaired fasting glucose (IFG). Individuals with IFG and IGT are at risk of developing diabetes because it indicates a level of insulin resistance. One is placed in a category depending on whether only fasting glucose or fasting glucose and 2h glucose values were obtained. If the value(s) falls into two different categories, the higher one applies (Unwin *et al.*, 2002).

Table 2.1: Values for the diagnosis of diabetes mellitus, impaired fasting glucose (IFG) and impaired glucose tolerance (IGT) (World Health Organisation, 1999).

	Glucose concentration, mmol/l		
	Plasma	**Whole blood**	
	Venous	**Venous**	**Capillary**
Diabetes Mellitus			
Fasting concentration	≥7,0	≥6,1	≥6,1
2h post- glucose load	≥11,1	≥10,0	≥11,1
IGT			
Fasting concentration	<7,0	<6,1	<6,1
2h post-glucose load	7,8-11,0	6,7-9,9	7,8-11,0
IFG			
Fasting concentration	6,1-6,9	5,6-6,0	5,6-6,0
2h post glucose load	<7,8	<6,7	<7,8

2.2.3 Complications

Complications in type 2 diabetes are a result of various factors such as poor glucose control, insulin resistance, impaired lipid profile and oxidative stress. Acute complications generally include diabetic ketoacidosis (DKA) and hyperosmolar hyperglycaemia state (HHS) (ADA, 2001). These complications usually occur when various aggravating, causative factors are present, such as: infection, cerebrovascular accident, alcohol abuse, pancreatitis, myocardial infarction, trauma and certain pharmaceuticals that influence carbohydrate metabolism such as corticosteroids, thiazides and sympathomimetic agents. Discontinuation or omission of insulin use may also cause DKA or HHS (ADA, 2002).

Long-term complications as described by the American Diabetic Association (ADA) include micro-vascular complications and macro-vascular complications (ADA, 2001). Microvascular complications can be caused by hyperglycaemia (Lebovitz, 2001) and include:

- Retinopathy (with potential loss of vision),
- Nephropathy (leading to renal insufficiency and renal failure),
- Peripheral neuropathy (results in pain, loss of sensation and muscle weakness that increase the risk of foot ulcers, gangrene and Charcot joints),
- Autonomic neuropathy (that affects gastrointestinal, cardiovascular and genitourinary function)(ADA, 2001; The expert committee on the diagnosis and classification of diabetes mellitus, 2002; Parillo & Riccardi, 2004).

Macrovascular complications in general comprise of cardio-vascular diseases (CVD) that include myocardial infarctions and stroke (Parillo & Riccardi, 2004). Type 2 diabetes increases the risk of cardiovascular mortality from 40% to 200%. It is considered as an independent risk factor for CVD mortality regardless of other risk factors (Unwin *et al.*, 2002) such as obesity, smoking, hypertension, dyslipidaemia, physical inactivity and poor regulated glycaemic control (ADA, 2001; DECODE Study group & the European Diabetes epidemiology group, 2001; Saydah *et al.*, 2001).

The Diabetes Control and Complications Trial (DCCT) have established a definite causal relationship between chronic hyperglycaemia and microvascular disease (DCCT, 1993; DCCT, 2000). However, the relationship between chronic hyperglycaemia and macrovascular complications is less well defined. Most of the evidence however, confirms a relationship between glycaemic control and the extent of macrovascular complications (Boyne & Saudek, 1999; Haffner, 1999).

Although both IGT and IFG are associated with in an increased CVD risk (Unwin *et al.*, 2002), a study done by Kuusisto *et al.* (1994) found no statistically significant increase in CVD among subjects with impaired glucose tolerance (Kuusisto *et al.*, 1994). Several other studies (Haffner *et al.*, 1997; Ito *et al.*, 1996; Meigs *et al.*, 1997) also failed to show an association between glycaemia and macro-vascular disease.

The cause of macrovascular complications is multifactorial, with hyperglycaemia being one of the responsible factors. Several mechanisms by which hyperglycaemia may cause tissue damage that ultimately leads to vascular disease have been proposed (Lebovitz, 2001). These include:

- Increased hexosamine pathway activity (Brownlee, 2005).
- Increased flux through the polyol/ sorbitol pathway (Gugliucci, 2000; Brownlee, 2005).
- Activation of the diacylglycerol-protein kinase C (PKC) second messenger system (Gugliucci, 2000; Lebovitz, 2001).
- Protein glycation and advanced glycated end products (AGE) formation Gugliucci, 2000; Lebovitz, 2001).

Of these mechanisms only protein glycation will be discussed in more detail as it pertains to the topic of this dissertation.

Glycated end products such as glycated fibrinogen, presents an important pathogenic mechanism underlying long-term complications of type 2 diabetes (Osei *et al.*, 2003), because hyperglycaemia increases the concentration of AGEs (Gugliucci, 2000; Dunn *et al.*, 2005; Jaleel *et al.*, 2005; Coppola *et al.*, 2006). The formation of AGEs involves non-enzymatic glycation

reactions between amino acids of extra-cellular proteins and glucose. A Schiff base forms through a condensation reaction of the carbonyl group of sugar aldehydes, with the free amino groups or NH_2 terminus of proteins (Arocha-Piňango, 1987; Singh *et al.*, 2001; Jaleel *et al.*, 2005). In the case of fibrinogen, it seems as if glycation occurs at the free amino group of lysine (Arocha-Piňango, 1987).

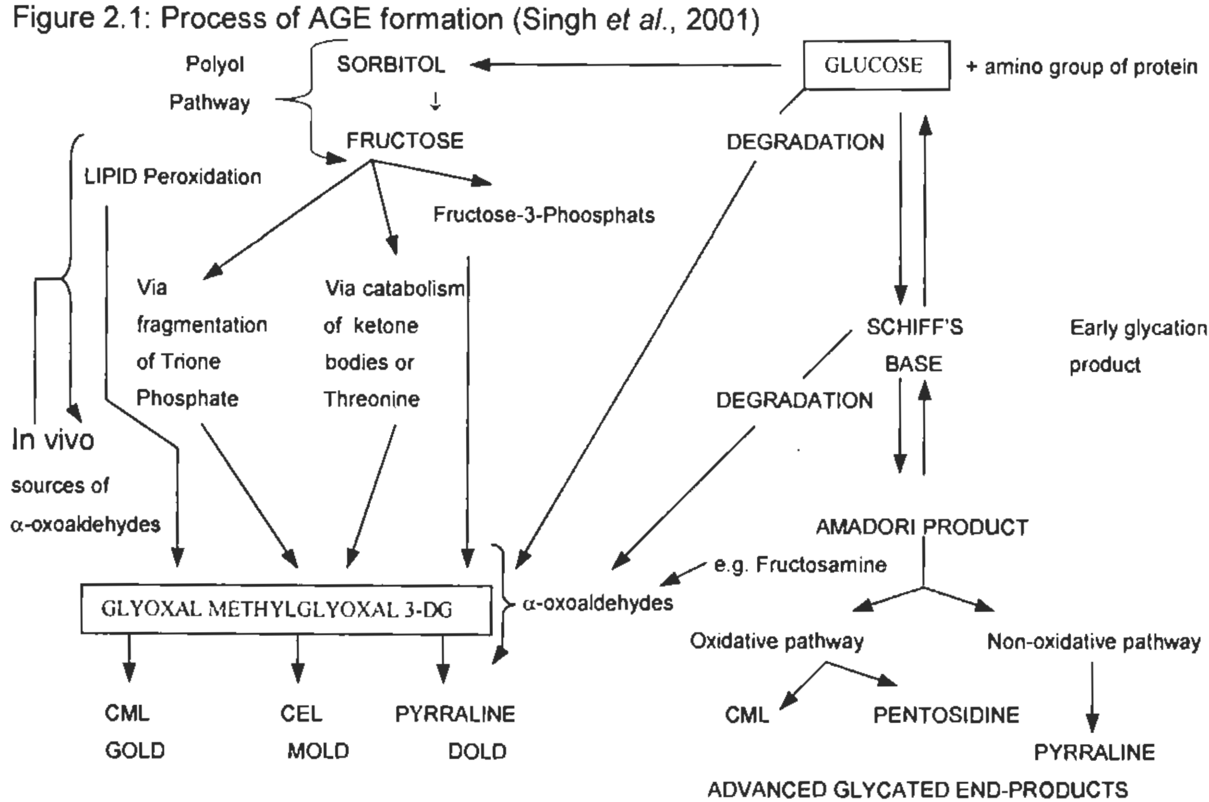
The Schiff base undergoes rearrangement through reversible acid-based catalysis to form intermediate Amadori adducts. Amadori adducts are the first stable product of the Maillard reaction. These compounds are known as α-dicarbonyls or oxoaldehydes. Examples hereof are HbA1c, fructosamine, 3-deoxyglucosone (3-DG) and methylglyoxal (MGO). 3-DG is formed from fructose-3-phosphate (a product of the polyol pathway that has been associated with hyperglycaemia induced diabetic complication) by non-oxidative rearrangement and hydrolysis of Amadori adducts. MGO may be derived from fructose, ketone bodies and threonine that may induce oxidative stress. AGEs form through irreversible chemical reactions of the Amadori adducts. Because it remains irreversibly attached to proteins and continue to accumulate over the entire lifespan of proteins, it may be a valuable marker for long-term hyperglycaemia (Arocha-Piňango, 1987; Singh *et al.*, 2001;Jaleel *et al.*, 2005).

The process of AGE formation is illustrated in figure 2.1. The structure of some AGE have been identified as N-ε-(carboxymethyl)lysine (CML), pentosidine, pyralline, deoxyglucasone-lysine dimer (DOLD), glyoxal-lysine dimer (GOLD) and methyl glyoxal lysine dimer (MOLD) (Singh *et al.*, 2001).

AGE formation is catalysed by transitional metals and is inhibited by reducing agents such as ascorbate. Glycation is enhanced in diabetes because it is concentration dependant in the early stages of the Maillard reaction rather than in the later stages, and intracellular sugars such as fructose have a much faster glycation rate than glucose.

AGEs may modify the functional group of proteins that produce abnormal interactions between molecules that may lead to their distorted functions (Arocha-Piňango, 1987; Jaleel *et al.*, 2005). Table 2.2 below, adapted from Singh *et al.* (2001), shows the biological effects of AGEs at cellular level. The formation of AGEs are usually endogenous but can be derived from tobacco smoke and food. Heat applied to food can lead to the Maillard reaction that is responsible for the non-enzymatic browning. Both the effects of smoking and ingesting AGEs on glycation in vivo have not been studied extensively especially amongst diabetic individuals. Thus, more studies are required to differentiate between exogenous and endogenous derived AGEs and their relation to histological damage (Singh *et al.*, 2001).

Figure 2.1: Process of AGE formation (Singh *et al.*, 2001)



Furthermore, could the incidence of macrovascular disease be explained by the glucose hypothesis. According to this hypothesis poor glycaemic control is the cause of the accelerated atherosclerosis. Hyperglycaemia might cause atherosclerosis through the production of oxidized low-density lipoprotein (LDL) cholesterol, haemorrheological changes, changes in vascular reactivity and glycation of end products that include proteins of blood vessel walls (Feener & King, 1997). However, the effects of uncontrolled diabetes on macro-vascular diseases go beyond hyperglycaemia and hyperinsulinaemia. It could be as a result of associated dysmetabolism, abnormalities of clotting factors, hypertension, or other ill-defined conditions, that accompany under-insulinazation (Boyne & Saudek, 1999). Nevertheless, optimal glycaemic control is important because it could improve cardiovascular outcome in type 2 diabetes (Opie *et al.*, 2006).

Table 2.2: Biological effects of AGEs at cellular level (Singh *et al.*, 2001).

AGEs causes:	
Lipid peroxidation causing	-Endothelial dysfunction -↑ Reactive oxygen species production -↓ Superoxide Dismutase function -↓ Nitric Oxide -↑ Endothelin-1
Stimulation of inappropriate cellular activity that may ↑ tissue remodelling and thickening of the basement membrane	-↑ Vascular cell adhesion molecule-1 (VCAM-1) -↑ Secretion of cytokine interleukin-1 (IL-1), tumor necrosis factor-β (TNF-β), IGF-1A -↑ Mitogenesis -↑ Chemotaxis of mononuclear cells -↑ T-cell stimulation and Interferon-γ production
Structural changes	-↑ Disruption of molecular order and changes in surface charge eg. by causing conformational changes in collagen leading to premature ageing in diabetes mellitus -↑ Irreversible cross-linking between proteins -↑ Cell membrane and matrix changes as in diabetic kidney disease -↑ Interference of cell-matrix interaction affecting adhesion and spreading
Thrombosis and Fibrinolysis	-↑ Tissue factor -↓ Thrombomodulin -↑ Platelet aggregation and fibrin stabilisation that - ↓ Platelet survival - ↑ Platelet "stickiness" following glycation of platelet glycoprotein receptors IIB and IIIA - ↓ Reduced sensitivity of fibrin/ fibrinogen to plasmin following glycation - ↓ Reduced heparin catalysed thrombin activity following glycation of anti-thrombin III

2.3 Glycaemic control

The objective of diabetes therapy is attaining optimal glycaemia. Optimal glycaemia is defined as preprandial blood glucose level of 4,4 to 6,7 mmol/L and a bedtime blood glucose level of 5,6 to 7,8 mmol/L (Lipkin, 1999). These values are generalised, thus patients with comorbid

diseases, very young or older patients, pregnant women and patients with unusual conditions may need different treatment goals or target values (ADA, 2001).

Currently, there are two markers of long-term glycaemic control namely HbA1c and fructosamine.

2.3.1 Markers and target values

2.3.1.1 HbA1c

HbA1c is formed when glucose combines, non-enzymatically with haemoglobin in erythrocytes (Osei *et al.*, 2003). Erythrocytes have an average half-life of 120 days and therefore, haemoglobin within the erythrocyte has a relatively long life in blood. HbA1c has a half-life of approximately 29 days and decays slowly (Jaleel *et al.*, 2005). It reflects glycaemic control over the preceding 6-12 weeks and is a significant predictor of long-term complications of type 2 diabetes (The Diabetes Control and Complications Trail Research Group, 1993; Singh *et al.*, 2001; Brand-Miller *et al.*, 2003;Osei *et al.*, 2003; Miciagna *et al.*, 2004).

Although, HbA1c represents both fasting and postprandial glycaemic states, it cannot be used independently for screening or diagnosing diabetes because it has a 65% sensitivity and a 94% specificity for the diagnosis of diabetes (ADA, 1999; Rohlfing *et al.*, 2002; Yates & Laing, 2002; Monnier *et al.*, 2003). It also correlates more with early or late postlunch glucose levels than with fasting glucose levels (Heine *et al.*, 2004) and does not reflect the quality of diabetes control or fluctuating hyperglycaemia due to its long half-life (Hammer *et al.*, 1989). A short period of hypo- or hyperglycaemia before HbA1c measurement will not alter the results and therefore it is possible that such conditions may be masked by a normal or near normal measurement. This might be problematic, especially among insulin treated diabetic individuals because, nocturnal hypoglycaemia is common among them (Peacock, 1984).

Nevertheless, HbA1c is a surrogate marker for biological products of hyperglycaemia that is associated with diabetic complications and therefore it is a valuable tool in the monitoring of diabetes. Sustained elevated plasma glucose values of more than 10mmol/L corresponds to an HbA1c concentration of 10% or more (Lipkin, 1999). The goal for most patients is to achieve an HbA1c concentration of <7% in order to reduce complications (Lipkin, 1999; ADA, 2001; DeWitt & Hirsch, 2003). The American Association of Clinical Endocrinologists (AACE) recommends a value of <6,5% (AACE, 2002). The non-diabetic range is 4-6% (ADA, 2001).

2.3.1.2 Fructosamine

Fructosamine levels are related to glycaemic control in the preceding 2-4 weeks (Winocour *et al.*, 1988; Gugliucci, 2000; Miciagna *et al.*, 2004). The fructosamine assay reflects glycation of

total serum proteins and has a half-life of approximately 16,5 days. Albumin is the major component measured by the fructosamine assay. Due to its half-life of 19 days (Jaleel *et al.*, 2005), the assay provides an indication of glycaemic control over a relatively long period of time. Futhermore, fructosamine is based on reducing chemistry and therefore, any reducing substance present in serum can affect the value measured (Hammer *et al.*, 1989).

Because both HbA1c and fructosamine are makers of glycaemic control for a relatively long preceding period, other glycated plasma proteins with shorter half-lives may be more effective to indicate the short-term effects of treatment. This will allow earlier intervention strategies, that will eventually assist in the prevention of long-term complications of hyperglycaemia.

Fibrinogen has a half-life of four days. Glucose binds non-enzymatically to it (Mirshahi *et al.*, 1987), causing it to become a glycated plasma protein. Therefore, fibrinogen may be a valuable short-term indictor of glycaemic control that also correlates well with HbA1c levels (Hammer *et al.*, 1989).

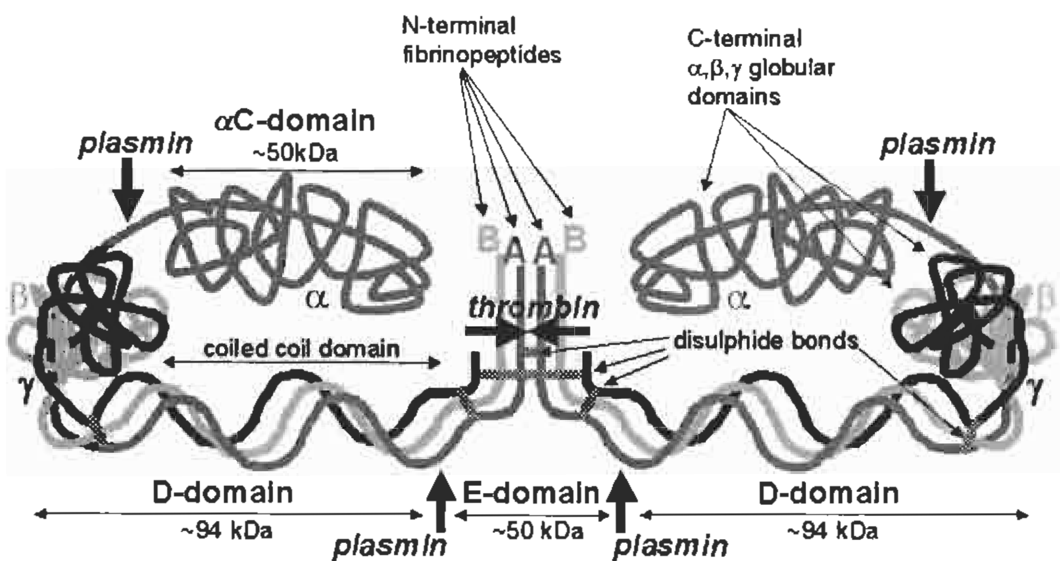
Rakhimova *et al.* (1999) found glycated fibrinogen to be an indicator of glycaemia levels during 3-4 days before measurement. They also found that glycated fibrinogen, in contrast with HbA1c, can be used to express carbohydrate metabolism after starting a new treatment strategy (Rakhimova *et al.*, 1999). Other studies that provide some evidence for the use of fibrinogen as marker of glycaemic control is discussed in section 4.4.

2.4 Fibrinogen and cardio-vascular disease

2.4.1 Structure and function of fibrinogen

Fibrinogen is a large fibrous glycoprotein (Weisel, 2005) with a molecular weight of 340,000 DA (Manten *et al.*, 2004). It is synthesized in the liver (Ko *et al.*, 1997) and has three pairs of polypeptide chains (Aα, Bβ and γ) linked together with 29 disulfide bonds and is 45nm in length. The molecule consists of three main structural regions, a central region (E) and two distal regions (D) connected to the E region by two α-helical coiled-coil rods (Scott *et al.*, 2004; Dunn *et al.*, 2005; Weisel, 2005). Region E contains fibrinopeptides A and B and the amino acid termini of all six polypeptide chains. The two D regions contain the carboxyl termini of the Bβ and γ chains and those from the Aα chain that extent to form flexible αC-domains (Figure 2.2) (Scott *et al.*, 2004). Calcium ions are important for the maintenance of its structure and function (Dunn *et al.*, 2005; Weisel, 2005).

Figure 2.2: Fibrinogen (Wellcome Trust Centre for Human Genetics, 2005).



Soluble fibrinogen can be converted to insoluble fibrin polymers via intermolecular interactions where two fibrinopetides, Aα and Bβ (at the central region of fibrinogen molecule) are cleaved by trombin at region E. Fibrin monomers polymerise to yield a three-dimensional network, the fibrin clot, which is essential for hemostasis. Factor XIIIa, a transglutaminase, covalently binds specific glutamine residues in one fibrin molecule to lysine residues in another fibrin molecule via isopeptide bonds. This stabilises the fibrin clot against mechanical, chemical and proteolytic insults (Standeven *et al.*, 2002; Scott *et al.*, 2004; Weisel, 2005).

Fibrinogen is an acute phase protein, that also binds to activated αIIbβ3 integrin on the platelet surface, forming bridges necessary for platelet aggregation in hemostasis. It has important adhesive and inflammatory functions through specific interactions with other cells, because it specifically binds to other proteins such as fibronectin, albumin, thrombospondin, von Willebrand factor, fibulin, fibroblast growth factor-2, vascular endothelial growth factor, and interleukin-1 (Standeven *et al.*, 2002; Weisel, 2005).

Infection, inflammation, hypertension, smoking, advanced age, female gender, LDL cholesterol and triglycerides are associated with elevated fibrinogen levels (Scott *et al.*, 2004). Fibrinogen levels are in general higher in type 2 diabetic individuals, than in non-diabetic individuals (Missov *et al.*, 1996; Reid *et al.*, 2000; Mills *et al.*, 2002; Scott *et al.*, 2004; Coppola *et al.*, 2006). The Rotterdam Study found no significant difference in fibrinogen levels between individuals with type 2 diabetes and those without diabetes, but that fibrinogen levels are significantly elevated among individuals requiring insulin therapy (Missov *et al.*, 1996).

However, there are conflicting results regarding the effect of insulin on plasma fibrinogen levels. These differences could be related to different experimental techniques or differences in the insulin activity between type 1 and type 2 diabetic individuals, that may be related to underlying insulin resistance in type 2 diabetic individuals. The effect of elevated hormones such as glucagons may also contribute to an increase in fibrinogen levels (Dunn & Ariëns, 2004).

There are some evidence that improving metabolic control causes fibrinogen levels to fall (Ceriello, 1997), whereas other studies found that improvement in glycaemic control was not significantly associated with reduced plasma fibrinogen levels (Dunn & Ariëns, 2004). Fibrinogen levels in diabetic individuals may also result from the interaction between an individual's fibrinogen genotype and their glycometabolic control. That may also in part explain the conflicting results (Dunn & Ariëns, 2004). It has been found that glycaemic control has an effect on high fibrinogen levels in diabetic subjects with $\alpha 2 \alpha 2$ genotype (Ceriello *et al.*, 1998).

2.4.2 Hypercoagulable state of diabetes and CVD

There are various causes for the hypercoagulable state seen in diabetes mellitus (Table 2.3) of which only those related to the topic of this dissertation will be discussed.

Table 2.3: Lipid coagulation and fibrinolytic abnormalities seen in diabetes associated with increased cardiovascular risk adapted from Sowers & Lester (1999).

1. Elevated plasma levels of very low-density lipoprotein (VLDL), LDL, and lipoprotein(a).
2. Decreased plasma high density lipoprotein (HDL) cholesterol.
3. Increased lipoprotein oxidation.
4. Increased lipoprotein glycation.
5. Increased small dense LDL cholesterol products.
6. Decreased lipoprotein lipase activity.
7. Elevated plasma levels of factor VII and VIII.
8. Increased fibrinogen and plaminogen activator inhibitor 1 levels.
9. Elevated thrombin-antithrombin complexes.
10. Decreased antithrombin III, protein C and S levels.
11. Decreased plasminogen activators and fibrinolytic activity.
12. Glycation of haemostatic proteins.

2.4.2.1 Elevated plasma fibrinogen

Elevated plasma fibrinogen levels are an independent risk factor for CVD. Mechanisms by which fibrinogen may increase cardiovascular complications are:

- Fibrinogen specifically binds to activated platelets via glycoprotein IIb/IIa that contributes to platelet aggregation.
- High levels of fibrinogen promote fibrin formation that leads to clot formation.
- Fibrinogen contributes to plasma viscosity (Stec *et al.*, 2000).

Hyperglycaemia and/or insulin resistance may cause oxidative stress, a condition where high levels of free radicals are present. These free radicals activate thrombin formation that causes an increase in the production of prothrombin fragments (F1+2) and an increase in fibrinogen turnover that leads to an increase in fibrin and consequently the production of D-dimer. Both F1+2 and D-dimer regulate fibrinogen production in the liver and their increased release into the circulation may produce an increase in circulating fibrinogen. This suggests that high fibrinogen levels in plasma might be a risk marker for CVD because it reflects increased thrombin formation and thus an increased risk for a thrombotic event to occur (Ceriello, 1997).

2.4.2.2 Endothelial dysfunction

Endothelial function plays a role in regulating vascular tone, controls matrix protein synthesis, stimulates cell growth and migration, controls permeability, regulates thrombogenesis and modulates inflammatory responses (Lebovitz, 2001; Nettleton & Katz, 2005). Endothelial dysfunction is caused by insulin resistance, hypertriglyceridaemia, increased levels of LDL cholesterol, hyperglycaemia and hypertension (Rattan *et al.*, 1997; Tooke *et al.*, 1995. Lebovitz, 2001). Alterations in vascular endothelium associated with diabetes in turn may cause:

- Impaired fibrinolytic activity.
- Increased endothelial cell procoagulant activity.
- Increased endothelial cell surface thrombomodulin.
- Impaired plasmin degradation of glycated fibrin.
- Increased levels of advanced glycated end products (Sowers & Lester, 1999).

2.4.2.3 Glycation of haemostatic proteins

Glycation and/or the formation of AGEs is another mechanism responsible for the pro-coagulant state that often exists in individuals with diabetes (Sowers & Lester,1999).

Fibrinogen was found to be glycated in diabetic patients (Lütjens *et al.*, 1985). Despite it's relatively short half-life of four days, its clearance is exponential. It is therefore assumed that a small amount of fibrinogen may remain for a long time in circulated plasma leading to the presence of highly glycated fibrinogen. This may lead to poorly degradable fibrin that may be responsible for capillary occlusion and therefore also atherosclerotic complications in diabetic patients (Arocha-Piňango, 1987; Mirshahi *et al.*, 1987). Glycated fibrinogen is preferentially distributed in the extravascular compartment whereas non-glycated fibrinogen is found in the

intravascular compartment. This will result in an increased uptake of fibrinogen into vessel walls that could contribute to atherosclerotic disease progression in diabetic individuals (Dunn & Ariëns, 2004). Fibrinogen synthesis is also increased during inflammatory conditions like atherosclerosis (Ganda & Arkin, 1992; Ridker *et al.*, 2001).

Apart from fibrinogen, other haemostatic proteins have also shown to be glycated in individuals with diabetes. This structural change could possibly lead to an alteration in function. Examples of such proteins are:

- Plasminogen (Geiger & Binder, 1986).
- Antithrombin III (Brownlee *et al.*, 1984).
- Plasminogen activator inhibitor (PAI) (Cortizo & Gagliardino, 1991).

2.5 Determination of glycated fibrinogen

Various methods have been used to determine glycated fibrinogen in vitro and in vivo. The most frequently used methods will be discussed below.

Before glycated fibrinogen can be measured, fibrinogen needs to be isolated out of plasma. Some of the fibrinogen purifying methods are:

- Glycine precipitation (Hammer *et al.*, 1989).
- Fibrinogen antibody column such as the IF-1 antibody (Kamiya Biomedical company, Seattle, USA) bound to a cyanogen-bromide (CNBr) activated resin (Pharmacaeia bought over by Pfizer, New York, USA) (Takebe *et al.*, 1995).
- Precipitation with polyethylene glycol 6000 (Vila *et al.*, 1985).

Methods generally used for the determination of glycated fibrinogen include:

- Thiobarbuturic acid method for measuring S-hydroxymethyl furfural (Flückiger & Winterhalter, 1976).
- HPLC method for measuring furosine (Schleicher & Weiland, 1981; Lütjens *et al.*, 1985; Arocha-Piňango, 1987).
- Glucose incorporation into fibrinogen can be measured using scintillation counting. This was done for in vitro glycation (Ney *et al.*, 1985).
- Measurement of 1-deoxy-1-morpholino-D-fructose (DMF) (Suzuki *et al.*, 1990).
- GlyPro® assay (Genzyme Diagnostics, Cambridge, MA) that provides a direct measurement of glycated protein.

These methods express glycation as mol glucose bound per mol fibrinogen. Fibrinogen glycation can also be expressed as percentage glycated fibrinogen from total fibrinogen. This can be done by using an affinity chromatography column containing immobilised m-

aminophenyl boronic acid on cross-linked 6% beaded agarose (Glyco-Gel B, Pierce (UK) Ltd) (Hammer *et al.*, 1989).

In order for glycated fibrinogen to be used as a diagnostic tool, these methods need to be improved or new methods need to be developed as these methods are time consuming and labour intensive.

2.6 Evidence for the use of fibrinogen as marker of glycaemic control

There is some evidence that fibrinogen glycation can be used as a marker of glycaemic control, due to its short half-life. Studies (Hammer *et al.*, 1989; Bruno *et al.*, 1996; Ko *et al.*, 1997; Ceriello *et al.*, 1998; Lam *et al.*, 2000; Reid *et al.*, 2000; Jain *et al.*, 2001) found that glycated fibrinogen levels correlate well with HbA1c levels. Since HbA1c is currently an accepted marker for glycaemic control, this evidence suggests that fibrinogen may also be a marker thereof.

A cross-sectional study was undertaken by Hammer *et al.* (1989) where they measured glycated fibrinogen and HbA1c on a single occasion in non-diabetic subjects and well-controlled diabetic individuals. They also measured glycated fibrinogen and HbA1c in newly diagnosed diabetic patients receiving treatment for three to four consecutive weeks. In pregnant diabetic individuals deteriorating control is expected after delivery. Thus, in order to predict deteriorating diabetic control, Hammer *et al.* (1989) measured glycated fibrinogen and HbA1c in women after delivery at frequent intervals for seven weeks. They found that in subjects with stable diabetic control the levels of glycated fibrinogen corresponded closely with the level of HbA1c. In the newly diagnosed diabetic individuals, fibrinogen glycation dropped more rapidly than HbA1c after pharmaceutical treatment. Glycated fibrinogen levels rose rapidly after delivery when diabetic control deteriorates. Their results indicated that short-term (days) fluctuations in glucose levels resulted in changes in the degree of fibrinogen glycation. Glycated fibrinogen correlates better with short-term glycaemic control than HbA1c (Hammer *et al.*, 1989).

Ardawi *et al.* (1990) compared glycated fibrinogen as a marker of short-term glycaemic control to HbA1c and glycated albumin. They found that after 6 days of treatment in newly diagnosed diabetic individuals, only glycated fibrinogen was significantly decreased. This suggested that glycated fibrinogen provides earlier objective evidence for glycaemic control and may be regarded as a short-term (2-3days) indicator thereof (Ardawi *et al.*, 1990).

As already mentioned Rakhimova *et al.* (1999) found glycated fibrinogen to be an indicator of glycaemia levels during 3-4 days before measurement (Rakhimova *et al.*, 1999). Suzuki *et al.* (1990) found that glycated fibrinogen was significantly higher in diabetic patients, than in normal subjects and that the glycated fibrinogen value correlated with blood glucose levels (Suzuki *et*

al., 1990). Kitamura *et al.* (1992) also indicated that glycated fibrinogen levels depend on plasma glucose levels. In their study non-treated diabetic patients' glycated fibrinogen levels were significantly higher than non-diabetic or well-controlled diabetic individuals (Kitamura *et al.*, 1992).

Thus, glycated fibrinogen correlates well with HbA1c and is suggested to be an indicator of short-term glycaemic control. However, most of the mentioned evidence is from cross-sectional data and/or does not clearly state whether the study was done on type 1 or 2 diabetic individuals. This study is the first study investigating the effect of insulin treatment on fibrinogen glycation among outpatient diabetic patients not on intensive hospital treatment. This would make the results applicable to the majority of diabetic individuals as it reflects real life situations.

2.7 Conclusion

Some evidence exists for the relationship between glycaemic control and macrovascular disease and non-enzymatic glycation of fibrinogen may partly explain this relationship. Fibrinogen has been shown to be an independent risk marker for CVD. The increased CVD risk amongst individuals with diabetes may be related to the increased fibrinogen levels found in diabetic patients as well as, the glycation of fibrinogen in the presence of uncontrolled blood glucose levels. This glycation may alter the structure/ function of fibrinogen.

Glycated fibrinogen correlates well with HbA1c, an accepted marker of glycaemic control, and plasma glucose levels. Measuring glycated fibrinogen and determining how glycation changes with glycaemic control whilst also, comparing the ability of glycated fibrinogen to monitor glycaemic control with HbA1c may help explain the relationship between glycaemic control and CVD.

Based on the literature studied, it was determined that there it is a need to study the relationship, between blood glucose levels and fibrinogen. Because fibrinogen is a protein involved in vascular disease, knowledge regarding the effect of glycaemic control on fibrinogen glycation may help explain the association between CVD and chronic hyperglycaemia.

- Access to telephone to enable telephonic follow up.

Exclusion criteria of diabetic and non-diabetic subjects:

- Major surgery within the last 6 months prior to the study or at any time during the study.
- Acute infection during the study.
- Macro-vascular complications e.g. stroke, deep vein thrombosis, MI, gangrene, diabetic foot, ischaemic heart disease, peripheral arterial disease.
- Diseases that could influence haemostasis such as thrombocytopenia, cancer, liver disease.
- Patients on aspirin, warfarin, steroids, hormone replacement therapy or non-steroidal anti-inflammatory drugs.
- Visual impairment severe enough to restrict self-glucose monitoring (diabetic subjects only).
- Proteinuria on urine dipstick (>300mg/day).

Inclusion criteria for non-diabetic subjects:

With the exception of type 2 diabetes, the same inclusion criteria were adhered to. Non-diabetic subjects with matching anti-hypertensive drug use (hidrochlorothiazide, ACE-inhibitor – Perindopril and Nifedipine- Adalat), age, gender and BMI were recruited. Venesection (baseline and end) for the non-diabetic subjects was done within a week of venesection of the matched diabetic subject. A fasting blood glucose and a 2-hour post glucose challenge blood glucose (OGTT) was done in order to rule out diabetes.

3.3 Study design

In this, parallel, controlled intervention study, twenty black African type 2 diabetic subjects were included. They were treated with insulin, whilst continuing the use of metformin until glycaemic control was achieved (4 out of 5 subsequent readings within normal glucose range). The patients then remained controlled for 8 days, before end blood sampling was done. Because fibrinogen has a half-life of 3-4 days, an 8-day period was chosen in order to provide enough time for unglycated fibrinogen to be produced, after glycaemic control had been achieved. Twenty non-diabetic black African subjects with the same socio-economic background than the diabetic subjects were included as a reference group in order to control for variation over time.

3.3.1 Study protocol

The intervention period for the diabetic subjects consisted of 3 phases.

Phase 1:

Upon the first visit, diabetic subjects were seen by a diabetes educator who taught them how to do self-glucose monitoring (SGM). Additional education included co-ordination of insulin-use

CHAPTER 3: METHODS

3.1 Introduction

The study included 20 type 2 diabetic subjects on insulin therapy and 20 non-diabetic subjects. There were two study aims namely:

- To determine whether intensive treatment of type 2 diabetes lowers fibrinogen glycation
- To determine whether glycated fibrinogen could be used as a diagnostic short-term indicator of glycaemic control in type 2 diabetic patients.

The study was done on outpatients as it reflects real life situations. Volunteers with type 2 diabetes were recruited from the Kalafong and Mamelodi diabetic clinics. They received Lantus after recruitment, while continuing the use of Metformin during the study period. They were also taught to do self-glucose monitoring twice daily. Upon their visits to the clinics, the physicians determined how their insulin needed to change in order to achieve optimal blood glucose control. Baseline blood sampling was done before insulin treatment was started. Patients stayed on Lantus for the following nine days where after end blood sampling were done. Fibrinogen has a half life of 4 days, therefore a treatment period of 8 days, after glucose control have been reached, will suffice to result in the formation of normal (unglycated) fibrinogen.

In this chapter subject recruitment and characteristics are discussed. The study-design and how blood sampling, analytic procedures and statistics were done are explained. In chapter 4 the results of the study is discussed and in chapter 5 follow the conclusions and recommendations regarding the results obtained.

3.2 Subjects

3.2.1 Recruitment

Volunteer type 2 diabetic subjects were recruited from the Kalafong and Mamelodi Diabetic Clinics. These diabetic subjects were matched with a non-diabetic subject for anti-hypertensive drug use, age, gender and BMI. All subjects came from the same socio-economic background.

3.2.2 Inclusion and exclusion criteria

Inclusion criteria of diabetic patients:

- Uncontrolled type 2 diabetic patients (HbA1c > 9%).
- Male and female.
- BMI > 25kg/m^2.
- Age: 40-65 years.
- Blood pressure sufficiently controlled as not to necessitate treatment change in the following 4-6 weeks (<140/90 mmHg).

with meals, management of hypoglycaemia and the use of glucagon (each patient was issued with a Glucagen hypokit and glucagons for intra-muscular (IM) administration). Fasting capillary glucose was measured and charted in a diary daily for 1 week.

Hypoglycaemic events were also recorded. Hypoglycaemia was categorized as symptomatic if clinical symptoms were confirmed by measurement of a blood glucose value < 2.8 mmol/l or as asymptomatic in the case of any event without symptoms but with a confirmed blood glucose level < 2.8 mmol/l. Severe hypoglycemia was defined as an event, with symptoms consistent with hypoglycaemia, for which the subject required assistance of another person and which was associated with a blood glucose level < 2.8 mmol/l or prompt recovery after oral carbohydrate, intravenous glucose, or glucagon administration. Nocturnal hypoglycaemia was defined as hypoglycaemia occurring while the subject were asleep between the evening injection and getting up in the morning, i.e. before the morning determination of fasting blood glucose.

Phase 2:
Subjects continued the use of metformin, while the use of sulfonylureas was stopped. 10 IU Glargine (Lantus, Sanofi-Aventis Pharmaceuticlas, Paris, France) daily at 22:00 was then added. Metformin treatment stayed unchanged throughout the study period. Insulin was up-titrated every third day according to the fasting blood glucose values (FBG) in order to achieve 4 out of 5 FBG values less than 7.2 mmol/l. Subjects visited the clinics once weekly and had telephonic follow-ups every third day. During that time period, fasting and post-meal glucose levels were stored electronically on each subjects' glucometer.

Phase 3:
Depending on the documented post-prandial glucose values (after breakfast, lunch and supper), short acting insulin, Insulin Aspart (Novo Nordisk, Bagsværd, Denmark), was given pre-meal, if needed, to ensure post-prandial glucose values of less than 10 mmol/l. Once achieved, post supper and FBG were again targeted with pre-meal Novorapid as well with adjustment of the Lantus, if needed, to achieve both post supper and FBG goals. Once the majority of fasting values were <7.2 and the post meal values <10mmol/l, the patient remained treated for 8 days after which blood sampling was done. All capillary glucose values were downloaded electronically and documented.

Baseline samples and anthropometrical measurements were taken at the end of Phase 1. End samples and measurements were taken at the end of Phase 3. Blood samples of non-diabetic subjects were drawn within one week of their matched diabetic subject's blood sampling.

3.4 Blood sampling

A medical doctor did fasting venous blood sampling with minimal stasis before 10 AM. For the determination of insulin and lipids, blood was left to clot for preparation of serum. Blood was collected into sodium fluoride tubes for venous glucose determination. For the determination of HbA1C, Ethylene-diamine-tetra-acetic acid (EDTA) blood was collected, whilst citrate blood was collected for the determination of fibrinogen and fibrinogen glycation. Blood was centrifuged for 15 minutes at 2000g at 4°C within 30 minutes of collection. Both, serum and plasma were stored at -82°C until analysis.

3.5 Anthropometry

Height and weight for both the diabetic and non-diabetic subjects were measured at baseline and end (weight). A stadiometer and precision health scale were used to measured height and weight respectively. Measurements were taken whilst subjects were standing in the correct position wearing light indoor clothing without shoes. BMI were calculated as kg/m^2.

3.6 Analytical procedures

3.6.1 Insulin, glucose, lipid and fibrinogen measurements

Fasting insulin was measured with an enzyme-linked immunosorbent assay (ELISA) method on the Immulite 2000 Analyzer (Diagnostic Products Corporation, Los Angeles, California, USA). Capillary glucose was measured with glucometers (Accu-Chek Active, Roche Diagnostics, Mannheim, Germany). Plasma glucose, baseline HbA1C and serum lipids were measured on a Synchron LX clinical System (Beckman Coulter Inc., Fullerton, CA, USA). LDL cholesterol was calculated by using the Friedewald formula (Friedewald *et al.*, 1972). Insulin resistance was calculated using the homeostasis model assessment (HOMA). (HOMA = (fasting insulin x fasting venous glucose)/22.5 (Katz *et al.*, 2000)). Plasma fibrinogen (modified Clauss method) was measured on an Automated Coagulation Laboratory 2000 (Instrumentation Laboratories, Milan, Italy) (between run variation coefficient volumes (CV) = 3%).

3.6.2 Fibrinogen purification

Fibrinogen was purified from the plasma for each subject using IF-1 affinity chromatography. For the preparation of the chromatography column a washing solution, coupling buffer, blocking buffer and washing buffer was prepared.

- **Washing solution:** The washing solution is 1mM hydrochloride (HCl) that is used to remove the preservatives and salt from the CNBr activated resin (Pharmacia, cat#17-0430-01, 15g package). For its preparation, the pH of 1000ml distilled water was adjusted to 3.0 with HCl.

- **Coupling Buffer:** The Coupling Buffer is used to maintain proper pH during coupling reaction. It was prepared by adjusting the pH of a 0.1M solution of sodium bicarbonate ($NaHCO_3$) (Molecular weight (MW)=84.01, 500 ml, 4.2 g) to 8.3 with a 0.1M solution of sodium carbonate (Na_2CO_3) (MW=105.99, 500 ml, 5.3 g). Sodium chloride (NaCl) (14.6g) was added to 500ml of this solution to have a final concentration 0.5M.

- **Blocking Buffer:** The Blocking buffer was made by adding 40ml distilled water to 3.017ml Ethanolamine. Sodium hydroxide (NaOH) was used to adjust the pH to 8.0.

- **Washing Buffer:** 6.804g Sodium acetate (0.1M) and 14.6 g NaCl (0.5M) were mixed together in 500ml distilled water. The pH was adjusted to 4.0 with acetic acid.

The procedure for preparing the IF-1 affinity chromatography column is described below:

- 1.5g of CNBr activated Sepharose powder was weighed and added to 50ml of the Washing Solution. Thereafter it was incubated for 10 minutes that allowed the resin to swell.
- The swollen resin was placed into a 30ml sintered glass funnel and washed with 900ml of Washing Solution.
- Coupling Buffer and 1.15ml of IF-1 antibodies were combined in a 50ml cylinder.
- The resin in the glass funnel was now washed with 60ml of Coupling Buffer and immediately transferred into the 50ml cylinder. Thereafter the volume was adjusted to 20ml with the Coupling Buffer. Resin was never allowed to dry.
- The 50ml cylinder was sealed with parafilm and rotated upside down for an hour at room temperature.
- After rotation, 20ml of Blocking Buffer was added to the suspension in the cylinder. The cylinder was sealed and rotated once again for an hour at room temperature.
- The resin was again placed on the 30ml sintered glass funnel and was washed alternatively with the Coupling Buffer and Washing Buffer. Thereafter it was washed with 100ml of 20mM Tris pH 7.4, 0.3M NaCl, 0.05% Sodium azide (NaN_3), transferred to a 50ml tube and stored in the refrigerator (Takebe *et al.*, 1995).

During the purification of plasma fibrinogen by the IF-1 affinity chromatography column several buffers were used:

- **Equilibration Buffer:**

  0.02M Tris

  0.3M NaCl

  1mM Calcium chloride ($CaCl_2$)

0.02% NaN_3

pH = 7.4 (titrate to pH with 5M HCl)

- **Dilution Buffer:**

  50mM Tris

  100mM NaCl

  pH = 7.4

- **Wash Buffer I:**

  0.02M Tris

  1M NaCl

  1mM $CaCl_2$

  pH = 7.4 (titrate to pH with 5M HCl)

- **Wash Buffer II:**

  0.05M Na acetate

  0.3M NaCl

  1mM $CaCl_2$

  pH = 6 (titrate to pH with 5M NaOH)

- **Elution Buffer:**

  0.02M Tris

  0.3M NaCl

  5mM EDTA

  pH = 7.4 (titrate to pH with 5M HCl)

- **Dialysis Buffer I:**

  0.05M Tris

  0.1M NaCl

  1mM $CaCl_2$

  pH = 7.4 (titrate to pH with 5M HCl)

- **Dialysis Buffer II:**

  0.05M Tris

  0.1M NaCl

  pH = 7.4 (titrate to pH with 5M HCl)

Citrated plasma (2ml) was thawed in a water bath at 37°C until thoroughly defrosted. Heparin (Final concentration (FC) =IU/ml), benzamidine (FC=5mM) and $CaCl_2$ (FC=20mM) were added to plasma. $CaCl_2$ was added last. Plasma was diluted to 5ml with the dilution buffer and was filtered through a 0.2µm syringe filter.

For plasma purification, 5ml of the diluted and filtered plasma was loaded onto the IF-1 affinity chromatography column equilibrated with the equilibration buffer. The column was washed with 6 column volumes (cv) wash buffer I and 6cv wash buffer II. Fibrinogen was eluted with 3cv elution buffer and ±18ml were collected. The column was now equilibrated in 5cv equilibration buffer. The collected fibrinogen fractions was concentrated using an Amicon Centriplus® Centrifugal Filter Device according to the manufacturer's instructions. Samples were concentrated at 25°C to achieve a final volume of ±2ml. The concentrated sample was dialysed twice with Dialysis Buffer I and three times with Dialysis Buffer II at 4°C. The dialysed sample was stored at -80°C.

3.6.3 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) analysis

The purified fibrinogen was run on 10% SDS-PAGE gels to confirm purity and the absence of degradation of the fibrinogen preparations. The running gel was prepared as follow:

Acrylamide 40%	3 ml
1.5M Tris pH 8.8	3.75ml
SDS 10%	0.15ml
Double distilled water	8.10ml
10% Ammonium persulfate	0.05ml (APS stock is stored at -20°C)
TEMED*	0.01ml
	15 ml

APS and tetramethylethylene diamine N,N,N^1,N^{1-} (TEMED) were added before the gel was thawed because they activate clotting of gel. Stacking gel was prepared according to the following recipe:

Acrylamide 40%	0.49 ml
0.5M Tris pH 6.8	1.25 ml
SDS 10%	0.05 ml
ddWater	3.15 ml
10%Ammonium persulfate	0.025 ml
TEMED	0.005 ml

The sample (6mg) was loaded onto the gel. After electrophoresis was finished, the gel was stained with Coomasie Blue.

3.6.4 Fibrinogen glycation

Finally, fibrinogen glycation was measured with a two-reagent enzymatic assay (GlyPro® assay, Genzyme Diagnostics, Cambridge, MA, see Annexure B for package insert) using the purified fibrinogen. This assay provides a specific method for the direct measurement of glycated serum proteins in plasma or serum.

Two reagents were used in this assay. Among the content of the first reagent is Proteinase K and among the second, the enzyme, ketoamine oxidase (KAO). The first reagent provides an on-line digestion of the sample and the subsequent release of glycated fibrinogen fragments. KAO in the second reagent facilitated the specific oxidation of the ketoamine bond of the glycated protein fragment substrate. Hydrogen peroxide is then released so that the amount of glycated fibrinogen could be calorimetrically determined in an end-point reaction.

The first step of the test procedure involves adding 250µL of reagent 1 to 20µL of the test sample. After incubation at 37°C for 5 minutes, the absorbance at 550nm was again measured to obtain value A1. The second step involved the addition of reagent 2 and then incubation at 37°C for 3-5 minutes where after the absorbance at 550nm was measured to obtain value A2. The total change in absorbance (ΔA) was determined by subtracting A1 from A2. The increase in absorbance at 550nm, is proportional to the glycated fibrinogen concentration in the sample.

Glycated fibrinogen was then calculated using the following formula:

$$\text{Glycated fibrinogen } (\mu mol/L) = \frac{\Delta A \text{ sample}}{\Delta A \text{ calibrator}} \times \text{Calibrator value } (\mu mol/L) \text{ provided on the vial lable}$$

Results were reported as µmol/L of glycated fibrinogen.
See package insert included in annexure B.

3.7 Statistics

An investigator blinded to the treatment groups captured the data in an Excell data sheet. Once the datasheet has been completed, the data was transferred to the Statistica (Statsoft Inc., Tulsa, Oaklahoma, USA) and Medcalc statistical software packages with which all statistical analyses were conducted.

There is not a defined clinical significant difference known for the main outcome variable, fibrinogen glycation. Therefore a power calculation was done using 1 standard deviation (SD)

as a clinical significant difference. To achieve a difference of 1SD at 80% power, 5% significance, each group should have consisted of at least 16 individuals.

Computer software packages as mentioned earlier were used for statistical analysis. Data was tested for normality. Normally distributed data is presented as the mean (95% confidence interval) and not normally distributed data as median (25th; 75th percentile). Baseline characteristic differences and the differences in changes during the intervention between the two groups were determined using the t-test for independent samples for parametric data and the Mann-Whitney U test for non-parametric data. Differences from baseline to end within each group were determined using the t-test for dependent samples. Spearman-correlation was used for all the correlations that were done. Receiver Operator Characteristic (ROC) curves were used to determine how well glycated fibrinogen compare to HbA1c in monitoring glycaemic control. Agreement was assessed between glycated fibrinogen and the current standard indicator of glycaemic control, HbA1c as well as fasting venous glucose. ROC curves were also used to determine the optimal threshold level of glycated fibrinogen to indicate glycaemic control.

CHAPTER 4: RESULTS

4.1 Introduction

The study's aims were to determine whether intensive treatment of type 2 diabetes lowers fibrinogen glycation and whether glycated fibrinogen could be used as a short-term indicator of glycaemic control. In this chapter the results of the intervention are presented to help explain whether these aims were met.

SDS-PAGE analysis was done to check the purity and absence of degradation of the purified fibrinogen. To ensure that the diabetic and non-diabetic subjects were comparable, baseline characteristics were compared between the two groups. In order to determine whether the intervention was successful, baseline and end values of the diabetic subjects were compared. Baseline and end values were determined within the non-diabetic group in order to determine variation over time. To determine whether changes in the diabetic group differed significantly from the changes in the non-diabetic group the deltas (change from baseline to end) were compared. Correlations between baseline characteristics were done in order to determine whether any relationship existed between the variables. Change in glycation during the intervention was correlated, with baseline levels as well as changes during the intervention in other metabolic variables in order to determine the possible role of baseline levels, in the perceived changes and to determine whether the changes in the different variables were inter-related.

Receiver Operator Characteristic (ROC) curves (also called relative operating characteristic curves) was used to compare glycated fibrinogen with HbA1c in their ability to predict glycaemic control. A cut-off value was also determined with the best trade off between sensitivity and specificity of glycated fibrinogen to indicate glycaemic control.

To estimate for which time period glycated fibrinogen corresponds to glucose concentrations, end glycated fibrinogen values were correlated, with the average fasting capillary glucose of different time intervals preceding the date of end blood sampling, using the daily capillary values.

In this chapter the SDS-PAGE results are described in section 4.2, baseline characteristics are discussed in section 4.3, differences between baseline and end values as well as deltas in section 4.4 and ROC curves in section 4.5. Glycated fibrinogen was correlated with the average fasting capillary glucose concentration for different time-intervals as discussed in section 4.5.

4.2 SDS-PAGE gel

Figure 4.1 shows the SDS-PAGE analysis of purified fibrinogen from plasma samples. Lane 1 shows a commercial human fibrinogen preparation (5 µg)(MP Biologicals cat no. 151123), which was used for comparison. Lane 2 is fibrinogen isolated from a diabetic subject before the glucose control intervention period and lane 3 after the glucose control intervention period. Fibrinogen of a non-diabetic subject before the intervention period and after the intervention is shown in lane 4 and 5 respectively. The positions of a molecular weight marker (x 10^3) and the respective peptides, Aα, Bβ, and γ are indicated on the left and right hand side, respectively.

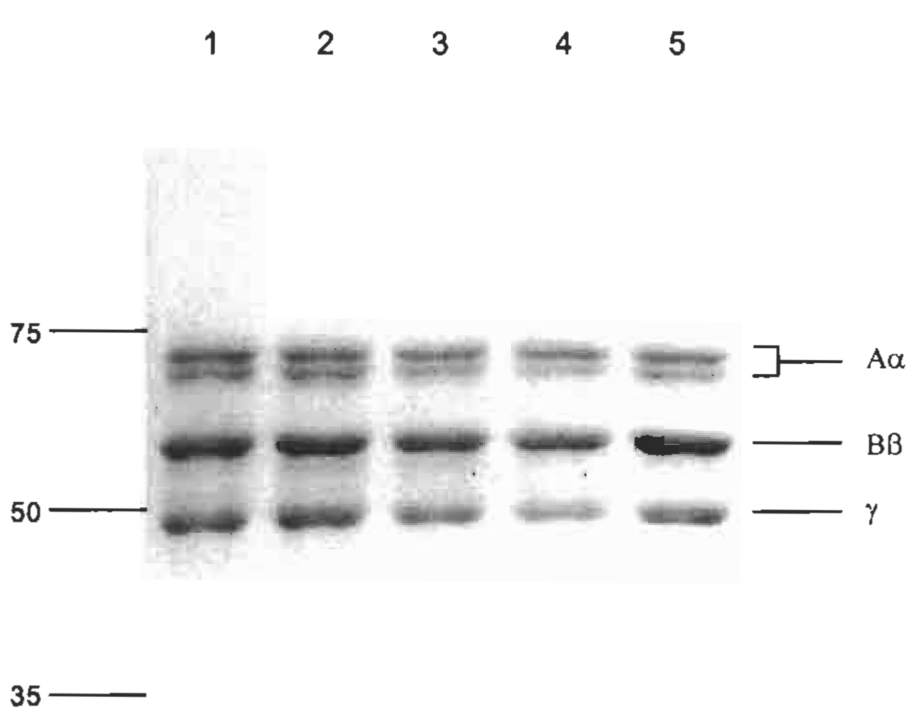


Figure 4.1: SDS-PAGE analysis of purified fibrinogen from plasma samples.

4.3 Baseline characteristics

Two non-diabetic subjects failed to return for end blood sampling and were excluded from the study. Only one of the subjects smoked and alcohol consumption was uncommon in both groups.

Table 4.1 indicates the baseline characteristics of the diabetic and non-diabetic subjects. The two groups were comparable regarding gender, age, BMI, fasting insulin, fibrinogen concentration, blood pressure (systolic and diastolic), total cholesterol, LDL and HDL

cholesterol. Non-diabetic subjects were also specifically chosen, to be comparable with the diabetic subjects regarding gender, age, BMI and blood pressure. All the subjects were therefore hypertensive and either overweight or obese.

The diabetic group had significantly higher fasting venous glucose, HbA1c, insulin resistance, triglycerides and fibrinogen glycation. Both, the high baseline fasting venous glucose and HbA1c levels confirms that the subjects had diabetes and that their blood sugar levels were uncontrolled. The higher insulin resistance levels seen in the diabetic group was expected since insulin resistance is the major pathogenic factor of type 2 diabetes (Lipkin, 1999). Mean duration of diabetes in the type 2 diabetic subjects was 11 years, were the minimum was 5 years. Thus, none of the diabetic subjects were newly diagnosed.

Table 4.1: Baseline characteristics of subjects

Variables	**Type 2 diabetic subjects**	**Non-diabetic subjects**
Patients (n)	20	18
Sex (male/female)	6 / 14	7 / 11
Age (years) [c]	53.0 (49.1 ; 56.9)	52.9 (49.2 ; 56.6)
Body mass index (kg/m^2) [c]	30.8 (28.0 ; 33.7)	31.8 (28.8 ; 34.7)
Venous glucose (mmol/L) [c]	14.6 (10.8 ; 18.4)[a]	5.18 (4.56 ; 5.80) [a]
Fasting Insulin (mU/L) [d]	11.0 (6.70 ; 15.8)	13.7 (8.9 ; 28.8)
HbA1c [d]	11.7 (9.50 ; 13.8) [a]	5.60 (5.30 ; 5.90) [a]
Insulin resistance (HOMA) [c]	5.18 (3.99 ; 6.95)[b]	3.11 (2.29 ; 7.42)[b]
Fibrinogen (g/L) [c]	4.25 (3.88 ; 4.63)	4.02 (3.59 ; 4.45)
Fibrinogen glycation (mol glucose/mol fibrinogen) [c]	7.84 (6.59 ; 9.10) [a]	3.89 (3.46 ; 4.32) [a]
Systolic blood pressure (mmHg) [c]	140.5 (129.6 ; 151.3)	143.3 (130.2 ; 156.4)
Diastolic blood pressure (mmHg) [c]	86.6 (82.7 ; 90.6)	89.8 (83.3 ; 96.3)
Total cholesterol (mmol/L) [c]	4.84 (4.14 ; 5.53)	4.54 (4.16 ; 4.92)
Triglycerides (mmol/L) [d]	1.80 (1.25 ; 2.50)[b]	1.05 (0.80 ; 1.25)[b]
High density lipoprotein cholesterol (mmol/L) [d]	0.9 (0.75 ; 1.3)	0.9 (0.8 ; 1.25)
Low density lipoprotein cholesterol (mmol/L) [c]	2.86 (2.37 ; 3.34)	2.88 (2.43 ; 3.32)
Duration of diabetes (years)	11.0 (8.00 ; 15.0)	

Note: [a] $P < 0.001$; [b] $P < 0.05$; [c] Data normally distributed therefore reported as the mean (95% confidence interval); [d] Data not normally distributed and therefore reported as median (25, 75 percentile); Insulin resistance = (fasting insulin x fasting venous glucose)/22.5; HOMA = Homeostasis Model Assessment.

Baseline glycated fibrinogen for the total group correlated significantly with baseline HbA1c (r = 0.8), fasting venous glucose (r = 0.69) and the average of the preceding four days fasting capillary glucose (r = 0.5).

4.4 Baseline to end changes (Deltas)

The average duration between baseline and end blood sampling for the diabetic subjects were 69.5 (range: 15 - 148) days and 75 (range: 8 - 154) days for the non-diabetic subjects.

Table 4.2 indicates the differences between the diabetic and non-diabetic subjects of selected variables for the intervention period. Except for a minor increase in fasting glucose levels (p = 0.05), there were no significant changes in the non-diabetic group during the intervention for variables measured. There was, however a significant increase in BMI from baseline to end in the diabetic group (p = 0.001). Fasting venous glucose and fibrinogen glycation decreased significantly during the intervention (p = 0.0008 and p = 0.0002 respectively) in the diabetic group while no changes were seen in the non-diabetic group.

Table 4.2: Differences between diabetic and non-diabetic subjects of selected variables for the intervention period

Variable	Type 2 diabetic subjects (n = 20)				Non-diabetic subjects (n = 18)				Between group comparison [b]
	Baseline	End	Baseline to end changes	p	Baseline	End	Baseline to end changes	p	p
Body mass index (kg/m²)	30.8 (28.0; 33.7)	32.2 (28.9; 35.5)	1.79 (0.79; 2.79)	0.001	31.7 (28.8 ; 34.7)	32.0 (28.9; 35.1)	0.30 (-0.11; 0.72)	0.14	0.009
Fasting glucose (mmol/L)	14.6[a] (10.8; 18.4)	6.72 (5.51; 7.92)	-7.88 (-12.04;-3.72)	0.0008	5.18[a] (4.56 ; 5.80)	5.63 (4.95; 6.31)	0.46 (0.00; 0.91)	0.05	0.0003
Fibrinogen (g/L)	4.25 (3.88; 4.63)	4.36 (3.99; 4.73)	0.11 (-0.34; 0.55)	0.62	4.02 (3.59 ; 4.45)	3.85 (3.43; 4.28)	-0.16 (-0.52; 0.19)	0.35	0.33
Fibrinogen glycation (mol glucose/mol fibrinogen)	7.84[a] (6.59; 9.10)	5.24 (4.47; 6.01)	-2.60 (-3.82;-1.39)	0.0002	3.94[a] (3.46 ; 4.32)	3.89 (3.43; 4.07)	-0.19 (-0.55; 0.17)	0.27	0.0007

Data represented as the mean (95% confidence interval)

[a] Values differed significantly at baseline (P<0,05); [b] Between group comparison was made between the baseline to end changes of each group

Fibrinogen levels did not differ significantly between the two groups at baseline. These levels were slightly higher than normal fibrinogen levels in both groups (2 - 4g/L)(Lancet, 2006). There was no significant change in fibrinogen levels from baseline to end in either group.

The increase in BMI in the diabetic group differed significantly ($p = 0.009$) from that of the non-diabetic group. The drop in venous glucose concentration and fibrinogen glycation in this group also differed significantly ($p = 0.0003$ and $p = 0.0007$ respectively) from that in the non-diabetic subjects. Although end fibrinogen glycation levels of the diabetic subjects decreased with achieving glucose control, the end glycation levels (5.24 mol glucose/mol fibrinogen) is still higher than those of the non-diabetic patients (3.94 mol glucose/mol fibrinogen) at baseline.

A significant correlation ($r = 0.52$, $p = 0.001$) between the decrease in fibrinogen glycation and venous glucose was found amongst the total group but not for the diabetic group alone. The decrease in fibrinogen glycation for the total group did not correlate with the increase in BMI ($p = 0{,}635$), nor with changes in fibrinogen levels ($p = 0.768$). Within the diabetic group, the correlation between the decrease in fibrinogen glycation and the decrease in capillary glucose was significant ($r = 0.6$, $p = 0.005$). The value of capillary glucose used for the correlation was obtained by calculating the average of 4 days' values measured at baseline and end. This decrease in fibrinogen glycation within the diabetic group did not correlate significantly with changes in BMI ($p = 0.825$), venous glucose ($p = 0.254$) nor fibrinogen levels ($p = 0.508$) or with the respective baseline values.

4.5 ROC curves

The comparison between the accuracy of glycated fibrinogen and glucose control were investigated by using ROC curves. ROC curves can be used to determine the cut-off glycated fibrinogen level that indicates controlled (mean glucose < 6.4mmol/L) or uncontrolled (mean blood glucose >6,4 mmol/L) blood glucose levels. This cut-off value relates to the point were the best trade-off between sensitivity and specificity exist. The higher the sensitivity and specificity of the test (glycated fibrinogen in this case), the better the accuracy of the test for the variable investigated (glycaemic control).

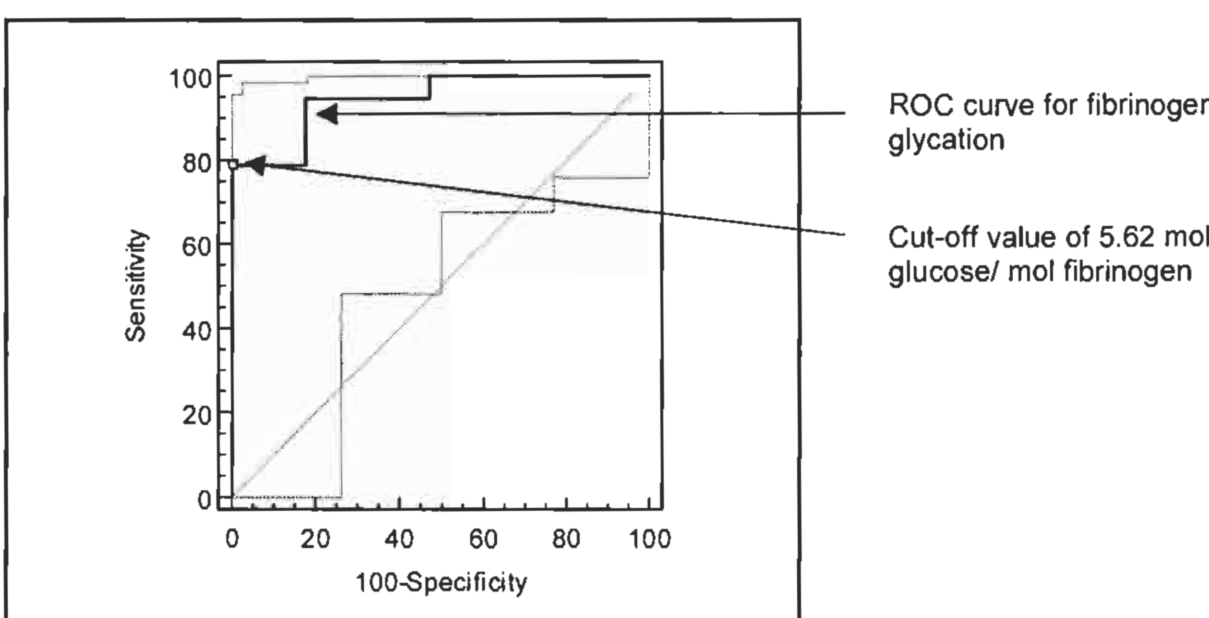
Sensitivity can be defined as the ability of the test to correctly classify uncontrolled patients as uncontrolled. Thus, sensitivity gives the proportion of cases detected by the test to have poor glycaemic control relative to all cases that actually have poor control. Specificity is the ability of a test to identify patients who are well controlled.

The area under the curve (AUC) provides an indication of how well the test (glycated fibrinogen) performs in discriminating between glycaemic controlled and uncontrolled. A value of 1.0

indicates that the test performance is equal to the reference or gold standard (in this case HbA1c) with 100% sensitivity and 100% specificity.

The ROC curve of fibrinogen glycation with HbA1c as reference value is shown in figure 4.2. The AUC of baseline glycated fibrinogen is 0.95 (95% confidence interval between 0.817 and 0.992), which indicates that glycated fibrinogen has a very good ability to indicate glycaemic control.

Figure 4.2: ROC curve for fibrinogen glycation with HbA1c as reference value

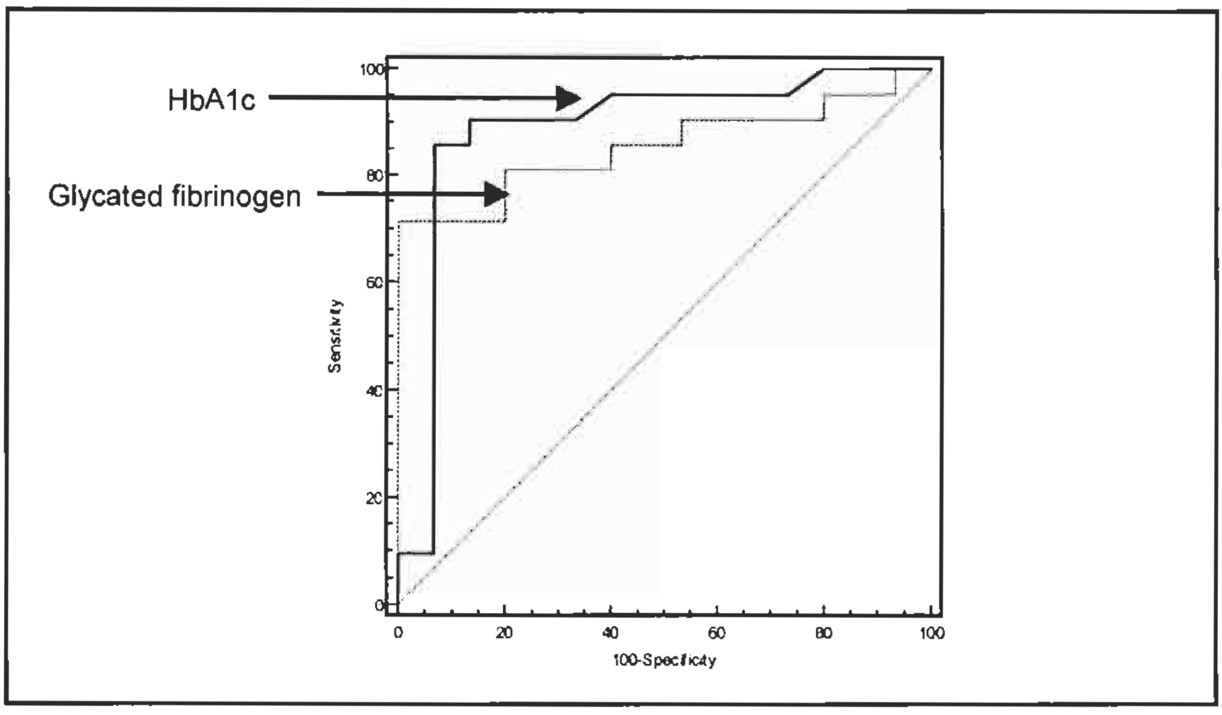


A glycated fibrinogen value of 5.62 mol glucose/ mol fibrinogen had the best trade-off between sensitivity and specificity. It has a sensitivity of 78.9% (54.4, 93.8) and specificity of 100% (80.3, 100.0) to discriminate between glycaemic controlled and uncontrolled with HbA1c as reference. This relates to a positive likelihood ratio of 13.42 for glycated fibrinogen with HbA1c as gold standard. This means that the probability of having a glycated fibrinogen level above 5.62 mol glucose/ mol fibrinogen is 13.42 times as high to be present in a patient with poor glycaemic control than in a patient with good glycaemic control. Thus the positive likelihood ratio relates to the tests ability to identify those individuals who truly have poor glycaemic control from those individuals who have optimum glycaemic control.

Figure 4.3 shows the comparison of the ROC curves of glycated fibrinogen and HbA1C using fasting venous glucose as reference. The AUC for glycated fibrinogen is 0.85 (0.696,0.949) and that of HbA1c is 0.89 (0.739,0.968). Since the AUC for HbA1c is closer to 1.0 than that of glycated fibrinogen, it is suggested that HbA1c has a higher sensitivity and specificity. However, this difference between the AUC of glycated fibrinogen and HbA1c is only 3.5%. This is not

statistically significant (p = 0.537), suggesting that no statistically significant difference in sensitivity and specificity trade-off could be demonstrated between glycated fibrinogen and the current gold standard, HbA1c.

Figure 4.3: Comparison of ROC curves of glycated fibrinogen and HbA1c using fasting venous glucose as reference



4.6 Fibrinogen glycation and fasting capillary glucose at different time-intervals

The end glycated fibrinogen value was correlated with the average fasting capillary values of different time intervals preceding the date of end blood sampling. Time-intervals of 4 days were chosen as that which corresponds with fibrinogen half-life. As shown in table 4.3, there were 5 different time intervals. Day 0 were assigned to the day upon which end blood sampling was done. Days -4, -5, -8, -9 and -12 corresponded then to the number of days preceding end blood sampling. These values were obtained from the daily patient recorded fasting capillary blood glucose values.

Table 4.3: Correlation of glycated fibrinogen with the average fasting capillary glucose concentration over different time intervals

Time Intervals	Fibrinogen glycation	
	r	P
0 to -4 days	0.31	0.74
-5 to -8 days	0.54	0.014
-9 to -12 days	0.33	0.16
0 to -8 days	0.47	0.038
0 to -12 days	0.53	0.017

The weakest correlation existed between fibrinogen glycation and the first time interval (day 0 to -4) (r = 0.31, p = 0.74), whereas the –5 to -8 day time interval showed the strongest correlation with glycated fibrinogen (r = 0.54; p = 0.014). The correlation between fibrinogen glycation and days –9 to -12 were also not significant (r = 0.33 and p = 0.16). When the 0 to -4 day time interval group were combined with the preceding 4 day time intervals to form days 0 to -8 and 0 to -12, significant correlations (r = 0.47; p = 0.038 and r = 0.53 and p = 0.017 respectively) were still observed. This is however not as strong as for the –5 to -8 day time interval. Overall, fibrinogen glycation showed a strong correlation with the preceding 12 days (r = 0.53; p = 0.017).

CHAPTER 5: DISCUSSION

5.1 Introduction

This study had two main aims. The first was to determine whether controlling blood glucose levels in outpatient type 2 diabetic subjects would affect fibrinogen glycation. The other was to determine how well glycated fibrinogen compares to HbA1c in its relation to glycaemic control. In this chapter the results with regards to the two aims are discussed in more detail, thus by elaborating on the factors related to the results obtained. Recommendations for further research are also given.

5.2 Fasting glucose, lipid profile, insulin resistance and body mass index

High fasting venous glucose, HbA1c and insulin resistance levels are commonly associated with diabetic individuals because they struggle with uncontrolled blood sugar levels. Hypertension, increased adiposity and abnormalities of lipoprotein metabolism are also often found amongst diabetic subjects (The Expert Committee on the diagnosis and classification of diabetes mellitus, 2002). Thus, the diabetic subjects in this study had typical characteristics associated with diabetes.

Serum triglycerides are known to be elevated in type 2 diabetic subjects. This is reflected in the baseline characteristics indicating that the serum triglycerides of the diabetic subjects are significantly higher, than the non-diabetic subjects. Although no significant differences in LDL- and HDL-cholesterol were found between the two groups, type 2 diabetic subjects are known to have smaller and denser LDL-cholesterol. This makes LDL-cholesterol more susceptible to oxidation, which is central to atherogenesis (Goldstein & Müller-Wieland, 2003).

The diabetic group was significantly more insulin resistant than the non-diabetic group, while fasting insulin concentrations were similar. Higher insulin resistance levels seen in the diabetic group was expected since insulin resistance is the major pathogenic factor of type 2 diabetes (Lipkin, 1999). The lower fasting insulin levels may be due to the fact that ß-cell production of insulin in the pancreas are diminished due to progressive destruction of the ß-cells over several years (The Expert Committee on the diagnosis and classification of diabetes mellitus, 2002). None of the diabetic subjects were newly diagnosed. The shortest duration between diagnosis and the start of the study intervention is 5 years.

During the intervention there was a significant increase in BMI in the diabetic group. This is probably due to the exogenous insulin they received during the intervention. In the United

Kingdom Prospective Diabetes Study (UKPDS) patients on insulin therapy gained significantly more weight than those treated with sufonylureas or metformin (DeWitt & Hirsch, 2003). Type 2 diabetic patients generally experience weight gain as they attempt better glycaemic control with the use of insulin, because of reduced glycosuria leading to reduced urinary calorie loss and intermittent over-insulinization which can result in hypoglycaemia, hunger and subsequently increased energy intake (Bell, 2002; DeWitt & Hirsch, 2003; Gottesman, 2004). Weight gain in the setting of insulin treatment might also be due to increased adiposity without any changes in lean body mass. This is due to reduced glycosuria and reduction in daily energy expenditure. The reduction in daily energy expenditure may be caused by a decrease in metabolic futile cycling involving protein, free fatty acids and glucose fuels (Boyne & Saudek, 1999).

As already mentioned, baseline fasting glucose levels of the diabetic subjects were above the recommended levels of 4.4 to 6.7 mmol/L (Lipkin, 1999), which differed significantly (P = 0.0003) from the non-diabetic subjects. These levels decreased significantly (P VISSER, M.E., STEYN, N., SETSUBI, O. 2003. Reducing chronic diseases of lifestyle and managing HIV using an evidence-based approach – what every clinician should know. *CME,* 21:516-522. = 0.0008) during the intervention to levels (6.72 mmol/l) were optimal blood glucose control is defined. No significant changes within the non-diabetic group were found.

5.3 Fibrinogen concentration and glycation

Plasma fibrinogen levels are associated with some conventional risk factors for cardiovascular disease, including age, gender, smoking, hypertension and diabetes mellitus (Kain *et al.* 2002). It increases with age and obesity, is higher in smokers than non-smokers, and fall with moderate alcohol consumption. In women the concentrations are higher in those using oral contraceptives (Meade *et al.*, 1979; Lee *et al.*, 1990).

In this study the subjects were matched for age and gender. They were all borderline hypertensive. Smoking and alcohol consumption was infrequent. Thus, they were similar except for having type 2 diabetes or not. Because there is some evidence suggesting that diabetic subjects have high fibrinogen levels, it was expected that the diabetic subjects' baseline fibrinogen levels would differ from the non-diabetic subjects. The diabetic subjects had relatively high fibrinogen concentrations on average (4.25g/L) as expected, since Type 2 diabetic subjects have been shown to have high plasma fibrinogen levels (Lütjens *et al.*, 1985; Ford *et al.*, 1991; Donders *et al.*, 1993; Asakawa *et al.*, 2000). However, there was no difference between the mean fibrinogen concentration in the diabetic and non-diabetic group. Both had high fibrinogen concentrations.

There are several possible explanations, for why fibrinogen glycation is not further elevated in the diabetic subjects. Increased fibrinogen levels are related to hyperinsulinaemia and it has been suggested that this relationship is not independent of the accompanying inflammatory reaction whereby acute-phase proteins such as fibrinogen are formed. This independent relationship has been found in non-diabetic subjects (Juhan-Vague *et al.*, 1993; Mertens & Van Gaal, 2002). Because the diabetic and non-diabetic subjects in this study had similar fasting insulin levels it may explain the similar fibrinogen concentration that has been found.

The release of free fatty acids in insulin resistance could also cause a rise in fibrinogen levels (Juhan-Vague *et al.*, 1993). Since free fatty acids were not measured in this study, its link to insulin resistance and fibrinogen cannot be exemplified.

Variation in fibrinogen levels is also determined by genetic factors (Kain *et al.*, 2002). Higher fibrinogen levels have also been found in black individuals compared to Caucasians (Folsom *et al.*, 1992; Jerling *et al.*, 1994). This may account for the relatively high fibrinogen levels found in the non-diabetic group. It may also be due to one of many other factors as already mentioned such as age, hypertension and obesity. Because the groups were similar regarding these matters, these might be responsible for the increased fibrinogen levels in both the diabetic and non-diabetic subjects. It is also considered that obesity *per se* rather than insulin resistance (that usually cause type 2 diabetes) results in high fibrinogen levels (Juhan-Vague *et al.*, 1999; Mertens & Van Gaal, 2002). Ditschuneit *et al.* (1995) found that a reduction in BMI has been accompanied by a fall in fibrinogen levels. Since the subjects were matched according to BMI, this provides a further explanation for the similar fibrinogen levels.

Although a number of studies (Ford *et al.*, 1991; Donders *et al.*, 1993; Crook *et al.*, 1996; Asakawa *et al.*, 2000; Streja *et al.*, 2003) found an increase in fibrinogen levels in type 2 diabetic subjects, some controversy remains as to whether fibrinogen levels are increased in diabetic subjects in general or only in those with CVD. Missov *et al.* (1996) found no increase in fibrinogen levels in type 2 diabetic subjects. Other studies however Danesh *et al.* (2005) demonstrated that fibrinogen is associated with the incidence of CVD. A study done by Ganda & Arkin (1992) revealed that fibrinogen is one of only three independent predictors of vascular complications. The others are diabetes duration and hypertension (Ganda & Arkin, 1992). The subjects in the study were chosen not to have CVD, which may explain why their fibrinogen levels were not further increased.

The average glycation of fibrinogen at baseline in the diabetic group was almost twice as high as that of the non-diabetic subjects (7.48 and 3.75 mol glucose/ mol fibrinogen). This significantly decreased ($P = 0.0002$) after achieving glycaemic control (with almost 33%),

although not to the level of glycation seen in the non-diabetic group. No significant fibrinogen glycation changes were found in the non-diabetic group.

Other studies (Hammer *et al.*, 1988; Ardawi *et al.*, 1990) also showed a decrease in fibrinogen glycation with improvement of glycaemic control in diabetic subjects. However, it is not clear from their results whether the results were obtained from type 1 or 2 diabetic subjects. Hammer *et al.* (1988) and Ardawi *et al.* (1990) reported their results after intensive treatment of 3 and 6 days respectively. Results found with this study shows that a decrease in fibrinogen glycation can also be achieved in the majority of type 2 diabetic subjects that attend out-patient clinics.

A significant correlation ($r = 0.52$, $p = 0.001$) between the decrease in fibrinogen glycation and venous glucose were found amongst the total group but not for the diabetic group alone. However, there was a significant ($r= 0.6$, $p = 0.005$) correlation between the decrease in fibrinogen glycation and capillary glucose within the diabetic group. The reason why there is a significant correlation between glycated fibrinogen and capillary glucose and not between glycated fibrinogen and venous glucose within the diabetic group is probably because venous glucose measurement was done at one point in time. The value of capillary glucose used for the correlation was however obtained by calculating the average of 4 days' values. This is a better reflection of the true fasting glucose levels around the time of blood sampling as opposed to one single venous glucose measurement. It can be further validated by the fact that a significant correlation was found between the decrease in fibrinogen glycation and the decrease in fasting venous glucose, when the sample size was increased by including the results of the total group.

The level of fibrinogen glycation found in the study is 7.84 (6.59; 9.10) mol glucose/ mol fibrinogen for the diabetic group and 3.89 (3.46; 4.32) mol glucose/ mol fibrinogen for the non-diabetic group. These values are similar to that found by Suzuki *et al.* (1990). They found fibrinogen glycation to be 8.1 ± 3.64 and 3.13 ± 1.29 mol glucose/ mol fibrinogen in diabetic and non-diabetic subjects respectively (Suzuki *et al.*, 1990). A study done by Lütjens *et al.* (1985) found the level of glycation to be 1.3 ± 0.19 and 1.33 ± 0.21 mol glucose/ mol fibrinogen for uncontrolled type 1 diabetic patients and 1.06 ± 0.08 and 0.95 ± 0.17 mol glucose/ mol fibrinogen for non-diabetic volunteers (Lütjens *et al.*, 1985). In this study and in the study done by Suzuki *et al.* (1990), a commercially available kit for measuring fibrinogen glycation was used. Lütjens *et al.* (1985), however used the thiobarbituric acid method (Flückiger & Winterhalter, 1976) and high performance liquid chromatography according to the method of Schleicher & Wieland (1981) for the determining of fibrinogen glycation (Lütjens *et al.*, 1985). Thus, the different methods used may explain the differences in glycation levels measured.

Hammer *et al.* (1988) and Ardawi *et al.* (1990) also measured fibrinogen glycation in diabetic subjects. Their results are however expressed as glycated fibrinogen as a percentage of total fibrinogen and not as the number of glucose molecules bound to one molecule of fibrinogen. It is therefore not comparable to the results found in this study.

Glycated fibrinogen has previously been suggested as a short-term indicator of glycaemic control (Hammer *et al.*, 1989; Ardawi *et al.*, 1990; Suzuki *et al.*, 1990; Kitamura *et al.*, 1992; Rakhimova *et al.*, 1999). How well it compares with HbA1c, the current gold standard for determining glycaemic control, has until now not yet been determined. This study indicated that glycated fibrinogen is comparable to HbA1c in monitoring glycaemic control in type 2 diabetic individuals. ROC curves were used to assess the discriminatory value of a diagnostic test. When using the HbA1c as the gold standard, the area under ROC curve of glycated fibrinogen is 0.95. This indicates that in ninety five percent of cases, based on the glycated fibrinogen value, individuals will be correctly classified as either having good (HbA1c < 7%) or poor (HbA1c > 7%) glycaemic control with a 95% confidence interval between 0.817 and 0.992.

There was no significant difference between the ROC curve for glycated fibrinogen and HbA1c in predicting glycaemic control, when using fasting plasma glucose as the reference. The AUC for glycated fibrinogen is 0.85 (0.70, 0.95) and that of HbA1c is 0.89 (0.74,0.97). This small difference is most likely due to the shorter half-life of fibrinogen compared to haemoglobin. Fibrinogen is also an acute-phase protein and plasma levels may change noticeably during illness (Hammer *et al.*, 1988).

The level of fibrinogen glycation as indicated by the ROC curves with the highest combination of sensitivity and specificity for predicting glycaemic control was 5.62 mol glucose/ mol fibrinogen. This level of glycation has a 78.9% (54.4, 93.8) sensitivity, 100% (80.3, 100.0) specificity and a 13.42 times positive likelihood ratio to predict glycaemic control of 13.42 with HbA1c as reference. Thus, a glycated fibrinogen value above the optimal threshold level of 5.62 mol glucose/ mol fibrinogen is 13.42 times more likely to occur in subjects with uncontrolled HbA1c (>7%) than in individuals who are well controlled.

HbA1c was used as a reference because it is the current gold standard. It is however important to mention that a gold standard or treatment paradox exists. Because HbA1c is the gold standard, it was accepted that it has a 100% sensitivity and specificity. If a test or parameter then do better than the gold standard, it will seem as if the test are weaker than what it actually is. It will seem as if the test falsely predicts more subjects to have a disease. In this case it will seem as if there are more subjects with increased glycated fibrinogen values than what is really the truth.

Therefore, in this study the AUC for glycated fibrinogen was compared with the AUC of HbA1c with fasting plasma glucose as reference. The difference between these two AUCs was 3.5%, which is not statistically significant suggesting that glycated fibrinogen compares well with HbA1c. Based on the available literature, HbA1c has 65% sensitivity and 94% specificity for the diagnosis of diabetes (ADA, 1999; Rohlfing *et al.*, 2002; Yates & Laing, 2002; Monnier *et al.*, 2003). These results on HbA1c can however not be compared with the results found in this study. The results of this study compare glycated fibrinogen with HbA1c while using fasting plasma glucose as a reference. It therefore does not relate to diagnosis of diabetes.

Fibrinogen is an ideal short-term marker for monitoring glycaemic control due to its short half-life of 3-4 days. It could therefore be useful in the early assessment of the effectiveness of a treatment regime. This is especially important in developing countries where daily home glucose monitoring is not an option as very few of these diabetic patients have their own glucometers and HbA1c cannot be used for monitoring short-term (days) fluctuations in glucose control. It could also be successfully applied in research studies with a shorter duration, in order to determine the effectiveness of a treatment. Evidence for this exists from cross-sectional studies, which found that glycated fibrinogen correlated with blood glucose levels 3 to 4 days prior to measurement (Rakhimova *et al.*, 1999) and with blood glucose at the same time or one day earlier (Suzuki *et al.*, 1990).

Although it was not the main aim of the study, end fibrinogen glycation level was correlated with the average fasting capillary glucose of different 4-day time-intervals (fibrinogen has a half-life of 4 days) in order to assess with which time-interval fibrinogen correlated best. By correlating end fibrinogen glycation levels with the average fasting capillary glucose of different 4-day time-intervals the study indicated that fibrinogen could be used as a short-term indicator of glycaemic control. The weakest correlation existed between fibrinogen glycation and the first time interval (day 0 to -4) ($p = 0.74$). This may be due to high levels of circulating glycated fibrinogen still present in the circulation that was formed before optimal glycaemia was achieved. Clearance of glycated fibrinogen has been shown to be exponential. Therefore it is assumed that a small amount of glycated fibrinogen may remain for a long time in circulating plasma despite its short half-life. This may lead to the presence of highly glycated fibrinogen in plasma (Arocha-Piňango, 1987; Mirshahi *et al.*, 1987) for periods longer than its half-life. The –5 to -8 day time interval showed a much stronger correlation with glycated fibrinogen ($r = 0.54$; $p = 0.014$). Overall, fibrinogen glycation showed a strong correlation with the preceding 12 days ($r = 0.53$; $p = 0.017$) of which the –5 to -8 day time interval made the most important contribution.

These results are comparable to that of Hammer *et al.* (1998) and Ardawi *et al.* (1990). Hammer *et al.* (1998) reported a 15% decrease in fibrinogen glycation after 3 days of treatment in 12

newly diagnosed diabetic patients. The HbA1c values of the same diabetic subjects decreased with only 3.5%, which shows that glycated fibrinogen reflects changes in diabetic control at an earlier stage than glycated haemoglobin (Hammer *et al.*, 1998).

Ardawi *et al.* (1990) reported that there was a 33.4% decrease after 6 days in fibrinogen glycation in ten newly diagnosed diabetic subjects, without any significant decrease in HbA1c levels. Despite the similar fasting blood glucose levels of these two studies (Ardawi *et al.*, 1990; Hammer, *et. al.*, 1998) (9.9 ± 2.3 mmol/L and 9.7 ± 1.4 mmol/L respectively), a more pronounced decrease in fibrinogen glycation was seen after the longer study period of 6 days (33%) compared to the 3-day period (15%). This is comparable to the better correlation that was found for the longer 8-day period ($p = 0.038$) than the initial 4-day period ($p = 0.74$).

The extent of in vivo glycation of proteins is not only dependent on the extent and duration of glucose exposure but also on the half-life of the protein and its chemical characteristics such as the intrinsic susceptibility to non-enzymatic glycation.

Acidic proteins have been found to be less susceptible to glycation than basic proteins (Austin *et al.*, 1987). Fibrinogen was shown to have a low intrinsic susceptibility to *in vivo* glycation. Austin *et al.* (1987) demonstrated that fibrinogen of five diabetic subjects was on average 5.9% glycated while fibrinogen that was *in vitro* exposed to 0.5 mol/L glucose for two days was 65.1% glycated. A possible explanation for this seemingly low intrinsic susceptibility to *in vivo* glycation may be the fact that fibrinogen is an acidic protein. This relatively low intrinsic susceptibility to non-enzymatic glycation may further explain why it was found that fibrinogen correlates better with a longer time-interval than just its half-life.

Results from this study can be used to help explain the link between hyperglycaemia and CVD. Glycated fibrinogen is preferentially distributed in the extravascular compartment whereas non-glycated fibrinogen is found in the intravascular compartment. This will result in an increased uptake of fibrinogen into vessel walls that could contribute to atherosclerotic disease progression in diabetic individuals (Dunn & Ariëns, 2004). Results from this study show that glycation of fibrinogen is indeed sensitive to fluctuations in blood glucose. Uncontrolled glucose levels may therefore contribute to the development of CVD through the production of glycated fibrinogen, which in turn has been shown to have an increased level of uptake into the vessel wall, and in doing so contributes to atherosclerotic disease progression.

5.4 Limitations

The study had some limitations worth mentioning. Determining fibrinogen glycation levels and fasting capillary glucose every fourth day would have provided better correlations to help

determine for which time-interval fibrinogen correlates best. This was not done since determining whether glycated fibrinogen could be used as a short-term indicator of glycaemic control was not the main study aim. The results of this study in this regard should therefore be handled circumspectedly.

Measuring HbA1c at the end of the intervention would have also helped to better explain the relationship between glycated fibrinogen and glycaemic control. This was not done because the planned study duration was shorter than three months and HbA1c is not as sensitive for fluctuations in glycaemic control in shorter than three month time-intervals.

Because there is a genetic variation in fibrinogen levels, repeating the study in another ethnic group may help confirm the results found in this study. Not all type 2 diabetic are obese and thus it may be helpful to repeat the study in non-obese individuals to exclude the factors related to obesity.

5.5 Conclusion

The methods for determining glycated fibrinogen that are currently used, are time consuming and labour intensive. In this study, a commercially available kit was adapted for the fast and easy determination of glycated fibrinogen. This was used to show that fibrinogen is significantly more glycated in type 2 diabetic individuals than in non-diabetic individuals. Fibrinogen glycation can also be successfully reduced in outpatients through optimal glycaemic control.

Glycated fibrinogen correlates with and compares well with HbA1c in monitoring glycaemic control. By correlating end fibrinogen glycation levels with the average fasting capillary glucose of different 4-day time-intervals (fibrinogen has a half-life of 4 days) the study indicated that fibrinogen could be used as a short-term indicator of glycaemic control. Because fibrinogen is involved in vascular disease itself, glycated fibrinogen may be a better long-term predictor of CVD than the current markers of glycaemic control (HbA1c and fructosamine). It may also aid in the elucidation of the relationship between hyperglycaemia and CVD. The results of this study showed that fibrinogen glycation is indeed sensitive to fluctuations in glycaemic control.

CHAPTER 6: REFERENCES

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ANNEXURE A: Article: Glycation of fibrinogen in uncontrolled diabetic patients and the effects of glycaemic control on fibrinogen glycation.

Original Article

GLYCATION OF FIBRINOGEN IN UNCONTROLLED DIABETIC PATIENTS AND THE EFFECTS OF GLYCAEMIC CONTROL ON FIBRINOGEN

Running title: Glycaemic control and glycated fibrinogen

Marlien Pieters[1,] Danie G. van Zyl[2], Paul Rheeder[3], Johann C. Jerling[1], Du Toit Loots[1], Francois H. van der Westhuizen[4], Louise T. Gottsche[1], John W. Weisel[5]

[1] Department of Nutrition, North-West University, South Africa

[2] Department of Internal Medicine, University of Pretoria, South Africa

[3] Division of Clinical Epidemiology, University of Pretoria, South Africa

[4] School of Biochemistry, North-West University, South Africa

[5] Department of Cell and Developmental Biology, University of Pennsylvania, USA

Corresponding author:

Dr. M. Pieters

School of Physiology, Nutrition and Consumer Science

Department Nutrition

North-West University

Potchefstroom Campus

Private Bag X6001

Potchefstroom

2520

South Africa

Tel no: +27 18 299 2467

Fax no: +27 18 299 2464

E-mail address: vgemp@puk.ac.za

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Abstract

Introduction

Evidence exists for a relationship between glycaemic control and macrovascular disease. Non-enzymatic glycation of proteins may explain this relationship in part. We investigated the effect of blood glucose control, under out-patient conditions, on fibrinogen glycation as well as the relationship between glycated fibrinogen and glycaemic control using a new sensitive method for the measurement of glycated fibrinogen.

Materials and Methods

Blood samples were taken from twenty subjects with uncontrolled Type 2 diabetes (HbA1c>7%) to determine the levels of glycation. The subjects were then treated with insulin in order to control blood glucose. Twenty age and BMI matched non-diabetic subjects were included as a reference group.

Results

The subjects with diabetes had significantly higher mean fibrinogen glycation at baseline than the non-diabetic subjects (7.84 vs 3.89 mol glucose / mol fibrinogen; $p < 0.001$). After control of blood glucose, fibrinogen glycation was reduced significantly in the subjects with diabetes (7.84 to 5.24 mol glucose / mol fibrinogen; $p < 0.0002$). The change in glycation during the intervention correlated significantly with the change in capillary glucose in the diabetic group (r=0.6, p=0.005). Fibrinogen glycation was comparable to HbA1c in predicting glycaemic control (p=0.54). Fibrinogen glycation correlated best with the average fasting capillary glucose of the preceding 5 – 8 days (r=0.54, p=0.014).

Conclusion

We conclude that glucose control under out-patient conditions decreases fibrinogen glycation in subjects with Type 2 diabetes and that glycated fibrinogen compares well with HbA1c in its relation to glycaemic control.

Keywords: glycation; glycated fibrinogen; Type 2 diabetes; glycaemic control

Abbreviations

HbA1c – glycated heamoglobin; CVD – cardiovascular disease; LDL – low density lipoprotein; BMI – body mass index; EDTA - Ethylenediaminetetraacetic acid; ROC curves – receiver operator characteristic curves

Diabetes mellitus is a worldwide pandemic with a projected increase in prevalence from 171 million people in 2000 to 366 million in 2030 (1). Both Type 1 and 2 diabetes are powerful and independent risk factors for cardiovascular disease (CVD), such that patients with diabetes have a two to fourfold increased risk for developing CVD along with increased atherosclerosis (2;3).

The major vascular complications of diabetes are atherosclerosis and microvascular disease, constituting the main cause of mortality and morbidity in diabetes mellitus. Data from the Diabetes Control an Complications Trial have unequivocally established a causal relationship between chronic hyperglycemia and microvascular disease (4;5). The relationship between chronic hyperglycemia and macrovascular complications is not as well defined. The bulk of the evidence does however, suggest a relationship between glycaemic control and the extent of macrovascular complications (6-8).

Pathophysiologically, hyperglycemia might cause atherosclerosis by several mechanisms, the most important of which is most likely the non-reactive enzymatic glycation of plasma proteins and lipids and the formation of reactive advanced glycation end products (AGE's) mediated by oxidative stress mechanisms (9). Advanced glycation end products potentially modulate initiating steps in atherosclerosis through blood-vessel wall interactions, triggering of inflammatory-proliferative processes, propagation of inflammation and vascular perturbation as well as through promotion of the formation of oxidized LDL (10).

Fibrinogen has been shown to be an independent risk marker for CVD (11). Fibrinogen may contribute significantly to the increased CVD risk in patients with diabetes both in that they generally have elevated fibrinogen levels and that fibrinogen undergoes non-enzymatic glycation in the presence of uncontrolled blood glucose levels in the diabetic subject (12-15). This glycation can alter fibrinogen structure / function, which may then result in the formation of a tight and rigid fibrin network that is resistant to lysis (16-18). Measuring glycated fibrinogen and determining how glycation changes with glucose control together with comparing the ability of glycated fibrinogen to monitor glycaemic control with that of HbA1c may shed more light on the relationship between glycaemic control and macrovascular complications.

Although glycated fibrinogen has been suggested as a short term index of glycaemic control based on its short half life of three to four days (14;19;20), this is the first study to determine the effect of glycaemic control on fibrinogen glycation in out-patient subjects and to demonstrate how well it compares with HbA1c in its relation to glycaemic control using a new sensitive method for the determination of fibrinogen glycation. The study was done under

out-patient conditions, in order to determine the applicability of these measurements to the majority of subjects with diabetes and not only to those receiving intensive in-hospital treatment.

Materials and methods

Study design:

In this, parallel, controlled intervention, twenty black African subjects with Type 2 diabetes, uncontrolled (HbA1c > 7%) on oral hypoglycaemic agents, were included and treated with insulin, until glycaemic control was achieved (four out of five subsequent readings within normal glucose range). The patients then had to remain controlled for eight days before end blood samples were drawn. This eight day period was chosen in order to provide enough time for unglycated fibrinogen to be produced (half-life 3-4 days) after glycaemic control had been achieved. Twenty non-diabetic black African subjects were included as a reference group in order to control for variation over time. All subjects signed informed consent and ethical approval was obtained form the ethics committees of both the University of Pretoria and the North-West University, South Africa.

Subjects

Type 2 diabetic subjects: Inclusion criteria: Patients had to be uncontrolled (HbA1c > 9%) on maximum dose combination oral hypoglycaemic medication; BMI > 25kg/m^2; 40-65 years of age; blood pressure sufficiently controlled not to necessitate treatment change during intervention (< 140/90 mmHg).

Exclusion criteria: Major surgery in the preceding six months; macrovascular complications; disease that can influence haemostasis (e.g. thrombocytopenia, cancer, liver disease) patients on aspirin, warfarin, steroids, hormone replacement therapy or non-steroidal anti-inflammatory drugs; proteinuria on urine dipstick (> 300mg/day) or acute infection.

Non-diabetic controls: Non-diabetic subjects with matching anti-hypertensive drug-use (hidrochlorothiazide, ACE-inhibitor - Perindopril and nifedipine – Adalat), age, gender and BMI were recruited. The same inclusion and exclusion criteria as for the subjects with diabetes were adhered to. Baseline oral glucose tolerance tests were done to rule out diabetes.

Study protocol

The intervention in the diabetic group consisted of 3 phases.

Phase 1: On the first visit, the subjects were seen by a diabetes educator. They were taught how to do self glucose monitoring, co-ordination of insulin use with meals, symptoms

and management of hypoglycaemic events and the use of glucagon. Fasting capillary glucose was measured daily for 1 week.

Phase 2: Subjects received 10 IU basal analogue insulin Glargine (Lantus, Sanofi-Aventis Pharmaceuticals, Paris, France) daily at 22:00 in addition to current treatment of maximum dose oral hypoglycaemic treatment. Metformin use was unchanged from before and during the intervention. Insulin administration was adjusted individually until four out of five subsequent fasting glucose values were less than 7.2 mmol/L (21). Sulphonylureas was stopped

Phase 3: Post-prandial glucose was now controlled with pre-meal administration of short-acting insulin Aspart (Novo Nordisk, Bagsværd, Denmark) as required. Once both fasting and post-prandial (glucose < 10mmol/l (21)) glycaemic control was achieved, the subjects remained on treatment for eight days. Capillary glucose was measured twice daily, fasting and once post-prandially throughout the intervention. Baseline blood samples and anthropometric measurements were collected at the end of phase 1 and end samples and measurements at the end of phase 3. Blood samples of non-diabetic subjects were drawn within one week of their matched diabetic subject's blood sampling.

Blood sampling

Fasting venous blood samples were drawn with minimal stasis by a medical doctor before 10 AM. For the determination of insulin and lipids, blood was left to clot for preparation of serum. For venous glucose determination, blood was collected into sodium fluoride tubes. EDTA blood was collected for the determination of HbA1c. Citrate blood was collected for the determination of fibrinogen and fibrinogen glycation. Blood was centrifuged for 15 minutes at 2000g at 4°C within 30 minutes of collection. Serum and plasma were stored at -82°C until analysis.

Analytical procedures:

Capillary glucose was measured with glucometers (Accu-Chek Active, Roche Diagnostics, Mannheim, Germany). Fasting insulin was measured with an enzyme-linked immunosorbent assay (ELISA) method on the Immulite 2000 Analyzer (Diagnostic Products Corporation, Los Angeles, California, USA). Plasma glucose, baseline HbA1c and serum lipids were measured on a Synchron LX clinical System (Beckman Coulter Inc., Fullerton, CA, USA). Low-density lipoprotein cholesterol was calculated by using the Friedewald formula (22). Insulin resistance was calculated using the homeostasis model assessment (HOMA) as: (fasting insulin x fasting venous glucose)/22.5 (23). Plasma fibrinogen (modified Clauss method) was measured on an Automated Coagulation Laboratory 200 (Instrumentation

Laboratories, Milan, Italy) (between run CV = 3%). Fibrinogen was purified from the plasma of each subject using IF-1 affinity chromatography as described previously (24). Purified fibrinogen was run on 10% SDS PAGE gels to confirm purity and the absence of degradation of the fibrinogen preparations. Fibrinogen glycation was measured with a two-reagent enzymatic assay (GlyPro® assay, Genzyme Diagnostics, Cambridge, MA (between run CV = 5%). This is a specific enzymatic method for the direct measurement of glycated proteins in serum or plasma. The first reagent digests the proteins and subsequently releases glycated protein fragments. Ketoamine oxidase in the second reagent facilitates the specific oxidation of the ketoamine bond of the glycated protein fragment substrate. Liberation of hydrogen peroxide allows a colorimetric determination of the amount of glycated protein in an end-point reaction. Absorbance at 550nm is measured after the addition of reagent 1 and again after reagent 2. Results are calculated as follows:

Glycated protein (µmol/L) = ΔA sample / ΔA calibrator x calibrator value (µmol/L).

Statistical analysis:

A power calculation was done using 1 standard deviation as a clinical significant difference, as a defined clinical significant difference is not known for the main outcome variable, fibrinogen glycation. To achieve a difference of 1 standard deviation at 80% power, 5% significance, each group should consist of at least 16 individuals. The computer software package, Statistica (Statsoft Inc., Tulsa, Oaklahoma, USA) was used for statistical analysis. Data was tested for normality. Normally distributed data is presented as the mean (standard deviation) and not normally distributed data as median (25th ; 75th percentile). Differences in baseline characteristics as well as differences in changes during the intervention between the two groups were determined using the t-test for independent samples for parametric data and the Mann-Whitney U test for non-parametric data. Differences from baseline to end within each group were determined using the paired t-test. Spearman-correlation was used for all correlations. In order to determine how well glycated fibrinogen compared to HbA1c in monitoring glycaemic control, agreement was assessed between glycated fibrinogen and the current standard measure for blood glucose control - HbA1c, as well as fasting venous glucose, utilizing the area under the Receiver Operator Characteristic (ROC) curves. ROC curves were also used to determine the optimal threshold level of glycated fibrinogen to indicate glycaemic control. The sensitivity and specificity for this glycated fibrinogen value was determined, for both HbA1c and fasting venous glucose level as reference.

Results

Two non-diabetic subjects failed to return for end blood sampling and were hence excluded from the study. Table 1 presents the baseline characteristics of the two groups. They were

comparable regarding age, BMI, blood pressure, fibrinogen concentration and fasting insulin. The diabetic group had significantly higher fasting venous glucose, HbA1c, insulin resistance, triglycerides and fibrinogen glycation. Only one of the subjects smoked and alcohol consumption was infrequent. Baseline glycated fibrinogen correlated significantly with baseline HbA1c ($r = 0.8$, $p < 0.001$), fasting venous glucose ($r = 0.69$, $p < 0.001$) and the average of the preceding four days' fasting capillary glucose ($r = 0.5$, $p < 0.035$) for the total group.

The average duration between baseline and end blood sampling was 69.5 (range: 15 – 148) days and 75 (range: 8 – 154) days for the diabetic and non-diabetic subjects respectively. There was a significant increase in BMI from baseline to end in the subjects with diabetes ($p = 0.001$) (Table 2). Fasting venous glucose and fibrinogen glycation decreased significantly during the intervention ($p < 0.001$ and $p < 0.001$). No significant changes were seen in the non-diabetic group, except for a slight increase in glucose ($p = 0.05$). The increase in BMI and the decrease in venous glucose concentration and fibrinogen glycation in the diabetic group differed significantly from the responses in the non-diabetic group. There was a significant correlation ($r = 0.52$; $p = 0.001$) between the decrease in fibrinogen glycation and venous glucose in the total group. This correlation was not significant for the diabetic subjects alone, however the correlation between the decrease in fibrinogen glycation and the decrease in capillary glucose (an average of 4 days' values at baseline and end) was significant ($r = 0.6$; $p = 0.005$).

The ability of glycated fibrinogen to predict blood glucose control as controlled (mean blood glucose < 6.4 mmol/L) or uncontrolled (mean blood glucose > 6.4 mmol/L) was assessed by utilizing ROC curves with fasting venous glucose values and HbA1c (control < 7%) as reference variables. The area under the ROC curve of baseline glycated fibrinogen is 0.94 (0.82, 0.99) with baseline HbA1c as reference (Figure 1). A glycated fibrinogen value of 5.62 mol glucose / mol fibrinogen has a 78.9 % (54.4, 93.8) sensitivity and 100 % (80.3, 100.0) specificity to predict glycaemic control with HbA1c as reference. This relates to a positive likelihood ratio of 13.42 for glycated fibrinogen to predict glycaemic control with HbA1c as gold standard. If the area under the ROC curve for glycated fibrinogen is compared with that of HbA1c with fasting plasma glucose as reference, the area under the ROC curve for glycated fibrinogen is 0.85 (0.70, 0.95), and that of HbA1c is 0.89 (0.74, 0.97) (Figure 2). This difference is not statistically significant.

In order to estimate for which time period glycated fibrinogen corresponds to glucose concentrations, the end value of glycated fibrinogen was correlated to the average fasting capillary glucose of different 4-day time intervals preceding the date of end blood sampling,

using the daily recorded capillary values (Table 3). Glycation of fibrinogen showed a strong correlation with the preceding 12 days ($r = 0.53$; $p = 0.017$), with the preceding 5-8 days as the most important contributor ($r = 0.54$; $p = 0.014$) of the 12 day period.

Discussion

This study had two main aims. The first was whether controlling blood glucose levels of out-patients subjects with Type 2 diabetes would result in decreased fibrinogen glycation using a sensitive method for measurement of glycated fibrinogen. The second aim, which has never been tested before was to determine how well glycated fibrinogen compares to HbA1c in its relation to glycaemic control. We were also able to provide information regarding the time-interval at which glycated fibrinogen correlated best with fasting capillary glucose.

Although there was no difference between the mean fibrinogen concentration of the two groups, both had high fibrinogen concentrations on average. Subjects with Type 2 diabetes have been shown to have high fibrinogen (25). The high fibrinogen concentration in the non-diabetic subjects is also in agreement with previous work from our group (26) as well as others (27) who have reported higher fibrinogen concentrations in black people compared to Caucasians. The diabetic group was significantly more insulin resistant than the non-diabetic group, while fasting insulin concentrations were similar. This is most likely a result of beta cell failure due to insulin resistance present in subjects with diabetes. Body mass index increased significantly in the diabetic group during the intervention. This is probably due to the exogenous insulin they received at the beginning of the study. There are several explanations for why insulin treatment results in weight gain: reduced glucosuria, leading to reduced urinary calorie loss and increased glucose metabolism, accumulation of adiposity or increased appetite (6).

The average glycation of fibrinogen in the subjects with diabetes was approximately twice as high as that of the non-diabetic subjects (7.84 vs 3.89 mol glucose / mol fibrinogen). This significantly improved after achievement of glycaemic control (33% decreased glycation) although not to a level seen in the non-diabetic subjects. Two other studies have reported decreased fibrinogen glycation after treatment of uncontrolled diabetic subjects, Hammer *et al.*(28) and Ardawi *et al.*(29). It is not clear, however, from their results whether Type 1 or type 2 diabetes was investigated. Their results are reported after intensive treatment of three and six days. Our results show that this decrease in fibrinogen glycation can also be achieved in the majority of diabetic subjects who attend out-patient clinics at hospitals. The reason why the decrease in glycated fibrinogen correlated significantly with the decrease in fasting capillary glucose but not with fasting venous glucose, is probably because venous glucose measurement is a single measurement at a point in time. The capillary glucose

values used for the correlation was an average of four days' values, which is a better reflection of the true fasting glucose levels around the time of blood sampling, as opposed to the single measurement of venous glucose. This is further substantiated by the fact that a significant correlation was obtained between the decrease in fibrinogen glycation and the decrease in fasting venous glucose, when the samples size was increased by including also the results of the non-diabetic subjects.

The level of glycation reported in this study, is in agreement with levels found by Suzuki *et al.*(30) (8.1 ± 3.64 mol glucose / mol fibrinogen in diabetic subjects and 3.13 ± 1.29 mol glucose / mol fibrinogen in normal subjects). Lütjens *et al.*(31) however, found lower levels in both Type 1 diabetic subjects and healthy volunteers. Using two different methods, they found 1.3 ± 0.19 and 1.33 ± 0.21 mol glucose / mol fibrinogen for subjects with uncontrolled Type 1 diabetes and 1.06 ± 0.08 and 0.95 ± 0.17 mol glucose / mol fibrinogen for healthy volunteers. A possible explanation for the different levels of glycation may be that both our group and the group of Suzuki *et al.*(30) used commercially available kits for the determination of glycated protein, where as Lütjens *et al.* (31) used the thiobarbituric acid method (32) and high performance liquid chromatography according to the method of Schleicher and Wieland (33). Other groups have also measured fibrinogen glycation *in vivo*, but they report their results as glycated fibrinogen expressed as percentage of total fibrinogen (28;34).

Glycated fibrinogen has been suggested as a predictor of glycaemic control (28-30;35). Exactly how well it compares to HbA1c in monitoring glycaemic control has, however, not yet been determined, until now. Results from this study indicated that glycated fibrinogen is comparable to HbA1c in its relation to glycaemic control in subjects with Type 2 diabetes. When using HbA1c as the gold standard, the area under the ROC curve of glycated fibrinogen is 0.94. The ROC curve is used to assess the discriminatory value of a diagnostic test. In ninety four percent of cases, subjects will be correctly classified as either having good or poor control based on the HbA1c. A glycated fibrinogen value above the optimum threshold level of 5.62mol glucose / mol fibrinogen is 13.42 times more likely to occur in subjects with HbA1c uncontrolled (> 7 %) than in subjects who are well controlled (positive likelihood ratio). There was also no significant difference between the ROC curves of glycated fibrinogen and HbA1c in predicting glycaemic control with fasting venous glucose as a reference. It has to be kept in mind that fibrinogen is an acute phase protein and plasma levels may change considerably during acute infections.

Due to its short half life, glycated fibrinogen is an ideal marker for monitoring short term glycaemic control and therefore the early assessment of the effectiveness of a treatment regime or a research study with a short duration. This is especially important in developing countries where home daily glucose monitoring is not an option as very few diabetic patients have their own glucometers. In cross sectional data glycated fibrinogen has been shown to correlate with blood glucose at the same time or one day earlier (30) or three to four days prior to measurement (36). Although not the main aim of the study, by correlating the end fibrinogen glycation level with the average fasting capillary glucose of different 4-day time-intervals (comparable to fibrinogen half-life) we were able to ascertain with which time-interval fibrinogen glycation correlated best. Measurement of glycated fibrinogen at each time-interval would have provided stronger evidence. Our results suggest that glycated fibrinogen correlated best with the preceding 12 days of which the preceding 5 – 8 days made the most important contribution. This is somewhat in agreement with the results from Hammer *et al.* (28) and Ardawi *et al.* (29), who respectively reported a 15 % decrease in fibrinogen glycation after three days and 33.4 % after 6 days of treatment of newly diagnosed diabetic subjects to similar levels of fasting blood glucose (9.9 ± 2.3 mmol/L and 9.7 ± 1.4 mmol/L respectively). Even though similar blood glucose levels were achieved, a more pronounced decrease in glycation was seen over the somewhat longer period of 6 days.

The extent of *in vivo* glycation of proteins is furthermore a complex function of the glucose concentration it is exposed to, the duration of exposure, the half-life of the protein as well as the intrinsic susceptibility to non-enzymatic glycation. Austin *et al.* (34) have shown that overall acidity or basicity relates fairly well with the extent of glycation. Fibrinogen, being an acidic protein, is therefore glycated much less *in vivo* than *in vitro*. This relatively low intrinsic susceptibility to non-enzymatic glycation may further explain why we found fibrinogen correlating better with a longer time-interval than just its half life.

We have adapted and implemented a sensitive method for the determination of glycated fibrinogen. With this we were able to show that fibrinogen is significantly more glycated in subjects with Type 2 diabetes than non-diabetic subjects and that even in out-patient subjects fibrinogen glycation can successfully be reduced through control of blood glucose. Glycated fibrinogen correlates with and compares well with HbA1c in monitoring glycaemic control. The results from this study aid in the understanding of the relationship between glycaemic control and macrovascular disease in that glycation of plasma proteins, specifically fibrinogen, is indeed sensitive to fluctuations in glycaemic control.

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Table 1 Baseline characteristics of subjects

Variables	Type 2 diabetic subjects	Non-diabetic subjects
Patients (n)	20	18
Sex (male/female)	6 / 14	7 / 11
Age (years)	53.0 (8.29)	52.9 (7.13)
Body mass index (kg/m^2)	30.8 (6.16)	31.8 (5.89)
Fasting venous glucose (mmol/L)	14.6 (8.12)[a]	5.18 (1.24)[a]
Fasting Insulin (mU/L)[c]	11.0 (6.70 ; 15.8)	13.7 (8.9 ; 28.8)
Fibrinogen (g/L)	4.25 (0.80)	4.02 (0.86)
Fibrinogen glycation (mol glucose/mol	7.84 (2.68)[a]	3.89 (0.86)[a]
fibrinogen)	11.7 (9.50 ; 13.8)[a]	5.60 (5.30 ; 5.90)[a]
HbA1c (%)[c]	140.5 (22.5)	143.3 (26.4)
Systolic blood pressure (mmHg)	86.6 (8.15)	89.8 (13.1)
Diastolic blood pressure (mmHg)	4.84 (1.49)	4.54 (0.71)
Total cholesterol (mmol/L)	1.80 (1.25 ; 2.50)[b]	1.05 (0.80 ; 1.25)[b]
Triglycerides (mmol/L)[c]	0.9 (0.75 ; 1.3)	0.9 (0.8 ; 1.25)
High density lipoprotein cholesterol (mmol/L)[c]	2.86 (1.03)	2.88 (0.83)
Low density lipoprotein cholesterol (mmol/L)	5.18 (3.99 ; 6.95)[b]	3.11 (2.29 ; 7.42)[b]
Insulin resistance (HOMA)[c]	11.0 (8.0)	
Duration of diabetes (years)		

Note: [a] $P < 0.001$; [b] $P < 0.05$; [c] Data not normally distributed and therefore reported as median (25 , 75 percentile); Insulin resistance = (fasting insulin x fasting venous glucose)/22.5

Table 2 Differences between diabetic and non-diabetic subjects of selected variables for the intervention period

Variable	Type 2 diabetic subjects (n = 20)				Non-diabetic controls (n = 18)				Changes between groups (deltas)
	Baseline	End	Delta	p	Baseline	End	Delta	p	p
Body mass index (kg/m^2)	30.8 (6.16)	32.2 (6.80)	1.79 (2.08)	0.001	31.7 (5.89)	32.0 (6.02)	0.30 (0.81)	0.14	0.009
Fasting glucose (mmol/L)	14.6 (8.12)	6.72 (2.57)	-7.88 (8.90)	0.0008	5.18 (1.24)	5.63 (1.37)	0.46 (0.91)	0.05	0.0003
Fibrinogen (g/L)	4.25 (0.80)	4.36 (0.79)	0.11 (0.95)	0.62	4.02 (0.86)	3.85 (0.86)	-0.16 (0.72)	0.35	0.33
Fibrinogen glycation (mol glucose/mol fibrinogen)	7.84 (2.68)	5.24 (1.65)	-2.60 (2.60)	0.0002	3.89 (0.86)	3.75 (0.62)	-0.19 (0.70)	0.27	0.0007

Table 3: Correlation of glycated fibrinogen with the average fasting capillary glucose concentration over different time intervals

Time Intervals	Fibrinogen glycation	
	r	p
0-4 days	0.31	0.74
5-8 days	0.54	0.014
9-12 days	0.33	0.16
0-8 days	0.47	0.038
0-12 days	0.53	0.017

Day 0 = day of end blood sampling. Days 4,5,8,9,12 – the amount of days preceding end blood sampling

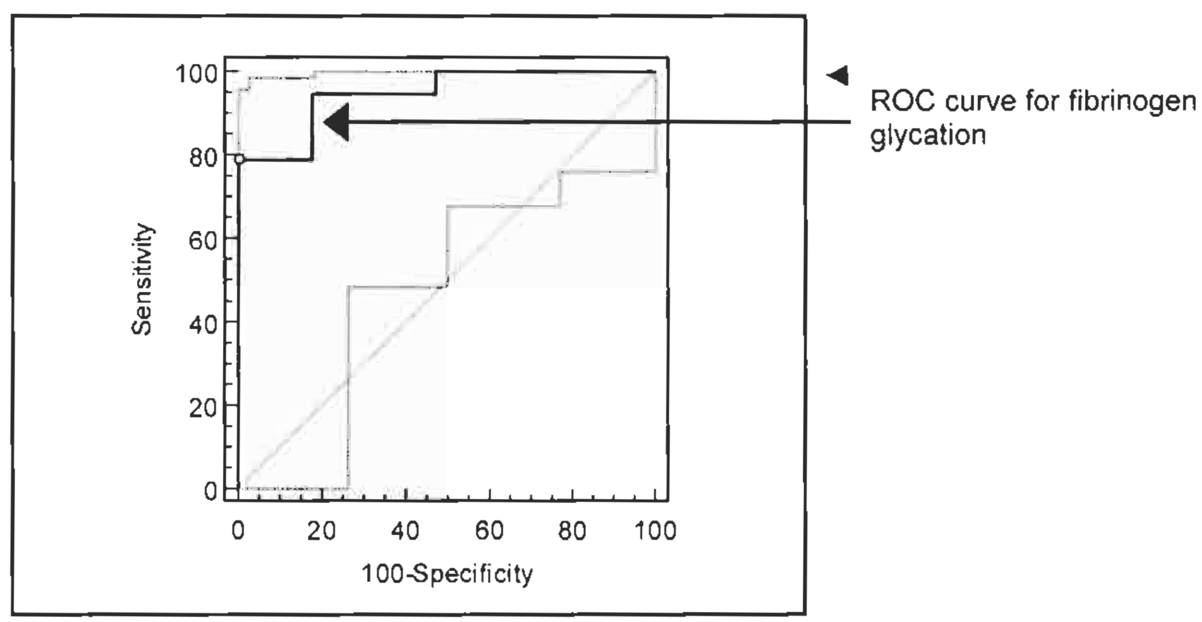

Figure 1 ROC curve for fibrinogen glycation with HbA1c as reference value

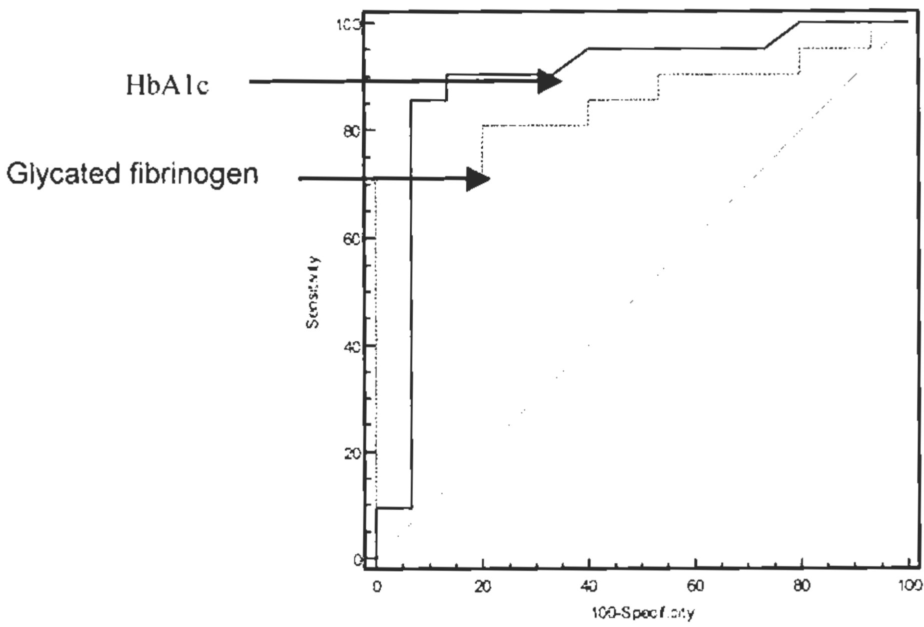

Figure 2 Comparison of ROC curves of glycated fibrinogen and HbA1c using fasting venous glucose as reference

Legends

Table 1 Baseline characteristics of subjects

Note: [a] P < 0.001; [b] P< 0.05; [c] Data not normally distributed and therefore reported as median (25 , 75 percentile); Insulin resistance = (fasting insulin x fasting venous glucose)/22.5

Table 2 Differences between diabetic and non-diabetic subjects of selected variables for the intervention period

Table 3: Correlation of glycated fibrinogen with the average fasting capillary glucose concentration over different time intervals

Day 0 = day of end blood sampling. Days 4,5,8,9,12 – the amount of days preceding end blood sampling

Figure 1 ROC curve for fibrinogen glycation with HbA1c as reference value

Figure 2 Comparison of ROC curves of glycated fibrinogen and HbA1c using fasting venous glucose as reference

ANNEXURE B: GlyPro® assay, Genzyme Diagnostics, Cambridge, MA, package insert

GB

GlyPro® Reagent

Catalog number 70-5125-05 or 70-5126-05

INTENDED USE
For the quantitative determination of glycated serum proteins.

SUMMARY

Control of the glycemic state is considered to be a fundamental aspect of Diabetes Mellitus care.[1] Numerous clinical studies, including the Diabetes Control and Complications Trial, have shown that long term monitoring and tight control of blood glucose levels can help to decrease diabetes related complications.[2,3]

Glycated proteins are complexes formed by a slow, non-enzymatic, chemical reaction between proteins and reducing sugars described as a Maillard reaction. This occurs when amino acids combine with glucose to form labile Schiff bases which, over time, are converted to stable ketoamines via an Amadori rearrangement.[4] In instances where blood glucose levels are abnormally elevated, such as Diabetes Mellitus, the concentration of glycated serum proteins also increases.[4,5]

Over the course of a day, blood glucose levels vary significantly. Consequently, they cannot be used to provide a reliable picture of glucose control over an extended period. The measurement of a variety of glycated proteins including albumin, total serum protein and hemoglobin are employed for medium to long term monitoring of the diabetic condition.

The degree of protein glycation is proportional to the sugar concentration in the serum and the half-life of the protein being glycated. Glycated hemoglobin (HbA_1 or HbA_{1c}) provides a measure of the average glucose level over a 6-8 week period. Measurement of glycated serum protein is representative of the mean blood glucose levels over the preceding 2-3 weeks.[1,2,4] The shorter half-life of glycated serum proteins indicates an improvement or deterioration of glucose control earlier than HbA_{1c}.[2]

Existing methods for the measurement of glycated serum proteins such as furosine (HPLC), affinity chromatography, thiobarbituric acid (TBA) and nitroblue tetrazolium (NBT) may be time consuming, difficult to automate and / or suffer from non-specificity.[6,7]

The measurement of both glycated hemoglobin and glycated serum proteins has been suggested as the most useful method for monitoring long-term control of diabetes.[4]

PRINCIPLE
The GlyPro® assay is a specific enzymatic method for the direct measurement of glycated serum proteins in serum or plasma.

The first reagent containing Proteinase K provides an on-line digestion of the sample and subsequent release of glycated protein fragments. A novel enzyme, ketoamine oxidase (KAO), in the second reagent facilitates the specific oxidation of the ketoamine bond of the glycated protein fragment substrate. Liberation of hydrogen peroxide allows a colorimetric determination of the amount of glycated protein in an end-point reaction.

Results are obtained by measurement of the increase in absorbance at 550nm, which is proportional to the concentration of glycated protein in the sample.

Reaction Sequence

$$\text{Glycated Protein} \xrightarrow{\text{Proteinase K}} \text{Glycated protein fragments}$$

$$\text{Glycated protein fragments} \xrightarrow{\text{KAO}} \text{Amino acids} + H_2O_2$$

$$H_2O_2 + \text{Chromogens} \xrightarrow{\text{Peroxidase}} \text{Color} + H_2O$$

REAGENTS

Composition

Component	Ingredient	Source	Contains
Reagent 1	Proteinase K	T. album	786 U/mL
	Peroxidase	Horseradish	60 U/mL
	EPPS buffer		60 mM
	Calcium acetate		5 mM
	Potassium - hexacyanoferrate(II)		90µM
	Copper acetate		30µM
	BPS		144µM
	TOOS		2.8 mM
	Cholic acid	Bovine or sheep	18 mg/mL
	POETE		2.5 mg/mL
Reagent 2	Ketoamine oxidase	F. oxysporum	9 U/mL
	EPPS buffer		50 mM
	Stabilizer		30 mg/mL
	EDTA		50 mM
	AAP		10.5 mM

BPS = Bathophenanthroline disulphonic acid.
TOOS = N-Ethyl-N-Sulphohydroxypropyl-m-toluidine, sodium salt
POETE = polyoxyethylene 10 tridecyl ether.
AAP = 4-Aminoantipyrene.

Precautions And Warnings

1. For In Vitro Diagnostic Use.
2. Do not pipette by mouth.
3. Do not use the reagents after the expiration date printed on the label.

Preparation

Reagent 1: Reconstitute each vial with 25.0 mL of purified water using a Class 'A' volumetric pipette. Replace the cap/stopper and mix gently by inversion. Allow to stand for a minimum of 10 minutes at room temperature. Mix gently by inversion before use.

Reagent 2: Reconstitute each vial with 5.0 mL of purified water using a Class 'A' volumetric pipette. Replace the cap/stopper and mix gently by inversion. Allow to stand for a minimum of 10 minutes at room temperature. Mix gently by inversion before use.

Storage And Stability

Unopened reagents are stable until the expiration date printed on the reagent bottle label when stored at 2-8°C.

Reagent 1: once reconstituted is stable up to 42 days at 2-8°C.
Reagent 2: once reconstituted is stable up to 42 days at 2-8°C.

Indications of Deterioration

Presence of extreme turbidity or growth may indicate deterioration.

SPECIMEN COLLECTION AND PREPARATION

Serum, or EDTA treated plasma are the recommended specimens. Fasting samples are not required.

Serum: Collect whole blood by venipuncture and allow to clot. Centrifuge and remove the serum as soon as possible after collection (within 3 hours).[8]

Plasma: Specimens may be collected using EDTA anticoagulant. Centrifuge and remove the plasma as soon as possible after collection (within 3 hours).[8]

Specimens can be collected as per the National Committee for Clinical Laboratory Standards Guidelines H4-A3.[8] If not analyzed promptly, specimens may be stored at 2 - 8°C for up to two weeks or at -20°C for up to five weeks. Samples may be frozen once.

EDTA plasma samples will give a mean difference of 6% less than serum samples.[9] The same sample type should be used for all comparative analyses on the same patient.

PROCEDURE

Assay

Below is a general example of the GlyPro® test procedure for an automated analyzer. All analyzer applications should be validated in accordance with CLIA recommendations. Genzyme has validated applications for several automated analyzers. This information is available through your distributor.

Step 1 :

Sample + Reagent 1 —(37°C, 5 min)→ Measure Absorbance (A_1)
20µL 250 µL (550nm)

Step 2 :

Reagent 2 —(37°C, 3 - 5 min)→ Measure Absorbance (A_2)
50 µL (550nm)

Materials Provided

Any of the following items may be included in the package you receive.

Description	Configuration	Catalog Number
GlyPro® Reagent 1	5 x 25 mL	70-5125-05
GlyPro® Reagent 2	5 x 5 mL	70-5126-05

Materials Required but not Provided

Description	Configuration	Catalog Number
GlyPro® Low Control	5 x 1 mL	70-5128-05
GlyPro® High Control	5 x 1 mL	70-5129-05
GlyPro® Calibrator	5 x 1 mL	70-5127-05

1. Class 'A' volumetric pipettes (5.0 mL and 25.0 mL).
2. Purified water.

Calibration

The Genzyme GlyPro® Calibrator (70-5127-05) is required for calibration. The value of the calibrator, which can be found on the vial label, was assigned relative to human serum glycated with ^{14}C-glucose and is expressed as µmol/L of glycated protein.[10] Refer to the instrument operator's manual for analyzer specific calibration procedures and for guidance in determining calibration frequency. See the GlyPro® Calibrator package insert for instructions on calibrator preparation.

Quality Control Procedures

Reliability of test results should be routinely monitored using the GlyPro® Low (70-5128-05) and High (70-5129-05) Controls. Use only control sera made specifically for the GlyPro® assay. Each laboratory should establish an acceptable range of values for the GlyPro® Controls by repeat analysis. The laboratory established mean should be within the range printed on the vial label of the controls.

Quality control requirements should be performed in conformance with local, state, and/or federal regulations or accreditation requirements.

RESULTS

The total change in absorbance (ΔA) is determined by subtracting A_1 from A_2.

$$\text{Glycated protein } (\mu mol/L) = \frac{\Delta A \text{ sample}}{\Delta A \text{ calibrator}} \times \text{Calibrator value } (\mu mol/L)$$

Results are reported as µmol/L of glycated protein.

Limitations / Interfering Substances

No interference was detected in the GlyPro® assay up to and including the concentrations stated below, using a Roche Cobas® Mira S.

Interfering Substance	Concentration with no interference *	
Triglyceride (Avian)	750 mg/dL	(8.5 mmol/L)
Ascorbic acid	8 mg/dL	(500 µmol/L)
Bilirubin	29 mg/dL	(500 µmol/L)
Hemoglobin	200 mg/dL	(200 mg/dL)
Uric acid	33 mg/dL	(2.0 mmol/L)
Glucose	1800 mg/dL	(100 mmol/L)

*See analyzer specific applications for information regarding other chemistry systems.

Samples with levels of interfering substances higher than the upper limit should be diluted 1 part sample with 1 part saline before assaying. Multiply the result by two to correct for the dilution.

The labile Schiff base fraction does not interfere with the measurement of glycated serum protein using the GlyPro® method.

In an *in vitro* study of commonly used drugs dobesilate, gentisic acid and methampyrone produced artificially low values at therapeutic levels. Refer to the work of Young for a review of other drug affects on glycated serum protein levels.[11]

EXPECTED VALUES

The expected values for glycated serum protein are 122-236 µmol/L.[12] The normal range for serum was determined by a study of 466 non-diabetic adults between the ages of 20 and 60 years old. Among the exclusion criteria were: pregnant women, fasting blood glucose > 200 mg/dL, triglycerides > 400 mg/dL, hemolyzed and icteric samples and individuals with an immediate familial history of Diabetes. Each laboratory must establish its own range of expected values. GlyPro® values should be used in association with patient history results, clinical data and other laboratory tests.

SPECIFIC PERFORMANCE CHARACTERISTICS

Accuracy

The GlyPro® method was compared to the Roche Unimate Fructosamine methodology on a Roche Cobas® Mira S using diabetic and normal subjects giving the following results:

Method	Roche Unimate Fructosamine
Number of samples	61
Sample range (µmol/L)	169.0 - 675.5
Slope	1.33
Intercept (µmol/L)	-127.45
Correlation coefficient (r)	0.9915

Precision

Within Run Precision

Within run precision of the GlyPro® Reagent was determined using three levels of frozen, in vitro glycated human serum pools analyzed 20 times in a single run on a Roche Cobas® Mira S.

	Low	Medium	High
n	20	20	20
Sample range (µmol/L)	179-184	403-408	656-670
Mean (µmol/L)	180.7	405.3	663.6
SD (µmol/L)	1.34	1.25	4.55
CV (%)	0.74	0.31	0.69

Between Run Precision

Between run precision of the GlyPro® Reagent was determined using three levels of frozen, in vitro glycated human serum pools. Each pool was run in duplicate, twice per day for ten days on a Roche Cobas® Mira S.

	Low	Medium	High
n	20	20	20
Sample range (µmol/L)	175-185	396-408	644-665
Mean (µmol/L)	179.6	402.3	654.6
SD (µmol/L)	2.99	3.17	5.73
CV (%)	1.66	0.79	0.88

Limit of Detection

The limit of detection of the GlyPro® assay, quantified as 3SDs plus the mean of twenty replicate measurements of saline, is 3.5 µmol/L on a Roche Cobas® Mira S.

Linearity

The GlyPro® method is linear to 1734 µmol/L on a Roche Cobas® Mira S.

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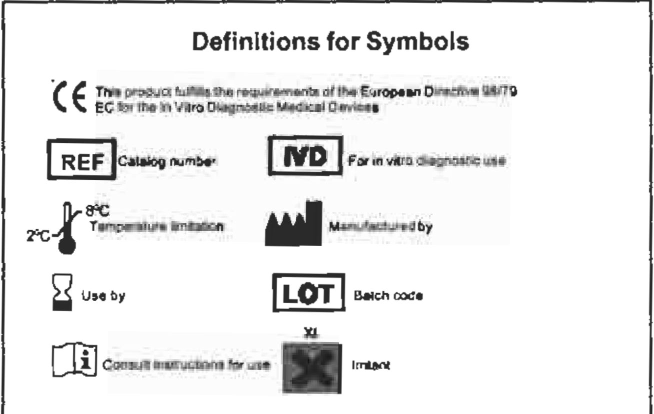

50 Gibson Drive
Kings Hill, West Malling
Kent, UK, ME19 4AF

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