

## The role of a deglycating enzyme 'fructosamine-3-kinase' in diabetes and COPD

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# The Role of a Deglycating Enzyme 'Fructosamine-3-Kinase' in Diabetes and COPD

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**A thesis submitted in partial fulfilment of the requirements of the University of Wolverhampton for degree of Doctor of Philosophy.**

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## Abstract

Recent statistics show that approximately 415 million people worldwide have diabetes. Glycated haemoglobin (HbA1c) measurements were introduced many years ago as the gold standard tool for detecting and monitoring treatment as well as making management decisions for diabetic patients. Glycated haemoglobins are formed by the non-enzymatic glycation of haemoglobin molecules. This non-enzymatic glycation process has been strongly related to pathogenesis of chronic complications associated to diabetes. It was suggested that this glycation process may be moderated by an enzymatic deglycation process thought to involve a deglycating enzyme known as Fructosamine-3-kinase (FN3K), an enzyme that deglycates the glycated haemoglobin in erythrocytes and other glycated proteins in other tissues.

FN3K acts through phosphorylation of fructosamines on the third carbon of their sugar moiety, making them unstable and consequently causing them to detach from the protein. The degree of deglycation is thought to depend on the activity of the FN3K enzyme. Moreover, variation in the activity of FN3K between individuals is hypothesised to lead to apparent differences in glycated haemoglobin levels: some individuals have high rates of deglycation so that they tend to have lower average glycaemia than actually the case, while others with low rates of deglycation appear to have higher than actual glycaemia (known as the glycation gap, G-gap). The G-gap has been reported to be associated with alteration of diabetic complications risk. The G-gap reflects the discrepancy between average glycaemia as determined from glycated haemoglobin (measured as HbA1c) and that from the determination of fructosamine. The positive G-gap is defined as a higher level of glycation of proteins than expected whereas a negative G-gap means a lower level of glycation than expected. To explore the role of FN3K in diabetes and other associated morbidities, we decided to divide our research into 3 studies. Each study was categorised according to the type and the source of samples involved.

The first study explored the correlation between FN3K activity and protein level with G-gap data; it involved 148 diabetic patients who were recruited at New Cross Hospital, Wolverhampton, selected as having a consistent positive G-gap  $> +0.5$  and a consistent negative G-gap  $> -0.5$  over a minimum of 2 estimations. Age, gender, race and BMI were collected from patients in this study. Blood samples were also

collected to measure FN3K activity, protein levels, and markers of CVD in relation to G-gap.

The second study involved 23 AECOPD patients who were recruited from St George's Hospital (London) and were treated with either metformin or a placebo. Serum samples were collected from these patients for a larger study: we assayed those 23 serum samples for FN3K protein levels to explore any possible correlation between FN3K with metformin therapy in COPD patients.

The third study utilised 36 human peripheral lung samples from healthy individuals, asymptomatic smokers and stable COPD patients (GOLD 2) who were recruited at The Section of Respiratory Medicine, University Hospital of Ferrara, Italy. Those samples were assessed for FN3K expression by means of immunohistochemistry to explore the difference in FN3K activity between those three categories.

It was found that the intracellular activity and protein expression of the FN3K enzyme in diabetic patients negatively correlated with the values of G-gaps where FN3K activity was high in patients with negative G-gap. FN3K serum protein levels were shown to be enhanced with metformin administration in COPD diabetic patients, suggesting a protective role for FN3K enzyme against protein damaged caused by the non-enzymatic glycation of proteins. Therefore, patients with positive G-gap have lower FN3K activity than those with negative G-gap, and in turn they are more susceptible to diabetes related complications. Our data also indicate that metformin has a beneficial effect in reducing damage caused by carbonyl stress from cigarette smoking in COPD patients by the action of FN3K.

Our research has demonstrated that FN3K contributes to the protein repair system which protects against damage caused by non-enzymatic glycation. The high activity for the FN3K enzyme was associated with low levels of AGEs and low carbonyl stress levels in observed among patients with diabetes and COPD. In contrast, COPD patients tend to have low FN3K-mediated protection against protein damage in comparison to the normal population. These patients tend to be at risk for developing more complications, particularly CVD complications, than normal, healthy individuals. Treatment with metformin enhances FN3K action in COPD diabetic patients, possibly as a protective enzyme against the damaged caused by the non-enzymatic glycation.

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## Abbreviations:

AECOPD	An acute exacerbation of chronic obstructive pulmonary
Antipain	[(S)-1-Carboxy-2-phenylethyl] carbamoyl-L-arginyl-L-valyl-argininal]
AGEs	Advanced glycation end products
ATP	Adenosine tri phosphate
BzGFruK	N $\alpha$ -hippuryl-N $\epsilon$ -(1-deoxy-D-fructosyl) lysine
BzGpFruK	N $\alpha$ -hippuryl-N $\epsilon$ -(3-phosphofructosyl) lysine
B.i.d	twice (two times) a day
COPD	Chronic obstructive pulmonary disease
DTT	Dithiothreitol
ECM	Extracellular Matrix
ED	Endothelial dysfunction
EDTA	Ethylendiaminetetraacetate
ELISA	Enzyme linked immunoassay
FEV1	Forced expiratory volume
FN3K	Fructosamine-3-kinase
G-gap	Glycation Gap
GLO-1	Glyoxalase-1
Hb	Haemoglobin
HbA1C	Glycated haemoglobin
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPLC	High-performance liquid chromatography
IHC	Immunohistochemistry
IKCa	Intermediate-conductance Calcium-sensitive potassium
KS	Kolmogorov–Smirnov test
PAI-1	Plasminogen activated inhibitor-1
PBS	Phosphate buffered saline

RAGEs	Receptor for Advanced glycation end products
RD	Reagent diluent
SBMG	Self blood glucose monitoring
SD	Standard deviation
(SKCa)	Small-conductance Calcium-sensitive potassium
SLS buffer	Sodium dodecyl sulfate
TMB	3,3',5,5'-Tetramethylbenzidine

# **Chapter 1**

## **Introduction**

## 1.1 Diabetes

Diabetes mellitus is a chronic condition characterised by an abnormal elevation of glucose in the blood (hyperglycaemia) due to an absolute or relative deficiency of insulin. It is associated with reduction in life expectancy, significant morbidity, increased risk of macro-vascular complications, and low quality of life (World Health Organisation (WHO), 1999).

### 1.1.1 Epidemiology of Diabetes

In 2015, it was estimated that approximately 415 million people around the world are living with diabetes, and it is predicted that this number will continue to increase, reaching approximately 642 million people by the year 2040 (International Diabetes Federation (IDF), 2015). The WHO anticipate that the number of deaths attributed to diabetes will double between the years 2005 and 2030 (WHO, 2011). A further 193 million diabetics are undiagnosed and are at risk of developing complications (IDF, 2015). Diabetes can be classified into two main types: Type 1 (insulin-dependent diabetes mellitus) and Type 2 (non-insulin-dependent diabetes mellitus) (Williams, 2002).

It was estimated that there are 3.8 million people living with diabetes in the UK alone, and according to the NHS, the total direct cost of diabetes was £9.8 billion in 2010-2011 (£1 billion for Type 1 and the remaining £8.8 billion for Type 2 diabetes). Approximately 80% of this cost can be attributed to complications associated with diabetes (Diabetes UK, 2014). In addition, the indirect costs associated with diabetes from 2010-2011 were calculated to be £13.9 billion (comprising £0.9 billion for Type 1 diabetes and £13 billion for Type 2). The estimated projected cost for this disease is expected to rise to £39.8 billion by 2035-2036: £16.9 billion of this is assigned for

direct costs (£1.8 billion for Type 1 and £15.1 billion for Type 2 diabetes, respectively), and the remaining £22.9 billion for indirect treatment (£2.4 billion to be spent on Type 1 diabetes and £20.5 billion on Type 2 diabetes) (Hex *et al.*, 2012).

In 2012, The American Diabetes Association (ADA) reported that the total expenditure for diabetes had exceeded their \$174 billion budget for 2007 and \$245 billion budget for 2012, excluding unforeseen social costs. These include factors such as “pain and suffering, additional care provided by nonpaid caregivers, excess medical costs associated with undiagnosed diabetes, and diabetes-attributed costs for health care expenditures categories excluding health care system administrative costs, over-the-counter medications, clinician training programs, and research and infrastructure development” (ADA, 2012).

Zhuo *et al.* (2014) concluded that patients with diabetes are subjected to a lifetime of costly medical expenditures and a lower life expectancy compared with the non-diabetic population. They proposed that a reduction in long-term medical costs associated with diabetes could be achieved if preventive costs were kept low.

Diabetic patients usually fail to control their blood glucose, lipid, and/or blood pressure levels. This is because the majority of diabetic adults seek their treatments from primary care and physicians (Crosson *et al.*, 2010). This failure to control blood glucose leads to an increased risk for cardiovascular diseases and other related complications (American Heart Association, 2016). The prevention of cardiovascular diseases can be achieved by providing optimal control for glycaemia, blood pressure and lipids, which is itself an important step in managing diabetes since much of diabetes-related morbidity and mortality is the result of cardiovascular disease (Crosson *et al.*, 2010).

### 1.1.2 Glycaemic control

The main therapeutic objective for prevention of acute and chronic diabetes and other associated complications is thought to be glycaemic control (Koro *et al.*, 2004). Many randomised control trials have shown that a reduction in glycaemia, lipids and blood pressure levels has a positive outcome for diabetes-related complications (Skyler, 2004). A number of studies have shown that a major decrease in glycaemic levels reduces the risk of cardiovascular disease and death (Cefalu and Watson, 2008). There is growing recognition that an improvement in intensive blood glucose monitoring will reduce the progression of diabetic macro-vascular diseases (UKPDS, 1998). Furthermore, Mazzone (2010) reported that intensive glycaemic control can be conducive to preventing long-term cardiovascular complications among diabetic patients.

According to Chan *et al.*, (2000) several independent factors contribute to poor glycaemic control: inappropriate treatment in relation to pancreatic  $\beta$ -cell function, increasing insulin resistance, lack of therapeutic patient education and smoking. A study conducted by Murata *et al.* (2003) involved a large cohort of Type 2 diabetic veterans who underwent stable insulin treatment. It was shown that intensive self-blood glucose monitoring (SBMG) improved glycaemic control level among each test subject, which in turn leads to a sustained decrease in glycated haemoglobin levels.

Type 2 diabetes, however, is characterized by peripheral insulin resistance and deterioration in  $\beta$ -cell function (Kahn, 2003). Insulin resistance can be described 'as the inability of insulin hormone to be biologically effective at circulating concentrations as in normal individuals' (Goke, 1998, s18). The core metabolic

defect in Type 2 diabetic patients is insulin resistance and it presents as hyperglycaemia (Taylor, 2012). It was estimated that up to 90% of Type 2 diabetic patients have a combination of insulin resistance and inadequate compensatory insulin secretion (Colberg *et al.*, 2010). Numerous cardiovascular risk factors are associated with Type 2 diabetes (Martín-Timón *et al.*, 2014) which are caused by insulin resistance (Steinberger and Daniels, 2003). According to Henry (1996), it was reported that intensive insulin therapy can be associated with improvement in glycaemic control in patients with Type 2 diabetes. This can be achieved by decreasing hepatic glucose output and improving its uptake. It may also improve insulin resistance and secretion by decreasing the glucotoxicity that results from hyperglycaemia.

One factor that contributes to  $\beta$ -cell dysfunction is glucokinase (GCK) gene dysfunction. GCK is encoded in mitochondrial DNA and is a pro-hormone convertase. Glucokinase is an important regulatory enzyme that plays a crucial role in regulation of glucose metabolism by maintaining insulin secretion in pancreatic  $\beta$ -cells and hepatocytes, and is referred to as the “glucose sensor” (Osbak *et al.*, 2009). However, the frequency of mutations in GCK is low compared with other causes of Type 2 diabetes (Goke, 1998).

There is a growing acceptance that for hyperglycaemia to present in Type 2 diabetes, pancreatic  $\beta$ -cell dysfunction must exist. The relationship between reduced  $\beta$ -cell secretory function and a fast-rising glucose level can be described as an inverse nonlinear relationship (Kahn, 2001).

### 1.1.3 Diagnosis of Diabetes

Davidson (2001) stated that “In 1979 the criteria for diagnosis of diabetes were based on levels of glycaemia during an oral glucose tolerance test (OGTT) associated with the subsequent development of retinopathy”. Later, glycated haemoglobin (HbA1c) measurements were introduced and became the gold standard tool for detecting and monitoring treatment, as well as making management decisions regarding diabetic patients (WHO, 2011). HbA1c levels are a preferable measure for glucose levels over OGTT because firstly they reflect the overall mean blood glucose level during the preceded period of 6 to 8 weeks rather than a single instance of time (Nathan *et.al.*, 2007). Secondly, many studies have reported an association between HbA1c and microvascular complications in diabetics (Davidson, 2001). WHO recommends the use of HbA1c as a diagnostic test for diabetes because it ensures that “stringent quality assurance tests are in place and assays are standardised to criteria aligned to the international reference values, and there are no conditions present which preclude its accurate measurement” (WHO, 2011, p.6).

Many studies were carried out to show the association between glycated haemoglobin levels and glycaemic control. A study carried by Koenig *et al.*, (1976) involved five poorly controlled diabetic patients to determine whether changes in glycaemic control were associated with changes in glycated haemoglobin levels. It was found that the reductions in glycated haemoglobin levels were associated with improved glycaemic control. The above finding is consistent with Ditzel and Kjaergaard (1978) who focused on 10 newly-diagnosed non-acidotic diabetic patients initially exhibiting elevated levels of HbA1c. They observed a gradual decrease in HbA1c in the weeks following weeks of initiation of dietary and insulin

therapy. It can therefore be concluded from both studies that glycated haemoglobin is an indicator for the long-term average blood glucose concentrations. To detect a significant concentration for blood glucose for diabetes an HbA1c value  $\geq 6.5\%$  (48 mmol/mol) was introduced as a preferred diagnostic test for diabetes (WHO, 2011). ADA (2010) established a diagnostic range of 5.7-6.4 % for HbA1c to identify subjects at risk of developing diabetes in the future.

Glycated haemoglobins are “haemoglobins with an attached sugar moiety, they constitute the HbA1 fraction of the adult haemoglobin (HbA). HbA1c is the predominant fraction of HbA1 and gives an estimate of the blood sugar levels of an individual over the last three months” (Nitin, 2010). They are formed by the glycosylation of haemoglobin molecules. Haemoglobin A (HbA) is the most common type of haemoglobin; it accounts for more than 95% of haemoglobin in adults and it is typically present in both healthy adults and those with certain diseases such as severe thalassemia (Yudkin *et al.*, 1990, The London North West Healthcare NHS Trust, 2015). HbA1c is typically present in non-diabetic individuals and comprises approximately 4-6%, of total haemoglobin (Yudkin *et al.* 1990). However, several factors have been reported to affect measurements of HbA1c levels such as: the age of an individual, decreased survival rate of red blood cells, haemoglobinopathies and a lack of standardised method for measuring HbA1c level (Kilpatrick, 1997). In a study conducted in Japan by Tsuki and Kobayashi (1995), it was demonstrated that glycated haemoglobin values in non-diabetic individuals appeared to increase with age, as well with diseases of the liver and kidneys. In addition, Soranzo (2011) showed that HbA1c levels vary among individuals without diabetes due to both biological and hereditary factors. Additionally, Simonos *et al.*, (2008) indicated that in healthy non-diabetic adults, the genes controlling HbA1c and FPG reflect different

aspects of glucose metabolism, and also reported that 75% of HbA1c level was heritable. Leslie and Cohen (2009) reported that differences in the survival rates of red blood cells, due to genetic and ethnic causes, can alter the HbA1c level by more than 1%. Furthermore, Soranzo (2011) stated that the utility of HbA1c in diagnosing diabetes can also be altered by inherited haemoglobinopathies. HbA1c level was found to be influenced by ethnic factors. In a study conducted by Herman *et al.* (2007), racial and ethnic differences in glycated haemoglobin levels were studied among people with impaired glucose tolerance (IGT). It was reported that the mean of HbA1c levels was 0.4% higher in Afro-Caribbeans compared with white Europeans exhibiting the same glucose tolerance. Similarly, Likhari and Gamma (2009) also reported that the mean HbA1c levels are higher in South Asians than in whites living in the UK. Therefore, it can be concluded that age and ethnic factors need to be taken into consideration when using HbA1c diagnosis for diabetes.

Despite these discoveries, HbA1c measurements are not appropriate for the diagnosis of diabetes in all cases John (2012). This method of diagnosis is not advised for:

- Children and young people
- People suspected of having Type 1 diabetes
- Patients exhibiting diabetic symptoms for less than two months
- Patients at high risk for diabetes who are acutely ill
- Patients taking medication that may cause rapid increases in glucose levels, e.g. the use of steroids and antipsychotics
- Patients with acute pancreatic damage or those who have undergone pancreatic surgery

- Individuals who exhibit haemoglobinopathies (e.g. Hbs, Hbc, etc.)
- Those who suffer from anaemia (haemolytic and iron deficiency)
- Persons experiencing renal failure
- Individuals infected with HIV infection and finally
- Women who have recently been or are currently pregnant

Glycated haemoglobin concentration can be used to predict the risk of developing cardiovascular diseases in people living with and without diabetes (Khaw & Wareham, 2006). It can also be used to identify people who are at high risk for cardiovascular diseases and in need of imminent medical intervention. Moreover, the same study found that a 10-20% increase in cardiovascular disease was correlated with a 1% increase in mean HbA1c concentration. It was also reported that a reduction of 14% of the incidences involving acute myocardial infarction among Type 2 diabetic patients can be attributed to only a 1% reduction in their glycated haemoglobin levels (Syed and Khan, 2011). In summary, Khaw and Wareham (2006) and Syed and Khan (2011) showed that efforts to reduce glycated haemoglobin, not only in diabetic patients but also within the general population, could be considered important in decreasing the risk of mortality (Diabetes.co.uk, 2013).

Paynter *et al.*, (2011) confirmed these findings, and also reported HbA1c levels alongside scores obtained for the risk of likelihood of suffering from cardiovascular diseases. They showed that it could be possible to predict which patients at the baseline are most susceptible to developing diabetes when this disease is considered a derivative of cardiovascular disease.

## 1.2 Glycation

Glycation is defined as a non-enzymatic reaction by which carbohydrates (such as glucose) bind to proteins (such as haemoglobin). This reaction was first studied by Louis Camille Maillard in the early 1900s and later came to be known as the Maillard reaction (Popova *et al.*, 2010). In this reaction, sugars spontaneously react with the NH<sub>2</sub> terminal residue of the f3 chain (f3-NA1 valine) in proteins, lipids and nucleic acids to form the advanced glycation end products (AGEs) (Schalkwijk *et al.*, 2004). The process can be summarised in 3 stages: (See figure 1.2.1):

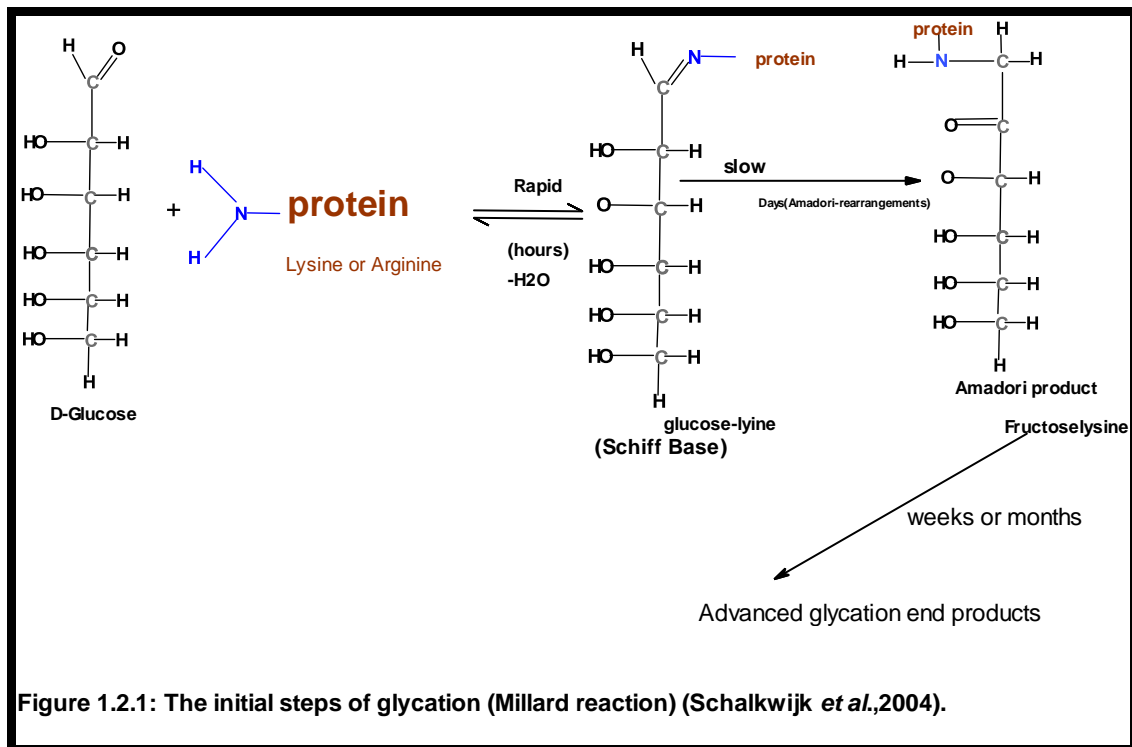
Stage (1): This involves the formation of a sugar- protein complex which is called the Amadori rearrangement. It is considered a precursor to all later compounds.

Stage (2): This stage includes the formation of numerous intermediate compounds, some of which very reactive and others that are not. The reactive compounds continue to take part in the reaction, advancing to the third and final stage.

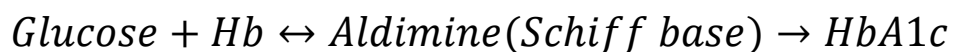
Stage (3): the reaction products that were formed in the previous stage undergo polymerisation, which results in the formation of the AGEs (Miller *et al.*, 1980).

The initial step of the first stage of this reaction includes the formation of a compound called a “Schiff base”, which undergoes spontaneous rearrangements to form the “Amadori product”, a keto-amine (Popova *et al.*, 2010). The initial step is rapid and highly reversible. The speed with which the reaction occurs depends on the concentration of the reactant (glucose). For instance, when glucose levels are low, this results in the breaking apart of the bonds between the sugar molecules and the amino groups that are attached to them. In contrast, when the glucose level is high, this will result in the opposite effect (Peppia *et al.*, 2003). Peacock (1984) reported that glycation begins during the erythropoiesis process and occurs slowly

during the life cycle of haemoglobin in the circulation.

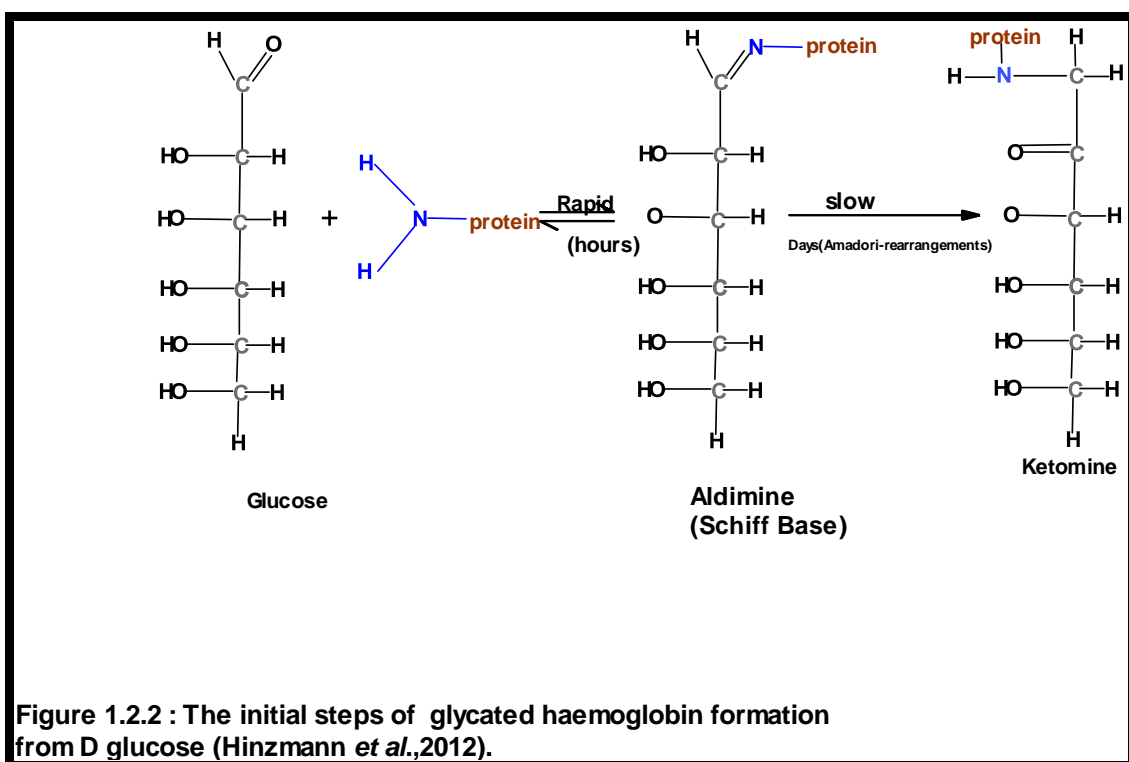


As discussed above, the glycation reaction can occur in proteins, lipids and nucleic acids. Glycation also occur in plasma; where glucose glycates proteins including albumin (50% of the plasma protein), immunoglobulin, and fibrinogen, and in the extracellular matrix (ECM) where glucose glycates collagen (the predominant protein in ECM) (Wautier & Schmidt, 2004). In blood the glycation of haemoglobin can be summarised by the simple equation below:



The following steps briefly describe the synthesis of glycated haemoglobin as discussed by Hinzman *et al.*, (2012):

1. The first step of this reaction is a reversible step (Peppia *et al.*, 2003). It includes the formation of a compound identified as aldimine (also known as the Schiff base) which results when glucose reacts with an amino-group ( $-NH_2$ ) of a valine residue located at the N-terminal of a globin chain (Lazareva *et al.*, 2008).
2. The second step is irreversible. The aldimine undergoes spontaneous self-rearrangements to yield a stable keto-amine [1 -deoxy- 1 (N-valyl-f-globin) fructose] which persists for the duration of the protein's lifespan (Figure 1.2.2):



### 1.2.1 Advanced Glycation End Products

AGEs can be defined succinctly as a heterogeneous group of compounds formed via non-enzymatic glycation between reducing sugars and free amino group of proteins, lipids and nucleic acids (Ahmed *et al.*, 2005). Since these complex groups of compounds are formed slowly, it was once believed that AGEs accumulated solely from long lived extracellular proteins (Goh and Cooper, 2008).

However, by the mid-1990s it was reported that AGEs often accumulate intracellularly. AGEs can also originate from exogenous sources such as tobacco smoke and food (Giardino *et al.*, 1994). Uribarri *et al.*, (2010) state that AGEs can originate either from endogenous or exogenous sources. Although AGEs are known as products of hyperglycaemia, they can be formed from food during cooking. A number of studies have reported that dietary AGEs can be partially absorbed and excreted in the urine, and they are considered as a major source of circulating AGEs in the body (Uribarri *et al.*, 2003; Vlassara *et al.*, 2002). Furthermore, smoking is considered as one of the main exogenous source of dietary AGEs (Uribarri *et al.*, 2003).

Sustained hyperglycaemia contributes to many of the complications associated with diabetes (Brownlee *et al.*, 1984, Cerami *et al.*, 1987 and Merimee *et al.*, 1990). AGEs Chronic hyperglycaemia has been reported to cause the accumulation of non-enzymatic glycosylated products in which glucose initially forms the Amadori product, then after a series of chemical reactions some of these Amadori products are converted to AGEs. The AGEs were reported to accumulate more in the tissues of diabetic patients where they are found to be connected to a number of complications (Brownlee *et al.*, 1988. Vishwanath *et al.*, 1988 and Monnier *et al.*, 1984).

Brownlee (2001) reported that the formation of AGEs *in vivo* can result from highly reactive carbonyl groups called alpha-dicarbonyls or oxoaldehydes, including 3-deoxyglucosone, glyoxal, and methylglyoxal. According to Hammes *et al.* (1999) AGEs accumulate within various sites where diabetic complications readily develop. These include the kidney, retina and atherosclerotic plaques. The rate at which these AGEs accumulate is accelerated by hyperglycaemia. Moreover, it has been reported

that the increase in diastolic and systolic hypertension seen among diabetic patients is caused by AGEs.

The UK Prospective Diabetes Study (UKPDS) (1998) established that hyperglycaemia is the main instigator of tissue damage in Type 2 diabetic patients. However, this process is dependent on individual variation and genetic factors, taking into consideration that AGEs formed by the non-enzymatic glycation are also promoted by hyperglycaemia (Fowler, 2008). Moreover, the intracellular formation of AGEs can be detrimental to the normal functioning of cells, whereas tissue alterations by AGEs can result from the modification of proteins. This can occur by stimulating the formation of cross-links between molecules in the membrane of extracellular matrices and by the involvement of receptors for advanced glycation end-products (RAGE receptors). These phenomena can serve to explain the contribution of AGEs in micro and macrovascular complications (Tan et al., 2007).

It has been suggested that AGEs are essential pathogenic mediators of most complications that are related to diabetes. For example, they can be found in the retinal vessels of diabetic patients and the severity of retinopathy is correlated with their levels in retinal vessel levels, in addition to their levels in serum (Peppia et al., 2003). The extent to which AGEs are formed depends on two key factors: the degree of hyperglycaemia and the degree of oxidative stress in the environment (Goldin et al., 2006). More recent research provides increasing evidence that hyperglycaemia is the initiator of tissue damage such as macrovascular and microvascular complications in patients with diabetes either via long-term accumulation of glycated products and AGEs, or through severe changes in glucose metabolism in cells (Stirban et al., 2014; Gkogkolou and Böhm, 2012; Schurman et al., 2008).

Some mechanisms in which AGEs contribute to diabetic complications, particularly the vascular effects of AGEs, are related to tissue inflammation and oxidative stress, as well as the increased glycation of LDL and HDL (Cai et al., 2002). Such mechanisms can be summarised into the following steps:

1. Cellular proteins such as those involved in the regulation of gene transcription are modified (Giardino *et al.*, 1994).
2. Precursors of AGEs diffuse from cells and modify nearby ECM thus modifying the signalling between matrix and cells and resulting in cellular dysfunction (Charonis *et al.*, 1990).
3. AGEs alter circulating proteins in the bloodstream, thereby modifying their function (Tan *et al.*, 2007).
4. Finally, these modified circulating proteins bind to RAGEs, altering the production of inflammatory cytokines and growth factors, which in turn leads to tissue damage (Vlassara, 2001; Goldin *et al.*, 2006).

Nevertheless, measurements of glycated haemoglobin levels do not give a strong indication of mean plasma glucose levels as is often assumed. This is because there are numerous analytical problems associated with glycated haemoglobin measurements, such as the lack of assay standardisation, various problems that affecting HbA1c measurement, haemoglobinopathies, fetal haemoglobin, renal failure, and haemolytic diseases (John, 2012). It has also been proven that there are discrepancies between estimates of the average glycaemia levels when calculated from glycated haemoglobin levels or from fructosamine measurements (Kilpatrick, 2000; Nayak *et al.*, 2011).

It can be concluded from the above observations that acceleration of the glycation process and increased incidences of diabetes related complications are strongly linked to the damaged protein by glycation reaction (Brownlee *et al.*, 1984).

Fructosamine is an index of the glycated fraction of all plasma proteins, predominantly albumin. Glycated albumin is considered to be the main glycated protein that is related to extracellular fructosamine in the circulation while HbA1c is the main intracellular fructosamine by product of the glycation reaction (Cohen *et al.*, 2003).

### 1.2.2 Glycation Gap

There are discrepancies between estimates of the average glycaemic levels when calculated from glycated haemoglobin levels or from using fructosamine measurements. A study by Cohen *et al.*, (2003) was conducted to test whether this discrepancy between HbA1c and an integrated measure of plasma glucose was a reliable indicator of physiological processes that cause variations between individuals. It was also conducted to determine whether these factors demonstrated a relationship with the frequency of diabetic nephropathy. The authors developed a measure of discordance between HbA1c and circulating fructosamine (FA) which they gave the name "glycation gap" in order to conduct a systematic evaluation. It was concluded that the glycation gap may be a useful tool in clinical research for estimating the physiological reasons behind the variation in diabetic complications among the individuals.

Glycation gap (G-gap) is defined as "the difference between measured HbA1c and HbA1c predicted from the FA based on the HbA1c-FA regression equation  $G\text{-gap} = \text{measured HbA1c} - \text{predicted HbA1c}$ " Cohen *et al.*, (2003).

However, several authors reported that the G-gaps varied among individuals. Hempe *et al.*, (2002) investigated the individual differences in the relationship between glycosylated haemoglobin (HbA1c) and mean blood glucose (MBG) levels among diabetic participants. They reported that these differences in the relationship between MBG and HbA1c were not due to the age of erythrocytes but rather that these differences were related to differences in the rate of glycation of haemoglobin, resulting in the categorisation of patients according to their G-gaps as high and low glycosylators. In addition, the research studies carried out by Hempe *et al.* (2002), Gould *et al.*, (1997) and Yudkin *et al.* (1990) showed that 42 out of 223 non-diabetic participants were categorised as high and low glycosylators based on the discrepancy between the centile ranking of their mean glycosylated haemoglobin and their mean blood glucose level after OGTT. Nayak *et al.*, (2013) proposed that the G-gap explained differences in the incidence of diabetic complications such as (nephropathy, neuropathy, retinopathy, CVD and mortality); the concept of G-gap has also received more interest in recent decades from many researchers since this discordance is between the two principal methods of estimating glycaemic levels, HbA1c and FA (Nayak *et al.*, 2011; Kilpatrick, 2000).

### 1.2.3 Deglycation

Szwergold *et al.*, (2002) proposed that intracellular non-enzymatic glycation is controlled by an enzymatic process thought to involve a key enzyme called Fructosamine-3-Kinase (FN3K). FN3K can deglycate the glycosylated haemoglobin in erythrocytes and also other glycosylated proteins in other tissues. Thus, the variations in the activity of FN3K between individuals may lead to the apparent differences in glycosylated haemoglobin levels. Some individuals have a high rate of deglycation and tend to have low glucose levels, whilst those with a low rate of deglycation tend to

have high glucose levels (Mohas *et al.*, 2010).

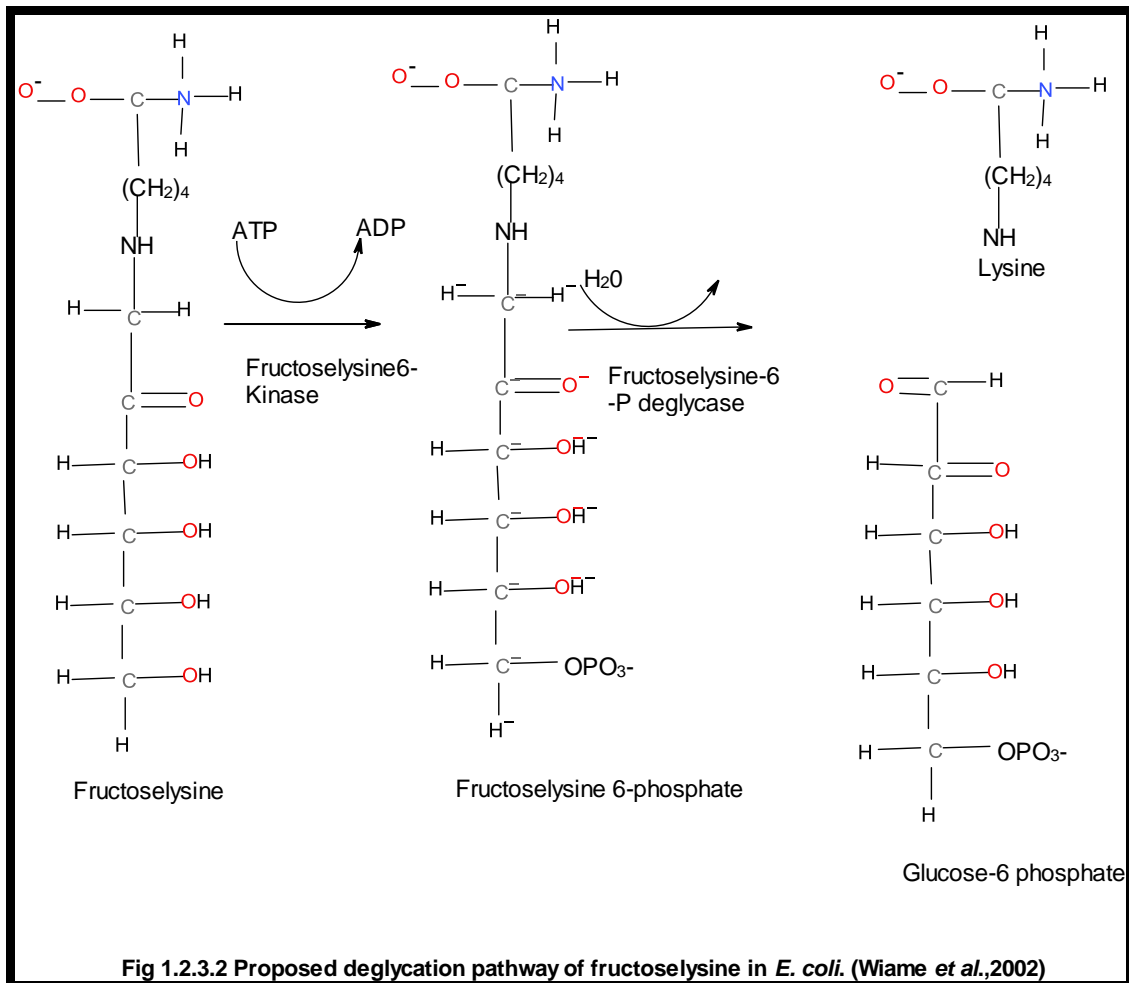
The concept of enzymatic deglycation catalysed by the FN3K enzyme can be illustrated by Figure 1.2.3.3: FN3K phosphorylates fructoselysine (FL) into fructoselysine-3-phosphate (FL3P), which is naturally unstable. This molecule then spontaneously decomposes into unmodified lysine, inorganic phosphate and 3-deoxglucosone (3DG) (Delpierre *et al.*, 2000 & Szwegold *et al.*, 2001).

Unlike the non-enzymatic glycation reaction which is slow and dependent on glucose levels, the rate of the deglycation reaction greatly depends on the activity of deglycating enzymes (Delpierre *et al.*, 2006). As the glycation reaction is accompanied by the accumulation of the AGEs and carbonyl stress which are likely to have a deleterious impact on tissues (Arasteh *et al.*, 2014), a natural defence mechanism is initiated to protect the body's tissues against the excessive build-up of AGEs. This is accomplished by deglycation enzymes (Monnier and Sell, 2006).

Popova *et al.*, (2010) described three types of deglycating enzymes. The first group is made up of enzymes called amadoriases, while the second group called fructosamine-3-kinases. The third group consists of fructose lysine-6-kinase and fructose-lysine-p-deglycase.

According to Takahashi *et al.*, (1997) the role of the amadoriase enzymes is to block the glycation reaction from moving forward during the early stages via oxidative deglycation of Amadori products yielding glucosone, a primary amine, and hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) (Figure 1.2.3.1). After undergoing oxidation, the Amadori product spontaneously decomposes by way of hydrolysis in the presence of fructosyl amine oxidases (isolated by Horiuchi and Kurokawa (1991) from *Corynebacterium spp.* and *Aspergillus spp.*). Two deglycating enzymes were named amadoriase I and

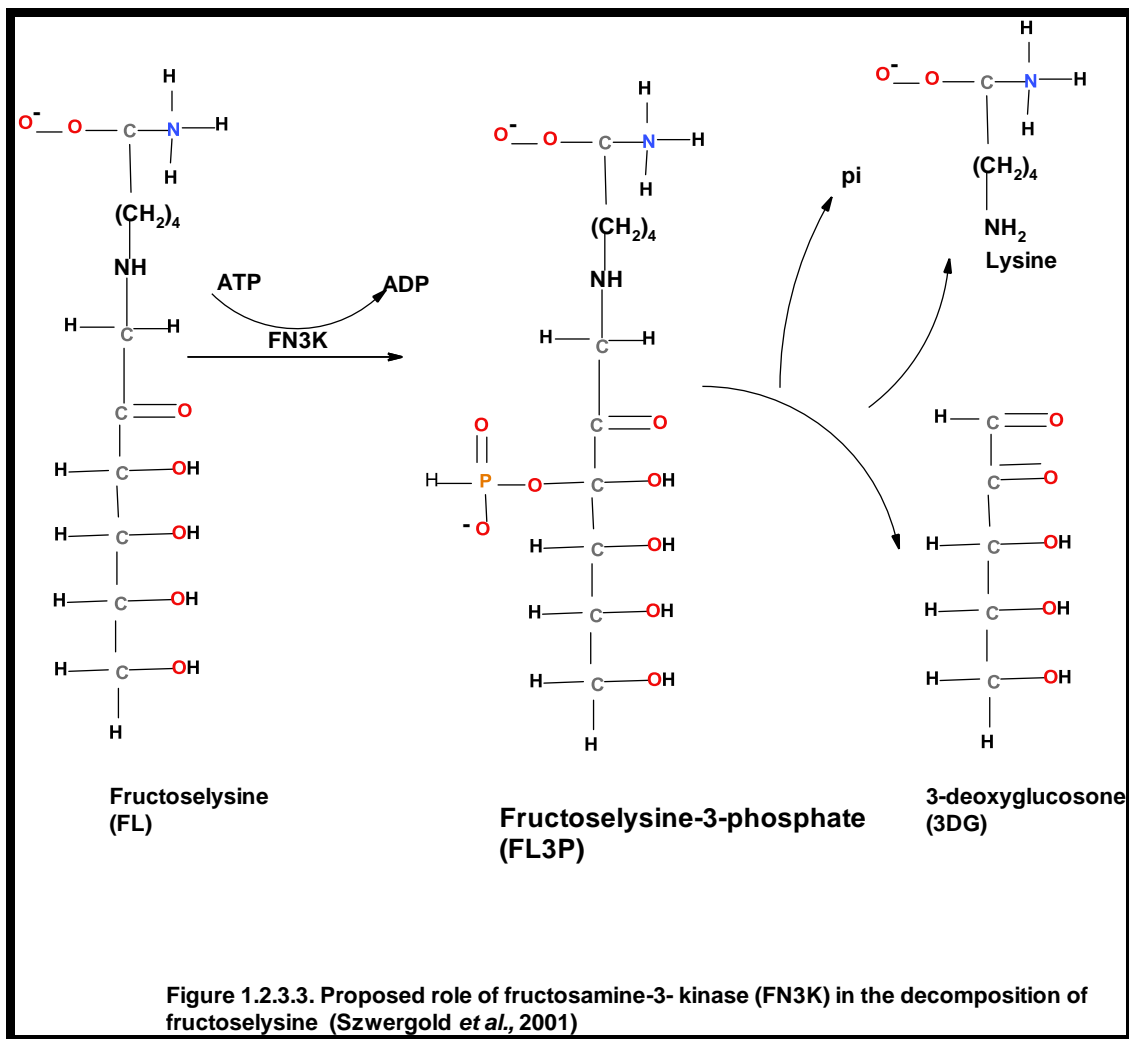




The third group of deglycating enzymes consists of the enzyme fructosamine 3-kinase (FN3K) (Figure 1.2.3.3), which was discovered by Delpierre et al., (2000). Based on early NMR research carried out by Szwegold et al. in 1990, they reported the presence of fructose 3-phosphate, a novel monosaccharide phosphate, in the lens of diabetic rats, as well as an enzyme that catalyses the phosphorylation of the Amadori product into fructosamine 3-phosphate. Delpierre et al., (2000) also cloned, characterised, and identified the FN3K enzyme in erythrocytes. It is suggested that FN3K maintains deglycating activity by catalysing the phosphorylation of both low molecular mass and protein-bound fructosamines. This leads to the formation of fructosamine 3-phosphate which is unstable and decomposes spontaneously to yield

3-deoxyglucosone and inorganic phosphate while generating a free lysine residue. Szwergold et al., (2001) suggested that protein bound FL can be a relevant substrate for the FN3K enzyme because of its high affinity for FL and other glycated protein that was detected in vivo. The subsequent phosphorylation of the third carbon for fructosamines by FN3K leads to the destabilization of the molecule and its partial removal from protein (Delpierre et al., 2000).

In addition to these observations, studies conducted by Collard *et al.*, (2004) and Fortpied *et al.*, (2005) reported the existence of another deglycating enzyme called fructosamine-3-kinase-related protein(FN3K-RP). FN3K-RP is also found in human erythrocytes and other vertebrates. This enzyme can phosphorylate psicosamines and ribulosamines located on the third carbon of the sugar moiety into psicosamine-3-phosphates and ribulosamine-3 phosphates. These products are both unstable and decompose at a pH of 7.1 and 37<sup>0</sup>C to form allose and free lysine. Psicosamine-3- phosphates and ribulosamine-3-phosphates have half-lives of 8.8 h and 25 min, respectively.



The physiological presence of enzymatic deglycation was demonstrated by showing that the decrease in FN3K activity leads to an increase in intracellular protein glycation (Delpierre *et al.*, 2002). This also was confirmed by Veiga-da-Cunha *et al.*, (2006) who demonstrated that the concentrations of haemoglobin-bound fructosamine and other intracellular proteins within the tissue and erythrocytes of FN3K-deficient mice were 2.5 times higher than those of healthy control mice. Additionally, haemoglobin-bound fructosamine was approximately 1.8-2.2 times higher in FN3K- deficient mice than other control intracellular proteins compared with healthy controls. These results show that fructosamines are removed from intracellular proteins by FN3K *in vivo*.

#### 1.2.4 FN3K

Fructoseamine 3 Kinase (FN3K) is an enzyme that was first identified by Szwergold and colleagues (Szwergold *et al.*, 2001) from its ability to phosphorylate fructosamines that are formed by the non-enzymatic glycosylation of D-glucose (Ahmed and Furth, 1992). Given that its enzymatic product, fructose-3-phosphate (F3P) is a key marker of the onset of diabetes, by virtue of its appearance within the lens of diabetic rats (Szwergold *et al.*, 1990), its role as a key enzyme within the aetiology of human diabetes has become of substantial interest within the field (Szwergold and Beisswenger, 2003). This is especially pertinent given that F3P spontaneously degrades to form 3-Deoxyglucosone (3DG), a highly reactive sugar which is produced by the body both when excessive sugar is consumed and in diabetes (Singh *et al.*, 2014), and which has been identified as a key metabolic precursor in the formation of AGEs (Dyer *et al.*, 1991; Kostolanská *et al.*, 2009), which have been implicated in the onset of diabetic.

Szwergold *et al.* (1997) subsequently observed deglycating enzymatic activity within human erythrocytes, thereby identifying an enzyme that is capable of metabolizing protein-bound Amadori products. This discovery implied that *in vivo* glycation is, in essence, reversible owing to the existence of an ATP-dependent mechanism which is catalysed by deglycating enzymes, thereby preventing glycated proteins from further reaction processes which lead to the formation of AGEs. Taken together these findings, the discovery of the deglycating enzyme FN3K by Delpierre *et al.* (2000) opened an entirely new window into our understanding of cellular metabolism and shed light upon the enzymatic role of protein glycation in the aetiology of diabetes. It also identifies a direct link between glucose metabolism and key complications in advanced diabetes, as FN3K is an enzyme that is uniquely capable

of the phosphorylation of the Amadori product into fructosamine 3-phosphate. Delpierre and colleagues (2000) went on to clone, characterise, and identify the presence of FN3K enzyme within erythrocytes. Further, FN3K was proposed to maintain deglycating activity by catalysing the phosphorylation of both low molecular mass and protein bound fructosamines (Delpierre *et al.*, 2000). The phosphorylation of fructosamine, catalysed by FN3K, has a  $k_m$  4-5 mmol/L, which is an order of magnitude lower than that for fructose at 50-100 mmol/L (Delpierre *et al.*, 2000). This suggests that this is a highly specific reaction and one which occurs naturally in healthy cells. In 2006, Krause and coworkers measured FN3K activity within human erythrocytes by means of a reverse-phase high-performance liquid chromatography (RP-HPLC). Their assay used an erythrocyte lysate that was diluted without enzyme purification and which was based upon the FN3K-dependent conversion of the synthetic substrate N $\alpha$ -hippuryl-N $\epsilon$ -(1-deoxy-D-fructosyl) lysine (BzGFruK) to the N $\alpha$ -hippuryl-N $\epsilon$ -(phosphofructosyl) lysine (BzGpFruK) (Krause *et al.*, 2006).

FN3K activity was consequently found to be high in erythrocytes isolated from humans, mice and rats, but was barely measurable in erythrocytes derived from chicken and pig haematocrit. Intriguingly, this variation in distribution correlated with the low intracellular concentrations of glucose that were present within erythrocytes in these animals. FN3K activity was subsequently found to be highest in the brain, heart, kidneys and skeletal muscles (Delplanque *et al.*, 2004). FN3K mRNA was also detected in numerous human and rodent tissues, especially within the bone marrow, brain, kidneys and spleen, and was also present at lower levels in other tissues, including the heart, liver and skeletal muscle (Monnier, 2006).

Given the role of FN3K in the enzymatic conversion of fructose into fructose-3-phosphate in the lens and erythrocytes of diabetic rats, its role in the production of deoxyglucosone, and the presence of fructose-3-phosphate in both human and animal tissues (Petersen *et al.*, 1990), a possible causal link between FN3K activity and the level of glycation of proteins was explored. It was found that, when the FN3K gene was silenced in animal models, levels of protein glycation increased (Veiga da-Cunha *et al.*, 2006). This is of special interest given the role of non-enzymatic glycation in the pathogenesis of the complications of diabetes. As FN3K plays a pivotal role in the removal of fructosamine from proteins, it has been postulated that it may serve to protect against diabetes (Mosca *et al.*, 2011) and even the aging process (Chondrogianni *et al.*, 2014) under certain metabolic conditions, especially given the predisposition of the FN3K 'knockout' mouse to developing symptoms which are characteristic of diabetes (Monnier, 2006).

The metabolic intermediates and glycated products are inherently complex, especially given that FN3K also catalyses the synthesis of Fructoselysine 3-phosphate from fructoselysine, a compound which then decomposes to 3DG. This has led to the proposition that 3DG production might in theory be reduced by the specific inhibition of FN3K (Brown *et al.*, 2003).

A study carried out by Delpierre *et al.*, (2006) examined the relationship between FN3K activity and the levels of glycated haemoglobin in 57 individuals, 31 of whom were Type 1 diabetics while the remaining 26 were normo-glycaemic. This study aimed to determine whether variations in FN3K activity among individuals existed, what genetic factors might affect variability in FN3K activity and to determine whether there was an inverse association with HbA1c. A non-significant difference was discovered in the mean erythrocyte FN3K activity between subjects with Type1

diabetes and the control group. However, there was a wide inter-individual variation in both groups ranging from 1 to 4mU/g of haemoglobin ( $\rho=0.8031$ ). Furthermore, there was no inverse correlation between FN3K activity and levels of glycated haemoglobin. These findings may be due to the fact that a substantial fraction of the fructosamines found in haemoglobin is bound to the N-terminal valine of the  $\beta$ -chain. Like free fructosevaline and other  $\alpha$ -glycated amino acids, the N-terminal fructosevaline residue is a poor substrate for FN3K, and therefore it escapes deglycation. Moreover, an earlier study by the same group (Delpierre *et al.*, 2002) showed that HbA1c is a poor substrate for FN3K. Therefore, this substrate is classified as being deglycation-resistant. The authors also reported that fructoselysine, which is poorly utilized by erythrocytes, is not likely to be a substrate for FN3K, but rather a substrate for other glycated proteins.

Recently, research conducted by Avemaria *et al.*, (2015) confirmed that free fructosevaline behaved much more poorly as a substrate for FN3K than free fructoselysine. On the other hand, Delplanque *et al.*, (2004) disagree with the results presented by Delpierre *et al.*, (2002) and reported that FN3K plays an extremely active role in the erythrocytes of human, rat and mouse when the intracellular glucose concentration is equal to the concentration of plasma. This is due to the fact that the protein is poorly or not renewed in the presence of an aggravating agent, which is identified as glucose. Thus, FN3K activity in erythrocytes was found to be non-essential when glucose was absent.

The presence of FN3K was shown by Szwegold *et al.*, (1990) who demonstrated the occurrence of fructose-3-phosphates (F3P) in the lens of diabetic rats. They reported that the development of some diabetic complications could be

partially attributed to an increase in the concentration of F3P and its hydrolysis to (3-deoxyglucosone) in the lens of diabetic rats.

Identification of FN3K was initially achieved through studies in human erythrocytes. Its capacity to phosphorylate fructose and sorbitol substrates, thereby producing F3P and sorbitol-3-phosphate, made its detection possible (Payne *et al.*, 2008). Since FN3K was purified, sequenced and cloned, the idea of enzymatic deglycation received an additional confirmation (Szwergold *et al.*, 2002). In fact, the whole concept of protein deglycation began when F3P was first identified by Szwergold and colleagues in 1990. By conducting NMR analyses of tissue extracts, Petersen *et al.* (1990) revealed the presence of F3P in the lenses of diabetic animals and in erythrocytes of both diabetic and non-diabetic subjects. Later, Petersen *et al.* (1992) reported that F3P could be induced by incubating erythrocytes with fructose, or by incubating fructose with erythrocyte lysates in the presence of ATP. Due to the high  $k_m$  values the fructose-3-kinase had shown for fructose, the lower enzymatic activity it exhibited in comparison to other enzymes involved in fructose metabolism, and the lack of information about additional metabolic activities for F3P, the theory that fructose-3-kinase was able to metabolize fructose was rejected. This led Szwergold *et al.*, (1990) and Schaftingen *et al.*, (2007) to assume that fructose-3-kinase was instead capable of catalysing the phosphorylation of fructosamines since they are similar to fructose in terms of molecular structure, apart from having an amino-group present on the first carbon of the hexose sugar fructose instead of a hydroxyl group. For this reason, Delpierre *et al.*, (2000) chose to use Fructosamine-3-kinase as the indicator of choice for the putative fructose -3-kinase. Moreover, FN3K strongly prevents the conversion of fructose to F3P and bears a  $k_m$  lower than that of fructose-3-kinase.

Owing to the apparent contribution that FN3K makes to the process of non-enzymatic glycation, the enzyme was purified and characterised to using a human erythrocyte model (Szwergold *et al.*, 2001). For the past decade, human erythrocytes have been used as a convenient model system for the purification of FN3K. The reasons for this owe to the fact that they are readily obtained, easy to incubate, extremely permeable to glucose (Lacko *et al.*, 1973), and contain relatively high amounts of FN3K (Szwergold *et al.*, 2002). Using electrospray tandem mass spectrometry (MS/MS), an analytical method used to detect molecules such as amino and fatty acids, the functionality and sequence of the FN3K enzyme was characterised by Szwergold *et al.*, (2001).

FN3K is a 35-KDa monomeric enzyme consisting of 309 amino acids that are able to phosphorylate fructose, fructoselysine and protein-bound Amadori products. However, it has a much higher affinity for Amadori products than for fructose as the relative  $k_m$  values range from 10 $\mu$ M for fructoselysine to more than 50mM for fructose (Delpierre *et al.*, 2000). The FN3K enzyme shares no significant homology with any other known proteins except with FN3K-RP. In humans FN3K appears to be encoded by genomic sequences that are located on chromosomes 1 and 17 (Szwergold, 2002).

Szwergold *et al.* (2001) reported that the FN3K enzyme may be part of an ATP-dependent system for removing carbohydrates from non-enzymatically glycated proteins due to its high affinity for fructoselysine on proteins and the liability of fructoselysine-3-phosphate. Deppe *et al.* (2011) also indicate that the FN3K enzyme and its homologue FN3K-RP might overcome protein damage arising from intracellular glycation by conferring protective and repair functions in *E. coli* and *B. subtilis*. To test whether pancreatic  $\beta$ -cell functions can be protected from

glucotoxicity by FN3K, Pascal *et al.* (2010) carried out a study to investigate the possible effects of FN3K deficiency on the function and survival of mouse pancreatic islets after prolonged culture in high glucose or ribose concentrations. They assumed that FN3K might play a partial role in protecting pancreatic  $\beta$ -cells from glucotoxic alterations so as to maintain their survival and functionality. Pascal *et al.*, (2010) found that FN3K is capable of decreasing the rate of glycation of intracellular islet proteins, but cannot protect  $\beta$ -cells from glucotoxicity. They also discovered that FN3K is not involved in maintaining the survival and function of  $\beta$ -cells under control conditions. Nevertheless, they demonstrated that a reduction in FN3K levels was the result of an increase in glycated ketoamines in the pancreatic islets of FN3K-deficient mice. Therefore, this argument supports the concept that FN3K is acting as a deglycating agent in the pancreas and in the other tissues (Veiga-da-Cunha *et al.*, 2006).

With regards to the genomic context of the FN3K and FN3K-RP sequences, Delplanque *et al.*, (2004) reported that the human genome contains two genes that are related to FN3K and FN3K-RP. They also found that these genes were presented in a tandem repeat in the telomeric region of chromosome 17q.

A small number of studies have reported a relationship between genetic variants in FN3K and its enzymatic activity. Delpierre *et al.* (2006) mentioned an association between the enzymatic activity of FN3K in erythrocytes and some polymorphisms in the FN3K gene in a Belgian cohort that included 31 Type 1 diabetic patients and 26 controls. They identified two single nucleotide polymorphisms (SNPs) in addition to other gene variants. The CC of the c.900C/G (rs1056534) in exon 6 and the G-gap of the c.-385A/G (rs3859206) in the promoter region were related to the low enzymatic activity exhibited by FN3K measured

erythrocytes. Nonetheless they could not find any correlation between FN3K SNPS and HbA1c concentrations. In a later study by Mohas *et al.*, (2010) involving a larger cohort of Type 2 diabetic subjects, the authors reported that the C allele of polymorphism c.900 C/G (rs1056534) in exon 6 was associated with lower glycosylated haemoglobin levels and a later onset of the Type 2 diabetes mellitus disease in comparison to the G allele, but they failed to find an association between this variant and diabetic complications such as nephropathy, neuropathy or retinopathy. Furthermore, a study was carried out in 2014 that involved 314 subjects with Type 2 diabetes who were screened for a total of 19 SNPs in six candidate genes encoding enzymes of metabolic pathways. The aim was to investigate whether genetic variability in such genes might stimulate the progression of diabetic nephropathy and the morbidity and mortality associated with diabetes. The authors discovered an association between the polymorphism in exon 6 (rs1056534) with the progression of diabetic nephropathy and cardiovascular morbidity and mortality (Tanhäuserová *et al.*, 2014).

Recently, Skrha *et al.*, (2014) conducted research to investigate the association between FN3K polymorphisms (rs1056534, rs3848403) and glyoxalase I polymorphisms (rs 4746) with soluble RAGE and parameters of endothelial dysfunction involving 595 diabetic and non-diabetic subjects. Skrha *et al.* (2014) found significant differences existed in s-RAGE of diabetic subjects in relation to their rs1056534 and rs3848403 genotypes. GG and CG genotypes of rs1056534 with mutated G allele were associated with a significant decrease of sRAGE (GG=1055±458 and CG= 983±363 vs. CC= 1796±987 ng/l,  $p < 0.0001$ ). On the other hand, the rs3848403 polymorphism, presented s-RAGE levels that were significantly increased (TT=1365±852 vs. CT= 1016±401 and CC= 1087±508 ng/l,  $p = 0.05$ ).

Significant differences in endothelial dysfunction markers were observed in genotype subgroups of GLO1 rs4746 polymorphism ( $p < 0.05$ ).

Glyoxalase I (GLO1) is an enzyme that removes the AGE methylglyoxal and is involved in the detoxification pathway which breaks down toxic reactive dicarbonyls to prevent the development of carbonyl stress. This prevents the formation of AGEs in proteins, lipids and nucleic acids. However, methylglyoxal, the primary substrate of glyoxylase, is generated mainly by the triose phosphate and pentose phosphate pathways rather than from glycated proteins, therefore there is no molecular link between FN3K and GLO1, each enzyme has its own independent mechanism in preventing formation of AGEs. GLO1 can be found in humans, mice, yeast, plants, insects, protozoa, fungi, and many bacterial strains (Thornally, 2003; Rabbani and Thornalley, 2011). In our project, we focused on the FN3K enzyme which acts on ketoamines in glycated proteins, rather than GLO1.

Unlike previous findings (Delpierre *et al.*, 2006; Tanhäuserová *et al.*, 2014; Skrha *et al.*, 2014), Avemaria *et al.*, (2015) did not observe any significant associations between certain SNPs and diabetic conditions, although they found that the genotype containing c.900 CC alleles (rs1056534) was associated with low concentrations of HbA1c.

As discussed earlier in this chapter, the FN3K enzyme appears to catalyze a repair mechanism for protein damage caused by glucose. An association between high levels of fructosamine in serum and colorectal adenomas was observed by Misciagna *et al.*, (2004). Additionally, Ross *et al.*, (2001) reported an existence of a common pathogenic pathway between diabetes and cardiovascular diseases coupled with colorectal cancer. To investigate such a pathway, Caruso *et al.*, (2007)

included 31 patients with colorectal cancer (CRC) in their study to evaluate FN3K gene expression via Reverse Transcription-PCR. They found a significant decrease in FN3K gene expression when compared with its expression in the surrounding healthy mucosa. Later it was reported that when 30 CRC patients underwent colon surgery, the FN3K activity and mRNA FN3K levels were downregulated in tumors located on the left side of colon compared to tumors situated on the right side (Notarnicola *et al.*, 2010). A year later in 2011, the same research group carried out a study that involved examining FN3K activity in erythrocytes obtained from patients with colorectal adenomas and cancer. Of the 33 individuals enrolled, 16 of them were diagnosed as having colorectal cancer (non-adenomatous), 7 had adenomas, while 11 patients served as the control group; exhibiting normal colonoscopy results. The activity of FN3K was measured by means of radiometric assay; it was found that FN3K activity was significantly lower in patients with non-adenomatous tumors than in patients with adenomas ( $19.55 \pm 6.4$  pmol/min per ml in patients with colorectal cancer, whereas 31.7 pmol/min per ml of red blood cells in adenoma,  $p = 0.04$ , Tukey's test. (Notarnicola *et al.*, 2011)).

### **1.3 Cardiovascular diseases**

Cardiovascular disease (CVD) is defined according to NHS (2014) as "a general term that describes a disease of the heart or blood vessels". The main types of CVDs are: coronary heart disease (angina and heart attack), congenital heart disease, stroke and peripheral arterial and aortic diseases.

#### **1.3.1 Epidemiology of Cardiovascular diseases**

The WHO states that: 17.5 million people die each year from CVD, which accounts for 31 % of all deaths worldwide. More than 75% of deaths from CVD occur in low

and middle income countries. Furthermore 80% of all deaths caused by CVD are due to heart attacks and strokes (WHO, 2014).

Fuster and Kelly (2010) reported that the total number of deaths caused by CVD (mainly coronary heart disease, stroke, and rheumatic heart disease) in 2005 had increased from the estimated 14.4 million in 1990 to 17.5 million. 7.6 million of these deaths were attributed to coronary heart disease and 5.7 million to stroke.

In the United Kingdom, a report by the British Heart Foundation (BHF) (2015) stated that more than 27% of all deaths in the UK (approximately 155,000 deaths annually) are due to CVD. In addition to the 7 million people living in the UK with CVD, the BHF also estimated that the economic burden of CVD would generate indirect costs caused by premature death and disability amounting to approximately more than £15 billion each year, despite the fact that healthcare alone costs up to £11 billion.

### 1.3.2 Pathophysiology of CVD

The majority of CVD is attributable to coronary heart disease (CHD) and stroke (Bhatnagar *et al.*, 2015). CHD accounts for 45% of CVD while stroke accounts for 30% (Fuster and Kelly, 2010). It can be concluded that, as most CVD pathology is within the arteries that are lined by endothelial cells, any decline in endothelial cell function leads to “endothelial cell dysfunction”, a well-recognized response to cardiovascular risk factors (Hadi *et al.*, 2005; Libby and Theroux, 2005).

The thin layer of endothelial cells lining blood vessels is called the endothelium and produces both vasodilators and vasoconstrictors. In normal conditions, continuous regulatory nerve signals and a continual supply of vasodilating nitric oxide are sent from the endothelium to the vascular smooth muscles (Lewis, 1998). It was proposed

that the endothelium enables the normal homeostasis of vessels via production of extracellular matrix components such as collagen, mediators of regulatory processes, nitric oxide, endothelin-1 (ET-1), Von Willebrand factor (VWF), angiotensin II, tissue-type plasminogen activator (t-PA), plasminogen activator inhibitor-1 (PAI-1), prostanoids and tumour necrosis factor (TNF) (Ambrose and Barua, 2004). Barrett *et al.*, (2010) also reported that the fibrinolysis process is controlled by the endothelium throughout its function in sustaining the integrity of the vascular wall. This can be achieved by producing t-PA and PAI-1, and in addition the endothelium altering the function of heparin sulphates/anti-thrombin, thrombomodulin/protein C and tissue factor/tissue factor inhibitor interactions, all of which limit the coagulation cascade. Moreover, it was found that vascular remodelling can be affected by endothelium through the release of factors that promote/inhibit the growth of blood vessels such as TIMPs and MIMPs, platelet-derived growth factor (PDGF) and angiotensin II (ANG II). A balance between MIMPs can influence the repair and maintenance of the extracellular matrix throughout breakdown of the ECM and the TIMP that inhibits MIMPs (Libby and Theroux, 2005). Furthermore, Betsy (2008) reported a contribution of diabetes in the defects in the autonomic nervous system, the endothelium, and local metabolism, all of which can result in microvascular disease. In conclusion, any decline or defects in endothelial cells normal function of endothelial cells may lead to cardiovascular disease (Hadi *et al.*, 2005). The endothelium mediates several vasoprotective effects such as vasodilation and suppression of smooth muscle cell growth and inhibition of inflammation via nitric oxide (NO). Any defect in the production of NO can lead to endothelial dysfunction and may initiate atherosclerosis (Feldman *et al.*, 2000).

### 1.3.3 Diabetes as a risk factor for CVD

The major risk factors for CVD are: diabetes mellitus, ethnic background, family history of heart disease, hypertension, high blood cholesterol levels, lack of exercise, smoking and obesity/being overweight (Grundy *et al.*, 1999). According to the American Heart Association (2016), people with diabetes are at high risk for CVD because diabetic patients, especially those with Type 2 diabetes may additionally have other risk factors for CVD. These include hypertension, abnormal cholesterol and high triglycerides levels, obesity and lack of exercise. Nathan *et al.*, (2005) found that hyperglycaemia in diabetic adults increases the risk of heart attack, stroke, angina, and coronary artery disease. In addition, Betsy (2008) also mentioned that hyperglycaemia as a symptom of diabetes is a factor that contributes to myocardial damage after ischemic events. Moreover, it was reported by The Center for Disease Control and Prevention (CDC, 2014) that death rates from CVD among adults aged 18 years or older with diagnosed diabetes were about 1.7 times higher than rates among adults without diagnosed diabetes. The risk of CVD in people with diabetes can further be doubled by smoking (NDEP, 2007).

Intensive glycaemic control in diabetic patients has been reported to reduce the risk of any CVD event by up to 42% and the risk of heart attack, stroke and death from CVD by 57% (Nahtan *et al.* 2005). In addition, the risk of any diabetic complications can be reduced by 12% with a reduction of 10mm Hg in systolic blood pressure (Adler *et al.*, 2000). Poornima *et al.*, (2006) indicate that the risk for development of CVD is elevated 2-4 times in diabetic patients.

It was reported by Ewing *et al.*, (1980) that diabetes contributes to defects in the autonomic nervous system as well as in the endothelium and in all conditions

that result in microvascular disease. They also describe a factor called diabetic autonomic neuropathy (DAN) that is associated with impaired autoregulation of blood flow in a variety of vascular beds such as the skin and heart. A year later Smith *et al.*, (1981) added that patients with diabetic autonomic neuropathy were reported to have an increase in the rate of sudden cardiac death and had a higher cardiovascular mortality rate than those without. Moreover, in patients with diabetes, a decrease in the bioavailability of NO, which acts as a potent vasodilator, was seen, in addition to an increase in the secretion of vasoconstrictor endothelin-1 (Koh *et al.*, 2005). This decrease in the bioavailability of NO in diabetic patients might be due to insulin deficiency or resistance in endothelial cells, plus hyperglycaemia was proven to inhibit NO production in arterial endothelial cells (Williams *et al.*, 1998).

#### 1.3.4 AGEs and CVDs

AGEs were found to accumulate at a much higher rate in individuals suffering from diabetes than among the non-diabetic population (Peppas *et al.*, 2003). Kilhovd *et al.*, (1999) found that serum AGEs were significantly increased in patients with diabetes and CHD compared with diabetic patients without CHD (8.1U/ml vs 7.1 U/ml, respectively). In another study related to AGE levels in tissue, Makita *et al.*, (1991) reported a significantly higher level of AGE content in the arterial wall collagen of patients with diabetes in comparison to samples from non-diabetic individuals. In addition to this study, the same group reported that the AGE content found in the renal tissues of diabetic patients with end-stage renal disease was approximately double the AGE content for diabetic patients without renal disease ( $21.3 \pm 2.8$  U/mg vs  $11.5 \pm 1.9$  AGE U/mg).

Pentosidine is a biomarker for AGEs that is constantly being formed under normal circumstances and more rapidly under a variety of situations that pose stress,

for example oxidative stress and hyperglycaemia (Dyer *et al.*, 1991). Yoshida *et al.*, (2005) observed that serum levels of pentosidine in patients with diabetes were significantly higher than those in nondiabetic individuals ( $64.4 \pm 21.0 \mu\text{g/L}$  vs  $22.8 \pm 7.0 \mu\text{g/L}$ ). Furthermore, in diabetic patients with CVD, the pentosidine serum levels were significantly higher than those without CVD ( $72.3 \pm 23.7 \mu\text{g/L}$  vs  $62.3 \pm 19.8 \mu\text{g/L}$ ). These serum levels for pentosidine were correlated with arterial wall stiffness in diabetic patients.

More recent findings by Koyama *et al.* (2007) investigated 141 patients with heart failure and 18 control subjects. This study reported that serum pentosidine levels were higher in patients with heart failure than those without ( $p < 0.001$ ). This suggests that serum pentosidine can be considered as an important risk factor in predicting of heart failure as it relates to the severity of heart failure. Likewise, Lapolla *et al.*, (2007) examined the association between AGEs, malondialdehyde, total reactive antioxidant potentials (TRAPs), and vitamin E in Type 2 diabetic patients with and without peripheral artery disease. They found that the levels of AGEs, pentosidine and malondialdehyde were significantly higher in patients with peripheral artery disease than in patients without and compared with control subjects. In conclusion, this study demonstrated that pentosidine can be considered a good predictor for peripheral artery disease in diabetes, although it would be essential to perform an appropriately-designed longitudinal study to further support this finding.

As discussed, many studies have investigated the association between AGEs and diabetes-related macro- and microvascular complications, in order to understand how AGE levels correlate with the development of diabetes related cardiovascular complications. Obayashi *et al.*, (1996) reported the presence of AGEs in

atheromatous lesions located in the coronary arteries of patients with diabetes. They proposed that AGEs can accelerate the development of atherosclerosis as was reported for patients diagnosed with diabetes. In support of this finding, Yeboah *et al.* (2004) showed that the levels of serum AGEs in Type 2 diabetic patients can be considered biomarkers for detecting the severity of coronary artery atherosclerosis. Moreover, Kiuchi *et al.*, (2001) reported the detection of higher levels of AGEs in Type 2 diabetics with obstructive coronary artery disease with respect to diabetic patients who did not exhibit obstructive coronary disease. A correlation between levels of AGEs and the degree of coronary atherosclerosis in the patients with obstructive coronary disease was also observed. Furthermore, it was reported that increased levels of AGEs in the serum of patients who underwent percutaneous coronary intervention served as an independent risk marker for restenosis in patients with diabetes (Choi *et al.*, 2005).

Hartog *et al.*, (2007), detected an association between serum AGEs levels and the development and progression of heart failure whereby increased serum levels of AGEs in diabetic patients accelerated the severity and development of heart failure. This can be utilized via two pathways. The first is the indirect pathway that occurs via their vascular effects (coronary dysfunction, atherosclerosis, and thrombosis). The second pathway occurs through direct actions on the myocardium. In contrast, Zieman and Kass (2004) and Bidasee *et al.*, (2004) reported that AGEs directly impacted the myocardium independent of effects imposed by the vascular tree. This is partially caused by cross linking of extracellular cardiac proteins and via actions utilized by RAGEs expressed on the myocardium.

However, endothelial dysfunction is considered to be an independent key predictor of side events associated with cardiac trauma and the hospitalization of

patients with CHF, and possible death (Fischer *et al.*, 2005). AGEs are known to impair the vascular functions of diabetic patients by influencing both endothelial function and vascular compliance (Fischer *et al.*, 2005); for example, endothelial dysfunction can be triggered by AGEs by reducing the availability of the vasodilator nitric oxide (Quehenberger *et al.*, 2000). Moreover, the production of endothelin-1, a potent vasoconstrictor, might also be enhanced by AGEs (Quehenberger *et al.*, 2000 & Sanders *et al.*, 2000). A correlation was observed between the levels of circulating AGEs and arterial compliance (Yoshida *et al.*, 2005). In general, it has been reported that for patients that exhibit vascular stiffness, treatment with AGE breaking medication such as ALT-711 can improve arterial compliance (Kass *et al.*, 2000).

### 1.3.5 Endothelial dysfunction

Endothelial dysfunction is a condition where the endothelium of blood vessels functions abnormally (Endemann and Schiffrin, 2004). The endothelium is “a thin monocellular layer that covers all the inner surface of the blood vessels, separating the circulating blood from the tissues. It works as a receptor-effector organ and responds to each physical or chemical stimulus with the release of the correct substance with which it may maintain vasomotor balance and vascular-tissue homeostasis” (Esper *et al.*, 2006). The endothelium plays a role in controlling vessel tone, haemostasis, fluid filtration, immune cell recruitment and transportation of hormones to the target organs and is a vital part of vascular system (Endemann and Schiffrin, 2004).

Endothelial dysfunction is characterised by an imbalance between vasodilative and vasoconstrictive agents (Muniyappa and Sowers, 2013). According

to Fieldman *et al.*, (2000) the endothelium mediates many vasoprotective effects such as vasodilation, suppression of smooth muscle cell growth and inhibition of inflammation via nitric oxide (NO). Defective production of NO can result in endothelial dysfunction. A reduction bioavailability of NO, an increased oxidative stress, an elevation in expression of pro-inflammatory and pro-thrombotic factors, and abnormal vasoreactivity are all characteristics of endothelial dysfunction (Barac *et al.*, 2007). Endothelial dysfunction is considered an important incident in an array of cardiovascular disorders (Ramesh and Shenovy, 2003). It is associated with many cardiovascular complications such as coronary artery diseases, coronary heart failure, coronary renal failure, hypertension and peripheral artery disease (Endemann and Schiffrin, 2004). Endothelial cells play a major role in vasoconstriction and vasodilation of blood vessels: they secrete several mediators that can induce both vasoconstriction, such as endothelin-1 and thromboxane A<sub>2</sub>, and vasodilation, such as endothelium-derived hyperpolarizing factor, NO and prostacyclin (Avogaro *et al.*, 2011). NO is one of the most important substances released by the endothelium; it has anti-aggregation effects on platelets and can inhibit growth and inflammation (Esper *et al.*, 2006). According to Muniyappa and Sowers (2013), NO is an important vasodilator that causes a relaxation of vascular smooth muscle, reducing intracellular Ca<sup>2+</sup> levels in response to vasoconstricting agents such as angiotensin II. The intracellular concentration of Ca<sup>2+</sup> increases and this increase promotes binding of calmodulin and activation of endothelial NO synthase to increase NO production.

Endothelial dysfunction also appears to be an important, consistent finding in diabetic patients: impairments to NO production and activity can result from hyperglycaemia and diabetes (Avogaro *et al.*, 2011). Brownlee (2001) and Meigs, *et*

*al.* (2004) both describe endothelial dysfunction as a defining event that precedes cardiovascular pathology such as atherosclerosis, and so it is considered a precursor of future pathogenic cardiovascular events. Endothelial dysfunction has proved some prognostic value for these events. Fischer *et al.*, (2005) also report that endothelial dysfunction is an independent key predictor for cardiac side events, hospitalization in patients with CHF and death.

Fischer *et al.* (2005) reported that AGEs can impair vascular function of diabetic patients by influencing both endothelial function and vascular compliance. Endothelial dysfunction can be induced by AGEs by reducing the availability of the NO (Quehenberger *et al.*, 2000). It was reported by Falk *et al.* (2008) that tobacco smoking, obesity and sleep disorders together contribute to endothelial dysfunction and atherosclerosis, creating a high risk for CVD in patients with COPD and diabetes. The contribution of smoking to cardiovascular events is believed to occur via induction of endothelial dysfunction; as well as the fact that cigarette smoking itself is linked to oxidative stress, widely considered to be a mediator of endothelial dysfunction (Morrow *et al.*, 1995; Cai and Harrison, 2000; Sambola *et al.*, 2003). Additionally, AGE was shown to bind with endothelial RAGE resulting in endothelial dysfunction (Schmidt *et al.*, 1994). According to Muniyappa and Sowers (2013), many methods have been used to assess both endothelial function and dysfunction, either directly or indirectly.

Indirect measurement involves measuring levels of markers of endothelial dysfunction, whereas direct measurement involves imaging techniques such as PET, which have been used to assess changes in capillary blood flow in response to intravenous infusion of vasodilatory agents, although these techniques are considered less reliable (Muniyappa and Sowers, 2013). Nadar *et al.*, (2004) and

Muniyappa and Sowers, (2013) have found indirect assessment of endothelial dysfunction to be more reliable as it requires less patient contact, less complicated procedures and is relatively cheaper than direct assessment.

#### **1.3.5.1 Thrombomodulin as a marker of endothelial dysfunction**

Thrombomodulin is "a glycoprotein that can bind to thrombin and activate protein C, thus mitigating the effects of cytokines produced by inflammatory and immunological processes" (Califano *et al.*, 2000). It is one of the important markers for endothelial dysfunction with antithrombotic action mediated via the thrombin-thrombomodulin complex. An increase in thrombomodulin levels can be observed in many neoplastic diseases (lymphoma, leukaemia and cancer) and in hepatitis (Takahashi *et al.*, 1992). Thrombomodulin levels are quantified in plasma by using ELISA techniques in patients with collagen disease, diabetes, haematological malignancies, liver disease and thrombotic disease (Takahashi *et al.*, 1992; Eng *et al.*, 2000).

Thrombomodulin is a key molecule as an anticoagulant in the blood vessel wall (Jansson *et al.*, 1996).

#### **1.3.5.2 E-selectin as a marker of endothelial dysfunction**

E-selectin (CD62E) is a member of the selectin family of cell surface glycoproteins. It is an endothelial cell-specific adhesion protein involved in the initial vessel wall binding and rolling of polymorphonuclear leukocytes (Pober and Cotran, 1990). E-selectin production occurs during stimulation of TNF- $\alpha$  since it is not stored in the endothelial cells, and thus takes around two hours to be expressed on the cell surface, with its peak expression lasting up to 12 hours after stimulation (Fries *et al.*, 1993). Expression of E –selectin is a result of cytokine activity such as tumor necrosis factor (TNF $\alpha$ ) and interleukin 1 $\beta$  (Bevilacqua *et al.*, 1989). Rahman *et al.*

(1998) concluded that TNF $\alpha$  induces generation of ROS, which in turn mediates E-selectin mRNA expression. Furthermore, Ley *et al.* (1998) showed that the expression of E-selectin is induced by TNF- $\alpha$ . E-selectin is widely recognised as an important endothelial cell product which leads to plaque formation in atherosclerosis and some other CVD events (Malatino *et al.*, 2007).

Harari *et al.*, (2001) speculated that the induction of E-selectin expression in endothelial cells might be a protective response to endothelial damage, in this case the levels of cytokines are increased and this stimulates the expression of E-selectin. The stimulation attracts leukocytes to move to the site of the damage and to bind to E-selectin on the surface of endothelial cells in a protective role. However, this only occurs when the inflammation is acute: if the inflammation becomes chronic then a negative response might develop. When endothelial injury occurs, E-selectin is more likely to be expressed at high levels, so that it can be considered as an indicator of endothelial damage and oxidative stress. It was reported that when there is endothelial dysfunction, there are alterations in E-selectin levels (Muniyappa and Sowers, 2013). As the level of NO is reduced in the presence of endothelial damage, endothelial cell adhesion molecules increase as a result of cytokine induction, and therefore the risk of developing CVD increases. In addition, any reduction in NO level causes constriction of blood vessels which in turn increases the probability of having an injury from high blood pressure (Vachharajani and Granger, 2009).

### 1.3.5.3 Plasminogen activated inhibitor (PAI-1)

Fibrosis is an abnormal fibroblast activation-related or fibroproliferative disease. Any deregulation in the process of wound healing can result in hyperactivation of fibroblasts and extra accumulation of ECM proteins in the wound area, which is recognised as the pathophysiological manifestation of fibrosis (Nadar *et al.*, 2004). It is considered as a final common stage of the pathological manifestation of several diseases such as alcoholic liver disease, cardiac and pulmonary hypertension, myocardial infarction, and non-alcoholic steatohepatitis (Brunt, 2004; Berk *et al.*, 2007; Ellmers, 2010; Ghosh, 2010; Mastuzaki, 2010; Yang *et al.*, 2010).

PAI-1 is a member of the serine protease inhibitor (serpin) gene family and is recognised as a major physiologic inhibitor of the serine proteases, uPA and tPA (tissue plasminogen activator) (Potempa *et al.*, 1994). According to Barrett *et al.* (2010) endothelium sustains the integrity of the vascular wall, which controls fibrinolysis when it produces tPA and PAI-1, which blocks tPA. Moreover, it was found that the endothelium affects the function of heparin sulphate with antithrombin, thrombin/protein C and tissue factor, with tissue factor inhibitor interactions resulting in limitation of the coagulation cascade. PAI-1 is therefore widely considered as a marker of endothelial damage and is also linked to thrombosis as it is partially responsible for inhibition of fibrinolysis (Nadar *et al.*, 2004).

## **COPD: Chronic obstructive pulmonary disease**

### **1.4.1 Introduction**

COPD is a major public health problem that is defined as a preventable and treatable entity which is usually progressive. Its main feature is a systemic inflammatory response of the airways and lungs to noxious gases, such as tobacco and biomass fuel smoke, that are associated with a poorly and progressively irreversible obstruction of airflow particles and causes an accelerated decline in ability of the lungs to function (GOLD, 2015).

### **1.4.2 Epidemiology of COPD**

The prevalence of COPD, morbidity and mortality varies from country to country and from group to group within the general population (Vermeire, 2002). A direct association between the prevalence of COPD and tobacco smoking has been reported; in addition to outdoor and indoor air pollution that results from the burning of fuels and woods. These issues are classified as the major COPD risk factors (Salvi and Barnes, 2009). The burden and prevalence of COPD are likely to increase in the next decades as a result of continuous exposure to risk factors associated with COPD and the aging global population (Chan-Yeung *et al.*, 2004). Meta-analysis and systematic review of previous studies conducted by Helbert *et al.*, (2006) revealed that the remarkable variation in COPD prevalence can be attributed to differences in survey methods, diagnostic criteria and analytic approaches. Several studies conducted across countries such as Asia and Latin and Meso-America, showed that the use of biomass fuel for cooking has increased the prevalence of COPD and has also resulted in a greater decline in lung function in women compared with women who do not use biomass fuel (Dutt *et al.*, 1996; Dossing *et al.*, 1994; Sungu *et al.*,

2001; Regalado *et al.*, 2006; Pandey, 1984). A report by the Global Burden of Disease Study states that COPD will be ranked the fourth leading cause of death worldwide by 2020; it was ranked as the sixth leading cause of death in 1990 (Mathers *et al.*, 2006).

### 1.4.3 Pathophysiology of COPD

The main signs and symptoms of COPD are limited airflow, chronic coughing, increased sputum production and dyspnoea. In general, the progression of COPD is strongly associated with a thickening of the walls of the small airways as a result of an increase of epithelium, lamina propria and muscle tissues and adventitial compartments (Chung, 2005).

Despite the fact that cigarette consumption is considered the number one risk factor for acquiring COPD, evidence showed that non-smokers can also develop chronic airflow limitation. Nevertheless, not all smokers develop COPD. The reasons for this irregularity are not yet clear. However, this is likely due to genetic differences and other exposure to dissimilar environmental settings (Celli *et al.*, 2005; Behrendt, 2005). Smokers inherently have a higher predisposition for lung abnormalities and prevalent respiratory symptoms, not to mention higher rates of decline in FEV<sub>1</sub> (forced expiratory volume<sub>1</sub>) than non-smokers (Kohansal *et al.*, 2009).

Chronic inflammation of the lungs can result from inhaling particles of smoke, air pollutants or other comparable sources. This type of inflammation is present among individuals who develop COPD. An inflammatory response of this nature can trigger destruction of the parenchymal tissue which, in turn, causes a disruption in normal repair and defence mechanisms. Disturbances of this type may lead to small areas of fibrosis that cause air trapping and airflow limitation (Chung, 2005). Chronic

inflammation of the lungs and the narrowing of peripheral airways cause a decrease in FEV1. In the case of emphysema, parenchymal destruction occurs, which results in reduced elastic recoil. Subsequently, this results in the limitation of airflow together, inflammation, the narrowing of peripheral airways, and parenchymal destruction, leading to a progressive trapping of gas during expiration and consequent hyperinflation (Hogg *et al.*, 2004).

#### **1.4.4 Oxidative stress and COPD pathogenesis**

Smoking is known to contribute to the development of COPD and studies have demonstrated its effects on systemic inflammation and oxidative stress (Yanbaeva *et al.*, 2007). It is widely accepted that oxidative stress is a major aetiological factor in the pathogenesis of COPD. Oxidative stress results from impaired or overwhelmed endogenous antioxidant defences caused by the presence of reactive oxygen species (ROS), such as those found in cigarette smoke (Mirrakhimov, 2012). Recent evidence suggests that an increase in oxidative stress may pose a risk by initiating Type 2 diabetes; at the same time, it can be considered a consequence of Type 2 diabetes. This increase in oxidative stress can be due to the generation of ROS that results from hyperglycaemia (Evans *et al.*, 2002). Stapleton *et al.*, (2008) reported that when there is oxidative stress, an increase in the generation of oxidants occurs which can result in either a failure to repair oxidative damage or a reduction in antioxidant damage due to the generation of ROS. Several other risk factors that are linked with oxidative stress are cigarette smoking, dyslipidemia, insulin resistance and inflammation.

There is also considerable evidence that injury is found to be caused or exacerbated by pro-inflammatory cytokines that occur through many different mechanisms; including enhancement of vascular permeability, apoptosis, and recruitment of invasive leukocytes and promotion of ROS (Chung and Barnes, 1999). The term ROS refers to a subset of molecules that are identified as free radicals. These free radicals are defined as any molecule that has unpaired electrons in the outer orbital shell. Each unpaired electron is responsible for making each molecule highly reactive rendering it capable of donating an electron to another molecule or compound, or withdrawing a proton from another molecule or compound to gain stability. The highly reactive molecule results in the formation of bonds between the ROS and other compounds and thus leads to impairment of tissue function (Cheeseman and Slater, 1993). Under normal situations ROS are manufactured by the immune system to overcome or destroy pathogens. Normal metabolism also produces ROS, but oxidative stress is presented only when the production of ROS exceeds the capacity of cellular antioxidant defences. Several findings proved that chronic oxidative stress in patients with diabetes is related to an excess of substrates in the metabolism (i.e. glucose and fatty acids). In addition, an association between mitochondrial dysfunction and insulin resistance was discovered (Nourooz-Zadeh *et al.*, 1997). As the majority of ROS are derived from mitochondria, any defect in mitochondrial function can increase the risks of developing diabetes-related complications; since the aetiology of insulin resistance and diabetes are related to mitochondrial dysfunction (Blake and Trounce, 2014). In brief, oxidative stress originates from the overproduction of ROS and is recognised as a main unifying factor for developing of complications associated with diabetes.

Many studies have explored possible correlations between oxidative stress and biomarkers of COPD. Most of these COPD biomarkers were found in highly significant levels among patients with COPD and in individual smokers compared with healthy individuals (Rezaeetalab *et al.*, 2014). As discussed, oxidative stress results from impaired or overwhelmed endogenous antioxidant defences caused by the existence of ROS. Consequently, this leads to the development of “carbonyl stress” in which oxidants damage the surrounding tissues and in turn forms highly reactive organic molecules that are responsible for modifying proteins. This occurs through a non-enzymatic pathway, forming carbonyl adducts, more commonly known as AGEs on the surface of proteins (Yao and Rahman, 2011). The formation of AGEs is historically linked to high glucose levels and the presence of oxidative stress in diabetes through a process known as glycation. In diabetes, an increase in carbonyl stress can be due to oxidative stress which is expressed as an increase in the levels of reactive carbonyls which are linked to the severity of COPD which leads to the formation of AGEs (Kirkham and Barnes, 2013).

#### 1.4.5 Carbonyl Stress

*In vivo*, modified AGEs were reported as being involved in the development of vascular lesions, which contribute to macro and microvascular pathologies (DCCT, 1993). HbA1c is widely considered the gold standard tool for diagnosing diabetes and measuring glycaemia (Genuth, 2002).  $\alpha$ -oxoaldehydes are recognised as potent glycating agents that play a key role in creating carbonyl stress. These compounds are produced endogenously (Thorpe *et al.*, 2000). An imbalance between the formation of carbonyl intermediates and the effectiveness of scavenger pathways leads to the formation of carbonyl stress (Miyata *et al.*, 1999). While dicarbonyls such as  $\alpha$ -oxoaldehydes are considered as AGE precursors, they form in one of

three ways: as glycolytic intermediates by conversion of glucose via metabolic pathways, by Millard reaction via degradation of glycated protein, or by peroxidation of lipids (Thornalley, 2005). Figure 1.4.5 shows that the  $\alpha$ -oxoaldehydes compounds are potent glycating agents with extremely high chemical activity, up to 50,000-fold as reactive glucose. Therefore, they can form AGEs even at low concentrations (Latruffe *et al.*, 1982). In fact, during the process of the glycation reaction glucose is manufactured into the amino group of lysine and N-terminal amino acids from proteins that react with glucose. In the case of dicarbonyls the glycation product is presented as an arginine residue (Thornalley, 2005).

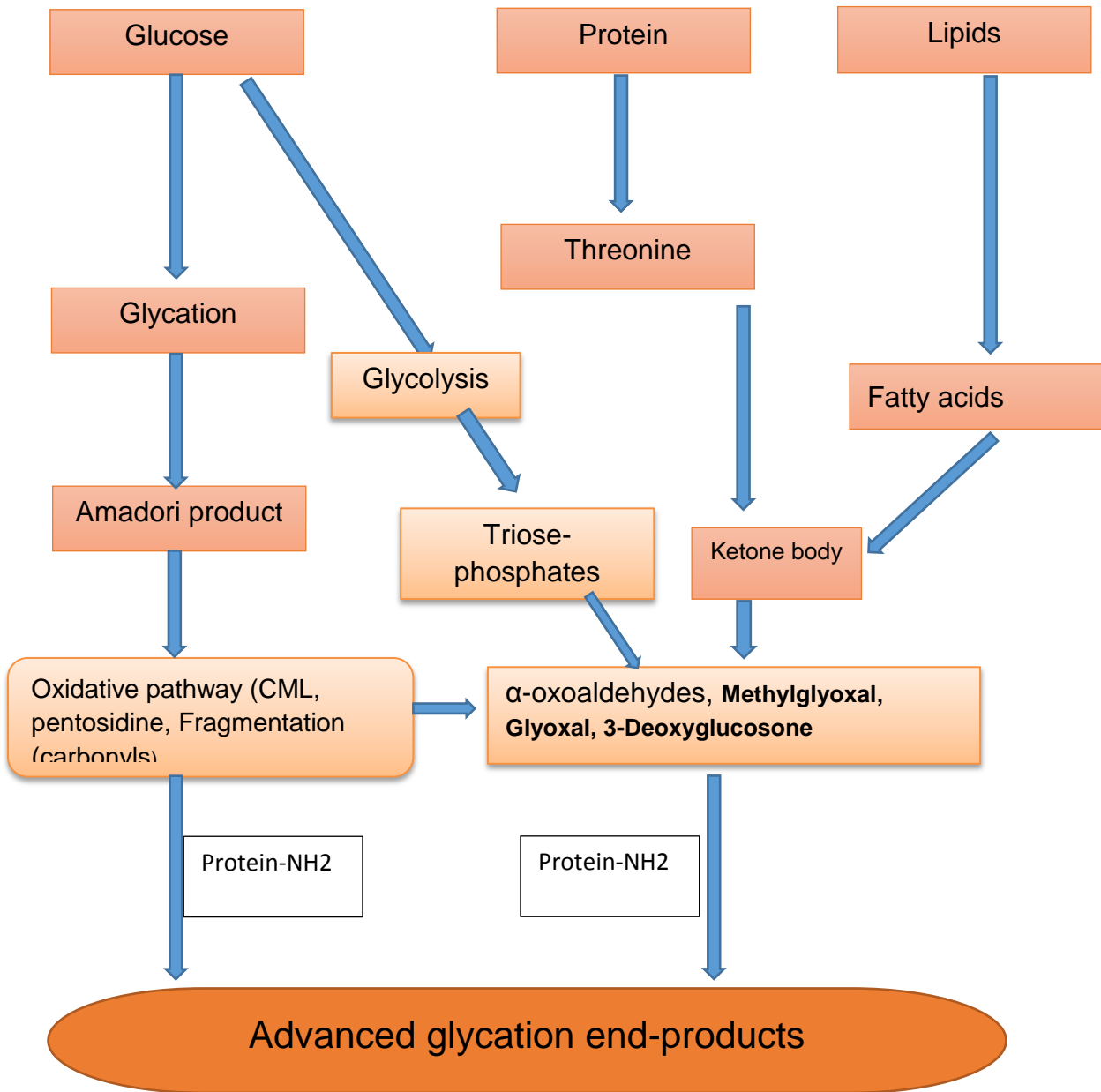


Figure 1.4.5: Pathways for reactive carbonyl compounds biogenesis.

#### 1.4.6 AGE and RAGE and the association with COPD

As mentioned in section 1.2.1, AGEs are the final products of the non-enzymatic glycation and the oxidation of proteins and lipids (Goldin *et al.*, 2006). Their production is accelerated in the presence of hyperglycaemia and oxidative stress (Schmidt *et al.*, 2001). Forbes *et al.*, (2001) reported that AGEs accumulate in the brain and contribute to the development and progression of Alzheimer's disease via cellular responses to ROS.

The two major risk factors of COPD are smoking and ageing, although it is unclear exactly how these two factors contribute to the development of COPD (Fletcher and Peto, 1977). It is known, however, that they may accelerate the formation of AGEs (Nicholl *et al.*, 1998). The binding of AGEs to RAGEs activate NF- $\kappa$ B (nuclear factor kappa- B cells) and enhances inflammation so that AGEs can play an important role in the development and progression of COPD (Bierhaus *et al.*, 2000). Demling *et al.* (2005) reported the expression of RAGE at low levels in tissues and blood vessels, although its expression becomes high in sites where its ligand accumulates. The same research group found that RAGE is well expressed in normal lung tissue. RAGE was identified as a specific differentiation marker of human ATI cells by analysing the alveolar epithelial Type 2 cells. In addition, Demling *et al.*, (2005) noted that RAGE was expressed in endothelia and alveolar macrophages. RAGEs also promote the adherence of epithelial cells to collagen. Despite these findings, the role of AGEs in the development of COPD has not yet been fully investigated (Mukherjee *et al.*, 2008).

#### 1.4.7 COPD comorbidity with Diabetes

Several studies have reported an association between diabetes and COPD. It has been estimated that the prevalence of diabetes amongst stable COPD patients is as high as 18% (Cazzola *et al.*, 2010). This number rises to 30-34% in acute exacerbating COPD patients (Kinney *et al.*, 2014), with the risk of developing diabetes increasing along with disease severity in COPD (Mannino *et al.*, 2008). It has been reported that for women with COPD, the presence of Type 2 diabetes is 1.8 times higher than for women without COPD; when age, activity, MI, diet and exercise, family history and smoking status are taken into consideration (Rana *et al.*, 2004). Moreover, the presence of diabetes in COPD patients has been associated with worse lung function (Van den Borst *et al.*, 2010), with smoking being a known risk factor for developing Type 2 diabetes in the absence of COPD (Xie *et al.*, 2009). The interaction between diabetes and COPD was shown throughout demonstrating that the decline in lung function is a risk factor for the development of diabetes (Lazarus *et al.*, 1998; Engstrom *et al.*, 2003). In fact, smoking has been considered a risk factor for diabetes (Manson *et al.*, 2000 & Rimm *et al.*, 1995).

Both COPD and diabetes are influenced by inflammation and oxidative stress after exposure to tobacco. The net outcome is downstream complications that are often associated with these diseases; particularly after an exacerbation in COPD, namely vascular complications brought about by high levels of AGEs (Lavi *et al.*, 2007).

In fact, in stable COPD patients it was reported that the levels of pro-inflammatory cytokines IL-6, IL-1 $\beta$ , TNF- $\alpha$  and IL-8 are increased in sputum (Chung, 2001). In COPD, the cytokine mediators are increased chronically and are thought to

contribute to the development of COPD. It was reported that in diabetic individuals, the TNF- $\alpha$ , IL-6 and C reactive protein (CRP) levels were higher than in non-diabetic individuals and can be considered predictors for risk of diabetes (Hu *et al.*, 2004). In addition, insulin resistance was found to be increased in patients with COPD than those without and this increase was related to plasma IL-6 and TNF-levels (Bolton *et al.*, 2007). However, the relationship between increased insulin resistance and chronic inflammation may be due to disruption of insulin receptor signaling by inflammatory mediators (Grimble, 2002). Such a relationship was demonstrated by Liu *et al.* (1998). In their research study, they cultured human adipocytes and noted that TNF- $\alpha$  caused a decrease in the insulin-stimulated autophosphorylation of the insulin receptor, which blocked the signaling of insulin.

Treatment of COPD with glucocorticoids has impacted on diabetes management. Diabetes control also affects COPD outcomes; although it is currently unclear whether improving glycaemic control can improve COPD outcomes (Luijckx *et al.*, 2015). The use of systematic corticosteroids as a form of therapy for COPD affects glucose intolerance and diabetes mellitus. A study carried out on patients with COPD (Wood-Baker *et al.*, 2007) compared patients treated with oral corticosteroids and those who received a placebo. Results indicated that those who received oral corticosteroid therapy were 7.7 times more likely to experience adverse effects than those who received the placebo. The adverse effects mostly commonly experienced were glucose intolerance and mild hypertension. In addition to these findings, other studies have also shown that inhalation of steroids can have slight negative effects on glucose metabolism. In a large, randomised control study, it was shown that patients who inhaled budesonide 400 $\mu$ g bid were more susceptible to developing diabetes within three years of follow up than those who received placebo

(Canis *et al.*, 2007; Faul *et al.*, 2009). Additionally, mortality rates were reported to be significantly higher in patients with poor glycaemic control who were admitted to hospital for acute exacerbation of COPD (Baker *et al.*, 2006). Even after discharge, hyper glycaemia remained as a risk factor for increasing the death rate for diabetic patients. It is still unclear whether improvement in glycaemic control can improve COPD outcomes (Gudmundsson *et al.*, 2006).

#### **1.4.7.1 Metformin as an antidiabetic drug in COPD**

Metformin treatment is well known for improving the outcomes of complications associated with COPD. During COPD, exacerbated glucose tolerance worsens because of the physiological stresses of acute illness including an increase in inflammation, acidosis and corticosteroids therapy. Thus, most COPD patients with diabetes are found to be overweight and insulin resistant (Baker *et al.*, 2009). Such patients are required to be treated with drugs that increase insulin sensitivity and encourage weight loss, for example metformin.

Metformin (dimethylbiguanide) is an anti-diabetic drug that is administered orally. It lowers blood glucose levels in Type 2 diabetic patients due to its role in improving insulin sensitivity, which in turn decreases insulin resistance. It also causes a significant reduction in plasma fasting insulin levels and so is widely considered as an insulin sensitizer (Violet *et al.*, 2012). It can be administered alone as an initial therapy to lower glucose or with sulphonylureas as an additional therapy (Bailey *et al.*, 1996). Metformin's main effect is reducing the production of hepatic glucose. This can be due to its action in activation of AMP-activated protein kinase (AMPK). AMPK is a major cellular regulator of lipid and glucose metabolism. This activation of AMPK stimulates glucose uptake in muscle, fatty acid oxidation in

muscle and liver, and results in reduced production of hepatic glucose, cholesterol and triglyceride (Zhou *et al.*, 2001).

Several studies show that administration of metformin has beneficial effects on cardiovascular events (Sasali and Leahy, 2003; Inzucchi, 2005; Lamanna *et al.*, 2011). Yin *et al.* (2011) reported that, in Sprague-Dawley rats, metformin treatment improves cardiac function and decreases the size of infarct after myocardial infarction. In addition, it was found that administration of metformin alone or with sulphonylurea can decrease mortality and morbidity rate in patients with Type 2 diabetes and heart failure, compared to administration of sulphonylurea alone (McDonald *et al.*, 2010, Eurich and McAllister, 2010). Metformin was also found to decrease the rate of atherothrombosis in a cohort of patients with atherothrombosis for continued health (REACH) Registry (Roussel *et al.*, 2010).

AGEs which result from non-enzymatic glycation are formed *in vivo* from highly reactive carbonyl groups called alpha-dicarbonyls or oxoaldehydes, including 3-deoxyglucosone, glyoxal, and methylglyoxal (Brownlee *et al.*, 2006). Several studies have shown that methylglyoxal (MG) is significantly increased in the plasma of diabetic patients (Knecht *et al.*, 1992; McLellan *et al.*, 1992) and that this increase in plasma MG levels correlates with glycaemic control (Beisswenger *et al.*, 1995). MG levels can cause increases in AGEs levels as they are one of the main source for AGEs. This has been proven to correspond to occurrences of diabetic vascular and neuropathic complications and diabetic nephropathy (Beisswenger *et al.*, 1995, Monnier *et al.*, 1986 and Biesswenger *et al.*, 1997).

Many investigators have shown that metformin has beneficial effects in reducing vascular risks associated with diabetes; alongside its main role as an anti-

hyperglycaemic drug (Beisswenger *et al.*, 1999). In addition, metformin has shown reductions in MG levels in the tissues of diabetic patients (Beisswenger *et al.*, 1999), which in turn reduce AGEs levels in the lens, kidney, and nerves of diabetic animals (Tanaka *et al.*, 1999). This reduction of MG levels by metformin occurs via formation of specific condensation products that result from the reaction of metformin with MG. One example of those compounds is triazepinone (TZP) (Ruggiero-Lopez *et al.*, 1999). Regan *et al.*, (2001) reported that functional and structural alterations of diabetic myocardium associated with glycation can be prevented by metformin treatment. They also reported that metformin can completely prevent myocardial AGE formation.

#### 1.4.8 COPD, Diabetes and CVD

Diabetes, hypertension, ischaemic heart disease and heart failure are the main comorbidities that are linked to COPD (Moussas *et al.*, 2008). Cardiovascular disease (CVD), cancer, chronic obstructive pulmonary disease (COPD) and diabetes mellitus (DM) are listed as the most prevalent among chronic morbidities; it was reported that hyperglycaemia, infections and cardiovascular complications are linked with exogenous hypercorticism which is most frequent among patients suffering from extra severe lung disease with endocrine alteration (Martin *et al.*, 2009).

Several studies have reported that Type 2 diabetes is a risk factor for macrovascular disease (Fowler, 2008). Some of the side effects of diabetes may alter the outcomes for COPD. These effects of diabetes may be related to an increase in cardiovascular morbidity and mortality, taking into account that the lung itself is targeted by microvascular diseases (Mirrakhimov, 2012). Weynand *et al.* (1999) showed that the thickening of alveolar epithelial and endothelial capillary

basal lamina can be observed in the lungs of patients with diabetes when compared to healthy people; using electron microscopy. Moreover, the lung's capacity is reduced in patients with diabetes mellitus and is expected to further diminish when coupled with macrovascular complications. The presence of pulmonary microvascular disease in patients with COPD and diabetes may lead to respiratory impairment (Strojek *et al.* 1992; Mori *et al.*, 1992).

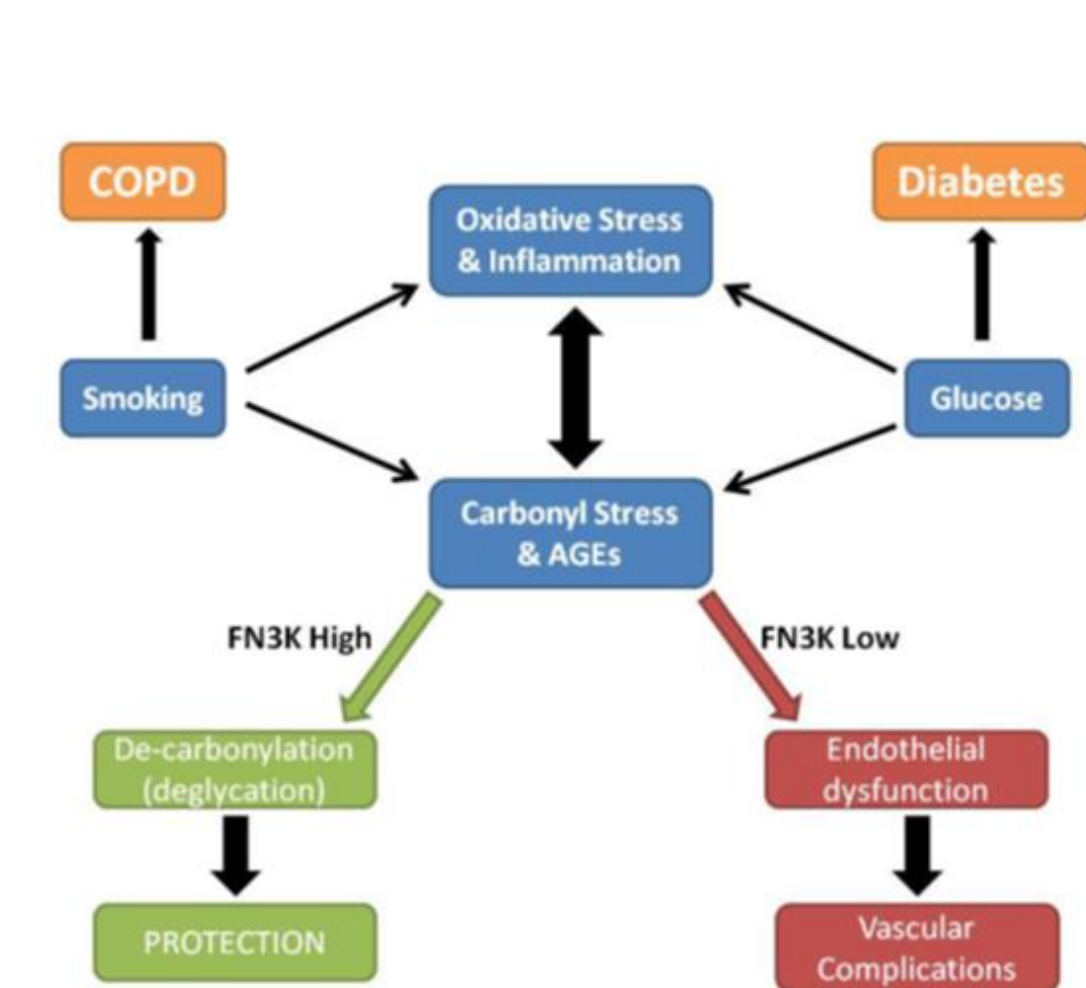
The relationship between cardiovascular diseases and respiratory diseases has received much attention (Sin and Man, 2005; Curkendall *et al.*, 2006). The reason for this association is unclear but may be due to systemic inflammation, chronic infections, shared risk factors (i.e. smoking) or other unknown factors (Le Jemtel *et al.*, 2007; Naunheim *et al.*, 2006). The Atherosclerosis Risk in Communities Study (ARIC) data demonstrate that respiratory impairment predicts the progression and development of cardiovascular disease. It was also discovered that this relationship decreases when fibrinogen, a marker of systematic inflammation is accounted for. This finding proposes that the association between COPD and cardiovascular disease may be partially related to other factors (Johnston *et al.*, 2008).

In patients with COPD, the plasma fibrinogen levels were increased. It is possible that this rise in fibrinogen levels may lead to an increased CVD morbidity and mortality rate among patients with COPD. As serum IL-6 levels rise in COPD patients, plasma fibrinogen levels increase in response. In turn, this increase in plasma fibrinogen results in the development of acute infection which plays a role in pre-occurrence of coronary heart disease (CHD) and stroke (Wedzicha *et al.*, 2000).

Furthermore, smoking may increase susceptibility to infection and causes the levels of inflammatory markers to intensify (Arcavi and Benowitz, 2004; Gan *et al.*, 2005).

In fact, relevant features in their development and their course of action demonstrated similarities between diabetes and COPD. These similarities include hypoxia, insulin resistance, inflammation and oxidative stress. Collectively, tobacco smoking, obesity and sleep disorders promote endothelial dysfunction and atherosclerosis, thereby placing diabetic and COPD patients at high risk (Falk *et al.*, 2008). As mentioned in section 1.4.4, this phenomenon may be caused by an increase in carbonyl stress brought on by oxidative stress. The latter may be expressed as levels of reactive carbonyl that are linked to the severity of COPD, which results in the formation of AGEs (Kirkham and Barnes, 2013). Oxidative stress is the result of free radicals present in cigarette smoke; the levels of isoprostanes, a marker of oxidative stress, were notably increased in smokers (Seet *et al.*, 2011).

The mechanism behind the contribution of smoking to cardiovascular events may be explained through the catabolic pathway of induction of endothelial dysfunction. Cigarette smoking is linked to oxidative stress; which is widely considered a mediator of endothelial dysfunction. In the presence of oxidative stress, the inflammatory response as well as blood thrombogenicity (both features of endothelial dysfunction) increase (Morrow *et al.*, 1995; Cai & Harrison, 2000; Sambola *et al.*, 2003). Due to the apparent contribution of FN3K in the process of non-enzymatic glycation and the assumed effect it has on the formation of AGEs, it was proposed that the enzyme might play a role in the association between COPD, CVD and diabetes. Figure 1.4.8 provides a summary of these possible links.



**Figure 1.4.8. The proposed role of FN3K in diabetes, COPD & CVD.**

In contrast, it has been shown that AGEs in the circulatory system bind with endothelial RAGE, thereby causing endothelial dysfunction. This can be explained by the activation of a variety of signaling pathways, such as nicotinamide adenine dinucleotide phosphate oxidase activation, which stimulates the manufacturing of ROS (Schmidt *et al.*, 1994). In support of this finding, Evans *et al.*, (2002) reported that ROS contribute to majority of the cardiovascular damage seen in patients with diabetes mellitus by modifying and altering the structure of cellular lipids, nucleic acids and proteins, ultimately affecting the physiological functions of these constituents.

## 1.5 Summary of Gaps in the Previous Studies

1. Most of the previous studies have reported the presence of FN3K in animal models but very few of them have examined human models. Many have looked at whether HbA1c levels reflect mean blood glucose (MBG) levels in Type 2 diabetes.
2. Previous studies have neglected to look at the relationship between the FN3K activity and glycated haemoglobin levels in human models.
3. No studies have investigated the presence and activity of the FN3K enzyme and its relation to G-gap values.
4. Studies have failed to look at any possible relationships between plasma AGEs and the activity and/or expression of FN3K enzyme in human erythrocytes.
5. Very few studies have clarified the role of AGEs in aging and diabetic complications.
6. No studies have been conducted to measure FN3K levels in human erythrocytes of patients with variety of G-gap values. Thus, there have been no reports on any association between Human FN3K levels and its activity in erythrocytes with the markers of endothelial dysfunction.
7. Few studies have considered the association between COPD, diabetes and CVD and even fewer studies have clarified how these comorbidities are linked together.
8. There are no studies that investigate the role of FN3K enzyme in patients with COPD, diabetes and/ or CVD.
9. Minimal studies have explored whether the discrepancies in FN3K activity

and/or concentration may affect the outcomes of diabetes in the presence or absence of accompanying complications.

10. There is no research to possible links between metformin therapy and FN3K enzyme activity and/or expression.
11. Past studies have overlooked the possibility for associations between carbonyl stress and the role of FN3K enzyme in patients with COPD and diabetes.
12. A limited number of studies have investigated the occurrences of isoenzymes for FN3K exhibiting different kinetic properties.

## 1.6 Research Questions, Aims and Objectives

### 1.6.1 Research Questions

The questions below have been formulated based on the gaps created in previous studies:

1. Is the FN3K enzyme responsible for deglycating the glycated haemoglobin within erythrocytes?
2. Are the differences in G-gap levels related to FN3K activity and/or levels in human erythrocytes or vice versa?
3. Can variations in the FN3K activity affect the levels of AGEs in individuals that display discrepancies in glycation gaps?
4. Are markers of endothelial dysfunction affected by FN3K enzyme activity and/ or levels in erythrocytes?
5. Does FN3K enzyme expression control the levels of carbonyl stress and AGEs in COPD patients with diabetes? If so, does it affect the occurrence of associated CVD complications?
6. What are the factors behind the discrepancies in FN3K levels among individuals?
7. Does FN3K activity in human erythrocytes associate with FN3K levels inside and outside erythrocytes?
8. Is the FN3K the only enzyme responsible for the deglycation of glycated haemoglobin in erythrocytes and are polymorphisms in the FN3K genes associated with this event?

### 1.6.2 Research Hypothesis

The following hypotheses were developed from the above research questions:

1. FN3K deglycates glycated haemoglobin in erythrocytes of diabetic patients
2. FN3K is an important enzyme that plays a role in maintaining of blood glucose levels in humans
3. Variations in the activity of FN3K between individuals may lead to apparent discrepancies in glycated haemoglobin levels, causing people with high FN3K activity to have low glucose levels while those with low FN3K activity present high glucose levels
4. Patients with positive G-gaps are assumed to exhibit lower FN3K activity in comparison to patient with negative G-gaps and are expected to have higher activity of FN3K enzyme
5. Patients with low G-gaps and high FN3K activity are assumed to be less at risk for developing diabetic complications, such as cardiovascular events, while those with a high G- gap value and low FN3K activity are expected to be at high risk for developing complications associated with diabetes
6. High FN3K activity plays a role in decreasing the levels of AGEs as well as decreasing carbonyl stress levels in patients with diabetes and COPD
7. Metformin, an antidiabetic drug, can have positive and beneficial effects in the frequency and severity of diabetic complications.

Validation of the above hypotheses would serve to pave the way for further complementary investigations to take place in the future. Such studies may contribute to improving the managements of diabetes. More research on this area of

diabetes is needed to achieve a better understanding of the FN3K enzyme and its possible clinical utilities regarding the management of patients with diabetes.

### 1.6.3 Aims of the Research

The aim of this research is to investigate possible associations between differences in G-gap values among diabetic individuals with the individual discrepancies in their FN3K activity and levels as presented by each case. Furthermore, this study also aims to look at whether these discrepancies impact the risk of developing diabetes-related complications and to assess whether the G-gap could be used to predict whether complications associated with diabetes will arise. This possible association is examined by measuring the amount of FN3K activity and level in diabetic patients and correlating the results with their G- gaps levels; whilst measuring markers of endothelial dysfunction, since it is known as an important precursor for other cardiovascular complications in diabetic patients (Green and Turner, 2017; Meigs, *et al.*, 2004; Sena *et al.*, 2013; Hadi and Al Suwaidi, 2007). In summary, this research seeks to:

1. To test the hypothesis that the enzyme fructoseamine-3-kinase is responsible for the deglycation of glycated haemoglobin in erythrocytes and to obtain a better understanding of its potential and physiological role of in this process;
2. Investigate any associations between the activity and levels of the FN3K enzyme both erythrocytes and plasma with the G-gap value in each individual;
3. Examine whether the rate of deglycation reaction influences differences in the occurrence of diabetic complications between subjects with high G- gap values and those with low G-gap values. If so the FN3K enzyme could be considered as an important enzyme for maintaining glucose levels in patients

as it catalyses the phosphorylation of glycated proteins, such as glycated haemoglobin, since the variations in the activity of FN3K between individuals may lead to apparent discrepancies in the glycated haemoglobin levels;

4. Investigate the relationship between the variations in G-gaps with FN3K levels in diabetic patients and the possibility of assessing development endothelial dysfunction by measuring markers of endothelial dysfunction in each of those patients;
5. Determine whether variations in G-gap values affect the levels of AGEs in diabetic patients and the involvement of FN3K enzyme in this association;
6. Understand the concept of oxidation/carbonyl stress and its association with FN3K levels in patients who have COPD and diabetes
7. Investigate the impact of metformin treatment therapy on FN3K levels in COPD patients with diabetes;
8. Investigate the impact of smoking on FN3K expression in COPD diabetic patients as FN3K may control the levels of carbonyl stress and AGEs formation, along with the vascular complications that can arise in COPD and diabetes.

#### 1.6.4 Objective of Studies

To achieve the above aims for this research, this work was divided into three separate studies, each study was categorised according to the type and the source of samples involved:

1. The first study: fructosamine-3-kinase (activity and protein levels) in relation to G-gap and AGEs in diabetic patients. This study was divided into five parts:

- I. Study 1 Part 1: FN3K enzyme activity in Erythrocyte lysate: The purpose of this part of the study is to compare FN3K enzyme activity between subjects with high G-gap values to those with low G-gaps values; using human erythrocytes from those subjects for this assay (Krause *et al.*, 2006).
- II. Study 1 Part 2: FN3K levels in erythrocyte lysate. The purpose of this study is to know if there are differences in the levels of FN3K enzyme between those with high and low G-gap values and if these differences in FN3K protein levels are correlated with its activity.
- III. Study 1 Part 3: Plasma FN3K protein levels in relation to G-gap. This part was set to investigate whether FN3K enzyme is presented in plasma and if it can deglycate glycosylated proteins in plasma such as glycosylated albumin.

- IV. Study 1 Part 4: Plasma AGEs levels in relation to G-gap. This part was aiming to investigate whether there is an association between differences in glycation gaps value and plasma levels of AGE. And to find out if there is a link between difference in plasma AGEs levels and the activity and levels of FN3K enzyme.
- V. Study 1 Part 5: The association between endothelial dysfunction and G-gap. The objective behind this part is to investigate whether any changes in G-gap values are associated with presence of endothelial dysfunction.
- VI. Study 1 part 6 Plasminogen activated inhibitor (PAI-1) relation as a marker of thrombosis and endothelial dysfunction to G-gap. The objective of this part is to explore whether changes in the G-gap values are associated with any risk of both thrombosis and endothelial dysfunction.

In summary, the objectives of this study comprise:

- A. FN3K enzyme levels were measured in plasma from the same diabetic patients who displayed difference in glycation gap levels.
- B. With regards to the possibility of having endothelial dysfunction in individuals with high glycation gap values, markers of endothelial dysfunction, such as, thrombomodulin and E-selectin molecules were measured in plasma samples obtained from the same patients using the ELISA technique (Eng *et al.*, 2000, Meigs *et al.*, 2004 & Nadar *et al.*, 2004). In case of fibrinolysis and endothelial dysfunction, the plasminogen activator inhibitor 1 (PAI-1), a protein primarily produced by the endothelium which is partially responsible for the inhibition of

fibrinolysis and thus is linked to thrombosis that contributes to endothelial dysfunction, was also measured using the ELISA technique (Nadar *et al.*, 2004).

c. AGEs levels in plasma were measured by performing a competitive ELISA technique on the same diabetic patients who exhibited high and low glycation gaps values.

2. The second study involved measuring of FN3K levels in acute exacerbating COPD (AECOPD) patients treated with metformin and placebo) the reason behind this study is to examine the association between COPD and diabetes and the impact of metformin therapy. This was achieved by investigating whether metformin treatment can enhance FN3K expression compared with placebo treatment in subjects with AECOPD. Serum FN3K enzyme levels were measured using the ELISA techniques in serum samples obtained from patients with AECOPD who were admitted to hospital and treated with either metformin or placebo.

3. The third study involves measurement of FN3K protein levels in COPD airways. The reasoning behind this study is to investigate whether the presence of COPD may enhance the expression of FN3K enzyme and to examine the impact of smoking on FN3K expression in COPD patients.

FN3K expression was assessed by examining bronchial biopsies from smokers, stable COPD patients, and healthy individuals using immunohistochemistry on paraffin-fixed biopsies; obtained following lung resection surgery.

# **Chapter 2**

## **Materials and Methods**

## **2.1 Study 1: FN3K, AGEs and Markers of Endothelial Dysfunction Assay**

### **2.1.1.1 Study Design**

A double blind and single-centre study was conducted using 148 plasma samples and 100 samples of erythrocytes obtained from patients diagnosed with diabetes. Samples were collected from New Cross Hospital (Wolverhampton, UK), a member of the West Midlands NHS trust.

### **2.1.1.2 Ethical Approval**

Ethical approval was obtained from the University of Wolverhampton and the local UK Health Service Research Ethical Committee - West Midlands prior to the collection of test samples.

### **2.1.1.3 Selection of Patients and Data Collection**

After receiving ethical approval for conducting this study, glycated haemoglobin and fructosamine measurements were taken from carefully selected patients over a three-year period (2006-2009) at New Cross Hospital. These patients were adults who had been recruited for a wider range of diabetes research which involves the investigation of glycated haemoglobin and various elements of G-gaps (Nayak *et al.*, 2013). In total, 148 samples were gathered from the corresponding number of patients involved in this study.

These patients were subjected to two different tests that involved taking two measurements for the levels of glycated haemoglobin and two for G-gaps which were presented as consistent. These values were categorised as positive or negative for G-gaps.

Of the 147 patients who met the inclusion criteria (one sample was excluded due to mishandling), 80 were found to have negative G- gaps while the remaining 67 had positive G-gaps. These patients had been diagnosed with either Type 1 or Type 2 diabetes although the majority of them had Type 2 diabetes. The 150 patients selected were made of the first 75 had 2 pairs of simultaneous estimations of HbA1c and 2 calculated G-gaps that were consistently positive and the first 75 patients that had 2 pairs of simultaneous estimations of HbA1c and 2 calculated G-gaps that were consistently negative and not from random selections.

The only known information during the analysis process at the time experiments were conducted was each patient's gender and ethnicity as the study was double-blinded (Table 2.1a and 2.1b). Variables, such as age and length of disease were unknown.

Table 2.1a: The known variables in the cohort in each glycation gap group of (Study 1).

Glycation gap values	Gender		Ethnicity		
	Male	Female	White	Black	Asian
<b>Negative G gaps (n=80)</b>	52	29	58	7	10
<b>Positive G gaps (n=67)</b>	40	27	48	3	16

Table 2.1b: Descriptive characteristics of patients involved in (study 1).

Gap Category>	Negative (mean ± SD)	Positive (mean ± SD)	P (unpaired t)	P (MWU)	P μ2
n	81	67			
Age (years)	61.3±10.4	64.4±9.3	ns	ns	
Gender (% male)	64%	60%			ns
Ethnicity (% white)	72%	72%			ns
Smoking status (% never smoked)	56%	49%			ns
Body mass index (kg/m2)	30.2±5.2	35.4±6.7	□ <0.001	□ <0.001	
Weight (kg)	87.8±18.3	99.1±21.4	□ <0.01	□ <0.01	
Type of Diabetes (% type 2)	84%	91%			ns
Duration of Diabetes (years)	15±10	15±9	ns	ns	
Insulin therapy (% yes)	69%	81%			ns
Retinal status (% with any retinopathy)	72%	71%			ns
Urinary albumin creatinine ratio (□g /□mol)	4.4±18.5	9.7±29.7	ns	□ <0.05	
Creatinine (umol /l)	86±22	82±21	ns	ns	
Vascular status (% macrovascular disease)	29%	31%			ns
Cholesterol (mmol /l)	4.3±1.2	4.2±1.3	ns	ns	
HbA1c (% glycated)	7.4±1.9	9.7±1.7	□ <0.001	□ <0.001	
Fructosamine	332±86	302±60	□ <0.05	□ <0.05	
Fructosamine derived HbA1c (% glycated HbA1c)	8.8±1.9	8.2±1.3	□ <0.05	□ <0.05	
Glycation gap (% glycated HbA1c)	-1.4±0.7	+1.5±0.7	□ <0.001	□ <0.001	

Tables 2.1a and 2.1b show that the total number of male participants was higher than females. Despite this observation, their G-gap numbers are approximately equal for both genders. Moreover, the distribution of ethnic groups appeared to be unequal with most patients being white, followed by Asians and blacks. This discrepancy might be because patients were recruited from a single area: the study is single centred in its design.

#### **2.1.1.4 Inclusion and Exclusion Criteria**

For individuals to be selected into this study they were required to meet the following criteria:

1. Be diabetic patients
2. Consistently produce measures for G-gaps that fall between  $\geq 0.5\%$  or  $\leq -0.5\%$
3. They should be 18 years of age or older
4. Should demonstrate variation in their glycated haemoglobin (HbA1c) levels.

Patients were excluded from this study if they were:

1. Non-diabetic
2. Displayed abnormal haemoglobin
3. Patients with electrophoretic pattern or were pregnant women
4. Patients with previously prescribed drugs affecting glucose levels, such as corticosteroids, for example
5. Patients under 18 years old
6. Patients with haemoglobinopathy

7. Patients with elevated creatinine levels of more than 200  $\mu\text{mol/L}$ .

#### **2.1.1.5 Consent Forms and Information Sheets:**

Each patient recruited was provided with information pertinent to this study, such as the research title, background information about the study and the research team, as well as the tasks to be carried out. Each participant was clearly informed about his/her right to withdraw from the study at any time without providing a reason, and assured that his/her privacy would be protected. Their written consent to willingly participate in this study was obtained before samples were collected.

#### **2.1.1.6 Samples**

Patients were given advance notice of the dates on which blood samples would be collected. Blood samples were collected by venepuncture using a safe vacutainer system. The heparinized blood samples were all taken at New Cross Hospital (Wolverhampton, UK). Samples were then centrifuged to separate plasma from the erythrocyte fraction and both fractions stored at  $-80^{\circ}\text{C}$  in order to maintain their bio-stability over a long period. Plasma samples were prepared using heparin, an anticoagulant, and then centrifuged at  $1,000\times\text{G}$  for 30 minutes after collection. These plasma and erythrocytes samples were then aliquoted to avoid repeated freeze-thaw cycles and stored for assays that were to be conducted later. All aliquoted samples were then moved and stored at  $-20^{\circ}\text{C}$  in the Diabetes Research Group laboratory at the University of Wolverhampton under the care of Dr Simon Dunmore's research group.

### 2.1.1.7 Analysis of Samples

In order to conduct this study, FN3K activity levels, AGE levels, and levels of markers of endothelial dysfunction were compared between subjects with positive and negative G-gap values. The following information was used as standards for evaluating these variables:

1. The normal range of glycated haemoglobin (HbA1c) is 4-6 % (20-42 mmol/mol); individuals with values higher than this range (> 6.5%; 48mmol/mol) were deemed diabetics (DCCT, 1993).
2. The identification of positive and negative G-gaps should fall within a range of >0.5% and > -0.5% (Cohen *et al.*, 2003). HbA1c level was measured using high performance liquid chromatography (HPLC) (Nayak *et al.*, 2013).

The performance scores used by the UK National External Quality Assurance Scheme (UK NEQAS) were applied to this study and are as follows: A (accuracy) score <100 and B (bias) score <2%, which are within the acceptable limits of the UK NEQAS standards for glycated haemoglobins (maximum limits: A score <200 and B score less than  $\pm 7.5\%$ ), whereas the between batch coefficient of variation was 1.8 for an HbA1c of 5.7% and 1.4% for an HbA1c of 9.5%. This was achieved in collaboration with Nayak *et al.*, (2011). Fructosamine was measured by nitroetrazolium blue reduction (Nayak *et al.*, 2013).

Since HbA1c is the key element of this analysis, any factors and/or conditions that altered its level were noted and excluded from the study. Examples of such anomalies include:

1. Abnormal haemoglobin levels. This can be due to high levels of Hbs in diseases such as sickle cell anaemia or deficiency of HbA in conditions such as thalassemia (Mediterranean anaemia).
2. Any changes in HbA levels can affect the glycation process since HbA accounts for 95% of all types of haemoglobin. Thus, if HbA levels are low, this leads to a reduction in the formation of HbA1c which in turn affects the glycation process. Alternatively, an increase in HbA level can lead to an increase in the levels of glycated haemoglobin (HbA1c), which requires that haemoglobin electrophoresis be carried out (Hempe *et al.*, 2002).
3. Many drugs, such as corticosteroids,  $\beta$ -blockers, diuretics, etc. affect blood glucose levels (Ginsberg, 2009). As glycated haemoglobin levels reflect the levels of glucose in blood, any medications that increase or decrease blood glucose levels should be considered. Corticosteroids, for instance, are well known to cause hyperglycaemia, while some  $\beta$ -blocker drugs can mask hypoglycaemia, which in turn, affects the degree of glycation and affects the results. Therefore, any patients undergoing treatment with such medication were excluded from the study (Ginsberg, 2009; Unnikrishnan *et al.*, 2012).

#### **2.1.1.8 Calculations of Fructosamine-predicted HbA1c (FHbA1c) and the G-gap**

Nayak *et al.*, 2013 stated that the predicted FHbA1c was calculated from simultaneously measuring fructosamine that was standardized to the distribution of HbA1c. This is illustrated by the equation below:

$$FHbA1c = \left( \frac{\text{fructosamine} - \text{mean fructosamine}}{SD \text{ fructosamine}} \times SDHbA1c \right) + \text{mean HbA1c}$$

According to (Nayak *et al.*, 2013) the glycation gap can be calculated as follows:

$$Ggap = HbA1c - FHbA1c$$

which simply means the true HbA1c value minus the predicted HbA1c. According to this equation, a negative G-gap means that the true HbA1c value is lower than the value for FHbA1c, while a positive G-gap means that the value of the true HbA1c reading is higher than that for FHbA1c. For those results with a second paired HbA1c-fructosamine estimation, a consistent G-gap was identified by calculating the product of two G-gaps (if consistent) so that the G-gap product would be positive (positive x positive or negative x negative). However, any discordance in the G-gap direction among the pairs, with increasing time, would be considered negative; the negative G-gap would be negative x positive.

In order to categorise the G-gap values, the G-gap (unit = HbA1c %) was categorized as negative when less than or equal to -1, neutral if greater than -1 and less than +1, or positive when greater than or equal to +1.

The use of G-gap in this study is based on Nayak *et al.*, (2003) to measure the deviation of HbA1c from its expected value: a negative G-Gap means lower level of glycation than expected whereas a positive G-gap relates to a higher level of glycation of proteins. An increase in the level of glycation is linked to hyperglycaemia in patients with diabetes and hyperglycaemia is assumed to be a strong predictive indicator for diabetes complications (Nayak *et al.*, 2013).

## 2.1.2 Study 1: Materials and Methods

### 2.1.2.1 Part 1: Determination of FN3K Activity in Erythrocytes

Based on previous studies, human erythrocytes were selected as a suitable model for detecting FN3K activity due to the fact that erythrocytes are easily obtained, incubated and are extremely permeable to glucose. Also, there are easy and robust methods exist for measuring glycated haemoglobin (Lacko *et al.*, 1973).

Furthermore, the presence of FN3K in human erythrocytes could be detected at high levels as reported by many previous studies (Delplanque *et al.*, 2004; Miller *et al.*, 1980). The activity was measured by means of HPLC, following the protocol of Krause *et al.* (2006) with a few slight modifications to fit with our HPLC machine. The activity of FN3K was measured following the formation of a product, BzGpFruK (N $\alpha$ -hippuryl-N $\epsilon$ -(3-phosphofructosyl)-lysine) from the substrate, BzGFruK (N $\alpha$ -hippuryl-N $\epsilon$ -fructosyl-lysine). This assay is based on FN3K-dependent conversion of the synthetic UV-active fructosamine N $\alpha$ -hippuryl-N $\epsilon$ -(1-deoxy-Dfructosyl) lysine (BzGFruK) to N $\alpha$ -hippuryl-N $\epsilon$ -(phosphofructosyl) lysine (BzGpFruK). The assay was performed on lysates of erythrocytes collected from diabetic patients exhibiting strongly positive and negative G-Gaps (Krause *et al.*, 2006). The chemicals and parts required for this assay were ordered according to Krause *et al.*, (2006).

Chemicals used for preparation of hypotonic lysis buffer which used for lysis of erythrocytes, were purchased from Sigma-Aldrich Company Ltd. Dorset, England (for more details about manufacturers of these chemicals see Appendix I):

- HEPES Buffer (pH=7.5): (5 mM 4-(2-hydroxyethyl)-piperazine-1-ethanesulfonic acid)
- Dithiothreitol (DTT): 1 Mm (HPLC grade)

- Leupeptin hemisulfate salt (1 µg/ml) (HPLC grade)
- Antipain (1 µg/ml) (HPLC grade)

Consumables needed to prepare the HEPES-buffered erythrocyte lysate were sodium chloride powder.

Chemicals required for final preparation of the product (N $\alpha$ -hippuryl-N $\epsilon$ -(3-phosphofructosyl)-lysine (BzGpFruK) were

- 25 µL of 22 mM ATP solution
- 170 mM of sodium chloride
- 10% (w/v) trichloroacetic acid (diluted in H<sub>2</sub>O).
- The substrate, N $\alpha$ -hippuryl-N $\epsilon$ -fructosyl-lysine, was gift from Dr Anne Hellwig from the Institute of Food Chemistry (Technische Universität Dresden, Dresden, Germany).

Two mobile phases were used to run the HPLC system: mobile phase A, a 0.02M ammonium acetate buffer at pH 6.5 and mobile phase B, methanol (HPLC gradient, both purchased from Sigma-Aldrich Company Ltd. Dorset, England). A stainless-steel column from Knauer Eurospher (Aberdeen, UK) was used to separate out the product, BzGpFruK.)

A stainless-steel column from Knauer Eurospher (Aberdeen, UK) was used to separate out the product, BzGpFruK: a Vertex Plus Column (250 x 4.6 mm; Eurospher II 100-5 C18A) was filled RP18 material of 5 µm particle size, with integrated pre-column (5 mm x 4 mm) filled with the same material.

The injection volume of the prepared sample was 25µl rather than 50 µl as in Kraus et al., (2006) as a different injection loop was used (Rheodyne/Loops/8125 / 8126 25µl SS Loop for 8125/8126 (EA) from Kinesis Company). A MBI Refrigerated Centrifuge (max 24 x 2.0 MI, Montreal, Biotech, Inc), and a micro Centrifuge (PT. Nutrilab Pratama, Indonesia) were also used.

#### 2.1.2.1.1 Preparation of HEPES-buffered Erythrocyte Lysate

This assay was set up with advice from Dr Anne Hellwig (Institute of Food Chemistry, Technische Universität Dresden, Dresden, Germany). The was as follows:

1. Preparation of erythrocyte lysate: the lysis process was performed according to Krause *et al.*, (2006) and Hellwig *et al.*, (2014) with some minor modifications. From each sample, 0.2 ml of erythrocyte isolate was mixed with 0.8ml of hypotonic lysis buffer, consisting of (5 mM 4-(2-hydroxyethyl)-piperazine-1-ethanesulfonic acid (HEPES), pH=7.5, 1 mM dithiothreitol, 1 µg/ml leupeptin, and 1 µg/ml antipain). This was stored for 10 minutes in ice. The mixed solution was centrifuged for 10 minutes at 4°C at a RCF 4500g; the membrane pellet was then discarded and the supernatant was then diluted 1:1 (v/v) with 200mM HEPES buffer (pH=7.5) containing 170 mM NaCl, resulting in HEPES-buffered erythrocyte lysate.
2. To assay FN3K activity, a 500-µL aliquot of HEPES-buffered erythrocyte lysate was mixed with 25 µL of 22mM ATP solution + 25 µL BzGFruK (substrate) solution (11 mM in HEPES buffer). Then the resulting mixture (550 µL) was incubated in a water bath at 37°C for 120 minutes with its final concentration being 0.5 mM BzGFruK, 2 mM ATP, erythrocyte dilution 1:11. This reaction was stopped by adding 550 µL 10% trichloroacetic acid to the same volume of the incubated sample. After

vigorous shaking, the samples were stored for one hour in the fridge and then centrifuged (3000 g, 20°C, 10 min), and then the supernatant was membrane-filtered using a regenerated cellulose filter of 0.45 µm. After that 0.25 µl of the prepared sample was then injected immediately into the HPLC system.

Separation of the assay products (including BzGpFruK) was performed by using PerkinElmer Series 200 HPLC Systems UV/Vis Detector. This incorporated a stainless-steel column (250X4.6 mm) filled with Knauer Eurospher 100, RP18 material of 5-µm particle size, with integrated guard column 5×4 mm filled with the same material purchased from (Knauer UK).

The mobile phase A used 0.02 M ammonium acetate buffer, pH=6.5 and the mobile phase was 100% methanol. The injected volume was 25 µL, column temperature was set to 20 °C and ultraviolet detection was performed at  $\lambda=230$  nm.

The elution gradient programme was set as in the two following tables (2.1.2.1.1a and 2.1.2.1.1b) for both the product and the substrate (as standard), following recommendations from Dr Ann Hellwig (personal communication):

Table 2.1.2.1.1a: shows gradient Programme for BzG-phospho-FruK (Product) according to Krause *et al.*, (2006).

<b>Time (minute)</b>	<b>Mobile phase B %</b>	<b>Flow rate (ml/min)</b>
0	5	0.5
0-15	5	0.5
15-15.1	5	0.3
15.1-37	11	0.3
37-37.1	11	0.5
37.1-40	80	0.5
4--45	80	0.5
45-48	5	0.5
48-57	5	0.5

Table 2.1.2.1.1 b: Gradient Program for BzGFruK (substrate and calibrant) according to Krause *et al.*, (2006), with 250 mm x 4.6 mm column: with same eluents, peak of BzGFruK at 14.5 minute.

<b>Time (minute)</b>	<b>Mobile phase B (%)</b>	<b>Flow rate (mL/min)</b>
<b>0</b>	14	0.5
<b>0-15</b>	14	0.5
<b>15-15.1</b>	14	0.3
<b>15.1-37</b>	20	0.3
<b>37-37.1</b>	20	0.5
<b>37.1-40</b>	80	0.5
<b>40-45</b>	80	0.5
<b>45-48</b>	14	0.5
<b>48-57</b>	14	0.5

### 2.1.2.1.2 Determination of Haemoglobin

According to Krause *et al.*, (2006), the activity of FN3K enzyme is usually expressed as (mU/g) of haemoglobin, in which each 1 unit (U) represents the amount of FN3K enzyme that catalysed the formation of 1  $\mu\text{mol}$  BzGpFruK/min. To know the amount of haemoglobin into each sample, we measured the amount of haemoglobin using the procedure described by Oshiru *et al.*, (1980) with slight modifications as recommended by Dr James Vickers (Diabetes research group, University of Wolverhampton). The equipment and reagents used were:

- Spectrophotometer (Model: SB038, Cadex, Quebec, Canada) available at School of Biomedical science, University of Wolverhampton
- SLS buffer (700 mg sodium dodecyl sulphate, 1 ml Triton X-100 in 1 litre 0.033 M phosphate buffer)
- Haemoglobin standard (a gift from Dr James Vickers)
- Sample: Blood or erythrocyte isolate

The procedure can be summarized as follows. Two tubes were labelled for each of the following standard concentrations of haemoglobin, and two tubes for each unknown (patient) sample. The standards were prepared by using a stock solution of  $200 \text{ mg ml}^{-1}$  haemoglobin:

Tube number	Concentration of Hb (mgml <sup>-1</sup> )	Volume Hb stock (ml)	Volume diluent (SLS buffer, ml)
1	200 mgml <sup>-1</sup>	4 ml	0 ml
2	150 mgml <sup>-1</sup>	3 ml	1 ml
3	100 mgml <sup>-1</sup>	2 ml	2 ml
4	50 mgml <sup>-1</sup>	1 ml	3 ml
5	0 mgml <sup>-1</sup>	0 ml	4 ml

Each patient sample was prepared by adding 20 µl blood (erythrocyte isolate) to 4 ml diluent (SLS buffer). The tube was stopped and inverted gently several times to mix and left for 5 mins. The absorbance was read at 540 nm against a reagent blank in the spectrophotometer.

A standard curve for haemoglobin measurements was plotted from the mean of duplicate measurements. The absorbance of the patient sample was measured (in duplicate) and the haemoglobin concentration was read from the curve (figure 3.1.1b).

#### **2.1.2.2 Study 1, part 2: Measurement of FN3K levels in Human Erythrocytes**

In this part of the study, FN3K enzyme protein levels were measured in human erythrocytes; complementary to part 1 of this study. The FN3K assay was performed by using FN3K sandwich ELISA Kit (SEJ094Hu) manufactured by Cloud-Clone Corp/USA, purchased from (Hölzel Diagnostika/50672 Cologne-Germany). The procedure can be summarised as follows.

This ELISA assay was a sandwich ELISA; a series of five ELISAs were run according to the protocol supplied. All reagents, wash buffer, reagent diluents were

supplied and the microtiter plate provided in this kit had been pre-coated with an antibody specific for the FN3K enzyme.

The three reagents supplied in the kit, detection Reagent A (Detection Reagent A: biotin-conjugated antibody), detection antibody B (Avidin conjugated to Horseradish Peroxidase), and the standard (recombinant human FN3K), were all reconstituted and stored according to the instructions in the manual and were used within 60 days (storage time limit as instructed).

Eight standard solutions of recombinant human FN3K were made via a serial dilution: 10 ng/ml, 5ng/ml, 2.5 ng/ml, 1.25 ng/ml, 0.625 ng/ml, 0.312 ng/ml, 0.156 ng/ml and 0ng/ml, whereas samples were diluted 1:5 with the sample diluent provided. The standards and samples were prepared 15 minutes before running the experiment. Standard and samples were added into the appropriate wells and the plate was covered for two hours and incubated at 37°C. Then the diluted reagent A was added and the plate was incubated for one hour at 37°C. The plate was then washed, using a multi-channel pipette to aspirate each well three times with 350 µl of wash buffer (WB) that was supplied with the kit. 100µL of Detection B working solution was added into each well and the plate was incubated at 37°C for 30 min. After a second wash 90µL of Substrate Solution was added to each well and the plate was covered and incubated for 20 minutes at 37°C. During this incubation, TMB is oxidised to a dimine and the H<sub>2</sub>O<sub>2</sub> reduced to water, catalysed by Detection B, the substrate TMB dimine gives a blue colour which is a measure of the amount of FN3K antibody. The reaction was then stopped by adding the stop solution which immediately turned the mixture yellow.

Using a Thermo Scientific Multiscan Ascent micro-plate reader (Thermo-Fisher Scientific Inc, Illinois, USA) along with Thermo Scientific™ Ascent™ software (Thermo-Fisher Scientific Inc, Illinois, USA), the optical density of the yellow solution in each well of the microplate was measured at a wavelength of 450nm.

The sample concentration for FN3K in erythrocytes was calculated by using the standards with known concentration to draw a curve of best fit using the 4 Parameter Logistic nonlinear regression model on Microsoft Office Excel 2013. The standard curve was used to interpolate the concentrations of the samples, using the equation of the line of best fit (figure 3.1.2a).

#### **2.1.2.3 Study 1, part 3: Measuring of FN3K levels in Human Plasma**

As described in section 1.2.1, plasma fructosamine is a measure of the glycated fraction of all plasma proteins. The predominant glycated protein of extracellular fructosamines in the circulation is glycated albumin, while HbA1c is the predominant intracellular fructosamine product of glycation (Cohen *et al.*, 2003). According to Szwegold (2007) deglycation of glycated proteins can arise with specific enzymes occurring naturally to phosphorylate fructosamine residues (fructosamine-6-phosphates), which in turn are removed from the circulation by destabilizing them. A key enzyme is FN3K with its proposed deglycating role. The FN3K enzyme was reported by several studies to be existed intracellularly in human erythrocytes with its deglycating action on HbA1c (Delpierre, *et al.*, 2002 and Delpierre & Van Schaftingen, 2003). Therefore, this part of the study aimed to investigate whether the FN3K enzyme is present in plasma and if it can deglycate glycated proteins in plasma such as glycated albumin.

The FN3K assay was performed using FN3K sandwich ELISA Kit (CSB-EL008760HU) (Hözel Diagnostika-50672 – Cologne, Germany). The procedure can be summarised as follows:

This ELISA assay was a sandwich ELISA, a series of seven ELISAs were run, all reagents, wash buffer, reagent diluents were supplied, and the microtiter plate provided in this kit has been pre-coated with an antibody specific for the FN3K enzyme. The three reagents supplied in the kit (biotin antibody, HRP-avidin, standard - recombinant human FN3K) were all reconstituted and stored according to the instructions in the manual and were used within 60 days (storage time limit as instructed).

Eight standard solutions of recombinant human FN3K were made via a serial dilution; 20 ng/ml, 10ng/ml, 5ng/ml, 2.5 ng/ml, 1.25 ng/ml, 0.625 ng/ml, 0.312 ng/ml and 0ng/ml whereas samples were diluted 1:4 with the sample diluent provided inside the kit box. The standards and samples were prepared 15 minutes before running the experiment. Standards and samples were added to the appropriate wells and the plate was covered for two hours at 37°C. Then the liquid was removed from each well, the Biotin-antibody added and the plate was incubated for one hour at 37°C. The plate was then washed by using a multi-channel pipette to aspirate each well three times with 200 µl of the wash buffer (WB) supplied in the kit. 100µL of HRP-avidin (1x) working solution was added to each well and the plate was incubated at 37°C for 60 min. After additional washing, 90µL Substrate Solution was added to each well and the plate was covered and incubated for 20 minutes at 37°C in an incubator. During this incubation, the substrate is oxidised to a dimine and the H<sub>2</sub>O<sub>2</sub> reduced to water, catalysed by detection B, the substrate TMB dimine gives the solution its blue colour which is quantitative to the amount of FN3K antibody. Once

the liquid turned into blue colour by the addition of substrate the reaction was stopped by adding the stop solution (50  $\mu$ l) the blue colour was turned into yellow colour at once.

By using a Thermo Scientific Multiscan Ascent micro-plate reader (Thermo-Fisher Scientific Inc, Illinois, USA) along with Thermo Scientific™ Ascent™ software (Thermo-Fisher Scientific Inc, Illinois, USA), the optical density of the yellow solution in each well of the microplate was measured at a wavelength of 450nm first then at 540nm. The reading at 540 nm was subtracted from the reading at 450nm to correct for optical imperfections in the plate.

The sample concentration was calculated by using the standards with known concentration to draw a curve of best fit using the 4 Parameter Logistic nonlinear regression model on Microsoft Office Excel 2013. The standard curve was used to obtain the concentrations of the samples were calculated using the equation of the line of best fit (figure 3.1.4a).

#### **2.1.2.4 Study 1, part 4: Measuring of AGEs in Human Plasma**

Several methods may be adopted for measuring levels of AGEs. These are:

1. ELISAs: these can be run either by using monoclonal antibodies or polyclonal antibodies (Vlassara *et al.*, 2002).
2. Fluorescence spectroscopy dependent on the fluorescence characteristics of AGEs (Meerwaldt *et al.*, 2004).
3. HPLC or the most reliable forms of mass spectrometry (Mendez *et al.*, 2010).

We chose to measure plasma levels of AGEs by ELISA since it is simple and fast.

The assay was performed by using OxiSelect™ Advanced Glycation End Product (AGE) Competitive ELISA Kit (STA-817) which was purchased from Cambridge Bioscience Ltd. This assay was carried out according to the contained protocol supplied with the kit. This ELISA assay was a sandwich ELISA.

A series of eight ELISAs was run. The six reagents supplied in the kit; AGE-BSA standard, AGE-conjugate, 100X Conjugate Diluent, anti-AGE antibody (1000X) and the assay diluent were all reconstituted and stored and were used within 60 days (storage time limit as instructed). Ten standard solutions of recombinant human FN3K were made via a serial dilution; (100 µg/ml, 50 µg/ml, 25 µg/ml, 12.5 µg/ml, 6.25 µg/ml, 3.13 µg/ml, 1.56 µg/ml, 0.78 µg/ml, 0.39 µg/ml and 0 µg/ml) and samples were diluted 1:5 with the assay diluent provided in the kit. The plate was coated a day before running the assay with a 1:1 mixture of AGE Conjugate and 1X Conjugate Diluent by adding 100 µl of this mixture to each assigned well of the plate: after this, the mixture is referred to as the AGE conjugate.

The next day, the AGE-conjugate was removed and each well was washed with a PBS 1X Solution. Then 200 µL of Assay Diluent was added to each well to block the wells for 1 hr in the fridge. The assay diluent was then removed without washing. Standards and samples were prepared 15 minutes before running the experiment. Then they were added into the appropriate wells and the plate was covered and incubated on an orbital shaker for 10 minutes at 37°C. 50 µl of the

diluted anti-AGE antibody was added to each well and the plate was incubated for a second time for one hour.

The plate was then washed three times with wash buffer by adding 200 µL of the reconstituted wash buffer. 100 µL of secondary antibody-HRP conjugate was added to each well and the plate was incubated for one hour on an orbital shaker. After repeating the washing step again, 100 µl substrate was added to each well and the plate was incubated for 20 minutes on an orbital shaker at room temperature. 50 µl of stop solution was then added to each well, turning the solution yellow and the optical density was measured by using the same plate reader as in previous experiments, at a wavelength of 450nm. The sample concentration was calculated by using the standards with known concentration to draw a curve of best fit using the 4 Parameter Logistic nonlinear regression model on Microsoft Office Excel 2013. The standard curve was then used to obtain the concentrations of the samples were calculated using the equation of the line of best fit (figure 3.1.5a).

#### **2.1.2.5 Study 1, part 5: Measuring of Markers of Endothelial Dysfunction**

According to Nadar *et al.* (2004) and Muniyappa and Sowers (2013), indirect assessment of endothelial dysfunction is more reliable as it requires less patient contact, less complicated procedures and is relatively cheaper than direct assessment. We therefore chose to use the indirect approach to assess markers of endothelial dysfunction.

The thrombomodulin and E-selectin assays were performed by using sandwich ELISA kits purchased from R&D Systems, UK. These kits are Human Thrombomodulin/BDCA-3 DuoSet ELISA (catalogue number DY3947) and Human E-Selectin/CD62E DuoSet ELISA (Catalogue number DY724).

### 2.1.2.5.1 Thrombomodulin Assay

A series of seven ELISAs was carried out. The ELISA 96-well plate was incubated with 100µl of capture Ab for thrombomodulin at room temperature (RT) overnight. The plate was then washed by using a multi-channel pipette to aspirate each well three times with 400µl of wash buffer, followed by the addition of 300µl of reagent diluent (RD), comprised of 1% Bovine Serum Albumin (BSA) in PBS produced by dissolving 1g BSA (Sigma-Aldrich Co) in PBS. The plate was then incubated for 1.5 hours in an incubator. The samples were diluted 1:7 with the reagent diluent (RD) whilst seven standard solutions of recombinant human thrombomodulin were made via a serial dilution; 6000pg/ml, 3000pg/ml, 1500pg/ml, 750pg/ml, 375pg/ml, 187.5pg/ml and 0pg/ml in RD. The plate was then washed and 100µl of the standards and samples loaded into the appropriate wells in duplicate. After a further two hour incubation, the microplate was washed again before the detection Ab was diluted to its working concentration and added to each well and the plate incubated for another two hours. The washing process was then repeated. The Streptavidin conjugated HRP was diluted 1:100 in RD and 100µl added to each well and incubated for 20 minutes in an incubator. The washing process was repeated and a mixture of H<sub>2</sub>O<sub>2</sub> and TMB was added to the microplate. The plate was incubated for 20 minutes out of direct light. After the liquid in each well turned blue 50µl of a blocking solution of 2N HCL was added to each well to turn the colour of the liquid in each well yellow. Finally, the microplate was transferred to a microplate reader and the reading was measured at 450nm as described previously.

#### **2.1.2.5.2 E-selectin Assay**

A series of seven ELISAs was carried out as per the protocol supplied with the kit. The samples were diluted 1:7 with RD whilst seven standard solutions of recombinant human E-selectin were made via a serial dilution; 6000pg/ml, 3000pg/ml, 1500pg/ml, 750pg/ml, 375pg/ml, 187.5pg/ml and 0pg/ml in RD. The E-Selectin ELISA assay was performed as for the thrombomodulin assay in 2.1.2.5.1, with the kit-supplied standard solutions of E-selectin and E-selectin-detection antibody instead of thrombomodulin standard solutions and thrombomodulin-detection antibody. The microplate was transferred to a microplate reader and the optical density for E-selectin was measured at 450nm.

#### **2.1.2.6 Study 1, part 6: Measuring of Markers of Thrombosis (PAI-1 assay)**

This assay used a sandwich ELISA kit that was purchased from R&D Systems, UK: Human Serpin E1/PAI-1 DuoSet ELISA, DY1786.

A series of seven ELISAs was carried out to cover the experiment samples. The ELISA 96-well plate was incubated with 100µl of capture Ab at room temperature (RT) overnight. The plate was then washed by using a multi-channel pipette to aspirate each well three times with 400µl of wash buffer, followed by the addition of 300µl of reagent diluent (RD) (1% Bovine Serum Albumin (BSA) in PBS produced by dissolving 1g BSA (Sigma-Aldrich Co) in PBS). The plate was incubated for 1.5 hours. The samples were diluted 1:8 with RD whilst seven standard solutions of recombinant human PAI-1 were made via a serial dilution; 20 ng/ml, 10ng/ml, 5ng/ml, 2.5ng/ml, 1.25 ng/ml, 0.625ng/ml and 0.3125ng/ml (RD). The plate was then washed and 100µl of the standards and samples loaded into the appropriate wells in duplicate. After two hours of incubation, the microplate was washed again before the detection Ab was diluted to its working concentration with

normal goat serum and added to each well. The plate was incubated for a further two hours. The washing process was repeated and the Streptavidin conjugated HRP diluted 1:100 in RD and 100µl added to each well. After 20 minutes of incubation, the washing process was repeated and a mixture of H<sub>2</sub>O<sub>2</sub> and TMB was added to the microplate. The plate was incubated for 20 minutes out of direct light; after the colour of the liquid in each well turned to blue 50µl of a blocking solution of 2N HCL was added to each well to turn the colour of the liquid of each well yellow. Finally, the microplate was transferred to a microplate reader and the optical density measured at 450nm.

### 2.1.3 Study 1 Materials and Equipment

Materials and equipment used in each part of this study are:

1. Study 1, part1: Measurement of FN3K activity in human erythrocytes. As mentioned in section 2.1.2.1, the activity was measured by means of an HPLC system. Assays of this nature were conducted in the University of Wolverhampton Faculty of Science and Engineering, using a PerkinElmer Series 200 HPLC Systems UV/Vis Detector, MBI Refrigerated Centrifuge (max 24 x 2.0 mL), MiniMouse II Microcentrifuge (Cat #: C0801-NR), Red lid and FISHER SCIENTIFIC 220 DIGITAL WATER BATH.
2. Study 2, part 2: Measurement of FN3K levels in erythrocyte lysate. PBS solution (Phosphate buffered saline) 10x concentrated (P5493-1L) was used for all immunoassays in this study when required. It was purchased separately from (Sigma-Aldrich Company Ltd. Dorset, England). The optical density of the yellow solution inside each well of the assay plate was read by using a Thermo Scientific Multiscan Ascent micro-plate reader (Thermo-Fisher

Scientific Inc, Illinois, USA) along with Thermo Scientific™ Ascent™ software (Thermo-Fisher Scientific Inc, Illinois, USA), at a wavelength of 450nm. This microplate-reader was used in all immunoassays in this study.

3. Study 1, part 3: Measurement FN3K enzyme levels in human plasma. The optical density was read at wavelength of 450nm and 540nm to enable correction of any background readings. The corrected readings, reading at 450nm minus the reading at 540nm, were used to quantify the amount of FN3K in plasma samples.
  
4. Study 1, part 4: Measurement of AGEs in human plasma. The optical density was read at a wavelength of 450nm using the microplate reader described previously. This study requires an orbital shaker: the model used was a PST-60HL-4, Plate Shaker-Thermostat manufactured by Biosan Medical Biological research technologies, Riga, Latvia.
  
5. Study 1, part 5 and part 6: Measurements of markers of endothelial dysfunction and thrombosis. The following items were prepared separately:
  - Wash buffer, which was prepared by dissolving 0.05% Tween® 20 in PBS produced by diluting a 10x concentrated solution in distilled water. The Tween sachet was purchased from Salimetrics, Pennsylvania, USA.
  - The reagent diluent (RD) was prepared by dissolving 1% Bovine Serum Albumin (BSA) in PBS. The BSA which was purchased (Sigma-Aldrich Company Ltd. Dorset, England).

- The substrate solution was purchased from R&D Systems separately from the kits, catalogue number DY999 (as recommended by the kit manufacturer).
- The Stop solution was 1M H<sub>2</sub>SO<sub>4</sub>, prepared in our lab.
- Plate Sealers: ELISA Plate Sealers (Catalog # DY992) purchased from R&D Systems.
- Finally, the microplates were purchased from R&D systems (as recommended in the kit), R&D Systems (Catalogue # DY990).

All of microplates in these ELISA assays in study (1) were washed with wash buffer using a multi-channel pipette (purchased from Eppendorf UK Limited Eppendorf House-Stevenage).

#### **2.1.4 Statistical Analysis**

Statistical analyses of data were conducted using the statistical software, Graphpad Prism 6 (Graphpad software Inc., 2014; California, USA) to compare and analyse the difference between sample specimens with positive versus negative G-gaps.

According to an initial analysis performed, the data were not normally distributed, and so the appropriate test for the collected data was determined to be a non-parametric, two-tailed, unpaired test also known as the Mann-Whitney U test.

Results for this statistical test are considered significant when  $p \leq 0.05$  with 95% confidence interval.

## **2.2 Study 2: Measuring of FN3K levels in AECOPD patients treated with metformin and placebo**

### **2.2.1 Study Design**

A randomised double blind trial involving 23 patients with AECOPD and diabetes admitted to St George's Hospital (London) and treated with either metformin or placebo (16 treated with metformin and seven treated with placebo, Clinical trial # NCT01247870). Serum samples were collected at several different stages; upon entry (baseline), at discharge (7days after entry) and afterwards for follow up (29 days after discharge). These samples were immediately stored at -80°C.

### **2.2.2 Ethical Approval**

Ethical approval was obtained before samples were collected from St George's University Hospitals NHS Foundation Trust, London, UK.

### **2.2.3 Inclusion Criteria and Exclusion Criteria**

The patients recruited were required to be: patients with diabetes and COPD, smokers and healthy individuals. They were generally aged 40 and above with a well-documented record of their medication history and treatment.

Patients excluded from this study were those who were characterised as:

1. Individuals without diabetes and non-COPD patients;
2. Females who were pregnant at the time of the study ;
3. Those diagnosed with renal diseases or renal dysfunctions (e.g. as indicated by serum creatinine levels greater than, or equal to, a value of 1.5 mg/dL for males, and greater than or equal to 1.4 mg/dL for females).

Individuals with abnormal creatinine clearances which may result from conditions such as, cardiovascular collapse (shock), acute myocardial infarction, and septicaemia, were also excluded from the study;

4. Patients with a known hypersensitivity to metformin hydrochloride;
5. Those with acute or chronic metabolic acidosis, including diabetic ketoacidosis, with or without coma.

#### 2.2.4 Selection of Patients and Data Collection

The patients were recruited for a wider study into COPD exacerbations and Type 2 diabetes (study cohort n=130) who were admitted to St George's hospital, (London, UK) between 1998 and 2010 and assigned International Classification of Diseases codes (ICD-10) for both COPD (J41-J44) and Type 2 diabetes mellitus (E10, E11, E14). These patients were identified from clinical coding data with our study. From those 130 patients, 23 serum samples were collected for our study at each stage (Entry (baseline), discharge and follow up)). See Appendix II for patients' map.

Hitchings *et al.* (2014) stated that the extracted data were written and electronic case notes that related to the patients' co-morbidities, severity of illness, relevant investigations, treatment and survival. The quantification of comorbidity burden was achieved by utilising the Charlson comorbidity index and the severity of each patient's illness was gauged using the APACHE (Acute Physiology and Chronic Health Evaluation) II scores (Knaus *et al.*, 1985; Charlson *et al.*, 1985).

Glycated haemoglobin (HbA1c) levels were used to assess glycaemic control. This measurement was made daily for the 90 days of the index admission. The vital status and dates for each recorded death were obtained from the hospital electronic record system as a part of the national mortality data.

The follow-up period was identified as the time frame during which the patient was alive to record their last electronic review in September 2013.

Patients were categorised into one of two groups upon entry to the hospital, metformin or non-metformin treated patients, based on their recorded drug histories indicating the administration, or lack thereof, of this drug (Hitchings *et al.*, 2014).

When considering the number of patient deaths that occurred at the hospital, an inquiry was conducted to determine and analyse whether the prescription of metformin therapy during the last week leading to their decline was a contributing factor to their death. Death after discharge from the hospital resulted in group assignment that was based on whether metformin was prescribed according to records of the patient's prescription letter, written at the time of their discharge.

### **2.2.5 Consent Forms and Information Sheets**

According to Hitchings *et al.* (2014), informed consent was not sought, as the data were collected in parallel with an audit of prescribing practices. In addition, the National Information Governance confirmed that support under Section 251 of the NHS (National Health Service/UK) Act 2006 was not required (accessing the identified data without consent). However, the South West London Research Ethics Committee provided a favourable opinion for the study.

### **2.2.6 Samples**

As stated by Hitchings *et al.*, (2014), all patients were informed of the dates for which blood samples would be collected and that venepuncture would be used. After the collection of blood samples, the blood was left to clot by leaving it undisturbed at room temperature for approximately 20 minutes. The clot was removed by centrifuging at 2000xg for 10 minutes in a refrigerated centrifuge. The resulting

supernatant was designated as a serum. The liquid serum was transferred into a clean polypropylene tube using a Pasteur pipette and the samples were maintained at 2-8°C during the handling. The serum was apportioned into aliquots and stored at -20°C for performing assays on them later at St George's University hospital. For this study, the 32 samples of serum which were collected from patients at all three stages mentioned in section 2.2.1 were received from Prof Emma Baker and Dr Andrew Hitchings from St George's University Hospital in collaboration with Prof Paul Kirkham of the University of Wolverhampton. These samples were subjected to ELISA assays for measuring expression of FN3K.

### **2.2.7 Study (2) Materials and Equipment**

The FN3K assay was performed by using FN3K sandwich ELISA Kit (CSB-EL008760HU) manufactured by Cusabio Corp, purchased from: Hölzel Diagnostika-50672 Cologne/Germany. This assay was performed for serum samples obtained from St George's Hospital following the manufacturer's recommended guidelines as stated in the protocol that accompanied the kit.

This was a sandwich ELISA. A series of five ELISAs was run and the microtiter plate provided in this kit had been pre-coated with an antibody specific to the FN3K enzyme. Eight standard solutions of recombinant human FN3K were made via a serial dilution; 20 ng/ml, 10ng/ml, 5ng/ml, 2.5 ng/ml, 1.25 ng/ml, 0.625 ng/ml, 0.312 ng/ml and 0ng/ml whereas samples were diluted 1:5 with the sample diluent provided inside the kit box. The standards and samples were prepared 15 minutes before running the experiment. Standard and samples were added into the appropriate wells and the plate was covered for 2 hours at 37°C. Then the liquid was removed from each well and the Biotin-antibody was added and the plate was

incubated for 1 hour at 37°C. The plate was then washed using a multi-channel pipette to aspirate each well 3 times with 200 µl of wash buffer (WB) which supplied inside the kit. 100µL of HRP-avidin (1x) working solution was added into each well and the plate was incubated at 37°C for 60 min. Once washing was repeated, 90µL of Substrate Solution was added to each well and the plate was covered and incubated for 20 minutes at 37°C in an incubator. During this incubation, the substrate (TMB) is oxidised to a dimine and the H<sub>2</sub>O<sub>2</sub> reduced to water. Catalysed by detection B, the substrate TMB dimine gives the solution its blue colour which is quantitative to the amount of FN3K antibody. Once the liquid turned blue by the addition of the substrate the reaction was stopped by adding 50 µl of the stop solution. The blue colour was turned yellow at once.

The optical density of the yellow solution in each well of the microplate was measured by microplate reader at a wavelength of 450nm first then at 540nm. The reading at 540 nm was subtracted from the reading at 450nm to correct for optical imperfections in the plate.

The FN3K concentration of each sample was calculated by using the standards with known concentration to draw a curve of best fit using the 4 Parameter Logistic nonlinear regression model on Microsoft Office Excel 2013. The standard curve was used to obtain the concentrations of the samples were calculated using the equation of the line of best fit (graph 5.3.1).

### **2.2.8 Statistical Analysis**

The statistical analyses of the data were performed using Graphpad Prism 6 software (Graphpad software Inc., USA, 2014) to analyse differences in FN3K expression in serum samples for those patients who were treated with metformin at

each stage (entry, discharge and follow-up) and with patients who were treated with placebos at each of the three stages. According to the data collected for the initial analysis, FN3K expressions were not normally distributed. As a result, it was decided that the appropriate test for analysing the data collected would be the same Mann-Whitney U test described in section 2.1.4.

## **2.3 Study 3: Measurement of FN3K expression in human peripheral lung tissue**

### **2.3.1.1 Study design**

A double-blind study involving 36 human peripheral lung samples from healthy individuals, asymptomatic smokers and stable (GOLD 2) COPD patients were assessed for FN3K expression by means of immunohistochemistry on paraffin fixed lung tissue biopsies obtained following lung resection surgery.

### **2.3.1.2 Ethical committee approval**

Subjects were recruited from the Section of Respiratory Medicine of the University Hospital of Ferrara, Italy, with approval by the local Ethics Committee.

### **2.3.1.3 Inclusion and exclusion criteria**

Patients recruited were selected on the basis that they were patients living with diabetes and COPD, were smokers and cleared as healthy individuals, with their medication history and treatment available on record. Those patients that were excluded from this study were either:

1. Individuals negative for diabetes or non-COPD patients;
2. Pregnant females;

None of the participants had received glucocorticoids, theophylline, antibiotics or antioxidants within the month preceding surgery or bronchodilators within the previous 48 hours. Patients had no history of asthma or other allergic diseases. Former smokers were classed as those who had not smoked for more than one year. Each patient had a medical history taken and received a physical examination, chest radiography, electrocardiogram, routine blood tests, and pulmonary function tests during the week prior to surgery.

#### **2.3.1.4 Patients Consent**

Tissue samples were acquired after written informed consent to thoracic surgery was obtained and pulmonary and pulmonary function tests were performed according to Marwick *et al.*, (2009). All patients were informed about their right to withdraw from the study at any time without giving a reason.

#### **2.3.1.5 Samples**

COPD was defined according to international guidelines (post-bronchodilator FEV1/FVC ratio < 70%), and the severity of COPD was classed according to current Global Initiative for Chronic Obstructive Lung Disease (GOLD) criteria ([www.goldcopd.org](http://www.goldcopd.org)). All patients were stable at the time of the study and were free from acute exacerbations of symptoms and from upper respiratory tract infections in the 2 months preceding the study.

Twenty subjects undergoing lung resection surgery for a solitary peripheral neoplasm were recruited. A further 20 subjects who were lifelong non-smokers with normal lung function 20 who were smokers with normal lung function and 16 subjects who were smokers with COPD were also recruited. All former smokers had stopped smoking for more than one year. None of the subjects underwent

preoperative chemotherapy and/or radiotherapy and none had been treated with bronchodilators, theophylline, antibiotics, antioxidants and/or glucocorticoids in the month prior to surgery. Lung tissue processing was performed as previously described (Kirkham, 2011). Two to four randomly selected tissue blocks were taken from the sub pleural parenchyma of the lobe obtained at surgery, avoiding areas grossly invaded by tumour. Samples were fixed in 4% formaldehyde in phosphate-buffered saline at pH 7.2 and, after dehydration, embedded in paraffin wax. Serial sections 4 µm thick were first cut and stained with haematoxylin-eosin (H&E) in order to visualize the morphology and to exclude the presence of microscopically evident tumour infiltration. Tissue specimens were then cut for immunohistochemical analysis and were placed on charged slides as previously reported (Kirkham *et al.*, 2011).

After meeting inclusion criteria, the total number of patients who were recruited was 36. (Table 2.3.1.5) shows the characteristics of recruited patients as follows:

Table 2.3.1.5: shows characteristics of subjects for the IHC studies for FN3K.

Subjects	n	Age	Sex	Smoking history	Pack-years	Chronic bronchitis	FEV <sub>1</sub> %pred	FEV <sub>1</sub> /FVC%
Non-smokers	12	68.6 (2.3)	5M/7F	-----	-----	0	108.5 (5.0)	78.2 (1.4)
Smokers	12	69.8 (2.6)	9M/3F	11 Ex  1 Current	30.8(8.4)	1	100.6(6.2)	77.1(1.4)
COPD	12	71.3(1.5)	12M	9 Ex  3 Current	41.2(4.4)	4	67.4(4.5)	54.6(2.7)

According to Table 2.3.1.5, the number of participants is equal among each group of non-smokers, smokers & COPDs, although there were more males than females (M: male subjects; F: female subjects).

### 2.3.1.6 Materials and Equipment Used for Study 3

In order to carry out this assay we collaborated with Prof Gaetano Caramori at University of Ferrara, Italy. This assay was carried out at in CEMICEF, University of Ferrara, Italy laboratories. The prepared 36 slides were assayed using Immunohistochemistry (IHC) techniques.

Reagents and kits purchased:

1. FN3K Antibody (N-term) Catalogue (T1993): Rabbit Polyclonal Antibody from Epitomics - an Abcam Company, 863 Mitten Road, Suite 103 Burlingame, California 94010-1303.
2. FN3K Antibody (N-term), source from rabbit, catalogue (AP7083a), was purchased from Abgent, Maidenhead, Berkshire, UK.
3. Biotinylated Goat Anti-Rabbit IgG Antibody Catalogue Number: BA-1000, purchased from: Vector Laboratories Ltd, Peterborough, United Kingdom.
4. Vectastatin Elite ABC Kit (Rabbit IgG), Catalogue Number: PK-6101, purchased from: Vector Laboratories Ltd, Peterborough, United Kingdom.
5. Normal rabbit IgG: sc-2027, purchased from: Santa Cruz Biotechnology Inc, Bergheimer, Heidelberg, Germany.

The immunohistochemistry microscope for parts of study was an OLYMPUS BX 60-NRI-MCDP, at the University of Ferrara, Italy.

The procedure for detection of FN3K expression in human lung slides, the appropriate dilution was obtained after several trials which are summarised in table 2.3.1.6.

The slides were rinsed with Xylene for 5 minutes then with 100% ethanol for 3 minutes, then 95% ethanol for 3 minutes. After that they were transferred into a glass slide holder, and the rest of the rack was filled with empty slides to ensure even heating. Then the rack was placed in 600ml of 10mM sodium citrate (PH-6) in a glass beaker and the beaker then was put inside a microwave for 20 minutes (unmasking procedure). After cooling the slides for 20 minutes the slide were

washed four times with distilled water for 3 minutes and four times with 1x PBS for 3 minutes. After washing the slides were blocked with peroxidase for 15 minutes and then the wash process was repeated according to the protocol. The slides were immersed in a dish containing blocking buffer (serum from rabbit of secondary antibody to be used, diluted 1:10 in PBS), followed by an incubation step at 37°C for 20 minutes. The tissue section was covered with primary AB diluted in blocking buffer (1:300). The slides were then incubated for one hour at 37°C. The excess liquid was then blotted from slides and they were rinsed three times in PBS for 5 minutes each wash.

The tissue was covered with secondary antibody diluted in blocking buffer according to the protocol instructions, then they were incubated at 37°C for 30 minutes. The slides were then washed with PBS + Saponin for 5 minutes. After that the counterstaining step with Chromogen (DAB) was applied, and the slides were rinsed with tap water. After blotting the excess water from the slides, one drop of mounting medium was added to each slide and a coverslip applied. Then they were transferred to the microscope and the first measure was with 10x then 40x lenses.

Table 2.3.1.6 Summary of the immunohistochemical procedures used to detect FN3K in human lung.

DAB: 3, 3'-diaminobenzidine tetra hydrochloride.

Antibody specificity	Company Manufacturer	Catalogue code	Source/host	Dilution	Unmasking procedure	Secondary antibody and amplification step	Chromogen	Positive control
FN3K	Abgent Co.	AP7083a	Rabbit	1:300 (human lung)	Microwave, 10 mM citrate, pH 6	Vectastain Elite kit	DAB	Human bronchial rings (for human lung)

### 2.3.1.7 Statistical Analysis

The statistical analysis of our data was performed by using Graph-pad prism 6 (Graphpad software Inc., California, USA, 2014) to analyse the difference in FN3K expression in serum among healthy individuals, smokers and COPD patients. The initial data analyses were not normally distributed. Therefore, the Mann-Whitney U statistical test was used. The statistical test would be significant when  $p \leq 0.05$ , using the 95% confidence interval.

## **Chapter 3**

### **Study 1**

**FN3K (activity and protein levels) in relation to G-gap and AGEs in patients with diabetes**

### 3.1 Results of Study 1, Parts 1, 2, 3 and 4

#### 3.1.1 Results for Study 1, Part 1: FN3K enzyme activity in relation to glycation gap (G-gap)

According to Krause et al. (2006), the retention time for the product (BzG-phospho-FruK) was around 18 minutes using the specified column (250 mm x 4.6 mm), and with our settings the retention time for the product was 18.5 minutes. The substrate (BzGFruK) retention time appeared to be 26 minutes.

By calculating the area under the curve (AUC) of the eluted product at the specific retention time, the concentration of the product was obtained in relation to the known concentration of the standard (the substrate, BzGFruK). A calibration curve was drawn using Microsoft Excel (2013) for of BzGFruK, ranging from 6 to 100  $\mu\text{M}$ , and was used for quantification of formed BzGpFruK (Figure 3.1.1a).



Figure 3.1.1a: Standard curve for BzGFruk

After determining the concentration of BzGPFruk in each sample from the equation shown in Figure 3.1.1a, the activity of the enzyme FN3K was quantified

according to the method of Krause et al. (2006). FN3K activity was expressed as mU/g haemoglobin (Hb).

The amount of Hb (g) in each sample was measured according to the method of Oshiru et al. (1980) (see Section 2.1.2.1.2). The Hb in each sample was quantified in relation to the known concentration of the standard Hb and the standard curve was drawn (Figure 3.1.1b).

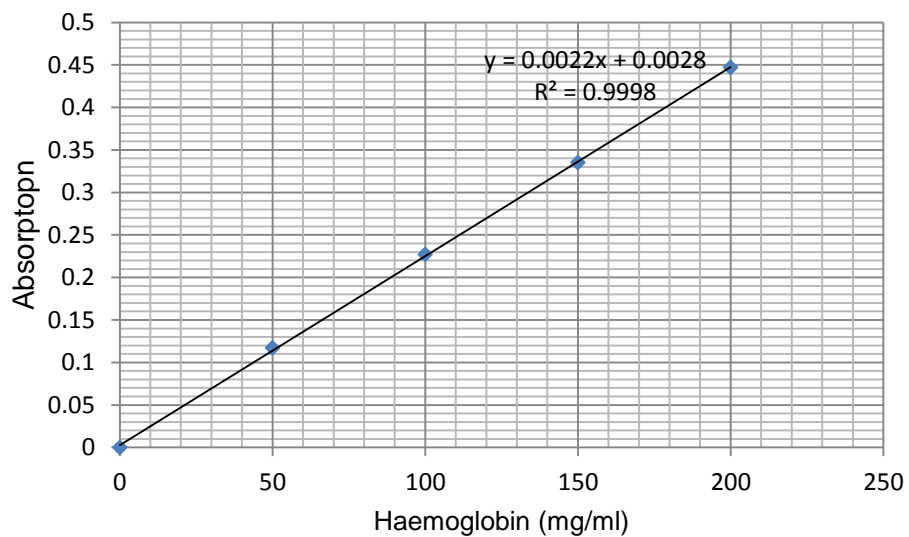


Figure 3.1.1b: Haemoglobin standard curve

The concentration of the product, BzGpFruk, was measured in  $\mu\text{M}$ . Each unit was defined as the amount of FN3K enzyme needed to catalyse the formation of 1  $\mu\text{mol}$  BzGpFruK/min. First, the concentration of the product was changed from  $\mu\text{M}$  to mU as follows: 1  $\mu\text{M}$  (equal to 1  $\mu\text{mol/L}$ ) was converted to  $\mu\text{mol}/\mu\text{L}$  (as our samples

sizes were in  $\mu\text{L}$ ) by dividing by 1,000,000. This was, in turn, converted to  $\mu\text{mol}$  by multiplying the value by the volume of the sample (200  $\mu\text{L}$ ). The result was the amount in  $\mu\text{mol}$  for each sample, thus the value was divided by 120 minutes (the incubation time) to obtain 1 unit ( $\mu\text{mol}/\text{min}$ ). Finally, to determine the activity, each unit was divided by 1000 to convert to mU which, in turn, was divided by the amount of Hb (in g), which was measured previously, to determine activity as mU/g.

After measuring FN3K activity in each sample, statistical analysis was performed to examine whether the activity in each sample was correlated with the G-gap value of each sample. As mentioned in Chapter 2, this statistical analysis was performed using GraphPad Prism 6, which was used to produce Figures 3.1.1c and 3.1.1d, and showed very significant differences in FN3K enzyme activity between subjects with high and low G-gap values ( $P < 0.001$ ). Figure 3.1.1c shows mean  $\pm$  standard deviation (SD) FN3K enzyme activity for the two G-gap groups.

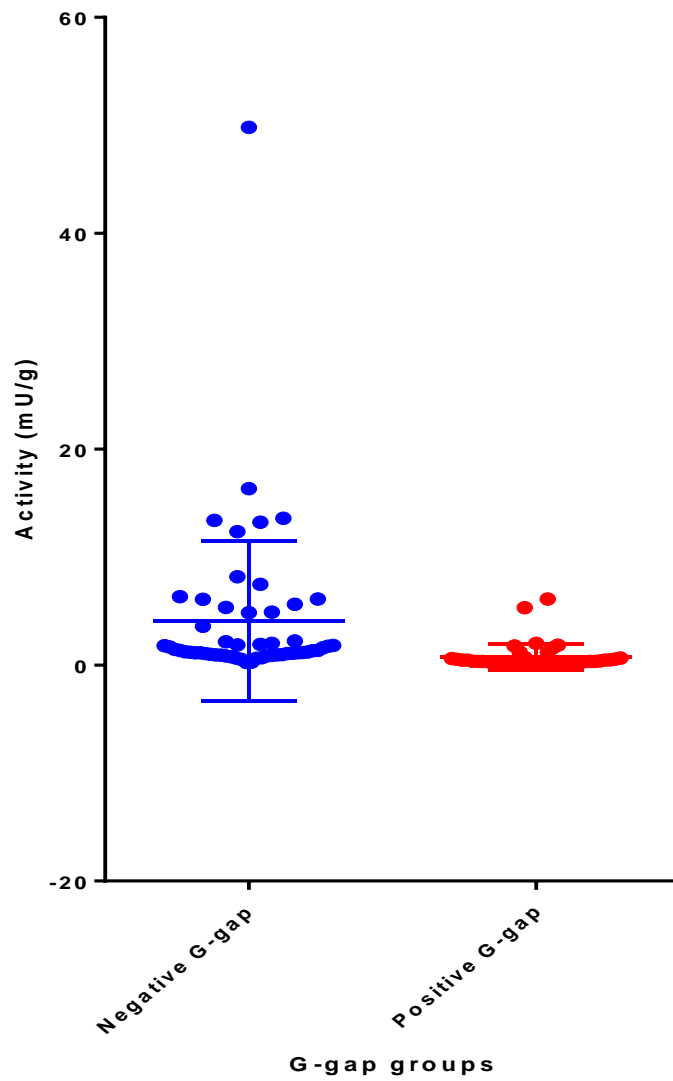


Figure 3.1.1c: Scatter plot of mean  $\pm$  SD FN3K activity by G-gap (negative and positive), and the median and quartiles of each group.

$P < 0.001$  negative ( $n = 55$ ) vs. positive ( $n = 43$ ).

Overall, Figure 3.1.1c shows a significant difference between the two G-gap groups: the negative G-gap group had a mean of 4.078 mU/g compared with 0.7769 mU/g for the positive G-gap group. The SD of the negative G-gap group was higher than that of the positive G-gap group ( $\pm 7.393$  mU/g vs.  $\pm 1.202$  m U/g); therefore, when considering these findings together it was clear that this difference was highly significant, which was confirmed in the statistical analysis ( $P < 0.001$ ).

Table 3.1.1d shows the descriptive statistics for the measurement of FN3K enzyme activity in erythrocytes in patients with low and high G-gaps. Descriptive statistics of the separated cohort by G-gap, along with the P-value and Kolmogorov–Smirnov (KS) normality test, were calculated in the analysis of the distribution of the two data sets. The KS normality test showed that the data were not normally distributed when considering the negative and positive G-gap groups with P-values  $< 0.05$  for both.

When taking into consideration the hypsographical plots of each data set, neither set followed the normal distribution curve and thus neither had a normal distribution. Therefore, a non-parametric test was carried out in the form of an unpaired, two-tailed Mann–Whitney U test which compares the median of the data as opposed to the mean which is compared in a Student's *t*-test if the data are normally distributed. A Mann–Whitney U test is shown in Table 3.1.1e which was produced by using GraphPad Prism 6 and gave a P-value of  $< 0.0001$  after adjustment for ties, with 95% confidence interval (CI). The result showed a highly significant difference in FN3K enzyme activity between subjects with high and low glycation, with those in the low G-gap group having higher mean and median FN3K enzyme activity than those with high G-gap values. Therefore, this finding confirms

our hypothesis that individuals with a low G-gap value have high FN3K activity and vice versa.

Table 3.1.1d: Descriptive statistics of the cohort by G-gap, together with the P-value and KS normality test calculated in the analysis of the distribution of the two data sets.

	Negative GG	Positive GG
Number of values	55	43
Minimum	0.2521	0.1975
25% Percentile	0.9618	0.2727
Median	1.368	0.33
75% Percentile	4.907	0.607
Maximum	49.82	6.11
Mean	4.078	0.7769
SD	7.393	1.202
Standard Error of Mean (SEM)	0.9969	0.1833
Lower 95% CI of mean	2.079	0.407
Upper 95% CI of mean	6.077	1.147
95% CI of median		
Actual confidence level	97.00%	96.85%
Lower confidence limit	1.136	0.2993
Upper confidence limit	1.916	0.4723
Sum	224.3	33.41
KS normality test		
KS distance	0.3075	0.3356
P-value	<0.0001	<0.0001
Passed normality test (alpha = 0.05)?	No	No
P-value summary	****	****

Table 3.1.1e: Mann–Whitney U test of normality in the form of a Student’s *t*-test.

Mann-Whitney U test (non-parametric)	
Table analysed	Study 1 Part 1
Column B	Positive G-gap
vs	vs
Column A	Negative G-gap
Mann–Whitney U test	
P-value	<0.0001
Exact or approximate P-value?	Exact
P-value summary	****
Significantly different (P<0.05)?	Yes
One- or two-tailed P-value?	Two-tailed
Sum of ranks in columns A and B	3579, 1272
Mann–Whitney U test	326
Difference between medians	
Median of column A	1.368, n=55
Median of column B	0.3300, n=43

### 3.1.2 Results of Study 1, Part 2: measurement of FN3K levels in erythrocytes in relation to G-gap

As mentioned in Chapter 2, this measurement was performed by enzyme-linked immunosorbent assay (ELISA). A standard curve was drawn to obtain the concentration of FN3K. Figure 3.1.2a shows the standard curve for the first experiment (samples #2–44). The concentrations of the unknown samples were obtained from the standard curve.

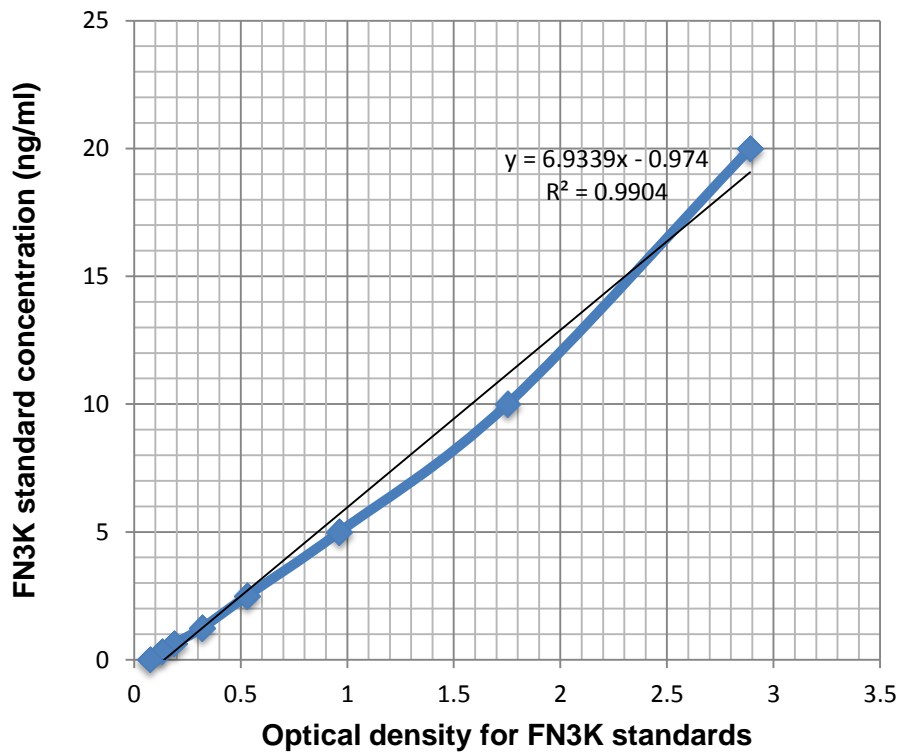


Figure 3.1.2a: The standard curve produced from the standard concentrations of human intracellular FN3K used in the first ELISA. The  $R^2$  value of the best fit curve is close to 1, which indicates a low level of deviation of the measurements plotted from the best fit curve to be the most accurate at predicting concentrations from the optical density.

Statistical analysis was performed using GraphPad Prism 6 software which was also used to produce Figures 3.1.2b and 3.1.2c, both of which showed highly significant differences in FN3K protein levels between subjects with low and high G-gap values ( $P = 0.0011$ ).

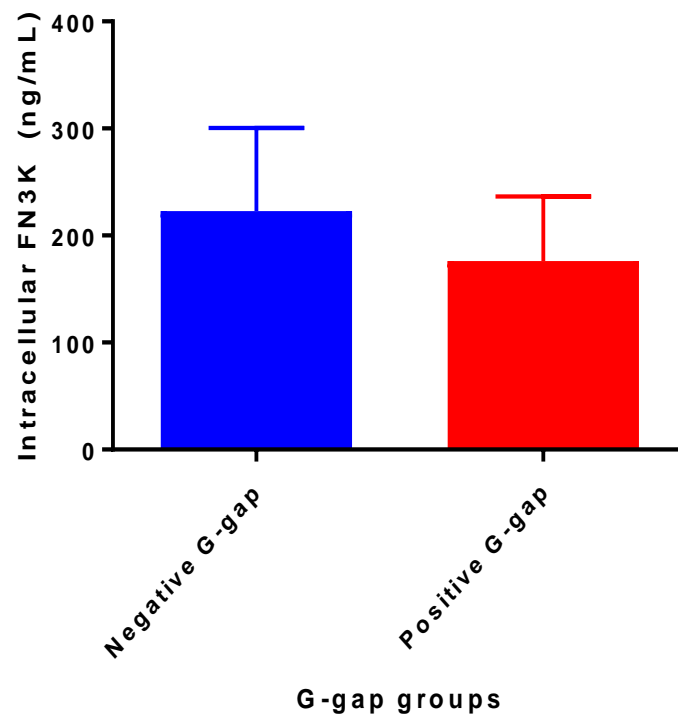


Figure 3.1.2b: Differences in mean  $\pm$  SD erythrocyte FN3K levels by G-gap ( $P < 0.0011$ ).

Overall, Figure 3.1.2b shows a significant difference between the two G-gap groups: mean FN3K levels of 223 ng/ml vs. 176 ng/ml for the negative and the positive G-gap groups, respectively. The corresponding SD values were  $\pm 77.5$  ng/ml vs.  $\pm 60.15$  ng/ml; therefore, it was clear that this difference was highly significant, which was confirmed in the statistical analysis ( $P < 0.0011$ ).

Table 3.1.2c shows the descriptive statistics for measurement of FN3K levels in erythrocytes in patients with low and high G-gap values. Descriptive statistics of the cohort by G-gap, along with the P-value, KS normality test and Wilcoxon signed rank test were calculated in the analysis of the distribution of the two data sets. The KS normality test showed a normal distribution of the data for the negative G-gap group ( $P < 0.0001$ ) whereas the data for the positive G-gap group were not normally distributed ( $P < 0.0001$ ). However, when considering both groups, the statistics showed that they were not normally distributed. A non-parametric Mann–Whitney U test (Table 3.1.2d) was carried out using GraphPad Prism 6, which showed, after adjustment for ties with 95% CI, a highly significant difference in FN3K enzyme levels between subjects with high and low glycation ( $P = 0.0011$ ). In addition, those with low G-gaps had higher mean and median FN3K enzyme levels than those with high G-gaps (this significant difference was clarified in Figure 3.1.2b, which shows the mean  $\pm$  SD). Therefore, this result confirmed our hypothesis that individuals with high G-gap values have low intracellular levels of FN3K and vice versa.

Table 3.1.2c: Descriptive statistics of the cohort by G-gap for FN3K levels, together with the P-value and KS normality test calculated in the analysis of the distribution of the two datasets.

	Negative G-gap	Positive G-gap
<b>n</b>	<b>55</b>	<b>43</b>
<b>Minimum</b>	<b>70.86</b>	<b>82.87</b>
<b>25% percentile</b>	<b>171.5</b>	<b>127.5</b>
<b>Median</b>	<b>212.3</b>	<b>166.1</b>
<b>75% percentile</b>	<b>288.0</b>	<b>201.7</b>
<b>Maximum</b>	<b>412.7</b>	<b>319.9</b>
<b>Mean</b>	<b>223.0</b>	<b>176.2</b>
<b>SD</b>	<b>77.50</b>	<b>60.15</b>
<b>SEM</b>	<b>10.45</b>	<b>9.173</b>
<b>Lower 95% CI</b>	<b>202.0</b>	<b>157.7</b>
<b>Upper 95% CI</b>	<b>243.9</b>	<b>194.8</b>
<b>KS normality test</b>		
<b>KS distance</b>	<b>0.1135</b>	<b>0.1478</b>
<b>P-value</b>	<b>0.0747</b>	<b>0.0192</b>
<b>Passed normality test (alpha=0.05)?</b>	<b>Yes</b>	<b>No</b>
<b>P-value summary</b>	<b>ns</b>	<b>*</b>
<b>Wilcoxon signed rank test</b>		
<b>Theoretical median</b>	<b>0.0</b>	<b>0.0</b>
<b>Actual median</b>	<b>212.3</b>	<b>166.1</b>
<b>Discrepancy</b>	<b>-212.3</b>	<b>-166.1</b>
<b>Sum of signed ranks (W)</b>	<b>1540</b>	<b>946.0</b>
<b>Sum of positive ranks</b>	<b>1540</b>	<b>946.0</b>
<b>Sum of negative ranks</b>	<b>0.0</b>	<b>0.0</b>
<b>P-value (two tailed)</b>	<b>&lt;0.0001</b>	<b>&lt;0.0001</b>
<b>Exact or estimate?</b>	<b>Exact</b>	<b>Exact</b>
<b>Significant (alpha=0.05)?</b>	<b>Yes</b>	<b>Yes</b>
<b>Sum</b>	<b>12265</b>	<b>7578</b>

Table 3.1.2 d: Mann–Whitney U test of normality in the form of a Student's *t*-test.

<b>Man–Whitney U test (non-parametric)</b>	
<b>Table analysed</b>	CFN3K-data
<b>Column B</b>	positive G-gap
<b>vs</b>	vs.
<b>Column A</b>	Negative G-gap
<b>Mann–Whitney U test</b>	
<b>P-value</b>	0.0011
<b>Exact or approximate P-value?</b>	Exact
<b>P-value summary</b>	**
<b>Significantly different? (P &lt; 0.05)</b>	Yes
<b>One- or two-tailed P-value?</b>	Two-tailed
<b>Sum of ranks in column A, B</b>	3173, 1678
<b>Mann–Whitney U</b>	732
<b>Difference between medians</b>	
<b>Median of column A</b>	212.3, n=55
<b>Median of column B</b>	166.1, n=43
<b>Difference: Actual</b>	-46.27
<b>Difference: Hodges–Lehmann</b>	-46.27
<b>Man–Whitney U test (non-parametric)</b>	
<b>Table analysed</b>	CFN3K-data
<b>Column B</b>	Positive G-gap
<b>vs.</b>	vs.
<b>Column A</b>	Negative G-gap
<b>Mann–Whitney U test</b>	
<b>P-value</b>	0.0011

### 3.1.3 Statistical analysis of FN3K activity vs. concentration in erythrocytes

This analysis was performed using SPSS software (IBM) to explore the relationship between FN3K activity and concentration by analysis of covariance (ANCOVA).

In order to normalise the otherwise skewed distribution, both measures were  $\log_{10}$  transformed, and the ANCOVA test of log transformed FN3K activity showed a significant difference in FN3K activity between the negative and positive G-gap groups after controlling for log transformed concentration ( $r^2 = 0.66$ ,  $F = 90.68$ ,  $P < 0.001$ ), with a significant slope parameter close to unity ( $B = 0.90$ ,  $t = 9.219$ ,  $P < 0.001$ ). Separate regression lines were fitted to the two groups which were significantly displaced from each other ( $F = 61.79$ ,  $P < 0.001$ ).

The difference between the negative G-gap (mean =  $\log_{10} 248$ ) and the positive G-gap groups (mean =  $\log_{10} -262$ ) was  $\log_{10} 51$  (SEM  $\pm 0.07$ ,  $t = 7.861$ ,  $P < 0.001$ ) and (since this difference is log minus log) this represents the ratio of increased enzyme activity in the negative G-gap group compared with the positive G-gap group of 3.23 (antilog) or 323% (see Figure 3.1.3.a).

The magnitude of that ratio is consistent with the raw data outcomes of FN3K activity unadjusted ( $2.4/0.5 = 4.8$ ) and adjusted ( $4.1/0.8 = 5.1$ ) to Hb concentration and then further adjusted to FN3K concentration ( $0.013 / 0.003 = 4.3$ ) (Table 3.1.3b).

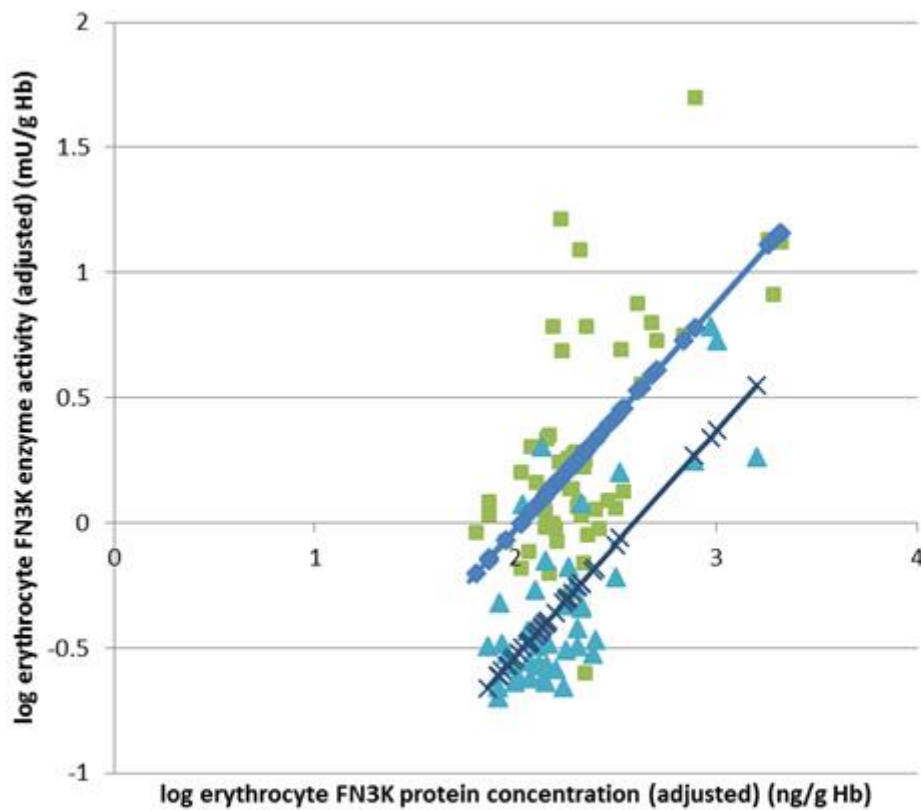


Figure 3.1.3a: Relationship of erythrocyte FN3K protein and enzyme activity in patients with diabetes with positive and negative G-gaps. Patients with a negative G-gap (■, n = 55) showed significantly higher FN3K enzyme activity in relation to FN3K protein compared with patients with a positive G-gap (▲, n = 43) ( $P < 0.001$ ).

Table 3.1.3b: ANCOVA test for FN3K expression vs. concentration separated by the two G-gap groups.

G-gap category	Negative G-gap	Positive G-gap	P-value
G-gap (% HbA1c)	-1.4±0.7	+1.5±0.7	0.001
Plasma FN3K concentration (ng/ml)	3.3±3.5	2.3±2.5	ns
Erythrocyte FN3K concentration (ng/ml)	223±78	176±60	0.01
Adjusted erythrocyte FN3K concentration (ng/g Hb)	351±481	238±295	0.05
FN3K activity (mU/ml)	2.4±2.4	0.5±0.4	0.001
Adjusted FN3K activity (mU/g Hb)	4.1±7.4	0.8±1.2	0.001
Ratio of FN3K activity/concentration	0.013±0.017	0.003±0.002	0.001

### 3.1.4 Results of Study1, Part 3: measurement of plasma FN3K protein levels in relation to G-gap

As mentioned in Chapter 2, this measurement was performed by ELISA and a standard curve was drawn to determine the concentration of FN3K. The standard curve for the first experiment is shown in Figure 3.1.4a (samples #2–40). The concentrations of the unknown samples were obtained from the standard curve.

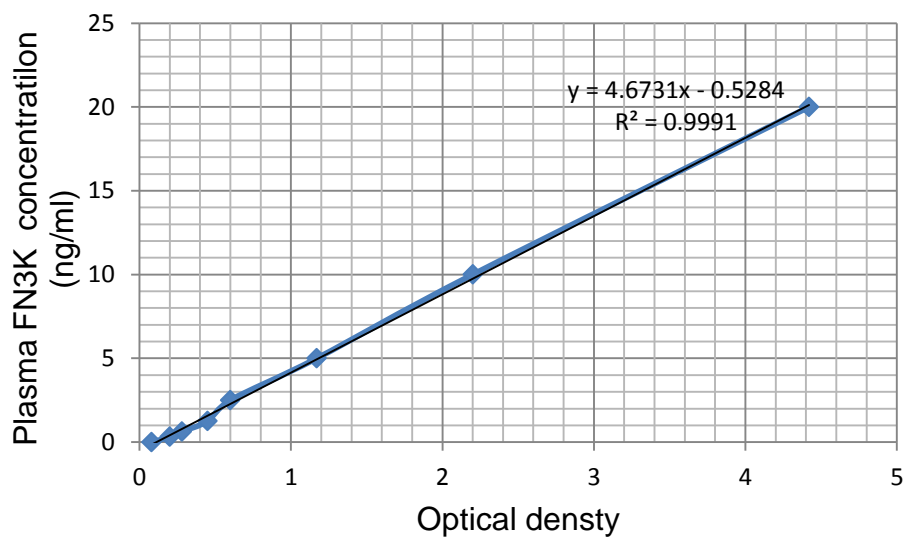


Figure 3.1.4a: The standard curve produced from the standard concentrations of human plasma FN3K levels used in the first ELISA. The  $R^2$  value of the best fit curve is close to 1, which indicates a low degree of deviation of the measurements plotted from the best fit curve to be the most accurate at predicting concentrations from the optical density.

Statistical analysis was performed using GraphPad Prism 6 software. Figure 3.1.4b clearly shows non-significant differences in mean  $\pm$  SD for plasma FN3K levels between subjects with low and high G-gap values.

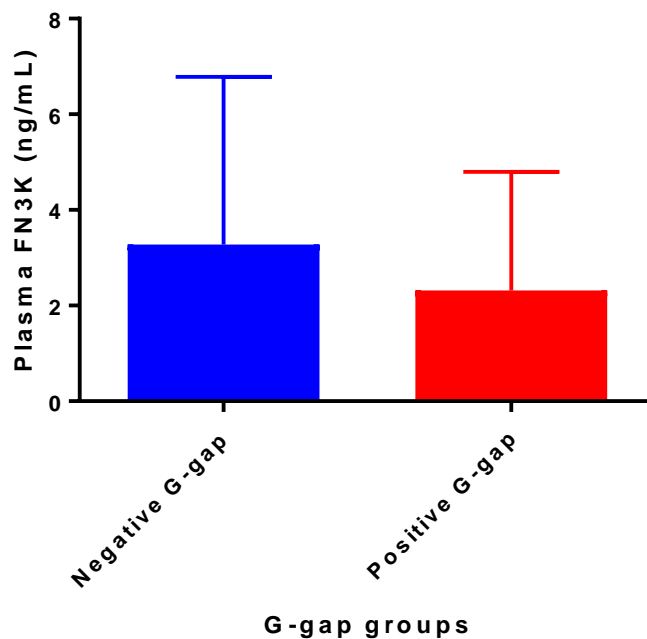


Figure 3.1.4b Differences in mean  $\pm$  SD plasma FN3K levels by G-gap.

Overall, Figure 3.1.4a shows a non-significant difference in mean plasma FN3K concentration between the two G-gap groups: 3.279 ng/ml vs. 2.317 ng/ml for the negative and positive G-gap groups, respectively. The corresponding SD values were  $\pm$ 3.505 ng/ml and  $\pm$ 2.478ng/ml. Therefore, when considering these findings together, it is clear that there is a non-significant difference in mean FN3K expression in plasma between the negative and positive G-gap groups, which was

confirmed in the statistical analysis ( $P = 0.0636$ ) using the Student's  $t$ -test (two-tailed) to compare the controls and samples.

The differences between the two groups are summarised in Table 3.1.4c, which shows the descriptive statistics for the measurement of FN3K levels in plasma in patients with low and high G-gap values. Descriptive statistics of the cohort by G-gap are shown along with the P-value, KS normality test and Wilcoxon signed rank calculated in the analysis of the distribution of the two data sets. The KS normality test showed that the data were not normally distributed when considering both negative and positive G-gap groups with P-values  $<0.0001$  for both. A non-parametric Mann–Whitney U test (Table 3.1.4d) was carried out using GraphPad Prism 6, and showed a non-significant difference after adjustment for ties with 95% CI ( $P = 0.0636$ ) in FN3K enzyme levels between subjects with high and low glycation with those in the low G-gap group. The mean and median FN3K concentration between the two groups were not significantly different (Figure 3.1.4b, Table 3.1.4d). Therefore, this result showed that the plasma levels of FN3K enzyme were not correlated with differences in G-gap values.

Table 3.1.4c: Descriptive statistics of the cohort by G-gap for plasma FN3K levels together with the P-value and KS normality test calculated in the analysis of the distribution of the two datasets.

	Negative G-gap	Positive G-gap
<b>n</b>	81	67
<b>Minimum</b>	0.0200	0.1645
<b>25% Percentile</b>	0.8314	0.6500
<b>Median</b>	2.270	1.440
<b>75% Percentile</b>	4.088	3.051
<b>Maximum</b>	17.35	14.26
<b>Mean</b>	3.279	2.317
<b>SD</b>	3.505	2.478
<b>SEM</b>	0.3894	0.3028
<b>Lower 95% CI of mean</b>	2.504	1.712
<b>Upper 95% CI of mean</b>	4.054	2.921
<b>KS normality test</b>		
<b>KS distance</b>	0.1860	0.2189
<b>P value</b>	< 0.0001	< 0.0001
<b>Passed normality test (alpha=0.05)?</b>	No	No
<b>P value summary</b>	****	****
<b>Wilcoxon Signed Rank Test</b>		
<b>Theoretical median</b>	0.0	0.0
<b>Actual median</b>	2.270	1.440
<b>Discrepancy</b>	-2.270	-1.440
<b>Sum of signed ranks (W)</b>	3321	2278
<b>Sum of positive ranks</b>	3321	2278
<b>Sum of negative ranks</b>	0.0	0.0
<b>P value (two tailed)</b>	< 0.0001	< 0.0001
<b>Exact or estimate?</b>	Exact	Exact
<b>Significant (alpha=0.05)?</b>	Yes	Yes
<b>Sum</b>	265.6	155.2

Table 3.1.4d: Mann–Whitney U test of normality in the form of Student’s t-test.

<b>Mann–Whitney U test (non-parametric)</b>	
<b>Table analysed</b>	Data (FN3K plasma)
<b>Column B</b>	Positive G-gap
<b>vs.</b>	vs.
<b>Column A</b>	Negative G-gap
<b>Mann–Whitney U test</b>	
<b>P-value</b>	0.0636
<b>Exact or approximate P-value?</b>	Exact
<b>P-value summary</b>	ns
<b>Significantly different? (P &lt; 0.05)</b>	No
<b>One- or two-tailed P-value?</b>	Two-tailed
<b>Sum of ranks in column A, B</b>	6516, 4510
<b>Mann–Whitney U test</b>	2232
<b>Difference between medians</b>	
<b>Median of column A</b>	2.270, n = 81
<b>Median of column B</b>	1.440, n = 67
<b>Difference: Actual</b>	-0.8300
<b>Difference: Hodges–Lehmann</b>	-0.5255

### 3.1.5 Results of study1 part 4: measuring of AGEs in plasma in relation to G-gap

As mentioned in Chapter 2, this measurement was performed by competitive ELISA; the standard curve was drawn to determine the concentration of AGEs. The standard curve for the first experiment is shown in figure 3.1.5a (samples #2–42). The concentrations of the unknown samples were obtained from the standard curve.

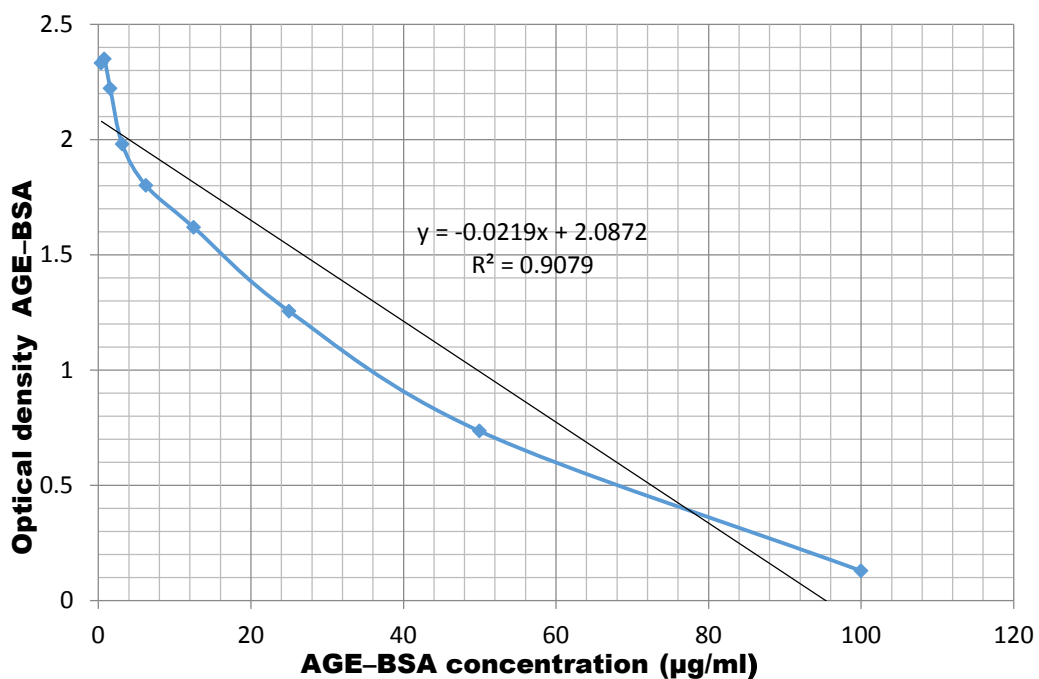


Figure 3.1.5a: The standard curve produced from the standard concentrations of human AGE–bovine serum albumin (BSA) used in the first ELISA. The  $R^2$  value of the best fit curve is close to 1, which indicates a low degree of deviation of the measurements plotted from the best fit curve to be the most accurate at predicting concentrations from the optical density.

The statistical analysis was performed using GraphPad Prism 6 software and showed significant differences in mean  $\pm$  SD plasma AGE-BSA levels by G-gap (Figure 3.1.5b).

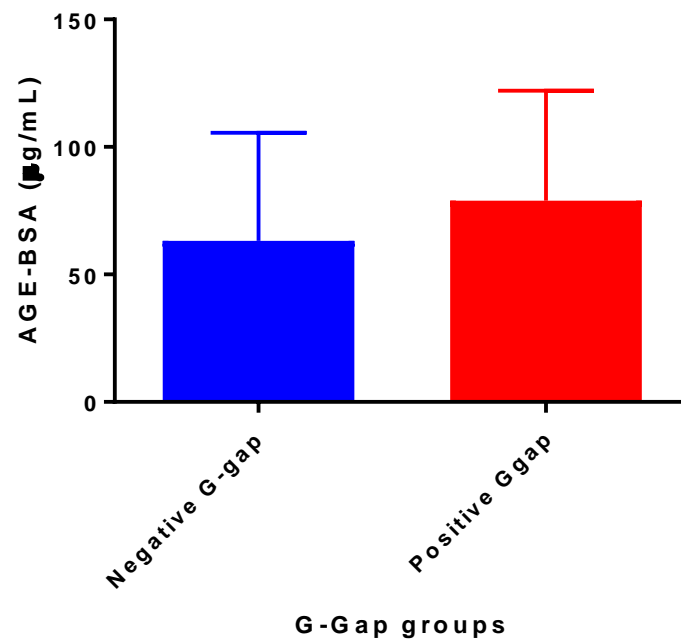


Figure 3.1.5b Differences in mean  $\pm$  SD plasma AGE levels by G-gap ( $P = 0.0109$ ).

Figure 3.1.5b shows a significant difference in mean plasma AGE concentration between the two G-gap groups: 63.13 µg/ml vs. 78.93 µg/ml for the negative and positive G-gap groups, respectively. The corresponding SDs were ±42.45 µg/ml and ±43.12 µg/ml. This significance in the differences of mean AGE levels between the two G-gaps was confirmed in the statistical analysis ( $P = 0.0109$ ) using a Student's *t*-test (two-tailed) to compare controls and samples.

This finding is summarised in Table 3.1.5c, which shows the descriptive statistics for measurement of AGE–BSA plasma levels in patients with low and high G-gap values. Descriptive statistics of the cohort by G-gap are shown along with the P-value, KS normality test and Wilcoxon signed rank calculated in the analysis of the distribution of the two data sets. The KS normality test showed that the data were not normally distributed when considering both negative and positive G-gap groups ( $P = 0.0030$  and  $P < 0.0001$ , respectively). A non-parametric Mann–Whitney U test (Table 3.1.5d) was carried out using GraphPad Prism 6, which showed a significant difference ( $P = 0.0109$ ), after adjustment for ties with 95% CI, in AGE–BSA levels between subjects with high and low glycation, with those in low G-gap group having higher mean and median values than those in the high G-gap group. Therefore, plasma levels of AGE–BSA are correlated with G-gap values and proved our hypothesis that subjects with high G-gap values and low FN3K enzyme activity have high levels of AGE–BSA, whereas those with low G-gap values and high FN3K enzyme activity have low levels of AGE–BSA.

Table 3.1.5c: Descriptive statistics of the cohort by G-gap for AGE–BSA plasma levels together with the P-value and KS normality test calculated in the analysis of the distribution of the two data sets.

	Negative G-Gap	Positive G-Gap
n	81	67
Minimum	0.8040	4.422
25% Percentile	30.57	54.27
Median	51.43	83.22
75% Percentile	91.81	98.69
Maximum	208.2	261.5
Mean	63.13	78.93
SD	42.45	43.12
SEM	4.717	5.268
Lower 95% CI of mean	53.75	68.42
Upper 95% CI of mean	72.52	89.45
KS normality test		
KS distance	0.1591	0.1376
P-value	<0.0001	0.0030
Passed normality test (alpha = 0.05)?	No	No
P-value summary	****	**
Wilcoxon Signed Rank Test		
Theoretical median	0.0	0.0
Actual median	51.43	83.22
Discrepancy	-51.43	-83.22
Sum of signed ranks (W)	3321	2278
Sum of positive ranks	3321	2278
Sum of negative ranks	0.0	0.0
P-value (two tailed)	<0.0001	<0.0001
Exact or estimate?	Exact	Exact
Significant (alpha = 0.05)?	Yes	Yes
Sum	5114	5288

Table 3.1.5d: Mann–Whitney U test of normality in the form of a Student’s *t*-test.

Table analysed	AGE–BSA
Column B	Positive G-gap
vs.	vs.
Column A	Negative G-gap
Mann–Whitney U test	
P-value	0.0109
Exact or approximate P-value?	Exact
P-value summary	*
Significantly different? ( $P < 0.05$ )	Yes
One- or two-tailed P-value?	Two-tailed
Sum of ranks in column A,B	5376, 5651
Mann-Whitney U	2055
Difference between medians	
Median of column A	51.43, n = 81
Median of column B	83.22, n = 67
Difference: Actual	31.79
Difference: Hodges–Lehmann	20.47

## 3.2. Results of Study1, Part 5: Markers of endothelial dysfunction in relation to G-gap

### 3.2.1 Result of the thrombomodulin assay

As described in Chapter 2, this assay was performed by ELISA and the standard curve was drawn to interpolate the concentration of thrombomodulin. Figure 3.2.1a shows the standard curve for the first experiment (samples # 2–40). The concentrations of the unknown samples were obtained from the standard curve.

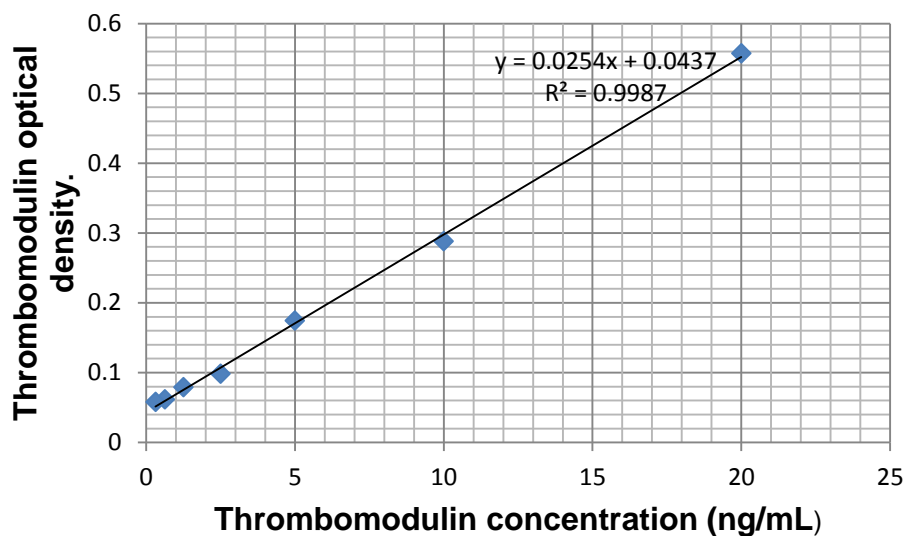


Figure 3.2.1a: The standard curve produced from the standard concentrations of human thrombomodulin levels used in the first ELISA. Regarding the goodness of fit model, the  $R^2$  value of the best fit curve is close to 1, which indicates low deviation of the measurements plotted from the best fit line. This suggests the predicted concentrations from the optical density will be interpolated accurately.

Statistical analysis of this assay was performed by using Graphpad Prism 6 software showed non-significant differences in mean $\pm$  SD for plasma thrombomodulin levels separated by G-gap.

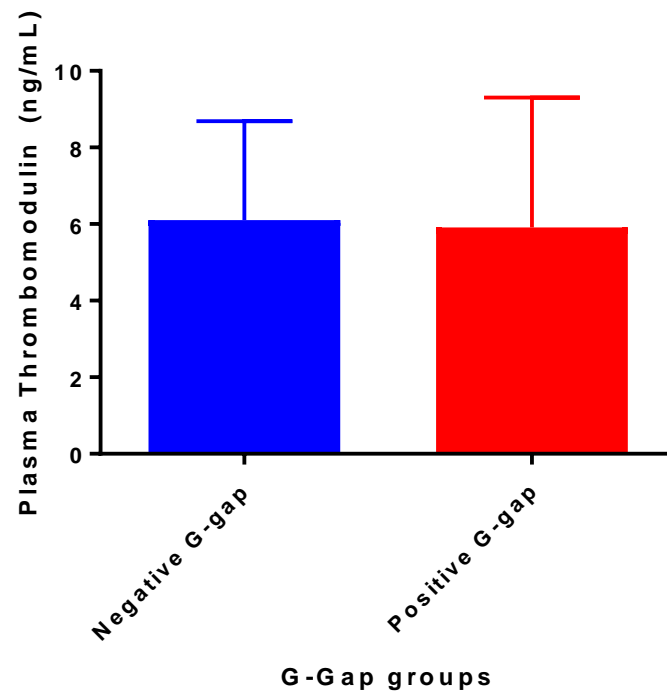


Figure 3.2.1b: Mean plasma  $\pm$ SD concentrations of thrombomodulin and the SD above the mean for the negative and positive for G-gap groups ( $P = 0.2351$ ).

Figure 3.2.1b indicates that there is a non-significant difference in mean plasma thrombomodulin concentration between the two G-gap groups. The negative G-gap had a mean of 6.108 ng/ml vs. 5.913ng /ml for the positive G-gap, although the SD of the negative G-gap group was higher ( $\pm 2.583$ ng/ml) than the SD for the positive G-gap ( $\pm 3.387$ ng/ml). This non-significant difference in mean plasma thrombomodulin levels between the two G-gap groups was confirmed with using a Mann-Whitney U test to compare samples ( $p = 0.2351$ ).

Table 3.2.1c shows descriptive statistics for measurement of plasma thrombomodulin levels in patients with low and high G-gap values, and a KS normality test calculated in the analysis of the distribution of the two data sets. The KS normality test showed that the data were not normally distributed for the positive G-gap group, whereas the negative G-gap group data were normally distributed ( $P = 0.0008$  and  $P > 0.1$  for the positive and negative G-gap groups, respectively). Therefore, a non-parametric Mann–Whitney U test was performed using Graph-pad prism 6 (Table 3.2. 1d), and showed  $P = 0.2351$  after adjustment for ties with 95% CI. The results showed a non-significant difference in plasma thrombomodulin levels between subjects with high and low glycation in the low G-gap group. The mean and median between the two groups were not significantly different, and therefore shows that the plasma levels of thrombomodulin were not correlated with differences in G-gaps values (see Figure 3.2.1a).

Table 3.2.1c: Descriptive statistics for thrombomodulin in the cohort separated by G-gap, together with the P-value and KS normality test calculated based upon the analysis of the distribution of the two data sets.

	Negative G-gap	Positive G-gap
<b>Number of values</b>	81	67
<b>Minimum</b>	1.400	1.347
<b>25% Percentile</b>	4.190	3.683
<b>Median</b>	6.304	5.151
<b>75% Percentile</b>	7.733	6.947
<b>Maximum</b>	12.76	21.00
<b>Mean</b>	6.108	5.913
<b>SD</b>	2.583	3.387
<b>SEM</b>	0.2870	0.4138
<b>Lower 95% CI of mean</b>	5.537	5.087
<b>Upper 95% CI of mean</b>	6.679	6.740
<b>KS normality test</b>		
<b>KS distance</b>	0.06390	0.1491
<b>P-value</b>	>0.1000	0.0008
<b>Passed normality test (alpha = 0.05)?</b>	Yes	No
<b>P-value summary</b>	ns	***
<b>Sum</b>	494.8	396.2

Table 3.2.1d: Mann–Whitney U test for thrombomodulin assay.

<b>Mann–Whitney U test</b>	
<b>Table analysed</b>	Data 1
<b>Column B</b>	Positive G-gap
<b>vs.</b>	vs.
<b>Column A</b>	Negative G-gap
<b>Mann–Whitney U test</b>	
<b>P-value</b>	0.2351
<b>Exact or approximate P-value?</b>	Exact
<b>P-value summary</b>	ns
<b>Significantly different? (P &lt; 0.05)</b>	No
<b>One- or two-tailed P-value?</b>	Two-tailed
<b>Sum of ranks in column A, B</b>	6344, 4683
<b>Mann–Whitney U test</b>	2405
<b>Difference between medians</b>	
<b>Median of column A</b>	6.304, n = 81
<b>Median of column B</b>	5.151, n = 67
<b>Difference: Actual</b>	-1.153
<b>Difference: Hodges–Lehmann</b>	-0.5914

### 3.2.2 Results of the E-selectin assay

As described in Chapter 2, this assay was performed by ELISA, and a standard curve was drawn to get the concentration of E-selectin. Figure 3.2.2a shows the standard curve for the first experiment (samples #2–40). The concentrations of the unknown samples were obtained from the standard curve.

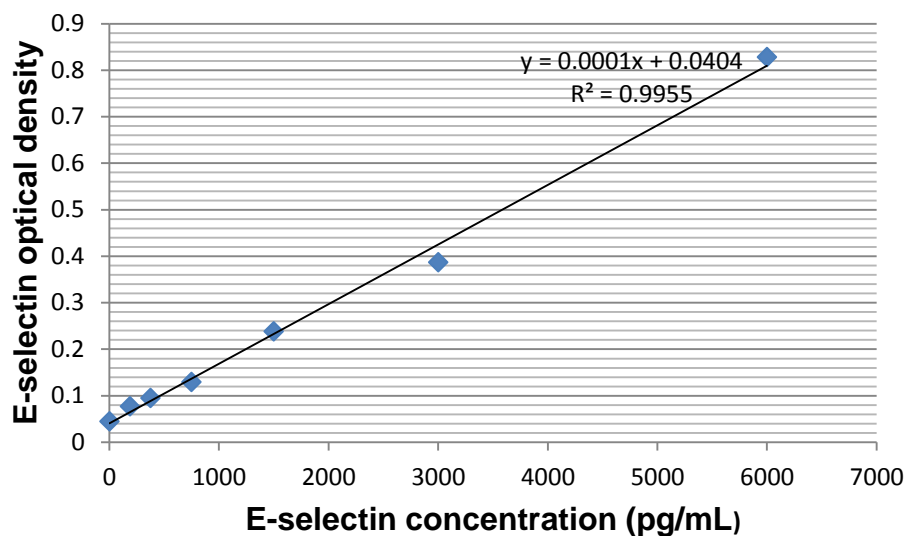


Figure 3.2.2a: The standard curve produced from the standard concentrations of human E-selectin levels used in the first ELISA. Regarding the goodness of fit model, the  $R^2$  value of the best fit curve is close to 1, which indicates low deviation of the measurements plotted from the best fit line. This suggests the predicted concentrations from the optical density will be interpolated accurately.

The statistical analysis for this assay was performed as in previous experiments by using Graphpad prism 6 software. This showed non-significant differences in mean +/-SD concentrations of E-selectin separated by G-gap, as shown in Figure 3.2.2b

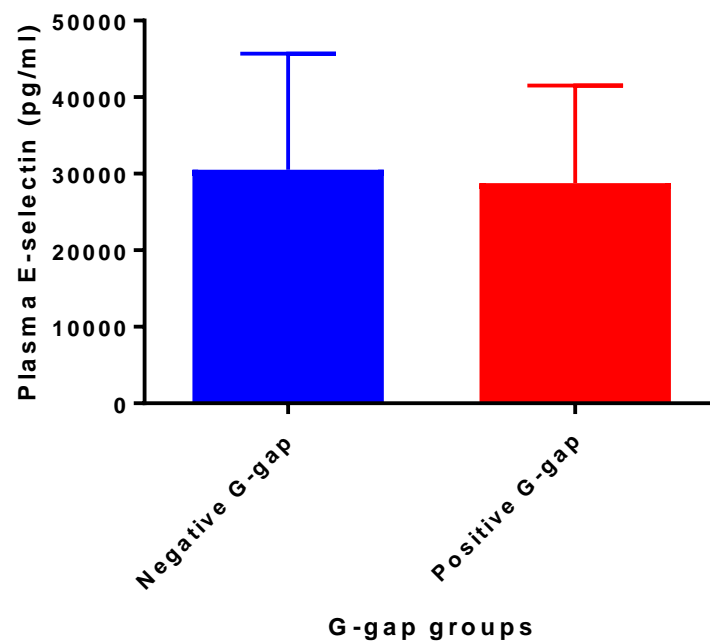


Figure 3.2.2b: Mean plasma E-selectin concentration of and the SD above the mean for the negative and positive G-gap groups.  $P = 0.7332$ .

Figure 3.2.2b shows minimal differences in the mean plasma E-selectin concentration; although the negative G-gap group had a slightly higher mean (30,527 pg/mL vs. 28,753 pg/mL). The SD of the group was also higher ( $\pm 1686$  pg/mL vs.  $\pm 1561$  pg/mL); however, this difference was not statistically significant ( $P > 0.05$ ).

The summary statistics of the two data sets are shown in Table 3.2.2c alongside the result of the KS normality test which was carried out for each data set. A Shapiro–Wilk normality test showed a normal distribution of the data when considering the positive G-gap group ( $P > 0.05$ ), and the data of the negative G-gap group was not normally distributed ( $P < 0.05$ ). Considering the histographical plots of each data set, neither set followed the normal distribution curve. Thus, a non-parametric test was carried out in the form of an unpaired, two tailed Mann–Whitney U test (Table 3.2.2d) to compare the median and mean between the two G-gap groups. A Mann–Whitney U test was carried out using Graphpad Prism 6 and showed  $P = 0.7332$  after adjusting for ties with 95% CI, and thus significance was taken at  $P \leq 0.05$  for  $H_1$  to be true. With  $P > 0.05$ ,  $H_1$  is unlikely to be true and any difference is due to chance, therefore,  $H_0$  cannot be rejected as it is likely true that the changes in G-gap value do not affect E-selectin concentration. Figure 3.2.3.2 shows the difference between the two groups.

Table 3.2.2c: Descriptive statistics for E-selectin assay with Shapiro–Wilk normality

	Negative G-gap	Positive G-gap
Number of values	81	67
Minimum	4560	8510
25% Percentile	18880	17010
Median	27280	28400
75% Percentile	40435	39088
Maximum	73680	58480
Mean	30527	28753
SD	15176	12776
SEM	1686	1561
Lower 95% CI of mean	27172	25637
Upper 95% CI of mean	33883	31870
Sum	2472710	1926477
KS normality test		
KS distance	0.09988	0.07474
P-value	0.0444	0.1000
Passed normality test (alpha=0.05)?	No	Yes
P-value summary	*	ns

Table 3.2.2d: shows Mann–Whitney U test for E-selectin assay.

Table analysed	E-selectin
Column B	Positive G-gap
vs.	vs.
Column A	Negative G-gap
Mann–Whitney U test	
P-value	0.7332
Exact or approximate P-value?	Exact
P-value summary	Ns
Significantly different? ( $P < 0.05$ )	No
One- or two-tailed P-value?	Two-tailed
Sum of ranks in columns A, B	6124, 4903
Mann–Whitney U	2625
Difference between medians	
Median of column A	27,280, n = 81
Median of column B	28,400, n = 67
Difference: Actual	1120
Difference: Hodges-Lehmann	-1063

### 3.2.3 Results of Study 1, Part 6: measurement of plasma plasminogen activator inhibitor-1 (PAI-1) in relation to G-gap

As described in Chapter 2, this assay was performed by ELISA and a standard curve was drawn to determine the concentration of PAI-1. The standard curve for the first experiment is shown in Figure 3.2.3.3a (samples # 2–40). The concentrations of the unknown samples were interpolated from the standard curve.

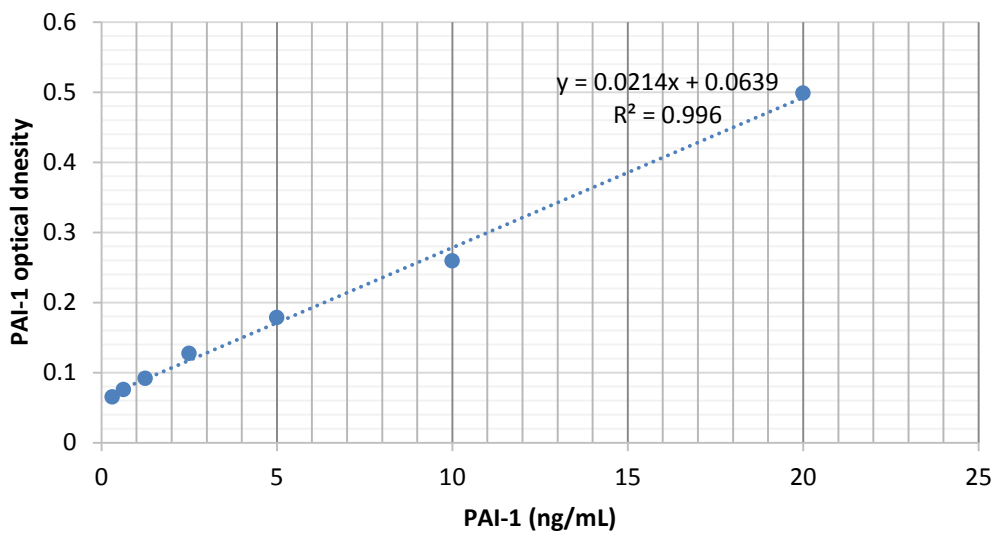


Figure 3.2.3a: The standard curve produced from the standard concentrations of plasma human PAI-1 levels used in the first ELISA. Regarding the goodness of fit model, the  $R^2$  value of the best fit curve is close to 1, which indicates low deviation of the measurements plotted from the best fit line. This suggests the predicted concentrations from the optical density will be interpolated accurately.

The statistical analysis showed highly significant differences in mean  $\pm$ SD for plasma PAI-1 concentrations separated by G-gap status ( $P < 0.0001$ ) (Figure 3.2.3b).

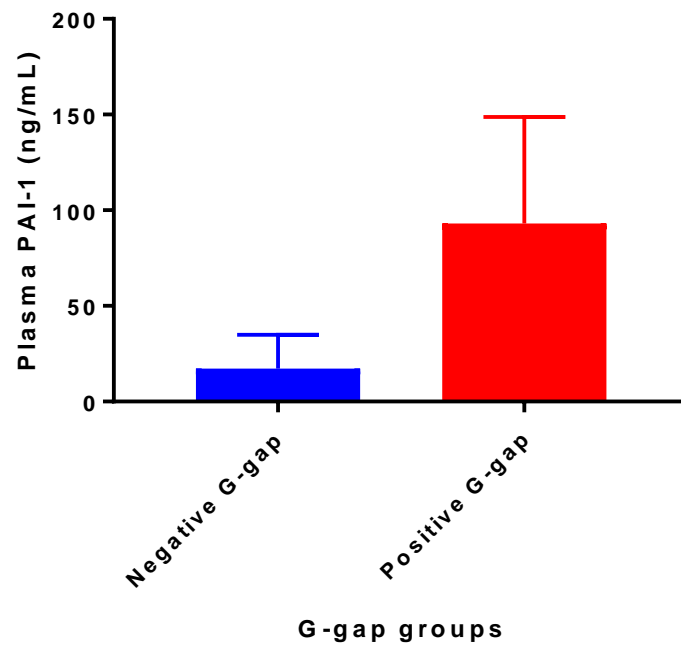


Figure 3.2.3b: Mean plasma concentrations of PAI-1 and the SD for the negative and positive G-gap groups.  $P < 0.0001$ .

Figure 3.2.3b shows a huge difference in the mean  $\pm$  SD for PAI-1 between the two G-gap groups. The positive G-gap had a higher mean than the negative G-gap group (93.06 ng/mL vs. 17.27 ng/mL). The SD of the positive group was also higher ( $\pm 55.72$  pg/mL) vs. ( $\pm 17.7$  ng/mL for the negative G-gap group). This result indicated highly significant differences in plasma PAI-1 levels between these two groups which was confirmed in the statistical analysis ( $P < 0.0001$ ).

The descriptive statistics are summarised in Table 3.2.3c alongside the result of the Shapiro–Wilk normality test which was carried out on each data set. The KS normality test showed both groups were unlikely to be normally distributed, and the histogram plots of each data set showed neither set followed the normal distribution curve. Therefore, a non-parametric test was carried out in the form of an unpaired, two tailed Mann–Whitney U test to compare the mean and the median of each data set. A Mann–Whitney U test was carried out using Graphpad prism which gave  $P < 0.0001$  (Table 3.2.3 d) after adjusting for ties with 95% CI. Thus, significance taken at  $P \leq 0.05$  for  $H_1$  to be true, with  $P < 0.05$ ,  $H_1$  is likely to be true and any difference is due to chance, therefore,  $H_0$  can be rejected as it is likely true that the G-gap affects plasma PAI-1 concentrations. This means PAI-1 levels are significantly correlated with difference in G-gap levels. Figure 3.2.3b shows the difference between the two groups. Elevated PAI-1 levels are associated with a positive G-gap status, suggesting that reduced deglycation may account for increased macrovascular disease and other diabetic complications in these patients.

Table 3.2.3c: Descriptive statistics for PAI-1 assay with KS normality test.

	Positive G-gap	Negative G-gap
Number of values	67	81
Minimum	20.04	0.4112
25% Percentile	53.5	5.527
Median	76.59	9.45
75% Percentile	125.8	22.87
Maximum	285.8	75.68
Mean	93.06	17.27
SD	55.72	17.7
SEM	6.807	1.967
Lower 95% CI of mean	79.47	13.35
Upper 95% CI of mean	106.7	21.18
95% CI of median		
Actual confidence level	95.02%	95.52%
Lower confidence limit	67.21	6.586
Upper confidence limit	87.2	16.2
Sum	6235	1399
KS normality test		
KS distance	0.178	0.1813
P-value	<0.0001	<0.0001
Passed normality test (alpha = 0.05)?	No	No
P-value summary	****	****

Table 3.2.3d: Mann–Whitney U test for plasma PAI-1 assay.

Table analysed	PA-1 plasma
Column B	Positive G-gap
vs.	vs.
Column A	Negative G-gap
Mann Whitney U test	
P-value	<0.0001
Exact or approximate P-value?	Exact
P-value summary	****
Significantly different? ( $P < 0.05$ )	Yes
One- or two-tailed P-value?	Two-tailed
Sum of ranks in column A,B	3531, 7495
Mann–Whitney U	210
Difference between medians	
Median of column A	9.450, n = 81
Median of column B	76.59, n = 67
Difference: Actual	67.14
Difference: Hodges–Lehmann	62.69

### 3.3. Discussion

#### 3.3.1 Discussion of study 1 parts 1 and 2: intracellular FN3K activity and levels in relation to G-gap

As mentioned in Chapter 1, non-enzymatic glycation is one of the major mechanisms by which hyperglycaemia contributes to cellular damage (Monnier *et al.*, 2008). An enzymatic defence mechanism reducing glycation may act to suppress this cellular damage. It has been proposed that FN3K acts as a protein repair enzyme that is responsible for intracellular protein deglycation which in turn may decrease the risk of developing diabetes-related complications due to its ability to convert protein-bound fructosamines into unstable 3-phospho derivatives (Delpierre *et al.*, 2000; Delpierre *et al.*, 2002). In addition, the G-gap has been reported to be associated with a variation in risk of diabetic complications, and is a measure of deviation of measured HbA1c levels from the expected value in relation to the average degree of glycaemia. A positive G-gap thus represents a higher level of glycation of proteins than expected whereas a negative G-gap relates to a lower than expected level (Nayak *et al.*, 2013). Previous studies suggest that a positive G-gap correlates with a higher incidence of diabetes-related complications, whereas a negative G-gap in diabetic patients correlates with a lower incidence of such complications (Cohen, *et al.*, 2003; Nayak, *et al.*, 2013). Thus, in our study, the G-gap represents an important predictor of risk of diabetes-related complications.

When comparing FN3K enzymatic activity and levels in erythrocytes of subjects with positive and negative G-gaps (figure 3.1.1c & figure 3.1.2b respectively), we observed very significant differences in the activity and protein expression of FN3K in the two groups ( $P < 0.0001$  for the mean FN3K activity and

P=0.0011 for FN3K expression for negative vs positive G-gaps). Therefore, whether the G-gap is negative or positive, it affects FN3K activity and expression. These results support our hypothesis that patients with a positive G-gap appear to have a greater level of intracellular erythrocyte glycation (higher HbA1c levels in relation to the average degree of glycaemia) which may result from lower FN3K activity and expression compared to those with a negative G-gap. Thus, patients with a positive G-gap have a greater potential for more negative effects of glycation with higher risk of developing diabetes-related complications than those with a negative G-gap. Hence our results are consistent with our prediction, namely that higher activity and expression of FN3K is associated with less diabetic complications whereas lower activity of FN3K is associated with a higher incidence of diabetic complications. These findings also provide support for the concept that FN3K acts as a protein repair enzyme against damage caused by glycation.

There have been many attempts over the years to find a good indicator that reflects glycaemic level and the presence of any complications related to diabetes; G-gap has been introduced as a good indicator that is associated with alteration of diabetic complications (Nayak *et al.*, 2013; Cohen *et al.*, 2008). However, Sack *et al.*, (2011) believed that in spite of the fact that the G-gap has been reported to be associated with various diabetic complications it is still not an ideal indicator because fructosamine is an indirect measure of glycation in plasma protein and does not predict glucose haemostasis if Hb is glycated.

The activity of FN3K has been reported to be higher in human erythrocytes than in those of other species such as chicken and pig when the concentration of intracellular glucose in erythrocytes is low. Taking into consideration the fact that hyperglycaemia is associated with a higher incidence of diabetes-related

complications (Monnier *et al.*, 2008) and since HbA1c is a standard indicator of hyperglycaemia, this means that higher intracellular glucose levels are associated with low FN3K activity and increased risk of diabetic complications (Delplanque *et al.*, 2004). This is consistent with our findings.

A study in FN3K gene knock-out mice showed an increase in glycation of proteins which provides support for the role of FN3K in the physiological deglycation process in erythrocytes (Veiga-da-Cunha *et al.*, 2006). The decrease in FN3K activity was shown to be associated with intracellular protein damage which in turn was associated with a higher risk of developing diabetes-related complications (Delpierre *et al.*, 2002). Such association was also confirmed by Veiga-da-Cunha *et al.*, (2006) in animal models by demonstrating that the concentrations of Hb-bound fructosamine and other intracellular proteins are higher in tissue and erythrocytes of FN3K-deficient mice than in those of healthy control mice.

This is the first time that this clear distinction has been reported and offers strong support for the hypothesis that FN3K may play a role in protein deglycation in patients with diabetes. It is likely therefore that the average degree of glycaemia would be underestimated in patients with a negative G-gap (high FN3K activity), and it is also possible that these patients, who we have shown to have a lower incidence of diabetic complications (Nayak *et al.*, 2013), would have lower levels of AGEs and of markers of diabetes-related complications. This was supported by the results of the assay of AGEs.

One possible explanation for the variations in the activity of FN3K might be differences in SNPs in the FN3K gene such as rs1056534 between patients in the different G-gap groups. Several studies have reported genetic variants in FN3K. For instance, Delpierre *et al.*, (2006) investigated a possible association between FN3K

enzymatic activity in erythrocytes and polymorphisms in the FN3K gene in a cohort of type1 diabetics and controls (n=58). They found that a decrease in FN3K activity in erythrocytes was associated with two SNPs in addition to gene variants {(CC of the c.900C/G (rs1056534) in exon 6 and GG of the c.-385A/G (rs3859206)}, although they did not find a direct correlation between FN3K SNPs and HbA1c levels. However, one of main limitations in this study was the small sample size. Later Mohas *et al.*, (2010), investigated large cohorts of type 2 diabetic patients who were analysed for the presence of the polymorphism rs 1056534 of FN3K gene, reported an association between the C allele of rs 1056534 and low HbA1c levels. However, these authors did not identify an association between this polymorphism and diabetic complications. In contrast, more recently, Tanhäuserová *et al.*, (2014) indicated that there is an association between polymorphism of the FN3K gene (rs 1056534) and the development of nephropathy and cardiovascular morbidity. Therefore, our study provides novel insights into exploring the relationship between variations in FN3K activity as well as intracellular levels and discrepancies in G-gap values among individuals.

### **3.3.2 Discussion of study 1 part 3: FN3K plasma levels in relation to G-gap values**

The results from the present assay showed a non-significant correlation between changes in G-gap values and variation in FN3K plasma levels (figure 3.1.4b, table 3.1.4d); in other words, patients with a negative G-gap who were previously shown to have higher intracellular activity and concentration of FN3K than those with positive G-gaps were unlikely to have a significant difference in their mean FN3K plasma proteins levels (2.317ng/ml and 3.279ng/ml for positive and negative G-gaps respectively, P=0.0636). We initially expected to have this result due to the fact that

G-gap reflects the intensity of glycation inside the erythrocyte rather outside it. Taking into consideration that the value of HbA1c is a principal measurement in the calculation of the G-gap due to the concept of G-gap is defined as:  $G\text{-gap} = \text{intracellular HbA1c} - \text{the standardized fructosamine-derived HbA1c equivalent (FHbA1c)}$ . Therefore, it can be concluded from this equation that when HbA1c level is high, the value of G-gap would be high (Nayak *et al.*, 2013). Along with what was proved in previous results of both part 1 and part 2 of this study, we can conclude that high level of HbA1c (or high G-gap value) is associated with low values of both intracellular FN3K activity and protein level rather than plasma FN3K protein level which is unlikely to be associated with changes in HbA1c levels. Thus, whether the G-gap is negative or positive, it is unlikely to be associated with plasma FN3K levels due to the fact that the FN3K concentration itself has been reported by several groups to be mainly active inside rather than outside erythrocytes (Avemaria *et al.*, 2015; Delpeirre *et al.*, 2002; Delplanque *et al.*, 2004; Payne *et al.*, 2008). Thus, we expected that there would not be much variation in FN3K protein levels in plasma since the G-gap value is associated more with intracellular glycation than with plasma protein glycation. This would lead us to suppose that the main effect of FN3K is to deglycate intracellular proteins.

The presence of FN3K in plasma might be understood as a response to glycation activity on plasma proteins such as albumins. However no previous studies have investigated the actions of FN3K in plasma. The reason for our measurement of FN3K in plasma in relation to G-gap was the significant correlation (found in study 2 which was performed at earlier time) between serum FN3K concentration and treatment with metformin in patients with AECOPD under different settings. Therefore, we assumed that there would be a possible link between G-gap and

FN3K levels in our plasma samples from the cohorts of study 1 despite the fact that the degree of deglycation in plasma and serum is less than in erythrocytes (Delpierre *et al.*, 2001). Therefore, in the present study, we attempted to investigate the presence of FN3K as a deglycating enzyme in the plasma and any correlation between both plasma and intracellular levels of FN3K and G-gap levels. Further studies are needed to investigate how FN3K in plasma is regulated and what factors affect its presence.

### **3.3.3 Discussion of study 1 part 4: plasma AGEs levels in relation to G-gap values**

When analysing the results of the AGE-BSA assay in plasma samples with positive and negative G-gaps (figure 3.1.5b, table 3.1.5d), there was a significant difference in the mean plasma levels of AGE-BSA in correlation with G-gaps (78.9 ng/ml vs 63.13 ng/ml for positive and negative G-gaps respectively,  $p=0.01$ ). This means the difference in G-gap values correlate positively with plasma AGEs levels. When considering the strong correlation between erythrocyte FN3K activity and protein levels with G-gap values in the results of study 1 parts 1 and 2 along with the results of this part, we concluded that changes in G-gap values correlate with both intracellular FN3K activity and levels along with plasma AGEs levels. This supports the hypothesis that a positive G-gap suggests a greater level of intracellular erythrocyte glycation of Hb than the predicted from the level of circulating fructosamine. This also means higher levels of AGEs with low activity and expression of FN3K with more negative effects of glycation with a higher risk of developing diabetic complications. Thus, our results confirm our prediction as well as previous findings that high levels of AGEs contribute to a higher risk of complications (Gkogkolou and Bohm, 2012; Peppas, *et al.*, 2003; Schurman *et al.*, 2008).

Considering that AGEs are the final products of the non-enzymatic glycation process that are formed from the amadori product via dicarbonyl intermediates (Ahmed *et al.*, 2005), a positive G-gap simply means higher glycated Hb than predicted from fructosamine which in turn means a higher degree of protein glycation (Nayak *et al.*, 2013). Thus, it seems intuitive that patients with positive G-gaps are likely to have higher levels of AGEs and lower FN3K activity than those with negative G-gaps. This was confirmed in our study. The higher activity of FN3K with lower levels of AGEs in patients with negative G-gaps compared to those with positive G-gaps can be explained by the role of FN3K as it is able to break down the second intermediate of the non-enzymatic glycation by phosphorylating FL to FL3P, which in turn prevents formation of AGEs when it is active (Szwergold *et al.*, 2001). Such correlation between FN3K activity and AGE levels was proven by Skhra *et al.*, (2014). These authors reported that there is a significant association between the FN3K SNPs rs1056534 and rs3848403 and sRAGE concentration in diabetic patients.

### **3.3.4 Discussion of study 1 part 4: endothelial dysfunction markers in relation to G-gap**

#### **3.3.4.1 Discussion of Thrombomodulin levels in relation to G-gap results**

When examining the results, there was no significant difference in plasma thrombomodulin levels between the two groups (negative or positive G-gap) with a p value >0.05 (figure 3.2.1b, table 3.2.1d), thus whether the value of G-gaps is positive or negative is unlikely to alter the levels of plasma thrombomodulin. Considering thrombomodulin is an important marker for endothelial dysfunction (Takahashi *et al.*, 1992) and the results of the assay suggest that there is no difference in endothelial

dysfunction occurrence with G-gap, this means G-gap may neither effect nor predict the occurrence of endothelial dysfunction.

As discussed in the introduction chapter, the rate of glycation is connected to the production of AGEs and ROS, both of which contribute to damage. Therefore, a positive G-gap means a higher level of intracellular glycated haemoglobin than the predicted level from fructosamine, which can be translated as greater chance of having endothelial dysfunction. Our findings are contrary to our prediction.

As thrombomodulin is only one marker of endothelial dysfunction, therefore it was recommended to measure more makers of endothelial damage which might show a different conclusion. In other words, it might show the expected relationship between incidence of endothelial dysfunction and G-gap values. Therefore we decided to measure other markers associated with endothelial damage such as E-selectin and PAI-1, although the result of this assay may have some power in the prediction of many cardiovascular events that are associated with diabetes, of which endothelial dysfunction is a precursor.

Identification of endothelial damage as an early precursor of several diabetic complications can help identify the predictive importance of G-gap on endothelial dysfunction and can also have important benefits for prediction of CVD complications.

#### **3.3.4.2 Discussion of E-selectin in relation to G-gap results**

In the E-selectin immunoassay there was no significant difference in plasma E-Selectin levels between the two groups (negative or positive G-gap) with a p value  $>0.05$  (figure 3.2.2b). This means G-gaps status is unlikely to affect the concentrations of plasma E-selectin.

As described in the introduction chapter, E-selectin is an important marker of endothelial dysfunction alongside thrombomodulin (Muniyappa & Sowers, 2013). Our findings showed a non-significant difference in E-selectin levels between the two glycation gap groups which means there will be no difference in the presence of endothelial dysfunction between those two groups. As we predicted that a positive G-gap means a higher level of intracellular glycated haemoglobin than the predicted level from fructosamine, which in turn can be translated as greater chance of having endothelial dysfunction, our findings are therefore contrary to our prediction. According to Endemann & Schiffrin (2004), E-selectin is a well know marker of endothelial dysfunction and an increase in its concentration is well connected to the presence of endothelial dysfunction, E-selectin is expressed on the surface of the cell for cell adhesion, a small proportion of it also found in the circulation which can be measured via immunoassays and this make it an important candidate of endothelial damage.

Despite our results showing no difference in Plasma E-selectin and thrombomodulin levels between the two glycation groups, many studies have reported contradictory results. For example, glycation gap status has been used in patients with diabetes for disease monitoring and prediction of diabetic complications, i.e. When the value is positive it is associated with certain diabetes-related complications, positive G-gap associated-retinopathy, nephropathy and some CVD diseases (Hempe *et al.*, 2002, McCarter *et al.*, 2000 and Cohen *et al.*, 2003).

In general, several studies have reported that the G-gap value is correlated with various diabetic complications. As an example, Cohen *et al.*, (2008) studied a cohort for 4 years with random measurements of G-gaps, blood glucose levels and glycated haemoglobin and found that an increased risk of diabetic nephropathy was

associated with positive G-gap but not with negative G-gap. In regard to other diabetes-related complications such as nephropathy, both Cohen *et al.*, (2003) and Rodriguez-Segade, *et al.*, (2011) found that the risk of nephropathy is higher with positive G-gap rather negative G-gap (positive G-gap was defined as >1% whereas the negative was defined <-1%): both studies show that G-gap is a predictor of nephropathy in NIDDM patients, taking into consideration that nephropathy is an important link to microvascular diseases.

Despite the previous studies found that positive G-gap was associated with diabetic complication such as nephropathy and neuropathy, Nayak *et al.*, (2013) found that the risk of mortality is increased in both positive and negative G-gaps, indicating a lack of correlation between mortality rate and the variation of G-gap values. This study describes a U shape quadratic relationship, proposing that neither positive nor negative G-gap status is linked with improving mortality rate. This result agrees with our findings that there is no link between difference in G-gap with plasma levels of thrombomodulin and E-selectin.

As both thrombomodulin and E-selectin are well known established markers of endothelial damage with previous studies showed that individuals exhibiting positive G-gaps have high risk of diabetes related complication and considering that ED is a well-known precursor of these complications, it could be assumed that those two markers of endothelial dysfunction are increased with positive G-gap (Cohen, *et al.* 2003, Nayak, *et al.*, 2013). However, our findings did not support this assumption. This finding suggests that G-gap is not associated with the early phases of developing CVD complications. Considering larger cohort might demonstrate a link between ED and G-gap variation, or these two markers alone are insufficient to support this assumption.

### 3.3.5 Discussion of PAI-1 in relation to G-gap results

When considering the results of PAI-1 immunoassay (figure 3.2.3b) there was a very significant difference in plasma PAI-1 levels between the positive and negative G-gap groups ( $p$  value  $< 0.0001$ ). This means the difference in G-gap values correlate positively with plasma PAI-1 concentration.

As discussed in the introduction chapter, PAI-1 is considered both as a marker of endothelial damage and is linked to thrombosis via its role as a partial inhibitor of fibrolysis (Nadar *et al.*, 2004). Our results show a highly significant change in plasma PAI-1 levels between the two groups in contrast to the previous two markers of endothelial dysfunction, which were not significantly different between the two G-gap groups.

This result agrees with previous studies which showed that individuals with positive G-gaps have high risk of diabetes related complication such as endothelial dysfunction with PAI-1 as a well-established marker of endothelial dysfunction. This contradiction between PAI-1 assay results with E-selectin and thrombomodulin assay results can be explained due to several factors, for example the small cohorts' size in our study might not be sufficient to support a positive correlation between positive G-gap and the chance of having endothelial dysfunction. A larger cohort might produce different results. However, the results of the PAI-1 assay showed a very significant difference in plasma PAI-1 level between positive G-gap and negative G-gap whereas the results of E-selectin and thrombomodulin did not produce a significant difference. This means the change in G-gap values might not influence the risk of having endothelial dysfunction, or that those two markers are insufficient to present such correlation between positive G-gap and endothelial

damage, unlike the result of PAI-1. Despite this, the powerful significance in the difference of PAI-1 levels between the groups is enough to support our prediction.

## **Chapter 4**

### **Study 2**

# **The effect of metformin therapy on FN3K expression in AECOPD patients**

## 4.1 Results of study2: Measuring of FN3K enzyme levels in serum of AECOPDs patients

### 4.1.1 Obtaining of FN3K enzyme serum concentration

As described in Chapter 2 this assay was performed by means of ELISA. The standard curve was drawn to get the concentration of FN3K; below is the standard curve for the first experiment (figure 4.1.1a: samples # 1-23). From the standard curve the concentrations of the unknown samples were interpolated.

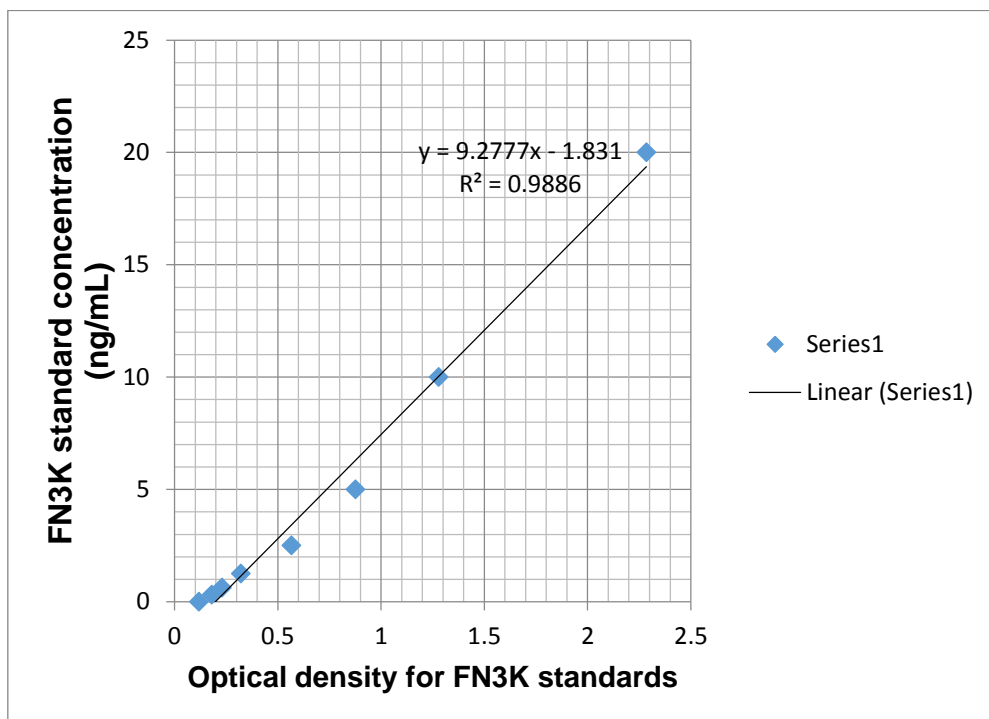


Figure 4.1.1a The standard curve produced from the standard concentrations of human serum FN3K levels used in the first ELISA. Regarding the goodness of fit model, the R2 value of the best fit curve is close to 1, which indicates low of deviation of the measurements plotted from the best fit curve. This indicates the accuracy of the interpolation for predicting concentrations from the optical density.

## 4.1.2 Statistical analysis

### 4.1.2.1 Metformin treatment

The statistical analysis performed by using Graph-pad Prism 6 software showed very significant differences in mean serum FN3K levels between discharge and follow up stages, which can be seen clearly in (Figure 4.1.2.1b).

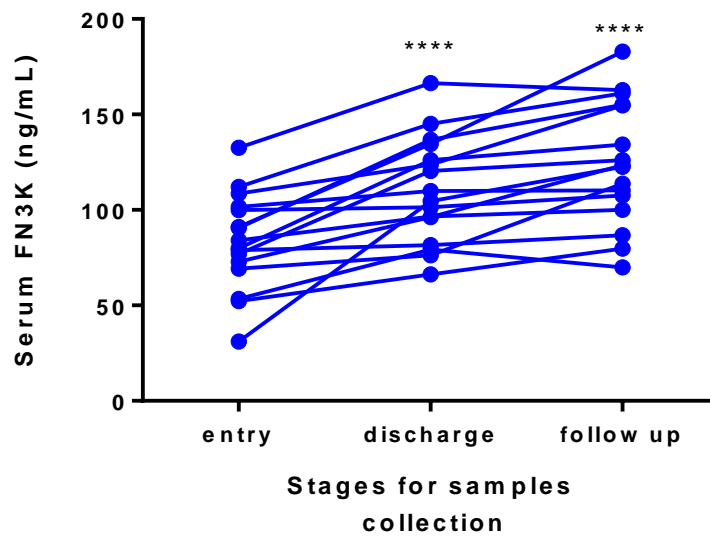


Figure 4.1.2.1b: Impact of metformin on FN3K expression for each subject (\*\*\*\* $p < 0.0001$  relative to entry) separated by the 3 stages (entry discharge- follow up) on x axis.  $P < 0.0001$ .

Figure 4.1.2.1b: shows the differences in mean FN3K levels  $\pm$  SD is plotted as a scatterplot to show the impact of metformin treatment in FN3K expression in each patient at every stage. This shows an increase in the mean of serum FN3K at each stage: (83.45, 110.3 & 124.4) ng/ml for entry, discharge and follow up, respectively. The standard deviation was also increased ( $\pm$ 25.27,  $\pm$ 27.78,  $\pm$ 32.44) ng/ml for entry, discharge & follow-up periods respectively. It can be concluded that metformin has an impact on FN3K expression in those patients. Therefore, when considering these together it is clearly show that this difference is significant, which was confirmed in the statistical analysis with  $P < 0.0001$ .

Table 4.1.2.1c shows the descriptive statistics for measurement of FN3K levels in serum of patients treated with metformin separated by 3 stages (entry-discharge-follow up). Descriptive statistics of the separated cohort by 3 stages along with the p-value and a KS normality test were calculated in the analysis of the distribution of the three data sets. The KS normality test showed a normal distribution of the data for the entry, discharge and the follow up groups with non-significant p values for each (0.9972, 0.9430, 0.8667). As we have FN3K serum levels values for each metformin treated individual taken at each point, we do need to compare the mean of FN3K concentrations among each point of entry. This was performed by the two- way ANOVA test table 4.1.2.1d: the test showed ( $p < 0.0001$ ), with R squared 66.68% and Geisser-Greenhouse's epsilon test (to correct the degrees of freedom of the F-distribution) value of 0.72, which indicates a very significant difference in FN3K expression between entry and discharge stages with homogenous variance between data in the two stages. When considering discharge, and follow up stages,  $p < 0.0001$  R squared was 59.16%, indicating a very significant difference in FN3K expression between discharge and follow up period. To then test

multiple variances (table 4.1.2.1e) a Tukey test with 95% confidence interval was used and this result showed a very significant difference in FN3K levels between all stages. This indicates that metformin treatment enhances FN3K expression (Figure 4.1.2.1b).

Table 4.1.2.1c: Descriptive statistics of the separated cohort by stages (entry-discharge-follow up) for treated patients with metformin together with the p-value and KS normality test calculated in the analysis of the distribution of the three data sets.

	entry	discharge	follow up
Number of values	16	16	16
Minimum	31.06	66.24	69.91
25% Percentile	70.12	85.19	101.9
Median	81.96	107.2	123.0
75% Percentile	101.2	132.5	155.1
Maximum	132.5	166.4	182.9
Mean	83.45	110.3	124.4
Std. Deviation	25.27	27.78	32.44
Std. Error of Mean	6.317	6.946	8.109
Lower 95% CI of mean	69.98	95.51	107.2
Upper 95% CI of mean	96.91	125.1	141.7
Actual confidence level	97.87%	97.87%	97.87%
Lower confidence limit	69.21	81.53	100.1
Upper confidence limit	101.6	134.6	155.1
KS normality test			
KS distance	0.09907	0.09985	0.1379
P value	0.9972	0.9430	0.8667
Passed normality test (alpha=0.05)?	Yes	Yes	Yes
P value summary	ns	ns	ns
Sum	1335	1765	1991

Table 4.1.2.1d: ANOVA test for analysing data of study2.

Repeated measures ANOVA summary					
Assume sphericity?	No				
F	30.26				
P value	< 0.0001				
P value summary	****				
Statistically significant (P < 0.05)?	Yes				
Geisser-Greenhouse's epsilon	0.72				
R square	0.6686				
Was the matching effective?					
F	8.741				
P value	< 0.0001				
P value summary	****				
Is there significant matching (P < 0.05)?	Yes				
R square	0.5916				
ANOVA table					
	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between columns)	13874	2	6937	F (1.440, 21.60) = 30.26	P < 0.0001
Individual (between rows)	30058	15	2004	F (15, 30) = 8.741	P < 0.0001
Residual (random)	6877	30	229.2		
Total	50810	47			
Data summary					
Number of treatments (columns)	3				
Number of subjects (rows)	16				

Table 4.1.2.1e: 2way ANOVA multiple comparisons for FN3K serum expression.

Number of families	1							
Number of comparisons per family	3							
Alpha	0.05							
Tukey's multiple comparisons test	Mean Diff.	95% CI of diff.	Significant ?	Summary				
entry vs. discharge	-26.86	-39.92 to -13.81	Yes	***		A-B		
entry vs. follow up	-40.99	-58.52 to -23.46	Yes	****		A-C		
discharge vs. follow up	-14.13	-24.24 to -4.016	Yes	**		B-C		
Test details	Mean 1	Mean 2	Mean Diff.	SE of diff.	n 1	n2	q	D F
entry vs. discharge	83.45	110.3	-26.86	5.027	16	16	7.557	15
entry vs. follow up	83.45	124.4	-40.99	6.749	16	16	8.590	15
discharge vs. follow up	110.3	124.4	-14.13	3.892	16	16	5.132	15

#### 4.1.2.2 Statistical analysis for placebo treated patients

The statistical analysis performed by using Graph-pad Prism 6 software showed non-significant differences in serum FN3K levels among patients treated with placebo, separated into 3 stages (entry discharge- follow up).

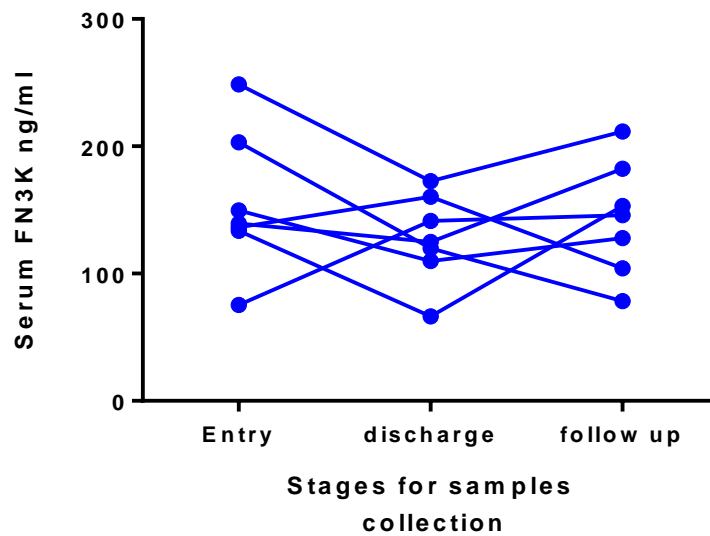


Figure 4.1.2.2a: Impact of placebo on FN3K expression in serum of patients treated with placebo separated by 3 stages (entry discharge- follow up) on x axis. It can be noticed that there is no significant change in FN3K expression among the three stages.

Figure 4.1.2.2a: shows the differences in mean FN3K levels  $\pm$  SD is plotted as a scatterplot to show the impact of placebo treatment in FN3K expression in each patient at all stages. It shows differences in the mean of serum FN3K at each stage: (155.1, 127.9 & 143.3) ng/ml for entry, discharge & follow up, respectively. The standard deviation was also increased ( $\pm$ 55.49,  $\pm$ 35.12,  $\pm$ 45.22) ng/ml for entry, discharge & follow-up periods respectively. From these data, it can be concluded that placebo is unlikely to affect FN3K expression in those patients. Therefore, when considering these together it is clearly shown that this difference is non-significant, which was confirmed in the statistical analysis with  $p=0.4692$ .

Table 4.1.2.2b summarises the descriptive statistics for measurement of FN3K levels in serum of patients treated with placebo separated by 3 stages (entry-discharge-follow up). Descriptive statistics of the separated cohort by 3 stages along with the  $p$ -value and a KS normality test were calculated in the analysis of the distribution of the three data sets. The KS normality test showed a normal distribution of the data for the entry, discharge and the follow up with non-significant  $p$  value for each (0.5214, 0.8411, 0.9934).

As we have FN3K serum levels values for each placebo-treated individual taken at each point, we do need to compare the mean of FN3K concentrations among each point of entry. This was performed by the two-way ANOVA test table 4.1.2.2c, the test showed ( $p=0.4692$ ), with  $R^2$  11.65%, Geisser-Greenhouse's epsilon test (to correct the degrees of freedom of the  $F$ -distribution) value of 0.9366, indicating a non-significant difference in FN3K expression between entry and discharge stages with non-homogenous variance between data in all two stages. When considering discharge, and follow up stages  $p=0.1697$  as with  $R^2$  45.1%, indicates a non-significant difference in FN3K expression between

discharge and follow up period. Then to test multiple variances table 4.1.2.2d a Tukey test with 95% confidence interval was used and this result showed a non-significant difference in FN3K levels between all stages. This indicates that placebo treatment has no effects on FN3K expression (figure 4.1.2.2a).

Table 4.1.2.2b: Descriptive statistics of the separated cohort by stages (entry-discharge-follow up) for treated patients with placebo together with the p-value and KS normality test calculated in the analysis of the distribution of the three data sets.

	Entry	discharge	follow up
Number of values	7	7	7
Minimum	75.4	66.44	78.44
25% Percentile	133.5	109.9	104.1
Median	139.3	124.8	145.8
75% Percentile	203.1	160.4	182.4
Maximum	248.5	172.5	211.5
Mean	155.1	127.9	143.3
Std. Deviation	55.49	35.12	45.22
Std. Error of Mean	20.97	13.28	17.09
Lower 95% CI of mean	103.8	95.44	101.5
Upper 95% CI of mean	206.5	160.4	185.1
95% CI of median			
Actual confidence level	98.44%	98.44%	98.44%
Lower confidence limit	75.4	66.44	78.44
Upper confidence limit	248.5	172.5	211.5
Sum	1086	895.5	1003
KS normality test			
KS distance	0.2532	0.1615	0.1292
P value	0.5214	0.8411	0.9934
Passed normality test (alpha=0.05)?	Yes	Yes	Yes
P value summary	ns	ns	ns

Table 4.1.2.2 c: Two-way ANOVA test for analysing data of study2

Number of comparisons per family	3							
Alpha	0.05							
Tukey's multiple comparisons test	Mean Diff.	95% CI of diff.	Significant?	Summary				
Entry vs. discharge	27.22	-37.58 to 92.01	No	ns		A-B		
Entry vs. follow up	11.84	-62.49 to 86.17	No	ns		A-C		
discharge vs. follow up	-15.37	-75.12 to 44.37	No	ns		B-C		
Test details	Mean 1	Mean 2	Mean Diff.	SE of diff.	n 1	n2	q	DF
Entry vs. discharge	155.1	127.9	27.22	21.12	7	7	1.823	6
Entry vs. follow up	155.1	143.3	11.84	24.22	7	7	0.6913	6
discharge vs. follow up	127.9	143.3	-15.37	19.47	7	7	1.117	6

Table 4.1.2.2d: Two-way ANOVA multiple comparison for FN3K serum expression of placebo.

Table analysed	Placebo				
Repeated measures ANOVA summary					
Assume sphericity?	No				
F	0.7913				
P value	0.4692				
P value summary	ns				
Statistically significant (P < 0.05)?	No				
Geisser-Greenhouse's epsilon	0.9366				
R square	0.1165				
Was the matching effective?					
F	1.860				
P value	0.1697				
P value summary	ns				
Is there significant matching (P < 0.05)?	No				
R square	0.4510				
ANOVA table	SS	D F	MS	F (DFn, DFd)	P value
Treatment (between columns)	2607	2	1303	F (1.873, 11.24) = 0.7913	P = 0.4692
Individual (between rows)	18381	6	3063	F (6, 12) = 1.860	P = 0.1697
Residual (random)	19767	12	1647		
Total	40754	20			
Data summary					
Number of treatments (columns)	3				
Number of subjects (rows)	7				

#### 4.1.2.3 Statistical analysis for placebo vs metformin treated patients

In order to confirm the impact of metformin on FN3K expression, a multiple comparison (two-way ANOVA) for FN3K expression was made between patients with metformin treatment with those with placebo. Figure 4.1.2.3a

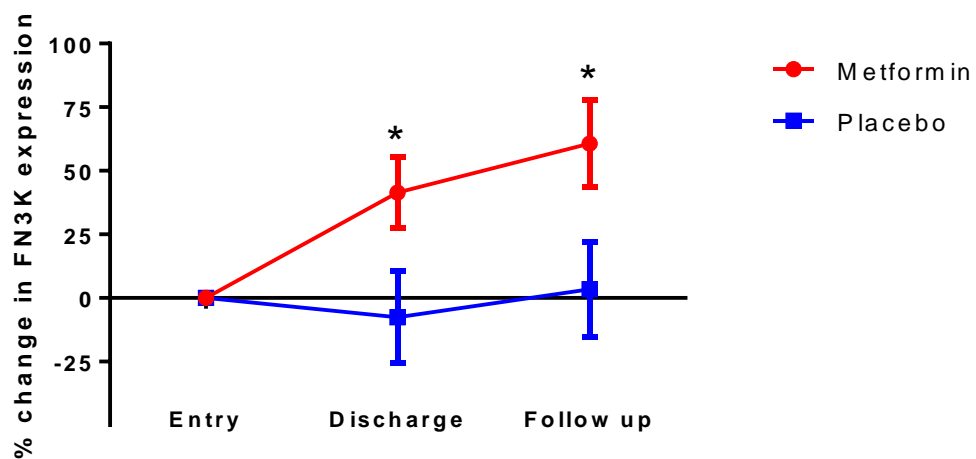


Figure 4.1.2.3a: Impact of metformin versus placebo on mean $\pm$ SD serum FN3K levels in AECOPD (\* $p$ <0.05 relative to placebo at each sampling point).

Figure 4.1.2.3a: shows the differences in mean FN3K levels +/- SD for the metformin treatment vs. placebo. It shows the differences in the mean of serum FN3K at each stage (entry, discharge & follow up) and it shows that these differences are significant at the discharge and follow up stages, which suggests that metformin has a relative impact of on FN3K serum expression in AECOPD patients. This was show as p <0.05.

Table 4.1.2.3b summarize this comparison by using two-way ANOVA test for multiple comparisons. The statistical analysis showed a significant difference between expressions of FN3K in metformin treatment at discharge vs. follow up stage compared with placebo figure 4.1.2.3a. For more powerful analysis the data was additionally analysed by Holm-Sidak method to allocate the differences at what stage table 4.1.2.3c. These results indicate that metformin may reduce this risk in COPD by raising FN3K levels.

Table 4.1.2.3b: Two-way ANOVA multiple comparisons for FN3K serum expression in metformin vs placebo.

Compare each cell mean with the other cell mean in that row.									
Number of families	1								
Number of comparisons per family	3								
Alpha	0.05								
Sidak's multiple comparisons test	Mean Diff.		95% CI of diff.	Significant?	Summary				
metformin - Placebo									
Entry	0.0		- 53.14 to 53.14	No	ns				
Discharge	49.01		- 4.133 to 102.2	No	ns				
Follow up	57.26		4.117 to 110.4	Yes	*				
Test details	Mean 1		Mean 2	Mean Diff.	SE of diff.	N1	N2	t	DF
metformin - Placebo									
Entry	0.0		0.0	0.0	21.67	16	7	0.0	63
Discharge	41.44		- 7.571	49.01	21.67	16	7	2.262	63
Follow up	60.69		3.429	57.26	21.67	16	7	2.643	63

Table 4.1.2.3c One unpaired *t* test (Holm-Sidak method) of FN3K expression for metformin vs placebo

	Significant?	P value	Mean 1	Mean 2	Difference	SE of difference	t ratio	df
Entry		1.0	0.0	0.0	0.0	21.6651	0.0	63.0
Discharge	*	0.0271491	41.4375	-7.57143	49.0089	21.6651	2.26211	63.0
Follow up	*	0.0103579	60.6875	3.42857	57.2589	21.6651	2.64291	63.0

## 4.2 Discussion

When analyzing the results of this study (figure 4.1.2.1b), there was a very significant difference in FN3K serum concentration when comparing FN3K serum concentration among all three stages (entry, discharge and follow up) with  $p < 0.0001$  for FN3K concentration in entry vs discharge &  $p < 0.0001$  for discharge vs. follow up. When performing multiple comparisons using an ANOVA test, there was a very significant difference in FN3K concentration among all stage, with highest mean at the follow up and lowest at the entry (table 4.1.2.1d). This means the admission of metformin increase the concentration of FN3K in AECOPD patients.

When considering placebo admission, the results showed a non-significant difference in FN3K levels after placebo treatment among all stages (entry, discharge, and follow up). When applying a Tukey post-hoc test with 95% confidence interval (table 4.1.2.2c) the result showed a non-significant difference in FN3K levels between all stages. This indicates that placebo treatment has no effects on FN3K expression (figure 4.1.2.2b).

The above result encouraged us to re-analyze the data by applying a multiple comparison of FN3K serum expression for metformin vs placebo treatment at all stages by using a two-way ANOVA test for multiple comparison. This showed a significant difference between expressions of FN3K in metformin treated patients at discharge and follow up stage compared with placebo-treated patients' (figure 4.1.2.3a). This means metformin has an impact on FN3K serum concentration, which might be attributed to a defensive mechanism against protein glycation in COPD, or as a part of its role in reducing the frequency and severity of diabetes related

complications along with formation of AGE (Beisswenger & Lopez, 2003 and Stirban *et al.*, 2014).

This study was designed to investigate the effect of metformin treatment on FN3K enzyme expression in AECOPD patients. We found that the serum FN3K concentrations increased with metformin treatment. This increase in FN3K concentration continued throughout the metformin treatment. Moreover, this relationship between metformin treatment and FN3K serum concentration was confirmed when we measured FN3K concentration in patients who underwent placebo treatment and found no correlation between placebo treatment and serum FN3K concentration. Thus, metformin treatment may raise FN3K concentration in patients with COPD as part of its beneficial effect in decreasing diabetes related complication (Zhou *et al.*, 2001).

Despite the known mechanisms behind the beneficial effects of metformin on protein glycation, it is still not clear how it has an impact on FN3K action in COPD patients. However, several authors have reported a role of metformin in inhibition of protein glycation through inhibition of AGEs formation (Beisswenger & Lopze, 2003, Tanaka *et al.*, (1997), Ishibashi *et al.*, 2012, and Ishibashi *et al.*, 2013).

The main effect of metformin is that of reducing hepatic glucose production throughout activation of AMPK, which is a major cellular regulator for glucose and lipid metabolism (Zhou *et al.*, 2001). Moreover, various authors have shown the beneficial effects of metformin administration in inhibiting adipose tissue lipolysis, decreasing formation of AGEs, reducing circulating levels of free fatty acids, and improving insulin sensitivity (Kirpichnikov *et al.*, 2002). Furthermore, metformin lowers blood pressure in patients with type2 diabetes and impaired glucose

tolerance (Verma *et al.*, 1994). Metformin was also reported to decrease morbidity and mortality rates in patients with type 2 diabetes when administered alone or with sulphonylurea (McDonald *et al.*, 2010 & Eurich and McAllister, 2010).

On the other hand, AGE is considered as one of the main pathogenic factors of diabetic vascular complications (Yamagishi *et al.*, 2005). AGEs are formed from non-enzymatic glycation of amino groups in proteins *in vivo*, which then produces highly reactive carbonyl groups (alpha-dicarbonyls or oxoaldehydes), including 3-deoxyglucosone, glyoxal, and methylglyoxal (Brownlee *et al.*, 2006).

As the methylglyoxal (MG) is one of the main sources of formation of AGEs, any changes in MG levels can alter AGE formation (Beisswenger *et al.*, 1995, Monnier *et al.*, 1986 and Biesswenger *et al.*, 1997). Metformin is thought to decrease AGE formation by reducing MG levels in the tissues of diabetic patients (Beisswenger *et al.*, 1999), which in turn decreases the incidence of diabetic vascular and neuropathic complications (Monnier *et al.*, 1986 and Biesswenger *et al.*, 1997). Metformin also decrease AGE levels in animal tissues such as in the eye lens, kidney and nerves of diabetic animals (Tanaka *et al.*, 1999). It is reported that metformin can reduce MG levels by formation of specific condensation products that result from the reaction of metformin with MG; an example of those compounds being triazepinone (TZP) (Ruggiero-Lopez *et al.*, 1999).

In a study by Zhao *et al.*, (2014) on rats, it was found that metformin significantly restored AGE-impaired IKca and Skca (intermediate-conductance and small-conductance Ca (2+)-activated potassium channels)-mediated vasodilators in mesenteric arteries of Streptozotocin-induced type 2 diabetic rats.

One of the important mechanisms by which AGEs contribute to diabetic complications is related to tissue inflammation and oxidative stress (Cai *et al.*, 2002). It is widely accepted that oxidative stress is a major aetiological factor in the pathogenesis of COPD (Mirrakhimov, 2012). Both oxidative stress and hyperglycaemia can accelerate the production of AGEs (Schmidt *et al.*, 2001).

As FN3K has been demonstrated to decrease levels of AGEs in Chapter 3 and the increase in oxidative stress levels in COPD patients leads to the formation of carbonyl stress that is linked to AGEs formation, it is therefore axiomatic that this increase in oxidative stress decreases FN3K expression. In summary, metformin treatment, which has been proved by many studies to inhibit glycation and formation of AGE, may enhance the expression of FN3K, and as a protective mechanism act against deleterious effects of oxidative stress, as was demonstrated in this study. Furthermore, metformin was reported to have more effects in reducing oxidative stress in COPD patients than lifestyle modifications (Esteghamati *et al.*, 2013).

## Chapter 5

### Measurement of FN3K protein levels in COPD airways

## 5.1 Results of study 3 measurement of FN3K expression in human peripheral lung tissue

The statistical analysis was performed using GraphPad Prism software; figure 5.1a shows FN3K expression in airway epithelial cells.

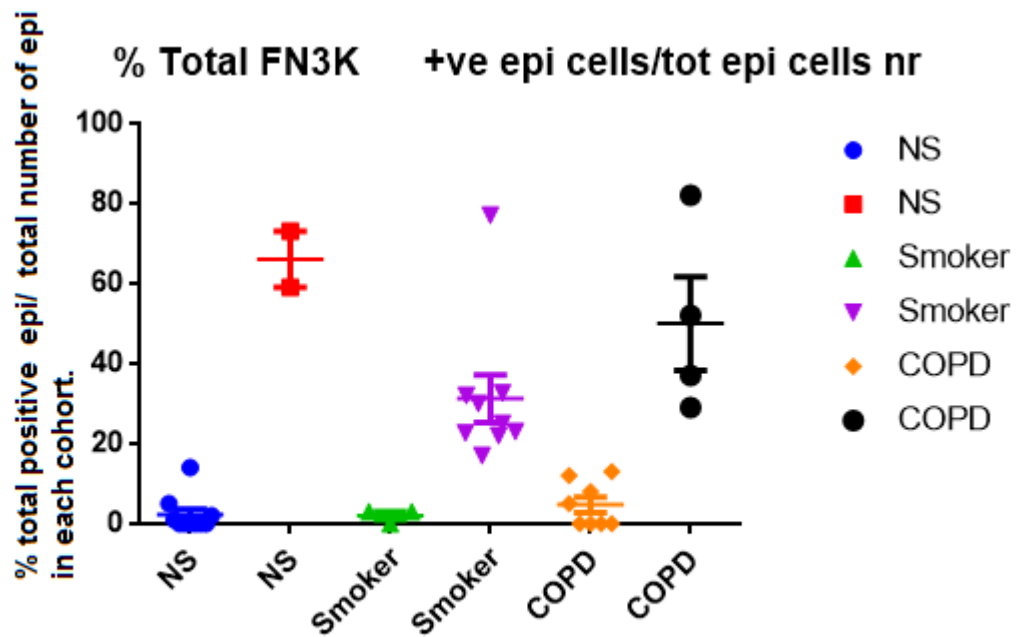
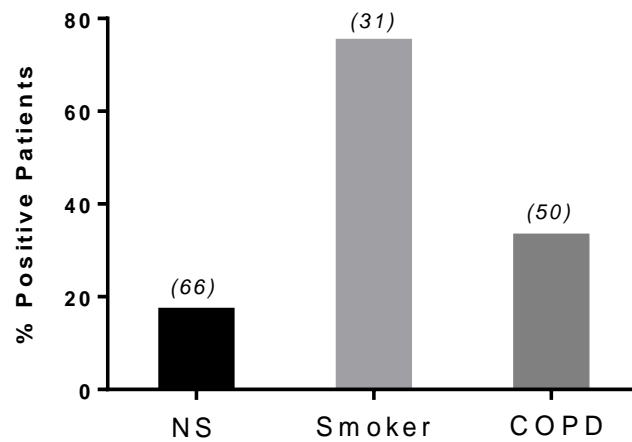


Figure 5.1a: FN3K expression in airway epithelial cells. Data were also used to calculate the percentage of positive (high) FN3K expressers.

Y-axis: percentage of epithelial cells staining positive for FN3K (epi) /total number of epithelial cells (epi) in each cohort.

**Frequency of subjects positive for FN3K expression  
in the airways**



Positive expression is present when >15% of cells stain positive  
*Numbers in brackets indicate % of cells staining positive for FN3K*

**NB: Subjects negative for FN3K expression had < 4% of cells staining positive for FN3K**

Figure 5.1b: Percentage of subjects positive for FN3K expression in airways. This figure was prepared by plotting the percentage of patients who were FN3K positive (as the number of FN3K patients' positive / total number patients).

Immunohistochemistry of bronchial biopsies was used to determine expression of FN3K in airway epithelial cells. In each cohort (healthy non-smokers, control smokers and patients with COPD) individuals were grouped in one of two categories, low or high FN3K expressers as described in figure 5.1a. In healthy non-smokers, 83% (10 of 12) of subjects expressed FN3K at low levels with only  $2.2 \pm 1.4\%$  of airway epithelial cells expressing FN3K. In contrast, 75% (9 of 12) of smokers exhibited a significant increase in FN3K expression with  $31.3 \pm 6.3\%$  of epithelial staining positive for FN3K. However, in the COPD group, 67% (8 of 12) of subjects had low levels of FN3K expression with only  $4.1 \pm 2\%$  of epithelial cells expressing FN3K (see table 5.1d). On the other hand, with regard to the percentage of subjects positive for FN3K expression in airways, the highest expression of FN3K is clearly observed in the control smokers followed by COPD patients and the healthy non-smokers (figure 5.1b).

Table 5.1c shows the descriptive statistics for the study. The Shapiro-Wilk normality test was applied and showed that the data were not normally distributed, with non-significant P-values ( $P < 0.0001$  for non-smokers,  $P = 0.0222$  for control smokers and  $P = 0.0084$  for COPD patients). Table 5.1d shows the descriptive statistics for the two categories in each cohort (high and low percentage FN3K expression, see figure 5.1a). In the newly categorised groups, the data were not normally distributed (table 5.1d).

In order to compare FN3K expression among all groups, a one-way ANOVA for multiple comparisons was applied (table 5.1e) and showed non-significant differences among all groups ( $P = 0.5222$ ). When applying the Tukey test for multiple comparison (table 5.1f) we also found a non-significant difference in FN3K expression among the three cohorts. However, the highest mean FN3K expression

was observed in the control smokers group (table 5.1c). This can be explained if FN3K expression is upregulated in the airways of smokers, presumably as a protective mechanism to prevent or limit the impact of increased carbonyl stress as a result of increased exposure to oxidative stress from cigarette smoking. In COPD, however, this protective mechanism of increased FN3K expression is lost. This can be confirmed in (table 5.1g) by categorising the individuals in each cohort into two categories high and low FN3K expressers. This conclusion confirms our hypothesis that patients with COPD may have low FN3K expression due to certain factors (oxidative and chronic inflammation).

Table 5.1c: Descriptive statistics for study 3.

	Non-smokers	Control smokers	COPD
n	12	12	12
Minimum	0.0	0.0	0.0
25% Percentile	0.0	6.500	0.0
Median	0.5000	22.85	10.00
75% Percentile	11.75	31.50	35.00
Maximum	73.00	77.00	82.00
Mean	12.83	23.95	19.83
Std. Deviation	25.33	20.22	25.80
Std. Error of Mean	7.313	5.838	7.447
Lower 95% CI of mean	-3.263	11.10	3.443
Upper 95% CI of mean	28.93	36.80	36.22
Shapiro-Wilk normality test			
W	0.5780	0.8320	0.7960
P value	< 0.0001	0.0222	0.0084
Passed normality test (alpha=0.05)?	No	No	No
P value summary	****	*	**
Sum	154.0	287.4	238.0

Table 5.1d: Descriptive statistics for the two categories (high and low FN3K expressers) in study 3. NS low (non- smokers/low FN3K expressers group), NS high (non-smokers /high FN3K expressers), CS low (controlled smokers/low FN3K expressers group), CS high (controlled smokers/high FN3K expressers group), COPD low (COPD patients with low FN3K expression group) and CS high (COPD Patients with high FN3K expression group).

	NS low	NS high	CS low	CS high	COPD low	COPD high
Total number of values	10	2	3	9	8	4
Number of excluded values	0	0	0	0	0	0
Number of binned values	10	2	3	9	8	4
Minimum	0	59	0	17	0	29
25% Percentile	0	59	0	22.35	0	31
Median	0	66	3	25	2.5	44.5
75% Percentile	2.75	73	3	32.35	11	74.5
Maximum	14	73	3	77	13	82
Mean	2.2	66	2	31.26	4.16	50
Std. Deviation	4.44	9.89	1.73	17.90	5.62	23.36
Std. Error of Mean	1.40	7	1	5.96	1.98	11.68
Lower 95% CI of mean	-0.97	- 22.94343	-2.30	17.49	0.047	12.818456
Upper 95% CI of mean	5.37	154.94	6.30	45.03	9.45	87.18

Table 5.1e: One-way ANOVA test for study 3 data.

Table analysed	% total FN3K +ve epi cells/tot epi cells nr				
ANOVA summary					
F	0.6626				
P-value	0.5222				
P-value summary	ns				
Are differences among means statistically significant (P<0.05)?	No				
r <sup>2</sup>	0.03861				
Brown–Forsythe test					
F (DFn, DFd)	0.2244 (2, 33)				
P-value	0.8002				
P-value summary	ns				
Significantly different SD (P<0.05)?	No				
Bartlett's test					
Bartlett's statistic (corrected)	0.7334				
P-value	0.6930				
P-value summary	ns				
Significantly different SD (P<0.05)?	No				
ANOVA table	SS	DF	MS	F (DFn, DFd)	P-value
Treatment (between columns)	758.1	2	379.1	F (2, 33)=0.6626	P=0.5222
Residual (within columns)	18879	33	572.1		
Total	19637	35			
Data summary					
Number of treatments (columns)	3				
Number of values (total)	36				

Table 5.1f: Tukey's multiple comparison test for study 3 data.

	1							
Number of comparisons per family	3							
Alpha	0.05							
Tukey's multiple comparisons test	Mean Diff.	95.% CI of diff.	Significant?	Summary	Adjusted P Value			
Non-smokers vs. Control smokers	-11.12	-35.08 to 12.84	No	ns	0.4977	A-B		
Non-smokers vs. COPD	-7	-30.96 to 16.96	No	ns	0.7553	A-C		
Control smokers vs. COPD	4.117	-19.84 to 28.08	No	ns	0.9070	B-C		
Test details	Mean 1	Mean 2	Mean Diff.	SE of diff.	n1	n2	q	DF
Non-smokers vs. control smokers	12.83	23.95	-11.12	9.765	12	12	1.61	33
Non-smokers vs. COPD	12.83	19.83	-7	9.765	12	12	1.014	33
Control smokers vs. COPD	23.95	19.83	4.117	9.765	12	12	0.5962	33

Table 5.1g: One-way ANNOVA for multiple comparisons with Dunn's test for each category test in each cohort of study 3 data.

Number of families	1				
Number of comparisons per family	3				
Alpha	0.05		2		
Dunn's multiple comparisons test	Mean rank diff.	Significant ?	Summary		
NS vs. NS	-23.65	Yes	**		A-B
Smoker vs. Smoker	-15.22	No	ns		C-D
COPD vs. COPD	-19.31	Yes	**		E-F
Test details	Mean rank 1	Mean rank 2	Mean rank diff.	n 1	n2
Non-smoker vs. Non -smoker	9.85	33.5	-23.65	10	2
Smoker vs. Smoker	11.67	26.89	-15.22	3	9
COPD vs. COPD	12.19	31.5	-19.31	8	4

## 5.2 Discussion

In the preceding chapter we explored the correlation between metformin treatment and FN3K serum concentration in AECOPD patients. The results showed that FN3K concentration was enhanced by metformin treatment with time. In this chapter, the statistical analysis using one-way ANOVA showed non-significant differences in FN3K expression among three groups: healthy non-smokers, control smokers and COPD patients ( $P=0.522$ ,  $r^2=0.03861$ ; table 5.1.e; figure 5.1b). However, there was a slight difference in the mean FN3K expression which was highest in control smokers followed by COPD patients then non-smokers (23.95, 19.83 and 12.83 respectively). This difference can be explained by upregulation of FN3K enzyme expression in the airways of smokers, presumably as a protective mechanism to prevent or limit the impact of increased carbonyl stress as a result of increased exposure to oxidative stress from cigarette smoking. In COPD, however, this protective mechanism of increased FN3K expression is lost.

It has been demonstrated that chronic inflammation, as a result of inhaled particles during smoking or from other sources, causes disruption in the normal repair and defence mechanism thus limiting airflow (Chung, 2005). In addition, AGEs and carbonyl stress formations can be resulted from the presence of both oxidative stress and systemic inflammation (Gopal *et al.*, 2014 & Goldin *et al.*, 2006), and FN3K is assumed to be the enzyme that deglycates glycated proteins in erythrocytes and other tissue including the lung (Delpierre *et al.*, 2000). Thus, in our study the mean FN3K expression in control healthy smokers is higher than that of COPD patients possibly due to the presence of two factors: chronic inflammation and oxidative stress in these COPD patients. Both factors lead to the formation of AGEs

which might affect FN3K expression due to the inability of FN3K to deglycate all elevated AGE levels. Another reason might be the fact that the level of NRF2 (which regulates the expression of antioxidant proteins that protect against oxidative stress) is decreased in COPD patients and therefore the level of oxidative stress is high (Boutten *et al.*, 2011). In healthy smokers, however, it seems that the FN3K enzyme expression might be enhanced to resist protein damage caused by chronic inflammation as a result of smoking. Thus, this enhances the expression of FN3K enzyme to resist disruption of the normal repair defence mechanism. Therefore, mean FN3K expression in control smokers was higher than in COPD patients due to the fact that in control smokers only one deleterious factor was developed (systemic inflammation) whereas in COPD patients, two factors were developed (chronic inflammation and oxidative stress). This explains why the mean FN3K expression was in COPD patient lower than that of smokers as the FN3K cannot overcome the presence of these two factors together in those COPD patients (see figure 1.4.8). However, one of the main limits in this study is the sample size, and perhaps using more samples of human peripheral lung tissue to detect FN3K expression might either lead to a different conclusion or confirm the present findings.

As mentioned in chapter 1, smoking is an important risk factor for diabetes and COPD, causing an increase in serum levels of inflammatory markers (Arcavi and Benowitz, 2004; Gan *et al.*, 2005). Moreover, smoking was demonstrated to contribute to cardiovascular events via induction of endothelial dysfunction. Cigarette smoking is linked to formation of oxidative stress which is widely considered to be a mediator of endothelial dysfunction (Morrow *et al.*, 1995; Cai and Harrison, 2000; Sambola *et al.*, 2003). ). Endothelial dysfunction itself is widely considered to be an

important precursor for other cardiovascular complications in diabetes (Green and Turner, 2017; Meigs, *et al.*, 2004; Sena *et al.*, 2013; Hadi and Al Suwaidi, 2007).

## **Chapter 6**

### **Final discussion and conclusion**

## 6.1 Discussion

The International Diabetes Federation (IDF) estimated that 415 million people around the world had diabetes in 2015 and this number was projected to increase to 642 million by 2040 (IDF, 2015). Several studies have shown an association between diabetes and COPD; the prevalence of diabetes among COPD patients is as high as 18% (Cazzola *et al.*, 2010). The presence of diabetes has been reported to be associated with poorer lung function (Van den Borst *et al.*, 2010) with smoking being a known risk factor for developing type 2 diabetes in the absence of COPD (Xie *et al.*, 2009). Moreover, smoking has been considered as a risk factor for both diabetes and COPD (Manson *et al.* 2000; Rimm *et al.* 1995). Diabetes, hypertension, ischaemic heart disease and heart failure are the main comorbidities associated with COPD (Moussas *et al.*, 2008). Several studies have reported that type 2 diabetes is a risk factor for macrovascular disease (Fowler, 2008), and COPD outcome might be altered by some of the effects of diabetes which may be related to increased cardiovascular morbidity and mortality. In addition, the lung itself is targeted by microvascular diseases (Mirrakhimov, 2012). Glycation is defined as a non-enzymatic reaction by which carbohydrates (such as glucose) can bind to proteins (Popova *et al.*, 2010).

AGEs are formed via non-enzymatic glycation. Their presence was reported to be associated with complications related to diabetes and ageing and have deleterious effects on several tissues such as skin and bones (Gkogkolou & Bohm 2012; Schurman *et al.*, 2008). It has been reported that an increase in the levels of glucose and its transporter (GLUT-1) can also glycate proteins in other cells such as those of the vascular endothelium which are unable to regulate intracellular glucose. Such glycation is a possible contributory factor in the development of complications

associated with diabetes such as CVD and COPD (Sing *et al.*, 2014). Glycation is controlled by an enzymatic process thought to involve a key enzyme (FN3K) which can deglycate the glycated haemoglobin in red blood cells and other glycated proteins in other tissues (Szwergold *et al.*, 2002). The main aim of our research is to investigate the role of FN3K enzyme in diabetes related complications.

The G-gap has been reported to be associated with an alteration of the risk of diabetic complications; a positive G-gap is defined as a higher level of glycation of proteins than expected whereas a negative G-gap denotes a lower than expected level of glycation (Nayak *et al.*, 2013). Therefore, it was assumed that a positive G-gap correlates with a higher incidence of diabetes-related complications, whereas a negative G-gap in diabetic patients correlates with lower incidence of such complications (Cohen, *et al.* 2003, Nayak, *et al.*, 2013).

No previous study has investigated the possible correlation between FN3K activity or expression and G-gap values. Few previous studies have measured the activity and expression of FN3K in human and animal models. Very few studies have reported the reasons behind the variations in the activity of FN3K among individuals; these reasons were owe to presence of two possible polymorphisms in FN3K gene (Delpierre *et al.*, 2006, Mohas *et al.*, 2010, and Tanhäuserová *et al.*, 2014). Furthermore, none of the previous studies has highlighted any possible correlation between metformin treatment and FN3K expression in either COPD or diabetic patients though it has been reported that metformin inhibits both glycation and AGE formation (Beisswenger & Lopze, 2003; Tanaka *et al.*, 1997; Ishibashi *et al.*, 2012; Ishibashi *et al.*, 2013). Finally, few studies have examined the relationship between variations in FN3K activity and AGE levels (Skhra *et al.*, 2014). Thus, this is the first study to highlight the role of FN3K in diabetes, to investigate any correlation between

FN3K and G-gap as an indicator of the risk of diabetes-related complications, and to explore the role of FN3K in COPD patients.

We have described for the first time a significant relationship between FN3K activity or levels and G-gaps, showing that erythrocyte activity and level of FN3K are inversely correlated with G-gaps. This striking result leads to new questions about the role of FN3K in diabetes-related complications as this interesting relationship between FN3K and G-gap values has not been investigated previously.

Moreover, the significant relationship between G-gap and plasma AGEs as show in figure 3.1.5b has provided further confirmation for the role of FN3K as a deglycating enzyme as the results of the AGE assay showed a direct correlation between AGEs and G-gaps, both correlating inversely with FN3K activity (study 1 parts 1 and 2).

However, results from the FN3K plasma assay showed a non-significant correlation between G-gap and plasma FN3K level because the G-gap value reflects the intensity of glycation inside the erythrocytes as the value of HbA1c is a principal measurement in the calculation of G-gaps (Nayak *et al.*, 2013).

No previous studies have examined the correlation between G-gaps and markers of endothelial dysfunction, therefore one of the aims of the present study was to explore the possibility of a correlation between G-gaps and markers of endothelial dysfunction which is considered an important precursor for other diabetes-related cardiovascular complications (Hadi & Al Suwaidi, 2007; Meigs *et al.*, 2004; Sena *et al.*, 2013). Although we showed non-significant differences in plasma thrombomodulin and E-selectin levels between positive and negative G-gaps (chapter 3), the PAI-1 assay showed a very significant difference in plasma PAI-1

between negative and positive G-gaps (figure 3.2.3b,  $p < 0.0001$ ). As mentioned in chapter 1, PAI-1 is also considered a marker of endothelial damage and partially controls inhibition of fibrinolysis so that it is linked to thrombosis (Nadar *et al.*, 2004). Therefore, the results of the PAI-1 assay suggest that patients with positive G-gaps are more likely to develop endothelial damage and/or thrombosis and tend to develop CVD complications associated with diabetes. It would have been assumed that these two markers of endothelial dysfunction (E-selectin and thrombomodulin) are increased with positive G-gaps but the results did not support this assumption (Cohen, *et al.* 2003; Nayak, *et al.*, 2013) suggesting that the G-gap is not associated with the early phases of development of CVD complications and/or these two markers are not enough to support this assumption. Perhaps investigating a larger cohort might demonstrate a link between endothelial dysfunction and G-gap on the one hand and FN3K on the other.

The results of the PAI-1 assay are consistent with the previous finding that individuals with positive G-gaps have a high risk of diabetes-related complication such as endothelial dysfunction, taking into consideration the fact that PAI-1 is a well-established marker of endothelial dysfunction. This contradiction between the PAI-1 and the E-selectin and thrombomodulin assay results suggests that the change in G-gap values might not affect the risk of endothelial dysfunction or that these two markers (E-selectin and thrombomodulin) might not reflect such correlation between positive G-gap and endothelial damage. By contrast, statistical analysis showed a highly significant difference in PAI-1 levels between the two G-gap groups can overcome the debate arising from both E-selectin and thrombomodulin results.

When examining the results of study 2, the statistical analysis showed very strong correlations between metformin treatment and FN3K serum levels in AECOPD patients for metformin vs. placebo treatment (figure 4.1.2.3a,  $p < 0.05$ ). This suggests that metformin may enhance FN3K levels in COPD patients; however how metformin acts on the deglycation process dictated by FN3K remains controversial. Even though metformin reduced MG levels, it can inhibit the formation of AGEs and thereby inhibit the glycation reaction. The underlying mechanism of this role is still not completely understood (Beisswenger *et al.*, 1999; Tanaka *et al.*, 1999). However, treatment with metformin was also shown to decrease oxidative stress (Esteghamati *et al.*, 2013), a key aetiological factor in the pathogenesis of COPD through which in turn formation of AGEs is decreased (Kirkham *et al.*, 2013). This is the first time that the correlation between metformin treatment and FN3K expression has been investigated. Our findings could suggest potential clinical approaches. Future studies are required to explore the link between metformin and FN3K activity and expression, and how to control FN3K expression in COPD patients in a way to prevent diabetes-related CVD complications.

With regards to the findings of study 3, FN3K expression was slightly increased in healthy control smokers compared to subjects with stable COPD. This might be due to an inability of FN3K to initiate protective mechanisms to resist the damage caused by chronic inflammation plus oxidative stress among those patients with stable COPD. This damage results in development of carbonyl stress that contributes to the formation of AGEs (Gopal *et al.*, 2014 & Goldin *et al.*, 2006). Moreover, in COPD the level of NRF2 (which regulates the expression of antioxidant proteins that protect against oxidative stress) declines, which raises the level of oxidative stress in patients (Boutten *et al.*, 2011). Whereas, FN3K expression in

control smokers was higher than in COPD patients due to the fact that in control smokers only one deleterious factor was developed (chronic inflammation) whereas in COPD patients, two factors were developed (chronic inflammation and oxidative stress). Therefore the FN3K enzyme was enhanced to resist disruption of the normal repair defence mechanism in control smokers. Further studies of this pathway are needed in COPD patients to investigate factors, especially genetic factors that regulate FN3K expression and their control.

Most studies on the FN3K were based on identifying its biochemical role either *in vitro* or *in vivo* using human or animal models rather than its involvement in diabetes and associated comorbidities (Delpierre *et al.*, 2000; Delplanque *et al.*, 2004; Monnier *et al.*, 2006; Szwegold *et al.*, 1990; Szwegold *et al.*, 2002; Veiga-da-Cunha *et al.*, 2006).

## 6.2 Conclusion

The primary aims of the present research were to answer the questions posed in chapter 1 that were raised through reviewing the literature and to investigate several issues that have not been explored previously. Thus, we sought to explore the role of FN3K in the development of diabetes-related complications by understanding the process through which the enzyme utilises the deglycation process. This was achieved by measuring FN3K activity and level with relation to G-gaps in diabetic patients and also by measuring levels of markers of endothelial dysfunction. The results demonstrated that FN3K plays an important role in the protein repair system which protects against damage caused by non-enzymatic glycation. Thus, patients with positive G-gaps exhibit lower FN3K activity than those with negative G-gaps, which in turn they are more susceptible to develop diabetes-related complications. The role of FN3K in phosphorylating fructosamines situated on the third carbon of their sugar moiety makes them unstable and therefore, results in their detachment from proteins. This process provides protection against protein damage. Moreover, we demonstrated that high FN3K enzyme activity was associated with low levels of AGEs as well as low carbonyl stress levels among patients with diabetes and COPD.

The lack of significant differences found for plasma concentrations of both E-selectin and thrombomodulin between the positive and negative G-gap groups in this research does not mean that an association between ED and the G-gap does not exist. On the contrary, it paves the way for future studies to determine whether an association between ED and G-gap exists by using other methods for detecting ED, and whether the G-gap is a useful tool for predicting ED and other diabetic complications. The G-gap is potentially useful for reflecting variation in the

occurrence of some diabetes-related complications when its predictive value is identified.

On the other hand, COPD patients tend to have a low degree of protection by FN3K against protein damage in comparison to the general population. They tend to be at risk of developing more complications, particularly CVD complications, than normal healthy individuals.

Metformin was also proven to have beneficial effects in reducing protein damage caused by carbonyl stress resulting from increased exposure to oxidative stress from smoking cigarettes by enhancing FN3K action, presumably as a protective enzyme, in addition to its role in decreasing lipid and glucose levels.

### **6.3 Limitations**

One of the major limitations of this research is that the participants in study 1 were recruited from a single NHS trust hospital situated in Wolverhampton which limits how translatable the results are to a wider population. Furthermore, working with a small population decreases the probability of accounting for the influences of genetic and lifestyle factors that are more apparent among wider populations in multicentre research. The idea of limited genetic variability is confounded when considering that the ethnic background of the cohort is limited, with a mainly white population and a relatively unspecific ethnic background with unknown country of origin. In addition to these considerations, the collection of data from participants of a single centre is not an accurate depiction of a country's ethnic diversity and thus limits the ability to make appropriate interpretations about the impact that genetic variability has on the medical conditions of diabetic patients. Ethnicity is an important factor that affects the variable expression of a medical condition, as well as responses to antidiabetic

drug management. For instance, it was reported that approximately 60% of the global diabetic population were of Asian ethnicity (Hu, 2011; Oldroyd *et al.*, 2005).

Another disadvantage of working with the small sample size of patients recruited for this study is the introduction of gender bias. In the present study, there was a disproportionate number of males compared to females (92 versus 65;  $p < 0.05$ ). Consequently, the results cannot be generally applied to both genders in this analysis as some measurements are related to gender-specific variation to some degree; however, there was a consistent gender bias in both groups of study 1 so that they were comparable. The lack of an ethnically diverse sample population and the effects of gender bias may affect variations in G-gap values as the findings of several studies suggest that gender and ethnicity are likely to be associated with potential variation in G-gap values (Cohen *et al.*, 2006; Nayak *et al.*, 2011). Therefore, the validity for the cohort to represent the global population may be questioned.

Another limitation of study 1 is the lack of details regarding patient age and the amount of time since diagnosis of the disease. Both are important as the longer the duration of diabetes, the greater the exposure of a patient to uncontrolled blood glucose levels and the higher the likelihood of developing diabetes-related complications such as endothelial dysfunction.

Previous authors have demonstrated that G-gap values are affected by the type of diabetes (Cohen *et al.*, 2002; Nayak *et al.*, 2013). Although the majority of the patients in study 1 had Type 2 diabetes, some had Type 1 diabetes. It was not clear in this study precisely how many and which patients had type 1 diabetes.

Because most the participants in studies 1 and 2 were classified as type 2 diabetics, the antidiabetic drug therapy should be taken into consideration. In particular, drugs such as metformin, an antidiabetic agent which lowers glycaemic and lipid levels (see chapter 1), can lower HbA1c levels (Jager *et al.*, 2014). This in turn affects the G-gap value. Thus, metformin can be a confounding factor in usefulness of G-gap. Moreover, it has been shown that metformin has beneficial effects on cardiovascular events (Inzucchi, 2005; Lamanna *et al.*, 2011; Sasali & Leahy, 2003). In addition, metformin may decrease levels of markers of endothelial dysfunction by decreasing AGE levels (Jager *et al.*, 2014). Furthermore, metformin has been shown to have anti-inflammatory effects by reducing levels of pro-inflammatory cytokines which in turn improves endothelial function (Fidan, *et al.* 2011; Jager, *et al.* 2014). Therefore, metformin can be considered a limiting factor in study 1.

Another potential limitation is the use of several freeze–thaw cycles which may alter the activity and protein levels of FN3K as well as the levels of other measured proteins (i.e. markers of endothelial dysfunction) in relation to G-gap value over time.

## 6.4 Future studies

Larger studies are necessary to obtain a better understanding of the role of FN3K in the deglycation process, including studies involving the measurement of FN3K genetic variants and their correlation with diabetes progression, and how this variation can be utilised in the management of the disease and its complications. Observing whether polymorphisms of FN3K (SNPs) can predict diabetic complications would also provide valuable information. Furthermore, assessment of the correlation between the activity of FN3K, markers of endothelial dysfunction and G-gap values through comparison of negative, positive and neutral values may be informative.

For study 2, it was important to examine whether levels of AGEs were significantly correlated with FN3K levels in subjects treated with metformin. Studies of such correlations are ongoing at St George's University Hospital. Moreover, further studies are needed to determine whether FN3K expression correlates with improvements in other clinical parameters of CVD and whether individuals with a high expression of FN3K have less severe exacerbated episodes and/or comorbidity with diabetes and other vascular disorders. Finally, further studies need to be carried out to identify the mechanisms responsible for promoter(s) that have an impact on the FN3K gene and activity for catalysing deglycation in larger cohorts.

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## Appendices

### Appendix I

List of purchased materials for preparation of HEPES buffered erythrocyte lysate solution:

Name of item	SKU-Pack Size	Company name
HEPES ≥99.5% (titration)	H3375-100G	Sigma-aldrich
Leupeptin microbial, ≥90% (HPLC)	L2884-.5MG	Sigma-aldrich
Dithiotreitol DTT	D0632-1G	Sigma-aldrich
Antipain 50000 U/mg	10791-5MG	Sigma-aldrich
Sodium chloride BioXtra, ≥99.5% (AT)	S7653-250G	Sigma-aldrich
Adenosine 5'- triphosphate disodium salt hydrate	A7699-1G	Sigma-aldrich
Trichloroacetic acid	Trichloroacetic acid	Sigma-aldrich

## Appendix II

Study 2 patients recruitment information:

<b>Metformin in COPD: serum sample map</b>							CI:	Prof Emma Baker				
<b>Box ID: 5</b>		<b>Visit 1 (study entry)</b>						Study contact:	Dr Andrew Hitchings			
								<a href="#">[e-mail address redacted]</a>				
<b>Samples for Paul Kirkham, University of Wolverhampton</b>								[tel.no. redacted]				
								[tel no. redacted]				
							Return address:	Andrew Hitchings				
<b>Please refreeze and return residual serum</b>								Clinical Research Facility				
								St George's, Univ of London				
<b>Key:</b>	1	Participant number						Cranmer Terrace				
	JG	Participant initials						London SW17 0RE				
							United Kingdom					
		<b>1</b>	<b>2</b>	<b>3</b>	<b>4</b>	<b>5</b>	<b>6</b>	<b>7</b>	<b>8</b>	<b>9</b>	<b>10</b>	
<b>A</b>		1	2	3	4	5	17	18	29	36	37	<b>Visit 1 (study entry)</b>
		JG	GC	BA	MM	FH	BRL	RP	GB	JP	IS	
<b>B</b>		41	51	52	53	54	58	63	69	70	72	
		JC	RW	LC	NJ	PJ	DG	SW	VS	GP	NC	
<b>C</b>		73	74	75	76	77	78	79	80	81	82	
		RR	RF	WKP	BP	DR	DS	BG	RB	PE	IW	
<b>D</b>		83	87	93	94	95	96	97	101	102	103	
		LA	AR	CAM	KT	JC	CR	JL	LCC	DJ	JK	
<b>E</b>		105	106	107	108							
		TLB	MF	PN	MW							
<b>F</b>												
<b>G</b>												
<b>H</b>												
<b>I</b>												
<b>J</b>												

Metformin in COPD: serum sample map							CI:	Prof Emma Baker			
Box ID: 6	Visits 2 (discharge) and 3 (follow-up)						Study contact:	Dr Andrew Hitchings			
							[e-mail address redacted]				
Samples for Paul Kirkham, University of Wolverhampton								[tel. no. redacted]			
							[tel. no. redacted]				
							Return address:	Andrew Hitchings			
Please refreeze and return residual serum								Clinical Research Facility			
							St George's, Univ of London				
Key:	1	Participant number						Cranmer Terrace			
	JG	Participant initials						London SW17 0RE			
							United Kingdom				
	1	2	3	4	5	6	7	8	9	10	Visit 2 (hospital discharge 7days after entry)
A	2	4	5	17	35	37	41	51	52	53	
	GC	MM	FH	BRL	RPW	IS	JC	RW	LC	NJ	
B	54	58	63	70	74	75	76	77	78	79	
	PJ	DG	SW	GP	RF	WKP	BP	DR	DS	BG	
C	80	81	83	87	93	95	96	97	105	107	
	RB	PE	LA	AR	CAM	JC	CR	JL	TLB	PN	
D	108										
	MW										
E											
F	1	4	5	17	18	29	35	37	41	51	
	JG	MM	FH	BRL	RP	GB	RPW	IS	JC	RW	
G	52	53	54	58	69	70	73	74	75	76	
	LC	NJ	PJ	DG	VS	GP	RR	RF	WKP	BP	
H	77	78	79	80	83	93	95	96	97	100	
	DR	DS	BG	RB	LA	CAM	JC	CR	JL	JT	
I	101	105	106	107	108						
	LCC	TLB	MF	PN	MW						
J											

## Appendix III

### Conference presentations

1. Abstract: 0048-PD:

A. Alderawi, S. Dunmore, A. Majebi, J. Brown, A. Narshi, A. Nayak, B. Singh. Risk markers of macrovascular disease are related to the "Glycation Gap" in patients with diabetes. DOI: 10.13140/RG.2.1.3426.6961 Conference: World Diabetes Congress-International diabetes federation, At Vancouver.

2. Abstract: 0017-PD

Simon Dunmore, Amr Alderawi, Aruna Narshi, James Brown, Paul Kirkham, Ananth Nayak, Baldev Singh. Fructosamine-3-kinase activity in erythrocytes is related to the "Glycation Gap" in patients with diabetes. Conference: World Diabetes Congress-International diabetes federation, At Vancouver.

3. Abstract: PA886 01 September 2015; volume 46, issue suppl 59.

Amr Alderawi, Simon Dunmore, Gaetano Caramori, Andrew Hitchings, Emma Baker and Paul Kirkham. Fructosamine-3-Kinase: A molecular link between COPD and diabetes regulating carbonyl stress and the impact of metformin treatment. Published in 2015 in European Respiratory Journal 46 (suppl 59).

4. Abstract : 468-P 08 June 2017; Simon J. Dunmore, Amr S. Alderawi, Aruna Narshi, Fakhra Naseem, James E.P Brown, Alan Nevill, Ananth Nayak, Baldev M Singh: Evidence of the Role of Fructosamine-3-Kinase in the Glycation Gap and in Protection against Complications in Patients with Diabetes.