Role of Advanced Glycation Endproducts in Breast Cancer

A Thesis Submitted in Partial Fulfilment of the Requirements of Manchester Metropolitan University for the Degree Doctor of Philosophy

Hanaa Sharaf

School of Healthcare Science

Manchester Metropolitan University

April 2014

Table of Contents

Abstract

Diabetes and cancer are major health problems because of their high incidences worldwide. A growing body of epidemiological evidence indicats a molecular link between diabetes and breast cancer. Patients with diabetes mellitus have an increased likelihood of developing various types of cancers including breast cancer through the formation of advanced glycation endproducts (AGEs) which lead to cellular and bio-molecular dysfunction. However, the effects of AGEs have been poorly investigated on breast cancer cells. This current study examined for the first time the biological effects of various concentrations of BSA-derived AGEs on the invasive and hormone–independent breast cancer cell line MDA-MB-231 and on non- invasive hormone-dependent human breast cancer cell line MCF-7.

Bovine serum albumin was glycated-using methylglyoxal for three days. Crosslinked AGEs were assessed using sodium dodecyl sulphate polyacrylamide gel electrophoresis followed by Coomassie blue staining. Different assays including cell proliferation, migration and invasion through the Matrigel^{TM} with assessment of matrix metalloproteinase (MMP) activity by using Zymogaraphy were performed to investigate the effect of different concentrations of BSA-AGE on both cell lines *in vitro*. Furthermore, signalling pathways were investigated by Western blotting and using kinexus^{TM} phspho-protein microarray. The expression of the main receptor for AGEs (RAGE) involved on the MDA-MB231 and MCF-7 breast cancer cell lines was assessed by Western blotting and Calibur Flow Cytometer System.

The results of this study demonstrated that BSA-AGEs increased MDA-MB-cell, proliferation, migration and invasion through the Matrigel^{TM} associated with an

enhancement of matrix metalloproteinase (MMP)-9 activities, in a dose-dependent manner, up-regulated the expression of the receptor for AGEs (RAGE) and of the key signalling protein, phospho-extracellular-signal regulated kinase (p-ERK)-1/2. In addition, the blockade of BSA-AGE/RAGE interactions using anti-neutralizing RAGE antibody reduced the expression of p-ERK1/2. Furthermore, in BSA-AGE-treated cells, phospho-protein micro-array analysis revealed the main enhancement of the over-phosphorylation of (ERK1/2), (p70S6K1), (STAT)-3 and (MAPK) p38, involved in cell survival, cell growth cell cycle and protein synthesis. In contrast, MCF-7 showed stimulatory effects of BSA-AGEs, on cell proliferation and migration, as compared to non-modified BSA. However, BSA-AGEs did not change the weak invasive capacity of MCF-7 cells to cross a reconstituted basement membrane. In addition, BSA-AGEs induced over-phosphorylation of RAGE in MCF-7 cells. The investigation of signalling pathways suggests that BSA-AGEs might contribute to breast cancer development through activation of MAPK pathway and activation of CREB1 transcription factor in MCF-7 cells. Throughout the study, the non-modified BSA had a negligible effect. In conclusion, BSA-AGEs might contribute to breast cancer development and progression of breast cancer. In addition, the up-regulation of RAGE and key phosphor-protein signalling expression induced by BSA-AGEs might be a promising target for therapy to prevent the development of breast cancer in diabetic patients.

Declaration

This thesis contains no material which has been accepted for the award of any other degree in any university, to the best of my knowledge and belief, contains no material previously published or written by another, except where due reference has been made in the text. In addition, no parts of this thesis have been copied from other sources. I understand that any evidence of plagiarism and/or the use of unacknowledged third party data will be dealt with as a very serious matter.

Signature: _____ Date: _____

Acknowledgments

This thesis contains my research work, which has taken nearly four years to complete. I am truly blessed by Allah (S.W.T) to have granted me the opportunity to conduct my research and for its successful conclusion. The Quran, as a guide to human kind, includes scientific facts that were only discovered in recent centuries. I am truly indebted to my beloved Prophet Muhammad, (Praise and Blessings Be upon Him), who 1400 years ago received guidance through the first word revealed to him by Arch angel Gabriel: *"Iqra!"* (Read). From that came my inspiration: Study, discovery, research and tireless learning are a beacon of light to a brighter future. Firstly, I want to express my heartfelt gratitude to my first supervisor, Dr. Nessar Ahmed. I was also very fortunate to have the support and patience of my second supervisor Dr. Qiuyu Wang. A whole lot of appreciation is due to my wonderful supervisor Dr. Sabine Matou-Nasri; your research expertise has provided me with a sound basis for developing my research potential. She is truly an amazing person who goes above and beyond and without whom I would have been truly lost. My supervisors were inspirational and left a significant impression on my life. I would like to thank Dr. Nasser Alshanti for the work we did in collaboration and I am deeply grateful to my friend Asima who contributed her knowledge and research skills to help me during my studies. On a personal note, to my Mum, special thanks, without you I would not be the person I am today. In addition, I am blessed for having a loving husband, Khalid, who encouraged me to complete my studies. It takes a special person to tolerate the long hours and stress associated with working on a thesis. I am also thankful to my dear loving kids Abdurrahman, Shahad, Jana and Sultan, to whom I owe a lot of lost time. Finally, I must thank my colleagues who stood by me throughout my studies. They provided critical moral support and encouragement over the past four years. Thank you all for your love and backing.

Dedication

This thesis is dedicated to the soul of my brother Atif who passed away a few months ago. My brother taught me the love of science, devotion and honesty. He always encouraged me to pursue higher education and helped me navigate through life especially when times were difficult: "*I miss you every day.*" I am also thankful for my loving husband Khalid for lending me his unflinching support and boundless confidence to pursue my dreams.

Publications

Hana Sharaf, Sabine Matou Nasri, Qiuyu Wang, Nessar Ahmed. (2012). Effect of Advanced Glycation Endproducts on Breast Cancer Cells. International Symposium on the Maillard Reaction (Nancy-France) (16th September 2012).

Hana Sharaf, Sabine Matou Nasri, Qiuyu Wang, Nessar Ahmed.(2014). Advanced Glycation Endproducts Increase Proliferation, Migration and Invasion of Breast Cancer Cell Line MDA-MB-231. *Biochimica et Biophysica Acta* (submitted on 21st March 2014).

List of Abbreviations

AD	Alzheimer diseases
AGEs	Advanced glycation endproducts
ASP	Ammonium persulfate
BSA	Bovine serum albumin
BSA-AGEs	Bovine serum albumin derived-AGEs
CML	Carboxymethyllysine
DM	Diabetes mellitus
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
EDTA	Ethylene diamine tetra acetic acid
ERK1/2	Extracellular signalling regulated kinase1/2
FBS	Foetal bovine serum
FGF-2	Fibroblast growth factor-2
GDM	Gestational diabetes mellitus
GOLD	Glyoxal-lysine dimer

H_2O_2	Hydrogen peroxide
HCl	Hydrochloric acid
HMBGB-1	High-mobility group box-1
IDDM	Insulin- dependent diabetes mellitus
IFG	Impaired fasting glucose
IgG	Immunoglobulin G
IGT	Impaired glucose tolerance
MCF-7	Michigan Cancer Foundation-7
MG	Methylglyoxal
mg/dl	Milligrams per deciliter
MMP	matrix metalloproteinase
NADPH	Nicotinic acid adenine dinucleotide phosphate
NDDG	The National Diabetes Data Group
NDS	National Diabetes Statistics
NIDDM	Non-insulin –dependent diabetes mellitus
PBS	Phosphate buffered sulphate
PKC	Protein Kinase C
ROS	Reactive oxygen species

SDS	Sodium dodecyl sulfate
TBS-Tween	Tris buffered saline and Tween 20
WHO	The World Health Organization

List of Figures

List of Tables

Chapter 1

Chapter 1. General Introduction

1.1 Diabetes Mellitus

Diabetes mellitus (DM) is a group of metabolic disorders that include, hyperglycaemia, dyslipidaemia and obesity characterised by increased blood glucose levels. Hyperglycaemia is the hallmark of DM and believed to be the crucial factor in the development of diabetic complications. DM is a common chronic health problem that affects people around the world (American Diabetes Association, 2006; Biessels *et al*, 2002). Long-term exposure to hyperglycaemia leads to chronic complications. For instance, the development of macrovascular complications includes atherosclerosis, cardiovascular diseases or microvascular complication that leads to retinopathy, nephropathy and neuropathy. Moreover, high blood glucose may lead to other diseases for example, depression and dementia (Biessels *et al*, 2002).

The National Diabetes Statistics estimates that diabetes was the seventh leading cause of death in the US in 2007, due to development of diabetic complications that lead to morbidity and mortality (National Diabetes Statistics, 2011; National Diabetes, 2011). The prevalence of diabetes has significantly increased since the last decade, whereby the World Health Organization (WHO) estimates that people with diabetes are expected to increase between 2000 and 2030 from 285 million to 439 million (Shwa *et al*, 2010). In addition, in 2011, the average prevalence of diabetes in adult population in the UK was 4.45% (Diabetes UK, 2012).

The National Diabetes Data Group (NDDG) and the WHO have recognized four types of diabetes (American Diabetes Association, 1979; Harris, 1998). The two major types that usually occur are Type 1 and Type 2. Type 1 DM, or insulin-dependent DM (IDDM), is usually a consequence of autoimmune failure that leads to the destruction of the pancreatic β-cells. This leads to the inhibition of insulin production, which counts for approximately

10% of all diabetic cases. Furthermore, Type 1 diabetes usually develops in early age (often before the age of 40) and the patients require regular insulin injections for life. However, Type 2 diabetes or non-insulin–dependent DM (NIDDM) is a multifactorial disease. Generally, this type is more common and counts for 90% of all diabetic cases. Type 2 diabetes leads to the production of inadequate levels of insulin or production of insulin that cannot functional correctly due to insulin resistance (Rolo and Palmeira, 2006). Third type of diabetes known as gestational (GDM) occurs spontaneously during pregnancy and could lead to development of Type 2 diabetes. GDM is caused by many factors such as hormonal changes, environmental factors and family history (Kuzuya *et al*, 2002). Fourth type of diabetes is classified under other specific types of diabetes mellitus (Table 1.1) (Maraschin, 2012).

Table 1.1: Diabetes mellitus classification

1. Type 1 Diabetes
2. Type 2 Diabetes
3. Gestational Diabetes
4. Other types Genetic defects of B cell function (MODY, Mitochondrial) Genetic defects in insulin action Diseases of exocrine pancreas Endocrinopathies Drug or chemical induced Infections Uncommon forms of immune mediated diabetes Other genetic syndromes (Down, Turner, other)

1.1.1 Causes of Diabetes

Specific etiology of diabetes is not defined, but there are numerous studies investigating the causes of diabetes, however it remains unclear what causes lead to the particular type of diabetes. Scientists have proposed the causes of DM to be environmental factors, obesity, genetic susceptibility, viral infection and autoimmune diseases (Notkins and Lernmark, 2001: Hossain *et al,* 2007). It is common consensus that genetic and environmental factors play a significant role. The mechanism of Type 1 DM consists of severely deficient insulin production due to destruction of islets cells within the pancreas (Bresson and Herrath, 2004). Insulin is a protein hormone produced by pancreatic islets cells of Langerhans (β-cells) with its secretion induced by increased blood sugar levels after meals and incretion (a group of gastro-intestinal hormones). Insulin act on receptors

virtually present on all body cells but highly concentrated in liver, muscles and adipose tissue. Stimulated insulin receptors act on glucose transport protein-4 (Glut-4) which gets translocated from cytoplasm to cell membrane allowing glucose to move from blood to inside the cell. Eventually glucose is metabolised to generate energy in the form of ATP or gets stored as glycogen in adipose cells to form triacylglycerol. According to the type of diabetes, different pathological processes are involved. Inheritance play a significant role in Type 1 DM and its clinical development is triggered by a viral infection, probably being coxsackie B4 virus, which destroy pancreatic cells. Furthermore, it has been suggested that Type 1 DM is an autoimmune response caused by a virus where the immune system attacks both virally infected and pancreatic beta cells (Fairweather and Rose, 2007). While, a cause of Type 2 DM is less defined but is commonly associated with different risk factors such as, obesity, age, gender, lifestyle and genetic make up. Some of these factors can be controlled (such as obesity), while others are uncontrollable such as age, genetics and gender. Obesity leads to the reduction of insulin receptors found on the cell membrane, thus preventing the muscle from using insulin sufficiently. Genetic factors may include family history of insulin resistance or genetic mutation (American Diabetes Association, 2010).

1.2 Hyperglycaemia and Diabetic Complications

Hyperglycaemia or high blood glucose is characterised by the presence of excessive amount of glucose that circulate in the blood. Numerous studies suggest that exposure to chronic hyperglycaemia plays a major role in the pathogenesis of diabetic complications and many chronic diseases. (American Diabetes Association, 2009; Biessels *et al.,* 2002). Notably, the concentration of glucose must excel above 11.1 mmol/l (UK units) or 200 mg/dl (USA units), but the symptoms are not recognised until the glucose value reaches

15-20 mmol/l (250-300 mg/dl) (Grundy, 2012). Pre-hyperglycaemia or pre-diabetes is the same condition described which was also classified by different health organizations at different periods. Moreover, pre-hyperglycaemia, which include impaired fasting glucose and impaired glucose tolerance could lead to the development of Type 2 DM, stroke and heart disease. Hyperglycaemia can cause alterations at the cellular level and increase the level of oxidative stress that promotes pro-inflammatory responses which leads to atherosclerosis and other diabetic complications (Fowler, 2008). Moreover, hyperglycaemia also leads to the formation of metabolic and haemodynamic derangement, which is considered to play an important role in vascular diabetic complications (Aronson, 2003; Wautier and Guillausseau, 2001). Chronic hyperglycaemia inflicts damaging effects on tissues through various biochemical mechanisms leading to the development of diabetic complications (Brownlee, 2001). Other causes that lead to hyperglycaemia, include medication, serious illness, physiological stress and endocrine diseases (Srinivasan, 2012). The particular role of hyperglycaemia in the molecular or cellular basis of long-term complications remains unclear and therefore needs further investigation. However, several theories have been devised, particularly one that has received considerable interest, is the role of protein glycation (Ahmed, 2005).

1.2.1 Metabolic Pathways of Hyperglycaemia

The biochemical pathways between chronic hyperglycaemia and tissue damage are not completely understood. Several studies suggest a relation between hyperglycaemia and diabetic complications induced through several mechanisms. These mechanisms include hyperactivity of the polyol pathway (Chung and Chung, 2005), increased polyol pathway flux through biosynthesis in hexosamine pathway (Fulop *et al*., 2007), activation of protein kinase C (PKC) (Das Evcimen and King, 2007) and glycation of proteins and lipids (Goh

and Cooper, 2008). Other studies have suggested that there is an alternative mechanism involved where the mitochondrial electronic transport generates an overproduction of superoxide (Brownlee 2001; Nishikawa *et al.*, 2000). However, glycation or protein glycation are known to be a major factor in long-term diabetic complications.

1.2.1.1 Glycation

One of main consequence of increased hyperglycaemia is the increasing interaction of glucose with proteins, which is called glycation. Glycation is generally accompanied by oxidation reactions. In the presence of molecular oxygen, and under conditions of enhanced metal-catalysed glycoxidation, protein dicarbonyl compounds are produced from Amadori products via a protein enediol generating free radicals.This radical reduces molecular oxygen to simultaneously generate oxidation. This consequence has a main role in damaging important biomolecules, such as lipids, proteins and nucleic acids (Baynes and Thorpe, 1999). The best examples of glycation are pentosidine and Carboxymethyllysine (CML) (Basta *et al.*, 2004). Glycation contributes to the development of diabetic nephropathy and other microvascular diseases (Ahmed, 2005). Furthermore, glycation affects the glomerular basement membrane and extracellular matrix molecules that change the level of soluble signals, such as cytokines, hormones and free radicals (Wautier and Guillausseau, 2001).

1.2.1.2 Hexosamine Biosynthesis Pathway

Commonly, when cells containing high volumes of glucose molecules will readily undergo glycolysis due to the excess volume of glucose-6-phosphate. However, the intermediate product (fructose-6 phosphate) of glycolysis may divert to signalling pathway. This leads to the production of glucosamine-6 phosphate and then uridine diphosphate *N*-

acetylglucosamine by glutamine fructose-6 phosphate amidotransferase (GFAT). The *N*-acetylglucosamine binds to the serine and/or threonine residues transcription messenger. This process is similar to phosphorylation and modification of *N*-acetylglucosamine may cause the gene expression to change. Cells under hyperglycaemic conditions will lead to increased flux in the hexosamine biosynthesis pathway that causes damage to blood vessels by increased expression of growth factor-β1 andpalsminogen activator inhihibitor-1 (Folli *et al.*, 2011).

1.2.1.3 PKC Activation

During hyperglycaemia, there is an overproduction of diacylglycerol (DAG) by the cell. DAG is a critical cofactor for protein kinase C (PKC) that induces several growth factors (Srivastava, 2002). Increased levels of PKC have an effect on gene expression, smooth muscle cells and vascular wall (Aronson and Rayfield, 2002). Moreover, PKC activation results in abnormalities in the capillary membrane by increasing the thickness of their basement and is the primary defect observed in vascular complications (Cade, 2008).

1.2.1.4 Polyol Pathway

High concentration of glucose within the cell is reduced to sorbitol and oxidised to fructose, this is called polyol or sorbitol-alose reductase pathway. Thus, the reaction causes increase consumption of the co-factor of aldose reductase nicotinic acid adenine dinucleotide phosphate (NADPH). NADPH is a vital molecule for the production of a critical intracellular antioxidant by reducing glutathione. The lower levels of reduced glutathione make the cell more susceptible to oxidative stress (Giacco and Brownlee, 2010). Moreover, high levels of glucose through the polyol pathway causes tissue damage of the lens, kidney and peripheral nerves (Chung *et al.*, 2003).

1.2.1.5 Mitochondrial Superoxide Production

Mitochondria are effective producers of cellular superoxide. However, the overproduction of superoxide by mitochondria leads to damage of molecules such as, lipids, proteins and nucleic acids. Mitochondrial role is particularly prone to oxidative damage, leading to decreased mitochondrial ATP synthesis, cellular calcium dyshomeostasis, and change of the mitochondrial permeability transition, all of which predispose cells to apoptosis or necrosis (Brownlee, 2004). Hyperglycaemia leads to the inhibition of the activity of the glycolytic enzyme (glyceraldehyde-3-phosphate dehydrogenase) contained within the mitochondria and this result in excessive superoxide production. Furthermore, superoxide species are considered to play an important role in activating different pathways implicated in diabetic complications such as, PKC, polyol and hexosamine pathway through glyceraldehye 3-phosphate (Sharma *et al*., 2002; Coughlan *et al.,* 2008).

1.3 Maillard Reaction

The discovery that sugars can react with proteins was made by a French scientist called Louis-Camille Maillard who was the first one observe a colour change in food from yellow to brown upon heating (Maillard, 1912). This was due to the endogenous reaction between the carbonyl group of a reducing sugar with an amino group of protein or other macromolecules (lipids and nucleic acids). This is reaction known as “Maillard reaction”, which has attracted a great deal of attention amongst researchers in the medical field to try understand the physiological and pathological consequences of protein glycation in biological systems (Nagai *et al*, 2012; Gerrard, 2006).

1.4 Chemistry of Protein Glycation

Protein glycation is a spontaneous reaction that occurs over a period of hours, days, months, and depends on the duration and severity of the glycaemia (Ahmed, 2005). Glycation reaction is subdivided into three stages, which result in various protein-protein cross-linked and non-crosslinked AGEs .A general pattern of the chemistry involved in the glycation reaction is outlined in Figure 1.1.

1.4.1 Protein Glycation: Early Stage

In 1953, Hodge proposed an early stage reaction that is initiated by the addition reaction between carbonyl groups on reducing sugars (i.e. glucose or fructose) and amino groups, on proteins (i.e. lysine or arginine) forming a Schiff base. Schiff base is formation rapid and reversible that occuring within a few hours (Hodge, 1953; Monnier *et al.*, 1981).

1.4.2 Protein Glycation: Intermediate Stage

Intermediate stage, which results from the Schiff base that rearrange giving product called Amadori. Amadori product undergoes further reactions to give highly reactive products such as glyoxal and methylglyoxal through a series of chemical rearrangements, oxidation and dehydration reactions (Thornalley, 2005).

1.4.3 Protein Glycation: Advanced Stage

Glycated protein can undergo further series of repeated dehydrations, condensations, fragmentations, oxidations and cyclisation reactions involving dicarbonyl intermediates, leading to stable and irreversible extremely heterogeneous group of compounds called advanced glycation endproducts (Ahmed, 2005; Schleicher *et al.*, 2001; Yim *et al.*, 2001).

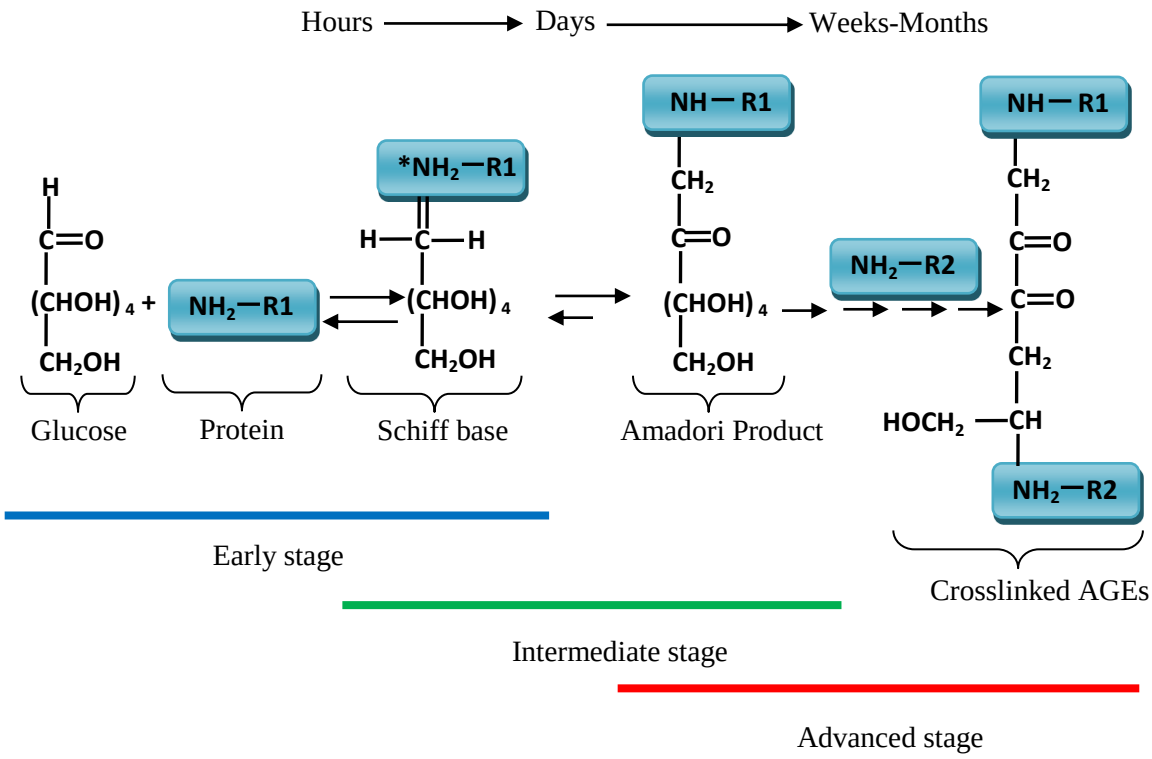


Figure 1.1: Schematic representation of the reaction of advanced glycation endproduct, where R1 and R2 represent different carbon alkyl chain (Aronson and Rayfield, 2002).

1.5 Characterisation of AGE

AGEs are complex molecules that have the ability to generate fluorescence and crosslinks between proteins. The role of AGEs has been extensively studied but their mechanism of formation is not fully understood (Bousova *et al*., 2005). Although many structures of AGEs have been identified in *vitro*, and *vivo*, AGE-structures are classified into four groups:

1.5.1 Fluorescent Crosslinking AGEs

There are minute amounts of AGEs 1% or less that are able to generate fluorescence when they crosslink with protein (Dyer *et al.*, 1991). For instance, when arginines reacts with lysine then produce pentosidine, which reacts with several carbohydrates to generate fluorescence and are detected in a variety of tissues, such as kidney, lung, liver, skin and

blood proteins. Pentosidine is a best example of a fluorescent crosslinking AGE and commonly used as a biomarker, another example is crossline (Salman *et al.*, 2009).

1.5.2 Fluorescent Non Crosslinking AGEs

Methylglyoxal can also produce a fluorescent non cross-linker of AGEs when it reacts with N-α-t-butoxycarbonyl-L-arginine, such as argpyrimidine. Moreover, this fluorescent product can develop and increase with age and diseases (Shipanova *et al.*, 1997).

1.5.3 Non-Fluorescent Crosslinking AGEs

The non-fluorescent cross-linkers are major structures that maybe detected by ELISA (Goh and Cooper, 2008). The best examples of a crosslinked AGE are glyoxal lysine dimer (GOLD) and methylglyoxal-lysine dimer (MOLD), which are believed to be highly reactive.

1.5.4 Non-Fluorescent Non Crosslinking AGEs

N-carboxymethyllysin (CML) is a non-fluorescent non cross-linked AGE that is utilised as a marker for oxidative stress (Linton *et al.*, 2001). This is formed during the metal-catalysed oxidation conditions in the presence of protein, and believed to contribute to pathological effects in complications of diabetes such as retinopathy and nephropathy.

1.6 Advanced Glycation Endproducts

AGEs may alter the extracellular matrix (ECM), cytokines and change the action of hormones through engagement of cell surface receptors, and which generate oxidative stress and have been implicated in the pathogenesis of diabetic complications. For instance, damaging and changing tissue structures, these alterations are accompanied by thickening

of the capillary basement membrane, increased permeability of capillaries, reducing the vessel walls, loss of pericytes and increased endothelial cell turnover. The formation of AGEs is via different pathways, one of which causes crosslink that lead to dysfunctions at the intracellular level. Alternatively, AGEs trigger many growth factors, chemokines, and cytokines and induce oxidative stress and free radicals, which cause aggregations of different molecules, such as proteins and lipids (Vlassara and Palace, 2002). AGEs form gradually and accumulate with time. Although, glucose is a major precursor of AGE formation, recent studies indicate that other reducing sugars such as methylglyoxal (MG), glyoxal and α-oxoaldehydes are considered more effective and reactive than glucose (Vander and Hunsaker, 2003; Singh *et al.*, 2001).

1.6.1 AGE Formation via Methylglyoxal or other Carbohydrates

Many carbohydrates can react with amino groups when they undergo the glycation reaction, while some reactions may also involve an oxidation process through the glycoxydation reactions. For instance, it is possible for the glucose molecules to readily react with amino groups under non-oxidative pathways to produce pyrraline or pentosidine and N6-carboxymethyllysine (CML) under oxidative pathway. MG is a reactive dicarbonyl compound (Figure 1.2) that is thought to contribute to the intracellular formation of AGEs, and in turn induces cellular oxidative stress and up regulation of cell adhesion molecules (Shipanova *et al*, 1997). This is due to its numerous origins causing it to be readily available during hyperglycaemia and other *in vivo* conditions. Furthermore, the three main factors that form MG are triosephosphate degradation, lipid peroxidation and side reactions caused by glucose-mediated glycation (Uchida *et al*., 1997; Lv *et al.*, 2011). MG increases the level of superoxide species that can quench nitric oxide and cause cellular damage to the DNA, proteins, endothelial cells or neutrophils (Shinohara *et al*, 1998). Moreover, MG

commonly reacts with amino acids (such as lysine, arginine and cysteine residues) (Grillo and Colombatto, 2008). Increased levels of MG have been detected in the cells of patients with Type 1 and Type 2 diabetes such as mesangial cells and, red blood cells. Furthermore, it is associated with other diabetic complications such as microvascular disease and atherosclerosis (Karachalias *et al*., 2003). More specifically, MG ultimately causes either reversible or irreversible protein modification. Notably, this is more likely to occur in the red blood cell, kidney and lens. A previous *in vivo* study under physiological conditions found that the maximum concentration of MG in a diabetic patient was 2.4 µM; and this level was sufficient in irreversibly changing plasma proteins (Lo *et al*., 1994).

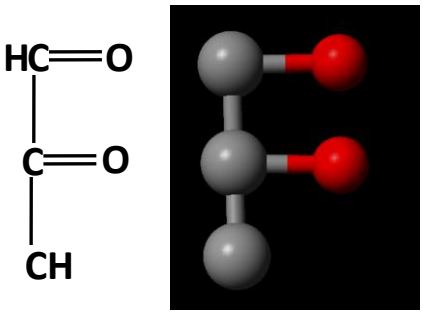


Figure 1.2: Methylglyoxal molecular structure (left hand side) and 3D structure (right hand side) is a compound that can cause glycation.

1.7 Intracellular Glycation and AGE Formation

It is now apparent that intracellular AGE formation is the key source of both intra- and extracellular AGE. During of hyperglycaemia a significant rise in concentration of intracellular sugars occurs therefore, the main sources of AGEs are derived from reactive metabolic intermediates. AGEs are formed from different carbohydrate such as, methylglycoxal, ascorbate, and glucose. Moreover, most intracellular precursors of AGEs such as, glyoxal, methylglyoxal and 3-deoxyglucosones, are particularly reactive due to the

presence of two carbonyl groups, as well as their high reactivity with arginine residues on proteins (Shinohara *et al.,* 1998; Thornalley, 1999). Furthermore, some other compounds containing free amino groups, including adenine and guanine bases in DNA are also prone to glycation and AGE formation, probably by reactive intracellular sugars. AGEs could also form on phospholipid and induce lipid peroxidation by a direct reaction between glucose and amino groups on phospholipids such as phosphatidylserine residue, which are important precursor for the glycation reaction (Ahmed, 2005; Mironova *et al.,* 2001).

1.7.1 Exogenous Source of AGEs

Despite the fact that there are different types of AGEs formed intracellularly from glucose and fatty acid oxidation, several studies confirm that AGEs are also derived from an exogenous source (Giacco and Brownlee, 2010). Exogenous sources have significant effect on biological systems and increase the risk of diabetic complications and other diseases (Peppa *et al.,* 2003; Vlassara and Palace, 2002). The common exogenous sources that generate AGEs are tabacco smoking and diet. Tabacco or cigarette smoke have high concentrations of specifiic components such as methylglyoxal that react with plasma and generate different adducts of AGEs. Moreover, high level of AGEs have been observed in the serum of smokers compared with non-smokers (Nicholl *et al.,* 1998). Diet or food have been shown as another source of highly reactive AGEs. However, the amount, type of diet such as protiens, heat processing and duration of processing time are factors that influences the formation of AGEs (Goldberg *et al*., 2004; Negrean *et al*., 2007). These AGEs have been implicated in vascular disease and ageing (Nicholl *et al.,* 1998: Vlassara *et al*., 2008).

1.8 Biological Effects of AGEs

Increasing evidence demonstrated that AGEs could have influence on the function of biological systems and cause damage to most tissues. First, by their modification of protein can alter cellular structure, enzymatic activity, and biological half-life, inhibit specific function of proteins, and cause damage to plasma proteins and lipoproteins. Secondly, via the interaction between AGEs and RAGE they can increase oxidant stress and induce a cascade of inflammatory reactions. The reactivity between the sugars and amino groups is dependent on the modified protein, where some modifications render a more reactive amino group (Sharma *et al*., 2002). This in turn has a serious impact on the cells during their life. Additionally, the formation of AGEs may increase oxidative stress in some molecules as compared to others. In addition, some modified proteins within the cell, for example, HSC-70 protein found within stem cells is known as the most modified protein (Hernebring *et al*., 2006).

The increased glycation and accumulation of tissue AGEs may contribute to the pathophysiology of diabetic complications. However, extracellular matrix proteins (EMP) contribute to the functions of the cell and basement membrane as it contains a wide array of growth factors. These growth factors play an important role in the synthesis of the cell and nucleus. The AGE accumulation causes the disruption of the matrix proteins and matrix-cell elements. However, this process may also affect and inhibit important molecules in the cell matrix this accumulation of AGE has the ability to change cellular adhesion thus, influencing the ability of cellular binding and moleculor charge leading to changes in cell-collagen interaction causing alteration of collagen (Basta *et al*, 2004). Karmakar and his college (2012) suggested that advanced glycation endproducts may cause tissue damage through several mechanisms include: modified intracellular proteins,

modified extracellular matrix proteins and by modified plasma proteins that interact with AGE-RAGE (Karmakar *et al.,* 2012). In addition, AGE accumulation on vascular ECM results in increased stiffness and decreased vascular elasticity (Smit and Lutgers, 2004; Tan *et al*., 2002).

The modification of the proteins has a critical effect on the intracellular proteins by changing their properties. However, this depends on the formation of AGEs and the presence of sugars like fructose or glucose. Furthermore, it is believed that the intracellular accumulation of AGEs may affect the activation of signalling pathways in the cells and cause alteration of vital proteins and enzymes. In addition, the intracellular AGEs cause changes to the mitochondrial proteins and leads to critical DNA damage (Ahmed, 2005).

1.9 Advanced Glycation Endproduct Receptors

Advanced glycation endproducts have non-specific cellular receptors, which belong to the immunoglobulin super-family of cell surface molecules (Hudson and Schmidt, 2004). The interaction that AGEs has with its receptors plays a significant role in diabetic complications. The first description of this receptor for advanced glycation endproducts (RAGE) was in 1992 (Neeper *et al,* 1992). RAGE consists of a single hydrophobic transmembrane helix, short highly charged cytoplasmic tail and an extracellular site. The extracellular region of RAGE is composed of three immunoglobulin-like domains, one V-type and two C-types. The V-type domain allows for ligand binding to occur, where there are interaction between RAGE and the extracellular ligands (Schmidt *et al.,* 1992). RAGE is anchored by a fourth transmembrane domain that is connected to a fifth intracellular domain. This fifth domain is highly charged that allows for the interaction between the domain and cytosolic transducer molecules to facilitate, as demonstrated in Figure 1.3 (Ahmed, 2005). RAGE behaves as signal transducer receptor for AGEs, in particularly for

CML as this is the most common type of AGE found in *vivo*. Other known receptors of AGEs are oligosaccharyl transferase-48, macrophage scavenger receptor types A and B1 (CD36), termed AGE (AGE-R1), 80K-H phosphoprotein (AGE-R2) and galectin-3 (AGE-R3) (Kalousova *et al.*, 2005; Ohgami *et al*., 2002). RAGE has the ability to trigger the activation of growth factors such as vascular endothelial growth factor (VEGF), which leads to angiogenesis and increased release of cytokines including tumour necrosis factor-α (TNF- α) and interleukin-1α (IL-1α) (Bierhaus *et al*., 2005; Ma *et al*., 2007). Furthermore, RAGE is highly expressed in a wide range of cell types such as, embryonic cells, lungs, macrophages, endothelial cells, epithelial cells, smooth muscle cells, astrocyte cells, renal mesangial cells and some malignant cells (Basta, 2008; Brett *et al*., 1993). Moreover, current studies have shown that high expression of RAGE is involved in chronic inflammatory conditions, vast amount of immune cells, and endothelial cells. In addition, RAGE interacts with a variety of ligands, such as S100/calgranulins that are expressed in chronic inflammation, amyloid-β peptide Mac-1 and HMGB1 (amphoterin) (Chuah *et al*., 2013). RAGE is associated with invasion and metastasis in many types of cancers, for example colorectal cancer. AGE induce oxidative stress generation in variety of cells through the interaction with RAGE, which leads to the release of inflammatory response especially when high glucose concentrations are present. Numerous studies have reported that RAGE is associated with many chronic diseases as they initiate intracellular signalling that leads to cellular disruption by altering the proteins structure (Ahmed, 2005; Chuah, *et al.*, 2013).

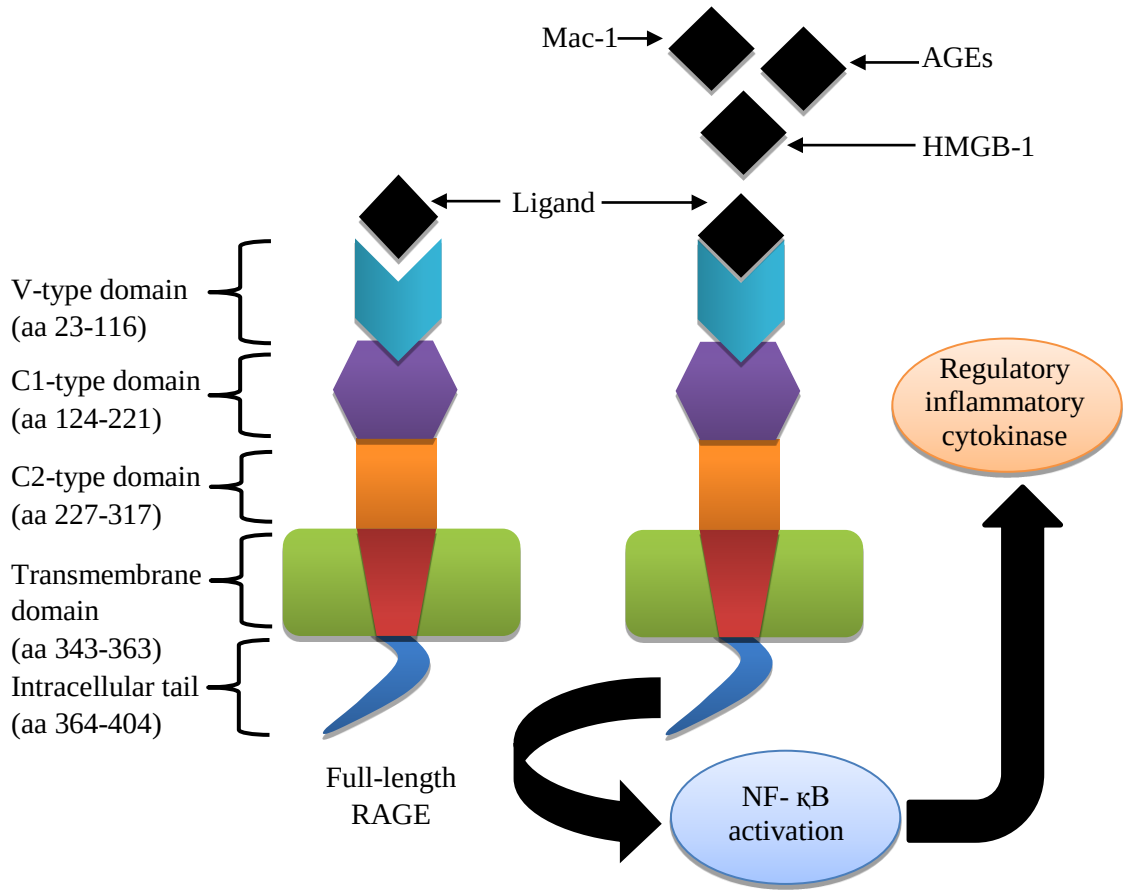


Figure 1.3: Schematic representation of the full-length of RAGE. It is composed of extracellular domain (one V-type domain and two C-types domains), transmembrane domain and intracellular tail (Chuah *et al.*, 2013).

1.10 Free Radicals and Oxidative Stress

The exposure of the human body to free radicals may occur both exogenously and endogenously. The generation of theses free radicals may be caused by numerous factors, such as exposure to radiation, smoking, pollution, sunlight, alcohol and some medicines. The cells require oxygen for the production of energy when undergoing the process of mitochondrial respiration. This process results in the production of free radicals. These molecules are highly reactive and contain one or more unpaired electrons. Thus, this unstable state causes the molecules to either accept or donate electrons in order to become

more stable, which leads to a chain reaction where new radicals are formed and can react with other macromolecules. Current research has proposed that AGEs play a crucial role in the generation of free radicals. Whereby, AGEs leads to the overproduction of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase that is an essential source of vascular oxidative stress in Type 2 DM (Tan 2007). The high level of NADPH oxidase activity leads to the formation of free radicals and reduction of cellular antioxidants. Moreover, AGEs interact with their receptors (RAGE) that modifies the enzymatic activity in the cells and induces free radicals. AGEs also induce the up regulation of specific genes through activating transcription factor NF-κB (Maritim, *et al.,* 2003). The overproduction of free radical species may result from numerous sources; such as mitochondrial respiration, PKC-dependent activation system and RAGE-triggered cellular oxidant stress (Lander *et al.*, 1997). The common free radicals that cause damage to essential biomolecules are oxygen-free radicals or reactive oxygen species (ROS). ROS are reactive molecule that contains oxygen. There are several ROS that are involved in the generation of oxidative stress under physiological conditions, such as superoxide ($O_2^{\cdot}$), hydroxyl radical ($OH_2^{\cdot}$), nitric oxide radicals ($NO^{\cdot}$), peroxyl radical ($ROO^{\cdot}$) and non-radical H_2O_2. The oxidation of glucose in diabetic patients is considered the major cause of ROS production in the form of reactive hydroxyl radicals. However, highly protective defence systems within the cell produce antioxidant that protects the cells and organs (Valko *et al*., 2007). Furthermore, there are different enzymatic and non-enzymatic antioxidants present in the body that fight against ROS. The enzymatic defence includes catalase, glutathione, glutathione reductase and peroxidase; whereas, non-enzymatic antioxidants includes vitamin E and C, carotenoid and flavonoid (Comhair and Erzurum, 2002; Jakus, 2000).

Oxidative stress occurs when there is an imbalance between the production of ROS and the ability of the biological system to detoxify using scavengers (Halliwell, 1994). This

imbalance may occur either because of reduced antioxidants or because of excessive free radical production. The disturbance in this balance may cause damage to lipids and DNA. The oxidative DNA damage has mutagenic and carcinogenic effects, which could significantly affect oncogene or tumour suppresser genes. Some studies have suggested the relationship between ROS and the progression of carcinogenesis. Furthermore, ROS potentially can cause tissue dysfunction and damage as implicated in the pathology of different diseases, such as inflammatory, neurodegenerative diseases, ageing and diabetes (Dickerson *et al.*, 2002; Atalay and Laaksonen , 2002; Giacco and Brownlee, 2010).

1.11 Diabetic Complications and Glycation

Diabetic patients are at high risk of developing serious complications, due to high blood glucose levels. This includes hyperglycaemia through the production of AGEs, dyslipidaemia that results from the aberrations in lipid metabolism and oxidative stress. In addition, the intracellular interaction between AGEs and RAGE, results in inadequate endothelial barrier function that may ultimately accelerate atherosclerosis (Aronson and Rayfield, 2002). For instance, Figure 1.4 demonstrates an interaction that leads to oxidative stress and finally increased levels of surface procoagulant activity and monocyte adhesion. The two main complications strongly associated with diabetes are macrovascular and microvascular diseases (Ahmed and Thornalley, 2007; O'sullivan and Dinneen, 2009). These complications affect several systems in the body, such as the heart, kidney, eye and brain. Furthermore, recent studies have shown that a major long-term complication of diabetes maybe cancer (Coughlin *et al.*, 2004; Giovannucci, *et al.*, 2010).

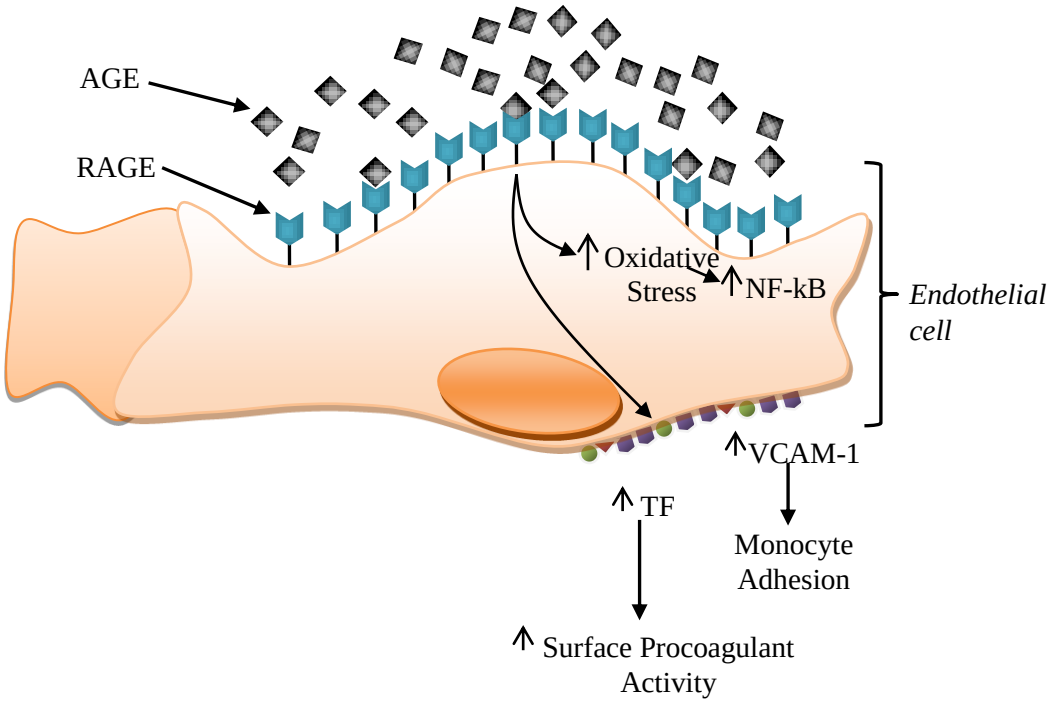


Figure 1.4: The interaction of AGE and RAGE on the endothelial cell. This schematic explains the effect of AGE-RAGE interaction on the endothelial cell result in the production of oxidative stress, transcription factor NF-κB, and VCAM-1 and consequently reduced barrier function (Aronson and Rayfield, 2002).

1.11.1 Microvascular Disease

Many studies have revealed that microvascular disease is a common consequence of diabetes and depends on the severity and duration of hyperglycaemia (Peppa and Vlassara, 2005). Moreover, there are many factors contributing to microvascular complications that include nephropathy, retinopathy and neuropathy, but the key factor is the accumulation of AGEs. The acceleration of AGEs leads to the morbidity in the final-stage of kidney failure and diabetic nephropathy has a 40-50% chance in all diabetic patients (Wolf, 2004). The accumulation of AGEs results in glomerular basement thickening and mesangial matrix expansion, which is responsible for the control of blood flow through the capillaries that leads to the reduction in renal clearance (Coughlan *et al.*, 2005). Diabetic retinopathy is one of the most common complications of diabetes that leads to blindness, and 10,000

cases develop every year in the United States (Fowler, 2008). Many studies indicate that the increased level of AGEs in diabetic patient plays an important role leading to the 2001; Chibber *et al.,* 1997; Stitt and Curtis, 2005; Zhang *et al,* 2011). Diabetic neuropathy is a common complication of diabetes, arising in more than 50% of all diabetic patients. It arises because of progressive damage to the peripheral sensory and autonomic nervous systems (Magalhães *et al.,* 2008). Many studies show that the formation of AGEs is considered as important biochemical pathways involved in diabetic neuropathy (Sugimoto *et al.,* 2008; Wada and Yagihashi, 2005). Further, the interaction of AGEs and RAGE is involved in nerve dysfunction, which may play an inflammatory role in peripheral nerve damage, although their precise role remains unidentified (Huijberts *et al.,* 2008).

1.11.2 Macrovascular Disease

Macrovascular disease is a major complication of diabetes and involves a wide range of pathological changes leading to functional and structural abnormalities. Macrovascular disease in diabetes includes an accelerated form of atherosclerosis, which is responsible for the high-incidence of several vascular diseases, such as, stroke, and peripheral vascular diseases (Laakso, 1999; Rahman *et al.,* 2007). Atherosclerosis leads to cardiovascular diseases that cause 70% mortality in Type 2 diabetes (Cade, 2008). The International Diabetes Federation confirmed that the postprandial hyperglycaemia (after diet intake) is a significant risk factor of macrovascular disease (Parkin and Davidson, 2009). However, meta-analysis showed that hyperglycaemic control significantly reduces the risk of macrovascular disease. To date, several studies on animals and humans indicate that AGEs are involved in hyperglycaemic progression, which increase the risk of atherosclerosis (Jandeleit-Dahm and Cooper, 2008; Vlassara and Palace, 2002; Peppa and Vlassara, 2005). AGEs have deleterious effects and cause change to the structural vessel wall

macromolecules and other abnormalities. AGEs also causes change in endothelial dysfunction, and increase stiffness of the arterial wall and human aortia (Laakso, 1999; Fowler, 2008). Moreover, AGE when added to nitric oxide (NO) leads to blocked NO activity, thus generate the production of ROS, these ROS reduce the half-life of endothelial synthesis and function (Pandolfi and De Filippis, 2007; Goh and Cooper, 2008). Moreover, AGE formation is associated with a significant acceleration of atherosclerosis through different mechanisms, including the cross-linking of proteins, the modification of matrix components and abnormal lipoprotein metabolism, for example, LDL is modified by AGE and this LDL–AGE has been found in the serum of diabetic patients and contribute to reduced clearance of LDL from the body (Goh and Cooper, 2008; Ahmed, 2005).

1.11.3 Other Complications Related to Glycation

Recently, it has become clear that AGEs are involved not only in diabetic complications but also in a number of other diseases (Xue *et al.*, 2011). Increased risk of hyperglycaemia and oxidative stress accelerate the rate of AGE formation. AGE formation is involved in pathophysiology of many diseases such as, Alzheimer diseases (AD), Parkinson disease cancer, and some types of liver disease (Hyogo and Yamagishi, 2008; Grillo and Colombatto, 2007). An *in vivo* study on normal rats has demonstrated that some model compounds have the ability to cause harmful effects on tissues and cause an increase in the level of mesangial volume that is related to neurodegenerative diseases (Vlassara *et al*, 1994). Moreover, AGEs are claimed to contribute to degenerative diseases by the AGE-RAGE up regulation and have been reported in the pathogenesis of AD. AD is characterised by a gradual and steady loss of memory. However, specific types of AGEs induce ROS that activate (NADPH) and lead to neuronal dysfunction. In addition, AGE accumulation increases the deposition of beta amyloid peptide (Aβ) in both plaques and

tangles, and is related to the progression of AD (Chuah *et al.*, 2013; Gella and Durany, 2009). Another complication related to AGE has been reported in certain liver disease such as cirrhosis through the interaction between AGE and RAGE (Semba *et al.*, 2010).

1.12 Introduction to Cancer

Cancer is a harmful disease that is characterized by rapid production of abnormal and uncontrolled cell division, which leads to malignant growth. Cancer cells have the ability to invade surrounding tissues and possess the ability to metastasize. Cancer also may spread through the blood stream or lymphatic system. Cancer is considered as a major cause of death in the world. During 2008, it was responsible for 7.6 million deaths worldwide (approximately 13% of all deaths) (World Health Organization, 2008). In UK 396.2 people per 100,000 of the population were diagnosed with cancer in 2011 (Cancer research in UK). In 2010, cancer caused the deaths of close to 1,500 Americans each day. Notably, it is the second most common cause of death in the US and responsible for nearly one in every eight deaths. The majority of cancer-related deaths each year are due to cancer of the breast, liver, lungs, stomach, and colon. Moreover, there is a rapid increase in the incidence of melanoma, prostate breast, and ovarian cancers (Ferlay *et al.*, 2010).

There is a difference between men and women with regard to most common types of cancer. Breast cancer is the most common female cancer, with approximately 1.4 million cases arising annually (Ferlay *et al.*, 2010). Furthermore, 22 million deaths worldwide because of cancer are expected to occur in 2030 (World Health Organization, 2014). Cancer is caused by various external risk factors such as, chemicals, radiation, tobacco, immunosuppressive treatment and infectious organisms and internal risk factors such as inherited mutations, declines in the immune function and hormones changes (Vajdic *et al.*, 2006; Beghe and Balducci, 2005).

1.13 Pathophysiology of Cancer:

Healthy cells have a particular size, morphology, functionality and growth rate that specific to the original tissues and serve the function of that organ. While cancer cells differ from healthy cells in morphology, cellular size, structure, function, and growth rate. The Malignant cells do not have the normal controls of growth seen in normal cells, and grow uncontrollably (Bekher, 1992). The uncontrollable growth leads to invasion of the cancer cells to the adjacent normal tissues and structures or migration of cancer cells to distant organs. When cancer cell loses its ability to differentiate like a normal cell, it will lose its normal function required by normal tissues and this resulted in a variety of tissue alterations and malfunctions: lower immunity, cachexia, anemia, leukopenia and thrombocytopenia (McCance and Roberts, 1998).

Histologically speaking, there are three characteristic features of cell abnormality. The first one called Dysplasia, which can be defined as cellular disorganization in terms of cell size, shape, or arrangement. While the second type is called Metaplasia, which is the primary level of dysplasia (early dysplasia). Metaplasia is a reversible process which is benign, but abnormal compared to healthy cells. The disorder in metaplasia results in changes of cells size, shape or orientation lining up, or in inappropriate or faulty tissue behaviors (McCance and Roberts, 1998). Anaplasia is complete absence of cellular differentiation and it is the most advanced stage of metaplasia and is a defining feature of malignant cells (Meng *et al.*, 2012). Pathophysiology of cancer depends on factor controlling cell growth and differentiation. These factors include immunity, cell growth rate. Normal cellular growth is affected by protein encoded by the genetic materials in the nuclei. Therefore, alteration of gentic materials or mutation by environmental or endogenous factor could results in tumour development. The altered or mutant genes are generally called oncogenes which allow the uncontrollable celluar growth leading to tumour development (Bekher, 1992).

Mechanism of cancer development is a complex process and it is not well understood yet. Therefore, studies have linked mechanism to the risk factors and hypotheses have been developed accordingly.

The important step of cancer development happens when the cellular DNA is altered or damaged by endogenous or exogenous source such as ionizing and non-ionizing radiation, hormones and viruses. Then, this will lead to an error in DNA synthesis or repair (Lee *et al.,* 2013). The damaged cells are required to be exposed to one or more promoting agent that facilitates development of uncontrolled cell growth. This stage is called promotion stage. Promoters may include environmental pollutants, drugs, and hormones. Failure to neutralise or regulate the cancer cells by apoptosis or cells death will lead to the progression of tumour development.

Environmental factors and unhealthy lifestyle practices have been shown to be major risk factors in the carcinogenesis of different types of Cancers (Irigaray *et al*., 2007). These factors involves: smoking, alcohol consumption, unhealthy diet, medications, and ultraviolet exposures.

Dietary factors and obesity have been linked to some types of cancers. Evidence shows that consumption of dietary fat may increase bile acids and cholesterol metabolites that may increase carcinogens in the body that are associated with colorectal cancers. Low dietry fibres also have been to be linked with increased risk of colonic cancer. Obesity is seen to be linked to an increased risk for breast, ovaries, and endometrium tumours in females (Forte *et al*, 2012; McTiernan, 2003). This effect is more linked to the high number of fat cells that produces oestrogen. Therefore, it has been suggested that high oestrogenic levels might increase cancer risk (McTiernan, 2003).

Medications like Androgen -anabolic steroids have been linked to liver, prostate and breast cancers (Grossmann and Wittert, 2012: Friedenreich, 2000). External or environmental

factors such as air pollutants, radiation, and radon gas also have been linked to high incidence of cancers. Cancer also can be caused by viruses which are generally termed as oncogenic viruses. They can cause alterations in the cellular genomic material; thus leading to oncogenic process in susceptible individuals (Hausen, 1991)

1.14 Breast Cancer

Breast cancer is the most common form of malignancy that has serious implications on the health of women worldwide, and ranks as the second leading cause of mortality in women. The main cause of breast cancer is still not completely understood (Ye *et al.*, 2004). Breast cancer may develop by different factors such as genetic alteration, life-style, and obesity, higher level of specific hormones, tobacco and smoking. For instance, several studies have suggested that chronic inflammation is derived from smoking and tobacco, which increases the risk of breast cancer (Mink *et al.*, 2002; Hecht, 2002). The pathology of breast cancer is classified into three categories according to its gene expression, receptors and immunohistochemistry characterisation (Table 1.2). This influences the prognosis of the disease and treatment response. The first type (Luminal subtype) is both estrogen receptor (ER) positive and progesterone receptor (PR) positive that constitutes for the majority of all breast cancer and is the least aggressive. Second type is human epidermal growth factor receptor-2 (HER2) positive that is approximately 25% and more aggressive than luminal. The final type is luminal triple-negative (basal-like) which is devoid of all receptors. This tumour or subtype is the most aggressive with worst prognosis (Gown, 2009; Schnitt, 2010).

Table 1.2: The main classification of breast cancer by gene expression profiling (Schnitt, 2010).

Molecular classification			
	Luminal	***HER2***	***Basal***
Gene expression pattern	High expression of ER and PR receptor and associated genes	High expression of HER2 and other genes.	High expression of basal epithelial genes, basal Cytokeratins.
Clinical features	The least aggressive. The majority of invasive breast cancers ER/PR positive.	~25% more aggressive than luminal. Invasive breast cancers (~15%) ER/PR negative	The most aggressive. Invasive breast cancers (~15%) Most ER/PR/HER2 negative ('triple negative')
Treatment response and outcome	Respond to endocrine therapy (but response to tamoxifen and aromatase inhibitors	Respond to trastuzumab (Herceptin) Respond to anthracyclinebased chemotherapy Generally. Worse prognosis.	No response to endocrine therapy or trastuzumab (Herceptin) This type of tumour is not carry same poor prognosis

1.15 Risk Factors for Breast Cancer

Many risk factors increase the chances of developing breast cancer. Notably, age is considered the primary risk factor related to breast cancer. For instance, women aged under 25 are less likely to develop breast cancer as there are 10 cases reported per 100,000 women and at the age of 45. These cases rise by 100-fold (Dumitrescu and Cotarla, 2005). Interestingly, women who conceive a child before the age of 20 are less likely get breast

cancer as compared to women who have a child after the age of 30 (McPherson *et al.*, 2000). Furthermore, the greater risk of developing breast cancer, as the women gets older may be due to the changes in their hormonal activities (McPherson *et al.*, 2000). There are different risks factors that have been found to play an important role in producing breast cancer, such as diet and obesity. The human diet consists of a combination of both carcinogenic and anti-carcinogenic compounds contained within various foods. Specific types of these compounds may lead to the production of free oxygen radicals that cause DNA damage (Dumitrescu and Cotarla, 2005). The connection between obesity and breast cancer related to menopause where it is due to the high levels of endogenous oestrogen found in obese women who are postmenopausal. On the other hand, women who are premenopausal and obese are less likely to develop breast cancer (McTiernan, 2003). An alternative known risk factor of breast cancer is mammography density, where it is irrelevant whether the women are in the pre- or postmenopausal stage. The family history is another important risk factor, where a meta-analysis has previously demonstrated that there is a 12% chance of having another family member affected and 1% chance of having more than one family affected by breast cancer (Dumitrescu and Cotarla, 2005; Biglia *et al.,* 2004). Moreover, up to 10% of breast cancer is referred as germline mutations. Two breast cancer mutations have been found the most common, *BRCA2* gene followed by *BRCA1* (McPherson *et al.,* 2000: Roy *et al.,* 2012). Exogenous hormones used after menopausal or the use of oral contraceptives increases the risk of breast cancer growth. Notably, there was a 24% greater risk of developing breast cancer when taking oral contraceptives and this risk reduces after 10 years of not taking them.

1.16 Diabetes as a Risk Factor for Cancer

Both diabetes and cancer are widespread diseases that are considered to be a major cause of death every year worldwide. Cancer is the second most common cause of death, while diabetes is the seventh (Habib and Rojna, 2013). Moreover, the likelihood of increasing diagnosis of cancer patients being diabetic cannot be by chance, suggesting a link. Reports observed by clinicians dating back more than 50 years have considered an association between the two diseases. Joslin *et al*, 1959 stated, "Studies of the association of diabetes and cancer have been conducted over a period of years, but evidence of a positive association remains inconclusive" (Joslin *et al.*, 1959). Many studies have suggested that the link between the two diseases is influenced by other factors such as type of diabetes. Several meta-analytical studies have suggested that some types of cancer are more likely to develop in diabetic patients than the general population (Giovannucci *et al.*, 2010; Vigneri *et al.*, 2009: Abe and Yamagishi, 2008). The Warburg effect is a theory that hypothesised high levels of glycolysis are found in many tumours. This is due to the tumours requiring high levels of energy in the form of ATP (Warburg, 1956). Recently, most studies indicate the link between diabetes and cancer via different mechanisms, such as hyperglycaemia, hyperinsulinemia and inflammation (Coughlin *et al.*, 2004; Griffiths *et al.*, 2012). In addition, several epidemiological studies have suggested that the increased level of insulin-like growth factor (IGF-1) is linked to many types of cancer such as liver, colon, kidney, pancreas and breast cancer (Coughlin *et al.*, 2004; Statin, 2007). Furthermore, many studies have proposed that female patients with both Type 1 and Type 2 diabetes are at high risk of developing breast cancer, especially through metabolism due to hyperglycaemia (Vigneri *et al.*, 2009; Xue and Michels, 2007; Michels *et al.*, 2003). However, the exact mechanism is still not fully understood. Notably, many studies have shown that there are four mechanisms that may contribute to the association between Type

2 diabetes and breast cancer, as demonstrated in Figure 1.5 (Wolf and Rubinek, 2008). Notably, many studies have found that increased level of glucoses above 7 mmol/L after fasting glucose test had overexpression of insulin receptor in breast cancer (Belfiore and Malaguarnera, 2011; Papa and Belfiore, 1996; Rapp *et al.*, 2006). Moreover, many studies have recently brought to light the benefits of metformin in diabetic patient in that it reduces both incidence and mortality of breast cancer (Ishibashi *et al.*, 2013).

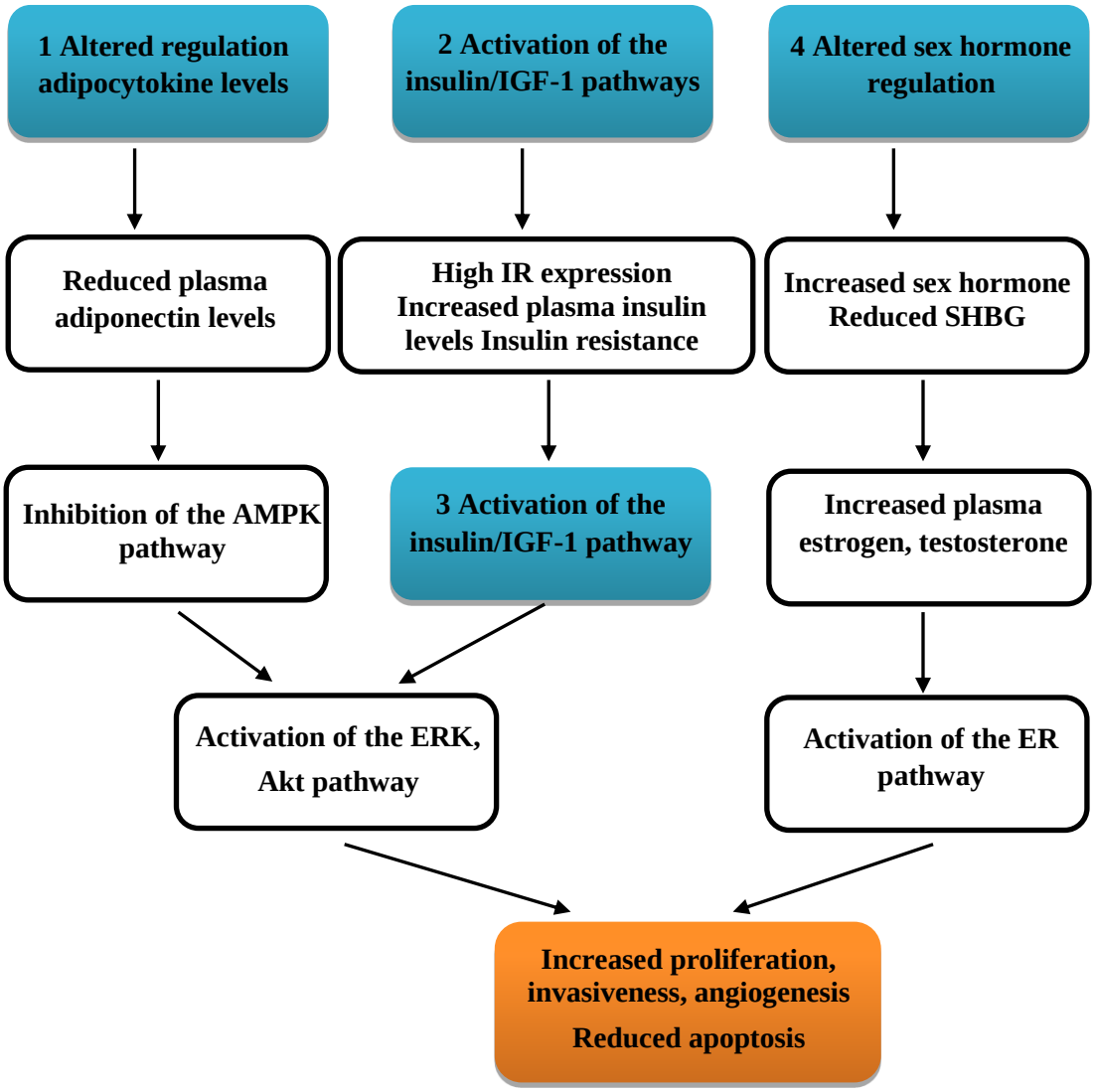


Figure 1.5: Mechanisms explain correlating type 2 diabetes and breast cancer. This Schematic explains the way insulin resistance develops (Wolf and Rubinek, 2008)**.**

1.17 Vascular complaications in breast cancer:

Breast cancer is known to have a high risk of developing vascular complications contributing to its development and its dissemination through metastasis. High incidence of venous thromboembolism (VTE) has been reported in many types of cancers including breast cancer particularly following chemotherapy and rises to approximately 18% in late stage of breast cancer (Kirwan *et al*, 2009; Caine *et al*, 2003).

Several studies suggested that this hyper-coagulable state reflected by VTE is associated with an increased level of plasma vascular endothelial growth factor (VEGF) a potent pro-angiogenic growth factor, released by activated platelets (Byrne *et al*, 2007) . Angiogenesis is tightly regulated process through which new blood vessels are formed. This process is strongly stimulated by the tumour and well known as a tumour angiogenesis, a process mainly induced by VEGF produced by the cancerous cells undergoing hypoxia (Filho *et al*, 2010). The abnormal new blood vessel formation will provide blood circulation to malignant cells assisting their growth and dissemination through metastasis. In addition, VEGF is also well known to increase vascular permeability, a critical event in angiogenesis also in inflammation which amplifies tumour angiogenesis. Different forms of VEGF have been detected at higher level of expression in the plasma or cancerous tissues and have been used as indicators of the stage of the progression of breast cancers (Heer *et al* 2001; Byrne *et al*, 2007; Kirwan *et al*, 2009). Other clinical studies have established the connections between the degree of VEGF expression with tumor aggressiveness reflected by microvessels density translated into patient's disease free and overall survival (Byrne *et al*, 2007).

AGEs have been reported to cause increased endothelial dysfunction through interaction with RAGE (Smit and Lutgers, 2004; Tan *et al.*, 2002) and are believed to be essentially responsible for the high-incidence of several vascular diseases leading to stroke and

peripheral vascular diseases for instance (Laakso, 1999; Rahman *et al.,* 2007). AGEs affect all main functions of vascular cells in both extracellular endothelial cells and supportive mesanchymal cells and increased vascular permeability. In addition, it has been demonstrated that AGE- RAGE interactions result in the stimulation of angiogenesis through up-regulation of VEGF expression and VEGF receptor activation which contribute to the onset and development of vascular angiopatheis. Moreover, AGEs have been found to stimulate the growth and tube formation of human microvascular endothelial cells which are considered to be the key steps of angiogenesis.

Similar changes of higher levels of VEGF expression is induced by AGEs affecting specifically pericytes leading to increasing vascular permeability and angiogenesis resulting into earlier changes seen in diabetic retinopathy ,more supportive evidence obtained by blocking this through blocking AGEs accumulation. Diabetic nephropathy have shown in *vivo* and *vitro* studies the pathological contribution of VEGF higher expression in mesangial cells and glomerular damage suggesting that AGEs may induce those changes and contribute to the development of increased permeability and clinical albuminuria which is an early evidence of diabetic nephropathy .other thrombogenic vascular changes is induced by reduction of nitric oxide NO which is known to possess an antithrombogenic pivotal role (Basta *et al*., 2004).

1.18 AGEs and Cancer

Numerous clinical and epidemiological studies have demonstrated that AGEs and their receptors are associated with various types of cancer through hyperglycaemia (Abe and Yamagishi, 2008; Jiao *et al.,* 2011). During the tumour progression, the uptake of glucose is increased and may result in increased levels of AGEs (van Heijst *et al.,* 2005). The modifications of AGEs have been found to contribute to malignancy, especially by their

influence on the DNA-glycation via oxidative stress and other various processes. Thus, the effect of AGEs on the DNA cause changes of gene expression and mutagenesis (Thornally *et al*, 2008). However, one recent study has shown that CML-AGEs increase the risk of colorectal cancers (Moy *et al.*, 2013). Another possible explanation is that hyperglycaemia enhances the oxidative stress via AGEs, which regulate chronic inflammation and changes the immune function that is associated with cancer. In addition, the interaction between AGEs and RAGE will lead to the activation of a diversity of cell signalling pathways that are expressed in various tumours (Kuniyasu *et al.*, 2002; Kim *et al.*, 2008).

1.19 Roles of AGE-RAGE in the Development of Cancer

RAGE is a multiligand receptor that have been expressed and up regulated in many types of cancer (Rojas *et al.*, 2011: Abe and Yamagishi, 2008). Many studies on animals and clinical data reinforce the belief that there is a direct link between RAGE activation that leads to progression of invasive and metastasis tumour cells (Riehl, *et al.*, 2009; Sparvero *et al*, 2009). Recent studies have suggested that there is an increased expression of RAGE during inflammation and tumour growth. For instance, this is demonstrated in colorectal, breast, lung, gastric, melanoma and prostatic cancer (Abe and Yamagishi, 2008; Abe *et al.*, 2004). This phenomena is considered to occur due to RAGE's ability to readily bind to many types of ligands, which then trigger the activation of signalling pathway and stimulate tumour growth. Then these cells proliferate, invade and metastasize that plays an important role in poor prognosis (Jing *et al.*, 2008). Among those receptor ligands that interact with AGEs and link to tumour growth, are high-mobility group box-1 (HMGB1, mphoterin) and S100. Furthermore, RAGE engagement with AGEs activates multiple signalling pathways such as reactive oxygen species, p38, ERK1, ERK2, JAK/STAT and SAPK/JNK (Rojas *et al*, 2011). The interaction between the AGEs and RAGE can

generate oxidative stress in the cells that contribute to cancer growth (Jiao *et al.*, 2011). The interaction between AGEs and RAGE, when induce the inflammation, results in the expression of NFκB that plays an important role in cellular processes and cell cycle as demonstrated in Figure 1.6 (Rojas *et al.*, 2011; Sinha *et al.*, 2008; Kim *et al.*, 2

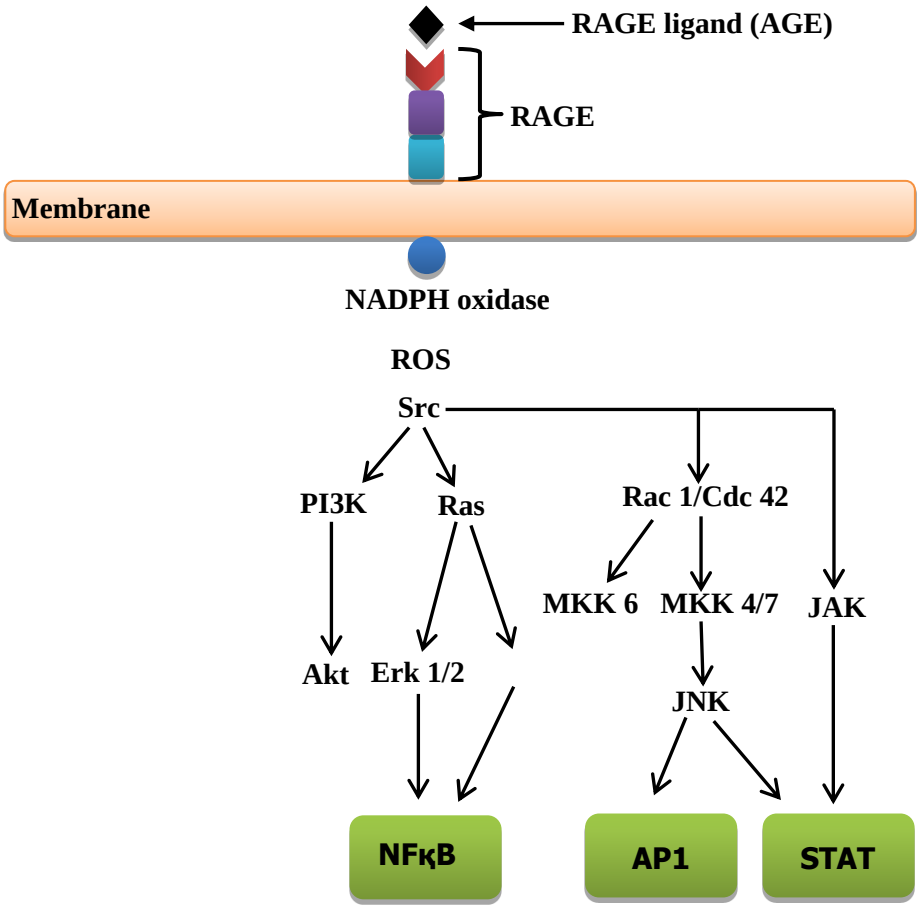


Figure 1.6: Schematic diagram of the interaction between RAGE ligands (AGE) and RAGE leading to numerous signalling pathways. These pathways are associated to tumours (Rojas *et al.*, 2011).

1.20 AGEs and Breast Cancer

In 2009, there were approximately 49,000 cases within the UK of women diagnosed with diabetes and breast cancer simultaneously (Cancer research, UK). Some studies have reported a correlation between diabetes and cancer (including breast cancer). However, underlying molecular link between diabetes and cancer remains to be elucidated.

There is a growing body of evidence to show that AGEs-RAGE interactions stimulated the growth of human cancer cells, but little is known regarding the molecular mechanism involved. Much attention has been given to the most aggressive phenotype of breast cancer with the identification of the roles that increasing RAGE expression, but AGE role in breast cancer is still unclear (Radia *et al.*, 2013; Korwar *et al.*, 2012). However, biological effects of AGE and AGE-RAGE in breast cancer cells are poorly investigated.

Aims and Objectives

The aim of this current study was to understand the role of AGEs in breast cancer. The main objectives are:

1. To prepare endotoxin and sugar free BSA-AGEs using glucose and methylglyoxal.
2. To assess the effects of BSA-AGE concentrations on the proliferation, migration and invasion of MDA-MB231 (non-hormone dependent) and MCF-7 (hormone dependent) breast cancer cell lines *in vitro*.
3. To determine the molecular signalling pathway mechanism that underlies the effect of BSA-AGEs on the MDA-MB231 and MCF-7 breast cancer cell lines *in vitro*.
4. To investigate the expression of RAGE-AGE involved on the MDA-MB231 and MCF-7 breast cancer cell lines *in vitro*.

Chapter 2

Chapter 2. Materials and Methods

2.1 Materials

- Breast cancer cell lines MDA-MB-231 and MCF-7 (American Type Culture Collection, Manassas, VA, USA)
- Conical centrifuge tubes, sterile (Scientific Laboratory Supplies, Nottingham, UK)
- Coverslips, Thermanox® plastic, sterile (Nunc™, Fischer Scientific, Loughborough, UK)
- Cryo-tubes, sterile (Nunc corporation, Roskilde, Denmark)
- Dialysis tubes (Camlab Limited, Cambridge, UK)
- Eppendorf tubes, sterile (Scientific Laboratory Supplies, Nottingham, UK)
- Gloves, sterile (Scientific Laboratory Supplies)
- Hypodermic needles, sterile Plastipak (Scientific Laboratory Supplies)
- Nitrocellulose membrane (Schleicher and Schuell, London, UK)
- Parafilm (Scientific Laboratory Supplies)
- Pierce™ high capacity endotoxin removal resin (Thermo Scientific™, Rockford, USA)
- Razor blades (Kratos Analytical Limited, Manchester, UK)
- Syringe, sterile Plastipak (Scientific Laboratory Supplies)
- Syringe filter, Acrodisc® 32 mm, 0.2 μm of porosity (Pall Corporation, Portsmouth, UK)
- Tissue culture flasks T25, T75 (Nunc™)
- Tissue culture plates in 6-well, 24-well, 96-well formats (Nunc™)
- Transwell® 24-insert plate (Nunc™)
- Universal tubes, sterile, 6 mL, 30 mL (Scientific Laboratory Supplies)

2.2 Chemicals and Reagents

- Acetic acid (Fisher Scientific UK Ltd, Loughborough, UK)
- Acrylamide/Bis solution 40% (Bio-Rad Laboratories, Hertfordshire, UK)
- Ammonium persulphate (Sigma-Aldrich, Dorset, UK)
- Antibiotics penicillin, streptomycin with L-glutamine in 0.9% NaCl (Sigma-Aldrich)
- Ascorbic acid (Sigma-Aldrich)
- Benzamidine (Sigma-Aldrich)
- Beta-glycerophosphate (Sigma-Aldrich)
- Bovine serum albumin, fraction V (Sigma-Aldrich)
- Bovine gelatine (Sigma-Aldrich)
- Brij® 35 (Sigma-Aldrich)
- Bromophenol blue stain (Sigma-Aldrich)
- Calcium chloride (Sigma-Aldrich)
- ColorBurst electrophoresis marker for SDS-PAGE (Sigma-Aldrich)
- Coomassie brilliant blue R-250 (Sigma)
- D-glucose (BDH Ltd, Dorset, UK)
- Dimethyl sulfoxide (Sigma-Aldrich)
- Dithiothreitol (Sigma-Aldrich)
- Dulbecco's modified Eagle's medium (Lonza Biowhittaker, Fisher Scientific)
- ECL chemiluminescent detection kit (Amersham Biosciences, Buckinghamshire, UK)
- Ethanol (Fisher Scientific UK Ltd)
- Ethylene diamine tetra acetic acid (Sigma-Aldrich)
- E-toxate kit (Sigma-Aldrich)
- Fibroblast growth factor basic (FGFb, R&D systems, Abingdon, UK)
- Foetal bovine serum (FBS, Lonza Biowhittaker, Fisher Scientific)

- Glyceraldehyde 3-phosphate dehydrogenase, mouse monoclonal antibody (Abcam, Cambridge, UK)
- Giemsa stain (Sigma-Aldrich)
- Glycerol (Sigma-Aldrich)
- Glycine (BDH Ltd)
- Growth factor-reduced Matrigel™ (Becton Dickinson, Oxford, UK)
- Horseradish peroxidase-conjugated secondary antibodies (Dako UK Ltd, Cambridgeshire, UK)
- Hydrochloric acid (Sigma-Aldrich)
- Isopropanol (Sigma-Aldrich)
- Isoton® II diluent (Fisher Scientific UK Ltd)
- Isotype control from murine myeloma, immunoglobulin IgG1 (Sigma-Aldrich)
- Lysozyme (Sigma-Aldrich)
- Leupeptin (Sigma-Aldrich)
- 2-Mercaptoethanol (Sigma-Aldrich)
- Methanol (Sigma-Aldrich)
- Methylene blue stain (Sigma-Aldrich)
- Methylglyoxal (Sigma-Aldrich)
- N, N, N′, N′-tetramethylethylenediamine (Sigma-Aldrich)
- Paraformaldehyde (Sigma-Aldrich)
- Pepstatin A (Sigma-Aldrich)
- Phenylmethylsulfonyl fluoride (Sigma-Aldrich)
- Phosphate-buffered saline (Sigma-Aldrich)
- Phospho-extracellular signal-regulated kinase, mouse monoclonal antibody (Santa Cruz Biotechnology)

- Protease inhibitor cocktail (Sigma-Aldrich)
- Protein estimation kit (Bio-Rad Laboratories, Hertfordshire, UK)
- Rabbit monoclonal anti-mouse antibody-fluorescein isothiocyanate (Invitrogen, Paisley, UK)
- Receptor for advanced glycation endproducts (RAGE, E-1), monoclonal antibody (Santa Cruz Biotechnology, Heidelberg, Germany)
- Skimmed milk powder (Local store, Manchester, UK)
- Sodium azide (Sigma-Aldrich)
- Sodium bicarbonate (Sigma-Aldrich)
- Sodium chloride (BDH Ltd)
- Sodium deoxycholate (Sigma-Aldrich)
- Sodium dihydrogen phosphate (Sigma-Aldrich)
- Sodium dodecyl sulphate (BDH Ltd)
- Sodium fluoride (Sigma-Aldrich)
- Sodium hydroxide (BDH Ltd)
- Sodium orthovanadate (Sigma-Aldrich)
- Sodium phosphate dibasic (Sigma-Aldrich)
- Sodium pyrophosphate (Sigma-Aldrich)
- Total-extracellular signal-regulated kinase, rabbit polyclonal antibody (Santa Cruz Biotechnology)
- Tris(hydroxymethyl)methylamine (BDH Ltd)
- Triton X-100 (Sigma-Aldrich)
- Trypan blue (Sigma-Aldrich)
- Trypsin/EDTA (Sigma-Aldrich)
- Tween 20 (Sigma-Aldrich)

2.3 Solutions and Buffers

- Activation buffer: 50 mM Tris-HCl, 0.2 M NaCl, 5 mM $CaCl_2$ and 0.02% Brij 35.
- Ammonium persulfate solution (10%): 0.1 g of ammonium persulfate dissolved in 1 mL of distilled water (dH_2O). The buffer was stored at 4°C.
- Blocking buffer (1% BSA in TBS-Tween): 1 g of bovine serum albumin (BSA) added to 100 mL of tris-buffered saline (TBS)-Tween, pH adjusted to 7.4. The buffer was stored at 4°C for one week.
- Blocking buffer (5% milk in TBS-Tween): 5 g of skimmed milk added to 100 mL of TBS-Tween, pH adjusted to 7.4. The buffer was stored at 4°C for one week.
- BSA solution (0.1%): 10 mg of BSA dissolved in 10 mL of dH_2O. The solution was stored at 4°C for one week.
- Cell lysis buffer for the extraction of RAGE: 10 mM Tris HCl (pH 7.4), 50 mM NaCl, 5 mM EDTA, 1% Triton X-100, 0.05% SDS, 50 mM sodium fluoride, 100 uM sodium orthovanadate, 10 mM beta-glycerophosphate, 10 mM sodium pyrophosphate, 100 ug/ml PMSF, 3 mM benzamidine, 1 mM dithiothreitol, 10 uM leupeptin, 5 uM pepstatin A and cocktail of protease inhibitors from Sigma.
- Destaining solution: 250 mL of methanol added to 70 mL of acetic acid to make a volume of 1 L with (dH_2O).
- Electrophoresis buffer: 12.02 g of Tris-base, 4 g of SDS and 57.68 g of glycine dissolved in 2 L of dH_2O. The buffer was kept at room temperature.
- Laemmli sample buffer: For 10 mL, mix 4 mL of 10% SDS, 1.2 mL of 1 M Tris-Cl (pH 6.8), 200 µL of 1% bromophenol blue, and 2.6 mL of H_2O. Add 2 mL of fresh DTT (1 M) from stock. (For experiments with radioactive methionine, use only 500 µL of fresh DTT [1 M], and boost the volume of H_2O to 4.1 mL.

- Radioimmunoprecipitation assay (RIPA) buffer: 25 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.5% sodium deoxycholate, 0.5% SDS, 1 mM EDTA, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1% Triton-X100 and 1 μM Leupeptin.
- Renaturation buffer: of 2.5% Triton X-100 (v/v).
- Sample buffer (non-reducing): 0.5 M Tris-HCL, pH 6.8 (2.5 mL), glycerol 2.0mL 10% (w/v) SDS (4.0 mL), 0.1% bromophenol blue (0.5mL) with (dH_2O) to 10.0 mL.
- Sample buffer: 1.51 g of Tris-base, 4 g of SDS and 0.004 g of bromophenol blue added to 20 mL of glycerol and 10 mL of 2-mercaptoethanol to make a volume of 100 mL of dH_2O. The pH was adjusted to 6.8 and the buffer was stored at -20°C.
- Separating buffer: 45.5 g of Tris-base and 1 g of SDS dissolved in 250 mL of dH_2O, pH adjusted to 8.8. The buffer was kept at room temperature.
- Separating gel (12.5% acrylamide): 2.5 mL of separating buffer, 4.2 mL of dH_2O were mixed with 3.3 mL of acrylamide/Bis solution 40%. The addition of 100 μL of 10% ammonium persulphate and 10 μL of TEMED allows the solution to polymerize within 20-25 minutes.
- Sodium phosphate buffer (0.1 M): 3.1 g NaH_2PO_4 and 10.9 g Na_2HPO_4 dissolved in 1 L of dH_2O, pH adjusted to 7.4. The sodium azide (3 mM) was added to prevent any contamination. The buffer was stored at 4°C for up to one month.
- Stacking buffer: 15 g of Tris-base and 1 g of SDS dissolved in 250 mL of dH_2O, pH adjusted to 6.8. The buffer was kept at room temperature.
- Stacking gel (5.5% acrylamide): 2.5 mL of stacking buffer, 6.1 mL of dH_2O were mixed with 1.45 mL of acrylamide/Bis solution 40%. The addition of 100 μL of 10% ammonium persulphate and 10 μL of TEMED allows the solution to polymerize within 10-15 minutes.

- Staining solution: 2.5 g of Coomassie blue added to 500 mL of methanol and 100 mL of acetic acid to make a volume of 1 L of dH_2O. The solution was filtered and kept at room temperature.
- TBS-Tween: 2.422 g of tris-base and 16.36 g NaCl were dissolved in 2 L of dH_2O and 2 mL of Tween 20. The pH was adjusted to 7.4 and the solution was kept at room temperature.
- Towbin buffer: 1.5 g Tris-base, 7.2 g glycine and 0.167 g SDS dissolved in 400 mL of dH_2O, pH adjusted to 8.3. The buffer was kept at room temperature.
- Transmembrane protein extraction buffer: 10 mM Tris-HCl (pH 7.4), 50 mM sodium fluoride, 100 μM sodium orthovanadate, 10 mM β-glycerophosphate, 10 mM sodium pyrophosphate, 100 μg/mL phenylmethylsulfonyl fluoride, 10 mM dithiothreitol, 3 mM benzamidine, 1 mM dithiothreitol, 10 μM leupeptin, 5 μM pepstatin A and a cocktail of protease inhibitors.

2.4 Equipment and Software

- Analytical balance (Sartorius Machatronics Ltd, Epson, UK)
- Automated cell counter TC10 (Bio-Rad Laboratories)
- Class II microbiological safety cabinet (Walker Safety Ltd, Derbyshire, UK)
- CO_2 incubator (Lab Impex Research Ltd, Abbey, UK)
- Coulter Counter (Beckman Coulter, Buckinghamshire, UK)
- Centrifuge 5415D (Eppendorf, Hamburg, Germany)
- FACS Calibur Flow Cytometer and FACScan analysis software (Becton Dickinson)
- Fujifilm FinePix S2 Pro 6.17 MP digital SLR camera (Fujifilm UK Ltd, Bedford, UK)
- G Box Chem HR 16 with GeneSnap software (Syngene, Cambridge, UK)

- Ice maker (Borolab Ltd, Abingdon, UK)
- Image J analysis software (http://rsbweb.nih.gov/ij/index.html)
- Inverted phase contrast microscope (TMS, Nikon, Tokyo, Japan)
- Laboratory freezer Bio cold (Scientific Laboratory Supplies)
- Laboratory fridge Bio cold (Scientific Laboratory Supplies)
- Laboratory pH/mV/temperature meter AGB-75 (Medical Scientific Instruments, Hertfordshire, England)
- LTE IP 30 incubator (Scientific Laboratory Supplies)
- Luminescence spectrometer model LS 30 (Perkin Elmer LAS Ltd, Buckinghamshire, UK)
- Microsoft office 2007 (Microsoft, USA)
- Sigma laboratory centrifuge 3K10 (Sigma Laboratories, Germany)
- Stuart Magnetic stirrer hotplate (Fisher Scientific UK Ltd)
- Trans-blot SD semi-dry transfer cell (Bio-Rad Laboratories)
- Ultrospec 2000 UV-VIS spectrophotometer (Amersham Pharmacia Biotech Ltd, Hertfordshire, UK)
- Water bath (Scientific Laboratory Supplies)
- Water purification system (Millipore UK Ltd, Watford, UK)

2.5 Methods

2.5.1 *In vitro* Glycation Reactions

The preparation of bovine serum albumin (BSA) derived advanced glycation endproducts (BSA-AGEs) was based on an established procedure previously described by Mashilipa *et a*l. (2011). Briefly, BSA or lysozyme (10mg/ml) was incubated with glucose (0.5M) or methylglyoxal (0.1 M) in 0.1M sodium phosphate buffer containing 3mM sodium azide, pH 7.4 at 37 °C for different incubation times or different concentrations with methylglyoxal (0.01 M, 0.5 M, 0.1 M). The sodium azide was added to prevent the growth of bacteria and fungi during the glycation reaction, which lasted for 3 days in the presence of methylglyoxal and 15 days in the presence of glucose at 37°C. Non-modified BSA and lysozyme underwent the same conditions of preparations but in the absence of glucose or methylglyoxal. All glycation reactions were carried out in triplicate.

2.5.2 Dialysis of BSA-AGEs

This method was used for the removal of free sugars from the glycated proteins via dialysis against distilled water (**Figure 2.1**). Special dialysis tubes were utilised and sterilized after being boiled for at least 30 minutes in a solution composed of 10 mM sodium bicarbonate /1mM EDTA. One end of the tube was tied then the protein samples were poured into the tube after which the other end of the tube was sealed. The dialysis tubing was transferred into a beaker containing 2 L of distilled water and 200 µL of chloroform were added to prevent any bacterial and fungi contamination. Dialysis was conducted by stirring the samples at 4 ºC. The water was exchanged over three days until equilibrium was reached. Finally, the samples were transferred into clean tubes and stored at -20°C until further use.

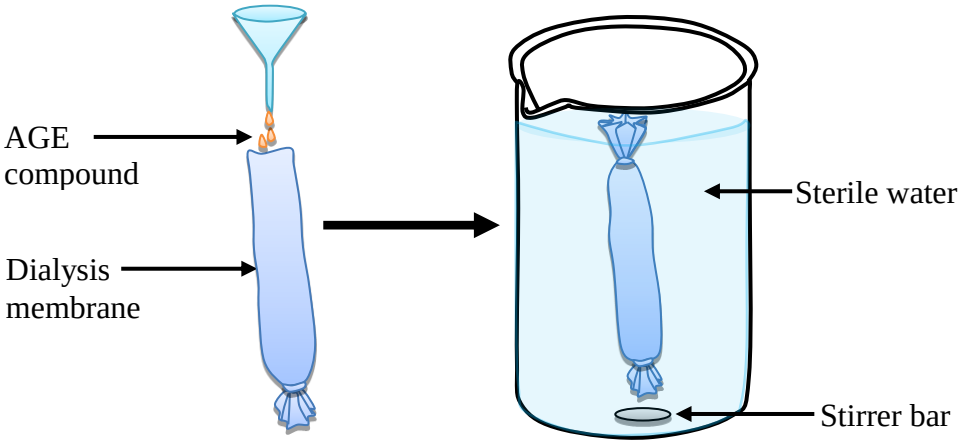


Figure 2.1: Illustration of the dialysis set-up

2.5.3 Endotoxin Removal from BSA-AGEs

The BSA-AGEs were prepared in non-sterile conditions with a high risk of bacterial endotoxin contamination. The bacterial endotoxins are small pro-inflammatory particles, which can alter the proper biological effects of BSA-AGEs on cells. Because of their small size, filtration of the BSA-AGE solution using a standard filter with 0.22 μm of porosity would be insufficient to get rid of the bacterial endotoxins from the solution. Therefore, in sterile conditions using the safety cabinet, the endotoxin removal was performed by subjecting the sample solution to Pierce™ high capacity endotoxin removing gel resin columns. These resin columns has a special filter containing molecules with affinity for endotoxin were regenerated by cycles of washes corresponding to 5 resin-bed volumes of 1% sodium deoxycholate. BSA-AGE and non-modified BSA solutions mixed with a basal medium with phenol red (tracking dye) were fulfilled the gel resin column with a total volume of 1 mL. After 2 hours at room temperature, the sample was collected in a sterile pyrogen-free tube and kept in the freezer -20 ºC.

2.5.4 Endotoxin Detection in BSA-AGEs

The remaining of bacterial endotoxins in the sample was detected by using E-toxate kit based on *Limulus Amebocyte* lysate assay. Briefly, 100 µL of samples, water and endotoxin standards were added to the sterile glass pyrogen-free tubes. E-toxate working solution (100 µL) was added to each test tube containing the sample. The tubes were then mixed gently and covered with Parafilm® for 1 hour at 37ºC. Subsequently, the tubes were gently inverted to 180º for the observation of a potential formation of a hard gel, which was considered as a positive test with high endotoxin concentration. The negative tests corresponded to the observation of soft gels, turbidity, viscosity or clear liquid. The endotoxin concentration was calculated using the following formula:

$$\text{Endotoxin (EU/mL)} = \frac{1}{\text{Highest dilution of sample (positive test)}} \text{ X } \text{Lowest concentration of endotoxin standard found positive}$$

2.5.5 Standard Curve for Protein Determination

BSA was used as a standard protein. Serial dilutions of BSA (1 µg/µL) were made with dH_2O up to 100 µL, establishing a range of protein varying from 0 µg to 40 µg/µL (See Table 2.1). Protein sample solution (10 µL) was mixed with dH_2O (90 µL). Bio-Rad protein assay reagent (2 mL) was added to each tube containing 100 µL of BSA or diluted BSA or protein sample. Absorbance of the protein solution was measured at 570 nm using a spectrophotometer. A standard curve required for the determination of sample protein concentration was established from the serial dilution of BSA as shown in (Figure 2.2).

Table 2.1 Prepatation of a Serial Dilutions of BSA for standard curve.

Stock 0.1% BSA (µL)	Distilled water (µL)	Bio-Rad reagent (mL)
0	100	2
10	90	2
20	80	2
40	60	2

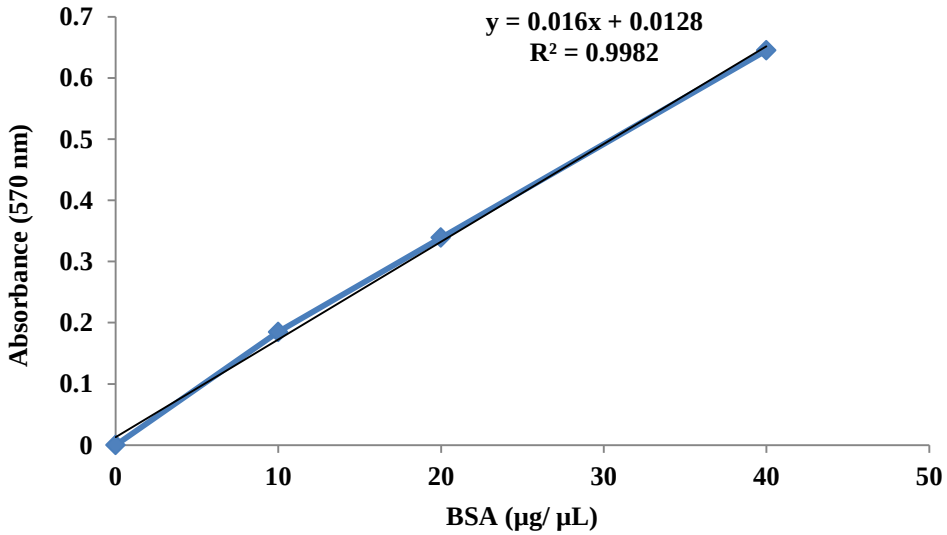


Figure 2.2: Standard curve for protein estimation.

2.5.6 Detection of Crosslinked BSA-AGEs using SDS-PAGE

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out to assess the integrity and cross-linking of glycated proteins. This method separates the denatured and negatively charged glycated proteins according to their molecular weight (Laemmli, 1970). The protein samples were denatured in 0.5 M Tris-HCl buffer (pH 6.8) containing 1% SDS (w/v), 1% 2-mercaptoethanol (v/v) and 20% glycine (v/v) and boiled for 5 minutes. The protein samples were loaded into the wells and migrated alongside a standard molecular weight marker. Bromophenol blue (3 µL) was added for protein tracking, and the electrophoresis was performed using the mini-Protean®3 apparatus. At the end of the complete protein separation, the gels were stained in a staining solution containing Coomassie brilliant blue R-250, methanol and acetic acid for 30 min at room temperature. The bands corresponding to the proteins were revealed after destaining the gels in the solution composed of methanol and acetic acid only for 15 min at ambient temperature.

2.5.7 Analysis of Crosslinked BSA-AGEs using G Box

After glycated protein separation by SDS-PAGE, followed by staining with Coomassie blue then destaining with methanol, the level of cross-linkage was assessed using an image analysis system. . Bands containing a mix of proteins two or more polypeptides may result from the occurrence of polypeptides that normally form noncovalent associations with each other or with other polypeptide types. This system used to estimate the relative quantities of monomeric, dimeric, trimeric and tetrameric species that resolved by SDS-PAGE. Briefly, the destained gels revealing the stained bands on a clear background were analysed using GeneSnap software with Gene tool image analyser, which allow transferring data directly into excel sheet.

2.5.8 Measurements of fluorescent BSA-AGEs

The formation of fluorescent BSA-AGEs was confirmed by the assessment of their characteristic fluorescence emission spectra at 420 nm in response to an excitation at 350 nm, measured with a fluorescence spectrophotometer. BSA-AGEs and non-modified BSA solution (0.2 mL) were diluted with dH_2O to a final concentration of 1 mg/mL. The reliability and accuracy of the instrument was checked by the use of fluorescent intensity standards to monitor its performance and by the use of quinine sulphate (1 ng/mL) in H_2SO_4 for its calibration. Fluorescence of BSA-AGEs was expressed in arbitrary units (AU) per mg of proteins.

2.6 Culture of Breast Cancer Cell Lines MDA-MB-231 and MCF-7 Cells

Cell culture is considered one of the most crucial techniques that have been discovered in the few decades. Notably, cell culture has the ability to control the physicochemical environment (such as the pH, temperature, carbon dioxide) to maintain constant environment during the experimentations All the procedure and experiment in this thesis were carried out in a sterile environment in culture room and safety cabinet. Ethanol (70% volume/ volume) was used for cleaning all surfaces.

2.6.1 Heat-Inactivation of the Foetal Bovine Serum (FBS)

The foetal bovine serum (FBS) was inactivated for 30 minutes in a water bath adjusted to 56°C. For a complete inactivation of the serum compliment, the bottle was regularly shaken. The serum was previously stored in the freezer; the frozen FBS was subsequently thawed at 37°C in a water bath before the heat-inactivation. The heat-inactivation of the serum avoided any cell activation or inflammation in response to serum proteins. To avoid

repeated freeze/thaw cycles, the heat-inactivated serum was aliquoted into sterile 30 mL universal tubes and stored at -20°C.

2.6.2 Preparation of Breast Cancer Cell Line Culture Medium

The complete medium prepared for the culture of both breast cancer cell lines MCF-7 and MDA-MB-231 was composed of Dulbecco's modified Eagle's medium (DMEM) containing 4.5 g/L glucose and supplemented with 10% heat-inactivated (FBS) and 100 U/mL penicillin, 100 μg/mL streptomycin), and 2 mM glutamine.

2.6.3 Culture of Breast Cancer Cells

MDA-MB-231 and MCF-7 breast cancer cell lines were obtained from American Type Culture Collection (Manassas, VA, USA) and cultured in T-25 flasks in complete medium at 37°C in 5 % CO_2 -incubator.

2.6.4 Trypsinization of Breast Cancer Cells

After MCF-7 and MDA-MB231 cells reached 80-90% confluence, the culture medium was removed and the cells were rinsed twice with 5 mL of sterile PBS. The cells were subsequently exposed to 1 mL of 0.05% trypsin 0.02% EDTA which allowed cell detachment by enzymatic digestion at 37°C. After 2-3 minutes of incubation, the floating cells were collected in a sterile 30 mL universal tube and the trypsin was neutralized by the addition of the complete medium. After 5 minutes of centrifugation at 300 g at room temperature, the supernatant was discarded and the cell pellet was re-suspended in a fresh complete medium for subculture or in serum-poor medium (SPM; basal medium supplemented with 2.5% FBS) according to the assay used throughout the study.

2.6.5 Freezing of Breast Cancer Cells

The MCF-7 and MDA-MB-231 confluent cells corresponding to 1 x 10^6 cells were detached from one T-25 flask according to the trypsinization method previously described. After the standard conditions of centrifugation, the supernatant was discarded and the cell pellet was re-suspended in 250 µL of complete medium and 750 µL of freezing medium composed of 10 % DMSO and 90 % FBS. The freezing medium containing the cells was equally distributed in 3 cryovials containing approximately 3 x10^5 cells. The cells were gradually frozen starting in the freezer at – 20 ºC for 30 minutes then placed in the freezer at -80 ºC overnight before being stored in liquid nitrogen (– 192 ºC).

2.6.6 Thawing of Breast Cancer Cells

The cryotube containing the frozen cells was taken from the liquid nitrogen (for long storage) or from the freezer -80 ºC (for short storage). The cryovial was gently defrosted at 37 °C in the water bath until the freezing medium was completely melted but remained cold because of the presence of DMSO, a cryo-protector reagent that becomes toxic to the cells at high temperatures. The cryovial was sprayed with 70 % ethanol inside the safety cabinet then the cells were transferred in a universal tube containing complete medium for centrifugation. The cell pellet was re-suspended in 3 mL of complete medium and the cells were seeded in a T-25 flask for incubation.

2.6.7 Cell Counting

The cells were counted using a Beckman-Coulter counter based on 50 μL of cell suspension diluted in 10 mL of isotonic solution and only 0.5 mL of diluted cell suspension were counted. The reading was taken three times and the cell concentration was calculated according to the following formulas:

Mean of cell number = Mean of cell counts X (10/0.05) = Mean X 200 (dilution factor) = N

Cell concentration (Mean of cell number/mL) = N / 0.5 mL (volume counted by the cell counter)

=Mean X 200 / 0.5 = Mean X 400 (dilution factor)

2.7 Effect of BSA-AGEs on Breast Cancer Cells

2.7.1 Cell Proliferation Assay

The MDA-MB-231 cells (2.5 x 10^4 / mL) and MCF-7 cells (5 x 10^4 / mL) in 0.5 mL of complete medium (10 % FBS) were seeded in 24-well plates. After 4 hours of incubation allowing the cells to attach to the bottom of the well, the medium was changed to a serum-poor medium (SPM) supplemented with 2.5% FBS in the presence or absence of different concentrations (25 – 200 μg/mL) of BSA-AGEs and non-modified BSA. Each condition was performed in triplicate. After 72 hours of incubation, the cells were detached in 250 μL of 0.05% trypsin / 0.02% EDTA then each cell suspension was diluted in 10 mL of isotonic solution and counted using a Beckman-Coulter counter.

2.7.2 Cell Viability Assay

The cell viability was assessed using a haemocytometer, a counting chamber analysed by TC10™ automated cell counter. MDA-MB-231 cells (2.5 x 10^4 / mL) and MCF-7 cells (5

x 10^4 / mL) in 0.5 mL of complete medium were seeded in each well of 24-well plate. After 4 hours of incubation, the culture medium was replaced with SPM in the presence or absence of different concentrations (25 – 200 μg/mL) of BSA-AGEs and non-modified BSA. Each condition was performed in triplicate. After 72 hours of incubation, the cells were washed with 0.5 mL of sterile PBS then detached by enzymatic digestion with 250 μL of 0.05% trypsin / 0.02% EDTA for 2-3 minutes. Then 20 μL of the cell suspension were mixed with 20 μL of 0.4% trypan blue, then, 10 μL of this mixture was loaded into each side of the haemocytometer. The cells were visualised by light microscopy. After staining, the viable cells appeared colourless and bright as they exclude the dye, while the dead cells appeared blue. The number of cells was counted and the percentage of viable cells was calculated using the equation below:

$$\text{Cell viability (\%)} = \frac{\text{Total number of cells} - \text{died number of cells} \times 100}{\text{Total number of cells}}$$

2.7.3 Cell Migration Assay using the Scratch Wound-Healing Method

The MDA-MB-231 cells (5 x 10^4 / mL) in 1 mL of complete medium were seeded on Thermanox® plastic coverslips (on the side treated for cells) in each well of a 24-well plate. After 24 hours of incubation, the cells reached the pre-confluence on the coverslips; the culture medium was changed to SPM for a further 24 hours of incubation. At confluence, the coverslip fully covered with the cells was removed from the well with a sterile forceps and the cells were bathed 3 times in a sterile universal tube containing sterile PBS. Each monolayer was subsequently wounded with a sterile razor blade on two sides of the coverslip, which gave the formation of two wound edges per coverslip with cell-denuded areas. Dislodged cells and cell debris were removed with sterile PBS. The coverslips with wounded cell monolayers were returned to the well containing SPM with or without

different concentrations (25 – 200 μg/mL) of BSA-AGEs and non-modified BSA for 24 hours of incubation. Each condition was performed in duplicate. At the end of the experiment, the cells were then fixed, rinsed with PBS then 100% ethanol was added for 5 minutes and the cells were left to dry at room temperature. The cells were stained with 0.1% methylene blue for 2 minutes and washed abundantly with distilled water to reveal the wound recovery. Four pictures from the wound edge of each side were taken to assess the cell migration by counting the number of the migrated cells and by measuring the distance of the cell migration using Image J software (http://rsbweb.nih.gov/ij/indix.html).

2.7.4 Cell Migration Assay using Boyden Chamber Method

In contrast to MDA-MB231 cells, which fully covered the coverslips, the MCF-7 cells grew mainly in clusters with the apparition of cell-denuded areas. Therefore, to assess the effects of BSA-AGEs on MCF-7 cell migration, the Boyden chamber method was used. The Boyden chamber, also called Transwell®, is composed of two chambers: the upper chamber is an insert, which has at the bottom a porous membrane with 8 μm of porosity and the lower chamber corresponds to the well of the plate. To avoid the increase of inactivated cell migration through the porous membrane because of the gravity, the porous membrane was covered with a thin layer of 0.1 % gelatine (20 μL) and left to dry in the safety cabinet (**Figure 2.3**).

MCF-7 cells (1 x 10^5 /mL) in 100 μL of complete medium were seeded on the porous membrane of the upper chamber and bathed in 400 μl of SPM in the presence or absence of different concentrations (25 – 200 μg/mL) of BSA-AGEs and non-modified BSA for 24 hours of incubation. Each condition was performed in duplicate. After 24 hours of incubation, the non-migrated cells on the upper side of the porous membrane were

removed using cotton swab soaked with PBS. The cells, which migrated across the porous membrane, were fixed with 4% paraformaldehyde (PFA) then stained with 0.1% Giemsa stain for cell counting of the membrane using a Zeiss optical microscope.

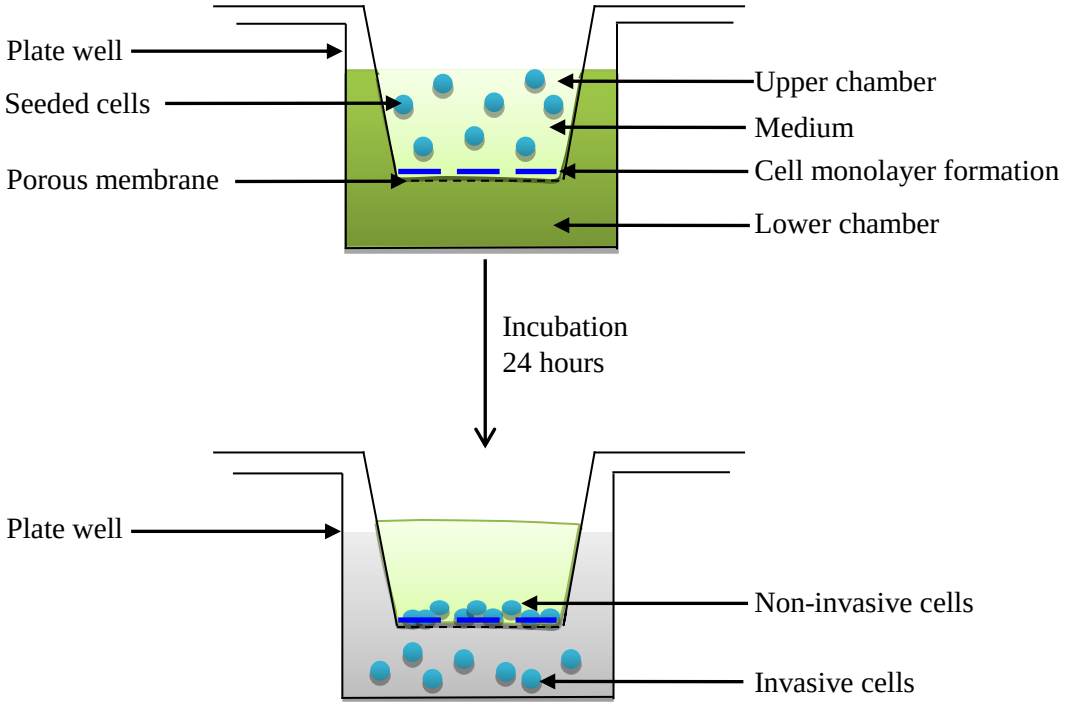


Figure 2.3: Schematic representation of Boyden Chamber.

2.7.5 Cell Invasion Matrigel™ Assay

The Matrigel™, a growth factor-reduced reconstituted basement membrane, was diluted (1:6) in serum-free medium then (20 µL) poured onto the porous membrane of a Transwell® 24-insert plate. The plate was incubated for 30 minutes to allow the gel to polymerise. MDA-MB-231 cells were seeded at 1 x 10^4 / 100 µL in SPM (in the upper chamber of the Transwell®) in the presence or absence of different concentrations (25 – 200 µg/mL) of BSA-AGEs and non-modified BSA (in the well corresponding to the lower chamber of the Transwell®). Each condition was performed in duplicate. After 24 hours of

incubation, the non-migrated cells located on the upper side of the porous membrane were removed using cotton swab soaked with PBS. The cells, which migrated across the porous membrane, were fixed with 4% PFA then stained with 0.1% Giemsa stain for cell counting using a Zeiss optical microscope.

2.7.6 Gelatine Zymography

The matrix metalloproteinase (MMP) activities in the cell-culture media, which had been used for cell invasion assay, were assessed. The medium was collected and centrifuged at 400 *g* for 15 minutes at 4°C. Protein concentration was determined using the Bio-Rad protein assay (see section 2.5.5), and the samples (100 μg of proteins) were mixed with an equal volume of non-reducing sample buffer. The samples were incubated for 10 minutes at room temperature and subjected to electrophoresis on 7.5% SDS-PAGE gels containing 1 mg/mL gelatine, used as a substrate. The gels were washed in renaturation buffer for 30 minutes at room temperature to remove SDS and to renature the MMPs in the gels. Then the gels were rinsed in activation buffer for 30 minutes to activate the MMPs. The gels were incubated overnight at 37°C with fresh activation buffer then stained with 0.5% Coomassie blue R-250 for 2 hours at room temperature. After destaining, MMP gelatinase activity was detected as a white band on a dark background and quantified by densitometry using Image J software.

2.7.7 Preparation of Cell Lysates and Western Blot Analysis

The MDA-MB-231 cells (3×10^5/mL) and MCF-7 cells (6×10^5/mL) in 2 mL of complete medium were seeded in 6-well plates. After 48 hours of incubation, the medium was renewed with SPM for a further 24 hours of incubation, and then BSA-AGEs or non-modified BSA were added for 5, 10, 30 and 120 minutes of incubation. Each condition was

performed in triplicate. After washing in cold PBS, all the intracellular proteins were extracted after lysing the cells with 120 µL/well of ice-cold radioimmunoprecipitation (RIPA) buffer (pH 7.5). The cell lysates were collected in cold eppendorf tubes then centrifuged at 20,000 *g* for 30 minutes at 4°C to get rid of the cell debris.For study of RAGE expression, the cells were treated with or without BSA or BSA-AGEs for 10 minutes and 24, 48 and 72 hours of incubation. Each condition was performed in triplicate. The cells were then lysed with 80 µL/well of ice-cold (RIPA) buffer. The cell lysates were collected in cold eppendorf tubes then centrifuged at 20,000 *g* for 30 minutes at 4°C to get rid of the cell debris.

Protein concentration of the intracellular and transmembrane proteins was determined using the Bradford protein assay and the samples (40 µg of intracellular proteins and 100 µg of transmembrane proteins) were mixed with an equal volume of 2X Laemmli sample buffer, denatured by boiling in a water bath for 15 minutes then briefly centrifuged. Samples were separated along with pre-stained molecular weight markers using12% SDS-PAGE. Proteins were electro blotted onto nitrocellulose membranes (1 hour) and the membranes were blocked for 1 hour at room temperature in TBS-Tween (pH 7.4) containing 1% BSA. Membranes were stained with the following primary antibodies diluted in the blocking buffer, overnight at 4°C on a rotating shaker: mouse monoclonal antibodies to phospho-extracellular signal-regulated kinase (p-ERK1/2, Tyr204 of ERK1, 1:1000), rabbit polyclonal antibodies to ERK1/2 (1:1000) and mouse monoclonal antibodies to RAGE (1:1000) and mouse monoclonal antibodies to GAPDH. After washing five times for 10 minutes in TBS-Tween at room temperature, the filters were stained with either rabbit anti-mouse or goat anti-rabbit horse-radish peroxidase-conjugated secondary antibodies diluted in TBS-Tween containing 5% de-fatted milk (1:1000) for 1 hour at room temperature with continuous mixing. After a further 5 washes in TBS-Tween, proteins

were visualised using ECL chemiluminescent detection and analysed using GeneSnap software with Gene tool image analyser.

2.7.8 Flow Cytometry Analysis

The MDA-MB-231 cells (3 x 10^5 / mL) and MCF-7 cells (6 x 10^5 / mL) in 2 mL of complete medium were seeded in 6-well plates. After 24 hours of incubation, the medium was renewed with SPM for a further 24 hours of incubation, and then BSA-AGEs or non-modified BSA were added for 10 minutes and 24, 48, 72 hours of stimulation at 37°C. Each condition was performed in triplicate. The cells were washed with PBS then the cells scraped to maintain intact the structure of the transmembrane protein RAGE. After centrifugation (300 *g* for 10 minutes), 10^6 cells were re-suspended in 20 µL of PBS then 20 µg/mL of mouse anti-RAGE antibody were added and the mixture kept on ice for 45 minutes. The excess antibody was removed by washing the cells twice with PBS followed by centrifugation. After the second centrifugation, the cells were re-suspended in 20 µL of PBS then 20 µg/mL of rabbit monoclonal anti-mouse antibody-FITC were added and the mixture was kept on ice for 30 minutes. The RAGE expression level was measured using FAC Scan analysis software from FACS Calibur Flow Cytometer (Becton Dickinson).

2.7.9 RAGE Neutralization

To investigate whether BSA-AGEs act through RAGE, the cells were treated with anti-RAGE antibody to neutralize all the RAGE receptors. Briefly, MDA-MB-231 cells (5 x 10^4 / mL) in 1 mL of complete medium were seeded in 24-well plates. In pre-confluent cultures, the medium was renewed with SPM, and then 20 µg/mL anti-RAGE antibody or 20 µg/mL IgG1 used as an isotype control were added to the medium. After 1 hour of incubation, the cells were treated with 100 µg/mL BSA-AGEs for 10 minutes stimulation

at 37°C. Each condition was performed in triplicate. Phospho-ERK1/2 expression was assessed by Western blotting as previously described.

2.7.10 Kinexus Phospho-Protein Array Analysis

The MDA-MB-231 cells (3 x 10^5 / mL) and MCF-7 cells (6 x 10^5 / mL) in 2 mL of complete medium were seeded in 6-well plates. After 48 hours of incubation, the medium was changed to SPM and after a further 24 hour-incubation, 100 μg/mL BSA-AGEs or non-modified BSA were added and the cells incubated for 10 minutes at 37°C. To determine the protein expression profile of signalling pathways downstream of RAGE, Kinexus Bioinformatics (Vancouver, Canada) performed a phospho-protein array analysis (Kinetworks PhosphoSite Screen, KPSS-1.3). Protein samples (500 μg) from BSA-AGEs or non-modified BSA-stimulated and un-stimulated cells were extracted according to the manufacturer's instructions. The samples were used for a multi-immunoblotting assay based on SDS-polyacrylamide mini-gel electrophoresis with 20-lane multi-immunoblotters using different primary antibodies. The protein expressions were visualized by chemiluminescence and relative expression was determined by Kinexus and expressed in normalized counts per minute (C.P.M.).

2.8 Statistical Analysis

Results are expressed as mean ± standard deviation (SD). Experimental points were performed with a minimum of three independent experiments using freshly prepared solutions. An unpaired Student's t-test was used for comparison of two groups. A significant value are given as *: $P < 0.05$, **: $P < 0.01$ and ***: $P < 0.001$.

Chapter 3

Chapter 3. Effects of BSA-AGEs on Breast Cancer Cell Line MDA-MB231

3.1 Introduction

The glycation process leads to the formation of AGEs and plays a crucial role in the pathogenesis of diabetic complications and cancer. Several studies conducted both *in vivo* and *in vitro*, have shown a possible link between AGEs and cancer. However, the presence of AGEs identified in human cancer tissues, and their expression is distinctly varied between different types of tumors (Rojas, 2011). Moreover, many studies have investigated whether AGE-RAGE interaction is a potential stimulant for growth proliferation, migration and invasion in different human cell lines (Yamagishi *et al*., 2005). For example, one of the studies on gylceraldehyde-AGEs (glycer-AGEs) was shown to enhance malignancy of melanoma cells (Takino *et al.*, 2010). However, the role of AGEs in breast cancer development is still not investigated. In this chapter, MDA-MB231 cell line was used, and this cell line is defined as tumors lacking estrogen receptor and shows remarkably high aggressive clinical behaviour. In order to study the impact of AGEs on MDA-MB231 cell line, several techniques have been performed to address the link between AGEs and breast cancer *in vitro.* Proliferation, migration and invasion are major techniques used in cellular and molecular biology in cancer. Moreover, RAGE expression was assessed and AGE-induce signalling pathways through RAGE were investigated to identify new therapeutic targets for diabetic patients diagnosed with breast cancer.

3.1.1 Aim and objectives:

The aim of this work is to examine the effects of BSA alone and BSA-AGE formation on breast cancer cell line MDA-MB231.

1. To investigate the effects of different concentration of non-BSA and BSA-AGE glycated using different concentrations of methylglyoxal on cell proliferation, migration and invasion of MDA-MB231 breast cancer cell lines in *vitro.*

2. To determine the changes in the cellular signalling after exposure to different concentrations of AGEs in *vitro.*

3. To investigate the expression of RAGE-AGE involved in MDA-MB231 breast cancer cells following interaction with AGEs.

3.1.2 Methods

BSA or lysozymes were glycated as described in section 2.5.1. Crosslinked AGEs were assessed as described previously in section 2.5.6 .The formation of fluorescent AGEs was assessed by their characteristic fluorescence as mentioned in section 2.5.8. The effects of increasing concentrations of BSA-AGEs and non-modified BSA on cell proliferation are described in section 2.7.1. Cell viability was assessed according to section 2.7.2. Effect of BSA-AGEs on cell migration using wound-healing essay was described in detail in section 2.7.3. Cell invasion was assessed using Matrigel as described in section 2.7.5. Western boltting was used to confirm the results as mentioned in section 2.7.7. Signalling pathway was investigated using RAGE neutralization as described in section 2.7.9. The effects of BSA-AGE on phosphorylation of protein were analysed by Kinexuse Bioinformatics as described in section 2.7.10.

3.2 Results

3.2.1 The Effect of Different Concentrations of Methylglyoxal on AGE Formation *in vitro*

The effect of varying concentration of methylglyoxal on glycation of lysozyme in *vitro* produced high amount of cross-linked AGEs within 3 days of incubation and caused the formation of AGEs Figure 3.1. These had molecular weight of approximately 42 kDa as indicated by molecular weight markers (lane M) in comparison with the lysozyme (lane a). The quantification of the effect of different concentrations of methyloglyoxal on cross-linked AGE formation shows significant increase ($P < 0.001$) in Figure 3.2. The effect of different concentrations of methylglyoxal (0.01– 0.1 M) on formation of fluorescente is AGEs shown in Figure 3.3.

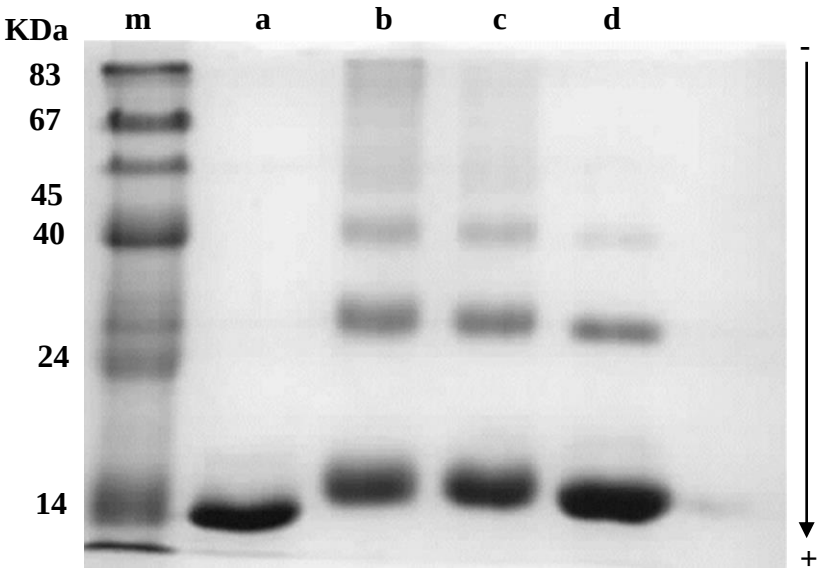


Figure 3.1: SDS-PAGE gel showing the effect of different concentrations of methylglyoxal on cross-linked AGE formation. Lysozyme (10 mg/mL) incubated alone (lane a) or in the presence of methylglyoxal (0.1 M lane b; 0.05 M lane c; 0.01 M lane d) respectively in 0.1 M sodium phosphate buffer (pH 7.4) at 37°C for 3 days. Lane M, represent the molecular weight markers. The cross-linked AGEs were analysed using SDS-PAGE and stained with Coomassie brilliant blue. Each value represents the mean ± SD (n = 3).

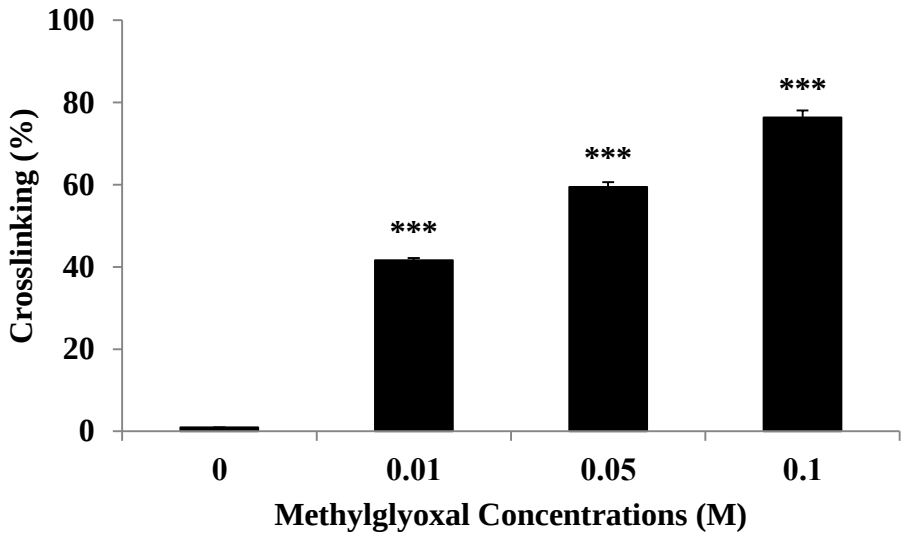


Figure 3.2: The effect of different of concentrations of methylglyoxal on cross-linked AGE formation. The graph shows gradual increases with each concentration. Lysozyme (10 mg/mL) incubated alone or in the presence of methylglyoxal (0.01M; 0.05M; 0.1 M) respectively. Each value represents the mean ± SD (n = 3). ***: $P < 0.001$.

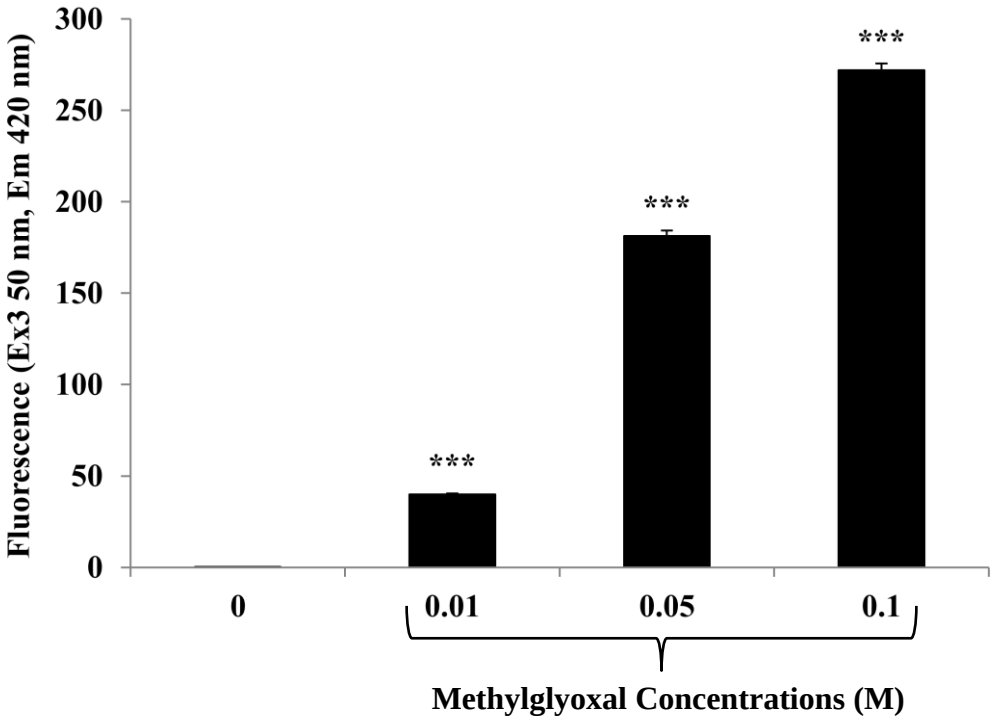


Figure 3.3: Different concentration of methylglyoxal on fluorescent AGE formation in lysozyme-methylglyoxal system. Lysozyme (10 mg/ml) was incubated with different concentrations of methylglyoxal (0 - 0.1M) in 0.1 M sodium phosphate buffer of pH 7.4 at 37 °C for 3 days. Each value represents the mean ± SD (n = 3). ***: $P < 0.001$.

3.2.2 Effect of Period of Incubation on AGE Formation *in vitro*

Adequate quantity of cross-linked AGEs was produced when BSA was allowed to react with glucose for varying incubation time during 15 days of incubation at 37ºC. The control sample contained BSA only (Figure 3.4, lane a). In comparison with the control, a gradual increase in the formation of the BSA-AGE with increasing time of glucose incubation, Figure 3.4 (lane b-f). The effect of different incubation times on the formation of cross-linked AGEs after scanning of this gel shows significant ($p< 0.001$) increase in comparison with BSA alon in Figure 3.5.

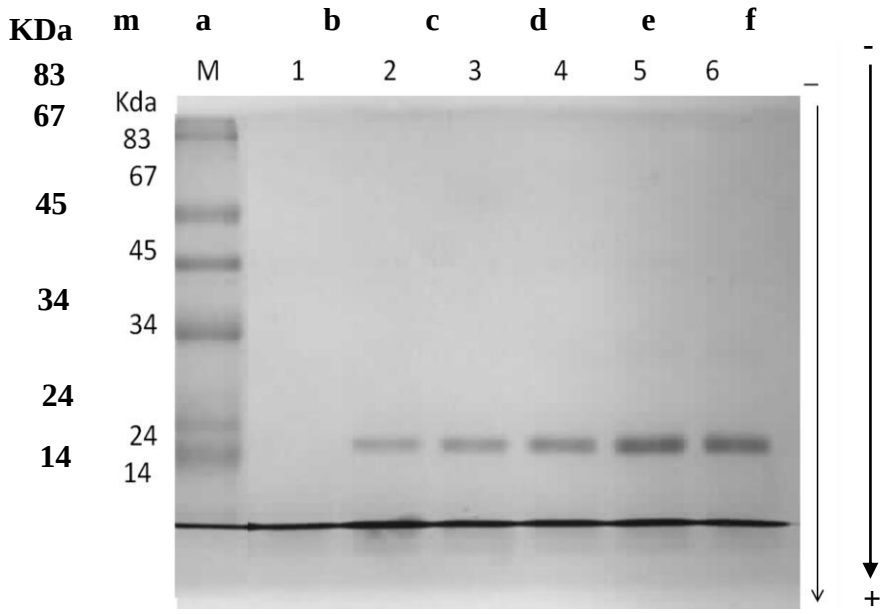


Figure 3.4: The effect of different incubation time on cross-linked AGE formation in the BSA-glucose system. Gel showing BSA (10 mg/ml) was incubated alone (lane a) or with glucose (0.5M) for different incubation time (3, 6 , 9 ,12, and 15 days) (lane b-f) respectively in 0.1 M sodium phosphate buffer of pH 7.4 at 37 °C for 15 days. Lane M, represent the molecular weight markers. The cross-linked AGEs were analysed using SDS-PAGE and stained with Coomassie brilliant blue. The result is a representative figure of at least three independent experiments.

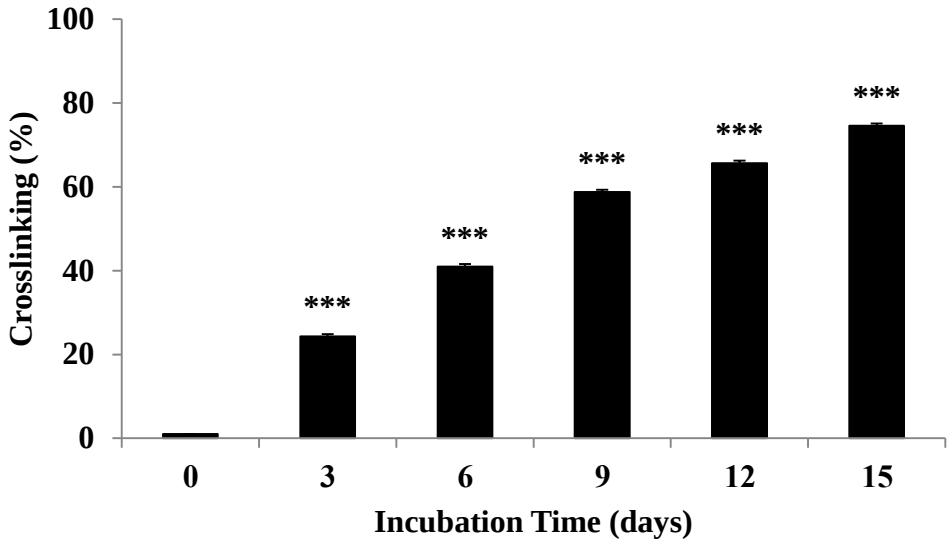


Figure 3.5: The effect of different incubation time on cross-linked AGE formation in the BSA-glucose system. BSA (10 mg/ml) was incubated with glucose (0.5 M) in 0.1 M sodium phosphate buffer of pH 7.4 at 37 °C for 15 days. Each value represents the mean ± SD (n = 3) ***: $P < 0.001$.

3.2.3 Effects of BSA-AGEs on MDA-MB-231 Cell Morphology, Proliferation and Viability

Representative photomicrographs showing the MDA-MB-231cell morphology in untreated conditions (the control) and those treated with BSA-AGEs or non-modified BSA (Figure 3.6 A-I). MDA-MB-231 breast cancer cell line cells, originally extracted from the tumor of breast epithelium tissue, displayed an epithelial-like morphology appearing as spindle shaped cells. At all concentrations used, BSA-AGEs and non-modified BSA did not change MDA-MD-231 cell morphology, compared to the control. As shown in Figure 3.6, BSA-AGEs induced a variation of the cell number in a dose-dependent manner, increasing then decreasing cell proliferation in a bell-shaped curve. The lowest concentration of BSA-AGEs (25 μg/mL) slightly but not significantly increased, while (50μg/mL) BSA-AGE significantly enhanced by 1.5-fold ($p<0.01$) cell proliferation as compared to the untreated control cells and BSA-treated cells. A peak of stimulation (2.0-fold increase) was reached

in the presence of 100 μg/mL BSA-AGEs ($p<0.001$), whereas the highest concentration (200 μg/mL) did not change the cell growth, compared to the untreated control cells and BSA-treated cells (Figure 3.7A). To estimate the specific effect of BSA-AGEs on cell proliferation, non-modified BSA was also tested. Compared to the control, BSA (50 μg/mL) slightly increased the cell proliferation ($p<0.05$) whereas no effect was observed with concentrations of BSA at 25, 100 and 200 μg/mL (Figure 3.7A). Moreover, for each condition, the percentage of cell viability was determined by the trypan blue exclusion method and revealed a high percentage of viability, indicating the absence of cytotoxicity at the 25. 50, 100 μg/mL concentrations of BSA-AGE and non-modified BSA used compared with control and compared as well with BSA. The percentage effects of viable cell numbers exposed to 200 μg/ml of BSA-AGEs and BSA only were reduced by 18 %, compared to the control (Figure 3.7B).

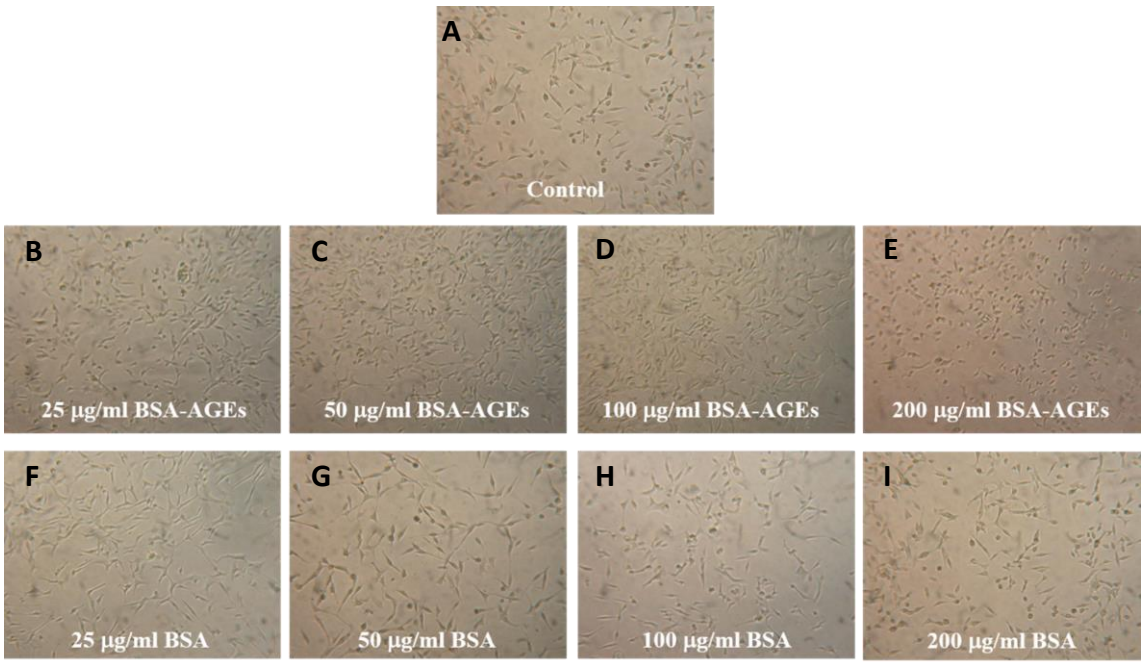


Figure 3.6: Effect of BSA-AGEs and non-modified BSA on the cell morphology of MDA-MB-231 cells. Representative photomicrographs (x400 magnification of phase contrast microscopy) showing the MDA-MB-231 cell morphology in non-treated conditions

(control) (A) or treated either with 25-200 μg/mL BSA-AGEs (B-E) or non-modified BSA (F-I) after 72 hours of incubation.

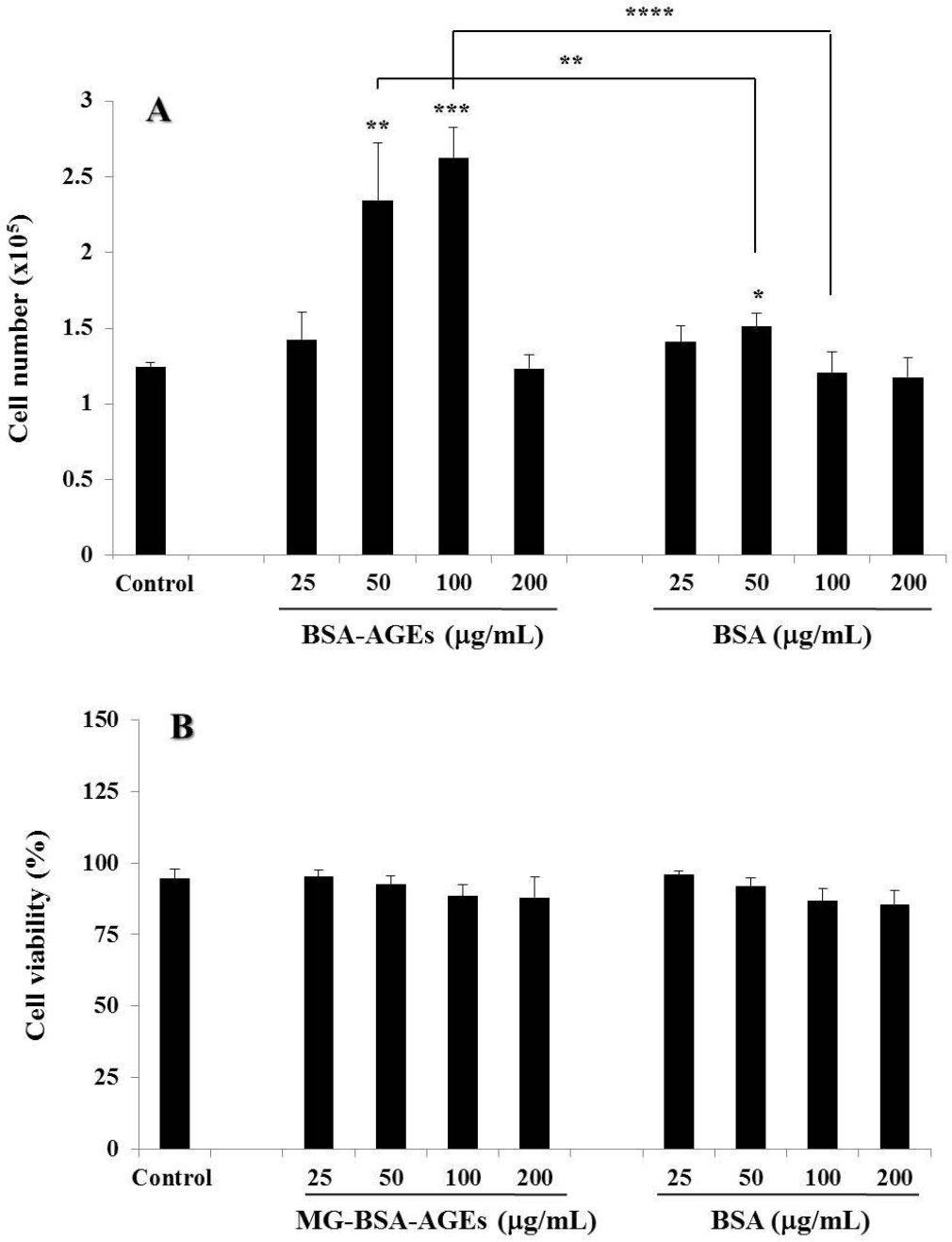


Figure 3.7: Effect of BSA-AGEs and non-modified BSA on (A) proliferation and (B) viability of MDA-MB-231. (B) were determined using an automatic cell counter and the trypan blue exclusion method, respectively. Control indicates non-treated cells**.** The cells were non-treated or treated either with (25-200 μg/mL) BSA-AGEs or 25-200 μg/mL BSA for 72 hours. Results are presented as mean ± SD (n=3). *: $P < 0.05$, **: $P < 0.01$ and ***: $P < 0.001$.

3.2.4 Effect of BSA-AGEs on MDA-MB-231 Cell Migration

The of the effects of BSA-AGEs on the migration of the breast cancer cell line MDA-MB-231 may indicate how AGEs potentially contribute to tumour progression. MDA-MB-231 cells were subjected to the wound-healing assay followed by a treatment with different concentrations (25-200 μg/mL) of BSA-AGEs or non-modified BSA. Representative photomicrographs show MDA-MB-231 cell migration under different conditions (Figures 3.8 A-C). Compared to the untreated control and to non-modified BSA. BSA-AGEs at 25, 50 and 100 μg/mL concentration increased the number of migrated cells by nearly 2.0-fold with a p-value for 25μg/mL ($p<0.01$) and 50-100 μg/mL ($p < 0.05$) while at 200 μg/mL, BSA-AGEs had no effect (Figure 3.8 D). On the distance of migration, BSA-AGEs significantly increased the cell migration in a dose-dependent manner with a peak of stimulation at 50 μg/mL 2.7 -fold increase ($p< 0.01$), compared to the control and compared with BSA-treated cells (Figures 3.8E). No change in a cell migration was observed with the highest concentration (200 μg/mL) of BSA-AGEs (Figure 3.8D, E). Non-modified BSA (25-100 μg/mL) had no effect on MDA-MB-231 cell migration (Figures 3.8 C and 3.8 D). However, 200 μg/mL BSA decreased the number of migrated cells in comparison to untreated control cells ($p <0.05$) and BSA-treated cells (Figure 3.8E).

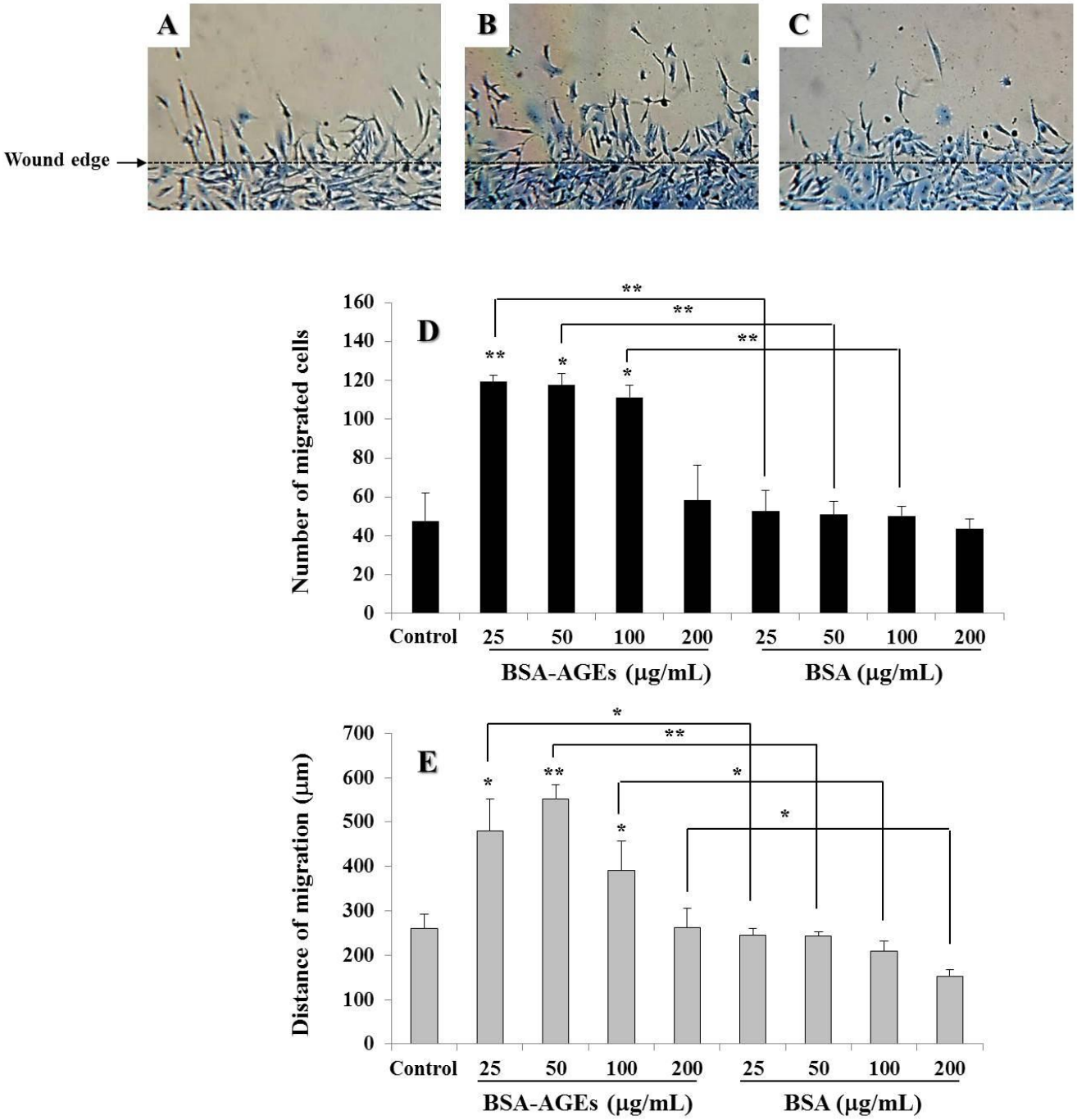


Figure 3.8: Effect of BSA-AGEs and non-modified BSA on MDA-MB-231cell migration. Representative photomicrographs (x400 magnification) show the MDA-MB-231 cell migration measured from the wound edge (indicated by the arrow) after 24 hours of incubation in the (A) absence or (B) in the presence of 50µg/mL BSA-AGEs or (C) non-modified BSA. (D) Shows the number of migrated cells, quantification of the number of migrated cells (black bars) and the distance of migration (grey bars) of MDA-MB-231 cells treated in the aforementioned conditions. (E) the distance of migration of MDA-MB-

231 treated in the conditions aforementioned. Results are presented as mean ± SD (n=3). *: $P < 0.05$, **: $P < 0.01$.

3.2.5 Effect of BSA-AGEs on MDA-MB Cell 231 Invasion

The effect of BSA-AGEs on the MDA-MB-231 cell invasion was estimated by counting the stained migrated cells, which passed across the porous membrane after invading the Matrigel™, a reconstituted basement membrane (Figure 3.9). Representative photomicrographs in Figure 3.9 A-C shows MDA-MB-231 cell invasion in untreated condition (Figure 3.9A), or treated either with 100 μg/mL BSA-AGEs (Figure 3.9 B) or non-modified 100 μg/mL BSA (Figure 3.9 C). Compared to untreated control cells, BSA-AGEs modulated MDA-MB-231 cell invasion in a dose-dependent manner. The variation of MDA-MB-231 cell invasion induced by different concentrations of BSA-AGEs described a bell-shaped curve with a peak stimulation at 100 μg/mL of BSA-AGE (2.39-fold increase $p< 0.01$), while at 50 μg/mL of BSA-AGE (2.35-fold increase $p< 0.01$) (Figures 3.9 D). Both were significantly increased compared to the control. The lowest 25 μg/mL and highest concentration 200 μg/mL had no significant effect. At all concentrations used (25-200 μg/mL), no effect of non-modified BSA on MDA-MB-231 cell invasion was observed (Figure 3.9 D).

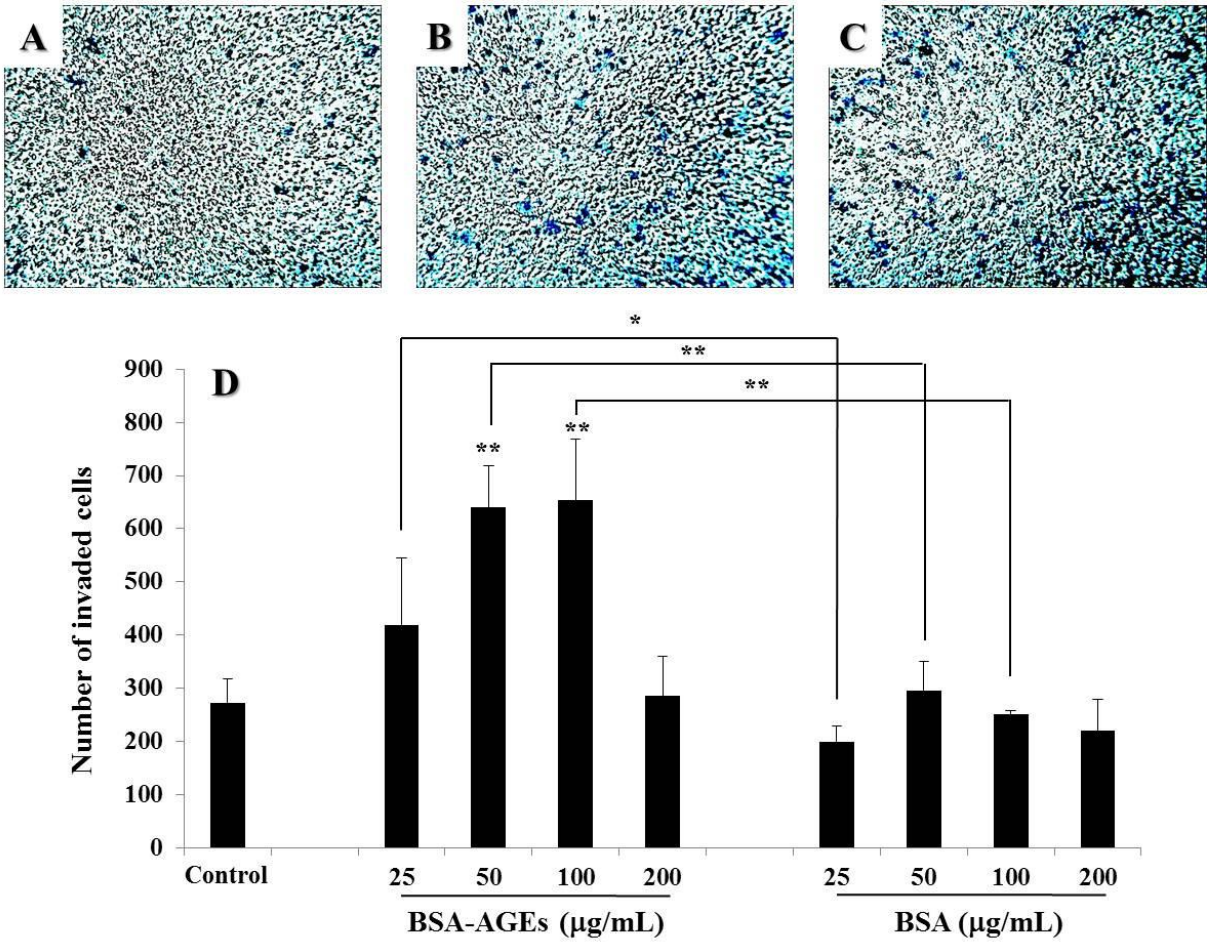


Figure 3.9: Effect of BSA-AGEs and non-modified BSA on invasion of MDA-MB-231 cells. Representative photomicrographs (x400 magnification) showing the effect of untreated MDA-MB-231 cells (A) and cells treated BSA-AGEs with 100 µg/mL (B) or non-modified BSA with 100 µg/mL (C) on invasion Matrigel™ assay for 24 hours of incubation. (D) The graph shows the number of invaded MDA-MB-231 cells treated with 25-200 µg/mL BSA-AGEs or non-modified BSA, compared to the untreated cells, the control. Results are presented as mean ± SD (n=3). **: $P < 0.01$.

3.2.6 Effects of BSA-AGEs on the Activities of Matrix Metalloproteinase

Compared to the MMP-9 activity in untreated MDA-MB-231 cell-condition medium (control) assessed by gelatine substrate zymography, pro-MMP-9 activities produced by cells treated with 50 and 100 μg/mL BSA-AGEs were significantly enhanced by 1.4-fold and 1.3-fold, respectively ($p<0.05$) compared to the control (Figures 3.10 A and 3.10 B). A decrease of pro-MMP-9 activity was noticed when the cells were treated with 200 μg/mL BSA-AGEs ($p<0.05$), compared to the control. While active-MMP9 was also significantly enhanced at all concentrations by nearly 1.2-fold ($p<0.05$) at 25, and 200 μg/mL, by 1.6-fold ($p<0.01$) at 50 μg/mL and by 2.2-fold ($p<0.001$) at 100 μg/mL (Figures 3.10 A and 3.10 B). At all concentrations of BSA-AGEs, used, no active MMP-2 was observed (Figure 3.10 C).

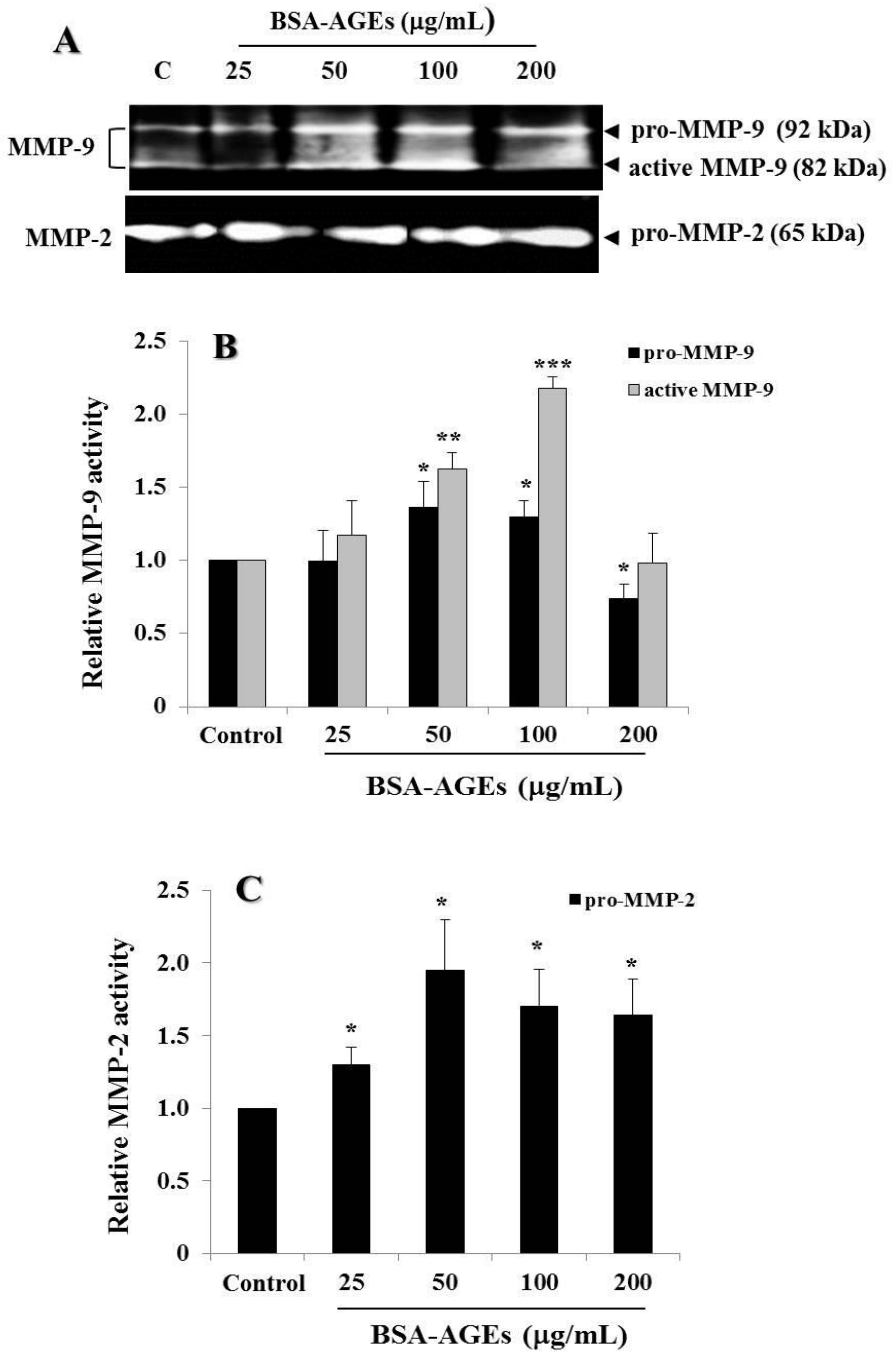


Figure 3.10: Effect of BSA-AGEs on MMP-9 and MMP-2 activities produced by MDA-MB-231 cells during invasion Matrigel^{TM} assay. (A) Representative gelatine zymograhic analysis showing the MMP-9 and MMP-2 activities from different BSA-AGE-treated MDA-MB-231cell-condition media, compared to untreated cell-condition medium (control). The gels revealed gelatinolytic activities of pro- and active MMP-9 and pro-MMP-2. (B) Shows the quantitative gelatinolytic activities of pro- and active MMP-9 and of (C) pro-MMP-2 expressed in relative activity calculated as a ratio to the control. (Results are presented as mean ± SD (n=3). *: $P < 0.05$, **: $P < 0.01$and ***: $P < 0.001$.

3.2.7 Effect of BSA-AGEs on RAGE Expression

A time-course for the expression of RAGE was established by Western blotting after MDA-MB-231 cells in SPM treated with 100 μg/mL BSA-AGEs were tested at different incubation times 10 min to 24 hours and 48 hours Figure 3.11 A1 shows that BSA-AGEs regulated RAGE protein expression in a time-dependent manner. Within 10 minutes incubation, a significant increase by 1.5-fold ($p<0.01$) of RAGE expression was detected compared to the basal level of RAGE expressed in untreated cells (Figure 3.11 A2). A peak of expression of RAGE was observed after 48 hours of incubation with BSA-AGEs 2.0-fold increase ($p<0.01$) compare with the control 48 hours, (Figure 3.11 A2). The over-expression of RAGE induced by BSA-AGEs in short incubation time (10 minutes) was also confirmed using flow cytometry analysis revealing a significant increase of RAGE expressed on the cell surface (Figure 3.11 B1 and B2).

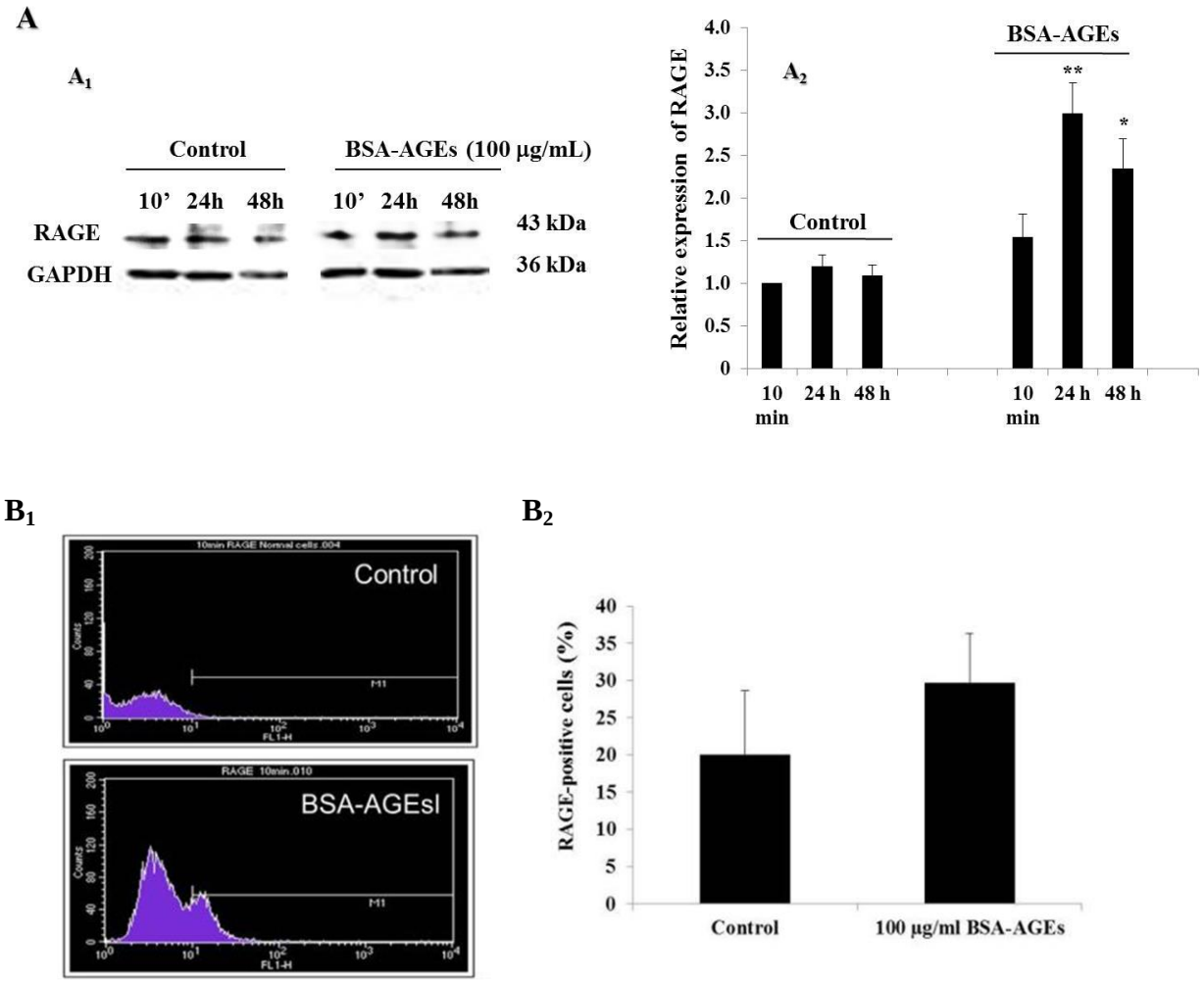


Figure 3.11: Time course of RAGE expression in MDA-MB-231 cells treated with BSA-AGEs. (A_1) Representative Western blot analysis showing the effect of 100 µg/mL BSA-AGEs on RAGE expression in MDA-MB-231 cells after 10 minutes, 24 hours, and 48 hours of incubation, compared to untreated cells (control). (A_2) shows the relative expression of RAGE calculated as a ratio to GAPDH expression, the loading control. B_1 and B_2 Photomicrographs showing a representative FACS analysis of the up-regulation of RAGE expression induced by 100 µg/mL BSA-AGEs after 10 minutes of incubation and IgG1 was used as an isotype control. Each value represents as mean ± SD (n = 3). **: $P < 0.01$.

3.2.8 BSA-AGEs Induced p-ERK1/2 Expression in MDA-MB-231 Cells through RAGE

To investigate whether the BSA-AGEs might induced cell signalling based upon the incubation time, a Western blot analysis was carried out to examine the expression of the phospho-extracellular-signal regulated kinase-1/2 (p-ERK1/2) in MDA-MB-231 cells treated with 100 μg/mL BSA-AGEs for 5, 10, 30 and 120 minutes (Figure 3.12 A). BSA-AGEs increased the expression of p-ERK-1/2 in the cells after 5 minutes of incubation. The maximum BSA-AGEs-induced pERK-1/2 expression was reached at 10 minutes of incubation then slightly decreased at 30 minutes, finally disappeared after 120 minutes of exposure (Figure 3.12 A). To study the effect of different concentrations of BSA-AGEs on p-ERK1/2 expression, the cells were treated with or without 25-50-100 and 200 μg/mL of BSA-AGEs or non-modified BSA for 10 minutes of incubation (Figure 3.12 B). Compared to untreated control cells, BSA-AGEs tested at different concentrations (25-50-100 and 200 μg/mL) modulated p-ERK1/2 expression in a dose-dependent manner with a peak of stimulation of 1.7 and 2.75 -fold for p-ERK1 and p-ERK2, respectively, ($p<0.01$) with 100 μg/mL of BSA-AGEs (Figure 3.12 B). Non modified BSA significantly increased the phosphorylation of p-ERK1/2 at lower concentrations (25-50 μg/ml) while at higher concentrations (100-200 μg/ml) non-modified BSA had no phosphorylation of p-ERK1/2 as compared to the control (Figure 3.12 B).

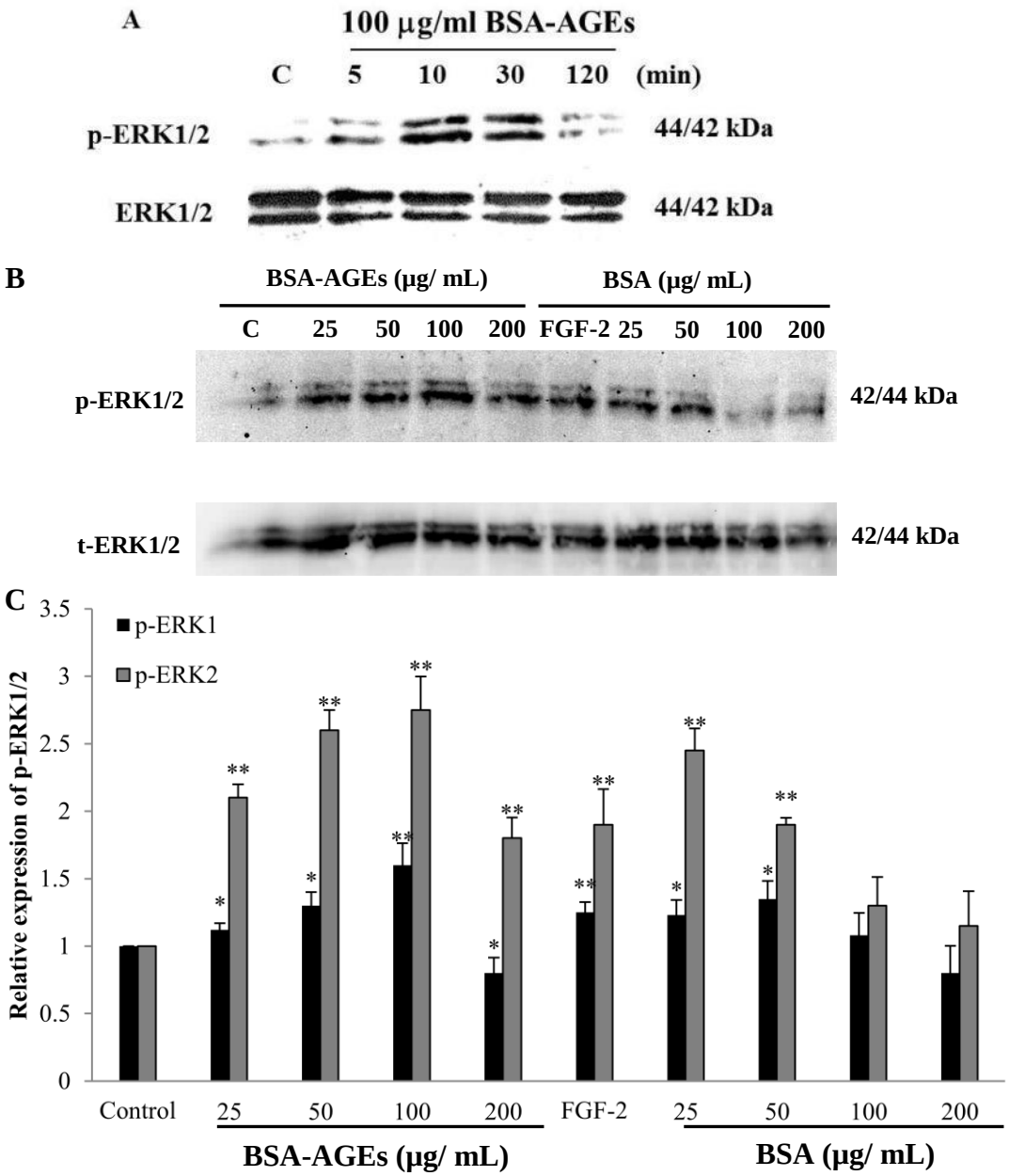


Figure 3.12: Effect of BSA-AGEs on phospho-ERK1/2 expression in MDA-MB-231 cells. (A) Representative Western blot analysis showing the variation of p-ERK1/2 expression induced by 100 µg/mL BSA-AGEs after different incubation times varying from 5 to 120 minutes, compared to untreated control cells. (B) Representative Western blot analysis showing the effect of 25-200 µg/mL BSA-AGEs or non-modified BSA on p-ERK1/2 expression, compared to untreated cells. (C) Relative expression of p-ERK1/2 calculated as a ratio to the total ERK1/2 (t-ERK1/2), the loading control. Each value represents the mean ± SD (n = 3). *: $P < 0.05$ and **: $P < 0.01$.

To check whether BSA-AGEs mainly induce signalling pathways including p-ERK1/2 activation in MDA-MB-231 through its major receptor RAGE, the cells were pre-treated with an anti-RAGE antibody blocking all the RAGE receptors or with an isotype control IgG1 (Figure 3.13 A). The isotype control did not affect BSA-AGEs-induced p-ERK1/2 over-expression; while there was no change in p-ERK1/2 expression in the cells treated with 100 μg/mL BSA-AGEs after blockade of RAGE by anti-RAGE neutralizing antibody when compared to basal level of p-ERK1/2 in untreated cells (Figure 3.13 B).

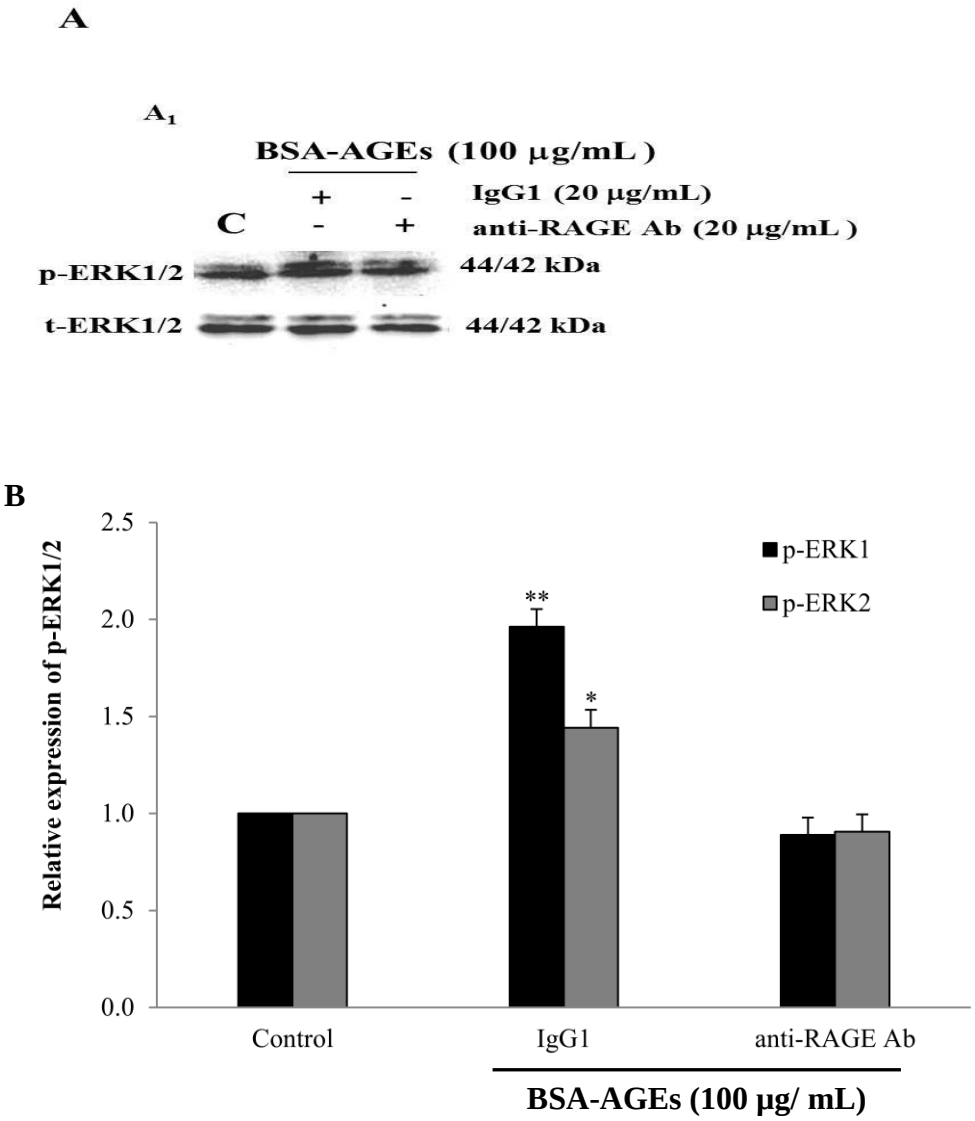


Figure 3.13: Effect of RAGE neutralization on BSA-AGEs-induced phospho-ERK1/2 expression in MDA-MB-231 cells. (A) Representative Western blot analysis showing the loss of BSA-AGE-induced p-ERK1/2 over-expression after the blockade of RAGE function utilising a neutralising blocking anti-RAGE antibody, compared to untreated cells (control) and an isotype control IgG1. (B) Relative expression of p-ERK1/2 calculated as a ratio to the total ERK1/2 (t-ERK1/2), the loading control. Value represents the mean ± SD (n = 3). **: $P < 0.01$.

3.2.9 Effects of BSA-AGEs on the Expression of Phosphorylated Proteins

To investigate the potential downstream phosphorylated proteins of BSA-AGEs/RAGE signalling pathways, the whole cell lysates from untreated MDA-MB-231 cells (Figure 3.14 A_1) and from cells treated with 100 μg/mL BSA-AGEs (Figure 3.14 A_2) or treated with 100 μg/mL non-modified BSA (Figure 3.14 A_3) for 10 minutes were analysed using the phospho-protein array KPSS-1.3 which contains 35 phospho-proteins including p-ERK2 (Figure 3.14 A). Consistent with previous Western blot data (Figure 3.12 C), the bio-informatic analysis of the phspho-protein array showed that BSA-AGEs caused a 3.0-fold increase of p-ERK2 expression (Figure 3.14 B). In addition, BSA-AGEs mainly induced the over-phosphorylation of the ribosomal protein serine S6 kinase (p70S6K)-beta 1 (25.4-fold increase), of the signal transducer and activator of transcription (STAT)-3 (8.0-fold increase), MAPK p38 and glycogene synthase-serine kinase (GSK)-3α (5.6-fold and 3.2-fold, respectively), and MAPK/ERK protein-serine kinase 1/2 (4.6-fold increase), compared to untreated control cells (Figure 3.14 B). The phosphorylation of proteins acting as transcription factors such as c-Jun including its kinase JNK were increased (2.0-fold) in BSA-AGE-treated cells, compared to untreated cells. The phosphorylation of tumour-suppressor protein named retinoblastoma-associated protein-1 (pRb) was also increased (2.6-fold) in BSA-AGE-treated cells, compared to untreated cells. While BSA-AGEs enhanced the expression of most of the phospho-proteins, the cell treatment with non-modified BSA displayed a similar profile of phospho-protein expressions as untreated control cells (Figure 3.14).

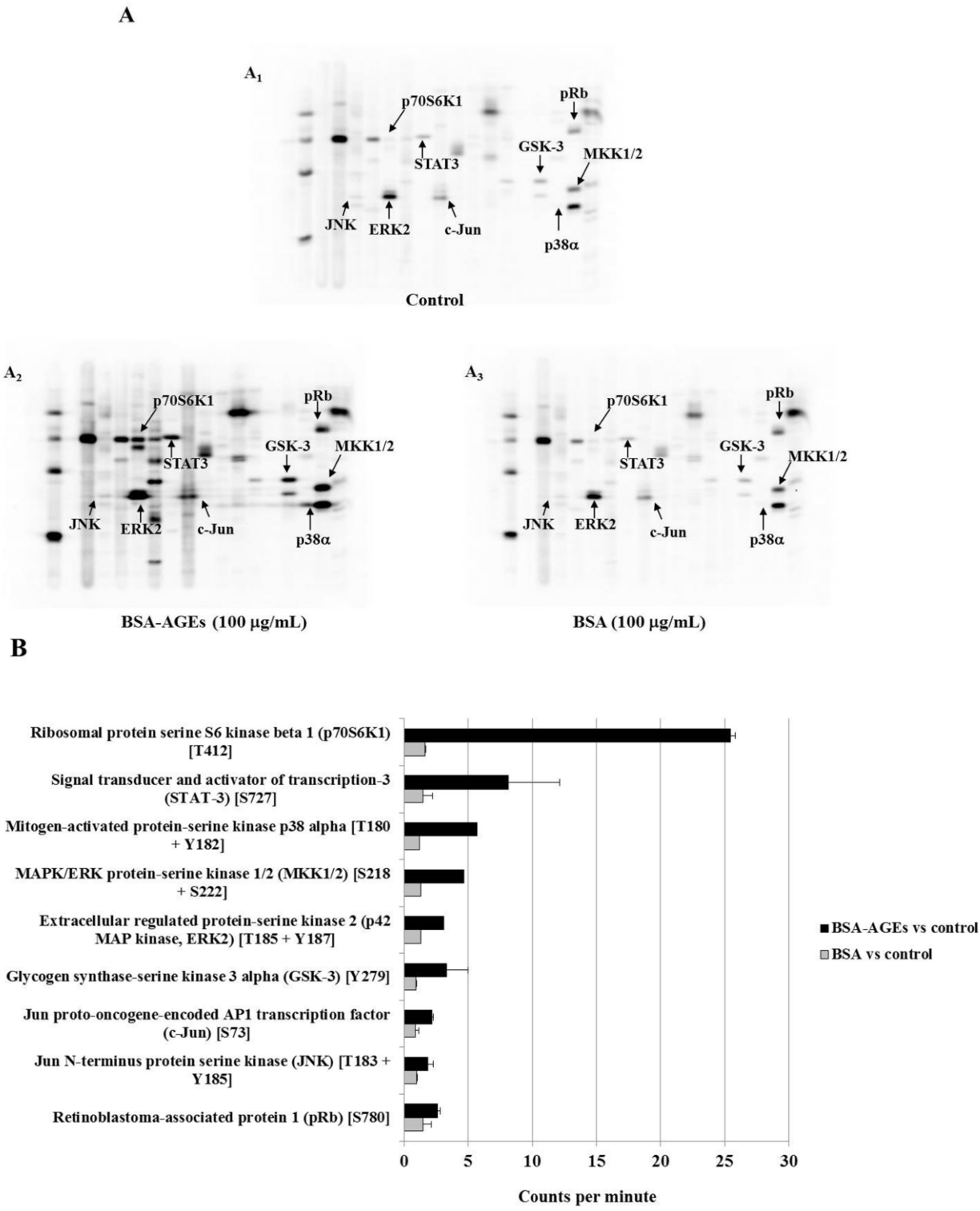


Figure 3.14: Effect of BSA-AGEs and non-modified BSA on phospho-protein expression in MDA-MB-231. (A) The multi-immunoblotting showing the expression of 35 phospho-proteins in untreated (A_1) MDA-MB-231 cells, cells treated with 100 µg/mL BSA-AGEs (A_2) or 100 µg/mL non-modified BSA (A_3) for 10 minutes. (B) Relative expression of the relevant phospho-proteins expressed in counts per minute and calculated as a ratio to the untreated control cells. Value represents the mean ± SD (n = 3).

3.3 Discussion

Diabetes and cancer are of major concern because of their high incidences in Western countries and more recently in developing countries. A growing body of epidemiological evidence has begun to indicate a molecular link between diabetes and breast cancer (Tesrasová *et al*, 2007; Logsdon *et al*, 2007). Moreover, in diabetic patients, the most circulating and abundant protein-derived AGE formed from a non-enzymatic oxidation of glucose is serum glycated albumin (Furusyo and Hayashi, 2013); furthermore, in patients with breast cancer, serum concentrations of AGEs were higher than in healthy controls (Tesrasová *et al*, 2007). To date, the biological effects of glycated albumin on human breast cancer cells have been poorly described.

This study examined for the first time the *in vitro* biological effects of different concentrations of BSA-derived AGEs on the invasive breast cancer cell line MDA-MB-231, compared to non-modified BSA that is commonly used as molecular model (Khan *et al*, 2013). We showed that BSA-AGEs at 100 µg/mL increased MDA-MB-231 cell proliferation, migration and invasion through growth factor reduced Matrigel™ (a reconstituted basement membrane). Cell invasion is a critical step during tumour progression because the basement membrane represents the last barrier for the cancer cells to reach the blood circulatory system and be disseminated via metastasis (Martin *et al*, 2000; Zijl *et al*, 2011*).* The effects of BSA-AGEs on elevated cell invasion might be associated with an enhancement of MMP-9 gelatinase activity. Furthermore, BSA-AGEs increased the expression of the main receptor for AGEs (RAGE) and induced an overexpression of phospho-ERK1/2 through RAGE. A wider investigation of BSA-AGE-induced signalling pathways in MDA-MB-231 cells highlighted the over-phosphorylation of key proteins involved in the control of protein synthesis and cell survival, including

p70S6K1 and STAT-3, compared to untreated cells. Throughout the study, non-modified BSA had almost no biological effects on MDA-MB-231 cells.

This present study showed that BSA-AGEs increased, in a dose-dependent manner, MDA-MB-231 cell proliferation, migration and invasion, while non-modified BSA had mostly no effect but a slight stimulatory effect on cell proliferation at 50μg/mL, this negligible mitogenic effect of non-modified BSA is correlated to the induction of ERK1/2 phosphorylation observed at short incubation time (10 minutes). In our present study and also reported by Chung and colleagues (2010), using tubular epithelial cells in similar conditions (Chung *et al.,* 2010). The bell-shaped curve is indicative of a bivalent bridging mechanism describing the cell response through the dimerization /oligomerization of specific cell-surface receptors after binding with the ligand (Posner *et al,* 1998). A recent study provides insights into the factors including glycation reagent like methylglyoxal and the RAGE structure that influence AGEs-RAGE binding (Indurthi *et al.,* 2012). Throughout this present study, the maximal cell response was obtained with 50 and 100 μg/mL BSA-AGEs, which seem to correspond to the optimal oligomerization of the AGE receptor such as RAGE to induce the maximal signalling stimulation. Recently, using an epitope-defined monoclonal antibody that specifically recognises oligomerization, Xu and colleagues (2013) demonstrated the importance of RAGE oligomerization for the formation of active signalling complex. Therefore, the loss of the cell response seen at high concentrations of BSA-AGEs may be explained by the impediment of AGE receptor oligomerization (Xu *et al,* 2013). Recently, using siRNA technology, RAGE has been demonstrated to play a critical role in the growth of human breast cancer cell lines, including MDA-MB-231 (Radia *et al,* 2013). Throughout most of the studies on BSA-AGEs including this present investigation, the biological effects of BSA-AGEs were

compared to native non-modified BSA and it showed no significant biological effect on MDA-MB231 (Yu *et al.*, 2013; Shi *et al*, 2013). Mainly used as an anti-inflammatory or anti-cancerous drug carrier with the benefit to prolong the half-life of the drug, native globular BSA even bound on the cell surface rarely triggers specific transduction signalling like BSA-AGEs (Arkin *et al*, 2012; Rasheed *et al*, 2011; Shi *et al*, 2013). Therefore, our results suggest that the mode of action of BSA-AGEs is dependent on signalling pathways mediated via RAGE rather than BSA-mediated pathways.

Matrix metalloproteinases (MMP) are enzymes considered as important regulators in tumour-cell invasion. In particular, MMP-2 and MMP-9, also called gelatinases, play a key role in the degradation of type IV collagen of the basement membrane, thus contributing to tumour invasion and metastasis (Klein *et al*, 2004). The results of this chapter also showed that BSA-AGEs enhanced the motility and invasive capacity of the MDA-MB-231 cells with increased MMP-9 gelatinase activity whereas MMP-2 gelatinase activity was not changed. Non-modified BSA had no effect on both MMP activities. It is known that invasion and metastasis are key cellular processes resulting in tumour progression; they are the hallmarks of cancer malignancy. Cancer cell metastasis represents the main cause of treatment failure and death in cancer patients (Abe *et al*, 2004). Contributing to metastasis, cancer cells degrade the extra cellular matrix by the production and secretion of MMPs which allowing their cell migration and invasion across the connective tissue and the basement membrane to finally reach the blood circulation, the main route of tumour dissemination towards vascularized organs. A recent study showed that human lung adenocarcinoma cells treated with glyceraldehyde-AGEs for 48 hours had promoted cell migration and invasion across Matrigel™ with the enhancement of MMP-2 activity but not MMP-2 mRNA (Takino *et al*, 2010). MMP-9 from body fluid has proved a putative biomarker in metastatic breast cancer (Noh *et al*, 2012). Hallet and colleagues (2013)

targeted MMP-9 mRNA in a mouse model using anti-MMP-9 DNAzyme transfected into MDA-MB-231 cells, and they reported decreased breast cancer metastasis and they confirmed the key role of MMP-9. Furthermore, using a murine macrophage cell line, AGEs have been demonstrated to mainly regulate MMP-9 production via RAGE and through activation of ERK1/2, p38 MAPK and NF-κB (Hallett *et al,* 2013; Zhang *et al*, 2011). Therefore, our results suggest that BSA-AGEs might promote breast tumour progression and metastasis by increasing cancer cell migration, invasion and MMP-9 activity. During treatment with BSA-AGEs, the breast cancer MDA-MB-231 cells became more sensitive to AGE, which is characterized by the up-regulation of RAGE expression from 10 minutes up to 48 hours of incubation. Shi and colleagues (2013) showed that AGEs up-regulate RAGE expression in various tissues reaching a maximum after a 24-hour treatment, facilitating the AGE-RAGE response by forming a positive feedback loop (Shi *et al*, 2013). Thus, these results suggest that RAGE up-regulation induced by BSA-AGEs may contribute to the increased cell response assessed in proliferation, migration and invasion.

The study of the signalling pathways induced by BSA-AGEs was analysed after a-10 minute BSA-AGEs treatment on MDA-MB-231 cells. The optimal signal transduction time was based on the increased expression of phospho-ERK1/2, a phospho-protein widely known to play a central role in most of the cellular responses including cell proliferation, migration and invasion. It was demonstrated that BSA-AGEs induced ERK1/2 expression through RAGE. After neutralization of the receptor using a specific monoclonal antibody. BSA-AGEs failed to stimulate p-ERK1/2 expression. Thus, this result eliminates the involvement of other AGE receptors such as CD36, scavenger receptors class a type II, class B type I and AGE receptors 1, 2 and 3 that are mainly implicated in the detoxification of AGEs rather than in signalling processes (Marsche *et al*, 2007).

Furthermore, non-modified BSA did not change the phospho-proteome profile of MDA-MB-231 cells compared to untreated cells, BSA-AGEs concomitantly increased the phosphorylation of p70 ribosomal protein serine S6 kinase beta 1 (p70 S6K1), the signal transducer and activator of transcription (STAT)-3, p38 MAPK, glycogen synthase kinase (GSK)-3 and of MAPK/ERK protein-serine kinase 1/2 (MKK1/2), which are mainly involved in the control of protein synthesis, cell cycle progression, cell survival and ERK1/2 activation, respectively (Sato *et al*, 2011; Uehara *et al*, 2012; Kim *et al*, 2013; Ko *et al*, 2011). Further, the p70 ribosomal S6K1 has been demonstrated to play a central role in tumour growth by regulating insulin pro-angiogenic effects and protein synthesis (Zhou et al., 2007; Dennis et al., 2012). Constitutively activated STAT proteins, which are critical in the regulation of cell cycle and cell growth, are found in various types of tumours including breast cancer (Chung *et al.,* 2013). However, there is still limited information about its role in cancer. Previous studies have reported the p38 MAPK as a central kinase in a common intracellular signalling pathway that plays an essential role in human breast cancer cell migration and invasion by modulating the expression and activity of MMPs (Wang *et al.,* 2013). Furthermore, several lines of evidences suggest a link between p38 MAPK activation and MMP-9 expression (Ranaivo *et al.,* 2012). Overall, our results suggest that BSA-AGEs mainly stimulated MDA-MB-231 cells in terms of protein synthesis machinery and survival ability. Among these over- expressed phospho-proteins, using pharmacological inhibitors of GSK-3 and of p38 MAPK in MDA-MB-231 cells and in a model mouse of breast cancer, some studies have lately demonstrated their key requirement in breast cancer development (Sukhtankar *et al*, 2011; Kim *et al,* 2013). In addition, targeting p70S6K1 gene expression by micro-RNA 145 could inhibit tumour growth (Xu *et al,* 2012). Thus, showing for the first time this distinctive over-expression of

phosho-p70S6K1 induced by BSA-AGEs, targeting phospho-p70S6K1 expression might prevent the progression and the development of breast cancer in diabetic patients.

In conclusion, we showed for the first time the dose-dependent stimulatory effects of BSA-AGEs on an invasive and non-hormone-dependent breast cancer cell line MDA-MB-231, regarding cell proliferation, migration and invasion with an enhancement of MMP-9 activity, compared to inactive non-modified BSA. Among the phospho-proteins involved in BSA-AGE-induced signalling pathways, we also highlighted a concomitant increase of the phosphorylation of ribosomal protein serine S6 kinase beta 1, which might be a new biomarker of the invasive subtype of the breast cancer and a promising targeted drug to prevent development and progression of the breast cancer in diabetic patients.

Chapter 4

Chapter 4. Effects of BSA-AGEs on Breast Cancer Cell Line MCF-7 cells

4.1 Introduction

AGE plays an important role in many pathological conditions including prostate cancer, ovarian cancer and colon cancer. Breast cancer is considered as the second leading cause of death in women, and may result from alteration of the normal cells, which causes chronic inflammation that promotes mammary tumours. Chronic inflammation is the underlying mechanism in breast cancer development and numerous studies have determined that inflammation can be induced due to accumulation of AGEs. Breast cancer is categorised into several sub-types according to immunohistochemistry markers. MCF-7 hormone-dependent breast cancer cell line is widely used in the investigation of tumour biology and the molecular biology of oestrogen action. MCF-7 is less aggressive, and has a better prognosis than other types. This cell line was isolated from a 69- year- old Caucasian in 1970, and known as malignant adenocarcinoma in a pleural effusion. Band and Sager (1989) have reported that MCF-7 cells were difficult to grow in culture due to their estrogen sensitivity. After investigating the effects of BSA-AGEs on MDA-MB-231 cells, the same studies were applied to MCF-7 cells, to described differences between MCF-7 and MDA-MB-231 cells, in terms of morphology, phenotypes and signalling pathways (Kirschmann *et al*, 2002; Gupta and Tikoo, 2013).

4.1.1 The Aim and objectives:

The aim of this work is to examine the effects of BSA alone and BSA-AGE formation on breast cancer cell line MCF-7.

The objectives are:

1. To investigate the effects of different concentration of BSA and BSA-AGE on cell proliferation migration and invasion of MCF-7 breast cancer cell line in *vitro.*

2. To determine the changes in the cellular signalling after exposure to different concentrations of AGE in *vitro.*

3. To investigate the expression of RAGE-AGE involved in the MCF-7 breast cancer cell line.

4. To investigate the effect of BSA-AGEs on downstream phosphorylation proteins in MCF-7 cell.

4.1.2 Methods:

Studies on the effects of increasing concentrations of BSA-AGEs and non-modified BSA on cell proliferation are described in section 2.7.1. Cell viability was assessed according to section 2.7.2. BSA-AGE on cell migration using Boyden chamber assay was described in section 2.7.4. Cell invasion was assessed using Matrigel as described in section 2.7.5 and the results confirmed by using gelatine zymography as outlined in section 2.7.6. Western boltting was used to confirm the results as mentioned in section 2.7.7. Signalling pathway was investigated using RAGE neutralization studies as described in section 2.7.9. The effects of BSA-AGE on phosphorylation of protein were analysed by Kinexuse Bioinformatics as described in section 2.7.10.

4.2 Results

4.2.1 Effects of BSA-AGEs on MCF-7 Cell Morphology, Proliferation and Viability

Representative photomicrographs (Figure 4.1) show the cobblestone-like morphology of the MCF-7 cells with the apparition of clusters at the end of the treatment. The addition of BSA-AGEs or non-modified BSA did not change the cell morphology observed in non-treated conditions (Figure 4.1). As shown in Figure 4.2.A, BSA-AGEs affected MCF-7 proliferation in a dose-dependent manner, with peak stimulation in the presence of 50 μg/mL BSA-AGEs (26% increase, $p< 0.001$), and 100 μg/mL BSA-AGEs (20% increase, $p<0.001$) compared to the untreated control cells. BSA-AGEs at the lowest concentration (25 μg/mL) had no effect on MCF-7 cell proliferation whereas a significant inhibition of the cell growth was observed (17.0% decrease, $p < 0.001$) at the highest concentration (200 μg/mL) compared to untreated control cells. At all concentrations used (25-200 μg/mL), non-modified BSA had no effect on MCF-7 cell proliferation (Figure 4.2.A). Moreover, the percentage of cell viability was determined by the trypan blue exclusion method. BSA-AGEs and BSA at all used concentrations produced similar patterns for percentage of viable cells as shown (Figure 4.2B). At concentrations of 50, and 100 μg/mL, BSA-AGE and BSA did not affect cell viability compared to untreated control cells. However, at the highest concentration used (200 μg/mL), BSA-AGE reduced cell viability by 15% ($p<0.05$). At the lowest concentration used (25 μg/mL), BSA-AGE increased the viability by 14% ($p<0.05$).

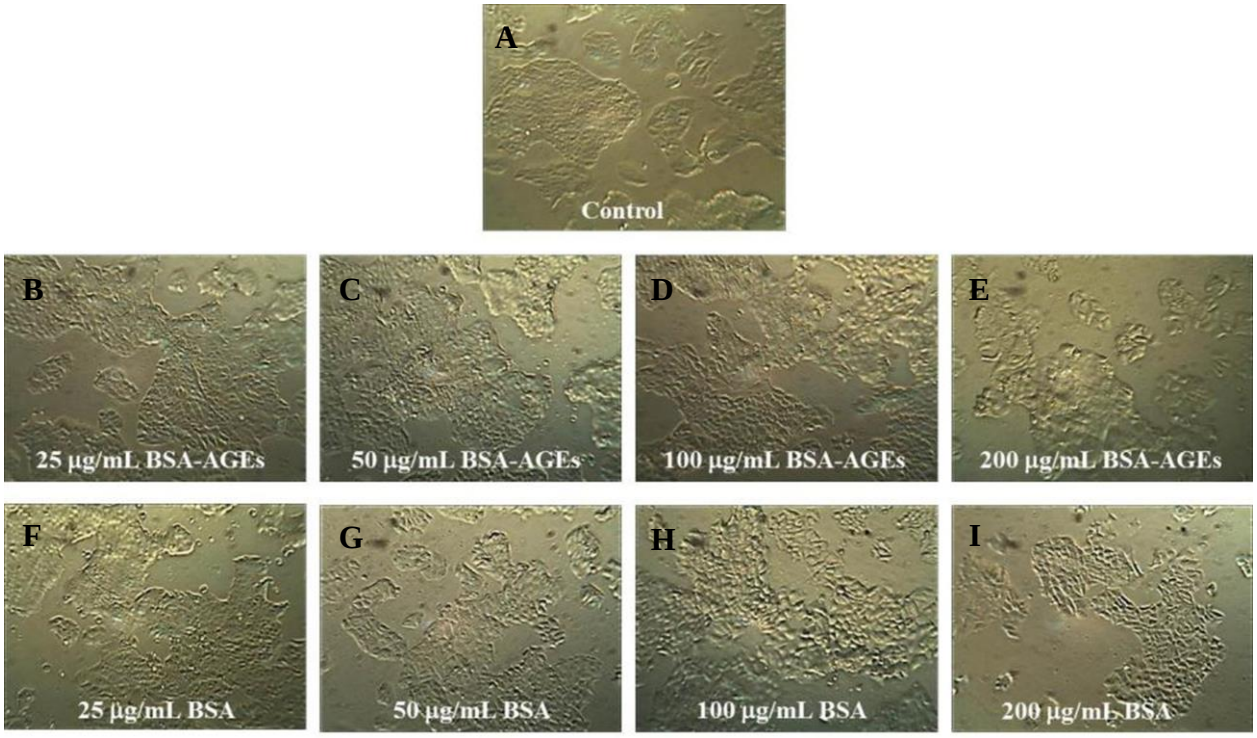


Figure 4.1: Effect of BSA-AGEs and non-modified BSA on MCF-7 cell morphology Representative photomicrographs (X400 magnification of phase contrast microscopy) showing the MCF-7 cell morphology in non-treated conditions control (A) or treated either with 25-200 µg/mL BSA-AGEs (B-E) or non-modified BSA (F-I) after 72 hours of incubation. At all conditions, BSA-AGEs and BSA did not change the cobblestone-like morphology of MCF-7 cells which formed clusters.

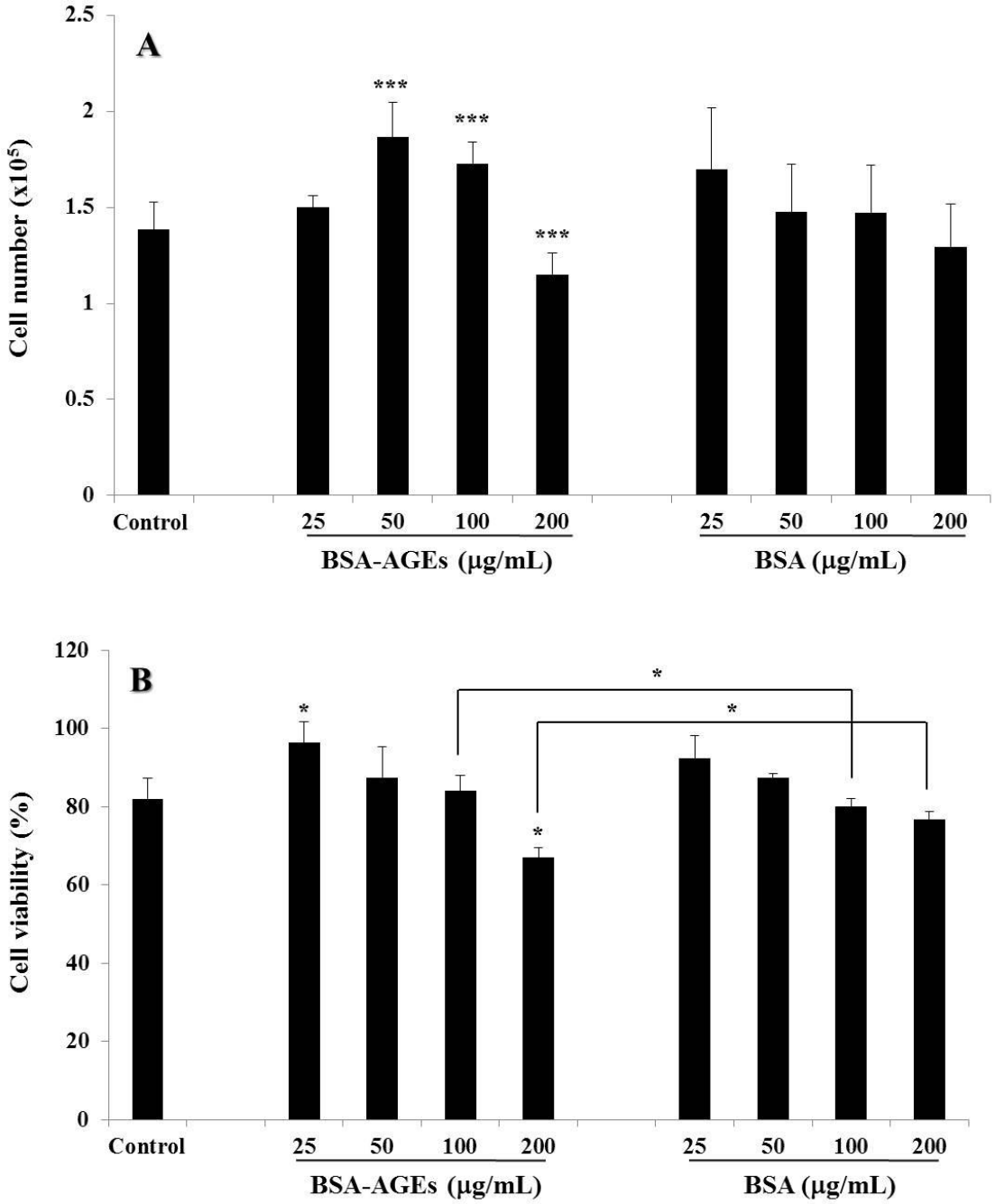


Figure 4.2: Effect of BSA-AGEs and non-modified BSA on (A) cell proliferation and (B) viability of MCF-7 cells. Cell viability was determined using an automatic cell counter and the trypan blue exclusion method, respectively. The cells were non-treated or treated either with 25-200 µg/mL BSA-AGEs or BSA for 72 hours. Results are presented as mean ± SD of three independent experiments. (*) and (***) signify a statistically significant difference ($p < 0.05$, and $p < 0.001$), compared with control and BSA-treated cells.

4.2.2 Effects of BSA-AGEs on MCF-7 Cell Migration

Due to the formation of clusters by MCF-7 cells, the Boyden chamber system was used to assess the effect of BSA-AGEs on MCF-7 cell migration. MCF-7 cells were incubated in SPM in the presence or absence of different concentrations (25, 50, 100, 200 μg/mL) of BSA-AGEs or non-modified BSA for 24 hours before the migrated cells were counted. Figure 4.3A-C showed the representative photomicrographs of the stained MCF-7 cells, which passed across the porous membranes of Boyden chambers. BSA-AGEs at 50 μg/mL and 100 μg/mL significantly increased cell migration by 1.7 -fold ($p < 0.001$) and 2.3 -fold ($p < 0.001$), respectively, as compared to the control (Figure 4.3). At the lowest (25 μg/mL) and the highest (200 μg/mL) concentrations used, BSA-AGEs did not significantly change the number of migrated cells, as compared to the untreated control cells. At all concentrations, used non-modified BSA had no effect on MCF-7 cell migration when compared to untreated control cells.

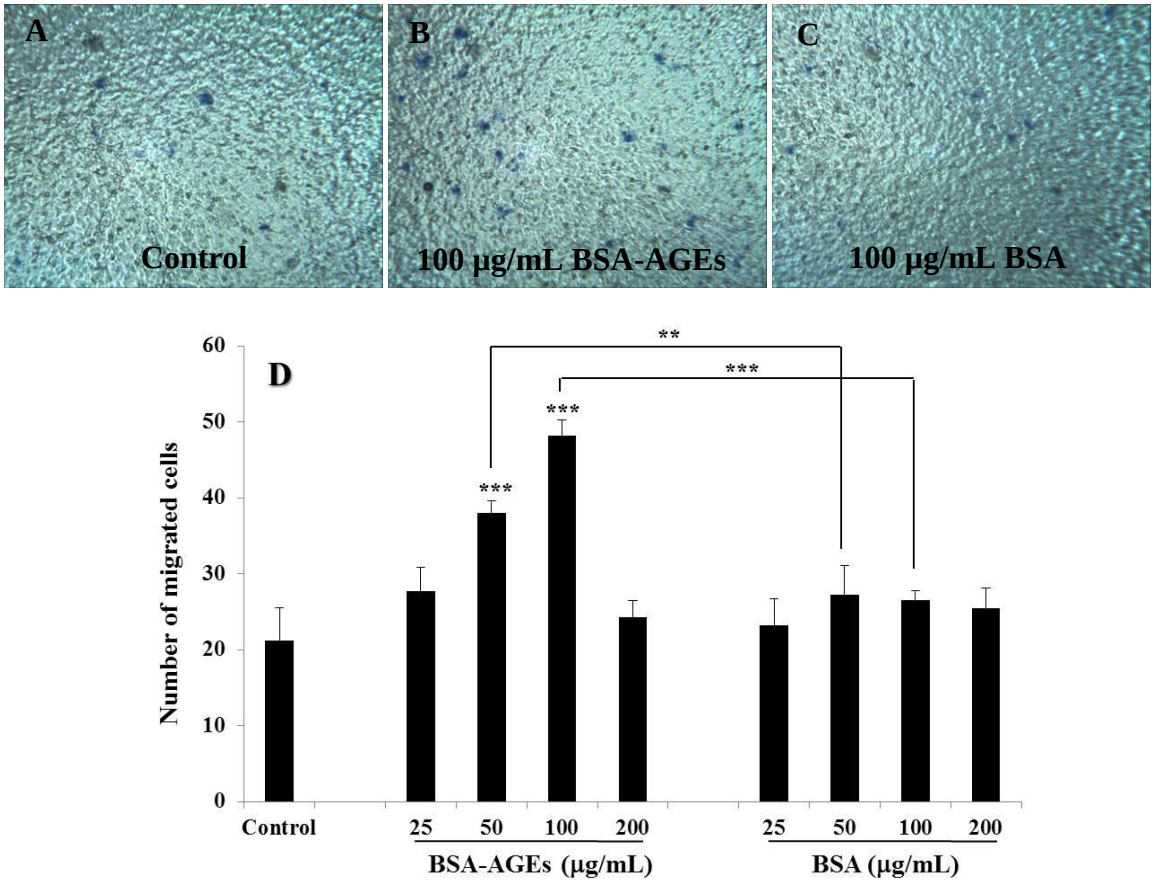


Figure 4.3: Effect of BSA-AGEs and non-modified BSA on MCF-7 cell migration. Representative photomicrographs (x400 magnification) showing the effect of (A) untreated MCF-7 cells and (B) cells treated with 100 µg/mL BSA-AGEs or (C) non-modified BSA on cell migration using the Boyden chamber method for 24 hours. Stimulated cells, which had migrated across the porous membrane, were stained with Giemsa dye for counting. (D) Shows the number of migrated MCF-7 cells. Results are presented as mean ± SD of three independent experiments. Results are presented as mean ± SD of three independent experiments. (**) and (***) signify a statistically significant difference ($p < 0.01$ and $p < 0.001$), compared with the control and BSA-treated cells.

4.2.3 Effects of BSA-AGEs on MCF-7 Cell Invasion and MMP-2/-9 Activities

In contrast to the highly aggressive MDA-MB-231 cells, the MCF-7 cells are well known for their low invasive and metastatic potential (Kirschmann *et al*, 2002). In this study, MCF-7 cells were unable to invade and pass across the Matrigel™ covered porous membranes of Boyden chambers in the presence or absence of different concentrations (25, 50,100 and 200 μg/mL) of BSA-AGEs or non-modified BSA. There were no stained cells observed under microscope.

The MMP-2 and MMP-9 activities were assessed using gelatine zymography. The cell-medium was collected after the MCF-7 invasion assay in which cells were incubated in the presence or absence of different concentrations of BSA-AGEs or non-modified BSA for 24 hours. As expected from the absence of cell invasion, at all conditions used, BSA-AGEs and non-modified BSA did not activate MMP-2 and MMP-9, which was indicated by the absence of the lysis of gelatine, on the zymogram gel (Figure 4.4).

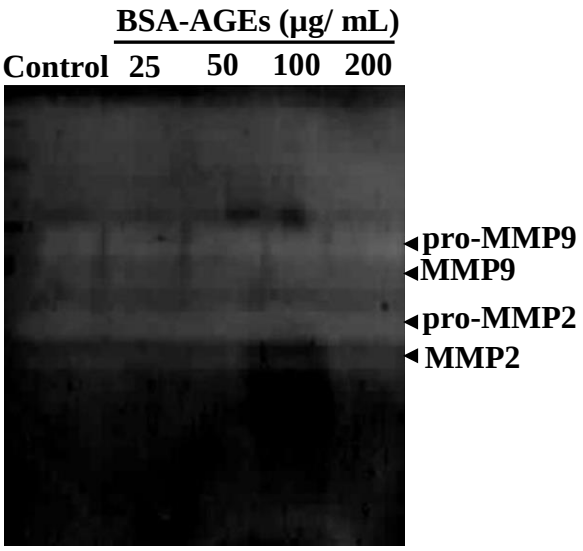
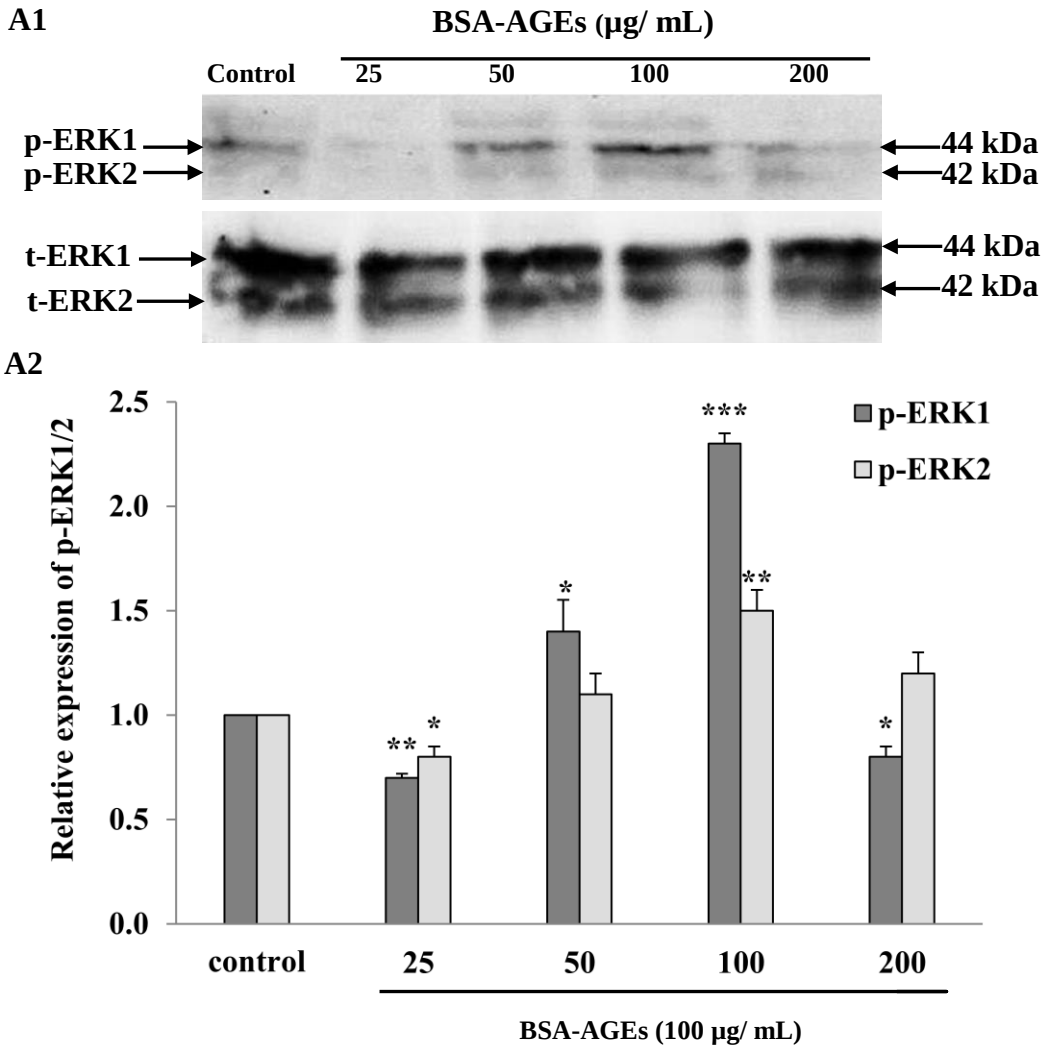


Figure 4.4: Effect of BSA-AGEs on MMP-9 and MMP-2 activities produced by MCF-7 cells during invasion Matrigel™ assay. The gel representative gelatine zymograhic analysis of MCF-7 cells showing the absence of MMP-9 and MMP-2 activities from different concentrations of BSA-AGE (25, 50, 100 and 200 µg/mL), compared to untreated cell-condition medium (control) after 24 hours. The gel has not showed any activation of MMP.

4.2.4 The Effects of BSA-AGEs on the Expression of p-ERK1/2 in MCF-7 Cells

To study the effect of different concentrations of BSA-AGEs on p-ERK1/2 expression or untreated control for 10 minutes in MCF-7 cells. The effects of BSA-AGEs on the expression of p-ERK1/2 were assessed by Western blotting (Figure 4.5). The results showed that BSA-AGEs modulated the expression of p-ERK1/2 in a dose-dependent manner (Figure 4.5A1). The addition of 50 µg/mL BSA-AGEs induced the expression of p-ERK1 by1.4-fold ($p<0.05$), wheres p-ERK2 expression was not changed when compared to the basel level of p-ERK1/2 expression in untreated control cells (Figure 4.5A2). The maximum stimulation was at 100 µg/mL BSA-AGEs, which resulted in a 2.2-fold increase of p-ERK1 ($p<0.001$) expression and 1.5-fold ($p<0.01$) increase of p-ERK2 expression, compared to untreated cells. At the lowest and highest concentrations used (25 and 200

μg/mL), BSA-AGEs mostly showed an inhibitory effect with approximately 30% decrease of p-ERK1/2 expression by 25 μg/mL of BSA-AGEs and 20% ($p<0.05$) decrease of p-ERK1 expression by 200 μg/mL of BSA-AGEs when compared to the untreated control cells (Figure 4.5A2). At all the concentrations used (25, 50, 100 and 200 μg/mL) non-modified BSA had no significant effect on p-ERK1/2 expression in MCF-7 cells (see Figure 4.5B1 for 100 μg/mL BSA), compared to untreated control cells. The t-ERK was used as a loading control (Figure 4.5A).


A1
BSA-AGEs (μg/ mL)
Control
25
50
100
200
p-ERK1
p-ERK2
44 kDa
42 kDa
t-ERK1
t-ERK2
44 kDa
42 kDa
A2
Relative expression of p-ERK1/2
2.5
2.0
1.5
1.0
0.5
0.0
p-ERK1
p-ERK2
control
25
50
100
200
BSA-AGEs (100 μg/ mL)

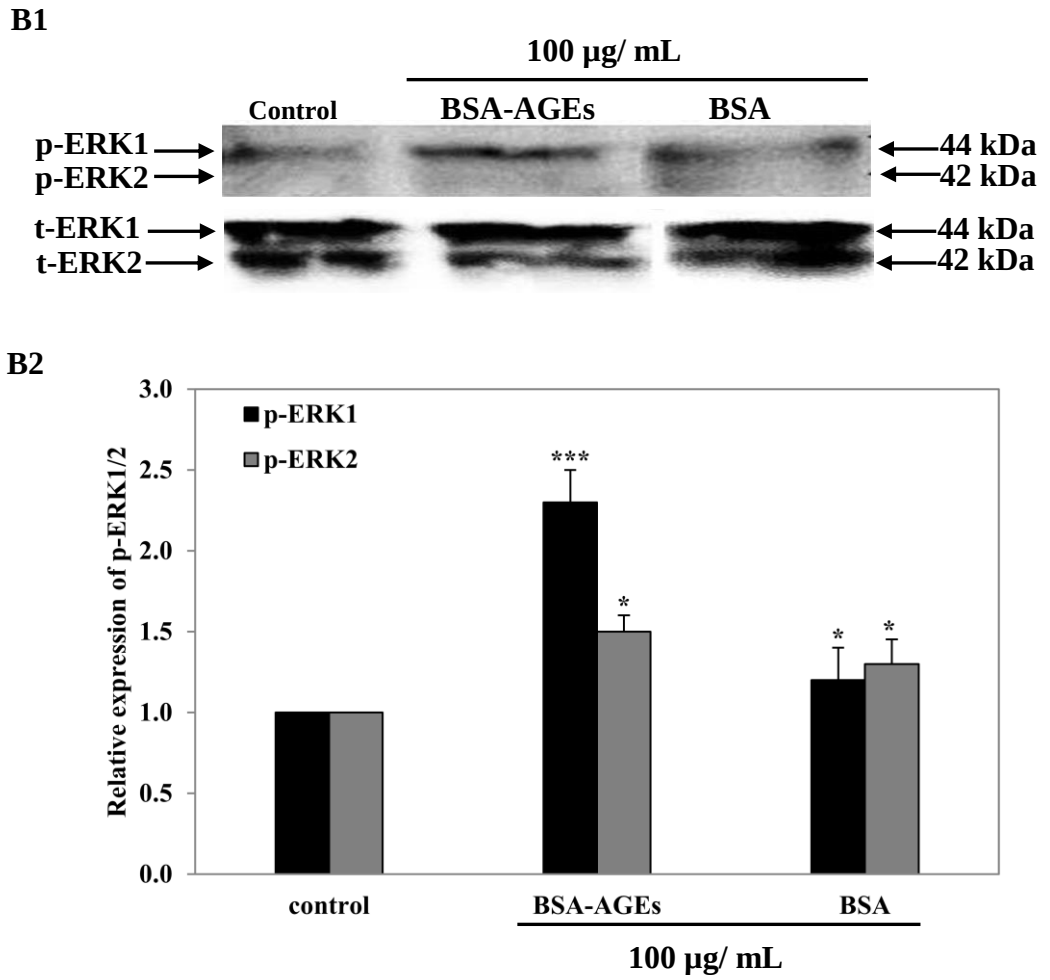


Figure 4.5: Effect of BSA-AGEs on-ERK1/2 expression in MCF-7 cells. (A1) Representative Western blot analysis, showing the variation of p-ERK1/2 expression induced by 25-200 µg/mL BSA-AGEs after 10 minutes incubation, compared to untreated control cells, in lane C. The graph (A2) shows the relative expression of p-ERK1/2 calculated as a ratio to the total ERK1/2 (t-ERK1/2), the loading control. (B1) Representative Western blotting analysis. (B2) Relative expression of p-ERK1/2 calculated as a ratio to the total ERK1/2 (t-ERK1/2), the loading control. Results are presented as mean ± SD (n=3).).*: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$.

4.2.5 The Effects of BSA-AGEs on RAGE Expression in MCF-7 Cells

A time-course of the expression of RAGE in MCF-7 cells following the treatment of 100 μg/mL BSA-AGEs was established by Western blotting. Figure 4.6 shows that BSA-AGEs up-regulated RAGE protein expression in a time-dependent manner. In the untreated conditions, RAGE expression was enhanced with the incubation time due to a quick synthesis of RAGE protein Figure 4.6A. Compared to the basal expression of RAGE in untreated cells, RAGE expression was increased in MCF-7 cells treated with 100 μg/mL BSA-AGE by 2.8-fold ($p<0.001$) after 10 minutes incubation time. On treating the cells with 100 μg/mL BSA-AGEs, the expression of RAGE was highly increased by 3.0-fold ($p<0.001$) at 24 hours compared to untreated cells (control) nd by 2.3- fold ($p<0.001$) after 48 hours incubation time (Figure 4.6B).

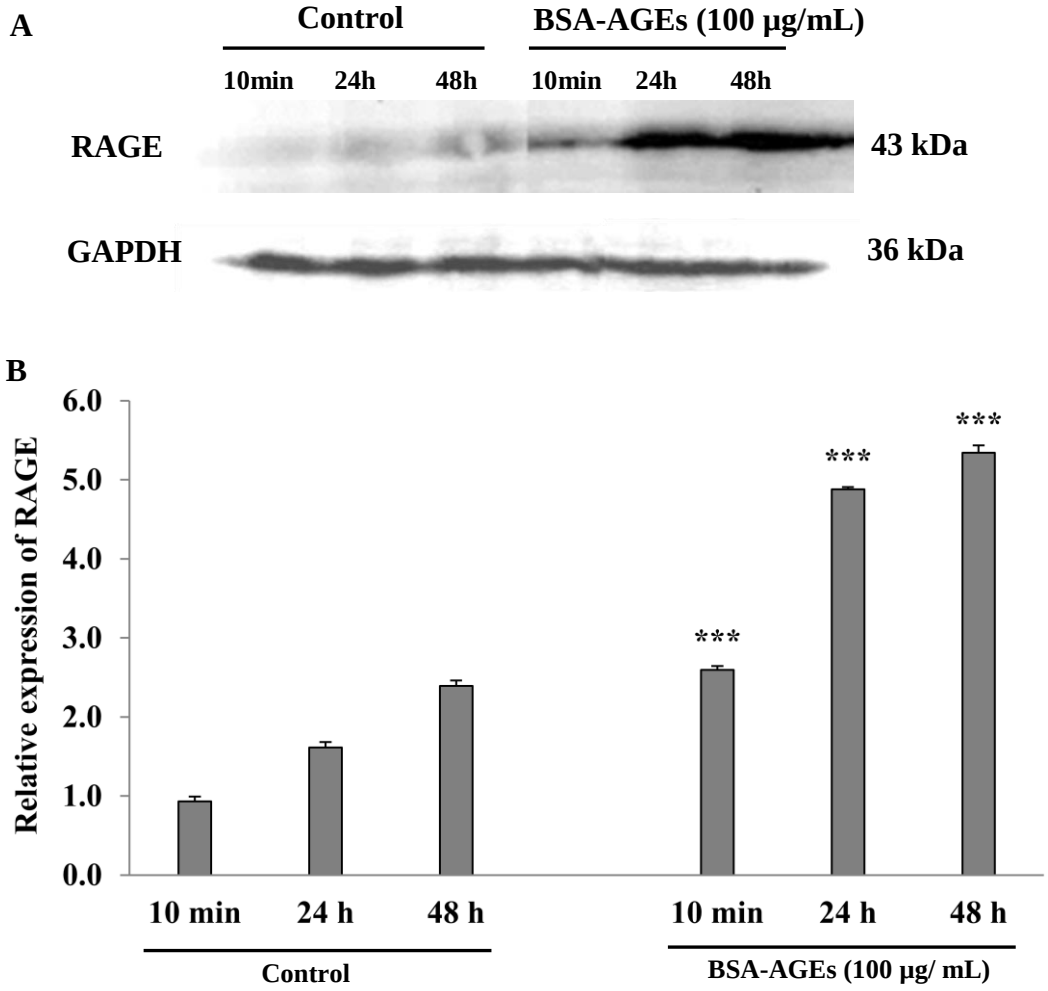


Figure 4.6: Time course of RAGE expression in MCF-7 cells treated with BSA-AGEs. (A) Representative Western blot analysis showing the effect of 100 µg/mL BSA-AGEs on RAGE expression in MCF-7 cells after 10 minutes, 24 hours, and 48 hours of incubation, compared to untreated cells (control). (B) Relative expression of RAGE calculated as a ratio to GAPDH expression, the loading control. Results are presented as mean ± SD (n=3). ***: $P < 0.001$.

4.2.6. The Effects of BSA-AGEs on Phosphorylation in MCF-7 Cells

4.2.6.1 BSA-AGE Induced Phosphorylation of MAPK/ERK Protein-Serine Kinase 1/2 and cAMP Response Element Binding Protein 1 (CREB1)

To investigate downstream phosphorylated proteins of BSA-AGEs/RAGE signalling pathways, the whole cell lysates from untreated MCF-7 cells (Figure 4.7A_1), or from cells treated with 100 μg/mL BSA-AGEs (Figure 4.7A_2) or 100 μg/mL non-modified BSA (Figure 4.7A_3) for 10 minutes were analysed using the phospho-protein array KPSS-1.3 (Figure 4.7A). The bio-informatics analysis of the immuno-blots showed that BSA-AGEs caused 1.17- and 1.3-fold increase of p-ERK1 and p-ERK2 expression respectively, as compared to the untreated control cells (Figure 4.7B). Compared to untreated control cells, BSA-AGEs increased by 2.04-fold phospho-protein expression of MAPK/ERK protein-serine kinase 1/2, which is an upstream kinase activator of p-ERK1/2 (Figure 4.7B). Compared to untreated control cells, BSA-AGEs also increased by 1.98-fold phospho-protein expression of cAMP response element binding protein 1 (CREB1), which was lately reported to be associated with breast cancer development (Son *et al*, 2010) (Figure 4.7B). In addition, non-modified BSA also increased by 50% phospho-protein expression of CREB1 and MAPK/ERK protein-serine kinase1/2 as compared to untreated cells (Figure 4.7B).

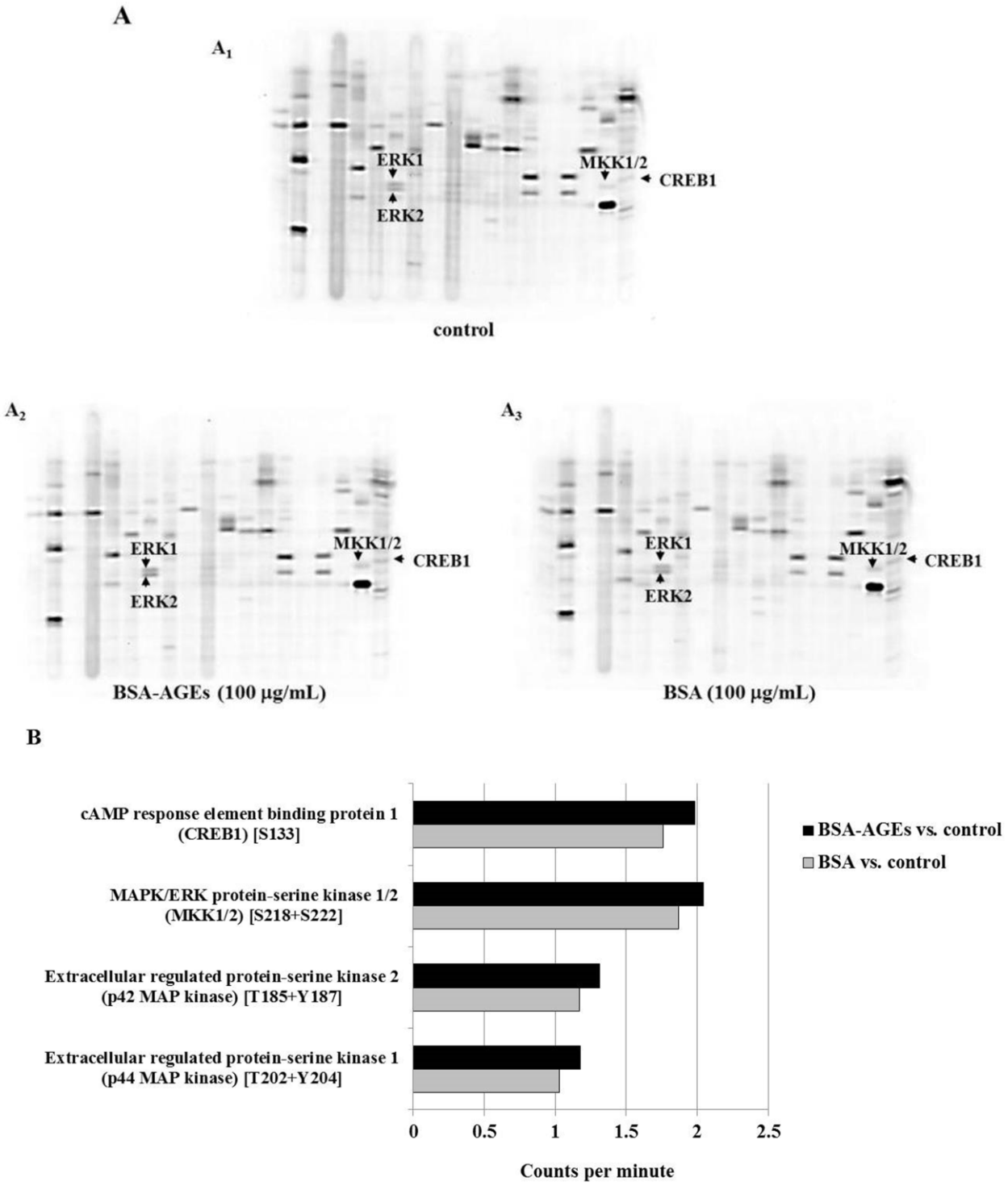


Figure 4.7: Up-regulatory effect of BSA-AGEs on phospho-protein expression in MCF-7 using phospho-protein-array. (A) The multi-immunoblotting showing the expression of 35 phospho-proteins in (A_1) untreated MCF-7 cells, (A_2) cells treated with 100 µg/mL BSA-AGEs or non-modified BSA (A_3) after 10 minutes of incubation. (B) shows the relative expression of the relevant overphosphorylation of CREB1, MKK1/2,/ERK1 and ERK2 expressed in counts per minute and calculated as a ratio to the untreated control cells.

4.2.6.2 BSA-AGEs Down-Regulated the Phosphorylation of Protein-Serine kinase C (PKC) Isoforms, Mitogen and Stress-Activated Protein-Serine kinase 1 (Msk1) and Adducin α

Phosphorylation of protein-serine kinase C (PKC) isoforms such as PKCα, PKCα/β2, PKCε, and PKCδ, have been described to play different roles in breast cancer progression (Urtreger *et al*, 2012). The phospho-protein array analysis demonstrated that BSA-AGEs induced a down-regulation of the phosphorylated PKCε by 20%, while non-modified BSA up-regulated PKCε phospho-protein expression by 1.75-fold as compared to the untreated control cells. Treatment with BSA-AGEs also caused a decrease in the phosphorylated PKCα (50%), PKCα/β2 (60%), and PKCε (30%) and PKCδ (60%). The phosphorylation of mitogen and stress-activated protein-serine kinase 1 (Msk1) was also assessed. Mski is an activated downstream protein of ERK1/2 and of p38 pathways and is involved in cell survival, proliferation and differentiation. The phosphorylation of Msk1 was down-regulated by 40% in MCF-7 cells after cell treatment with BSA-AGE treatment (Figure 4.8). The phosphorylation of adducin α, a cytoskeleton protein, which dissociates from F-actin and spectrin during cell migration, was down-regulated by 60% in MCF-7 cells after BSA-AGE treatment, as compared to the untreated control cells. However, the MCF-7 cell treatment with non-modified BSA induced a level of expression slightly higher than BSA-AGEs-down-regulated phospho-protein expression of PKCα, PKCα/β2, PKCδ, ADD1 and Msk1.

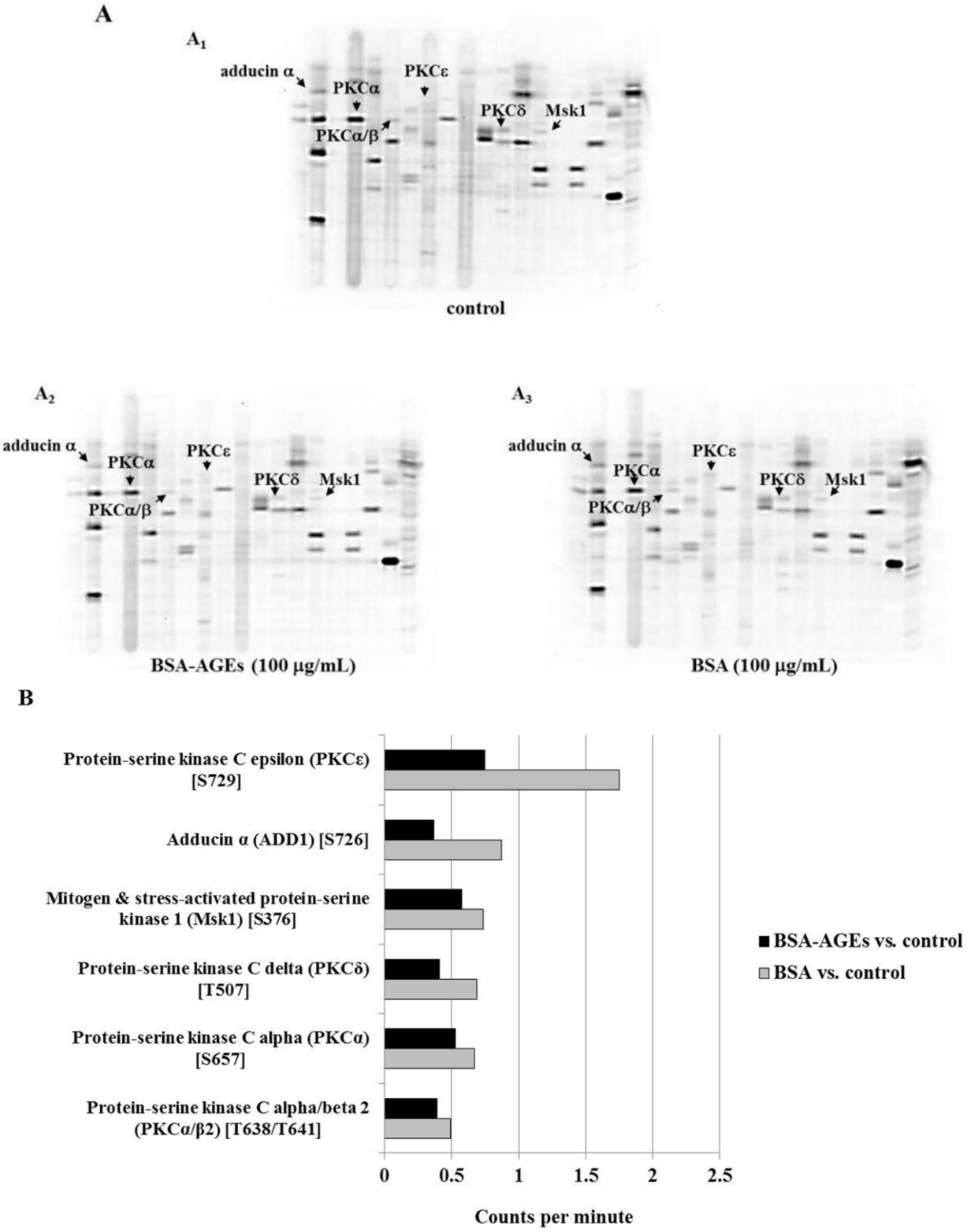
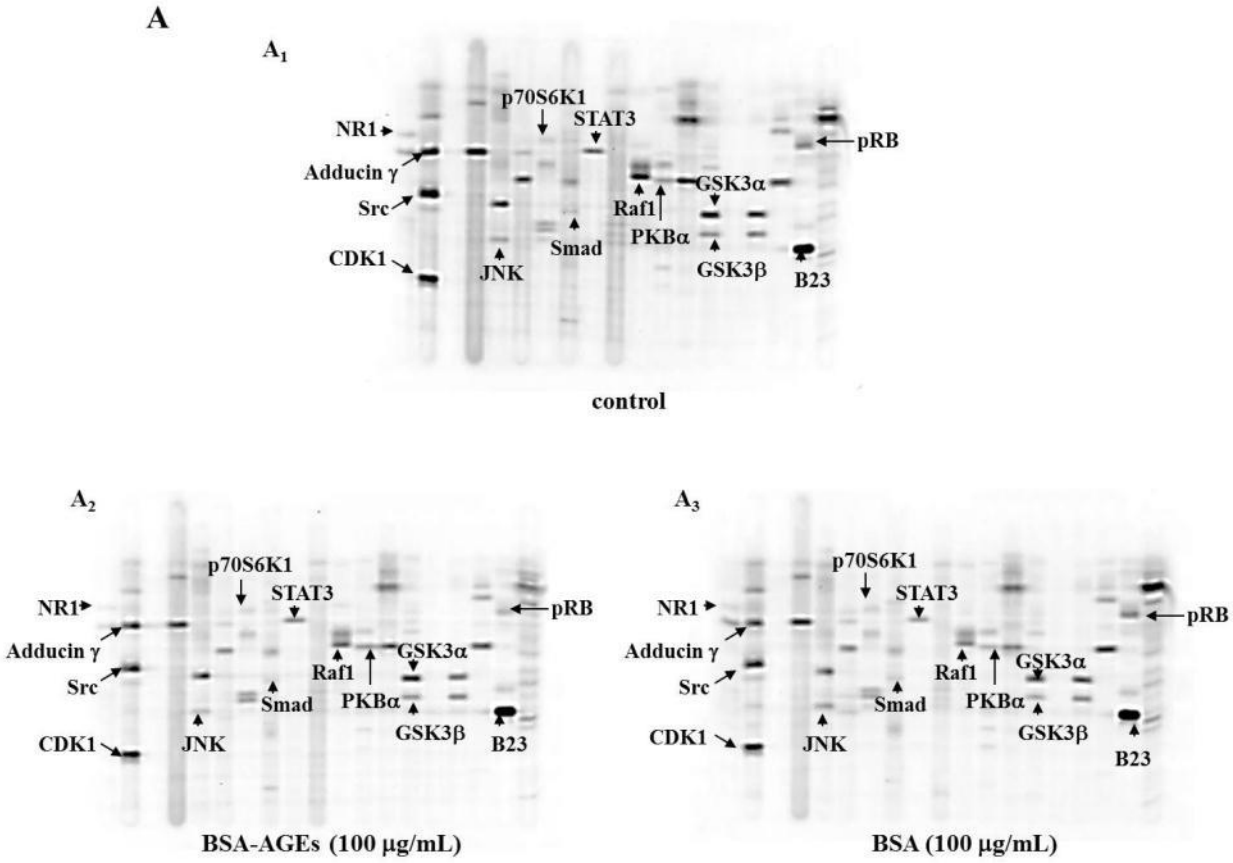


Figure 4.8: Down-regulatory effect of BSA-AGEs on phospho-protein expression in MCF-7 using phospho-protein-array. (A) Multi-immunoblotting showing the expression of 35 phospho-proteins in (A_1) untreated MCF-7 cells, (A_2) cells treated with 100 µg/mL BSA-AGEs or (A_3) non-modified BSA after 10 minutes of incubation. (B) shows the relative expression of the relevant phospho-proteins expressed in counts per minute and calculated as a ratio to the untreated control cells.

4.2.6.3 BSA-AGEs and Non-Modified BSA had Similar Effects on Phosphorylation of most of the Protein on KPSS-1.3 Array

For most of the proteins screened using the phospho-protein array analysis KPSS-1.3. The MCF-7 cells exposed to 10 minute-treatment with BSA-AGEs showed same variation of phospho-protein expression STAT3, CDK1,ADD3, S4, RB, SAPK, NR and p70S6K1 as those analysed after similar treatment with non-modified BSA (Figure 4.9). While BSA-AGEs and non-modified BSA-treated cells slightly up-regulated the phospho-protein expression of the nucleolar nucleocytoplasmic shuttling protein B23, all the other phospho-proteins were down-regulated and mostly by 50% (Figure4.9).



B

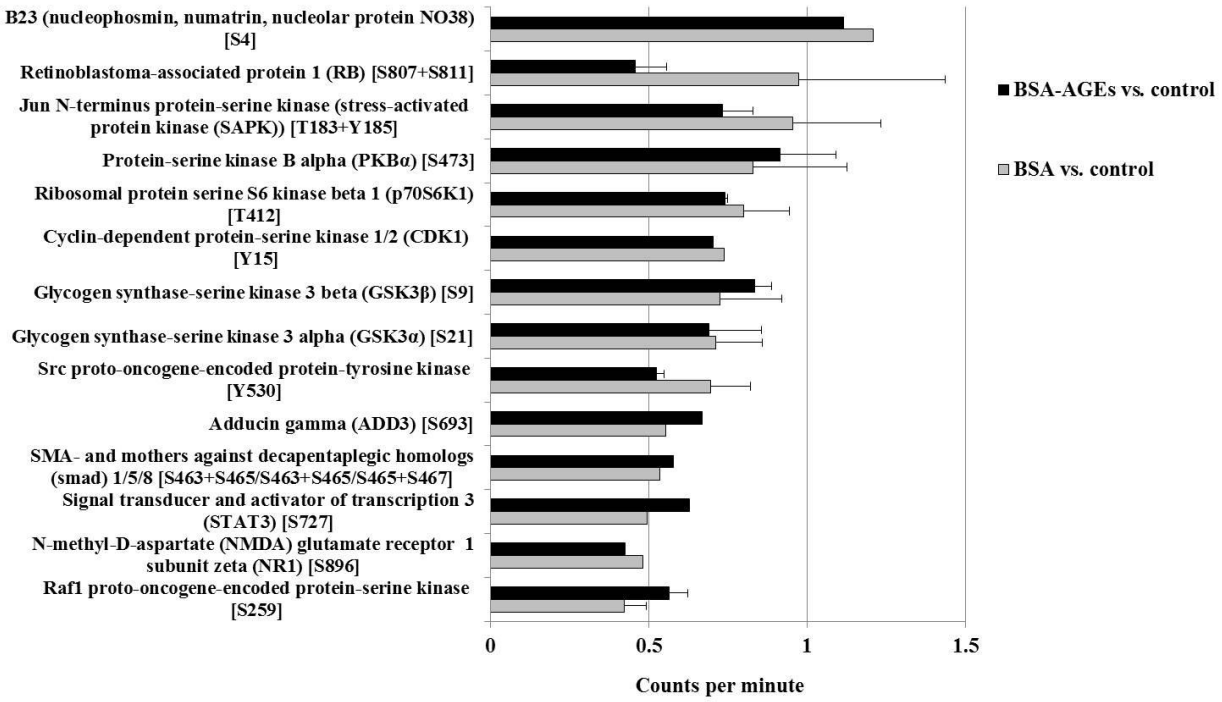


Figure 4.9: Effects of BSA-AGEs and non-modified BSA on MCF-7 phospho-protein expression from micro-array analysis. (A) Multi-immunoblotting showing the expression of 35 phospho-proteins in (A_1) untreated MCF-7 cells, (A_2) cells treated with 100 μg/mL BSA-AGEs or (A_3) non-modified BSA after 10 minutes of incubation. The graph (B) shows the relative expression of the relevant phospho-proteins expressed in counts per minute and calculated as a ratio to the untreated control cells. Results are presented as mean ± SD (n=3).

4.2.7 The comparison between the two cell lines:

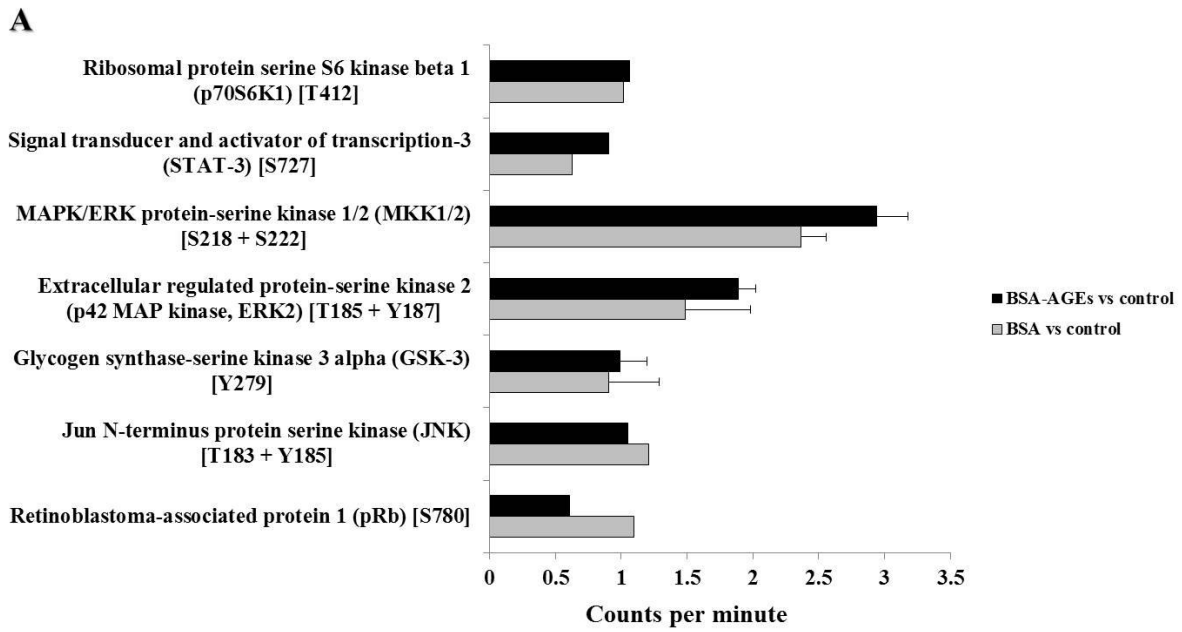
The heterogeneity of breast cancer has obliged scientists to use different cell lines, which serve as in *vitro* models to explain the mechanisms involved in its development and progression. In this study two main breast cancer cell lines have been used with different features in regards to antigenic and expression profiles, tumorigenicity and organ-specific metastatic potential (Nagaraja et al, 2006). Despite, the importance of using these different breast cancer cell lines, they are not representative of the diversity of the actual tumour. The two breast cancer cell lines usually used are MDA-MB231 and MCF-7. The first cell line MDA-MB231 was isolated from pleural effusion of a Caucasian breast cancer patient. MDA-MB-231 cells are highly invasive, oestrogen-independent and correspond to the late stage of breast cancer progression with high aerobic glucose intake rates (Gupta and Tikoo, 2013). In contrast to MDA-MB231 cell line, MCF-7 cell line is non-invasive, oestrogen-dependent and corresponds to early stage of breast cancer progression. In addition, MCF-7 cells respond to endocrine therapy with good prognosis.

This study examined in *vitro* biological effects of different concentrations of BSA-derived AGEs on MDA-MB231 and MCF-7 cell lines compared to non-modified BSA and non-treated cells (used as the control). Throughout this study same techniques and conditions were applied to both cell lines to establish a comparative study.

This study demonstrated that BSA-AGEs significantly increase the MDA-MB231 cell proliferation, migration, and invasion associated with increased MMP-9 activity and up-regulate RAGE expression. At the difference of MDA-MB-231 cells, MCF-7 cells exposed to BSA-AGEs undergo a slight increase of their proliferation and migration. We also showed that MCF-7 cells are unable to invade growth factor-reduced reconstituted basement membrane and to produce or activate the gelatinases such as MMP-2 and MMP-9. The significant positive biological effects in both cell lines was observed at 100µg/mL BSA-

AGEs compared to non-modified BSA and untreated condition (control). The key signalling proteins involved in BSA-AGE-mediated cell response were investigated by using the phospho-protein array analysis KPSS-1, following 10 minutes treatment of MDA-MB-231 and MCF-7 with 100 μg/mL BSA-AGEs. This phospho-protein array analysis revealed that BSA-AGEs enhance expression of ribosomal protein serine S6 kinase beta -1 (p70S6K1) in both cell lines BSA-AGEs induce the over-phosphorylation of (p70S6K1) (25.4-fold increase) in MDA-MB231 cells compared to the control and non-modified BSA, while in MCF-7 cells similar degree of p70S6K1 phosphorylation was observed in BSA-AGE-, non-modified BSA-treated cells and in untreated cells. The p70S6K1 has a central role in tumour growth by regulating pro-angiogenic effects and protein synthesis of insulin (Zhou et al., 2007; Dennis et al., 2012). Overall, the non-modified BSA has no effect on both cell lines except a slight stimulation of MDA-MB231cell proliferation at 50 μg/mL and an inhibitory effect of MDA-MB231 cell migration at the highest concentration (i.e. 200 μg /mL). This slight pro-mitogenic effect of non-modified BSA is correlated with the induction of ERK1/2 phosphorylation observed at short incubation time (10 minutes) at the same concentration 50 μg/mL, whereas no increase in phosphorylation of ERK1/2 was induced by 100 μg/mL of non-modified BSA. The pro-mitogenic effect of native BSA was also reported by Chung and colleagues using tubular epithelial cells in similar conditions. Furthermore, this positive effect might possibly be attributed to *in vivo* derived AGEs from the BSA used. Indeed, BSA purchased commercially is found modified by a low degree of glycation that has occurred *in vivo* Indeed, native globular BSA even bound to the cell surface rarely triggers specific transduction signalling unlike BSA-AGEs. These signalling investigations clarified the greater mitogenic effect of 100 μg /mL of BSA-AGEs on MDA-MB-231 cells than on MCF-7 cells and identified the impacts of BSA-AGEs on the high production of proteins (revealed by concomitant increase of (p70S6K1) required for increased growth of high invasive breast

cancer cells. Additional studies are necessary in order to identify these proteins specifically over-produced by these breast cancer cells exposed to BSA-AGEs and involved in cell proliferation such as cyclins. Thus, because of heterogeneity between MDA-MB231 and MCF-7 cells, it is important to deeper investigate and clarify the molecular mechanisms underlying the development of breast cancer contributed by AGEs.



B

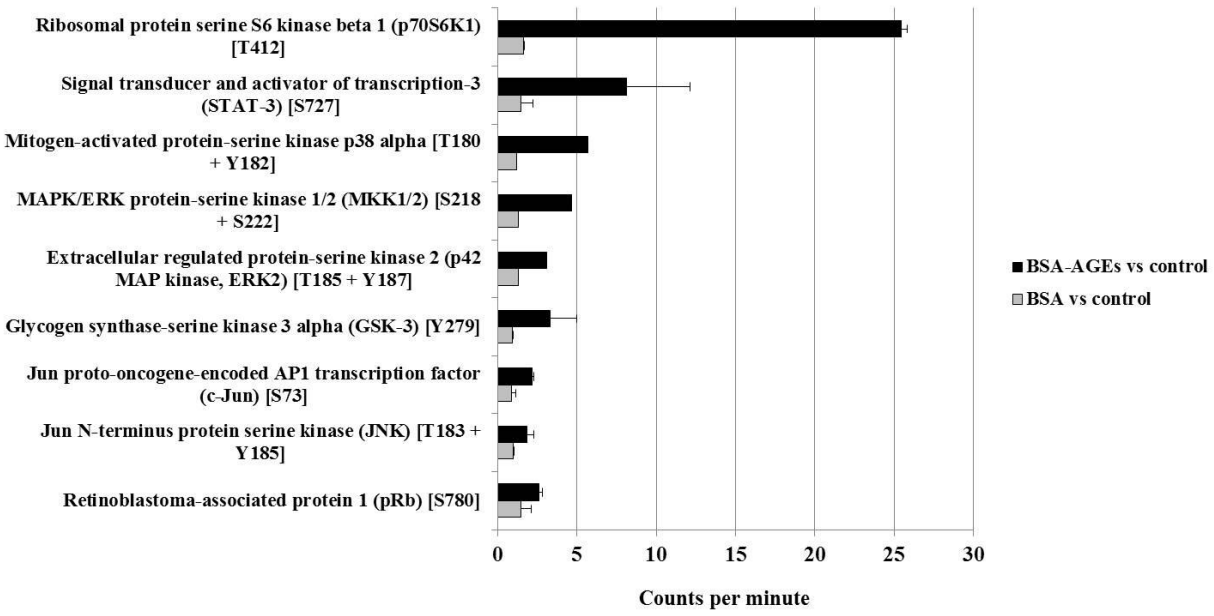


Figure 4.10: Micro-array analysis of MDA-MB231 and MCF-7: Effect of BSA-AGEs and non-modified BSA on phospho-protein expression in MCF-7 (A) and MDA-MB-231 (B) The graphs show the relative expression of the relevant and same phospho-proteins expressed in counts per minute and calculated as a ratio to the untreated control cells in both cell lines,. Value represents the mean ± SD (n = 3).

4.3 Discussion

MCF-7 is a hormone-dependent breast cancer cell line isolated from poorly invasive nonmetastatic cells which means that these cells have not developed invasive capacity yet, not like MDA-MB-231 cells (Kirschmann *et al,* 2002). In addition, many studies have described differences between MCF-7 and MDA-MB-231cells, in terms of morphology, phenotypes and signalling pathways (Kirschmann *et al,* 2002; Gupta and Tikoo, 2013; Mladkova *et al,* 2010). Therefore, after investigating the effects of BSA-AGEs on MDA-MB-231 cells, the same studies were applied to MCF-7 cells.

First, MCF-7 cells were slow to grow in contrast to MDA-MB-231 due to their sensitivity to hormones such as oestrogen and progesterone. Clinically, the suppression of these hormones or blocking their receptors is likely to inhibit the development of *in situ* mammary carcinoma (Miller *et al*, 2011). In this study, MCF-7 cells were treated with different concentrations of BSA-AGEs, in comparison to non-modified BSA. Regarding cell migration, the wound-healing assay used with MDA-MB-231 cells was not applied to MCF-7 cells because these latter did not cover the whole surface of the well. Therefore, the effect of BSA-AGEs on MCF-7 cells migration was assessed using the Boyden chamber. Like their effects on MDA-MB-231 cells, non-modified BSA had no effect on MCF-7 migration while BSA-AGEs had dose-dependent effects, showing a bell-shaped curve response. BSA-AGEs at the concentration of 50 μg/mL and 100 μg/mL significantly increased MCF-7 cell growth and motility while no effect on cell proliferation and migration were observed when BSA-AGE was at 25 μg/mL. On the contrary an inhibitory mitogenic effect on MCF-7 cells was noticed with 200 μg/mL BSA-AGE. This bell-shaped curve observed in response to BSA-AGEs suggests the activation of p-ERK1/2 through a receptor whose optimal oligomerization is reached in the presence of 50-100 μg/mL BSA-AGEs. With regards to their invasive capacity, MCF-7 cells treated with BSA-AGE or BSA only for different concentrations as expected for a non-metastatic cell line MCF-7, cells were unable to invade growth factor-reduced reconstituted basement membrane and to produce or activate the gelatinases, MMP-2 and MMP-9. This finding confirmed previous studies, which also demonstrated the absence of gene expression and protein activity of gelatinases in the cells derived from MCF-7 cells (Singer *et al*, 2002). However, other studies have reported MMP-2 production in MCF-7 cells (Azzam, *et al,* 1993). These controversies might be explained by the different passages or subclones used in their experiments. We also found that 100 μg/mL BSA-AGEs induced the expression of RAGE in MCF-7 cells, in particular after 24 and 48 hours of incubation.

These findings are supported by Tanaka and colleagues (2000) who showed an overexpression of RAGE at both mRNA and protein levels following human vascular endothelial cell treated with 50 μg/mL BSA-AGE for 24 hours, while non-modified BSA had no effect (just quick synthesis of RAGE protein at the post-translation level). They also identified an AGE- responsive element on *RAGE* gene, which is the region upstream from the transcription start site of *RAGE* (Tanaka *et al*, 2000). As previously shown in MDA-MB-231 in (section 3.4.5), BSA-AGE-induced RAGE up-regulation in MCF-7 cells suggesting a positive feedback loop enhancing the cell sensitivity to BSA-AGEs with risk of cancer development.One of an important study demonstrated that Glycer-AGEs, significantly stimulated the growth and migration of human melanoma cells through their interactions with RAGE (Takino *et al*, 2010). In addition, RAGE interaction with BSA-AGEs enhance pro-inflammatory and pro-oxidative reactions of the cells, assuming that RAGE might be involved in the complication of breast cancer (Yan *et al*, 1994; Jiao *et al*, 2011). In addition, recently, Lata and Mukherjee (2014) demonstrated the key role of RAGE in the stimulation of proliferation and survival of MCF-7 cells induced by a synthetic oestrogen named 17α-ethinyl-estradiol. Altogether, BSA-AGEs might contribute to the growth of the breast cancer through stimulation of cancerous cells located in carcinoma *in situ* with potential synergic actions with oestrogen.

An analysis of signalling pathways (mainly involved in cell proliferation) in MCF-7 cells following 10 minute treatment with 100 μg/mL BSA-AGEs, revealed that BSA-AGEs enhanced expression p-ERK1/2, MAPK kinase 1/2 (MKK1/2, an activator of phospho-ERK1/2) and (CREB1, a well-characterised ERK signalling transcription factor), while BSA-AGEs down-regulated the expression of different isoforms of phospho-PKC, (Msk1) and phospho-adducin α. However, like non-modified BSA, BSA-AGEs had no effect on

the phosphorylation of SATAT3, CDK1, ADD3 and S4in MCF-7 cells compared to untreated cells. It was previously demonstrated that BSA-AGEs up-regulated phospho-ERK1/2 expression mainly through RAGE/AGEs axis in MDA-MB-231 cells (section 3.4.6), BSA-AGEs up-regulate the increase of phospho-ERK1/2 and its upstream activator in MKK1/2 by BSA-AGE in MCF-7 cells were not as much as their counter parts in MDA-MB-231 cells. An increase of MAPK1/2 activity was also reported in rat pulmonary artery smooth muscle cells after treatment with 100 μg/mL AGE-albumin for 10-15 minutes (Lander *et al,* 1997). The difference in cell signal intensity between MCF-7 and MDA-MB-231 cells, for example on phospho-ERK1/2 overexpression induced by BSA-AGEs, was also observed after chemotherapy with doxorubicin or docetaxel (Taherian and Mazoochi, 2012).Thus, the heterogeneity of cell sensitivity to BSA-AGE between MCF-7 and MDA-MB-231 cells actually reflects the heterogeneity of breast cancers. Furthermore, BSA-AGEs up-regulated expression of phospho-CREB1, transcription factor involved in cell stress responses, cell survival and proliferation. Recently CREB1 has been demonstrated to contribute to malignancy of the breast by inducing transcription of aromatase in breast adipose mesenchymal cells, which leads to increased oestrogen levels and subsequently to cancer (Chhabra *et al,* 2007; Samarajeewa *et al,* 2013). These findings suggest that BSA-AGEs might contribute to breast cancer development through activation of MAPK pathway and activation of CREB1 transcription factor. On the other hand, in present study, BSA-AGEs down-regulated the expression of phospho-PKC, a key signal for injury, and phosphor-Msk1, an activated downstream molecule of MAPK signalling. The family of PKC, consisting of multiple isoforms, has been associated with enhanced activity of transcription factor NF-κB, which leads to gene expression encoding pro-inflammatory cytokines (Ye, 2001). With AGEs, well known as mediators of inflammation an increased PKC phosphorylation was expected (Zhang *et al,* 2011). Many studies have

reported PKC activation following RAGE ligation with AGEs in rat cardiomyocytes or mesangial cells but so far, not in breast cancer cells (Tuttle *et al.,* 2009; Yu *et al.,* 2013). An increase of Msk1 phosphorylation was expected as MAPK (phospho-ERK1/2) was significantly increased (Khan *et al.,* 2013).This surprising finding of down-regulation of Msk1 might be due to the level of MAPK activation was not sufficient to subsequently activate Msk1. The investigation of signalling pathways present a complex system which require additional study and the application of complementary techniques including the knock-down of cytoplasmic proteins such as PKC, Msk1 expression or even RAGE expression

In conclusion, we showed for the first time the dose-dependent stimulatory effects of BSA-AGEs on a hormone-dependent breast cancer cell line MCF-7 cells, with regards cell proliferation and directed migration, as compared to non-modified BSA. However, BSA-AGEs did not change the weak invasive capacity of MCF-7 cells to cross a reconstituted basement membrane. In addition, BSA-AGEs induced over-expression of RAGE in MCF-7 cells. The investigation of signalling pathways suggests that BSA-AGEs might contribute to breast cancer development through activation of MAPK pathway and activation of CREB1 transcription factor in MCF-7 cells. The knock-down of RAGE expression might be a promising targeted therapy to prevent the development of breast cancer in diabetic patient.

Chapter 5

Chapter 5.1 General Discussion

This study provides the first evidence that BSA-AGEs promote proliferation, migration and invasion of MDA-MB-231 cells probably by modulating MMP-9 activity, up-regulating RAGE expression and increasing phosphorylation of ERK1/2, STAT-3 and p70S6K1, which are involved in cell growth, cell cycle, motility, cell invasion and protein synthesis. In contrast to MDA-MB-231 cells, very weak stimulatory effects of BSA-AGEs were observed in MCF-7 cells, indicating lower cell sensitivity than MDA-MB-231 cells to BSA-AGEs. Furthermore, throughout the study, the non-modified BSA had negligible effect.

The morphological and phenotypic heterogeneity of breast cancer have led to the frequent use of MDA-MB-231 and MCF-7 cell lines in the determination of bioactivities of various compounds, in cytotoxicity measurements, and in studies of specific functions of protein and genes after manipulations (Mladkova *et al*, 2010). Although both cell lines are derived from pleural effusions of metastatic mammary carcinoma patients, MDA-MB-231 is an oestrogen-independent, highly invasive cell line isolated from poor prognostic late-stage breast cancer, which corresponds to basal-like subtype accounting for 10-17 % of all breast carcinomas (Badve *et al*, 2011).Whereas MCF-7 is an oestrogen-dependent, non-invasive cell line drived from good prognostic early-stage breast cancer corresponds to luminal subtype accounting for 60-70% of breast cancinoma cases (Blows *et al*, 2010). Despite the advantages of using these cell lines in cancer research, they are not representative of the actual tumour diversity. The differential biological effects of BSA-AGEs on MDA-MB-231 and MCF-7 cells demonstrated *in vitro* studies give important molecular insights into the roles of BSA-AGEs in the proliferation, migration and invasion of breast cancer cells, which reinforce the link between diabetes and breast cancer. In addition, to epidemiological studies which support

the pathological consequences of diabetes on the onset and development of cancer including breast cancer, a meta-analysis study has reported that breast cancer rates are increased by 23% for women who are diagnosed with type 2 diabetes (Liao *et al*, 2011). The use of these two cell lines has allowed us to suggest BSA-AGE-induced p70S6K1 overexpression in MDA-MB-231 cells as a potential invasive biomarker of breast cancer and as a promising therapeutic target to prevent breast cancer development and progression in patients diagnosed with diabetes. In addition, the suppression of RAGE expression because RAGE play key roles in the pathogenesis of diabetes, and in breast cancer development and progression (Ramasamy *et al,* 2005; Raida *et al,* 2013). A recent study found MDA-MB231 cells expressed higher level of RAGE than MCF-7 cells did (Radia *et al,* 2013).This present study demonstrated that BSA-AGEs induced the expression of RAGE in both MDA-MB231 and MCF-7 cell lines. Thus, suppressing RAGE expression may be a promising therapeutic method of treating diabetic pateints in danger of developing breast cancer and those with over breast cancer. Hence in future, *in vivo* mouse breast cancer models will be of interest to confirm the effects of BSA-AGEs on tumour growth and characterization of cancerous epithelial cells which respond to BSA-AGEs. This will help to fight against the cancer by targeting and specifically killing BSA-AGE stimulated breast cancer cells.

Despite their phenotypic differences, MDA-MB-231 and MCF-7 cells were cultured in similar conditions composed of culture media with normal glucose concentration (5 mM) and supplemented with 10% FBS and antibiotics. Although MCF-7 is a hormone-dependent cell line, the addition of insulin (10 μg/mL) in the media impeded cell growth. Therefore, the insulin was withdrawn from the culture medium of MCF-7 cells. The use of similar culture media (without the addition of insulin) for both MDA-MB-231 and MCF-7 cells were also reported in many studies (Mladkova *et al*, 2010; Gest *et al*, 2013).

In addition, although insulin is considered as a key element for MCF-7 cell growth, a recent study has reported that high insulin concentration (25 mM) inhibited MCF-7 cell proliferation, whereas MDA-MB-231 cell proliferation was stimulated (Gupta and Tikoo, 2013). These recent findings support our observation with regards to the withdrawal of insulin from the complete medium. The same study demonstrated also the differential modulation effect of high glucose concentration (25 mM) on the two breast cancer cell lines including a stimulation of MDA-MB-231 cell proliferation with an increased phosphorylation of ERK1/2, whereas no change was observed on MCF-7 cell growth (Gupta and Tikoo, 2013). A study showed the differences between human breast cancer cell lines in susceptibility towards growth inhibition by genistein, a phytoestrogen (Dampier *et al,* 2001). This difference was explained by the different oestrogen receptor expression involved including ER-β by MDA-MB-231 cells while MCF-7 cells express both ER-α and ER-β receptors (Vladusic *et al,* 2000; Ford *et al,* 2011). However, surprisingly they showed that genistein induced apoptosis in only MDA-MB-468 cells a non-hormone dependenat cell line. In addition, a recent study has reported that in fact MDA-MB-231 cells expressed both ER-α and ER-β receptors (Ford *et al,* 2011). This report published by Ford and colleagues could explain the reason why during cell treatment with genistein established by Dampier and colleagues (2001), hormone-dependent breast cancer cell lines were not distinguished from non-hormone dependent breast cancer cell lines. Therefore, despite the different phenotypic characteristics between MDA-MB231 and MCF-7 cells, it is essential to use them to identify the impacts of BSA-AGEs on the growth and metastatic features of different types of breast cancer cells, to investigate their associated heterogeneous spectrums to elucidate possible molecular mechanisms underlying the development of breast cancer contributed by AGEs.

In this study, BSA-AGEs modulated the expression of proteins involved in cell signalling pathways differently between in MDA-MB-231 and in MCF-7 cells. MDA-MB-231, previously described as a high RAGE expressing cell line (Radia *et al,* 2013), treated with BSA-AGEs had ERK-1/2 MAPK, p38 MAPK, GSK-3, STAT-3 and p70S6K1 highly over-phosphorylated; whereas MCF-7, previously described as the lowest RAGE expression cell line, treated with BSA-AGEs had ERK1/2 and CREB1 (ERK signalling transcription factor) had moderately over-phosphorylated and different isoforms (ε, δ, α, α/β_2) of PKC dephosphorylated. These signalling investigations justified the greater mitogenic effect of BSA-AGEs on MDA-MB-231 cells than on MCF-7 cells. With similar BSA-AGE concentration used (i.e. 100 μg/mL), Ishibashi and colleagues (2013) have demonstrated that metformin, a chemotherapeutic agent against breast cancer for diabetic patients, inhibits BSA-AGE-induced proliferation, VEGF and RAGE expression in MCF-7 cells by blocking BSA-AGE-RAGE axis (Ishibashi *et al.,* 2013). A recent study demonstrated that inhibition of RAGE using RAGE siRNA in breast cancer cell lines down-regulated the expression of transcription factor, NF-κB p65 which eventually led to the down-regulation of cyclin D1, a protein involved in normal cell cycle regulation and responsible for transition from the G1 phase to the S (DNA synthesis) phase (Radia *et al,* 2013). In addition, the expression of these two cytoplasmic proteins were also investigated in high glucose-treated MCF-7 and MDA-MB-231 cells (Gupta and Tikoo, 2013). High-glucose condition led to a decrease of cyclin D1 expression in MCF-7 cells, whereas an increased cyclin D1 expression was observed in MDA-MB-231 cells (Gupta and Tikoo, 2013). With regards to NF-κB expression, an over-phosphorylation was observed in MCF-7 cells, whereas a de-phosphorylation of NF-κB was noticed in MDA-MB-231 cells in high glucose condition. They also provided the evidence that high glucose promoted carcinogenesis in MDA-MB-231 cells and inhibited

cell proliferation in MCF-7 cells. In addition, activation of downstream intracellular signalling like MAPK pathway has also been linked to hormone resistance (Tomlinson *et al,* 2012). There is growing evidence that there also exists a cross-communication between ER and growth factor receptor signalling such as insulin-like growth factor-1, epidermal growth factor (EGF), transforming growth factor (TGF)-α and TGF-β (Ignar-Trowbridge *et al,* 1995; Thorne and Lee, 2003). However, so far, no cross-communication between ER and RAGE signalling has been described, which opens a new door of investigation.

With regards to MDA-MB-231 cell invasion, here we have shown that BSA-AGEs increased MMP-9 activity and not MMP-2. MMP-9 activity in MDA-MB-231 cells was also reported by Gest and colleagues (2013) who also demonstrated a decrease in MMP-9 activity after the knockdown of Rac3, a Rho GTPase with relevant function in cancer (Gest *et al.*, 2013). It is known that during breast cancer cell progression, late-stage breast cancer cells including MDA-MB-231 cells undergo some changes in the expression levels of cathepsin D and other matrix digesting enzymes with loss of ER, with increased expression of the mesenchymal marker vimentin and decreased expression of epithelial marker E-cadherin, which all are believed to be implicated in breast tumour invasiveness (Thompson *et al,* 1992). Vimentin acquisition has been associated with increased metastatic potential through enhanced invasiveness and epithelial-mesenchymal transition (Ford *et al,* 2011). Consequently, an understanding of the underlying causes of tumour cell metastasis is essential for the development of new treatments that can slow or arrest the progress of breast cancer. Therefore, it will be of interest to assess vimentin expression in MDA-MB-231 cells after BSA-AGE treatment. Moreover, this study has several limitations such as the difficulties in growing MCF-7 cells in the presence of insulin, which impeded the cell growth. Using qRT-PCR to validate AGE induced RAGE

expression observed using Westren blotting could be a beneficial step. A further limitation of this study was to use only one type of AGE. Future studies are needed to evaluate the potential roles of the other AGEs in breast cancer development.

As aforementioned, the proliferative and invasive abilities of cancer cells, particularly of MDA-MB-231 cells, are correlated with aggressiveness and poor clinical prognosis. Thus, our present study has improved our understanding of the molecular mechanisms involved in the growth and aggressiveness of breast cancer that may be applied for identification of new therapeutic targets.

5.1 Future Studies

Based on the results of this study, future studies could include:

- Detect in of highly glycated BSA in the serum of breast cancer patients with metastasis.
- Knock-down of RAGE expression using siRNA technology to confirm the lack of activity of RAGE in breast cancer cells, and to measure breast cancer in response to therapeutic intervention.
- *In vivo* research using animal models carrying different types of breast cancer cells which represent the distinctive phenotypic characteristics of MDA-MB231 and MCF-7 cells to and test the effects of different types of AGEs to identifiy which one play a pathogenic role, and which AGE receptors mediate the effects of AGEs on breast cancer cells.
- To address the roles of AGEs in breast cancer and type 2 diabetes by using anti-glycation methods on animal models and patients who have breast cancer and type 2 diabetes at the same time.

References

Abe, R. and Yamagishi, S. (2008). AGE-RAGE system and carcinogenesis. *Current Pharmaceutical Design*, **14**, 940-945.

Abe, R., Shimizu, T., Sugawara, H., Watanabe, H., Nakamura, H., Choei, H., Sasaki, N., Yamagishi, S., Takeuchi, M. and Shimizu, H. (2004). Regulation of human melanoma growth and metastasis by AGE-AGE receptor interactions. *Journal of Investigation Dermatology*, **122**, 461-467.

Ahmed, N. (2005). Advanced glycation endproducts-role in pathology of diabetic complications. *Diabetes Research and Clinical Practice*, **67**, 3-21.

Ahmed, N. and Thornalley, P. J. (2007). Advanced glycation endproducts: What is their relevance to diabetic complications? *Diabetes, Obesity and Metabolism*, **9**, 233-245.

American Diabetes Association. (2006) Diagnosis and classification of diabetes mellitus. *Diabetes Care*, **29**, 46.

American Diabetes Association. (1979). Classification and diagnosis of diabetes mellitus and other categories of glucose intolerance. *National Diabetes Data Group Diabetes*, **28**, 1039-1057.

American Diabetes Association. (2009). Diagnosis and classification of diabetes meltius. *Diabetes Care*, **32**, S62-S67.

American Diabetes Association. (2010). Diagnosis and classification of diabetes meltius. *Diabetes Care*, **33**, S62-S69.

Arkin, M. R., Glicksman, M. A., Fu, H., Havel, J. J. and Du, Y. (2012). Inhibition of protein-interactions: non-cellular assay formats. *Assay Guidance Manual*, 1-25.

Aronson, D. (2003). Cross-linking of glycated collagen in the pathogenesis of arterial and myocardial stiffening of aging and diabetes. *Journal of Hypertension*, **21**, 3-12.

Aronson, D. and Rayfield, E. J. (2002). How hyperglycemia promotes atherosclerosis: molecular mechanisms. *Cardiovascular Diabetology*, **1**, 1-10.

Assero, G., Lupo, G., Anfuso, C. D., Ragusa, N. and Alberghina, M. (2001). High glucose and advanced glycation endproducts induced phospholipid hydrolysis and phospholipid enzyme inhibition in bovine retinal pericytes. *Biochimica et Biophysica Acta,* **1533**, 12-140.

Atalay, M. and Laaksonen, D. E. (2002). Diabetes, oxidative stress and physical exercise. *Journal of Sports and Medicine,* **1**, 1-14.

Azzam**,** H. S., Arand, G., Lippman, M. E. and Thomson, E.W. (1993). Association of MMP-2 activation potential with metastatic progression in human breast cancer cell lines independent of MMP-2 production. *Journal of National Cancer Institute,* **85**, 1758-1764.

Badve, S., Dabbs, D. J., Schnitt, S. J., Baehner, F. L., Decker, T., Eusebi, V., Fox, S. B., Ichihara, S., Jacquemier, J., Lakhani, S. R., Palacios, J., Rakha, E. A., Richardson, A. L., Schmitt, F. C., Tan, P. H., Tse, G. M., Weigelt, B., Ellis, I. O. and Reis-Filho, J. S. (2011). Basal-like and triple negative breast cancers. A critical review with an emphasis on the implications for pathologists and oncologists. *Journal of Modern Pathology*, **24**, 157-167.

Baily, A. J. (2001). Molecular mechanism of ageing in connective tissues. *Mechanisms of Ageing and Development*, **122**, 735-755.

Basta, G. (2008). Receptor for advanced glycation endproducts and atherosclerosis: From basic mechanisms to clinical implications. *Atherosclerosis*, **196**, 9-21.

Band, V. and Sager, R. (1989). Distinctive traits of normal and tumor-derived human mammary epithelial cells expressed in a medium that supports long-term growth of both cell types. *National Academy of Sciences of the United States of America*, **86**, 1249-1253.

Basta, G., Schmidt, A. and Caterina, R. D. (2004). Advanced glycation endproducts and vascular inflammation: implication for accelerated atherosclerosis in diabetes. *Cardiovascular Research*, **63**, 582-592.

Baynes, J.W. and Thorpe, S. R. (1999). Role of oxidative stress in diabetic complications: a new perspective on an old paradigm. *Diabetes Journal,* **48**, 1-9.

Beghe, C. and Balducci, L. (2005). Biological basis of cancer in the older person. *Cancer Treatement and Research,* **124**, 189-221.

Belfiore, A. and Malaguarnera, R. (2011). Insulin receptor and cancer. *Endocrine- related Cancer*, **18**, 125-147.

Belcher, E. A. (1992). Cancer Nursing. Mosby's clinical nursing series, 13-14.

Bierhaus, A., Humpert, P. M., Morcos, M., Wendt, T., Chavakis, T., Arnold, B., Stern, D. M. and Nawroth, P. P. (2005). Understanding RAGE, the receptor for advanced glycation endproducts. *Journal Molecular Medicine,* **83**, 876-886.

Biessels, G. J., Van Der Heide, L. P., Kamal, A., Bleys, R. L. and Gispen, W. H.(2002). Ageing and diabetes: implications for brain function. *European Journal of Pharmacology*, **441**, 1-14.

Biglia, N., Defabiani, E., Ponzone, R., Mariani, L., Marenco, D. and Sismondi, P. (2004). Management of risk of breast carcinoma in postmenopausal women. *Endocrine-Related Cancer*, **11**, 69-83.

Blows, F. M., Driver, K. E., Schmidt, M. K., *et al.* (2010). Subtyping of breast cancer by immunohistochemistry to investigate a relationship between subtype and short and long term survival. A collaborative analysis of data for 10,159 cases from 12 studies. *PLoS Medicine,* **7**, 1-12.

Bousova, I., Vukasovic, D., Juretic, D., Palicka, V. and Drsata, J. (2005). Enzyme activity and AGE formation in a model of AST glycoxidation by D-fructose *in vitro. Acta Pharmaceutica,* **55**, 107-114.

Bresson, D and Herrath, M. V. (2004). Mechanisms underlying Type 1 diabetes. *Drug Discovery Today: Disease Mechanisms*, **3**, 321-327.

Brett, J., Schmidt, A. M., Yan, S. D., Zou, Y. S., Weidman, E., Pinsky, D., Nowygrod, R., Neeper, M., Przysiecki, C., Shaw, A. and Et Al. (1993). Survey of the distribution of a newly characterized receptor for advanced glycation endproducts in tissues. *American Journal of Pathology*, **143**, 1699-1712.

Brownlee, M. (2001). Biochemistry and molecular cell biology of diabetic complications. *Nature*, **44**, 813-820.

Brownlee, M. (2001). The pathology of diabetic complications. *American Diabetes Association,* **54**, 1615-1625.

Brownlee, M. (2004). The pathobiology of diabetic complications: A unifying mechanism. *Diabetes Journal,* **54**, 1615-1625.

Byrne, G. J., McDowell, G., Agarawal, R., Sinha, G., Kumar, S. and Bundred, N. J. (2007). Serum vascular endothelial growth factor in breast cancer. *Antiancer Research,* **27**, 3481-3488.

Cade, W. T. (2008). Diabetes-related microvascular and macrovascular disease in the physical therapy setting. *Physical Therapy*, **88**, 1322-1335.

Caine, J. G., Stonelake, S. P., Rea, D. and Lip, H. Y. G. (2003). Coagulopathic Complications in Breast Cancer. *American Cancer Society*, **98**, 1578- 1586.

Chhabra, A., Fernando, H., Watkins, G., Mansel, R.E. and Jiang, W.G. (2007). Expression of transcription factor CREB1 in human breast cancer and its correlation with prognosis. *Oncology Report,* **18**, 953-958.

Chibber, R., Molinatti, P. A., Rosatto, N., Lambourne, B. and Kohner, E. M. (1997). Toxic action of advanced glycation endproducts on cultured retinal capillary pericytes and endothelial cells: relevance to diabetic retinopathy. *Diabetologia*, **40**, 156-164.

Chuah, Y. K. Basir, R. Talib, H. Hing Tie, T. and Nordin, N.(2013). Receptor for advanced glycation endproducts and its involvement in inflammatory diseases. *International Journal of Inflammation*, **2013**, 1-15.

Chung, S. S. and Chung, S. K. (2005). Aldose reductase in diabetic microvascular complication. *Current Drug Targets*, **6**, 475-486.

Chung, S. S., M., Ho, E. C. M., Lam, K. S. L., and Chung, S. K. (2003). Contribution of polyol pathway to diabetes-induced oxidative stress. *Journal of the American Society of Nephrology*, **14**, S233-S236.

Chung, C. K., Zhang, H., Kong, Y. Z., Tan, J. J., Huang, X. R., Kopp, J. B. and Lan, H. Y. (2010). Advanced glycation endproducts induce tubular CTGF *via* TGF-β–independent smad3 signaling. *Journal of the American Society of Nephrology*, **21**, 249-260.

Chung, S. S., Aroh, C. and Vadgama, J. V. (2013).Constitutive activation of STAT3 signaling regulates hTERT and promotes stem cell-like traits in human breast cancer cells. *PLOS One*, **8**,1-10.

Comhair, S. A. A. and Erzurum, S. C. (2002). Antioxidant responses to oxidant-mediated lung diseases. *American Journal of Physiology-Lung Cellular and Molecular Physiology*, **283**, 246-255.

Coughlan, M. T., Cooper, M. E. and Forbes, J. M. (2005). Can advanced glycation end product inhibitors modulate more than one pathway to enhance renoprotection in diabetes? *Annals of the New York Academy of Sciences*, **1043**, 750-758.

Coughlan, M. T., Mibus, A.L. and Forbes, J. M.(2008). Oxidative stress and advanced glycation in diabetic nephropathy. *Annals of New York Academy of Sciences*, **1126**, 190-193.

Coughlin, S. S., Calle, E. E., Teras, L. R.., Petrelli, J. and Thun, M. J.(2004). Diabetes mellitus as a predictor of cancer mortality in a larg cohort of US adults. *American Journal of Epidemiology*, **12**, 1160-1167.

Dampier, K., Hudson, E.A., Howells, L. M., Manson, M. M., Walker, R. A. and Gescher, A. (2001). Differences between human breast cell lines in susceptibility towards growth inhibition by genistein. *British Journal of Cancer*, **4**, 618-624.

Das Evcimen, N. and King, G. L. (2007). The role of protein kinase C activation and the vascular complication of diabetes. *Pharmacological Research,* **55**, 498-510.

Dennis, M. D., Jefferson, L. S. and Kimball, R. S. (2012). Role of p70S6K1-mediated phosphorylation of eIF4B and PDCD4 Proteins in the regulation of protein synthesis. *Journal of Biological Chemistry,* **287**, 42890-42899.

Diabetes UK. (2012). Diabetes in the UK 2012 Key statistics on diabetes. 1-17.

Dickerson, T. J. and Janda, K. D. (2002). Aqueous aldol catalysis by a nicotine metabolite. *Journal of the American Chemical Society*, **124**, 3220-3231.

Dumitrescu, R. G. and Cotarla, I (2005). Understanding breast cancer risk- Where do we stand in 2005? *Journal of Cell Molecular Medicine,* **1**, 208-221.

Dyer, D. G., Blackledge, J. A., Katz, B. M., Hull, C. J., Adkisson, H. D., Thorpe, S. R., Lyons, T. J. and Baynes, J. W. (1991). The Maillard reaction *in vivo. Z Ernahrungswiss*, **30**, 29-45.

Fairweather, D. and Rose, N. R. (2007). Coxsackievirus-induced myocarditis in mice: a model of autoimmune disease for studying immunotoxicity. *National Institutes of Health*, **1**, 118-122.

Ferlay, J., Shin, H. R., Bray, F., Forman, D., Mathers, C. and Parkin, D. M. (2010). Estimates of worldwide burden of cancer in 2008: Globocan 2008. *International Journal of Cancer,* **127**, 2893-2917.

Folli, F., Corradi, D., Fanti, P., Davalli, A., Paez, A., Giaccari, A., Perego, C. and Muscogiuri, G. (2011). The role of oxidative stress in the pathogenesis of Type 2 diabetes mellitus micro- and macrovascular complications: Avenues for a mechanistic-based therapeutic approach. *Current Diabetes Review*, **7**, 313-324.

Forte, V., Pandey, A., Abdelmessih, R., Forte. G., Connell, W. A., Sowers, J. R. and McFarlane, S. I. (2012). Obesity, diabetes, the cardiorenal syndrome, and risk for cancer. *Cardio Renal Medicine*, **2**, 143-162.

Ford, C. H. J., Al-Bader, M., Al-Ayadhi, B. and Francis, I. (2011). Reassessment of oestrogen receptor expression in human breast cancer cell lines. *Anticancer Research*, **31**, 521-528.

Fowler, M. J (2008). Microvascular and macrovascular complications of diabetes. *Clinical Diabetes*, **26**, 77-82.

Filho, A. L., Lopes, J. M. and Schmitt, F.C. (2010). Angiogenesis and breast cancer. *Journal of Oncology*, 1-7.

Fulop, N., Marchase, R. B. and Chatham, J. C. (2007). Role of protein O-linked N-acetyl-glucosamine in mediating cell and survival in cardiovascular system. *Cardiovascular Research*, **73**, 288-297.

Furusyo, N. and Hayashi, J. (2013). Glycated albumin and diabetes mellitus. *Biochimaica et Biophysica Acta*, **1830**, 5509-5514.

Friedenreich, C. M. (2000). Review of anthropometric factors and breast cancer risk. *European Journal of Cancer Prevention*, **10,** 1532

Gella, A. and Durany, N. (2009). Oxidative stress in Alzheimer disease. *Cell Adhesion and Migration*, **3**, 88-93.

Gerrard, J. A. (2006). The Maillard reaction in food: Progress made, challenges ahead-Conference Report from the Eighth International Symposium on the Maillard Reaction. *Trends in Food Science and Technology*, **17**, 324-330.

Gest, C., Joimel, U., Huang, L., Pritchard, L.L., Petit, A., Dulong, C., Buquet, C., Hu, C. Q., Mirshahi, P., Laurent, M., Fauvel-Lafève, F., Cazin, L., Vannier, J. P., Lu, H., Soria, J., Li, H., Varin, R. and Soria, C. (2013). Rac3 induces a molecular pathway triggering breast cancer cell aggressiveness: differences in MDA-MB-231 and MCF-7 breast cancer cell lines. *BMC Cancer*, **13**, 1-14.

Giacco, F. and Brownlee, M. (2010). Oxidative stress and diabetic complications. *Circulation Research*, **107**, 1058-1070.

Giovannucci, E., Harlan, D. M., Bergenstal, R. M., Gapstur, S. M., Habel, L. A., Pollak, M., Regensteiner, J. G. and Yee, D. (2010). Diabetes and cancer. *Diabetes Care*, **33**, 1674-1685.

Goh, S.Y. and Cooper, M. E. (2008). The role of advanced glycation endproducts in progression and complications of diabetes. *Journal Clinical Endocrinology and Metabolism*, **93**, 1143-1152.

Goldberg, T., Cai, W., Peppa, M., Dardaine, V., Baliga, B. S., Uribarri, J. and Vlassara, H. (2004). Advanced glycoxidation endproducts in commonly consumed foods. *Journal of the American Dietetic Association*, **104**, 1287-1291.

Gown, A. M. (2009). Molecular vs. immunohistochemical classification of breast cancer. *Breast Cancer*, 28-30.

Griffiths, R. I., Danese, M. D., Gleeson, M. L. and Valderas, J. M. (2012). Epidemiology and outcomes of previously undiagnosed diabetes in older women with breast cancer: an observational cohort study based on SEER-Medicare. *BMC Cancer*, **12**, 1-14.

Grillo, M. A. and Colombatto, S. (2008). Advanced glycation endproducts (AGEs): involvement in aging and in neurodegenerative diseases. *Amino Acids*, **35**, 29-36.

Grillo, M. A. and Colombtto, S. (2007). Advanced glycation endproducts (AGEs): involving in aging and in neurodegenerative diseases. *Journal of Amino Acids*, **35**, 29-36.

Grossmann, M. and Wittert, G. (2012). Androgens, diabetes and prostate cancer. *Endocrine-Related Cancer*, **19**,F47–F62.

Grundy, S. M. (2012). Pre-diabetes, metabolic syndrome and cardiovascular risk. *Journal of the American College of Cardiology*, **7**, 635-643.

Gupta, C. and Tikoo, K. (2013). High glucose and insulin differentially modulates proliferation in MCF-7 and MDA-MB-231 cells. *Journal of Molecular Endocrinology,* **1**, 119-129.

Habib, S. l. and Rojna, M. (2013). Diabetes and risk of cancer. *ISRN Oncology,* **2013**, 1-16.

Hallett**,** M. A., Teng, B., Hasegawa, H., Schwab, L.P., Seagroves, T. N.and Pourmotabbed, T. (2013). Anti-matrix metalloproteinase-9 DNAzyme decreases tumor growth in the MMTV-PyMT mouse model of breast cancer. *Breast Cancer Research*, **15**, 1-12.

Halliwell, B. (1994). Free radicals, antioxidants, and human disease: Curiosity, cause, or consequence? *The Lancet*, **344**, 721-724.

Harris, M. I. (1998). Diabetes in America: epidemiology and scope of the problem. *Diabetes Care*, **21**, 11-14.

Hecht, S. S. (2002). Tabacco smoke carcinogens and breast cancer. *Environmental and Molecular Mutagenesis*, **39**,119-126.

Hernebring, M., Brolen, G., Aguilaniu, H., Sem, B. H., and Nystrom. (2006). Elimination of damage proteins during differentiation of embryonic stem cells. *Proceeding of the National Academy of Science of the United States of America*, **103**, 7700-7705.

Heer, K., Kumar, H., Read, G. R., Fox, J. N., Monson, J, R. T. and Kerin, M. J. (2001). Serum Vascular Endothelial Growth Factor in Breast Cancer: Its Relation with Cancer Type and Estrogen Receptor Status. *Clinical Cancer Research*, **7**, 3491–3494.

Hodge, L. E. (1953). Dehydrated food- chemistry of browing reaction in modle system. *Journal oF Agriculture, Food and Chemistry*, **1**, 928-943.

Hossain, P, Kawar, B. and EL, N. M. (2007). Obesity and diabetes in developing world- a growing challenge. *New England Journal of Medicine*, **356**, 213-215.

Hudson, B. and Schmidt, A.M. (2004). RAGE: a novel target for drug intervention in diabetic vascular disease. *Pharmaceutical Research*, **21**, 1079-1086.

Huijberts, M. S., Schaper, N. C. and Schalkwijk, C. G. (2008). Advanced glycation end products and diabetic foot disease. *Diabetes/Metabolism Research and Reviews*, **24**, 19-24.

Hausen, Z. H. (1991). Viruses in human cancers, *Science*, **254**, 1167-1173.

Hyogo, H. and Yamagishi, S. (2008). Advanced glycation endproducts (AGEs) and their involvement in liver disease. *Current Pharmaceutical Design*, **14**, 969-972.

Irigaray, P. Newby, A. J., Clapp, R., Hardell, L., Howard, V., Montagnier, L., Epstein, S. and Belpomme, D. (2007). Lifestyle-related factors and environmental agents causing cancer: *An overview Biomedicine and Pharmacotherapy*, **61**, Pages 640-658

Ignar-Trowbridge, D. M., Pimentel, M., Teng, C. T., Korach, K.S. and Mclachlan, J.A. (1995). Cross talk between peptide growth factor and estrogen receptor signaling systems. *Environmental Health Perspectives*, **7**, 35-38.

Indurthi, V. S. K., Leclerc, E. and Vetter, S.W. (2012). Interaction between glycated serum albumin and AGE-receptors depends on structural changes and the glycation reagent. *Archives of Biochemistry and Biophysics* .**528**, 185-196.

Ishibashi, Y., Matsui, T., Takeuchi, M. and Yamagishi, S. (2013). Metformin inhibits advanced glycation endproducts (AGEs)-induced growth and VEGF expression in MCF-7 breast cancer cells by suppressing AGEs receptor expression via AMP- activated protein kinase. *Hormone and Metabolic Journal*, **5**, 387-390.

Jakus, V. (2000). The role of free radicals, oxidative stress and antioxidant systems in diabetic vascular disease. *Bratisl Lek Listy*, **101**, 541-551.

Jandeleit-Dahm, K. and Cooper, M. E. (2008). The role of AGEs in cardiovascular disease. *Current Pharmaceutical Design*, **14**, 979-986.

Jiao, L., Taylor, Ph., R. Stephanie., J. Weinstein., Graubard, B. I., Virtamo, J., Albanes, D. and Stolzenberg-Solomon, R. Z. (2011). Advanced glycation endproducts, soluble receptor for advanced glycation endproducts, and risk of colorectal cancer. *Cancer Epidemiology, Biomarkers and Prevention*, **7**, 1430-1438.

Jing, R., Wang, H., Jiang, Sh.and Zhang, Z. (2008). Up-regulation of the receptor for advanced glycation endproduct (RAGE) in esophageal cancer and down- regulation in lung cancer and their relationship to clinicopathological features. *Science*, **39**, 661-667.

Joslin, E. P., Lombard, H. L., Burrows, R. E. and Manning, M. D.(1959). Diabetes and cancer. *New England Journal of Medicine*, **260**, 486-488.

Kalousova, M., Zima, T., Tesar, V, Dusilova-Sulkova, S. and Skrha, J. (2005). Advanced glycoxidation endproducts in chronic diseases-clinical chemistry and genetic background. *Mutation Research*, **579**, 37-46.

Karachalias, N., Babaei-Jadidi, R., Ahmed, N. and Thornalley, P. J. (2003). Accumulation of fructosyl-lysine and advanced glycation endproducts in the kidney, retina and peripheral nerve of streptozotocin-induced diabetic rats. *Biochemical Society Transactions*, **31**, 1423-1425.

Karmakar, P. S., Siliguri, Goswami, R. P and Kolkata. (2012). advanced glycation end products (AGEs): its role in the pathogenesis of diabetic complications. *Medicine Update*, **22**, 277-282.

Khan, M. Sh., Dwivedi, S., Priyadarshini, M., Tabrez, Sh., Siddiqui, M. A., Jagirdar, H., Al-Senaidy, A. M., Al-Khedhairy, A. A. and Musarrat, J. (2013). Ribosylation of bovine serum albumin induces ROS accumulation and cell death in cancer line (MCF-7). *European Biophysics Journal*, **42**, 811-818.

Khan, P., Drobic, B., Cadahı´a, P. E., Healy, Sh., He, Sh. and Davie, J. R.(2013). Mitogen- and stress-activated protein kinases 1 and 2 are required for maximal trefoil factor 1 induction. *PLOS One*, **8**, 1-11.

Kim, H.M., Kim, C. S., Lee, J.H., Jang, S.J., Hwang, J.J., Ro, S., Choi, J. (2013). CG0009, a novel glycogen synthase kinase 3 inhibitor, induces cell death through cyclin D1 depletion in breast cancer cells. *PLOS One*, **8**, e60383.

Kim, J. Y., Park, H. K., Yoon, J. S., Kim, S. J., Kim, E. S., Ahn, K. S., Kim, D-S., Yoon, S. S., Kim, B. K. and Lee, Y. Y. (2008). Advanced glycation endproduct (AGE)-induced proliferation of HEL cells via receptor for AGE-related signal pathways. *International Journal of Oncology*, ***33,*** 493-501.

Kirschmann, D. A., Seftor, E. A., Fong, S. F. T., Nieva, D. R. C., Sullivan, G. M., Edwards, E. M., Sommer, P., Gsiszar, K. and Hendrix, M .J. C. (2002). A molecular role for lysyl oxidase in breast cancer invasion. *American Association for Cancer Research Journal*, **62**, 4478-4483.

Kirwan, C. C, Byrne, J .G., Kuma,Sh. and McDowelI, G.(2009). Platelet release of Vascular Endothelial Growth Factor (VEGF) in patients undergoing chemotherapy for breast cancer. *Journal of Angiogenesis Research*,**1:7**, 1-9.

Klein, G., Vellenga, E., Fraaije, M. W., Kamps, W. A. and de Bont, E. S. J. M. (2004). The possible role of matrix metalloproteinase (MMP)-2 and MMP-9 in cancer, e.g. acute leukemia. *Critical Review in Oncology Haematology*, **50**, 87-100.

Ko, J. C., Tsai, M. S., Chiu, Y. F., Weng, S. H., Kuo, Y. H. and Lin, Y.W. (2011). Up-regulation of extracellular signal-regulated kinase 1/2-dependent thymidylate synthase and thymidine phosphorylase contributes to cisplatin resistance in human non-small-cell lung cancer cells. *Journal Pharmacology Experimental Therapeutics*, **338**, 184-194.

Korwar, A. M., Bhonsle, H. S., Chougale, A. D., Kote, S. S., Gawai, K. R., Ghole, V. S., Koppikar**,** C. B. and Kulkarni, M. J. (2012). Analysis of AGE modified proteins and RAGE expression in HER2/neu negative invasive ductal carcinoma. *Biochemical and Biophysical Research Communications*, **19**, 490-494.

Kuniyasu, H., Oue, N., Wakikawa, A., Shigeshi, H., Matsutani, N., Kuraoka, K., Ito, R., Yokozaki, H. and Yasui, W. (2002). Expression of receptors for advanced glycation end-products [RAGE] is closly associated with the invasive and metastatic activity of gastric cancer. *The Journal of Pathology*, **196**, 163-170.

Kuzuya, T., Nakagawa, S., Satoh, J., Kanazawa, Y., Iwamoto, Y., Kobayashi, M., Nanjo, K., Sasaki, A., Seino, Y., Ito, C., Shima, K., Nonaka, K. and Kadowki, T. (2002). Report of the committee on the classification and diagnostic criteria of diabetes mellitus. *Diabetes Research and Clinical Practice,* **55**, 65-85.

Laakso, M. (1999). Hyperglycaemia and cardiovascular disease in type 2 diabetes. *Diabetes*, **48**, 937-942.

Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, **227**, 680-685.

Lander, H. H., Tauras, J. M., Ogiste, J. S., Hori, O., Moss, R. A. and Schmidt, A. M. (1997). Activation of the receptor for advanced glycation endproducts trigger a p21 ras-dependent mitogen-activated protein kinase pathway regulated by oxidant stress. *Journal of Biological Chemistry*, **272**, 17810-17814.

Lata, K. and Mukherjee, T. K. (2014). Knockdown of receptor for advanced glycation endproducts attenuate 17α-ethinyl-estradiol dependent proliferation and survival of MCF-7 breast cancer cells. *Biochimica et Biophysica Acta,* **1840**, 1083-1091.

Lee, L, W., Huang, Y. J., and Lie-Fen Shyur, F.L. (2013). Phytoagents for cancer management: regulation of nucleic acid oxidation, ROS, and related mechanisms. *Oxidative Medicine and Cellular Longevity* **1**, 1-22.

Liao, S., Wel, W., Wang, L., Zhang, Y., Li, J., Wang, C. and Sun, S. (2011). Association between diabetes mellitus and breast cancer risk: a meta-analysis of the literature. *Asian Pacific Journal of Cancer prevention,* **12**, 1061-1065.

Linton, S., Davies, M. J. and Dean, R. T. (2001). Protein oxidation and ageing. *Experimental Gerontology,* **36**, 1503-1518.

Lo, T. Westwood, M. E., McLellan, A. C., Selwood, T. and Thornalley, P. J. (1994). Binding and modification of proteins by methylglyoxal under physiological conditions. *American Journal of Biochemistry and Molecular Biology,* **269**, 32299-32305.

Logsdon, C. D., Fuentes, M. K., Huang, E. H. and Arumugam, T. (2007). RAGE and RAGE ligands in cancer. *Current Molecular Medicine,* **7**, 777-789.

Lv, L., Shao, X., Chen, H., Ho, Ch. and Sang, Sh. (2011). Genistein inhibits advanced glycation endproduct formation by trapping methylglyoxal. *Chemical Research in Toxicology*, **24**, 579-586.

Ma, W., Lee, S. E., Guo, J., Qu, W., Hudson, B. I., Schmidt, A. M. and Barile, G. R. (2007). RAGE ligand upregulation of VEGF secretion in ARPE-19 cells. *Investigative Ophthalmology and Visual Science,* **48**, 1355-1361.

Magalhães, P., Appell, H. and Duarte, J. (2008). Involvement of advanced endglycation products in the pathogenesis of diabetic complications: the protective role of regular physical activity. *European Review of Aging and Physical Activity*, **5**, 17-29.

Maillard, L. C. (1912) Action of amino acids on sugars. Formation of melanoidins in a methodical way. *Compte Rendu du roi*, **154**, 66-68.

Maraschin, J. De. F. (2012). Classification of diabetes. *Bioscience and Springer,* **2**, 12-19.

Maritim, A. C. Sanders, R. A. and Watkins, J. B.(2003a). Diabetes, oxidative stress, and antioxidants: A Review. *Journal of Biochemical and Molecular Toxicology*, **17**, 24-38.

Maritim, A. C., Sanders, R. A. and Watkins, J. B., 3rd (2003b) Effects of alpha-lipoic acid on biomarkers of oxidative stress in streptozotocin-induced diabetic rats. *Journal of Nutritional Biochemistry,* **14**, 288-294.

Marsche, G., Weigle, B., Sattler, W. and Malle, E. (2007). Soluble RAGE blocks scavenger receptor CD36-mediated uptake of hypochlorite-modified low-density lipoprotein. *FASEB Journal*, **21**, 3075-3082.

Martin, T. A., Ye, L., Sanders, A. J., Lane, J. and Jiang, W. G. (2000). Cancer invasion and metastasis: molecular and cellular perspective. *Madame Curie Bioscience Database [Internet]*. *Austin (TX): Landes Bioscience.*

Mashilipa, C., Wang, Q., Slevin, M. and Ahmed, N. (2011). Antiglycation and antioxidant properties of soy souces. *Journal of Medicinal Food,* **14**, 1647-1653.

Meng, M. Zhao, H. X., Ning, Q., Hqu, L., Xin, H. G., and Liu, F. L.(2012). Tumor stem cells: A new approach for tumor therapy. *Oncology Letters*. **4**, 187-193.

McPherson, K., Steel, G. M. and Dixon. (2000). Breast cancer-epdemiology, risk factors, and genetics. *ABC of Breast Diseases*, **321**, 624-628.

McTiernan, A. (2003). Behavioural risk factor in breast cancer: Can risk be modified. *The Oncologist*, **8**, 326-334.

Michels, K. B., Solomon, C. G., Rosner, B. A., Hankinson, S.E., Colditz, G. A., Manson, J. E. (2003). Type 2 diabetes and subsequent incidence of breast cancer in the Nurses' Health study. *Diabetes Care*, **26**, 1752-1758.

Miller, T. W., Balko, J. M. and Arteage, G. L. (2011). Phosphatidylinositol 3-Kinase and antiestrogen resistance in breast cancer. *Journal of Clinical Oncology*, **29**, 4452-4461.

Mink, p. j., Shahar, E., Rosamond, W. D., Alberg, A. J and R, A. (2002). Serum insulin and glucose level and breast cancer incidence. *American Journal of Epidemiology,* **4**, 349-353.

Mironova, R., Niwa, T., Hayashi, H., Dimitrova, R. and Ivanov, I. (2001). Evidence for non-enzymatic glycosylation in *Escherichia coli*. *Molecular Microbiology,* **39**, 1061-1068.

Mladkova, J., Sanda, M., Matouskova, E. and Selicharova, I. (2010). Phenotyping breast cancer cell lines EM-G3, HCC1937, MCF7 and MDA-MB-231 using 2-D electrophoresis and affinity chromatography for glutathione-binding proteins. *Academic Journal,* **10**, 1-10.

Monnier, V. M., Stevens, V. J. and Cerami, A. (1981). Maillard reactions involving proteins and carbohydrates *in vivo*: relevance to diabetes mellitus and aging. *Progress in Food and Nutrition Science*, **5**, 315-327.

Moy, K. A., Jiao, L., Freedman, N. D., Weinstein, S. J., Sinha, R., Virtamo, J., Albanes, D. and Stolzenberg-Solomon, R. Z. (2013). Soluble receptor for advanced glycation end products and risk of liver cancer. *The American Association for the Study of Liver Disease*, **57**, 2338-2345.

Nagai, R., Jinno, M., Ichihashi, M., Koyama, H. and Yamamoto, Y. (2012). Advanced glycation end products and their receptors as risk factors for aging. *Anti-Aging Medicine,* **4**, 108-113.

National Diabetes 2011. Diagnosed and undiagnosed diabetes in the United States, all ages, *Centres of Disease Control and prevention*.

National Diabetes Statistics (2011) http://diabetes.niddk.nih.gov/dm/pubs/statistics/.

Neeper, M., Schmidt, A. M., Brett, J., Yan, S. D., Wang, F., Pan, Y. C., Elliston, K., Stern, D. and Shaw, A. (1992). Cloning and expression of a cell surface receptor for advanced glycosylation endproducts of proteins. *Journal of Biological Chemistry*, **267**, 14998-15004.

Negrean, M., Stirban, A., Stratmann, B., Gawlowski, T., Horstmann, T., Gotting, C., Kleesiek, K., Mueller-Roesel, M., Koschinsky, T., Uribarri, J., Vlassara, H. and Tschoepe, D. (2007). Effects of low- and high-advanced glycation endproduct meals on macro- and microvascular endothelial function and oxidative stress in patients with type 2 diabetes mellitus. *American Journal of Clinical Nutrition*, **85**, 1236-1243.

Nicholl, I. D., Stitt, A. W., Moore, J. E., Ritchie, A. J., Archer, D. B. and Bucala, R. (1998). Increased levels of advanced glycation endproducts in the lenses and blood vessels of cigarette smokers. *Molecular Medicine*, **4**, 594-601.

Nishikawa, T., Edelstein, D. and Brownlee, M. (2000). The missing link: A single unifying mechanism for diabetic complications. *Kidney international*, **58**, S26-S30.

Noh, S., Jung, J. J., Jung, M., Kim, K. H., Lee, H.Y., Wang, B., Cho, J., Kim, T. S., Jeung, H. C. and Rha, S. Y. (2012). Body fluid MMP-2 as a putative biomarker in metastatic breast cancer. *Oncology Letters*, **3**, 699-703.

Notkins, A. L. and Lernmark, A. (2001). Autoimmune Type 1 diabetes: resolved and unresolved issues. *Journal of Clinical Investigation*, **108**, 1247-1252.

Ohgami, N., Nagai, R., Ikemoto, M., Arai, H., Miyazaki, A., Hakamata, H., Horiuchi, S. and Nakayama, H. (2002). CD36, serves as a receptor for advanced glycation endproducts (AGE). *Journal of Diabetes and its Complications*, **16**, 56-59.

O'sullivan, E. P. and Dinneen, S. F. (2009). Benefits of early intensive glucose control to prevent diabetes complications were sustained for up to 10 years. *Evidence-Based Medicine*, **14**, 9-10.

Pandolfi, A. and De Filippis, E. A. (2007). Chronic hyperglicemia and nitric oxide bioavailability play a pivotal role in pro-atherogenic vascular modifications. G*enes & Nutrition*, **2**, 195-208.

Papa, V. and Belfiore, A. (1996). Insulin receptors in breast cancer: biological and clinical role. *Journal of Endocrinological Investigation*, **19**, 324-333.

Parkin, Ch. G. and Davidson, J. A. (2009). Value of self- monitoring blood glucose pattern analysis in improving diabetes outcomes. *Journal of Diabetes Science and Technology*, **3**, 500-508.

Peppa, M. and Vlassara, H. (2005). Advanced glycation endproducts and diabetic complications: a general overview. *Hormones (Athens)*, **4**, 28-37.

Peppa, M., Brem, H., Ehrlich, P., Zhang, J. G., Cai, W., Li, Z., Croitoru, A., Thung, S. and Vlassara, H. (2003). Adverse effects of dietary glycotoxins on wound healing in genetically diabetic mice. *Diabetes*, **52**, 2805-2813.

Posner, R.G., Bold, J., Bernstein, Y., Rasor, J., Braslow, J., Hlavacek, W.S. and Perelson, A.S. (1998). Measurement of receptor crosslinking at the cell surface via multiparameter flow cytometry. *Proceeding of the Royal Society*, **3256**, 132-143.

Radia, A. M., Yaser, A. M., Ma, X., Zhang, J., Yang, C., Dong, Q., Rong, P., Ye, B., Liu, S. and Wang. W. (2013). Specific siRNA targeting receptor for advanced glycation end products (RAGE) decreases proliferation in human breast cancer cell lines. *Molecular International Journal of Science*, **14**, 7959-7978.

Rahman, S., Rahman, T., Ismail, A. A. and Rashid, A. R. (2007). Diabetes-associated macrovasculopathy: pathophysiology and pathogenesis *Diabetes, Obesity and Metabolism*, **9**, 767-780.

Ranaivo, H. R., Hodge, J. N., Choi, N. and Wainwright, M. S. (2012). Albumin induces upregulation of matrix metalloproteinase-9 in astrocytes via MAPK and reactive oxygen species-dependent pathways. *Journal of Neuroinflammation*, **9**, 1-12.

Ramasamy, R., Vannucci, S. J., Yan, S.S., Herold, K., Yan, S. F. and Schmidt, A. M. (2005). Advanced glycation endproducts and RAGE: a common thread in aging, diabetes, neurodegeneration, and inflammation. *Glycobiology*, **15**, 16R-28R.

Rapp, K., Schroeder, J., Klenk, J., Ulmer, H., Concin, H., Diem, G., Oberaigner, W. and Weiland, S. K.(2006). Fasting blood glucose and cancer risk in cohort of more than 140,000 adults in Austria. *Diabetologia*, **49**, 945-952.

Rasheed, Z., Aktar, N. and Haqqi, T. M. (2011). Advanced glycation endproducts induce the expression of interleukin-6 and interleukin-8 by receptor for advanced glycation end product-mediated activation of mitogen activated protein kinases and nuclear factor-κB in human osteoarthritis chondrocytes. *Rheumatology*, **50**, 838-851.

Rayfield, E. J.and Aronson, D. (2002). How hyperglycaemia promotes atherosclerosis: molecular mechanism. *Cardiovascular Diabetology*, **1**, 1-10.

Riehl, A., Németh, J., Angel, P. and Hess, J. (2009). The receptor RAGE: bridging inflammation and cancer. *Cell Communication Signaling journal*, **12**, 1-7

Rojas, A., Gonzalez, I., Morales, E., Perez-Castro, R., Romero, J. and Figueroa, H. (2011). Diabetes and cancer: looking at the multiligand/RAGE axis. *World Journal of Diabetes*, **7**, 108-113.

Rolo, A. P. and Palmeira, C. M. (2006). Diabetes and mitochondrial function: role of hyperglycaemia and oxidative stress. *Toxicology and Applied Pharmacology*, **212**, 167-178.

Roy, R., Chun, J. and Powell, S. N. (2012). BRCA1and BRCA2: different roles in a common pathway of genome protection. *Nature Review Cancer*, **12**, 68-78.

Salman, A. G., Mansour, D. E. A. A., Swelem, A.-H.A., AL-Zawahary, W.M.A.-R. and Radwan, A. A. (2009). Pentosidine-A new biochemical maraker in diabetic retinopathy. *Ophthalmic Research,* **42**, 96-98.

Samarajeewa, N. U., Docanto, M. M., Simpson, E. R. and Brown, K. A. (2013). CREB-regulated transcription co-ordinator family stimulate promoter II-driven aromatase expression in preadipocytes. *Hormones and Cancer,* **4**, 233-241.

Sato, T., Neilson, L. M., Peck, A. R., Liu, C., Tran, T. H., Witkiewicz, A., Hyslop, T., Nevalainen, M. T., Sauter, G. and Rui, H. (2011). Signal transducer and activator of transcription-3 and breast cancer prognosis. *American Journal of Cancer Research,* **1**, 347-355.

Schleicher, E. D., Bierhaus, A., Haring, H. U., Nawroth, P. P. and Lehmann, R. (2001). Chemistry and pathobiology of advanced glycation endproducts. *Contributions to Nephrology,* **131**, 1-9.

Schmidt, A. M., Vianna, M., Gerlach, M., Brett, J., Ryan, J., Kao, J., Esposito, C., Hegarty, H., Hurley, W., Clauss, M. and ET, Al. (1992). Isolation and characterization of two binding proteins for advanced glycosylation endproducts from bovine lung which are present on the endothelial cell surface. *Journal of Biological Chemistry*, **267**, 14987-14997.

Schnitt, S, S. (2010). Classification and prognosis of invasive breast cancer: from morphology to molecular taxonomy. *Modern Pathology*, **23**, S60-S64.

Sebeková, K., Podracká, L., Blazícek, P., Syrová, D., Heidland, A. and Schinzel, R. (2001). Plasma levels of advanced glycation endproducts in children with renal disease. *Paediatric Nephrology Journal,* **16**, 1105-1112.

Semba, R.D., Nicklett, E, J. and Ferrucci, L. (2010). Does accumulation of advanced glycation endproducts contribute to the aging phenotype. *Journal of Gerontology,* **9**, 963-975.

Sharma, S. D., Pandey, B. N., Mishra, K. P. and Sivakami, S. (2002). Amadori product and AGE formation during nonenzymatic glycosylation of bovine serum albumin *in vitro*. *Journal of Biochemistry, Molecular Biology and Biophysics*, **6**, 233-242.

Shi, L., Yu, X., Yang, H. and Wu, X. (2013). Advanced glycation endproducts induce human corneal epithelial cells apoptosis through generation of reactive oxygen species and activation of JNK and P38 MAPK. *Public Library of Science One*, **8**, 1-10.

Shinohara, M., Thornalley, P. J., Giardino, I., Beisswenger, P., Thorpe, S. R., Onorato, J. and Brownlee, M. (1998). Overexpression of glyoxalase-I in bovine endothelial cells inhibits intracellular advanced glycation endproduct formation and prevents hyperglycaemia -induced increases in macromolecular endocytosis. *Journal of Clinical Investigation*, **101**, 1142-1147.

Shipanova, I. N., Glomb, M. A. and Nagaraj, R. H. (1997). Protein modification by methylglyoxal: chemical nature and synthetic mechanism of a major fluorescent adduct. *Archives of Biochemistry and Biophysics*, **344**, 29-36.

Shwa, J. E., Sicree, R. A. and Zimmet, P. Z. (2010). Global estimates of the prevalence of diabetes for 2010 and 2030. *Diabetes Research and Clinical Practice*, **87**, 4-14.

Singer, C. F., Kronsteiner, N., Marton, E., Kubista, M., Cullen, K. J., Hirtenlehner, K., Seifert, M. and Kubista, E. (2002). MMP-2 and MMP-9 expression in breast cancer-derived human fibroblasts is differentially regulated by stromal-epithelial interactions. *Breast Cancer Research Treatment*, **72**, 69-77.

Singh, R. Barden., A. Mori, T. and Beilin, L. (2001). Advanced glycation endproducts: a review. *Diabetologia*, **44**, 129-146.

Singh, R. P., Sharad, Sh. and Kapur, S. (2004). Free radicals and oxidative stress in neurodegenerative diseases: Relevance of dietary antioxidants. *Journal Indian Academy of Clinical Medicine*, **5**, 218-225.

Sinha, P. Okoro., C. Foell, D. Freeze, HH, Ostrand-Rosenber, S. and Sikrishna, G. (2008) Proinflammatory S100 proteins regulate the accumulation of myeloid-drived suppressor cells. *Journal of Immunology*, **181**, 4666-4675.

Smit, A. J. and Lutgers, H. L. (2004). The clinical relevance of advanced glycation endproducts (AGE) and recent developments in pharmaceutics to reduce AGE accumulation. *Current Medicinal Chemistry*, **11**, 2767-2784.

Son, J. 1., Lee, J. H., Kim, H. N., Ha, H. and Lee, Z. H. (2010). cAMP-response-element-binding protein positively regulates breast cancer metastasis and subsequent bone destruction. *Biochemical Biophysological Research Communication*s, **398**, 309-14.

Sparvero, L. J., Asafu-Adjei, D., Kang, R., Tang, D., Amin, N., Im, J., Rutledge, R., Lin, B., Amoscato, A. A., Zeh, H. J. and Lotze, M. T. (2009). RAGE (Receptor for Advanced Glycation Endproducts), RAGE ligands, and their role in cancer and inflammation. *Journal of Translational Medicine*, **7**, 1-21.

Srinivasan, V. (2012). Stress hyperglycaemia in pediatric critical illness: the intensive care unit adds to the stress. *Journal of Diabetes Science and Technology*, **1**, 37-47.

Srivastava, A. K. (2002). High glucose-induced activation of protein kinase signalling pathways in vascular smooth muscle cells: A potential role in the pathogenesis of vascular dysfunction in diabetes. *International Journal of Molecular Medicine*, **9**, 85-89.

Statin, P., Bjor, O., Ferrari, P., Lukanov, A., Lenner, P., Lindahl, B., Hallmans, G. and Kaaks, R. (2007). Prospective study of hyperglycaemia and cancer risk. *Diabetes Care*, **3**, 561-567.

Stitt, A. W. and Curtis, T. M. (2005). Advanced glycation and retinal pathology during diabetes. *Pharmacological Reports*, **57**, 156-168.

Sugimoto, K., Yasujima, M. and Yagihashi, S. (2008). Role of advanced glycation endproducts in diabetic neuropathy. *Current Pharmaceutical Design*, **14**, 953-961.

Sukhtankar, D., Okun, A., Chandramouli, A., Nelson, M. A., Vanderah, T. W., Cress, A. E., Porreca, F. and King, T. (2011). Inhibition of p38-MAPK signaling pathway attenuates breast cancer induced bone pain and disease progression in a murine model of cancer-induced bone pain. *Molecular Pain*, **7**, 1-15.

Taherian, A. and Mazoochi, T. (2012). Different expression of extracellular signal-regulated kinases (ERK) 1/2 and phospho-ERK proteins in MDA-MB-231 and MCF-7 cells after chemotherapy with doxorubicin or docetaxel. *Iranian Journal of Basic Medical Science*, **15**, 669-677.

Takada, M., Hirata, K., Ajiki, T., Suzuki, Y. and Kuroda, Y. (2004). Expression of receptor for advanced glycation endproducts (RAGE) and MMP-9 in human pancreatic cancer cells. *Hepatogastroenterology*, **51**, 928-930.

Takino, J., Yamagishi, S. and Takeuchi, M. (2010). Cancer malignancy is enhanced by glyceraldehyde-derived advanced glycation endproducts. *Journal of Oncology*, **2010**, 1-8.

Tan, A. l. Y., Forbes, J. M. and Mark E. Cooper, M. E. (2007). AGE, RAGE, and ROS in diabetic nephropathy. *Nephropathy Journal*, **27**, 130-143.

Tan, K. C., Chow, W. S., Ai, V. H., Metz, C., Bucala, R. and Lam, K. S. (2002). Advanced glycation endproducts and endothelial dysfunction in type 2 diabetes. *Diabetes Care*, **25**, 1055-1059.

Tanaka, N., Yonekura, H., Yamagishi, S., Fujimori, H., Yamamoto, Y. and Yamamoto, H. (2000). The receptor for advanced alycation endproducts is induced by the glycation products themselves and tumor necrosis factor-α through nuclear factor-κB, and by 17β-estradiol through Sp-1 in human vascular endothelial cells. *Journal of Biological Chemistry*, **275**, 25781-25790.

Tesarová, P., Kalousová, M., Jáchymová, M., Mestek, O., Petruzelka, L. and Zima, T. (2007). Receptor for advanced glycation endproducts (RAGE)-soluble form (sRAGE) and gene polymorphisms in patients with breast cancer. *Cancer Investigation*, **25**, 720-725.

Thompson, E. W., Paik, S., Brünner, N., Sommers, C. L., Zugmaier, G., Clarke, R., Shima, T. B., Torri, J., Donahue, S. and Lippman, M. E. (1992). Association of increased basement membrane invasiveness with absence of estrogen receptor and expression of vimentin in human breast cancer cell lines. *Journal of Cell Physiology*, **3**, 534-544.

Thornalley, P. J. (2008). Protein and nucleotide damage by glyoxal and methylglyoxal in physiological system-role in ageing and disease. *Drug Metabolism and Drug Interactions*, **23**, 125-150.

Thornalley, P. J. (1999). The clinical significance of glycation. *Clinical Laboratory*, **45**, 263-273.

Thornalley, P. J. (2005) Dicarbonyl intermediates in the Maillard reaction. *Annals of the New York Academy of Sciences,* **1043**, 111-117.

Thorne, C. and Lee, A. V. (2003). Cross talk between estrogen receptor and IGF signalling in normal mammary gland development and breast cancer. *Breast Disease*, 17, 105-117.

Tomlinson, D. C., Knowles, M. A. and Speirs, V. (2012). Mechanisms of FGFR3 actions in endocrine resistant breast cancer. *International Journal of Cancer,* **130**, 2857-2866.

Tuttle, K. R., Anderberg, R. J., Cooney, S. K. and Meek, R. l. (2009). Oxidative stress mediates PKC activation and advanced glycation endproduct formation in a mesangial cell model of diabetes and high protein diet. *American Journal of Nephrology*, **29**, 171-180.

Uchida, K., Khor, O. Th., Oya, T., Osawa, T., Yasuda, Y. and Miyata, T. (1997). Protein modification by a Maillard reaction intermediate methylglyoxal immunochemical detection of fluorescent 5-methylimidazolone derivatives in *vivo*. *Federation of European Biochemical Societies*, **410**, 313-318.

Uehara, N., Kanematsu, S., Miki, H., Yoshizawa, K. and Tsubura, A. (2012). Requirement of p38 MAPK for a cell-death pathway triggered by vorinostat in MDA-MB-231 human breast cancer cells. *Cancer Letters*, **315**, 112-121.

Urtreger, A. J., Kazanietz, M. G., and Bal, E. D. (2012). Contribution of individual PKC isoforms to breast cancer progression. *IUBMB Life Journal*, **64**, 18-26.

Vajdic, C. M., McDonald, S. P., McCredie, M. R. E., van Leeuwen, M. T., Stewart, J. H., Law, M., Chapman, J. R., Webster, A. C., Kaldor, J. M. and Grulich, A. E. (2006). Cancer incidence before and after kidney transplantation. *Journal of American Medical Association*, **296**, 2823-2831.

Valko, M., Leibfritz, D., Moncol, J., Cronin, M. T., Mazur, M. and Telser, J. (2007). Free radicals and antioxidants in normal physiological functions and human disease. *The International Journal of Biochemistry and Cell Biology*, **39**, 44-84.

van Heijst, J. W., Niessen, H. W., Hoekman, K. and Schalkwijk, A. C. (2005). Advanced glycation endproducts in human cancer tissues: Detection of $N^{€}$-(Carboxymethyl) lysine and argpyrimidine. *Annals of the New York Academy of Sciences*, **1043**, 725-733.

Vander, J., D. L. and Hunsaker, L. A. (2003). Methylglyoxal metabolism and diabetic complications: roles of aldose reductase, glyoxalase-1, betaine aldehyde dehydrogenase and 2-oxoaldehyde dehydrogenase. *Chemico-Biological Interaction*, **143**, 341-351.

Vigneri, P., Frasca, F., Sciacca, L., Pandini, G. and Vigneri, R. (2009). Diabetes and cancer. *Endocrine-Related Cancer*, **16**, 1103-1123.

Vladusic, E. A., Hornby, A. E., Guerra-Vladusic, F. K., Lakins, J. and Lupu, R. (2000) .Expression and regulation of estrogen receptor beta in human breast tumors and cell lines. *Oncolgy Reports*, **7**, 157-167.

Vlassaa, H. Striker, L. J., Teichberg, S., Fuh, H. and LI, Y. M. (1994) Advanced glycosylation endproducts induce sclerosis and albuminuria in normal rats. *Medical Science*, **91**, 11704-11708.

Vlassara, H. and Palace, M. R. (2002). Diabetes and advanced glycation endproducts. *Journal of Internal Medicine*, **251**, 87-101.

Vlassara, H., Uribarri, J., Cai, W. and Striker, G. (2008). Advanced glycation endproduct homeostasis: exogenous oxidants and innate defenses. *Annals of the New York Academy of Sciences,* **1126**, 46-52.

Wada, R. and Yagihashi, S. (2005). Role of advanced glycation endproducts and their receptors in development of diabetic neuropathy. *Annals of the New York Academy of Sciences,* **1043**, 598-604.

Wang, X. F., Zhou, Q. M., Du, J., Zhang, H., Lu, Y. Y. and Su, S. B. (2013). Baicalin suppresses migration, invasion and metastasis of breast cancer via p38MAPK signaling pathway. *Anti-Cancer Agent in Medical Chemistry,* **13**, 923-931.

Warburg, O. (1956). On the origin of cancer. *Science,* **123**, 309-314.

Wautier, J. L. and Guillausseau, P. J. (2001). Advanced glycation endproducts, their receptors and diabetic angiopathy. *Diabetes and Metabolism*, **27**, 535-542.

Wolf, G. (2004). New insights into the pathophysiology of diabetic nephropathy: from haemodynamics to molecular pathology. *European Journal of Clinical Investigation,* **34**, 785-796.

Wolf, I. and Rubinek, T. (2008). Diabetes mellitus and breast cancer. *Epidemiological Evidence and Molecular Link,* **19**, 97-113.

Xu, D., Young, J. H., Krahn, J. M., Song, D., Corbett, K. D., Chazin, W. J., Pedersen, L. C. and Esko, J. D. (2013). Stable RAGE-heparan sulfate complexes are essential for signal transduction. *Biology Journal ACS Chemical*, **8**, 1611-1620.

Xu, Q., Liu, L. Z., Qian, X., Chen, Q., Jiang, Y., Li, D., Lai, L. and Jiang, B. H. (2012). MiR-145 directly targets p70S6K1 in cancer cells to inhibit tumor growth and angiogenesis. *Nucleic Acids Research,* **40**, 761-774.

Xue, F. and Michels, K.B. (2007). Diabetes, metabolic syndrome, and breast cancer: A review of the current evidence1′2′ 3′ 4′. *The American Journal of Clinical Nutrition,* **86**, 8235-8355.,

Xue, J., Rai, V., Singer, D., Chabierski, S., Xie, J., Reverdatto, S., Burz, D.S., Schmidt, A. M., Hoffmann, R. and Shekhtman, A. (2011). Advanced glycation endproduct recognition by the receptor for AGEs. *Structure Article,* **19**, 722-732.

Yamagishi, S., Nakamura, K. and Imaizumi, T. (2005). Advanced glycation endproducts (AGEs) and diabetic vascular complication. *Current Diabetes Reviews*, **1**, 93-106.

Yan, S. D., Schmidt, A.M., Anderson, G.M., Zhang, J., Brett, J. and Zou, Y. S. (1994). Enhanced cellular oxidative stress by the interaction of advanced glycation endproducts with their receptors/binding proteins. *Journal of Biological Chemistry,* **269**, 9889-9897.

Yang, Yu., Hennessy, S. and Lewis, J. D. (2005). Type 2 Diabetes mellitus and the risk of colorectal cancer. *Clinical Gastroenterology and Hepatology*, **3**, 587-594.

Ye, R. D. (2001). Regulation of nuclear factor κB activation by G-protein-coupled receptors. *Journal of Leukocyte Biology*, **70**, 839-848.

Ye, Y., Qiu, T. H., Kavanaugh, C. and Green, J. E. (2004). Molecular mechanism of breast cancer progression: Lessons from mouse mammary cancer models and gene expression profiling. *Journal of Breast Cancer*, **19**, 69-82.

Yim, M. B., Yim, H. S., Lee, C., Kang, S. O. and Chock, P. B. (2001). Protein glycation: creation of catalytic sites for free radical generation. *Annals of the New York Academy of Sciences,* **928**, 48-53.

Yu, L., Zhao, Y., Xu, S., Ding, F., Jin, C., Fu, G. and Weng, S. (2013). Advanced glycation endproduct (AGE)–AGE receptor (RAGE) system upregulated connexin43 expression in rat cardiomyocytes via PKC and Erk MAPK pathways. *International Journal of Molecular Sciences*, **14**, 2242-2257.

Zhang, F., Banker, G., Liu, X., Suwanabol, P. A., Lengfeld, J., Yamanouchi, D., Kent, K. C. and Liu, B. (2011). The novel function of advanced glycation endproducts in regulation of MMP-9 production. *Journal of Surgical Research*, **171**, 871-876.

Zhang, W., Liu, H., Al-shabrawey, M., Caldwell, R. W. and Caldwell, R. B. (2011). Inflammation and diabetic retinal microvascular complications. *Journal of Cardiovascular Disease Research*, **2**, 96-103.

Zhou, Q., Liu, L. Z., Fu, B., Hu, X., Shi, X., Fang, J. and Jiang, B. H. (2007). Reactive oxygen species regulate insulin-induced VEGF and HIF-1alpha expression through the activation of p70S6K1 in human prostate cancer cells.*Carcinogenesis,* **28**, 28-37.

Zijl, F. V., Krupitza, G and Mikulits, W. (2011). Initial steps of metastasis: Cell invasion and endothelial transmigration. *Mutation Research,* **728**, 23-34.