ANGLIA RUSKIN UNIVERSITY

FACULTY OF SCIENCE AND ENGINEERING

THE IMPACT OF ARTIFICIAL SWEETENERS ON VEGF-INDUCED PERMEABILITY IN GLOMERULAR MICROVASCULAR ENDOTHELIUM

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A thesis in partial fulfilment of the requirements of
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DECLARATION

I HEREBY DECLARE THAT THIS WORK IS MY ORIGINAL RESEARCH EXCEPT

WHERE REFERENCES AND ACKNOWLEDGEMENTS ARE MADE.

DEDICATION

This work is dedicated to my beloved mum, Ms Eugenia Onyeaghala for her passion in education and sacrifices she made in ensuring everyone around her view education as the key to unlocking doors of opportunities.

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ABSTRACT

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THE IMPACT OF ARTIFICIAL SWEETENERS ON VEGF-INDUCED PERMEABILITY OF

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Increasing economic and personal cost of diabetic kidney disease (DKD) globally, highlights the importance of developing alternative treatment strategies for the disease, since there are very limited treatment approaches. Characterised by increase in urinary albumin excretion and decrease in glomerular filtrate rate, individuals suffering from DKD at an advanced stage require kidney dialysis and organ transplantation. To manage DKD, maintaining close to normal blood glucose level is a prerequisite which has led to a shift towards the use of alternative sugar substitutes such as artificial sweeteners. Although considered safe by Food Standards Agency and well tolerated, the impact of artificial sweeteners in circulation and on vascular permeability associated with DKD is lacking. The discovery of sweet taste receptors in extraoral cells has led to questions on their physiological roles besides sweet taste satiation. This research, therefore, employed various experimental approach to better comprehend the effects of artificial sweeteners, aspartame, neotame, saccharin and sucralose on VEGF-induced permeability through activation of sweet taste-dependent signalling pathways.

To mimic endothelial permeability associated with DKD *in vitro*, glomerular microvascular endothelial cells were exposed to VEGF with and without artificial sweeteners, using FITC-dextran as a tracer to evaluate the amount of leak across the cell monolayer. An analytical technique, gas chromatography-mass spectrometry (GC-MS) was utilised to validate the binding of the sweetener to its receptor.

Findings demonstrated novel protective effects of saccharin and sucralose on VEGF-induced permeability through activation of the sweet taste receptor, T1R3, and maintenance of the cell-cell junctional protein, VE-cadherin. GC-MS results showed that sucralose was unable to cross the cell membrane of glomerular microvasculature.

Whilst controversy remains regarding consumption of AS, these novel findings, that some artificial sweeteners attenuate VEGF-induced vascular permeability, may represent an alternate treatment strategy towards ameliorating vascular permeability and improving renal function in individuals living with DKD.

Key words: Diabetic kidney disease (DKD), vascular endothelial growth factor (VEGF), artificial sweeteners (AS), sweet taste receptors, permeability, glomerular endothelium, gas chromatography-mass spectrometry (GC-MS).

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LIST OF ABBREVIATIONS

AGEs	Advanced glycation end products
AS	Artificial sweeteners
1,3-BPG	1,3-Bisphophoglycerate
DAG	Diacylglycerol
DKD	Diabetic kidney disease
DM	Diabetes mellitus
ECM	Extracellular membrane
ESKD	End-stage kidney disease
F6P	Fructose 6-phosphate
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GC-MS	Gas chromatography-mass spectrometry
GDM	Gestational diabetes
GFR	Glomerular filtrate rate
GMVEC	Glomerular microvascular endothelial cell
HbA1C	Glycated heamoglobin
IDF	International Diabetes Federation
LOD	Limit of Detection
LOQ	Limit of quantification

mg	Milligram
mg/L	Milligram per litre
mL	Millilitre
MODY	Maturity on-set diabetes of the young
MS	Mass spectrometry
Mg	Milligram
mg/L	Milligram per litre
mL	Millilitre
m/z	Mass-to-charge ratio
NADP$^+$	Nicotinamide adenine dinucleotide phosphate
NADPH	Reduced form of NADP$^+$
ng/L	Nanogram per litre
ng/mL	Nanogram per millilitre
NOX	NADPH oxidase
NOS	Nitric oxide synthase (NOS)
OGTT	Oral glucose tolerant test
PAR	Peak area ratio
PPP	Pentose phosphate pathway
PKC	Protein kinase C
PTR	Programmed translational readthrough
RAGE	Receptor Advanced glycation end products
ROS	Reactive oxygen species

RSD	Relative standard
R^2	Coefficient of determination
RT	Retention time
SIM	Selected ion monitoring
STR	Sweet taste receptors
T1DM	Type 1 diabetes mellitus
T2DM	Type 2 diabetes mellitus
UAE	Urinary albumin excretion
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor

CHAPTER 1

GENERAL INTRODUCTION

1.1 Research background

Globally, about 451 million people aged between 18-99 years lived with diabetes in 2017 with an anticipated increase to 693 million by 2045 (IDF, 2018). The estimated prevalence, healthcare expenditure and deaths attributed to diabetes presents social and financial burden across the globe (Cho, et al., 2018). The estimated global expenditure of people living with diabetes was 850 billion USD in 2017. In the UK, the cost of diabetes is predicted to reach 17% of the NHS budget by 2035 (Barr, 2018). The cost of diabetes in England and Wales is over £1.5m an hour and the cost of prescribing diabetic drugs is about 8% of total cost with medication for diabetic complications 3 to 4 times higher (Kanavos, Van den Aardweg and Schurer, 2012). Additionally, it is undeniable that the number of people living with diabetes will continue to increase, especially in developing countries where free access to healthcare and early diagnosis of diabetes is currently limited (Sarpong, et al., 2017; Animaw and Seyoum, 2017). Diabetes is therefore a global health concern.

Blood glucose level need to be maintained within a specific range, with any chronic increased level of blood glucose above that threshold resulting in diabetes, as highlighted in Table 1.1. As main regulator of cellular glucose utilisation, adequate and functioning levels of insulin are essential in maintaining glucose homeostasis. Diabetes develops mainly from the body's inability to manage blood glucose due to lack of or insufficient production of insulin (Evans and Scriven, 2015). Insulin is a peptide hormone made up of 51 amino acids, synthesised by the pancreatic beta (β) cells firstly as preproinsulin and cleaved to proinsulin in the rough endoplasmic reticulum with removal of the signal peptide (Fox, 2011). The proinsulin is then transported to the Golgi apparatus for cleavage into insulin and C-reactive peptide, and then packaged into secretory granules from where it is released through exocytosis when required (Evans and Scriven, 2015; Fox, 2011). In a normal physiological state, the secretion and breakdown of insulin balances out. However, when beta-cells in the pancreas are destroyed and are unable to produce insulin or the

body's inability to utilise the secreted insulin is decreased due to insulin resistance, dysregulation of blood glucose results, with an excess blood glucose level and consequently development of the two main type of diabetes, type 1 diabetes mellitus (T1DM) and type 2 diabetes mellitus (T2DM). Therefore, defects in intracellular responses to insulin is central in the pathophysiology of diabetes.

Table 1. 1: **Diagnostic criteria of diabetes**

Diagnostic criteria	Impaired glucose tolerance	Diabetes
Fasting plasma glucose	5.6-6.9 mmol/L or 100-125 mg/dL	>7 mmol/L or ≥126 mg/dL
2-hour plasma glucose during OGTT	7.8 mmol/L-11.1 mmol/L or 140-199 mg/dL	>11.1 mmol/L or ≥200 mg/dL
HbA1C	5.7-6.4%	≥ 6.5%.
Random plasma glucose		>11.1 mmol/L or ≥ 200 mg/dL

OGTT = oral glucose tolerance test; HbA1C = glycated haemoglobin (ADA, 2016) and (Diabetes UK, 2019).

1.2 Classification of diabetes mellitus

Diabetes is presently classified into two main forms, type 1 (T1DM) and type 2 diabetes mellitus (T2DM) (ADA, 2016; Ahlqvist, et al., 2018). Other forms of diabetes are gestational diabetes (GDM), usually diagnosed in pregnancy during the second or third trimester and the maturity on-set diabetes of the young (MODY), resulting from monogenetic defects in pancreatic β-cell function (Yi, et al., 2017; Lekva, et al.,2018; Temming, et al., 2016; ADA, 2016; Siddiqui, Musambil and Nazir, 2015). It is to be noted that the diagnostic criteria outlined in Table 1.1 differ from those used in diagnosing GDM. In GDM, fasting blood glucose level is 5.1 mmol/L and 8.5 mmol/L for 2-hour glucose threshold following a 75g OGTT (Wong, Lin and Russell, 2017). Whilst all these forms of diabetes affect glucose regulation, the focus of this literature review will be on T1DM and T2DM.

1.2.1 Type 1 diabetes mellitus (T1DM)

Type 1 diabetes mellitus (T1DM) results from an autoimmune destruction of the pancreatic β-cells (type 1A) through anti islet autoimmunity leading to an absolute insulin deficiency. However, some T1DM patients have no evidence of pancreatic β-cell autoimmunity but rather, defects in insulin secretion due to inherited pancreatic β-cell defects in glucose sensing and other acquired or genetic diseases. T1DM is characterised by excessive thirst (polydipsia), excessive production of urine (polyuria), and weight loss (Jefferies, et al., 2015).

1.2.2 Type 2 diabetes mellitus (T2DM)

Type 2 diabetes mellitus (T2DM) is characterized by progressive β-cell failure, insulin resistance and increased blood glucose levels (>11.1 mmol/L) resulting from dysfunctional insulin secretion to glucose stimulus and increased glucose production in the liver (Marin-Martinez, Molino-Pagan and Lopez-Jornet, 2019; Tam, et al., 2017; Despande, 2008). Although T2DM was traditionally assumed to affect only adults and T1DM triggered during puberty, this is no longer the case as both T1DM and T2DM are prevalent in both cohorts (Ahola, et al., 2019; Yang, et al., 2014). Therefore, subsequent discussions on diabetic complications would include all cases of T1DM and T2DM irrespective of age.

Amongst the types of diabetes, T1DM accounts for 5–10% of all cases while T2DM is by far the most common form of diabetes accounting for almost 90% of total number of sufferers (IDF, 2019). Although some risk factors associated with diabetes such as genetic make-up and age are non-modifiable, other preventable risk factors includes unhealthy diet, physical inactivity, obesity, excessive use of alcohol and smoking (Kartha, Ramachandran and Pillai, 2017). The surge in diabetes increases the propensity of developing microvascular (small blood vessel) and macrovascular (large blood vessel) diseases. Both microvascular and macrovascular diabetic complications are evident in T1DM and T2DM with any form of diabetic complication affecting the quality of life of its sufferers (Kartha, Ramachandran and Pillai, 2017). Therefore, it is vital to give adequate attention to these complications since there is continuous relationship between diabetes and development of its complications.

1.3 Diabetic complications

Individuals living with diabetes are more susceptible to developing macrovascular and microvascular complications within 5 to 10 years of diabetes occurrence (Huang, et al., 2017; Fowler, 2008). While microvascular complications affect the most intricately vascularized organs in the body such as peripheral nerves (diabetic neuropathy), the retina (diabetic retinopathy-DR) and kidney (diabetic kidney disease); the macrovascular complications affects the brain (cerebrovascular disease), heart (cardiovascular disease) and the peripheral arteries (peripheral vascular disease) (Marin-Martinez,Molino-Pagan and Lopez-Jornet, 2019; Gonzalez-Perez, et al., 2019; Adler, et al 2002).

1.3.1 Macrovascular complications

Complex molecular pathways resulting from insulin dysfunction and hyperglycaemia predispose diabetic individuals to developing macrovascular complications with the main cause of death in T1DM and T2DM patients being cardiovascular disease (Morrish, et al., 2001). Cardiovascular disease (CVD) linked with diabetes occurs about two decades earlier in comparison to non-diabetics (Fomison-Nurse, et al.,2018; Adler, et al., 2002). Another macrovascular disease linked with diabetes is peripheral vascular disease (PVD). PVD arises from narrowing of blood vessels which transports blood to upper and lower limbs, characterised by a gradual reduction in blood flow to one or more limbs with the disease occurring almost three times more in individuals living with diabetes compared to non-sufferers of diabetes (Lange, et al., 2004). While these macrovascular complications are debilitating, the current research was geared towards microvascular complications of diabetes.

1.3.2 Microvascular complications

Microvascular complications caused by diabetes occur in tissues where glucose uptake is independent of insulin activity such as microvasculature of the eyes and kidneys (Chawla, 2016). Exposure of these cells to high glucose levels causes altered blood flow and changes in extravascular protein deposition and coagulation resulting in dysfunction of affected organs. The three main diabetic microvascular complications are diabetic nerve (neuropathy), eye (retinopathy) and kidney (nephropathy) diseases (Tonnies, et al., 2018). Although diabetic neuropathy remains the least understood of the microvascular complications, it is known that diabetic patients who develop neuropathy suffer a progressive deterioration of nerves, resulting in autonomic and peripheral nerve dysfunction (Gatt, et al., 2018; Labad, et al., 2018). Diabetic retinopathy is characterised by pericyte loss, thrombogenesis and abnormal blood vessel formation (angiogenesis) in the retina, while diabetic kidney disease results in proteinuria, increased serum creatinine levels, enlargement of the kidney, glomerulosclerosis and renal failure (Ruiz-Ocaňa, et al., 2018; Zeng, et al., 2019; Alicic, et al., 2017).Therefore making it vital to study these microvascular complications.

As one of the most common forms of diabetic microvascular complications, diabetic kidney disease (DKD) was the focus of this research. DKD occurs due to damage to the capillaries in the kidney's glomeruli and is a major but under-recognised contributor to the global burden of kidney disease with a highest observed death increase of 94% reported between 1990 and 2012 (Lozano, et al., 2012; Zhang, et al., 2015; Gonzalez-Perez, et al., 2019). Hence, based on the large residual risk of this disease onset and progression, there is a great need for innovative therapeutics for its management and treatment.

1.4 Diabetic kidney disease

DKD is amongst the most prevalent forms of microvascular complications seen in patients with T1DM and T2DM and is the cause of end-stage renal disease (Xue, et al., 2017; He, et al., 2019; Groop, et al., 2009). The growing number of people with diabetes has a direct impact on DKD incidence. Approximately 30% and 40% of individuals with T1DM and T2DM respectively developed DKD over a median of 15 years from the time of disease diagnosis (Alicic, et al., 2017; Zhang, et al., 2018; Retnakaran, et al., 2006; Khalil, 2017; Leung, et al., 2016). As highlighted in Figure 1.1, an increase in the number of individuals diagnosed with diabetes leads to a rise in the number of people living with DKD. These data (Figure 1.1) suggest that the number of people with DKD will continue to rise as the incidence of diabetes surges. Therefore, urgent, novel and effective strategies in delaying its progression, management and treatment are required.
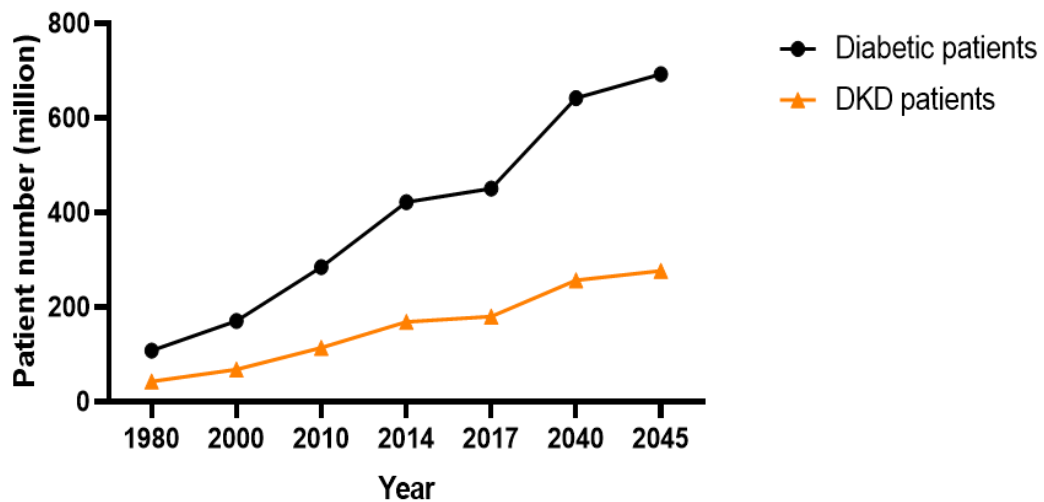
Figure 1.1:**The impact of diabetes in development of diabetic kidney disease**
The Figure represents the increasing number of individuals diagnosed with diabetes from 1980 to 2017 and estimated Figures for 2040 to 2045 (black line) with the correlating rise in people living with diabetic kidney disease (orange line) . The Figure was generated based on the knowledge that 40% of people living with diabetes would develop DKD within 5 to 15 years of disease diagnosis. The Figure was self-generated and plotted based on information from Khalil, 2017; Leung, et al., 2016 and International Diabetes Federation (IDF, 2019).

1.4.1 Diagnosis of diabetic kidney disease

DKD is clinically categorised by increasing urinary albumin excretion rates (UAER) and decreased renal function (Huang, et al., 2017; Sharaf El Din, 2017). Haemodynamic changes and impairment of the glomerular filtration barrier cause persistent high urinary albumin-to-creatinine ratio ≥ 30 mg/g and other clinical features such as a glomerular filtrate rate <60 mL/min/1.73m^2. Hence, annual spot urine albumin/creatinine ratio testing is used for early DKD screening, which is confirmed by repeated increases in urinary albumin excretion. Individuals suffering from DKD sometimes suffer from other microvascular complications such as diabetic retinopathy (Bilious, 2015; Retnakaran, 2006; Hung, et al., 2017; Barrios, et al., 2018). DKD is grouped into 5 progressive stages: normal albuminuria, micro and macro albuminuria, overt proteinuria and finally, end stage renal disease (ESRD) as shown in Figure 1.2 (Satirapoj and Adler, 2014; Usama, et al., 2017). Therefore, timely diagnosis of DKD especially in individuals living with diabetes for more than 5 years is vital for its management and delay in progression. Given the damaging consequences of DKD, there is a pressing need to understand the molecular mechanisms which regulate this disease in order to develop effective therapeutic approaches.
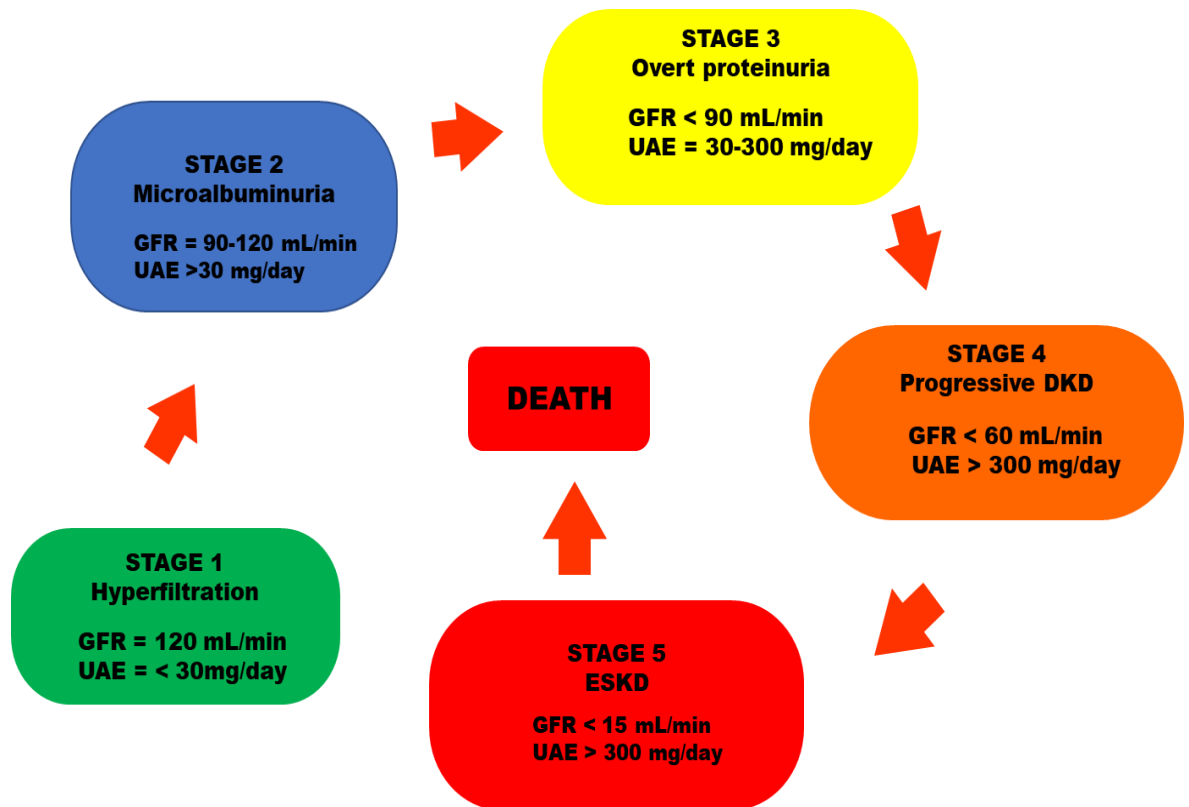
Figure 1. 2: **5-Stage clinical features of diabetic kidney disease**

The Figure shows the declining clinical stages leading up to DKD and kidney failure. Stage 1 (green) represents the normal kidney function while stage 2 (blue) is the precursor of DKD as excretion of urinary albumin reaches 30 mg/g/day. At stage 3 (yellow), the risk of DKD increases as the UAE is now between 30 to 300 mg/g/day with higher risk as it progresses to stage 4 (orange), culminating in kidney failure at stage 5 (red) and subsequently death. GFR = glomerular filtrate rate; UAE = urinary albumin excretion; ESKD = end-stage kidney disease (self-generated diagram and adapted from Usama, et al., 2017).

1.5 The kidney in health and disease

The kidneys are two bean-shaped organs, each located at the posterior abdominal wall of the spine with the right kidney slightly lower than the left kidney as it is located beneath the liver (Fox, 2011). The kidneys are the main functional unit of the urinary system with the other components being temporary storage areas and passageways (Verfasserln and Derrickson, 2017). The kidneys aid in maintaining homeostasis in the body through the regulation of blood pressure, pH and ion levels (sodium, potassium, calcium, phosphate and chloride ions) in the blood. In addition, the kidneys also regulate blood volume, production of hormones (calcitriol and erythropoietin) and the excretion of waste substances such as ammonia and urea (from amino acids), bilirubin (from haemoglobin), uric acid (from nucleic acid), and creatinine (from muscle creatine phosphate) as urine (Jang, et al., 2019, Verfasserln and Derrickson, 2017). These functions of the kidneys are vital in regulating fluid and electrolyte balance, acid-base balance, arterial pressure, hormone secretion and excretion of waste substances; both metabolic wastes and foreign chemicals (Verfasserln and Derrickson, 2017; Fox, 2011).

The main region of the kidney which aids in the excretion of waste substances as urine is the nephron as shown in Figure 1.3. There are approximately 1 million nephrons in each kidney (Barry, et al., 2019; Verfasserln and Derrickson, 2007). The kidneys generate about 120 mL/min of ultrafiltrate of which only 1-2 mL/min is generated as urine (Dahlquist, Stattin and Rudberg, 2001). This filtration ability of the glomerulus frequently observed as estimated GFR (eGFR) is used in measuring the degree of kidney damage. Hence, the important role of the kidney as a filtration unit is determined by the health of its glomerular compartment.
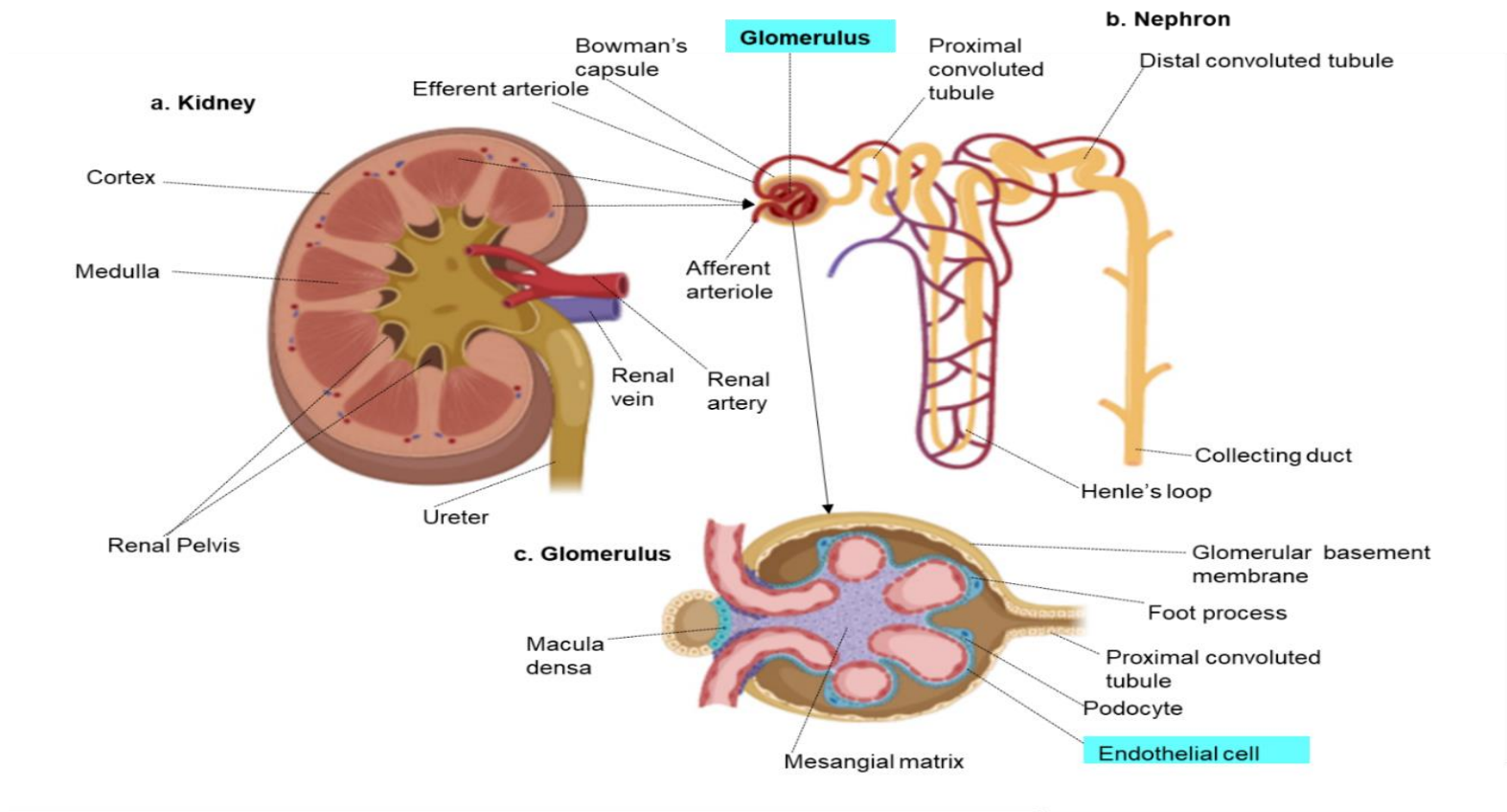
Figure 1. 3: **Structural representation of the kidney**

Figure represents parts of the kidney (a), the nephron (b) and glomerulus(c). The glomerulus is composed of mesangial matrix, podocytes and its foot processes and highly fenestrated endothelial cells. The nephron (1.3b) utilizes its filtrating unit, the glomerulus (1.3c) to filter water and small molecular size solutes from the blood while reabsorbing amino acids, proteins, electrolytes and glucose. The glomerulus is connected to the long tubular part of the nephron made up of the proximal tubule, the loop of Henle and distal tubule (Created with Biorender.com with adaptations from Tanabe, et al., 2017; Barry, et al., 2019).

1.5.1 The glomerular endothelium

The glomerular endothelial cells are unique in their feature due to their fenestrae and are amongst the most diversified endothelial cell (EC) populations found within any organ (Barry et al., 2019). This diversity can be attributed firstly, to its contribution in differential transport capabilities across various segments of the nephron. Secondly, renal endothelium withstands extreme environments in osmolality and oxygenation, with the outer cortex exposed to normal osmolality of 275–300 milliosmoles/kg (mOsm/kg) and oxygen tension of 40-50 millimetre of mercury (mm/Hg) whereas those in the inner medullary region are exposed to an O_2 content as low as 20 mm/Hg and osmolality of up to 1,200 mOsm/kg (Verma and Molitoris, 2015; Dasgupta and Wahed, 2014; Fu, et al., 2015).

The major phenotypic property of glomerular endothelium is its fenestrations and rich glycocalyx which differentiates it from other endothelial cells (Ballerman, 2019). Fenestrations are circular transcellular pores of about 60–80nm in diameter that cover about 20% of the endothelial surface (Satchell, et al., 2006). These fenestrations are vital for the selective permeability of the glomerular filtration barrier and efficient passage of high volumes of fluids. Additionally, the glomerular endothelial cells are lined by thick filamentous glycocalyx, enriched in a negatively charged network of hyaluronic acid made up of proteoglycans (mainly chondroitin sulfate and heparan) (Boels, et al., 2013). The glycocalyx hampers the adhesion of blood cells to the endothelium and aids in the regulation of vascular permeability and fluid balance (Zuurbier, et al., 2005). These unique properties of the glomerular microvasculature are essential for the filtration function of the kidney. The glomerular filtration barrier is a specialized blood filtration unit in the kidney, permeable to small solutes and water but impermeable to macromolecules such as albumins (Pajęckaa, et al., 2017; Satchell, et al., 2006). This ability of glomerular endothelial cells to prevent passage of macromolecules such as albumin is achieved mainly due to the filling of the endothelial fenestrae with densely packed hyaluronic acid. This acid also anchors the glycocalyx to the

glomerular basement membrane (GBM), another component of the glomerular endothelium.

As shown in Figure 1.4, glomerular barrier functionality is maintained by cooperation between its three major components – the podocytes on the apical side extracellular matrix and the glomerular endothelial cells with its glycocalyx on the luminal side (Qi, et al., 2017; Pajęckaa, et al., 2017).
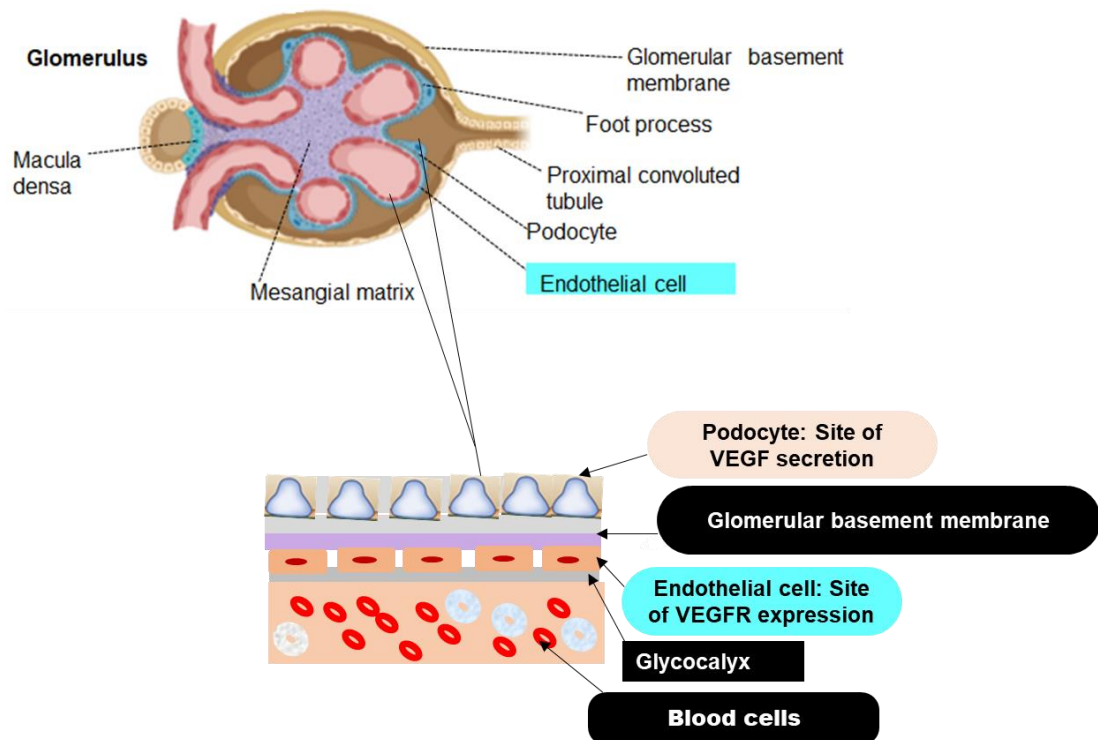
Figure 1. 4: **Structure of the glomerular endothelium compartments**

The glomerulus is composed of podocytes and its foot processes that wraps the capillaries and highly fenestrated endothelial cells. VEGF = vascular endothelial growth factor and VEGFR = vascular endothelial growth factor receptor.

The morphological and structural differences between normal and diseased glomerulus lies in the thickening of the glomerular basement membrane, fusion of foot processes, loss of podocytes and mesangial matrix expansion. These changes subsequently lead to fluid build-up, causing enlargement of the endothelium and barrier permeability. Hence, any alteration to the normal physiological state of the body, such as chronic increased levels of blood glucose evident in diabetes, leads to impairment in the functionality of the kidney and its glomerular endothelium.

1.6 Glucose homeostasis in the kidneys

The digestion and absorption of carbohydrate occurs mainly in the small intestine (SI) where the sugar oligomers and disaccharides are broken down into monosaccharides by an enzyme expressed on the apical membrane of the enterocyte, sucrase-isomaltase (O'Brien, 2016). Glucose homeostasis in the body involves several physiological processes which includes its absorption in the gastrointestinal tract, uptake into muscle, liver and adipose tissue, reabsorption in the kidneys, glycogenolysis and gluconeogenesis in the liver and kidney, and glucose excretion in the kidneys (Landau, et al., 1996). It is critical to maintain a balanced level of glucose in the blood to prevent several pathological states resulting from either too low (hypoglycaemia) or excess (hyperglycaemia) blood glucose levels. While hypoglycaemic conditions could result in behavioural changes, seizures, confusion, unconsciousness and even death, hyperglycaemia can lead to diabetes with its macrovascular and microvascular complications (Seaquisrt, et al., 2013; Maran, et al., 2000).

 Since maintaining a balanced blood glucose level is imperative, the digestive and transport mechanisms of sugar are tightly regulated in order to match assimilation capacity with the luminal carbohydrate load (O'Brien, 2016). This balance in blood glucose involves an increase in renal glucose of < 9.0 mmol/L after a meal while minimal concentration usually after a moderate exercise or fast > 3.0 mmol/L (Rizza, et al., 1980; ADA, 2016; Woerle, et al., 2003).

The kidneys play vital role in glucose regulation, releasing approximately 20% of the total body glucose following absorption, 40% through gluconeogenesis, and the release of approximately half of all circulatory glucose during the fasting state (Meyer, et al., 2002; Consoli, et al., 1989). The kidneys also filter and reabsorb about 180g of D-glucose from plasma per day (Wright, 2001; Szablewski, 2017). Therefore, the important role of the kidney in maintaining glucose homeostasis is evident in a pathological state such as DKD.

1.6.1 Renal glucose transport

Based on the polar property of glucose, it is impermeable to the lipid bilayer of the plasma membrane. Carrier proteins present in the plasma membrane facilitate the transport of glucose into cells. There are three families of glucose transporters in human: GLUT proteins, encoded by *SLC2* genes; SWEET, encoded by the *SLC50* gene and sodium-dependent glucose transporters (SGLT) encoded by *SLC5* genes (Szablewski, 2017). Glucose transport across cell membranes is accomplished through the facilitative diffusion by glucose transporters (GLUTs) and active transport against concentration gradient by sodium-dependent glucose co-transporters (SGLTs). Whilst the GLUTs transport molecules across the cell membrane in either direction, the SGLTs actively transport glucose only into the cells because of its dependant on the Na^+ gradient (Chen, et al., 2010). The predominant glucose transporters associated with the kidney are the low-affinity (K_m 1.1 mM) high-capacity transporter (SGLT2), the high-affinity (K_m 0.35 mM) low-capacity (SGLT1) and the facilitative glucose transporters, GLUT2 (Devaskar and Mueckler, 1992; Chen, et al., 2010). Whilst the SGLT1 and SGLT2 require an electrolyte (Na^+) gradient to transport glucose across the lumen (active transport), the GLUT does not and so operate as passive facilitated diffusion (Tabatabai, et al., 2009; Chen, et al., 2010). The SGLTs are localized on the apical membrane and reabsorb the filtered glucose using secondary active transport, while the facilitative GLUT glucose transporters efflux the reabsorbed glucose back into circulation (Alsahli and Gerich, 2017; Vallon, et al., 2011). According to a study by Masuda, et al. (2014), SGLT2 account for 97 % of renal glucose reabsorption and SGLT1 the remaining 3% in euglycemia. Under hyperglycaemic condition, selective SGLT2 inhibitors reduces

renal glucose threshold through inhibition of tubular glucose reabsorption (Abdel-Wahab, et al., 2018), thereby indicating their involvement in the pathogenesis of DKD.

The metabolic processes involved in renal release of glucose into circulation are gluconeogenesis and glycogenolysis (Owczarek, et al., 2020; Stumvoll, et al., 1995). Gluconeogenesis involves the production of glucose from pyruvate or lactate and glutamine and other amino acids. Conversely, glycogenolysis is the breakdown of glycogen to glucose. The metabolism of glucose in the kidney is carried out mainly in the medulla and the cortex as depicted in Figure 1.3a. The cortex of the kidney utilized glucose while the medulla is responsible for glucose release into circulation (Alsahli and Gerich, 2017). This vital role of the kidney in maintaining blood glucose level could be hampered in a pathological state such as diabetes and when not well managed or diagnosed early, leads to diabetic kidney disease.

1.6.2 Renal glucose transport in disease

The importance of the kidney in glucose homeostasis is evident in its role in the prevention of hypoglycaemia through glucose reabsorption and gluconeogenesis. Any dysfunction or mutation in the expression of the GLUTs and SGLTs proteins and other disease conditions such as diabetes could damage these renal glucose transporters, thereby affecting glucose regulation. In a pathological state such as diabetes, the expression and function of glucose transporters changes with direct impact on the absorption, reabsorption and release of glucose in the kidney. In the normal physiological state, glucose filtered by glomerulus is completely reabsorbed, while under hyperglycaemic condition such as diabetes, excess glucose reabsorption occurs, worsening the conditions (Meyer, 2004). Moreover, studies have shown changes to the expression of renal glucose transporters under diabetic condition (Rahmoune, et al., 2005; Kamran, Peterson and Dominguez, 1997). Additionally, an *in-vivo* study has linked metabolic dysregulation caused by diabetes with changes on renal glucose transporters (Chichger, et al., 2016; Guzman, et al., 2014). Therefore, altered renal glucose handling under diabetic conditions lead to increased glucose levels in post absorptive and prandial states, the consequence, abnormal function of other molecular

mechanisms associated with glucose metabolism, culminating in diabetic microvascular complication, DKD.

1.7 Pathophysiology of diabetic kidney disease

1.7.1 Diabetes-Induced biochemical and molecular mechanisms implicated in DKD
1.7.1.1 Hyperglycaemia in the onset of diabetic kidney disease

In individuals living with kidney disease, there are two main perturbations observed; hyperglycaemia (metabolic) and hypertension (haemodynamic). Both disruptions are modulated through different cellular pathways and drive the relentless decline in kidney function. Hyperglycaemia is the major systemic factor associated with diabetic complications (Gnudi, 2012). Hyperglycaemia indicates a sustained increase in blood glucose levels of 11.1 mmol/L (≥ 200 mg/dL) for people living with diabetes whereas normal level ranges from 4.0 to 6.0 mmol/L (72 to 108 mg/dL) when fasting and up to 7.8 mmol/L (140 mg/dL) 2 hours after food (Kahanovitz, et al., 2017).

Two landmark experimental and large-scale clinical studies conducted in patients with diabetes; the Diabetes Control and Complications Trial (DCCT) in T1DM patients and the United Kingdom Prospective Diabetes Study in T2DM patients showed hyperglycaemia as the main causative agent in diabetic complications (DCCT 1983-1993; UKPDS, 2008). These studies demonstrated that maintaining blood glucose level close to normal (intensive glucose control) delays the onset and progression of diabetic microvascular complications (Barret, et al., 2017). The UKPDS study was focused on reducing the blood glucose of individuals living with T2DM to HbA1c of 7.0 mmol/L rather than 7.9 mmol/L over a period of 10 years. Their results showed that the risk of developing diabetes-related microvascular disease reduced by 25% (UKPDS,1998).

The DCCT study was carried out over a 10-year period (1983-1993) involving 1441 participants between the ages of 13 to 39 living with T1DM for at least one year but not more than 15 years. The results demonstrated that individuals who used the intensive glucose treatment strategy lowered their risk of developing diabetic complications by up to

50% for diabetic kidney disease (Nathan, et al., 2009). These studies therefore, highlight the detrimental effects of hyperglycaemia and its contribution in the development of DKD.

Furthermore, hyperglycaemia upregulates multi-metabolic abnormalities in cell biology and physiology, cell signalling and gene expression with many of these abnormalities concurrently occurring during the development of diabetic vascular complications. These metabolic mechanisms, otherwise known as 'hyperglycaemic damage pathways' as shown in Figure 1.5, include the activation of protein kinase C (PKC), production of reactive oxygen species (ROS), signaling through the polyol and hexosamine pathway and the production of advanced glycation-end products (AGEs) (Sun, et al., 2013; Brownlee, 2001; 2004; 2005; Saulnier, et al., 2018; Kowluru and Chan, 2007; Mima, 2012; Rhee and Kim, 2018). Hyperglycaemia evident in diabetes is therefore an initiator of various signalling cascades and vascular complications.
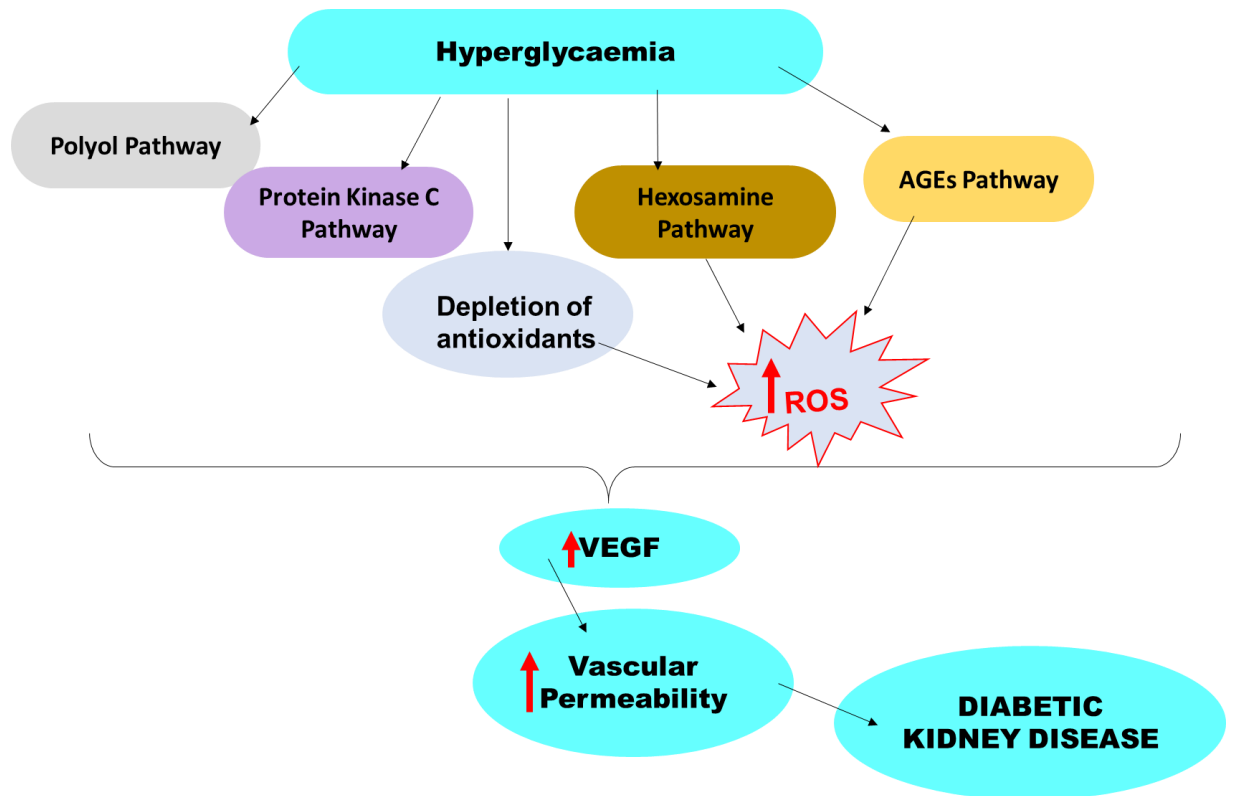
Figure 1. 5: **Relationship between hyperglycaemia and diabetic kidney disease**
The Figure highlights the relationship between hyperglycaemic damaging pathways, vascular permeability and DKD. AGEs= advanced glycation-end products, ROS= reactive oxygen species, VEGF = vascular endothelial growth factor.

1.7.1.2 **The polyol pathway**

This pathway involves the catalysis of glucose to sorbitol by the aldose reductase enzyme with sorbitol further oxidised by sorbitol dehydrogenase to form fructose (Lagies, et al.,2019). As depicted in Figure 1.6, hyperglycaemia increases the flux of glucose into this pathway thereby utilising nicotinamide adenine dinucleotide phosphate (NADPH) as cofactor, generating $NADP^+$. Under diabetic condition, almost 30% of blood NADPH could flux through the polyol pathway (Gonzolez, et al., 1984) causing further depletion of NADPH. Cytosolic NADPH depletion and reduced bioavailability affects the production of reduced glutathione (GSH) (Ghashghaeinia, et al. 2016), a reactive oxygen species scavenger, essential for maintenance of the body's intracellular antioxidant in its reduced state (Zhang, et al., 2012; Kowluru and Chan, 2007; Brownlee, 2001). Furthermore, the level of intracellular NADPH is diminished through the inhibition of glucose-6-phosphate dehydrogenase, an enzyme that catalyses the first intermediary reaction in the pentose phosphate pathway and the primary source of NADPH (Xu, 2009).
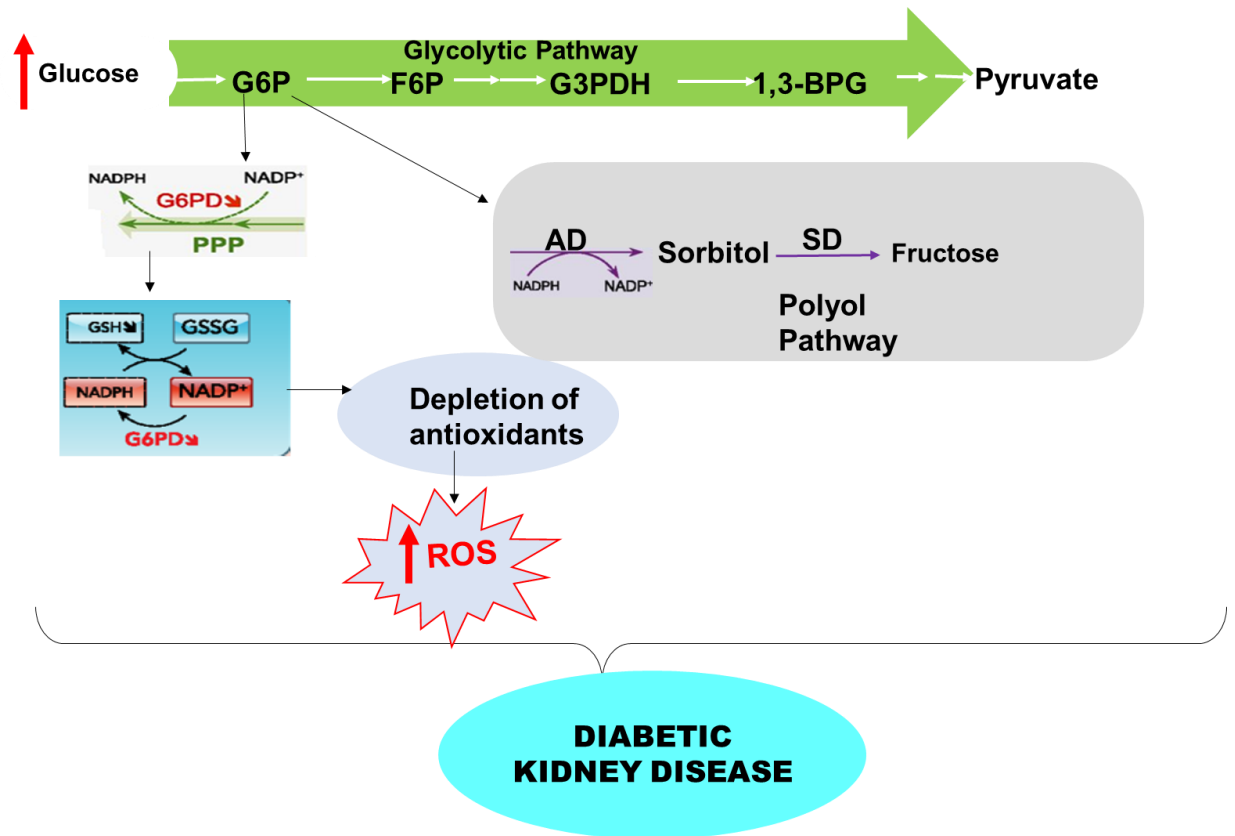
Figure 1. 6:**The polyol pathway and diabetic kidney disease**

The polyol pathway indicating postulated biochemical consequences relevant to the pathogenesis of DKD. SD=Sorbitol dehydrogenase, AD= aldose reductase, DAG= diacylglycerol, ROS= reactive oxygen species, GAPDH= glyceraldehyde-3-phosphate dehydrogenase, PPP=pentose phosphate pathway, G6P = glucose 6-phosphate, F6P =fructose 6-phosphate, 1,3-BPG = 1,3-bisphophoglycerate, NADP+ = nicotinamide adenine dinucleotide phosphate, NADPH = reduced form of NADP+, GSH = reduced glutathione, GSSH = oxidized glutathione.

The involvement of the polyol pathway in the development of DKD was further demonstrated by Lagies, et al. (2019). The authors used four cell lines representing the proximal tubule, glomerulus and collecting duct of the kidney to support the notion that under diabetic conditions, increased flux of glucose to the polyol pathway is activated in kidney cells. Another activation signal for the polyol pathway, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) inhibition, occurs through the addition of ADP-ribose moieties to GAPDH by the enzyme poly (ADP-ribose) polymerase (PARP), following activation by reactive oxygen species (Sun, et al., 2013; Du, 2003). Since GAPDH is necessary for the conversion of glyceraldehyde3-phosphate (G3P) in the glycolytic pathway, the inactivation of GAPDH leads to increased levels of G3P. In turn, causing *de novo* synthesis of diacylglycerol (DAG), a PKC activator and increased production of methylglyoxal, an AGE precursor (Brownlee, 2001). Therefore, the increased activation of the polyol pathway not only contribute to the development of DKD but also acts as an intermediary pathway to other metabolic mechanism; development of advanced glycation end products (AGEs).

*1.7.1.*3 Advanced glycation end products

The spontaneous nonenzymatic reaction of glucose and other reducing sugars with a wide spectrum of lipids and proteins, initiating a post-translational, nonenzymatic modification process is known as advanced glycation. This advanced glycation yields a heterogeneous group of irreversible adducts called advanced glycation end-products (AGEs) (Han, et al., 2009). Advanced glycation end products (AGEs) are stable, post-translationally modified proteins which are produced from non-enzymatic glycation of amino groups on proteins by glucose (Maillard reaction) and from the dicarbonyl compounds (Zabad, Amin and El-Shishtawy, 2019; Balistreri, et al., 2018). As shown in Figure 1.7, AGEs are formed through autoxidation and the degradation products of glucose, such as glyoxal and methylglyoxal or α-hydroxy aldehydes such as glycolaldehyde and glyceraldehyde (Rhee and kim, 2018; Rabbani and Thornalley, 2018). These glycation reactions lead to alteration in activity, function and degradation of proteins through chemical rearrangement, intramolecular and intermolecular crosslinking (Brownlee, 2004).

AGEs exert their damaging effect on the cells by binding to the AGE receptor, RAGE. This receptor is a 45-kDa transmembrane receptor and a multi-ligand receptor expressed in different cell types including immune cells such as monocytes/macrophages, dendritic cells and lymphocytes, cardiomyocytes, vascular cells, neurons and kidney podocyte epidermal cells and plays a role in inflammatory processes (Lv, et al., 2016; Basta, et al., 2005). This RAGE interaction with its ligand, AGE leads to a positive feedback activation and further increases receptor expression unlike other receptors that are usually downregulated by an increased level of their ligands (Basta, et al., 2005; Balistreri, et al., 2018). AGE binding to its receptor, therefore, generates by-products deleterious to the kidney.
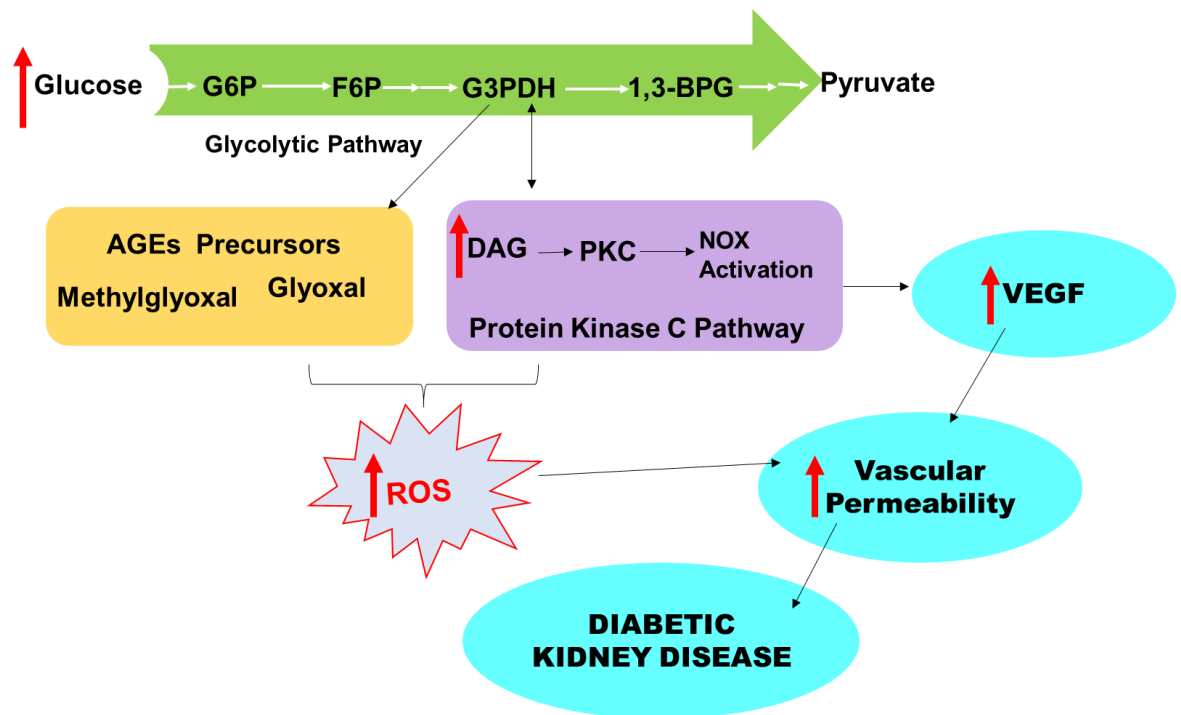
Figure 1. 7: **The relationship between DKD and advanced glycation endproducts**

The Figure illustrates the damaging consequences of AGEs in relation to DKD. AGEs = advanced glycation-end products, PKC = protein kinase C, DAG= diacylglycerol, ROS= reactive oxygen species, GAPDH = glyceraldehyde-3-phosphate dehydrogenase, PPP=pentose phosphate pathway, G6P = glucose 6-phosphate, F6P = fructose 6-phosphate, 1,3-BPG = 1,3-bisphophoglycerate, NADP$^+$ = nicotinamide adenine dinucleotide phosphate, NADPH = reduced form of NADP$^+$, NOX =NADPH oxidase, VEGF = vascular endothelial growth factor.

Increasing evidence demonstrates the pivotal role AGEs play in the development and progression of diabetic vascular damage with some studies suggesting an increased level of AGEs and glycoxidation products in patients with diabetes in comparison with healthy individuals (Saulnier, et al., 2016; Galler, et al., 2003; Rabbani and Thornalley, 2018). Linking the production of AGEs to the progression of DKD, a cross-sectional study on the relationship between the accumulation of AGEs and histological changes in the glomerular basement width of a T1DM population was carried out by Beisswenger, et al. (2013). This study implicates elevated methylglyoxal (an AGE) levels in the development of DKD as increased cellular production of methylglyoxal and increased oxidative stress were observed. This intracellular increase in methylglyoxal, a highly reactive α-dicarbonyl, thereby results in the formation of long-lived oxidative products which accumulate and subsequently lead to the changes in glomerular morphology (Beisswenger, et al., 2013). Additionally, an experimental study by Saulnier, et al. (2016), on early-stage diabetic kidney disease subjects highlights the correlation between the progressive retention of AGEs and declining renal function in DKD, demonstrating the deleterious effects of AGEs on kidneys. Another fluorescence spectroscopy study on serum levels of AGEs by Galler, et al. (2013) observed a similar association. In that study, comparing 99 children and adolescents up to 20 years of age with T1DM, and 60 control subjects, a significant increase in the serum levels of fluorescently-tagged AGEs was noted in patients with T1DM in comparison with control subjects. These studies therefore demonstrate that the accumulation of AGEs in the plasma, under chronic hyperglycaemic conditions, can lead to abnormal cross-linking of intracellular and extracellular proteins, disrupting their normal structure and function (Yan, et al.,2010).

Whilst these studies (Galler, et al. 2013; Beisswenger, et al., 2013; Saulnier, et al. 2016) suggest that increased concentrations of AGEs in diabetic state are directly correlated with the level of hyperglycaemia, it has been proposed that the level of AGEs could be primarily determined by changes in the metabolism of glucose-derived reactive intermediates, irrespective of blood glucose concentrations (Rask-Madsen and King, 2013; Fleming,

2012). Therefore, the abnormal modification of proteins by AGEs plays a role in development of DKD.

The binding of AGEs to its receptor, RAGE, has also been associated with diabetic complications. RAGE's ability to cause diabetic complications has been demonstrated in a study where soluble RAGE (sRAGE) was measured in serum samples from 3647 participants with diabetic kidney disease (Waden, et al., 2019). The results indicated that sRAGE concentrations are significantly higher in participants with DKD in comparison to control subjects. Whilst AGE production and accumulation has been shown to play a key role in the development and progression of DKD, there is also a well-established link between AGEs, increased cellular reactive oxygen species (ROS) and *de novo* synthesis of cytokines in glomerular endothelial cells (Yamagishi, et al., 1997). The involvement of AGE and its receptor in the induction of ROS production was further studied by Basta, et al. (2005) where the authors used an *in vitro* model of human umbilical vein endothelial cells (HUVEC). Their findings demonstrated that the induction of ROS by AGEs is specifically blocked by an anti-RAGE antibody. Therefore, hyperglycaemia-induced production of AGEs and its binding to RAGE do not only elicit other signalling cascades implicated in DKD but also increase intracellular ROS production.

1.7.1.4 Hyperglycaemic-induced oxidative stress

The term oxidative stress otherwise known as oxidant-derived tissue injury occurs when production of reactive oxygen species exceeds the local antioxidant capacity (Forbes, Coughlan and Cooper, 2008). Reactive oxygen species (ROS) such as superoxide (O_2^-) and hydrogen peroxide (H_2O_2), are a cluster of highly unstable compounds which are normal by-products of cellular metabolism formed through incomplete reduction of molecular oxygen (Boueiz and Hassoun, 2009).These generated ROS are maintained by intracellular antioxidant system. However, an impairment of this system (antioxidant) leads to excess ROS generation and consequently, oxidative stress, which can induce and propagate significant injury in affected cells (Boueiz and Hassoun, 2009; Chen, et al., 2016).

This redox imbalance initiated by hyperglycaemia plays critical role in the pathogenesis of DKD (Amorim, et al., 2019). The other reactive species linked with DKD are the nitrogen species which include nitric oxide (NO), nitrogen dioxide (NO_2^-) and peroxynitrite ($ONOO^-$) (Etoh, et al., 2003). While there are several sources of ROS in a normal physiological state, under diabetic condition, there are enzymatic and nonenzymatic sources of ROS in the kidney. These include advanced glycation end-products, polyol pathway flux, auto-oxidation of glucose, mitochondrial respiratory chain deficiencies, peroxidases, nitric oxide synthase (NOS), NAD(P)H oxidase and xanthine oxidase activity (Satoh, et al., 2005; Rizwan, et al., 2020). Therefore, the identification of ROS sources in the kidney is essential for targeted therapeutic strategies in managing DKD.

The cytosolic sources of ROS in the kidney emanates mainly from the glycolytic and sorbitol pathways as shown in Figure 1.5. and 1.6. The reduced level of this cofactor, NADPH, as previously mentioned in section 1.7.1.2 decreases the availability of glutathione in its reduced state, GSH (Kilanczyk, et al., 2016; Taniguchi, et al., 2015). Under normal physiological conditions, the measurement of GSH and GSSG ratios in blood has been used as an index for cellular oxidative stress and a useful biomarker of diseases in humans (Rossi, et al., 2002). In addition to reducing the bioavailability of GSH, the surge of NADPH to the sorbitol pathway under hyperglycaemic condition leads to the reduction of NAD^+ to NADH, a substrate to complex-I of the mitochondrial respiratory chain which augments the production of ROS in the mitochondrial respiratory chain.

Mitochondria play a vital role in oxygen metabolism, with over 90% of oxygen in humans metabolised during oxidative phosphorylation (Larsen, et al., 2011). In physiological conditions, the electrons from glucose and other fuels are transferred to molecular oxygen, utilizing respiratory chain complexes I–IV and finally ATP synthase. Protons are pumped across the mitochondrial membrane creating an electrochemical gradient, which is collapsed to generate ATP (Sourris, et al., 2012). These reactions are tightly regulated with an estimated 1-2% of oxygen partially reduced to $\cdot O_2^-$, rather than fully to water (Fisher-Wellman, et al., 2013). NADH and $FADH_2$ produced through the glycolytic pathway are

transported into the mitochondria through the glycerol phosphate shuttle or the malate-aspartate systems (Abbrescia, Piana and Lofrumento, 2012). Since NADH is the main electron donor to the mitochondrial respiratory chain, hyperglycaemia would therefore increase the ratio of NADH and $FADH_2$ and consequently increase the mitochondrial proton gradient (Poburko, et al., 2012). This increase in the mitochondrial proton gradient inhibits the transfer of electrons from reduced co-enzyme Q (ubiquinone) to complex III of the electron transport chain, causing electron transfer to molecular oxygen and subsequently production of superoxide ions (Brownlee, 2001, 2004). Another source of ROS is through the endothelial nitric oxide synthase (eNOS). A pro-oxidant redox state favours oxidation of the eNOS cofactor tetrahydrobiopterin (BH_4). The oxidation of BH_4 leads to uncoupling of electron transport in eNOS, thereby releasing superoxide ions (Laursen, et al., 2001).

The generation of superoxide and other ROS with resultant oxidative stress states in the vascular wall has been implicated in some studies to play a prominent role in the pathogenesis of vascular diseases (Ogura, et al., 2018; Qi, et al., 2017; Liles, et al., 2018). Sustained increase in renal blood glucose elevates the generation of ROS through various sources and reduces the cellular availability of antioxidant molecules, resulting in oxidative stress and consequently kidney injury. Therefore, developing novel agents that would either increase the availability of antioxidants, or reduce the generation of intracellular ROS might ameliorate ROS-induced kidney damage and delay the progression of DKD.

1.7.1.5 The hexosamine pathway

The next damaging route of sustained high blood glucose is the hexosamine pathway which results from the inhibition of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The GAPDH enzyme is a multifunctional protein that causes the diversion of glycolytic metabolite; fructose-6 phosphate into the hexosamine pathway. Fructose-6-phosphate is then converted by the enzyme glutamine, fructose-6-phosphate amidotransferase (GFAT) to UDP–N-acetylglucosamine (UDP-GlcNAc) (Ruegenberg, et al., 2020). The UDP-GlcNAc is a substrate in the post translational modification of intracellular factors such as growth factors (Schleicher and Weigert, 2000). Therefore, the hexosamine pathway although not

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very popular in the pathogenesis of DKD like the other hyperglycaemic damaging pathways such as AGEs and ROS, it does contribute to the increased activation of other metabolites associated with DKD such as protein kinases.

1.7.1.6 Protein kinase C

Protein kinase C (PKC) is a ubiquitous enzyme that plays a vital role in several intracellular signal transduction cascades such as cell proliferation, differentiation, cell cycle and apoptosis (Li and Gobe, 2006). As diagrammatically presented in Figure 1.7, PKC is activated by the increased *de novo* synthesis of diacylglycerol (DAG) from dihydroxyacetone phosphate (glycolytic pathway intermediate), through the reduction of glycerol-3-phospate (Abbrescia, Piana and Lofrumento, 2012). PKC activity is upregulated in both macro and micro vascular tissues under diabetic condition (Inoguchi, et al.,1992). There are 10 isoforms of PKC in humans (Li and Gobe, 2006), amongst which the α, β and δ PKC isoforms, have been consistently implicated in diabetic vascular complications (Thallas-Bonke, et al., 2008). Additionally, activation of PKC also leads to decreased nitric oxide production and increased expression of vascular endothelial growth factor (VEGF) (see Figure 1.5), a known growth factor associated with DKD (Sun, et al., 2013).

Interestingly, these hyperglycaemic damaging pathways; polyol, hexosamine, AGEs and PKC act in conjunction through several ways to induce vascular damage evident in DKD. Increased influx of glucose into the polyol pathway not only contribute to the development of DKD through development of advanced glycation end products (AGEs) but has also been linked to endothelial dysfunction. The involvement of polyol pathway in vascular permeability, was demonstrated by Obrosova, et al. (2003). The author implicated aldose reductase, an enzyme of the polyol pathway which catalyses the reduction of glucose to sorbitol, with overexpression of vascular growth factor, VEGF in early diabetes. As detailed in section 1.7.1.3, the accumulation of AGEs in the plasma under chronic hyperglycaemic conditions, can lead to abnormal cross-linking of intracellular and extracellular proteins, disrupting their normal structure and function (Yan, et al.,2010). In addition, UDP-GlcNAc is a substrate in the post translational modification of intracellular factors such as growth

factors (Schleicher and Weigert, 2000). *O*-linked *N*-acetylglucosamine (*O*-GlcNAc) modifies a variety of cytoplasmic and nuclear proteins through posttranslational modification, otherwise known as O-GlcNAcylation in the presence of a catalytic enzyme, *O*-GlcNAc transferase (OGT). The substrate for this reaction is the UDP-GlcNac, upregulated through the hexosamine pathway (Yang, et al., 2001). An increase of O-GlcNAcylation and transcriptional activity of specificity protein 1 (Sp1) has been implicated in the upregulation of VEGF-A under hyperglycaemic condition *in vitro* (Donovan et al., 2014). Therefore, the hexosamine pathway although not very popular in the pathogenesis of DKD, does contribute to the increased activation of metabolites associated with permeability in DKD such as VEGF and other protein kinases. An example of such kinases is the protein kinase C (PKC) and its activation can lead to increased gene expressions of various stress related genes like growth factors through mitogen-activated protein kinase (MAPK) activation and phosphorylation of several important transcription factors. This was demonstrated in a study by Xia, et al. (1994), where PKC-β was shown to have a role in the form of a signalling component for VEGF, a regulator of endothelial cell permeability. Another study has also shown attenuation of increased VEGF expression under diabetic conditions in the absence of PKC-β (Ohshiro, et al., 2006).

Therefore, it is evident that hyperglycaemia upregulates the expression of the growth factor, VEGF, which is a potent permeability inducing factor linked with DKD. Taken together, it is evident that a key downstream response to the hyperglycaemic-induced cellular pathways and mechanisms discussed herein are implicated in the regulation of vascular inflammation and altered gene expression of growth factors and cytokines associated with the development and progression of DKD.

1.8 Vascular endothelial growth factor and vascular permeability

One of the key growth factors upregulated in diabetes is the vascular endothelial growth factor. The vascular endothelial growth factor (VEGF) was discovered first in 1983 as the vaso-permeability factor (VPF) based on its permeability-inducing potency. VEGF is a protein with mitogenic and permeability-inducing activities specific for vascular endothelium (Peng, et al., 2010; Shulman, et al., 1996; Singh, et al., 2017; Jelkmann, 2001). The permeability -inducing effect of VEGF is about 50,000 times greater than that of histamine, a preformed mediator of allergic inflammation and strong inducer of vascular permeability (Ashina, et al., 2015; Koyama, 2002).

There are five members of the VEGF family (VEGF-A, B, C, D and placental growth factor), with two others, the para poxvirus proteins (VEGF-E) and (VEGF-F) from snake venom all of which are structurally related (Uciechowska-Kaczmarzyk, et al., 2018). The most studied amongst the five VEGF family is VEGF-A which has eight various exons encoding several protein isoforms produced by splicing. These VEGF-A isoforms are VEGF-A$_{121}$, VEGF-A$_{145}$, VEGF-A$_{165}$, VEGF-A$_{189}$ and VEGF-A$_{206,}$ with VEGF-A$_{165}$ being the most abundant in humans (Tanabe, et al., 2017). VEGF-A is the single most important regulator of blood vessel formation under normal physiological states and is vital for neovascularization in pathological states. As illustrated in Figure 1.8, all VEGF-A isoforms contain exons 1-5, with diversity created through alternative splicing of exons 6, 7 and 8 (Kilipatric, et al., 2017). Therefore, the differences in the VEGF-A isoforms permeability potency occurs due to diversity of the exon splicing.
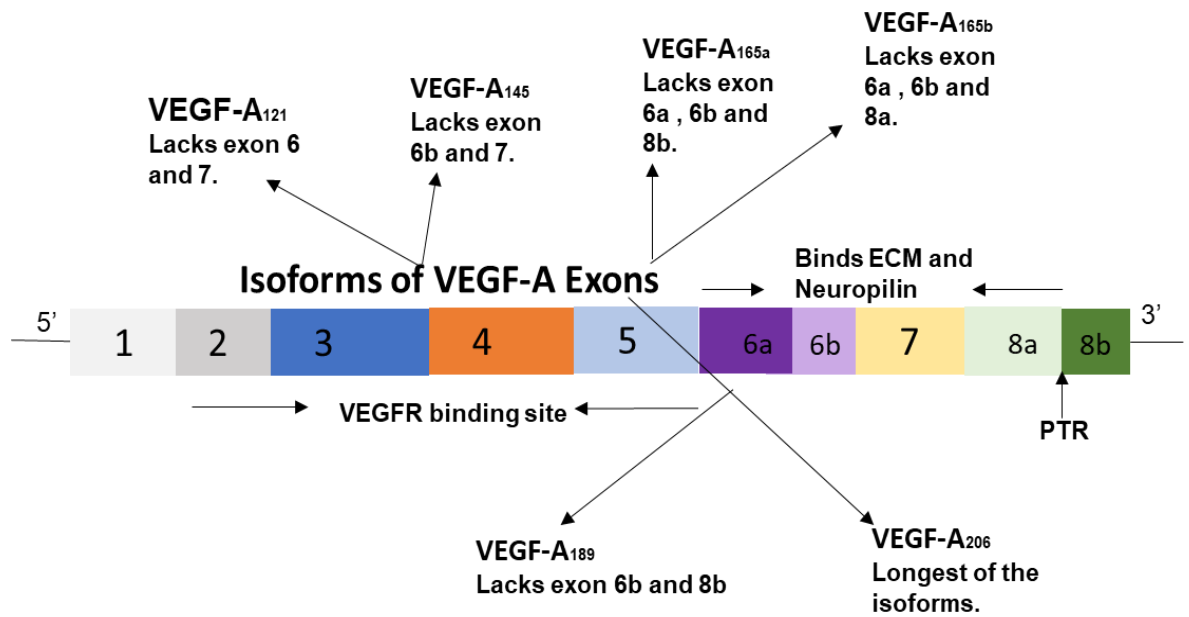
Figure 1. 8: **The VEGFA exons and its respective isoforms**

The VEGF-A has 5 main isoforms which vary in the number of exons they possess. The presence of the designated exons in each isoform determines its binding affinity. As an example, while VEGF-A$_{165a}$ is the prototypical agonist lacking exons 6a,6b and 8b and binds to both ECM and neuropilin, VEGF-A$_{206}$, the longest isoform, lacks only exon 8b. The signal sequence starts from the 5' end. VEGFR=Vascular endothelial growth factor receptor. PTR= programmed translational readthrough. ECM =Extracellular membrane.

Another splice variant of human VEGF-A known as VEGF-A$_{165b}$ has been identified. VEGF-A$_{165b}$ contains an alternative exon 8, encoding a carboxy-terminal sequence, and lacks exon 6 but has same number of amino acids as the VEGF-A$_{165a}$ variant (Bates, et al., 2013). VEGF-A is considered as the pro-angiogenesis ligand of VEGF, with the VEGF-A isoforms; VEGF-A$_{121}$, VEGF-A$_{165}$ and VEGF-A$_{189}$ being most dominant in humans (Shulman, et al., 1996; Clegg and Mac Gabhann, 2017).

VEGFs binds to their receptors, Flt-1 VEGF-receptor 1 (VEGFR1) and Flk-1/KDR VEGF-receptor 2 (VEGFR2), forming a complex with the VEGF-Rs to carry out their biological functions (Clegg and Mac Gabhann, 2017). Following the binding of VEGF to its receptor, as shown in Figure 1.9, dimerization of the intracellular domain of the VEGFRs occurs followed by activation of downstream signalling cascades which culminates in endocytosis of VE-cadherin. The VEGFR has seven immunoglobulin (Ig)-like domains (D1 to D7) at the extracellular part with D2 and D3 required for VEGFR2 and D2 for VEGFR1 binding. VEGF also requires the binding of other co-receptors; heparin (HE) or heparin sulphate (HS) and neuropilin (NRP1) for efficient signal transduction (Uciechowska-Kaczmarzyk, et al., 2018). In order to determine the expression and biological activity of VEGF and its binding sites in human glomerular endothelial cells, a study by Pala, et al. (2005) demonstrated that VEGF binds to VEGF-R2 with high affinity Kd of 0.17 ± 0.6 nM and with lower affinity to the heparan sulfate proteoglycans (HSPG) with a Kd of 9.1 ± 4.2 nM. The VEGF isoforms thus, vary in their binding affinity to VEGFR. The VEGF-A$_{165}$ and VEGF-A$_{189}$ isoforms possess the heparin-binding domain located in the extracellular matrix and the coreceptor, neuropilin-1 (NRP1) which influences and regulates VEGF-R2 affinity and trafficking (see Table 1.2). VEGF-A$_{145}$ is heparin-binding but does not bind to NRP1 while VEGF-A$_{121}$, neither binds to the ECM nor to NRP1 but rather diffuses away upon secretion into the extracellular space (Clegg and Mac Gabhann, 2017).

These VEGF isoforms mediate their action mainly through two of the transmembrane auto-phosphorylating tyrosine kinase protein receptors, Flt-1 (VEGF-R1) and Flk-1/KDR (VEGF-R2), with both possessing VEGF binding affinity depending on the VEGF isoform, as

illustrated in Table 1.2 (Carmeliet, et al., 2001; Aiello and Wong, 2000). Other characteristics of the various VEGF-A isoforms are detailed in Table 1.2.

Table 1. 2: **Characteristic of the VEGF-A isoforms**

Characteristics	VEGF-A$_{121}$	VEGF-A$_{145}$	VEGF-A$_{165}$	VEGF-A$_{189}$	VEGF-A$_{206}$
Exon splicing	Lacks exons 6 and 7		Lacks exons 6		Longest isoform
Free plasma VEGF (%)	7%	N/A	84%	9%	N/A
Basal production and secretion target	8%	N/A	77%	15%l	N/A
Binding affinity/ technique	93 nM / ITC (VEGF-R2)	1.82 nM /NanoBRET (VEGF-R2)	170 nM/ITC (VEGFR2)	1.02 nM /NanoBRET (VEGF-R2)	N/A
VEGFR1 Ligand binding	Binds to VEGF-R1	Binds to VEGF-R1	Binds to VEGF-R1	Binds to VEGF-R1	N/A
VEGFR2 Ligand binding	Binds to VEGF-R2	Binds to VEGF-R2	Binds to VEGF-R2	Binds to VEGF-R2	N/A
Heparin ligand binding	No binding	Binds heparin	Binds heparin	Binds heparin	Binds heparin
Neuropilin-1 and 2 Ligand binding	Do not bind to NRP1	Binds only to NRP2	Binds to NRP1 and NRP2	Binds to NRP1	Binds to NRP1 and NRP2
Diffusion Gradient	Readily diffusible	N/A	50–70% remains bound to ECM	The strongest ECM-binding isoform and not readily diffusible	Not readily diffusible

* Ligand binding affinity quantified as equilibrium dissociation constant (Kd) or competing ligand (Ki). Abbreviations: Bioluminescence resonance energy transfer (BRET) using NanoLuciferase (NanoBRET); isothermal titration calorimetry (ITC); surface plasmon resonance (SPR), ECM=extracellular matrix). N/A implies no data found on published literature. (Clegg and Mac Gabhann, 2017; Kilpatrick, et al., 2017; Ancelin, et al., 2004; Shulman, et al., 1996; Holmes and Zachary, 2005; Brozzo, et al., 2012).
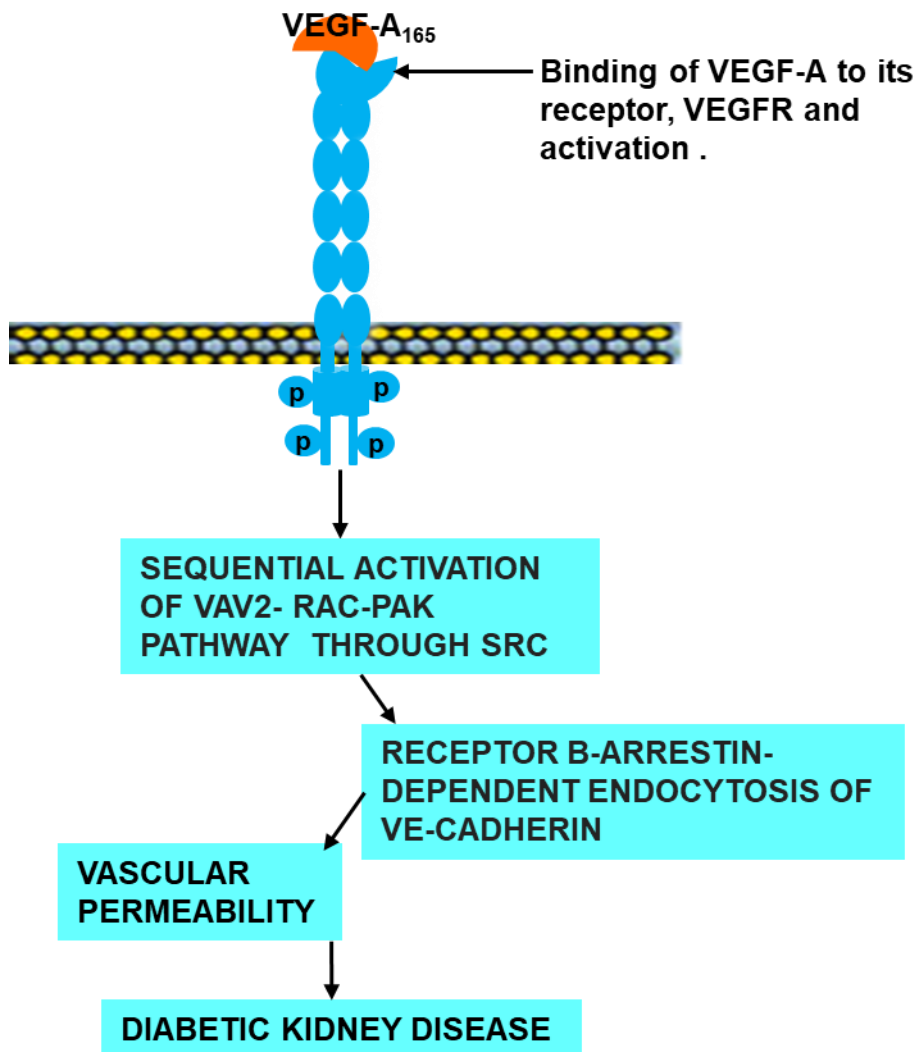
Figure 1. 9: **Representation of VEGF-A$_{165}$ binding to its receptor, VEGF-R2**

The binding of VEGF-A$_{165}$ to its receptor, VEGF-R2 leads to a cascade of signalling reaction, culminating in vascular permeability and subsequently endothelial barrier leak evident in diabetic kidney disease. VEGFR-2 = VEGF receptor 2, Src= (tyrosine kinase), p = phosphorylated, VEGF =Vascular endothelial growth factor, Vav2 = guanine nucleotide exchange factor, Rac1= Rho-GTPase, and PAK = p21-activated kinase. Therefore, since VEGF-A is a known permeability inducing agonist, it is vital to evaluate its effect in settings of diabetic kidney disease.

1.8.1 Vascular endothelial growth factor and diabetic kidney disease

Whilst VEGF-A is abundantly secreted in the podocytes, the glomerular endothelial cells express the corresponding VEGF receptors at the cell surface (Peng, et al., 2010) as previously highlighted in Figure 1.4. The VEGF-A intracellular signalling is facilitated by two tyrosine kinase-receptors, VEGF-R1 and -R2, with VEGF-R2 being the only receptor directly involved in both normal and pathological vascular permeability (Koch and Claesson-Welsh, 2012). Thus, upon expression of the VEGF-R2 in the glomerular endothelial cells, there are vital interactions between the podocytes and the glomerular endothelial cells through the VEGF-A-VEGF-R2 axis (Tanabe, et al., 2017; Satchell, et al., 2004).

Although produced in the podocytes, VEGF-A is thought to be transported across the glomerular basement membrane from podocytes to endothelial cells through diffusion, where it binds to its receptors (Tanabe, et al., 2017; Gnudi, 2012). These existing connections shows the dependency of cells on each other to regulate glomerular physiology. Hence, phenotypic alteration of these cells might lead to secondary changes to neighbouring cells. As an example, VEGF-A, which is involved in the maintenance of normal glomerular physiology is constitutively secreted in podocytes while its receptors, VEGFR-1 and VEGFR-2 are localized on the glomerular endothelial cells. However, as shown in Figure 1.9, over-secretion of VEGF-A in the podocyte and binding to its receptors in the endothelium has been implicated in diabetic glomerulopathy (Schrijvers, et al., 2004). These cell-cell interactions therefore indicate a crosstalk between the podocytes and the glomerular endothelial cells in VEGF-induced permeability. The role of VEGF in normal cells is vital but in a pathological state such as diabetes, it increases the barrier permeability of vascular endothelial cells (Sun, et al., 2014).

This permeability-inducing effect of VEGF has been demonstrated in landmark research (De Vriese, et al., 2001; Flyvbjerg, et al., 2002) with both studies pointing towards the over expression of VEGF in type 1 and type 2 diabetic animals, resulting in an increase in proteinuria and glomerular hypertrophy. An *in-vivo* study by Wanga, et al. (2019), further demonstrates the role of VEGF in the progression of DKD, with a significant difference in levels of serum VEGF and proteinuria in the case studies, when compared to the control

group. These studies therefore suggest that VEGF is also a marker of diabetic kidney disease (De Vriese, et al., 2001; Flyvbjerg, et al., 2000; Sun, et al., 2014; Wang, et al., 2019).

Renal expression of VEGF and its receptors has been upregulated in patients with T1DM and T2DM, especially at the onset of the disease (Schrijvers, et al., 2004). Increased levels of VEGF have also been observed in both human biopsy samples and in urine. In the biopsy samples, the number of extra vessels around the glomerular vascular pole was associated with upregulation of VEGF expression in the kidney. There was significant increase in urinary VEGF in T2DM patients in comparison with healthy control subjects, which correlates positively with urinary albumin-to-creatinine ratio and negatively with creatinine clearance (Nakagawa, et al., 2009). In determining the relationship between this growth factor, VEGF and DKD, other studies have shown that plasma concentration of VEGF is higher in DKD patients in comparison with healthy individuals and this increases as the disease progresses (Wasada, et al., 1998; Hovind, et al., 2000; Lenz, et al., 2003).

According to Hovind, et al. (2000) who investigated the potential role of VEGF as a predictor of progression in DKD in 155 patients with type 1 diabetes, an increase in plasma VEGF of 45.7 ng/L (22.0–410) in DKD patients was found in comparison to 27.1 ng/L (22.0–355) of normo-albuminuria subjects. Research by Santilli, et al. (2001) on adolescents and young adults with diabetes showed a 4.1% increase in VEGF serum levels (using 160 pg/ml as the arbitrary cut-off point) compared with controls at the beginning of the study; with about 10.9% of the 101 patients developing persistent microalbuminuria during an 8-year follow-up period. This body of research (Hovind, et al., 2000; Santilli, et al., 2001; Rodriguez, et al., 1998; Jelkmann, 2001; Wasada, et.al, 1998; Lenz, et.al, 2003) linking increased VEGF plasma and serum level to DKD suggests that the intracellular level of vascular endothelial growth factor is amongst the predictors for DKD. According to the work of Kanesaki, et al., (2005) and Satchell, et al., (2004), the assertion that the progression of DKD is dependent on the over-secretion of VEGF which in turn increases vascular permeability was further supported.

Few studies have targeted blocking VEGF activity as a therapy to prevent DKD, but normal endothelial cells require VEGF for other physiological activities. Therefore, substantial inhibition of VEGF could lead to endothelial injury (Nakagawa, 2009). Two studies, Eremina, et al. (2003) and Veron, et al. (2010), suggest that either too little or too much secretion of VEGF-A in the glomeruli could lead to significant renal pathology. While increased proteinuria was observed in conditional *in vivo* deletion of the *VEGF-A* gene from the podocyte (Eremina, et al., 2003), induced podocyte-specific over-secretion of VEGF-A resulted in glomerulomegaly, glomerular basement membrane thickening, mesangial expansion, permeability and proteinuria (Veron, et al., 2010). Thus, maintaining a "normal" level of VEGF in the glomeruli is important. In a pathological state, such as DKD, VEGF level has been postulated to be increased. Hence, since a balanced level of VEGF is required to halt the permeability-inducing effect of this growth factor, the development of novel agent that will neither block VEGF secretion below normal, nor lead to its over secretion is essential, as DKD continues to surge with more people developing diabetes.

In a quest to develop an alternative approach to attenuate VEGF-A induced permeability, given that inhibition of VEGF production in the podocytes exacerbates DKD, other researchers have studied other isoforms of VEGF-A to seek an alternative therapeutic mechanism. The VEGF-A$_{165b}$ variant was the isoform of interest (Oltean, et al., 2015), which was shown to have a protective effect in a DKD setting, as increasing VEGF-A$_{165b}$ levels effectively improves functional and histologic features of the disease. The study (Oltean, et al., 2015) further showed that the VEGF-A$_{165b}$ isoform achieved that result locally (through podocyte-specific over secretion) and systemically (via repeated intraperitoneal injections). Although this protective effect of VEGF-A$_{165b}$ is promising, its success in treating DKD is questionable because studies have shown that overexpression of VEGF-A could exacerbate DKD. The cost of giving repeated intraperitoneal injections of this growth factor could be another setback with rising healthcare cost, thereby making it available only to the affluent suffers of DKD. Thus, there is a need for a novel and effective therapeutic agent targeted at the attenuation of VEGF-induced permeability, rather than inhibition of VEGF in the glomerular endothelium.

Research and knowledge in the association of VEGF and DKD is not lacking. However, only few of these studies (Harrington, et al., 2018; Lizunkova, Enuwosa and Chichger ,2019) have focused on ameliorating VEGF-induced vascular permeability as a therapeutic strategy in the endothelium of other diseases characterized by barrier leak such as diabetic retinopathy. Therefore, since VEGF is an inducer of permeability in DKD, this study was focused on mimicking its induced vascular permeability associated with the disease *in vitro* whilst evaluating the protective effect of a novel therapeutic agent targeted at attenuating this leak in a cell model of the glomerular microvascular endothelium. As the effect of VEGF is mainly evident on the barrier of microvascular endothelium, it is therefore vital to consider a detailed function of the microvascular endothelium in normal and pathophysiological state.

1.8.2 The microvascular endothelium

The endothelium, a single-vessel layer that lies between the blood and vascular supply to other tissues is amongst the largest organs in the body (Xina, et al., 2019). Composed of a trillion cells, the endothelium regulates vasoconstriction and vasodilation, thereby maintaining effective vascular tone (Xina, et al., 2019). The entire human circulatory system is lined by vascular endothelial cells which actively maintains about 60,000 miles of blood vessels in the human body (Rajendran, et al., 2013; Fu, et al., 2014). The vasculature has several distinct functions ranging from hormone trafficking to organ-specific roles, such as fluid homeostasis and fluid filtration in the case of renal microvasculature. The microvascular endothelium allows the movement of the plasma constituents and restricts the passage of cellular blood components into the vascular space. These characteristic properties of the microvascular endothelial cells vary in morphology, function and behaviour depending on their location, microenvironment and shear stress of blood flow (Chi, et al., 2003; Aird, et al., 2012). Therefore, since the glomerular endothelial cells are the primary target for DKD, it is of importance to investigate their features in relation to glucose homeostasis and DKD.

1.8.3 The Impact of hyperglycaemia on glomerular endothelium

A typical example of a unique microvascular endothelium with peculiar features are those located at the glomerulus. The glomerulus filters about 180g of glucose in a day which are reabsorbed through the glucose transporters with a maximum capacity otherwise known as tubular maximum for glucose (TmG) ranging between 260-370 mg/min/1.73 m^2 ,corresponding to approximately to 11 mmol/L of plasma glucose level in a normal physiological state (section 1.6.1). However, under DKD setting, enhanced glucose uptake above the maximum capacity has been identified in many of the cell populations within the kidney such as the glomerular, mesangial and proximal tubule cells (Welsh, et al., 2010; Coward, et al., 2005). The inability of the glucose transport proteins of the glomerular endothelial cells to decrease excessive glucose entry into the cells is the plausible explanation to the susceptibility of the glomerular endothelial cells to injury resulting from hyperglycaemia (Heilig, et al., 1995; ; Alpert, et al., 2005).

Due to their constant interaction with circulation, directly receiving and responding to biochemical, metabolic and haemodynamic signals in the blood, glomerular endothelial cells have been the focus of studies into the development and progression of DKD (Wanga, et al., 2015). The glomerular endothelium exhibits several phenotypic changes in the presence of a high blood glucose concentration which includes but is not limited to altered cell membrane structures and cytoskeletal changes, causing enhanced cell monolayer permeability and disruption of the cell surface glycocalyx (Wanga, et al., 2015). These phenotypic changes in the glomerular endothelium, shows it is affected by kidney damage under hyperglycaemic condition.

Histologically, DKD involves glomerular alterations which include glomerular mesangial matrix accumulation, thickening of the glomerular basement membrane, fusion of the foot processes, expansion of mesangial cells, loss of podocytes and endothelial barrier leak when compared to normal cell (Weil, et al., 2012;Tanabe, et al., 2017; Lee, et al., 2019). Moreover, it is the primary responsibility of the glomerular endothelium to prevent all plasma proteins from extravasation. However, in hyperglycaemic conditions, an increase in the permeability of plasma proteins such as albumin occurs through a damaged glomerular

filtration barrier with the excess leakage of albumin in the urine (UAE) used as a biomarker for DKD (Figure 1.2). The glomerular microvascular endothelium thus, plays a vital role in the development and progression of DKD, thereby creating a research gap on investigating a novel agent, which will ameliorate this permeability in the glomerular endothelium.

1.8.4 Pathways involved in microvascular endothelial permeability

Amongst the damaging effect of hyperglycaemia in setting of DKD is increased barrier permeability of the microvasculature. The endothelium plays a vital role in maintaining the vasculature by regulating the transport of plasma proteins and solutes. However, when the integrity of the endothelial barrier is compromised and dysfunctional, it becomes leaky, resulting in increased vascular permeability as seen in metabolic diseases such as diabetes (Deissler, et al., 2013; Komarova and Malik 2010; Weil, et al., 2012). Microvascular endothelial permeability is mediated by two pathways, transcellular and paracellular. Transcellular permeability is mediated through specialised vesicles containing caveolin-1, which allow the passage of materials across the cell and contribute to the basal permeability of the endothelium. In contrast, paracellular permeability occurs across endothelial cell-cell junctions allowing flow of plasma, proteins and cells (Offeddu, et al., 2019). The paracellular pathway is a major contributor to pathophysiological conditions such as proteinuria associated with DKD (Kumar and Molitori, 2015; Dobrinskikh, et al., 2014). The rationale of this research is thus focused on paracellular permeability evident in glomerular endothelium.

Endothelial paracellular permeability is regulated by a large number of highly specialized transmembrane proteins (Kiuchi-Saishin, et al., 2002). These cell-cell junctions include tight junctions (TJ) and adherens junctions (AJ) proteins. The TJ transmembrane proteins include the claudin family proteins, occludin and junctional adhesion molecules (JAMs), whereas the AJ is made up of the vascular endothelial (VE)-cadherin (Ho, et al., 2018; Rincon-Choles, et al., 2006; Gavard and Gutkind, 2006). The main AJ transmembrane protein, VE-cadherin, is responsible for the intercellular cell-cell contacts between cadherins on neighbouring cells (Hartsock and Nelson, 2007). Moreover, the blocking of adherens

junction's formation inhibits correct organisation of tight junctions, implicating the importance of these adherens junctions in maintaining the barrier integrity of the microvascular endothelium (Azzi, et al., 2013; Ohsugi, et al., 1997). To further determine the importance of the VE-cadherin in endothelial barrier permeability, a study by Gory-Fauré, et al. (1999), demonstrated that the knockout of VE-cadherin *in vivo* causes death resulting from severe vascular defects. Therefore, while the tight junction proteins play a significant role in maintaining endothelial cells barriers, adherens junctions establish and maintain cell–cell adhesion, signal transduction, actin cytoskeleton remodelling and transcriptional regulation (Dobrinskikh, et al., 2014; Gavard, 2009). Hence, it is of importance to investigate the effect of the barrier-disruptive agent, VEGF on this adhesion protein, VE-cadherin, in the glomerular microvasculature.

1.8.4.1 VE-cadherin and microvascular endothelial permeability

The most important of the barrier-forming adhesive structures are the adherens junctions and an alteration in this membrane component of the endothelium underlies increased vascular permeability (Rodrigues and Granger; 2015). Although the main function of VE-cadherin is to maintain a tight barrier in the paracellular space, it has also been implicated in modulation of angiogenesis, and transcriptional regulation of claudin-5, a component of the endothelial tight junction (Gavard and Gutkind, 2008; Rodrigues and Granger; 2015). VE-cadherin comprises of one single-pass transmembrane domain, a short intracellular cytoplasmic tail and extracellular region as diagrammatically represented in Figure 1.10a. The extracellular region confers Ca^{2+} dependency and allow homophilic interaction between cadherin proteins (Azzi, et al., 2013). Whereas the cytoplasmic tail recruit's catenin proteins, mainly α-, β-, and p120-catenin to the adhesion complex, the α-catenin (actin-binding protein) of this complex anchors the VE-cadherin to the actin cytoskeleton which serves to strengthen adhesion (De Bruin, et al., 2016; Gavard, 2009; 2013; Azzi, et al., 2013).

The binding of an extracellular stimulus to the receptor causes VE-cadherin to undergo continuous assembly, disassembly, and remodelling. Additionally, as presented in Figure 1.10b, the binding of the extracellular stimuli results in downstream signalling from the receptor, triggering phosphorylation of VE-cadherin. The cell-cell adhesion property of VE-cadherin is regulated by phosphorylation and dephosphorylation of its intracellular tail. VE-cadherin phosphorylation is mediated by tyrosine kinase src protein and focal adhesion tyrosine kinase (FAK), whereas the dephosphorylating enzymes are vascular endothelial receptor protein tyrosine phosphatase, VE-PTP and Shp-2 (Timmerman, et al., 2012). Phosphorylation of the protein leads to dissociation of the AJ complex from the actin cytoskeleton and its internalisation from the cell surface (Xiao, et al., 2005; Deissler, et al., 2013).

As demonstrated by Wessel, et al. (2014), phosphorylation of VE-cadherin leads to vascular permeability. This *in vivo* study showed that tyrosine residues Tyr685 and Tyr731 of VE-cadherin selectively regulate the induction of vascular permeability through phosphorylation of these tyrosine residues. Additionally, the magnitude and duration of the extracellular stimuli determines the degree of VE-cadherin disruption and disassembly, either transient or prolonged and results in permeability. This barrier permeability is the leading cause of tissue oedema and leakage of proteins associated with DKD (Hara, et al., 2009; Kamarova, et al., 2017; Gavard, 2006; 2009; 2013; Azzi, et al., 2013). Therefore, it is vital to determine novel agents for the control of barrier function through modulation of this adhesion protein.
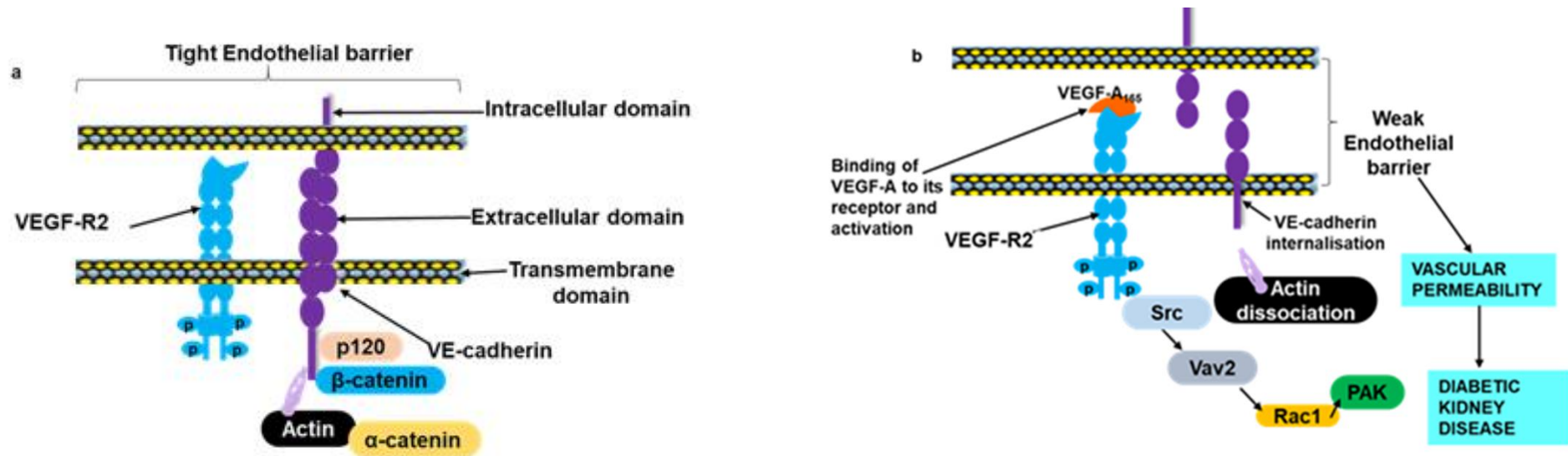
Figure 1. 10: **The assembly of the VE-cadherin cell-cell adhesion**

The diagram represents the maintenance of tight endothelial cell barrier (panel a) by the adherens protein, VE-cadherin while panel b details the

dissociation of the actin cytoskeleton through the phosphorylation of the Src-Vav-Rac-PAK pathway resulting in weak endothelial barrier and consequently

vascular leak associated with DKD. VEGFR-2 = VEGF receptor 2, Src= (tyrosine kinase), p = phosphorylated, VEGF =Vascular endothelial growth factor,

α-catenin, β-catenin,p120-catenin adhesion complex, Vav2 = guanine nucleotide exchange factor, Rac1= Rho-GTPase, and PAK = p21-activated kinase.

It is thus, evident that VE-cadherin supports the endothelium barrier integrity under normal physiological conditions. However, in a diseased state such as DKD, where hyperglycaemic condition leads to the over production of VEGF, the main extracellular stimuli in the disassembly of the AJs (Figure 1.10b), results in microvascular endothelial permeability. The vascular endothelial growth factor (VEGF) and its tyrosine kinase receptor plays a central role in the destabilisation of microvascular endothelial barrier as already detailed in section 1.8. VEGF increases the phosphorylation of VE-cadherin and associated catenin thereby inducing the disassembly of the VE-cadherin-catenin complex (De Bruin, et al., 2016). As previously mentioned in section 1.8, the binding of VEGF to its receptors (VEGF-R2) induces their dimerization, auto phosphorylation and signal transduction. VEGF-R2 also mediates phosphorylation of VE-cadherin and its accessory molecule, β-catenin and p120 through the activation of Src (tyrosine kinase), vav2 (guanine nucleotide exchange factor), Rac1, (Rho-GTPase) and PAK (p21-activated kinase), otherwise known as the Src-Vav2-Rac1-PAK pathway. Activation of the Src-Vav2-Rac1-PAK pathway leads to internalisation of VE-cadherin, endothelial barrier disruption and consequently an increase in vascular endothelial barrier permeability (Deissler, et al., 2013; Gavard, 2013; Shibuya, 2011; Koch and Claesson-Welsh, 2012). Endothelial microvascular integrity is thus maintained by the cell-cell adhesion of VE-cadherin (Garrett, et al., 2016). However, there are other molecular mechanisms associated with microvascular endothelial permeability such as cyclic adenosine monophosphate and reactive oxygen species.

1.8.4.2 Cyclic AMP and endothelial microvascular permeability

In addition to cell-cell adhesion molecules, another modulator of the endothelial barrier system is a second messenger, cyclic adenosine monophosphate (cAMP). Cyclic AMP is a secondary messenger constantly formed in most cells, including the endothelial microvasculature, that plays important role in the modulation of endothelial barrier function (Liu, Jiang and Grange, 2005). Cyclic AMP is generated by adenylyl cyclase, a membrane-associated enzyme following activation of G-protein coupled receptors (GPCR) as shown in Figure 1.11. Activation of the GPCR could be by exogenous (e.g., drugs, xenobiotics and germs) or endogenous (e.g., hormones, inflammatory mediators and neurotransmitters) stimuli while degradation of cAMP is modulated by phosphodiesterase (PDE) (Tsvetanova and Zastrow, 2014; Zhu, et al., 2004).

The accumulation of cAMP in endothelial cells can either destabilize or preserve the endothelial barrier, based on duration of cAMP generation (Perrot, Sawada and Masanobu, 2018). In addition, the level of intracellular cAMP depends on the balance between the activities of membrane-associated adenylyl cyclases (ACs) and the phosphodiesterases (PDEs) (Abaffy, Trubey and Chaudhari, 2003). Figure 1.11 shows that cAMP maintains the barrier integrity of the endothelial cell membrane through various signalling mechanisms that serve as vital modulators of vascular permeability. These modulators include the phosphorylation of myosin light-chain kinase (MLCK), activation of cAMP-dependent protein kinase A (PKA) and exchange protein activated by cAMP (EPAC1) (Fukuhara, et al., 2005). Cyclic AMP activation of EPAC leads to Rap1 activation through the PKA-independent pathway and subsequently results in reorganisation of the actin molecules and other junctional proteins associated with cell-cell adhesion (Figure 1.11) (Ramos, et al., 2018; Gunduz, et al., 2019). Therefore, since intracellular increase of cAMP promotes barrier integrity through structural organisation of VE-cadherin, agonists that elicit increased level of cAMP could attenuate endothelial barrier permeability and reduce the progression of DKD.
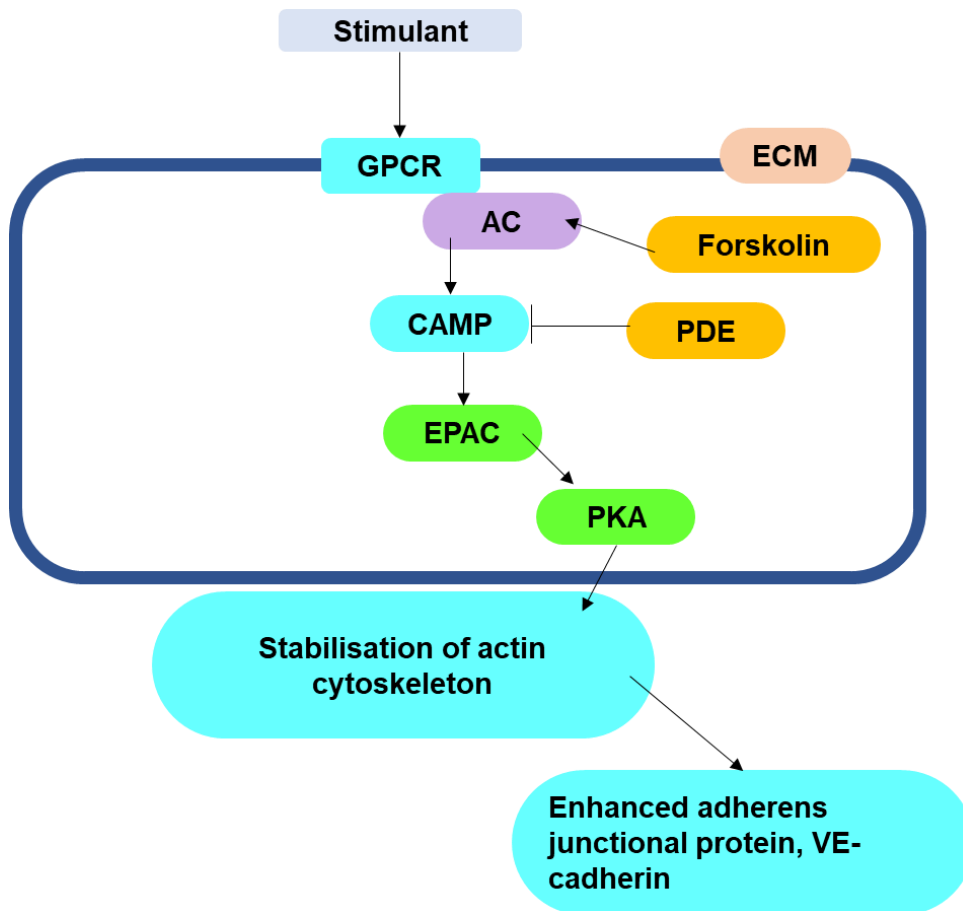
Figure 1. 11: **Cyclic AMP enhances endothelial cell-cell interaction**

A diagrammatic representation of enhanced endothelial cell junctional protein interaction by cyclic AMP through stimulation of the GPCR. A known activator of the adenylyl cyclase enzyme is forskolin while PDE modulates cAMP degradation. cAMP= cyclic adenosine monophosphate, PKA= cAMP-dependent protein kinase A, EPAC = exchange protein activated, PDE=, AC = adenylyl cyclase. GCPR = G-protein-coupled receptors, ECM= endothelial cell membrane.

1.8.4.3 Reactive oxygen species and microvascular endothelial permeability

Another molecular mechanism linked to the permeability of the microvasculature is increased level of reactive oxygen species which leads to oxidative stress. Reactive oxygen species (ROS) under physiological conditions, can act as intracellular second messengers through modulation of proangiogenic pathways such as VEGF-A signalling and postnatal vasculogenesis (Colavitti, et al., 2001). However, as discussed in section 1.7.1.4, an alteration of redox equilibrium through prolonged increase in ROS production or inefficient antioxidant mechanisms can lead to pathological processes that negatively affect vessel growth (Ebrahimian, et al., 2009).

The two major sources of ROS in the diabetic kidney are NADPH oxidase (Nox) (enzymatic) and mitochondrial electron transport chain (non-enzymatic). These sources of ROS have been implicated in the onset of protein leakage and progression to renal injuries through the signalling of other pathways (Lin, et al., 2010; Gorin, et al., 2005; Nakagawa, 2008). More so, ROS affects all layers of the glomerular barrier; the endothelium, glomerular basement membrane and the podocytes (Lin, et al., 2010). Under hyperglycaemic condition, the over production of NADH (in the cytosol) serves as a substrate to produce superoxide (section 1.7.1.4) and its metabolite, hydrogen peroxide (H_2O_2) (El-Daly, et al., 2018). As shown in Figure 1.12, any dysregulation in the transfer of these electrons by the complexes (in the mitochondria), results to electron leakage and reduces O^2 to superoxide (O_2^-) (St-Pierre, et al., 2002). Therefore, an impairment of the intracellular glucose homeostasis observed in DKD, results in excessive generation of the $NADH/FADH_2$, increased electron leakage, ROS production and consequently oxidative stress.
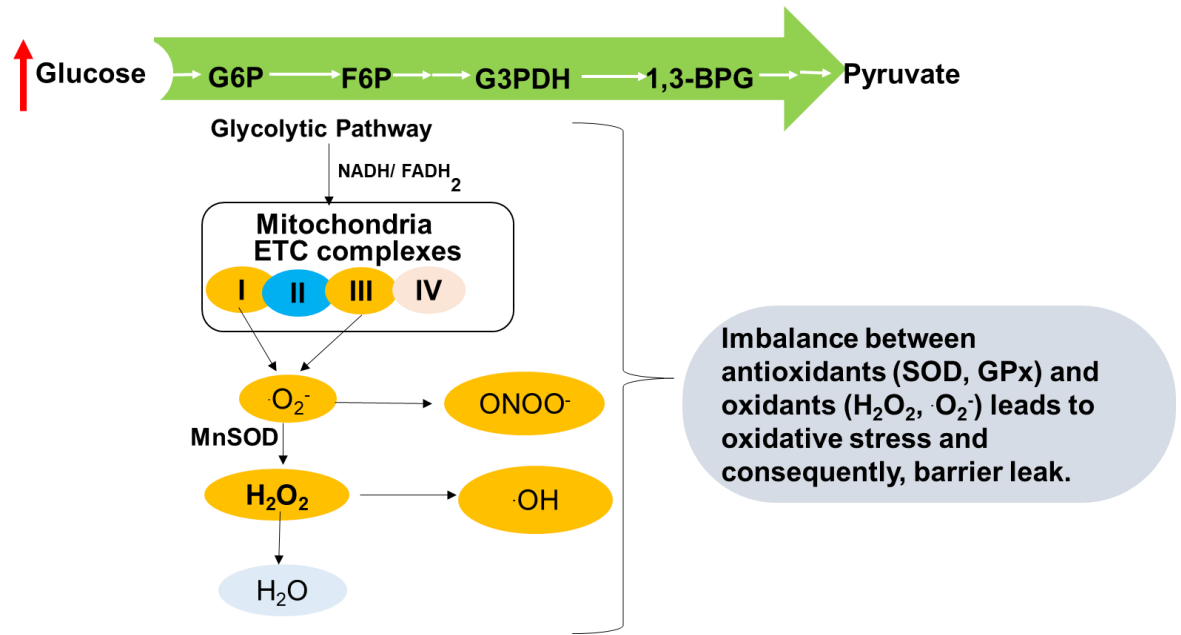
Figure 1. 12: **Renal generation of ROS and barrier leak in DKD**

The Figure highlights ROS generation and its effect in endothelial barrier permeability associated with DKD. FADH= flavin adenine dinucleotide; NADH = nicotinamide adenine dinucleotide (reduced state); GPx = glutathione peroxidase; $\cdot O_2^-$ = superoxide radical; H_2O_2 = hydrogen peroxide; ETC = electron transport chain; MnSOD = manganese superoxide dismutase; H_2O = water; $ONOO^-$ = peroxynitrite radical, $\cdot NO$ = nitric oxide; $\cdot OH$= hydroxyl radical.

Under settings of DKD, the glomerular endothelium is affected by oxidative damage. As earlier shown in Figure 1.4, the glomerular cell comprises of three layers which are in crosstalk with each other, working collectively in maintaining glomerular barrier integrity. Therefore, if a layer is dysfunctional, the others will be affected. Hence, the overproduction of ROS and depletion of antioxidants in the endothelial cells affects the luminar surface coating of the cell (glycocalyx). This glycocalyx possess the heparan sulphate which gives the endothelial surface layer its anionic charge (Rops, et al., 2014). The endothelial glycocalyx is affected by ROS, leading to a decrease in the anionic charges and increased permeability of macromolecules (Singh, et al., 2013). This effect of ROS on the endothelial glycocalyx was demonstrated by Singh, et al. (2013) *in vitro* where ROS causes significant loss of heparin sulphate and consequently, increases trans endothelial albumin passage. Another way in which ROS can affect vascular permeability associated with DKD is through the glomerular podocyte (Liu, et al., 2013). The podocyte aids in maintaining the endothelial barrier by repelling against anionic albumin, preventing easy passage of proteins due to its possession of anionic surface protein, podocalyxin (Hara, et al., 2012). The podocyte does not only repel against albumin, but also establishes the crosstalk between the other layers, GBM and endothelial cells through secretion of mediators such as angiopoietin-1 and VEGF (Satchell, Anderson and Mathieson, 2004). Hence, damage to the podocyte, impairs glomerular selective permeability and leads to dysfunction of the other layers and consequently progression of DKD.

The effect of ROS on the glomerular podocyte through dysregulation of the redox signalling causes podocyte detachment or apoptosis which precedes its depletion and urinary albumin excretion (UAE) as demonstrated in a study by Susztak, et al. (2006). Additionally, studies have linked ROS generation to elevated tyrosine-phosphorylation of the VE-cadherin and consequently the dissociation of the VE-cadherin catenin complex, resulting in loss of cell-cell adhesion (Monaghan-Benson and Burridge, 2009; Rhee, et al., 2000). The effect of oxidant stress on the adherens junctional proteins was further demonstrated by Gong, et al. (2014) wherein ROS activates Src-pathway, promoting dissociation of p120-catenin from VE-cadherin, internalization of VE-cadherin and consequently, barrier leak.

Hence, although, the adhesion protein VE-cadherin has the principal role of maintaining endothelial permeability, its integrity is dependent on the catenin complex of the actin cytoskeleton. This complex is in turn, dependent on other signalling cascade factors such as the GTPase (Rac1), which are in turn affected by oxidative stress (Gong, et al., 2014).

Taken together, ROS has been shown to play a role in pathogenesis and progression of DKD through alteration of adhesion protein signalling mechanisms in the glomerular endothelial compartments. Therefore, finding novel therapies that mitigate the effect of excessive ROS generation might improve cell-cell adhesion protein, VE-cadherin and subsequently reduce the glomerular microvascular endothelial leak observed in DKD.

1.9 Treatment for diabetic kidney disease

There are multifactorial strategies for the prevention, and or slowing the progression of diabetic kidney disease which hinges on "five carriages"; diet, exercise, weight control, lowering of blood glucose and medications (Barret, et al., 2017), with the first four referred to as nonpharmacological measures. As a diagnostic criterion for DKD, maintaining normal eGFR and decreasing proteinuria, remains the main target for slowing the progression of DKD to its advanced level, known as end-stage kidney disease (ESKD) (Barret, et al., 2017).

1.9.1 Novel therapies and approaches for the treatment of diabetic kidney disease

Despite many approaches to the management of diabetes and DKD, there is still a residual risk with novel agents targeting mechanisms, such as, inflammation and glucose reabsorption as outlined in Table 1.3. Some of these drugs are canagliflozin, ruboxistaurin, finerenone and atrasentan (Perkovic, et al., 2019; González-Blázquez, et al., 2018; Tuttle, et al., 2007; Heerspink, et al., 2019; De Zeeuw, et al., 2014).

Table 1. 3 : **Current and proposed treatment for diabetic kidney disease**

Drug/ Treatment	Molecular target	Trials	Efficacy	Side effects
Canagliflozin	SGLT2 inhibitor	CREDENCE	FDA approved (2019)	Increased risk of amputation
Atrasentan	Endothelin A receptor antagonist	Phase 3 clinical trial (SONAR project)	N/A	Peripheral oedema and worsening of heart failure
Ruboxistaurin	Protein kinase C-β inhibitor	N/A	N/A	N/A
Finerenone	Selective nonsteroidal mineralocorticoid receptor antagonist	Phase 2 clinical trial	N/A	N/A

(CREDENCE= Canagliflozin and Renal Events in Diabetes with Established Nephropathy Clinical Evaluation; SGLT2 =sodium glucose cotransporter 2).

Amongst these drugs outlined in Table 1.3, only Canagliflozin was recently approved for treatment of DKD in T2DM patients but not without adverse side effects such as increased bone fracture and amputation within 12 weeks of administration (Perkovic, et al., 2019). The other promising drug in slowing DKD progression is Atrasentan, which has gone through a phase 3 clinical trial (SONAR project) (Heerspink, et al., 2019). According to the SONAR project research, Atrasentan reduces risk of renal events in patients with DKD and those at high risk of developing end-stage kidney disease. The side effects present with the use of Atrasentan include significant increased weight gain, excess risk of peripheral oedema, reduction in haemoglobin and worsening of heart failure in test subjects compared to placebo (De Zeeuw, et al., 2014; Heerspink, et al., 2018; 2019). The remaining agents have demonstrated potential efficacy only in small studies and when tested in larger longer studies, some of their putative reno-protective effects were not reproducible. Thus, the search for novel and effective therapeutic agents for managing and treatment of DKD continues.

Other therapeutic studies in the management and treatment of DKD have focused on molecular markers linked to DKD such as VEGF as treatment options for the disease. VEGF is an inducer of permeability in the glomerular endothelium with increased levels observed in DKD patients compared to placebo. Hence, a VEGF antibody, bevacizumab was developed as a therapeutic agent for vascular diseases characterised by permeability such as diabetic retinopathy (Abdallah and Fawzi, 2009; Ferrara, et al., 2004). Considering the relative effect of VEGF-A in the development and progression of DKD, some studies (Advani, et al.,2007; Eremina, et al., 2003) investigated the effect of anti-VEGF agent (bevacizumab) and VEGF-receptor inhibitor (vandetanib) on preventing and delay of DKD. Whilst bevacizumab, caused renal thrombotic microangiopathy partly due to endothelial injury in patients (Eremina, et al., 2003), VEGFR-2 inhibitor, vandetanib exacerbated hypertension and renal disease rather than ameliorating DKD (Advani, et al., 2013; 2007). These studies therefore suggest that inhibition of VEGF signalling as a therapeutic strategy for the management and delay in progression of DKD accelerates endothelial injury and not a plausible treatment strategy.

Another approach to managing DKD is improving glycaemic control, and, because of the challenges in the treatment of DKD, intensive glycaemic control from early in the course of diabetes is essential for preventing its development and progression (Wanga, et al., 2015). Moreover, as demonstrated by the UKPDS, DCCT and subsequent Epidemiology of Diabetes Interventions and Complications (EDIC) trial studies, maintaining tight glycaemic control is a prerequisite in managing the disease. As previously discussed in section 1.7.1.1, the DCCT trial shows the delayed development and progression of microalbuminuria due to intensive glucose control in T1DM, whereas the UKPDS supports the finding in T2DM patients (Barret, et al., 2017). Another randomized controlled trial, EDIC, further demonstrated that better glycaemic control reduces the risk of developing DKD, while the UKPDS shows significant prevention of microvascular events during 10 years of post-trial follow-up (Gnudi, 2012). Based on these landmark studies, the primary target of managing and delaying the progression of DKD, therefore, is to control blood glucose level.

Since maintaining glycaemic control close to normal range has been proven to delay the progression of DKD, there has been a shift towards the use of sugar alternatives such as artificial sweeteners. The primary aim of using these sugar alternatives centres on lowering blood glucose level whilst enjoying the satisfaction of sweet taste sensation. The increase in the use of artificial sweeteners has prompted several studies into their effects as sweet taste agonist (Liu, et al., 2011). The discovery of these G-protein coupled receptor stimulants (artificial sweeteners) led to their targets as treatment for microvascular diseases associated with vascular permeability (Nuemket, et al., 2017; Pepino and Bourne, 2011; Kamal, et al., 2017). Hence, the need for an innovative therapeutic strategy for maintaining the barrier integrity of the glomerular microvasculature without effects linked with those of VEGF inhibition, such as targeting the sweet taste receptors for the management and treatment of leak in DKD.

1.10 Taste receptors

The sense of taste aids in the recognition of pleasurable and sweet substances around us while developing our discriminatory power to avert toxic and harmful substances. Although, taste signalling involves various cascades of events, it starts with the binding of tastant molecules and their recognition by the taste receptors. Sugars, amino acids, alkaloids and minerals in food act as tastants, binding to their corresponding taste receptors and inducing either sweet, sour, umami, bitter or savoury tastes. While the bitter taste receptors lead to aversion and discourage ingestion of toxic substances, sweet taste receptors aid in recognition, promotion and facilitation of nutrient utilization in nutritionally rich foods (Zhang, et al., 2003). The taste sensation begins in the papillae of sensory cells located at the surface of the tongue. The papillae are composed of 40-140 cells made up of precursor cells, taste receptor cells (TRCs) and support cells (Lindemann, 2001). In humans, the sweet taste receptor, belonging to the class C of the G-protein coupled receptor (GPCR) is made up of taste 1 (T1R) receptor (Mahalapbutr, et al., 2019). In contrast to the sweet taste receptors, the recognition of many bitter substances requires several class A GPCRs (Chandrashekar, et al., 2006). Therefore, the binding of any agonist, bitter or sweet has its

specific receptors and for the purpose of this research, the focus was on sweet taste receptors.

1.10.1 Sweet taste receptors

Glucose, fructose, sucrose, and other sugars elicit a distinctive perceptual quality termed sweetness in humans (Fujiwara, et al., 2012). The sweet taste receptor is a specialised network of transmembrane protein involved with the transmission of information from sweet molecules such as natural and artificial sweeteners into the intracellular domain (Welcome, Mastorakis and Pereverzev, 2014). In humans, the sweet taste receptor, belongs to the T1R class C of GPCRs as previously stated. The T1R has two subtypes, the T1R member 2 and member 3 (T1R2 and T1R3) which forms a heterodimer to act as sweet taste receptor (Mahalapbutr, et al., 2019).

The heterodimeric T1R2 and T1R3 sweet taste receptors binds to the large extracellular venus fly-trap domain (VFTD), linked by a short cysteine-rich domain (CRD) to an α-helical transmembrane domain (TMD) of the GPCR (Pin, Galvez and Prezeau, 2003; Kim, et al., 2017; Mahalapbutr, et al., 2019). Sweet taste receptors can act as either homo or hetero-dimeric receptors (Fujiwara, et al., 2012). These taste receptors are coupled to secondary messenger enzymes, transducin, gustducin and Gαi2. The gustducin comprises of 3 subtypes; α-, β- and γ-gustducin making it a heterotrimeric G-protein (DeVree, et al., 2016).

Upon the binding of sweet taste activators, heterotrimeric gustducin dissociates into its α and βγ subunits with the βγ-subunit activating the phospholipase $C\beta_2$ (PLC β_2) as Figure 1.13 depicts. The activation of phospholipase $C\text{-}\beta_2$ leads to the formation of 1,4,5-triphosphate (IP_3) through the hydrolysis of phosphatidylinositol 4,5-bisphosphate (Masubuchi, et al., 2013). The IP_3 triggers release of calcium from the endoplasmic reticulum, increasing intracellular calcium levels which triggers the transient receptor potential channel (TRPM5), a non-selective cation channel and consequently influx of sodium ions and membrane depolarisation. This then allows the release of ATP through the pannexin-1 hemichannels which is stimulated by the neighbouring presynaptic cells (type

III) leading to excitation of the afferent fibres, followed by taste sensation (Masubuchi et al., 2013).

Binding of sugars as shown in Figure 1.13 leads to activation of the α-gustducin which increases the intracellular level of cAMP through activation of adenylyl cyclase. Conversely, the binding of bitter tastants evokes the activation of a cAMP hydrolytic enzyme, phosphodiesterase, leading to a decrease in cAMP intracellular levels (Gilbertson, et al., 2000). Thus, elevated level of cAMP, following activation of the sweet taste receptor, aid in maintaining the barrier integrity of the microvasculature (section 1.8.4.2). Therefore, this provides a rationale for targeting sweet taste receptors as an alternative treatment approach for permeability in DKD.
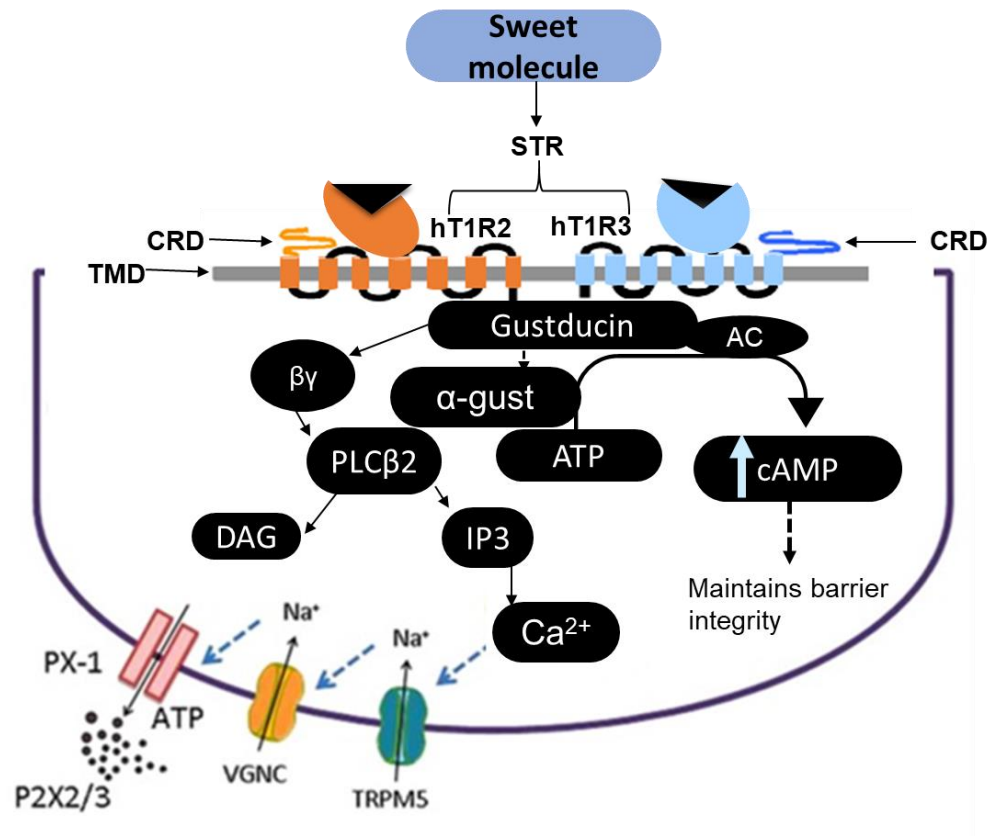
Figure 1. 13:**Representation of sweet taste perception**

The binding of sweet molecule to the sweet taste receptors (hT1R2/3) activates the G-protein (gustducin) followed by downstream signalling cascades that leads to taste perception. AC = adenylyl cyclase, CRD = Cysteine residue domain, STR =sweet taste receptor; ATP = adenosine triphosphate; ECM = Extracellular membrane; TMD = transmembrane domain; IP_3 = inositol 1,4,5-trisphosphate; PLCβ2 = phospholipase C isoform β2; DAG = diacylglycerol; PDE= phosphodiesterase, PX-1 = pannexin 1-hemichannel, hT1R2 = human sweet taste receptor, type 1 member 2, hT1R3 = human sweet taste receptor, type 1 member 3, VGNC = voltage-gated Na^+ channel, TRPM5 = transient receptor potential cation channel M5 (Adapted from Masubuchi, et al., 2013; Lee and Owyang, 2017).

Since the discovery of sweet taste receptors, they were thought to be localised in the tongue. However, subsequent studies have shown that the sweet taste receptors are expressed in many extraoral locations including the eyes, lungs, adipose tissue, colon, liver, leukocytes and gut (Lizunkova, Enuwosa and Chichger, 2019; Harrington, et al., 2018; Masubuchi, et al., 2013; Rozengurt, et al., 2006; Kiuchi, et al., 2006; Malki, et al., 2015; Margolskee, et al.,2007). These studies therefore suggest the existence of sweet-taste sensing in other parts of the body. It has been suggested that differences in sweet taste perception with molecular species depend on structural variations of the taste receptor (Jiang, et al., 2005). However, Acevedo and Temussi (2019) demonstrated that, irrespective of the high number of sweet molecules available, all are recognized by the class C GPCR heterodimer, T1R2 and T1R3, in humans. Therefore, while several processes are involved in taste sensing, from the binding of the sweet taste molecule to its perception as a sweet molecule, the initial step lies in recognition of the sweet taste agonist by the receptors.

1.10.2 Natural and artificial sweeteners as agonists of sweet taste receptors

The C-GPCR family can bind many of its agonists, be they natural or artificial. These agonists range from six-carbon simple sugars to large peptides, polypeptides and guanidino acetic acids. Whilst the GPCRs act as a T1R2/T1R3 heterodimer, the T1R3 subunit can act alone as a homodimer (Jiang, et al., 2005). The expressed T1R2/T1R3 heterodimer is activated by a broad spectrum of structurally diverse natural sugars such as glucose, fructose, sucrose and maltose, or sweet amino acids, D-serine, D-tryptophan and D-phenylalanine. In addition, sweet tasting proteins, brazzein, thaumatin and monellin, and artificial sweeteners; aspartame, acesulfame-K, cyclamate, sucralose, neotame and saccharin are all agonists for the T1R2/ T1R3 receptors (Jiang, et al., 2005).

Whilst both natural and artificial sweeteners could activate the sweet taste receptors, the binding of natural sugars to the sweet taste receptors requires very high concentrations >300 mM, which are detrimental to the viability of the target cells and organs. In contrast, lower concentrations (<1 mM) of artificial sweeteners activates the sweet taste receptors

(Li, et al., 2002; Fujiwara, et al., 2012). Although some of the natural sweeteners possess very low amounts of calories, their intake by individuals living with DKD could exacerbate the progression of the disease. In addition, there is evidence that natural sugars such as sucrose is a culprit in the development of diabetes and its complications, with other studies linking these sugars to increased vascular permeability (Hoshi, et al.,2002; Doronzo, et al., 2012). Therefore, it is impractical to use natural sweeteners such as sucrose as activators of the GPCR when seeking to develop a novel agent for ameliorating leak associated with DKD. Hence, the need to evaluate the impact of artificial sweeteners in setting of DKD.

1.11 Artificial sweeteners
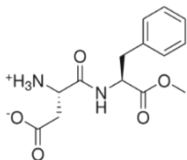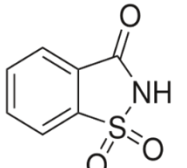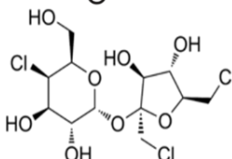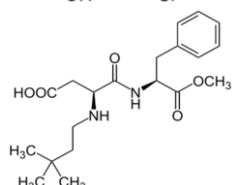
The term 'artificial sweeteners' (AS) refers to regulated substances obtained by chemical synthesis, subject to safety evaluation prior to market authorisation and used in the food industry for their high sweetening power and low caloric or no calorie content (EFSA, 2011; Bruyere, et al., 2015). Artificial sweeteners are readily absorbed into circulation with most excreted unmetabolized unlike normal sugars such as glucose (Roberts, 2000).

In recent years, the impact of added sugar on increasing incidence of diabetes and its complications, has led to a shift towards the use of sugar substitutes like artificial sweeteners (Montonen, et al., 2007; Schulze, et al., 2004). In 2011, the European Food Safety Authority (EFSA) endorsed the use of some non-nutritive sweeteners, including artificial sweeteners to reduce calories intake. Study has also shown that non-nutritive sweeteners does not affect glycaemic control when consumed in amounts below the acceptable daily intake (McKenzie, et al., 2013). These artificial sweeteners are widely used today and can be found in many soft drinks, chewing gums, yogurts, and are sometimes used as drug excipients (Lourdes, et al., 2019). The most commonly used and commercially available artificial sweeteners with their trade names are acesulfame–K (Sunnet), aspartame (NutraSweet), saccharin (Sweet N' Low), sucralose (Splenda) and a high potency sweetener, neotame (Newtame). These sweeteners vary in their structure, intensity of sweetness, after-taste and pharmacokinetics, as highlighted in Table 1.4. In

relation to this research, three commercially available artificial sweeteners; aspartame, saccharin, sucralose and a high-potency sweetener, neotame were investigated based on their non-cytotoxic effect *in vitro* as demonstrated by Rahiman and Pool, (2014).

Table 1. 4: **Comparison of commonly used artificial sweeteners**

adapted from Magnuson et al., (2016); Gardner, et al., (2012) , (Open chem. Database)

Sweetener/Brand name	Compound classification	Sweetener Structure	Sweetness intensity compared to sucrose	Molar mass (g/mol)	Representative amount of sweetener in 340 ml Soda, in mg (µM)
Aspartame/ Nutra sweet	Methylated dipeptide		200X	294.3 g/mol	187 (635 µM)
Saccharin/Sweet N'Low	Benzoic acid sulfimide		300X	183.19g/mol	8 (blended with aspartame) (43 µM)
Sucralose/Splenda	Chlorinated disaccharide		600X	397.64g/mol	68 (171 µM)
Neotame/Newtame	Methyl ester		6000X	378.5 g/mol	Not yet used in carbonated drinks

The equivalent amount in µM in table 1.4 was calculated by dividing the number of sweeteners present in a can of soda (mg) by the relative molecular mass (RMM) molar mass of the sweetener. As an example, 187 mg (187,000 µg of aspartame was present in a can of soda, and the RMM of aspartame is 294.3 = 187,000/294.3 = 635 µM.

1.11.1 Metabolism of selected artificial sweeteners
1.11.2 Artificial sweetener: Aspartame

The most common of these sweeteners that activates sweet taste receptor is aspartame. Aspartame (also codified as E951) is a white, odorless crystalline molecule, a methyl ester of a dipeptide containing 2 amino acids; *L*-aspartic acid and *L*-phenylalanine. This low-calorie sweetener is widely used as a tabletop sweetener and found in a variety of foods and beverages, including but not limited to yogurt, desserts, nutritional bars and chewing gums, with a calorie content of approximately 4 per gram (Magnuson, et al., 2016). The acceptable daily intake (ADI) of aspartame is 40 mg/kg. This number is generated by dividing the "no observable adverse effect level" (NOAEL) by an uncertainty factor of 100 following long-term toxicology studies. Hence, based on the amount of aspartame found in 340 ml soda (187mg), an average 60 kg adult would have to consume about 12 cans of aspartame sweetened soda per day to reach their ADI (Magnuson, et al., 2016 ; Gardner, et al., 2012). While it is rare to ingest that amount of aspartame in a day, if it does occur, then such an individual would only be consuming the lowest dose without any observed negative effect which is 1/100th of its NOAEL (Marina, et al., 2013).

Following ingestion, aspartame is hydrolyzed in the intestinal lumen into its components; aspartic acid (40%), phenylalanine (50%) and methanol (10%). Thus, aspartame does not reach the general circulation as the intact molecule (Butchko, et al., 2002; Magnuson, et al., 2016). These metabolites are then absorbed into the blood and each is metabolized accordingly. It has been hypothesized that metabolites of this sweetener do not accumulate in the body. The aspartame metabolite, methanol produced through the breakdown of aspartame enters circulation followed by oxidation in the liver to formaldehyde by an alcohol dehydrogenase enzyme (Trocho, et al., 1998). Formaldehyde dehydrogenase then converts formaldehyde to formic acid with no accumulation of formaldehyde since it has a half-life of less than 2 minutes (EFSA, 2014). Formic acid is converted to carbon dioxide (CO_2) and water (Dorokhov, et al., 2015, Magnuson, et al., 2007).

These metabolites of aspartame are utilized by the body in a similar manner as when they are derived from common foods such as non-fat milk and tomato juice, with a glass of tomato juice containing six times the amount of methanol derived from aspartame. (Aspartame Information Center, 2006; Marinovich, et al., 2013; Butchko, et al., 2002). Although there are controversies over the use of aspartame as sugar substitute, a study by Magnuson, et al. (2007) demonstrated that aspartame consumption has no effect on other blood constituents such as glucose, amino acids and methanol.

As an artificial sweetener, aspartame activates the heterodimeric sweet taste receptor, T1R2/T1R3, with studies indicating its specificity lies on the hT1R2 binding pocket in the VFTD extracellular domain of the taste receptors, as shown in Figure 1.14 (Temussi, 2007; Xu, et al., 2004; Maillet, et al., 2015). Two independent studies have shown that aspartame activates the sweet taste receptor at concentrations less than 1 mM (Masuda, et al., 2012; Fujiwara, et al., 2012). While aspartame elicits sweet taste in humans through activation of the T1R2, it is insensitive to the T1R2/T1R3 of other species (Xu, et al., 2004; Liu, 2011). Therefore, the availability, lack of effect on blood glucose and ability to activate the sweet taste receptor at low concentrations makes aspartame a suitable choice as an agonist of the sweet taste receptor.

1.11.3 Artificial sweetener: Saccharin

Saccharin, (also known as E954), the least expensive of the artificial sweeteners, is the worldwide market leader as a tabletop sweetener (Sylvetsky and Rother, 2016). Saccharin is produced through the oxidation of o-toluenesulfonamide, leaving a bitter and metallic aftertaste (Kuhn, 2004). The ADI for saccharin is 5 mg/kg and applying similar calculation to aspartame as shown in Table 1.4, a 60 kg individual would need to consume more than 37 cans of soda containing saccharin to achieve its ADI (Gardener, et al., 2012). As a sweet taste agonist, saccharin binds to T1R2/T1R3 with its specificity at the extracellular domain of the sweet taste receptor. According to Masuda, et al. (2012), < 0.2 mM of saccharin triggers activation of the T1R2/T1R3 receptors (see Figure 1.14).

Saccharin is usually utilized as a blend with other sweeteners to mask the after taste. In line with this, a study by Behrens, et al. (2004), indicates that saccharin is often blended with cyclamate because it inhibits the bitter taste receptors stimulated by cyclamate and in turn, cyclamate reduces the off taste of saccharin. While there are two commercially available forms of saccharin; pure and sodium saccharin (Rehn, et al., 2018), the one utilised in this research was pure saccharin. Although there are concerns over the safety of saccharin, its use as an artificial sweetener was approved by the ESFA after rigorous studies (ESFA, 2019) and other independent studies demonstrated the safety of saccharin as an alternative to table sugar.

Upon ingestion of saccharin, aproximately 85% to 95% is absorbed and eliminated in the urine, which is the principal method of plasma clearance, and the remainder is excreted in the faeces (Magnuson, et al., 2016). Therefore, based on these features of saccharin, very low concentrations are required to activate the T1R2/T1R3 and as a commonly used tabletop sweetener, it was selected to determine other physiological effects it might elicit, such as a potential agent for regulating endothelial barrier function.

1.11.4 Artificial sweetener: Sucralose

Discovered accidentally in 1976 by Shashikant Phadnis, sucralose is an artificial sweetener obtained from sucrose. Sucralose is formed by replacing three hydroxylic groups, from positions 4, 1 and 6 in sucrose with chlorine atoms (Ophardt, 2003; Knight, 1994). Sucralose, also known as E955 with an ADI of 15 mg/kg and a sweetening power 600 times that of sucrose (Table 1.4). Sucralose activates the sweet taste receptors T1R2/T1R3 at concentrations <0.08 mM (Magnuson, 2016; Masuda, et al., 2012).

In a similar manner to other artificial sweeteners, sucralose is not exempt from controversial studies about the effects of its consumption in humans (Sylvetsky, et al., 2017). However, some pioneer studies have shown that sucralose is not well absorbed in humans, undergoes little metabolism, and excreted primarily unchanged in faeces (Roberts, et al., 2000; Grotz, et al., 2000; 2009; Baird, et al., 2000).

According to a study by Baird, et al. (2000), sucralose does not accumulate in the blood even at prolonged ingestion and high doses, with complete elimination after 24 hours. Furthermore, sucralose ingestion does not affect other biochemical parameters such as glucose homeostasis and insulin levels (Grotz, et al., 2017; Brown, et al., 2011). In contrast to aspartame, which is metabolized in the body, sucralose cannot be cleaved by glycosidic enzymes that hydrolyze sucrose and other carbohydrates due to difference in the conformation of sucralose (Grotz and Munro, 2009; Grotz, et al., 2000; Magnuson, et al., 2016). Therefore, sucralose was selected for this research as it does not accumulate in the body following ingestion and binds with high affinity to the T1R2/T1R3 sweet taste receptor.

1.11.5 Artificial sweetener: Neotame

Neotame, a white/off-white powder, chemically related to aspartame and manufactured from aspartame and 3, 3-dimethylbutyraldehyde in a one-step reductive alkylation reaction, with chemical name, N-[N-(3,3-dimethylbutyl)-L-alpha-aspartyl]-L-phenylalanine-1-methylester. Upon oral administration, approximately 20–30% of neotame is absorbed and rapidly converted to its major metabolite, N- [N-(3, 3-dimethylbutyl)-L-alpha-aspartyl]-L-phenylalanine (de-esterified neotame) and an insignificant amount of methanol (JCEFA, 2004). Neotame, a high-potency sweetener with a sweetness intensity of about 6000 (Table1.3.) was cleared for use as an artificial sweetener in 2002 by US Food and Drug Administration (FDA) and in 2010 by the European Food Safety Authority (EFSA). However, it is not amongst household names for tabletop artificial sweeteners at the time of writing up this research possibly because of the high sweetening potency. Selection of neotame for this research was to decipher the physiological events it might have on the sweet taste receptors as it is the sweetest of the other artificial sweeteners (aspartame, saccharin and sucralose). Moreover, based on the high potency of neotame, it activates the sweet taste receptors T1R2/T1R3 at the extracellular domain like aspartame as depicted in figure 1.9. at concentrations < 0.01 mM (Fujiwara, et al., 2012).

Following ingestion, neotame and de-esterified neotame are rapidly cleared from the plasma and eliminated from the body in urine and faeces within 72 hours (European Food Safety Authority, 2007). The small amount of methanol produced is metabolised in the body as outlined in section 1.11.2. In contrast to aspartame, the 3,3-dimethylbutyl group present in neotame , blocks the activity of the peptidases which would typically break the peptide bond between the aspartic acid and phenylalanine moieties, thus, reducing the availability of phenylalanine upon neotame ingestion (Whitehouse, et al., 2008; Nofre and Tinti, 2000). Therefore, it is of interest to determine other physiological effects of neotame beyond its value as a sweet taste agonist. In addition, as the sweetest amongst the selected artificial sweeteners, this research would also determine whether the high sweetening intensity of neotame makes any difference on studied parameters in comparison to aspartame, saccharin and sucralose.
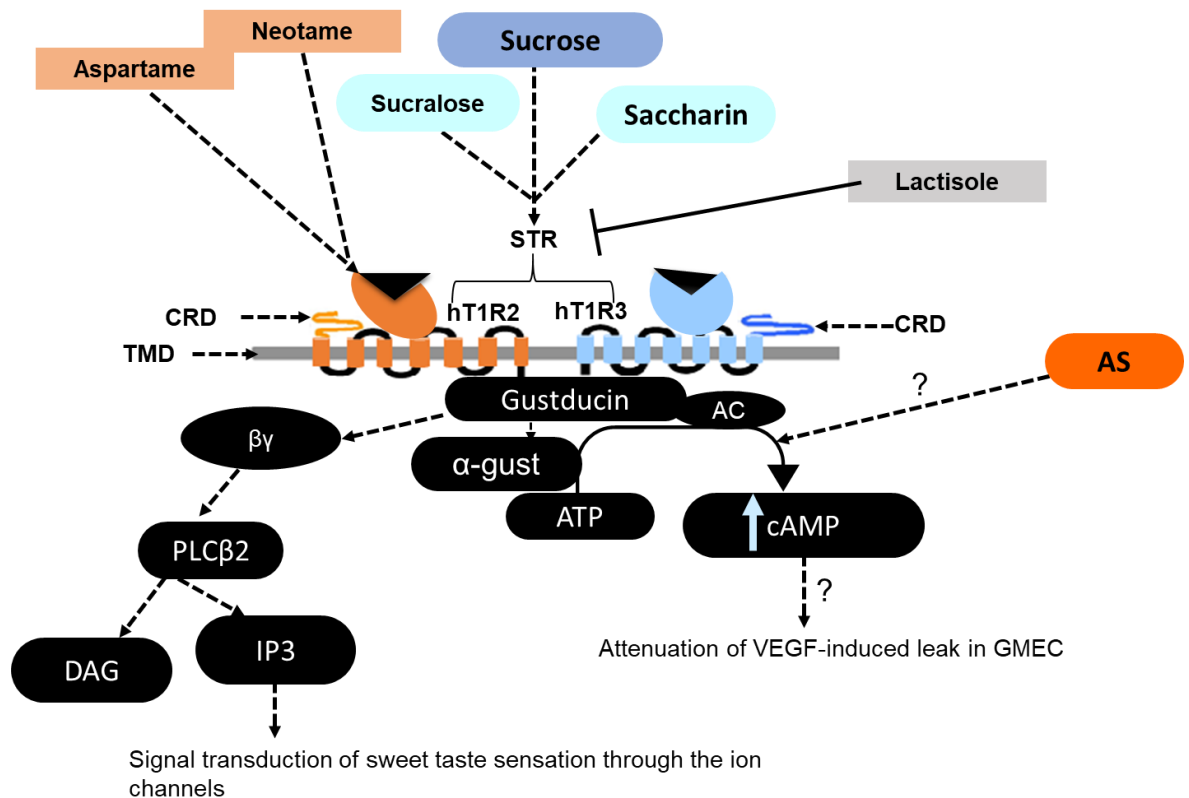
Figure 1. 14:**The working model of the sweet taste receptor with its binding ligands**
The diagrammatic representation of the sweet taste receptors depicting the binding sites of sucrose, saccharin, sucralose, aspartame and neotame. The sweet taste inhibitor, lactisole binds to the STR heterodimer to block the sweet taste sensation. While natural sugars trigger an increase in cAMP level, it is not yet known whether the studied artificial sweeteners (aspartame, saccharin, sucralose and neotame) would have any effect on intracellular cAMP in glomerular endothelium. AC = adenylyl cyclase, CRD = cysteine residue domain; STR =sweet taste receptor; AS = artificial sweeteners, ATP = adenosine triphosphate; ECM = extracellular membrane; TMD = transmembrane domain; IP_3 = inositol 1,4,5-trisphosphate; PLCβ2 = phospholipase C isoform β2; DAG = diacylglycerol; PDE= phosphodiesterase.

As previously discussed in section 1.10.1, the binding of the sweet taste agonist, activates sweet taste receptors, leading to dissociation of the gustducin complex into βɣ and α-gustducin, triggering a downstream signalling to generate the sense of sweet taste. Another downstream signalling effect upon the activation of the sweet taste receptors by artificial sweeteners is the activation of second messengers such as cAMP as shown in Figure 1.14. Hence, since cAMP is a known modulator of the microvascular endothelium (section 1.8.5.2), this research investigated the effect of the artificial sweeteners on intracellular cAMP level and possible attenuation of VEGF-induced leak through reinforcement of the endothelial barrier.

Collectively, these artificial sweeteners have been approved by the regulatory bodies, EFSA and FDA, as being safe with the majority licensed for use in different countries including UK at an acceptable daily intake (Berry, et al., 2016). Since these sweeteners (aspartame, saccharin and sucralose) are commercially available and commonly used today in many food and drink products, activates the sweet taste receptors T1R2/T1R3, at very low concentrations (<1 mM), it is therefore practical to determine their impact on glomerular microvascular endothelium as agonists of sweet taste receptor.

1.12 Sweet taste receptors as treatment target for microvascular permeability

The discovery of sweet taste receptors in extraoral cells has led to their use as novel treatment target in diseases characterised by sweet taste such as diabetes and its complications (Kyriazis, et al., 2014). However, there is no study targeting the leak in the microvascular endothelium linked with DKD. Microvascular endothelial permeability is the hall mark of DKD and as earlier mentioned in section 1.9.1, the available treatment to limit this leak associated with DKD; inhibiting VEGF expression has not been a success but rather resulted in further kidney injury. Thereby leaving a research gap for a new treatment approach that will not inhibit VEGF expression but rather focus on protecting glomerular microvasculature from its permeability-inducing effects.

The impact of artificial sweeteners on vascular bed has been controversial (Alleva, et al., 2011; Schiano, et al., 2020). The work of Alleva, et al. (2011) on HUVEC monolayer model suggests that the artificial sweetener, aspartame at low concentration might induce the production of angiogenic factor, VEGF thereby creating suitable environment for the development of diseases characterised by vascular permeability. On the other hand, a significant study regarding the physiological benefits of another artificial sweeteners, sucralose, in relation to vascular permeability was demonstrated by Lizunkova, Enuwosa and Chichger, (2019). Another pioneer study linking artificial sweeteners to attenuation of leak caused by the endothelial barrier-disruptive agent, LPS, was carried out by Harrington, et al. (2018). That study demonstrated that the artificial sweetener sucralose protects the pulmonary microvasculature *in vitro* and *in vivo* against LPS, through a sweet taste receptor-dependent pathway. However, the effect of artificial sweeteners on glomerular endothelial function is not yet known nor is the role of TIR2/TIR3 in other vascular beds well understood.

Considering the downstream signalling mechanism through which artificial sweeteners regulate the endothelium, the authors (Harrington, et al., 2018) blocked the key signalling molecule of the sweet taste receptor T1R3, gustducin using a molecular inhibitor, siRNA. The knockdown of the gustducin abolished the protective effect of the artificial sweetener, sucralose, thereby implicating activation of the sweet taste receptor, T1R3 by sucralose in the pulmonary microvascular endothelium (Harrington, et al., 2018). Additionally, it has been established that the endothelial barrier is maintained by the cell adhesion protein, VE-cadherin through tight regulation of the actin cytoskeleton (Deissler, et al., 2013; Gavard, 2013; Garrett, et al., 2016; Wetering, et al., 2002). The maintenance of this adhesion proteins through the activation of T1R3 by sucralose was evident in a study carried out by Harrington, et al. (2018). Therefore, since studies have associated artificial sweetener with attenuation of Src-phosphorylation in the presence of the barrier disruptive agents, LPS and VEGF, it would be interesting to assess the effects of artificial sweeteners on other cellular mechanisms that affect the barrier integrity of the microvascular endothelium such as ROS and cAMP.

Moreover, whilst natural and artificial sweeteners activate the same TIR2/TIR3 receptors resulting in generation of several second messengers (Zhang, et al., 2003), it is yet unclear how their downstream signalling pathways differs. Whilst sugars are believed to elevate cAMP (Bernhardt, et al., 1996), it is important to evaluate the effects of artificial sweeteners on intracellular cAMP levels in the glomerular microvasculature. Controversies on the role of artificial sweeteners in vascular permeability exist because signalling mechanisms, upon activation of the sweet taste receptors by its agonist, may vary depending on the cell type (Santos, et al., 2018; Alleva, et al., 2010; Harrington, et al., 2018). Therefore, with no study yet on the glomerular microvasculature it will be of added value and knowledge to assess the effect of artificial sweeteners, aspartame, saccharin, sucralose and neotame on vascular permeability. It is also important to determine whether these artificial sweeteners cross the glomerular microvascular endothelial cell membrane based on differences in their structural arrangement, conformation and metabolism. In order to investigate the effect of these sweeteners on the cell membrane and to assess if they can cross the endothelial barrier to get into the cells as an intact molecule, since some of the artificial sweeteners are thought to be amphiphilic, an effective but sensitive analytical technique is required. Based on the literature reviews carried out during this research, there was no published research on the detection of artificial sweeteners in the glomerular endothelium with the aim of determining whether sweeteners cross the cell membrane. Hence, as a potential therapeutic agent in managing DKD, it is essential to assess whether artificial sweeteners crosses the glomerular endothelium, as either way has valuable applications to our knowledge of these sweeteners and their physiological role besides satiation from sweet sensation.

1.12.1 Analytical detection of artificial sweeteners in biological cell samples

The safety of artificial sweeteners has been critically tested prior to endorsement by world's leading regulatory bodies in several countries and the Joint Expert Committee of Food Additives (JECFA) for its use in food and drinks. However, there are still debates over their physiological effects as well as their contribution as environmental and water contaminants (Stolte, 2013; Loos, et al., 2009) due to an increase in their incorporation into different dietetic foods and drinks. Thus, several studies have been deployed in the screening of these artificial sweeteners in foods, drinks and water samples using various analytical techniques such as ion chromatography, thin-layer chromatography, gas chromatography, high-performance liquid chromatography, capillary electrophoresis, flow-injection analysis, electroanalysis and biosensors (Berset and Ochsenbein, 2012; Stojkovic, Mai and Hauser, 2013; Niu, et al., 2012; Zhang, et al., 2014). Whilst the detection of artificial sweeteners in different matrices using the afore-mentioned techniques has been reported, there are very limited studies on cells. It is therefore important to investigate whether artificial sweeteners cross the endothelial cell membrane. More so, the analytical detection of all four named artificial sweeteners (aspartame, saccharin, sucralose and neotame) is not feasible considering differential constituents of these sweeteners and the limited time frame of this research. Hence, the decision to select one sweetener for its analytical detection in the glomerular endothelial cell was vital. Amongst these named sweeteners, aspartame and neotame does not remain intact molecules since they can be broken down to other metabolites such as methanol (section 1.11.2 and 1.11.5) and therefore not suitable for this type of test. The other sweetener, saccharin although not degraded in cells, but has differential binding mechanisms as it can activate sweet taste receptors and serves as agonist for bitter taste receptors at higher concentration (Pronin, et al., 2007). Therefore, the artificial sweetener sucralose possesses desired properties for its analytical detection in the glomerular endothelium since it binds only to sweet taste receptors and not metabolised upon ingestion. Sucralose was therefore, selected to assess if artificial sweeteners crosses the cell membrane of glomerular endothelial microvasculature using an analytical technique.

In addition, it has been shown that sucralose binds to the sweet taste receptor, T1R3 in microvascular cells, triggering a signalling cascade, thereby regulating vascular permeability (Harrington, et al., 2018). However, it is not yet known if sucralose exerts this effect by acting independently of sweet taste receptors. Thus, it is important to evaluate the uptake of sucralose (if any) and its quantification in the glomerular microvascular endothelial cells. While not yet developed or commercially available, a simple colorimeter test could be developed to give a qualitative yes or no answer as to the presence of sucralose in these cells, it is unlikely though that low concentration (< 1 mM) of sucralose would be detected using the technique. While it could also be argued that the use of biosensor might be another way to determine whether sucralose was transported across the endothelial microvasculature, such technique requires very high concentrations of sweetener of >10 mM (Zhang, et al., 2014). Therefore, considering the low doses of sucralose required to activate the sweet taste receptors and the type of cells used in this study, it is of importance to utilise a method with high sensitivity and a robust quantification technique such as gas chromatography coupled with mass spectrometry (GC-MS).

1.13 Gas chromatography-mass spectrometry-based detection of sucralose

1.13.1 Structural and chemical properties of sucralose

Sucralose is a substituted disaccharide synthesized by acetylation and selective chlorination of sucrose at three of the primary hydroxyl groups, inverting the configuration at carbon-4, from the gluco- to the galacto analogue as depicted in Figure 1.15 (Schiffman and Rother, 2013). Sucralose was first approved for use in 1991 (Canada), in 1993 (Australia), in 1996 (New Zealand), in 1998 (The U.S.) and in 2004 (the European Union) (Schiffman and Rother, 2013; Grotz, et al., 2009) following extensive toxicological studies. Sucralose is soluble in water and methanol, and maintains its sweetness at high temperature (Ferreira, et al., 2016; Magnuson, et al., 2016). Therefore, considering that this sweetener is commercially- available and can withstand a high temperature, it was suitable for the selected analytical technique; gas chromatography.

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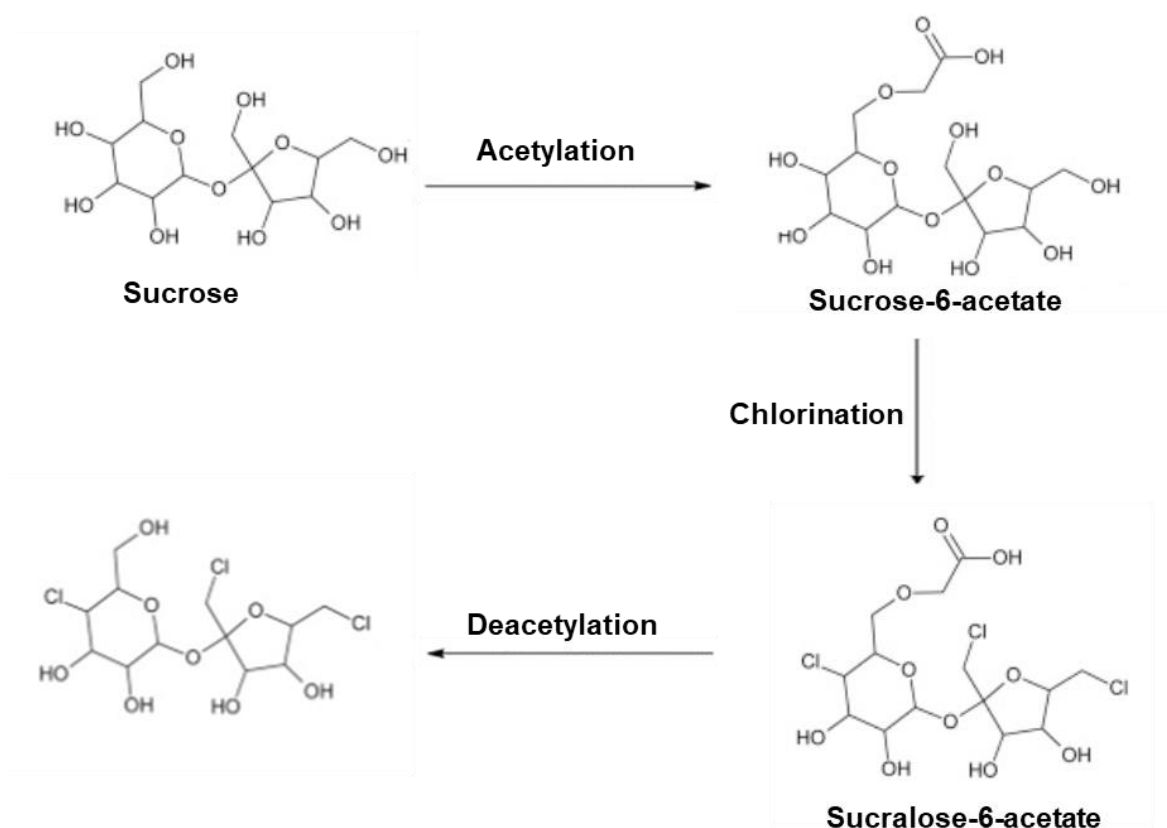
Figure 1. 15: **Formation of sucralose from sucrose through chlorination reaction**

Sucralose is synthesised from sucrose through a series of reactions. Since sucrose is chlorinated at carbon 4 of the glucose unit and carbon 1 and 6 (C1/6) positions on the fructose unit, the other hydroxyl group are then protected from chlorination through addition of an acyl group (acetylation reaction). The acetylated sucrose then undergoes chlorination reaction to insert the chlorine at the desired carbon positions. Following the selective chlorination, the molecule is deacetylated to produce sucralose (Figure adapted from Sun, et al., 2017).

1.13.2 A review of analytical techniques for detection of sucralose

The use of analytical techniques to identify and quantify artificial sweeteners is of great importance as we are living in a calorie-conscious, sugar-free frenzy and dieting society, with the shift towards the use of artificial sweeteners in everyday food and drink. There are several analytical methods for the analysis of artificial sweeteners including gas chromatography-mass spectrometry (Qiu, et al., 2007), ion chromatography (Zhu, et al., 2005), capillary isotachophoresis (Herrmannová, et al., 2006), high performance liquid chromatography and evaporative light scattering detection (Wasik, et al., 2007; Buchgraber and Wasik, 2009), and column-switching high-performance liquid chromatography (Yang and Chen, 2010). However, the two main chromatographic techniques employed in the detection of artificial sweeteners are liquid chromatography (LC) and gas chromatography-mass spectrometry (GC-MS) (Halket, et al., 2003).

Whilst various chromatographic techniques have been used to detect sucralose in foods (Zygler, et al., 2009), water (Ferrer, et al., 2013), urine (Farhdi, et al., 2003) and in table top sweetener, Splenda (Qiu, et al., 2007), some of these techniques are less sensitive and affected by interferences due to matrix effects (sample components besides the analyte of interest) (Darshi, et al., 2016). Additionally, no studies have been conducted to reflect the detection of sucralose in cells (*in vitro*) using GC-MS. Thus, this aspect of the research is not only aimed at developing a method for the detection of sucralose in the glomerular microvasculature but also to determine whether this sweetener is transported across the glomerular endothelial microvasculature, independent of the TIR2/TIR3 using GC-MS techniques. The utilisation of GC-MS as an analytical tool, do not only provide sensitivity but also structural information of the analyte of interest. However, the major drawback of GC-MS is the need for an additional sample preparation process, such as derivatisation to make the polar compounds more volatile (Zygler, et al., 2009). Therefore, as a polar compound, sucralose requires derivatisation as part of sample preparation prior to its analysis in GC-MS. Furthermore, the developed method could also be utilised in cell studies and other body fluids such as saliva, urine and blood. Hence, based on these research gaps, and the potential therapeutic effect of sucralose in the management of

DKD, there was a need to evaluate its uptake and quantification in the glomerular microvascular endothelium using GC-MS.

1.14 Introduction to chromatographic techniques

In modern analytical chemistry, the two main chromatographic techniques used are gas chromatography (GC) and liquid chromatography (LC) as earlier mentioned in section 1.13.2. The major difference between GC and LC is the mobile phase. While LC uses a liquid mobile phase to transport the samples through the column, GC uses gaseous mobile phase (Braithwaite & Smith, 1999) and for this research, the analytical technique utilised was GC-MS.

1.14.1 Gas chromatography-mass spectrometry

Heralded as a "gold standard" for substance identification due to its ability to perform specific tests through the identification of a substance in a given sample, GC-MS is a well-known technique in analytical chemistry (Himabindu, et al., 2013). The ability of GC-MS to elucidate structural information is essential when the analyte is unknown. Additional information on the advantages and disadvantages of using GC-MS as an analytical technique is highlighted in Table 1.5. GC-MS is composed of two main parts; gas chromatograph and mass spectrometer featuring a mobile phase which is a gas (helium or nitrogen).

Table 1. 5: **Advantages and disadvantages of GC-MS**

(Darshi, et al., 2016; Jens Rohloff, 2015).

Advantages	Disadvantages
Highly sensitive and specific	Limited to molecular ion mass to-charge ratio (m/z) of <1100
High efficiency and resolution	Analyte of interest need to be volatile and thermally stable
Small amount (< 1 ml) of samples required	Chemical derivatization is often required for polar samples.
Less matrix effects in comparison to LC-MS	
Use of same gaseous phase and column for different analytes	
Large linear range	
Abundant reference spectra libraries such as NIST	

A gas chromatograph utilizes a capillary column with different packing material and dimensions (length, diameter, film thickness) resulting in difference in various types of columns (Fishman, Lester and Lamparski, 2006). The main separation of the analyte of interest starts as the sample mixture travels the length of the column, with individual compounds separated based on their polarity and vapour pressure of analytes. The more the compound is retained by the column, the longer it takes to be eluted from the column. Thus, elution of molecules at different times (called the retention time), allows the mass spectrometer to capture, ionize and generate the mass ion of the individual compound (Bhardwaj, et al., 2016). In addition, the column is placed in a thermostatically controlled oven, as temperature influences the volatility of analytes. Therefore, the main separation of the analyte from the sample mix occurs in the column.

A reduced time of analysis can be achieved by changing operational parameters (faster temperature program rate, higher carrier gas flow rate or a combination of both approaches) and column parameters (shorter length, smaller column inner diameter, thinner film of stationary phase) (Bhardwaj, et al 2016). Since operational and column parameters affect the quality and sensitivity of the GC-MS technique, it is essential to consider the following; carrier gas, flow rate selection, column selection (dimension and packing material composition), temperature programming (initial temperature, initial hold, ramp rate, final temperature, and final hold), injector temperature and detector temperature when developing a method. Following separation of the analyte of interest in the gas chromatograph, the second part of GC-MS operational system is mass spectrometry.

1.14.2 Mass spectrometry

Mass spectrometry (MS) is based on the ionization of analyte molecules in the gas phase, where it is ionised and detected ions are graphically represented as mass spectrum according to mass-to-charge ratio (m/z) of the analyte (Thompson, 2017; Himabindu, et al., 2013). The performance of chromatography with mass spectrometer, increases the selectivity of analyte detection, thereby allowing the analysis of less pure samples (Fox, et al., 2000). The main components of the mass spectrometer include the ion source, mass analyser and the ion detector (Smith, 2013). The separated molecules from the chromatographic system are transferred first to the ion source for ionisation, followed by transfer of the ions and fragments to the mass analyser for m/z ratio separation and the separated ions are then detected by the ion detector (Smith, 2013).

1.14.2.1 Ion source

Following the passage of the molecules from the transfer line and entrance into the mass spectrometer, ionization takes place by various methods with typically only one method being used at any given time. The decision of choosing an ion source lies on the properties of the sample of interest and the desired fragmentation if known. Commonly used ion source in biological research today include chemical ionization, electron impact ionization, matrix-assisted laser desorption ionisation (MALDI) and thermal ionization (Finehout and Lee, 2004). In this research, electron impact ionisation was used.

1.14.2.2   Electron ionisation

According to Smiths (2013), electron ionisation occurs when the gaseous sample interacts in the gaseous phase with the high energy emissions from a heated MS filament. The molecules are bombarded by the electrons, causing the molecules to fragment in a characteristic and reproducible way. Electron ionisation yields extensive fragmentation of molecular ion (the first positively charged ion from the sample) with structural information

for sample identification (Thompson, 2017). The molecular fragmentation pattern depends on the electron energy applied to the system, and since the energy of electrons is typically 70 eV (electron Volts), the use of 70 eV is sufficient to overcome the first ionisation potential (removal of an electron) of the analyte (Himabindu et al., 2013; Smiths, 2003).The results from the molecular fragmentations of one sample injection to another are reproducible, enabling the generation of mass spectra library such as NIST and Wiley which are imperative resources in biochemical and chemical identification (Samokhin, Sotnezova and Revelsky, 2019). Therefore, the use of electron ionisation model for the analysis of analyte of interest is plausible.

1.14.2.3 Mass analyser

Following on from sample ionisation, analysis of the m/z produced in the ion source is carried out. The beam of ions is focused and directed into a mass analyser which separates the ions under the influence of an applied magnetics or electric field analyser (Erin and Lee, 2003). When scanning of the ions using voltage or field strength, only ions with a specific mass/charge (m/z) will reach the detector. The type of mass analyser for this research was a quadrupole mass analyser which is comprised of four parallel conducting rods, connected to radiofrequency (RF) and direct current (DC) generators. When the ions hit the rods, only ions with a stable trajectory will reach the detector and the other ions will be deflected (Smith 2013). The ion beams from the mass analyser are then converted to a series of electrons with the resulting current digitalised and sent to a computer system for the generation of mass spectrums as presented in Figure 1.16.
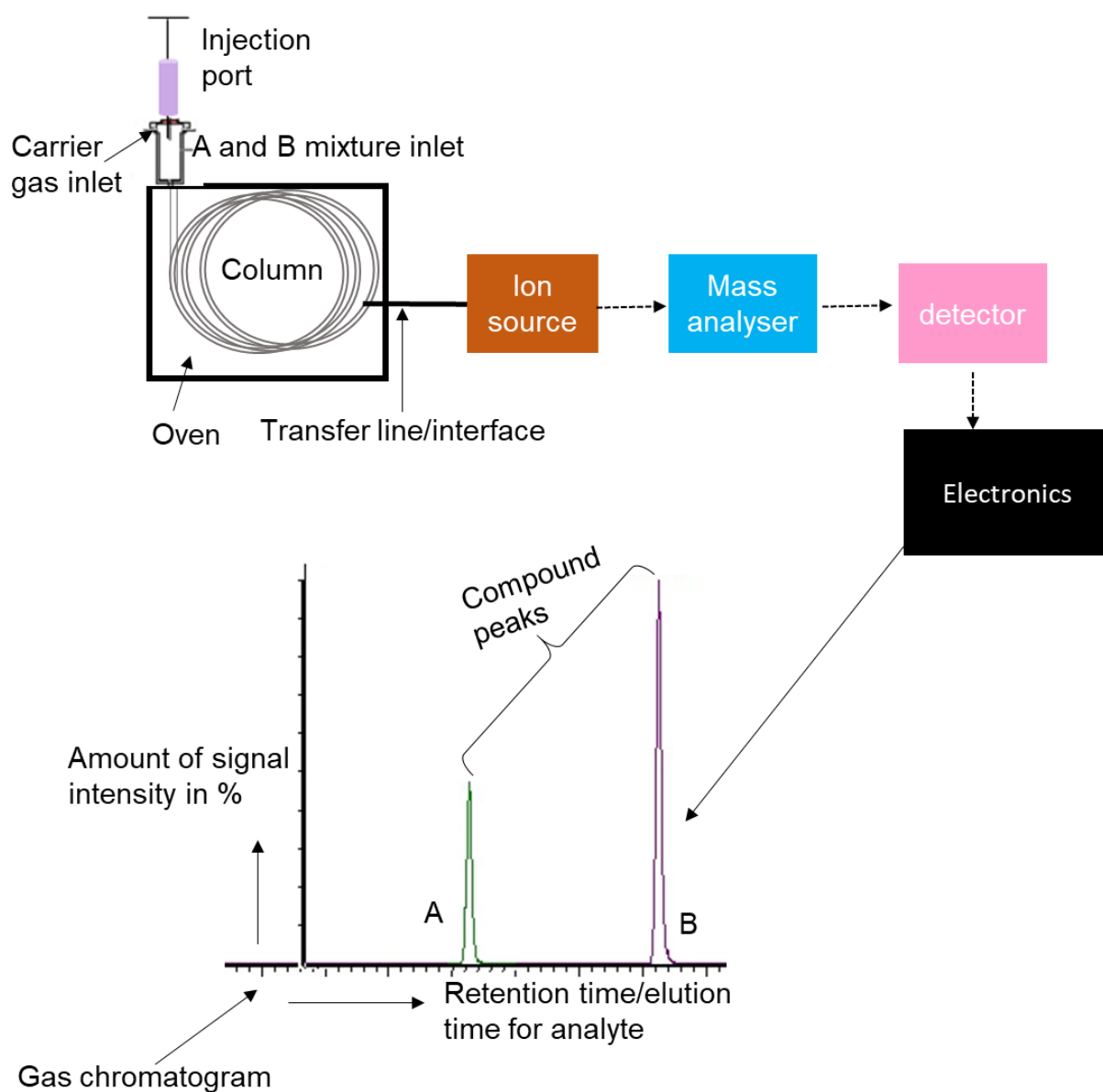
Figure 1. 16: **Gas chromatography-mass spectrometry working pattern**

The figure represents different parts of the GC-MS, as the samples are injected into the column, the high temperature of the oven with the carrier gas (helium) separates the samples based on their affinity and boiling point to the column and the molecules are subsequently fragmented and eluted into the MS where it is ionised, analysed, detected and represented as chromatograms (self-generated diagram).

1.15: The working pattern of the gas chromatography-mass spectrometry technique

The operational pattern of GC-MS is depicted in Figure 1.16 showing the internal instrumentation of the GC-MS used for this research. The GC-MS separation mechanism starts from the injection of analyte into the column driven by the mobile phase (gas), in this case helium. Once in the column, the samples are separated by differential partitioning which is based on their attraction level to the stationary phase. The separated compounds are eluted from the column into a detector (Mass spectrometer) where it is ionised and then processed as a signal and the amplifiers translate signals electronically into chromatograms and mass spectra (Stauffer, 2013). It is important to note that most GC-MS instruments follow the same pattern of sample separation, fragmentation and detection with little variation in the type of carrier gas, column packing material, MS detector amplifiers and programme software. The GC-MS model used during this research was the Clarus 500 Perkin Elmer with Turbomass software.

GC-MS was the technique of choice during this research based on but not limited to the advantages highlighted in Table 1.5. The GC-MS is both a qualitative and quantitative technique with a powerful combination of sample separation and identification (Stauffer, 2013). According to Harvey (2011), GC-MS has gained general acceptance for analysis of carbohydrates and sugars due to its high sensitivity and efficiency. Prior to developing the method for the detection of sucralose in the cell model of glomerular endothelial microvasculature, certain parameters in relation to method development and validation were considered. Taken together, it is evident that the use of GC in combination with MS is a powerful analytical technique with specificity, selectivity and sensitivity advantages which is a prerequisite if this method is to be valuable in the detection and identification of sucralose in the glomerular microvasculature. However, for any polar compound to be detected by GC-MS, it must undergo an essential sample preparation process, known as derivatisation, making it more volatile for the analytical procedure.

1.16 Derivatisation

Derivatisation is the chemical modification of a functional group, utilising one or multiple reactions for polar and less volatile samples. As an example, if large sugar molecules are injected into the GC without derivatisation, they will degrade and their OH-group would bind strongly with the inner coating of the columns, causing damage (Moldoveanu and David, 2018; Orata, 2012). Hence, sucralose needs to undergo derivatisation prior its analysis with GC-MS due to its polar nature and low volatility (Qui, et al., 2007; Ruiz-Matute, et al., 2011).

Although derivatisation could lead to thermal decomposition of certain compounds, the process improves the suitability, efficiency, and detectability of the derivatised product. In addition, upon derivatising a sample, a modified analyte is generated, known as the derivative. There are very few published articles on the derivatisation reagents and process for sucralose in GC-MS analysis and the one adapted to this research was a study by Qiu, et al. (2007). Therefore, there was a need to develop or amend the existing derivatisation protocol of sucralose for GC-MS purposes.

1.16.1 Types of Derivatization Reactions

The derivatisation reactions are classified according to their reaction process with the most commonly used methods being alkylation, acylation and silylation. The derivatisation by silylation is the most prevalent for sugars, including some artificial sweeteners (Baird, et al., 2000; Harvey, 2010).

1.16.1 .1 Alkylation

Commonly used derivatization reagents for the alkylation reactions are diazoalkales, pentafluorobenzyl bromide (PFBBr), tetrabutylammonium hydroxide (TBH) and Boron trifluoride (BF_3) in methanol among others. Alkylation reagents can be used in conjunction with other derivatisation reagents such as silylation and acylation reagents or alone to form ethers, esters and amides (Orata, 2012). The replacement of the hydrogen with an

alkyl group yields a less polar derivative compared to the parent compound. The drawback to this derivatisation reaction is that some of the reagents used pose health risk, such as diazomethane which is highly toxic and a carcinogenic reagent. Moreover, most alkylation reactions involve fatty acids, which is not the best derivatisation reaction for the analyte of interest, sucralose.

1.16.1 .2 Acylation

The introduction of an acyl group to an organic compound during a derivatisation reaction is known as acylation. Acylation is a popular choice for derivatisation reactions to produce volatile derivatives of highly polar and non-volatile organic compounds (Zaikin and Halket, 2003). Analytes containing active hydrogens (such as -SH, -OH, and -NH) can be converted into thioesters, esters and amides respectively, through acylation (Moldoveanu and David, 2018). In addition to providing volatility to organic analytes, acylation improves analyte stability by protecting unstable groups. Examples of acylation reagents are fluoracylimidazoles, pentafluorobenzoyl chloride (PFBCl), fluorinated anhydrides and pentafluoropropanol (PFPOH) (Orata, 2012; Zaikin and Halket, 2003). While acylation reaction has been used for the derivatisation of sucralose, the requirement of a catalyst for the reaction placed it behind other reagents that are capable of derivatising sucralose in a single step reaction without the need of a catalyst. Therefore, derivatisation by acylation reaction was not selected for this study.

1.16.1 .3 Silylation

The most prevalent derivatization method for carbohydrate and sugar molecules is silylation (Becker, et al., 2013; Kozar and Steinberg, 2000) as it readily derivatises the analyte and is very suitable for non-volatile compounds such as simple sugars and artificial sweeteners such as sucralose for GC-MS analysis (Rojas-Escudero, et al., 2004; Ruiz-Matute, et al., 2011). The silylation reaction involves the replacement of active hydrogen by a silyl group, thereby reducing the polarity of the compound and its hydrogen bonding (Pierce, 1968). According to a study by Chen, et al. (2007), derivatisation by silylation has been successfully used to derivatise some compounds regarded as non-volatile and unstable including many hydroxyl and amino compounds. Additionally, silylation improves the detectability and efficiency of the derivatives as they are more volatile, and stable with narrow and symmetrical peaks (Kataoka, 2005). Silylation reagents used for the derivatization process include hexamethyldisiloxane (HMDS), Bis (trimethylsilyl) acetamide (BSA), Trimethylsilyldiethylamine (TMS-DEA), Trimethylchlorosilane (TMCS), Trimethylsilylimidazole (TMSI), Bis-trimethyl-silyl-trifluoroacetamide (BSTFA) and N-methyl-trimethyl-silyl-trifluoroacetamide (MSTFA) (Orata, 2012). Silylation introduces a silyl group such as dimethylsilyl [$SiH(CH_3)_2$], chloromethyldimethylsilyl [$SiCH_2Cl(CH_3)_2$] and tert-butyldimethylsilyl chloride [$Si(CH_3)_2C(CH_3)_3$] to the sample analyte substituting the active hydrogen with the silyl group (Lin et al., 2008; Orata, 2012). The main caution in using silylation reagents for derivatisation is their sensitivity to moisture; thus, samples to be derivatised should be dry and stored in tightly sealed containers (Sobolevsky, et al., 2003). The silylation reagents used for this research in derivatising sucralose were Bis-trimethyl-silyl-trifluoroacetamide (BSTFA) and N-methyl-trimethyl-silyl-trifluoroacetamide (MSTFA). Although numerous derivatizations reagents are used for derivatising sugars, (Moldoveanu and David, 2018), just one study (Qiu, et al., 2007) has derivatised sucralose for GC-MS studies using Hexamethyldisilazane (HMDS) as a silylation reagent with other reagents in several stage reactions. Additionally, the study by Qiu, et al. (2007) was the primary article adopted in developing the methods for the current study.

However, their methodological approach in selection of derivatisation reagent and internal standards are not without critical drawbacks (Qiu, et al., 2007) which are further explained in chapter 5. In addition, an effective and efficient derivatisation reaction is vital for the generation of stable derivatives, improved analyte fragmentation pattern, better signal intensity and simultaneous detection of high and low compounds in a single analysis. Therefore, it is essential that an effective derivatisation methodological strategy is adopted when developing GC-MS analytical methods for polar compounds such as sucralose.

1.17 GC-MS method optimisation and validation parameters for this research

The very limited availability of published literature on sucralose analysis using GC-MS and the gap in knowledge using the same technique in the detection and identification of sucralose in microvascular cell type makes this study novel. Thus, optimisation of GC-MS analytical method for sucralose detection and validation was vital prior to its application to analysis of biological cell samples.

Validation of an analytical method is a prerequisite for a new method to ensure that the performance attributes of the method meets the requirements of the analytical application, thereby giving accurate, reliable and reproducible results irrespective of the operators, using same equipment in the same or different laboratory (Himabindu, et al., 2013; Bhardwaja et al., 2016). According to Gonzalez and Herrador, (2007), method validation ensures that every future measurement in routine analysis will be close enough to the unknown true value of the analyte and reliable. To further emphasize on the importance of method validation, Himabindu et al., (2013), stated that method validation gives the developer and user a degree of confidence in the analytical procedure, eliminates frustrating repetitions, saves time and absorbs any interference such as change in reagent supplier or grade. In developing the GC-MS method for the detection of an analyte of interest, several steps need to be considered, such as understanding the physicochemical properties of the sample analyte, selection of chromatographic conditions, method optimization parameters, sample preparation, and validation of selected parameters (Bhardwaja, et al., 2016). In this current study, selection of appropriate instrument

parameters and validation was essential to ensure that the developed sample preparation and analytical methods could identify and quantify sucralose correctly. The method optimisation and validation parameters were carried out following the general method validation criteria set by the International Conference on Harmonisation (ICH) (1995; 2005), the International Journal of Analytical and Bioanalytical Chemistry (2016) and the Scientific Working Group for Forensic Toxicology (SWGTOX, 2013). These regulatory bodies recommend the following parameters when developing and validating a method; specificity, precision, robustness, linearity and range, stability, limit of detection (LOD) and limit of quantification (LOQ).

1.17.1 Specificity and selectivity

Specificity clearly assesses the analyte in the presence of other components which may be expected to be present, such as matrix, degradants and impurities. These parameters are vital because the aim of this aspect of the research was to investigate whether sucralose crossed the cell barrier. To achieve this aim, the developed method should be able to differentiate from other sugars that might be present in a cell sample. The specificity procedures and evaluation criteria utilised for sucralose are described in chapter 2. It is not always feasible to develop an analytical procedure specific to an analyte in which case other identification tests can be used to identify the analyte (ICH,2005).

1.17.2 Precision and robustness

The analytical criteria, precision, is used to express the closeness between an acceptable conventional value and the new value from the developed method (ICH, 2005). An example of a precision tool is repeatability, which evaluates the degree of scatter between measurements obtained from same sample over multiple sampling time. Robustness, on the other hand, is used to measure the capacity of a method to withstand small variations in method parameters, such as different columns, temperature and flow rate when using gas chromatography (ICH, 2005). Robustness also enhances the reliability of the method. This analytical procedure can be evaluated by data generated from two separate analyses

using different columns, or through the stability of analytical solutions utilised, which are further explored in chapter 5.

1.17.3 Calibration model - linearity

There are various models of calibrations as a method validation tool (Paprotny, et al., 2019) with a linear relationship indicating results directly proportional to the concentration of the analyte. The popularity of the linear calibration curve is not limited to its wide use as acceptable analytical method but also the ease at which calibration curve equation, coefficients and standard deviations are computed (Wille, et al., 2017). Linearity of the calibration curve can be evaluated visually by inspecting the regression plot and if linearity is established, statistical methods can be used to validate results (Shrivastava and Gupta, 2011). The degree of linearity can further be obtained through mathematical estimates of the regression plot. For this research, the linearity model of sucralose standards was assessed using the linear regression plot with results compared to acceptable criteria as described in chapter 5. Therefore, it was essential that the degree of linearity of sucralose standards matches the criteria for method development and validation.

1.17.4 Limit of detection and quantification

The limit of detection (LOD) is the lowest amount of analyte in a sample which can be detected but is not necessarily quantified as an exact value. Several approaches for determining the limit of detection exist but the three main ways are visual evaluation, standard deviation of the response, and the slope and signal-to-noise ratio (ICH 2005; SWGTOX; 2013). Visual evaluation is determined by analysis of known sample concentrations of the analyte and visually establishing the minimum level of analyte detection. The signal-to-noise procedure is evaluated by comparing known low concentrations of analyte signals from samples with blank samples and the acceptable signal-to-noise ratio is 3:1 for estimating limit of detection (Sanagi, et al., 2009). The standard deviation of the response and the slope evaluation procedure is based on using the calibration curve to determine the limit of detection. This is based on the equation;

LOD = 3.3 σ/S, where LOD = detection limit, σ = the standard deviation of the response and S = the slope of the calibration curve (SWGTOX, 2013; Sanagi, et al., 2009).

In addition, the limit of quantification (LOQ) is the lowest amount of analyte in a sample which can be quantitatively determined in an individual analytical procedure. There are various ways of determining the quantitation limit and these include but are not limited to visual evaluation, signal-to-noise ratio, standard deviation of the response and the slope of calibration curve. The equation for the estimation of LOQ using the response and the slope method is; LOQ = 10 σ/ S, where σ = the standard deviation of the response, S = the slope of the calibration curve. Furthermore, the LOD and LOQ are vital parameters as the data generated from both parameter aids in the selection of appropriate concentrations of the analyte of interest (sucralose). To determine the LOD and LOQ, the standard deviation of the response and the slope evaluation equation was utilized in this research with the calculations of the other parameters mentioned in the equations such as standard deviation and findings further explained in chapter 5.

1.18 Application of GC-MS method for the detection of sucralose in glomerular microvascular endothelial cells

As previously discussed in section 1.13.3, the two main chromatographic techniques employed in the detection of artificial sweeteners are liquid chromatography (LC) and gas chromatography-mass spectrometry (GC-MS) (Halket, et al., 2004). However, none of the methods have been utilised in cell studies to detect the artificial sweetener, sucralose nor to determine whether sucralose crosses the cell membrane of glomerular endothelial cells. The developed GC-MS method in this research for the detection of artificial sweetener, sucralose was applied to cell studies to detect the presence or absence of sucralose in the glomerular endothelial microvascular cells. Hence, this aspect of the research not only addressed some of the aims outlined in section 1.19, but also added new knowledge in the application of GC-MS analytical technique in *in vitro* studies.

1.19 Research aims and hypothesis

The research presented in this thesis was based on the hypothesis that artificial sweeteners induced TIR2/TIR3-dependent signalling pathways to attenuate VEGF-induced glomerular microvascular permeability. The objective of the study was to assess the protective effect of commercially available artificial sweeteners; aspartame, saccharin, sucralose and a high-end sweetener, neotame; on VEGF mediated barrier leak and the associated molecular mechanisms of action. The study assessed the role of T1R2/T1R3 in regulating these processes. Lastly, the study developed an analytical technique to measure sweetener levels within the endothelial cell as a potential T1R2/T1R3-independent mode of action. To address these research questions, the aims for the research were:

Aim I: To establish the protective effect of artificial sweeteners on VEGF-induced barrier disruption and permeability in glomerular microvascular endothelial cells.

Aim II: To determine the molecular mechanisms, linked to T1R2/T1R3, cAMP signalling and oxidative stress, through which artificial sweeteners impact the glomerular endothelium.

Aim III: To develop and validate a GC-MS analytical technique for the detection and identification of the artificial sweetener, sucralose.

Aim IV: To apply the validated GC-MS method to assess whether sucralose crosses the cell membrane of the glomerular microvascular endothelium to exert these biological effects.

While this research aimed to fill in the knowledge gap on the role of artificial sweeteners in DKD, it was anticipated that the findings from this research would further our understanding of the physiological roles of artificial sweeteners beyond sweet taste satiation. The analytical method that was developed, optimised and validated for the detection of sucralose in the cell lysate provided a novel technique to address potential questions on the ability of sucralose as an artificial sweetener to cross the glomerular endothelial cell membrane. The study has the potential to provide the foundation for the

development of a novel therapeutic strategy in the management of the permeability associated with DKD, therefore improving outcomes for patients living with the disease.

CHAPTER 2:

MATERIALS AND METHODS

2.1 Introduction

To address the research questions highlighted in section 1.19, a range of materials and experimental procedures were used, highlighting various preliminary and optimisation studies investigated. Risk assessments and ethical considerations for both the cell biology and chemical analysis experiments were conducted and approved by the Department/School Research Ethics Panel of Anglia Ruskin University. All experiments were carried out under good laboratory practice for assessment of both biological and chemical samples.

2.2 Cell Biology materials and methods

2.2.1 Materials

All T-25, 75 and 150 cell culture flasks; 1, 5, 10- and 25-ml serological pipette tips; 10, 200 and 1000 µl pipette tips; 6, 12, 24 and 96 well plates and other hard consumables such as PPE were purchased from Thermo Fisher Scientific UK unless stated otherwise. Transwell inserts plates were supplied by Corning Incorporated. Reagents and cells were obtained from a range of suppliers (Table 2.1).

Table 2. 1: **Materials used and supplying company**

Material	Supplier
Cell Counting Kit (CCK-8)	Sigma-Aldrich UK
Primary human glomerular microvascular endothelial cells (GMVEC)	Cell systems Corporation (Kirkland, WA)
Complete Classic Medium	Cell systems Corporation (Kirkland, WA)
Complete Classic Culture Boost	Cell systems Corporation (Kirkland, WA)
Complete Classic attachment factor	Cell systems Corporation (Kirkland, WA)
Neotame, Sucralose, Aspartame and Saccharin	Sigma-Aldrich UK
Lactisole	Cayman Chemicals UK
FITC-dextran	Sigma-Aldrich UK
Paraformaldehyde (PFA)	Thermo-Fisher Scientific UK
Phosphate Buffered Saline (DPBS)	Thermo-Fisher Scientific UK
Dimethyl Sulphoxide (DMSO)	Thermo-Fisher Scientific UK
Penicillin-Streptomycin	Thermo-Fisher Scientific UK
4', 6-diamidino-2-phenylindole (DAPI)	Sigma-Aldrich UK
Trypsin EDTA	Sigma-Aldrich UK
VEGF$_{165}$	Thermo-Fisher Scientific UK

Table 2. 2: **List of antibodies used and supplying company**

Cat Number	Antibody	Company	Epitope
Sc398996	T1R3 antibody (N20, goat)	Santa-Cruz Biotechnology	Extracellular N terminal
Sc31017	Anti-VE-cadherin antibody (N14, goat)	Santa-Cruz Biotechnology	Extracellular N terminal
Sc2024	Fluorescent secondary antibody (Donkey anti goat FITC)	Santa-Cruz Biotechnology	
#4412186	anti-cAMP antibody	Applied Biosystems	cAMP-Screen Direct System

Table 2. 3: **Equipment used and supplying company**

Equipment	Supplier
Micro plate reader	Tecan Sunrise
Class III microbiological safety cabinet	Envair
Tissue culture incubator	HERA
AccuSpin 1R centrifuge	Thermo-Fisher Scientific
P2, P10, P200 and P1000 auto pipettes	Gilson
Haemocytometer	Thermo-Fisher Scientific
Zoe Fluorescent cell imager	BIO-RAD
Vortex mixer	Stuart
EVOM2 meter	World Precision, Herts, UK
Fluorescent plate reader	PerkinElmer 2030 Manager

2.2.2 Methods
2.2.2.1 Stock preparation of reagents and artificial sweeteners

Pure and analytical grade artificial sweeteners used in this research were aspartame, saccharin, sucralose and neotame. A sweet taste inhibitor, lactisole, was also used. A 10 mM stock solution of each artificial sweetener was prepared using the complete cell culture media as the solvent. The stock solutions of the artificial sweeteners were sterile-filtered and diluted to various working concentrations used for the experiments; 0.01, 0.1, 1, 10, 100 and 1000 µM. Whilst the ADI of these artificial sweeteners ranges between 5-40 mg/kg as shown in section 1.11.1, the sweeteners are poorly absorbed except for aspartame. Therefore, considering the cell type used in this research, these concentrations were selected to reflect those estimated to be seen in circulation which is about 2-20% of ingested sweeteners (Sylvetsky, et al., 2017; Sims, et al., 2000; Roberts, et al. 2000). The stock solution of lactisole, 10 mM, was prepared in 100% ethanol, sterile-filtered and diluted in complete media to working concentrations of 0.01, 0.1, 1, 10, 100 and 1000 µM. Each working concentration of artificial sweeteners and lactisole was prepared prior to each experiment and stock concentrations were stored for a maximum of one week at -20ºC to avoid excessive freeze-thaw cycles. The calculations used in preparing the artificial sweeteners, lactisole, and other reagents such as vascular endothelial growth factor (VEGF) and N-acetylcysteine (NAC) utilised in this research are detailed in the appendix.

2.2.2.2 Cell culture techniques
2.2.2.2.1 Culturing of primary glomerular endothelial cells
The complete classic medium was supplemented by adding 10 ml of culture boost to 500 ml of classic medium (with 10% serum), as per manufacturer's guidelines. Culture boost contains bovine growth factor and porcine heparin with a working concentration of 50 µg/ml. To culture the glomerular microvascular endothelial cells (GMVEC), tissue culture plasticware were first rinsed with attachment factor (supplied by manufacturer) for a minimum of 5 minutes at room temperature prior to plating.

Attachment factor was subsequently removed, and cells were plated at the required density. Cells were cultured in a humidified incubator at 37°C and 5% CO_2. At 90% cell confluence, used media was removed and cells were rinsed with 5 ml of Dulbecco's phosphate buffered saline (DPBS, a 1x balanced salt solution with no calcium and magnesium) by swirling the cell culture flask gently. Then, 3 ml of trypsin-EDTA (Hanks' balanced salt solution with phenol red) was added to the cells, incubated for 3-5 minutes at 37°C and 5% CO_2 and monitored under the microscope to confirm detachment of cells. The trypsinisation reaction was then stopped by adding 3 ml of pre-warmed supplemented classic cell medium and then transferred into a 50 ml centrifuge tube. The cells were centrifuged at 212 g force (RCF) for 5 minutes and the supernatant was discarded using a micropipette. Fresh complete media (10 ml) was added to the cell pellet and gently resuspended by pipetting up and down to mix cells thoroughly. In order to calculate the total number of cells and the amount of cells required for each experiment, a small sample of cells (10 µl) was taken from the cell mixture and counted using a haemocytometer. The required amount of GMVEC were then plated onto an appropriate cell culture flask already pre-washed with 2 ml of attachment factor, checked under the microscope and then incubated at 37°C and 5% CO_2. The passage number used for the entire study was from passage 3-15 and up to that passage number there was no change in the cell morphology as this was checked prior key experiments. This is because the GMVEC are primary endothelial cells and if used at higher passage number, they might begin to undergo senescence or transform into non-endothelial phenotypes (Pan, et al., 2009; Satchel, 2006).

2.2.2.2.2 Freezing down primary glomerular endothelial cells

To freeze the cells down for future use, two reagents were used to make up the freezing medium; the classic media and the dimethyl sulphoxide (DMSO) in a ratio of 9:1. To cryopreserve the cells, the growing cells were first harvested using the trypsin-EDTA and following the same protocol as counting the cells (section 2.2.2.2.1). Next, cells were counted to determine appropriate cell number for freezing the cells (recommended to be

between 500,000-1,000,000 cells/ml), the freezing media mix was then transferred into the cells and 1 ml of the mixture was pipetted into freezing vials and immersed into a "Mr. Frosty" (isopropanol solution) with each freezing vial containing 500,000-1,000,000 of cells. Mr Frosty was kept in a -80 $^{\circ}$C freezer for 24 hours and then the cryovials were transferred to liquid nitrogen for longer storage period.

2.2.2.2.3 Resuscitating the primary glomerular endothelial cells

To resuscitate the cells, a pre-warmed cell media was kept ready in a centrifuge tube. A freezing vial containing the cells was taken from the liquid nitrogen and kept in the hood cupboard to defrost. Once the cell solution defrosted, it was pipetted into the centrifuge tube with media and spun at 212 g force (RCF) for 5 minutes. The supernatant was discarded and replaced with 10 ml of warm complete classic media and the cell solution were transferred into the T75 plasticware (pre-rinsed with attachment factor) and incubated at 37°C and humidified 5% CO_2.

2.2.2.3 Establishing cell numbers for viability assay

To establish the appropriate cell number to use for the viability assay, the proliferation assay was performed. Varying cell numbers; 500, 800, 1000, 3000, 5000, 8000, 10,000 and 20,000 of GMVEC (100 µl) were plated onto 96-well plate (pre-rinsed with attachment factor) and cultured separately for a duration of 24, 48 and 72 hours. After the designated time of cell incubation, 10 µl of cell counting kit-8 (CCK-8) was added into each well and the plates were incubated between 1-4 hours. The absorbance was then measured at 450 nm using a micro plate reader. All samples were assayed in triplicate, with a minimum of three independent experiments. An average of each triplicate was considered as an n-number of 1.

2.2.2.4 Viability assay of glomerular microvascular endothelial cells

The effect of the artificial sweeteners and the sweet taste inhibitor, lactisole, on GMVEC viability was determined using the CCK-8 cytotoxicity assay. The CCK-8 cytotoxicity assay is a colorimetric assay, which utilises 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium monosodium salt (WST-8), a highly soluble tetrazolium salt to assess cell viability. The WST-8 gives an orange-colour formazan dye in the presence of dehydrogenases in viable cells (Han, et al., 2011). The formazan dye is soluble in cell media with the amount of dye produced directly proportional to the number of viable cells. Based on the results from the proliferation assay, the cell number (5000) and maximum incubation time of 48 hours and post CCK-8 incubation time of 2 hours was selected for the viability test (Figure 3.2b).

To perform the cell viability assay, 100 µl of GMVEC suspension (5000 cells per well) was dispensed into a 96-well plate (pre-rinsed with attachment factor and incubated for 24 hours. The used media was then removed and GMVEC were treated with 100 µl of 0.01, 0.1, 1, 10, 100 and 1000 µM concentrations of aspartame, saccharin, sucralose, neotame and lactisole separately. The cells were further incubated for an additional 24 hours. Then, 10 µl of used media was aspirated off and replaced with 10 µl of CCK-8 reagent, mixed gently and incubated for an additional 2 hours. Whilst adding the CCK-8 reagent, care was taken to prevent introduction of bubbles into the plate as they might interfere with absorbance reading. The plate was then measured at 450 nm using a multi-mode microplate reader equipped with Meglan software. The blank measurement for the assay was performed using only the CCK-8 and media with the exclusion of cells while the control was untreated cells (GMVEC) with CCK-8.

The following formula was used to calculate the amount of viable cell number from absorbance reading:

% viable cells = [(Absorbance of sample test – Absorbance of Blank) / (Absorbance control – Absorbance of Blank)] * 100

Sample test = GMVEC + artificial sweeteners + CCK-8

Blank = media + CCK-8

Control = GMVEC + CCK-8

All experimental repeats were as described in section 2.2.2.3, with a minimum of three independent experiments.

2.2.2.5 Permeability studies
2.2.2.5.1 Studying the effect of VEGF on GMVEC monolayer permeability

This experiment was performed in a 24-well transwell plate with 24 individual hanging cell culture inserts. The insert of the transwell features a 0.4 µm pore size within a thin translucent membrane (Figure 2.1). Each insert was pre-rinsed with an attachment factor prior to seeding of GMVEC. Transwell inserts were seeded with 20,000 cells per well. The base part, or receiver, of the plate was filled with 1.5 ml complete media to ensure that the bottom of the insert was covered, whilst the insert (where cells were seeded) was covered with 400 µl of media followed by incubation in a humidified incubator at 37°C and 5% CO_2 for 44 hours. Following incubation, used media was gently removed from the top of the insert and cells were treated with vascular endothelial growth factor A-165 (VEGF-A$_{165}$) at concentrations ranging from 10 to 100 ng/ml and kept in the incubator for an additional 4 hours. After cell exposure to VEGF-A$_{165}$, 80 µl of used media was aspirated off from the insert of the transwell and replaced with 80 µl of FITC-Dextran (5 mg/ml) of various molecular weights; FD4 – 4000kDa, FD20 – 20,000kDa, FD40 – 40,000kDa and FD70 – 70,000kDa. As diagrammatically represented in Figure 2.1a-c, the GMVEC tight monolayer (Figure 2.1a), was exposed to VEGF-A$_{165}$ and then FITC-dextran loading (Figure 2.2b), allows the fluorescent molecules to pass through the endothelial cell

monolayer at a rate proportional to the monolayer's permeability (Figure 2.1c). A timer was set following the FITC-Dextran loading for 60, 180 and 360 seconds. At the end of each time point, 100 µl of the solutions from both the insert well and the base/receiver well of the transwell plate were aliquoted into a separate clean 96-well plate respectively. The permeability of the GMVEC monolayer was determined by measuring the fluorescence levels (which serves as the permeability tracer) at different time points using a fluorescent plate reader at excitation and emission wavelengths of 485 nm to 535 nm. It is important to note that the cell number used for the permeability studies was 20,000 cells per well and this varied from the cell number used in the viability assay, because for permeability studies a tight monolayer was required. To achieve a tight monolayer of primary endothelial cells, the cells could either be cultured/grown for a longer period or cell number could be increased and then incubated for a shorter time. For this research, the latter was chosen; increased number of cells for a shorter time and because they are primary cells, the time they could be incubated for is limited unlike immortalised cells as demonstrated from the proliferation assay. The data generated from the permeability studies were analysed to determine the permeability ratio for each treatment. The permeability ratio was calculated as results from base reading/receiver of the transwell plate divided by results from the insert reading as exemplified below. This assay was carried out in four independent repeats performed with duplicates. Each experiment was performed with a FITC-dextran standard curve.

To exemplify the calculation of the permeability ratio;

Permeability ratio = Base value (BV)/Insert value (IV);

So, if BV was **70609** and IV was **123686**, BV/IV =**70609/123686**= **0.571.** Therefore, the permeability ratio would be 0.571. However, the data was not presented as a ratio but as normalised values. The permeability data was normalised using Excel to achieve a single figure. Thus, normalising the permeability ratio of 0.571 with Excel (=Standardize, x, mean, stdev), the permeability ratio would then be **6.6.**
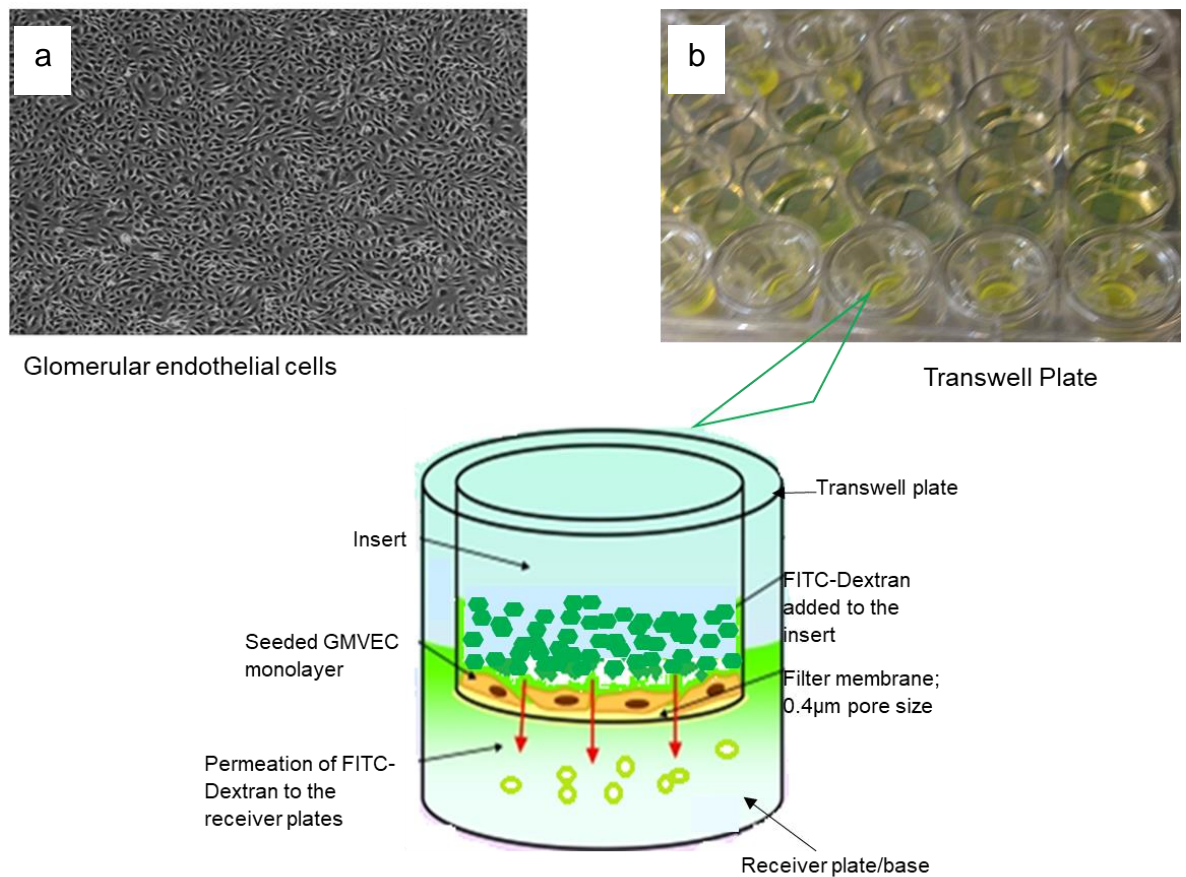
Figure 2. 1: **Schematic representative model of permeability assay**

Panel a represents the microscopic image of the human glomerular microvascular endothelial cells while in panel b, GMVEC are seeded onto the insert of the transwell plate to form a monolayer, followed by addition of VEGF-A$_{165}$, a permeability modulator and the FITC-dextran. The FITC-dextran permeates the cell monolayer into the receiver plate and the resulting ratio of fluorescence in the insert and base plates were used to measure vascular permeability.

2.2.2.5.2 Assay validation using trans-endothelial electric resistance (TEER)

To validate the effect of VEGF-A$_{165}$ on endothelial monolayer in GMVEC, an EVOM2 meter with an alternating current frequency of 12.5Hz was used to assess the trans-endothelial electric resistance (TEER) of the GMVEC monolayer. A 24-well transwell plates with inserts were pre-rinsed with attachment factor and seeded with 20,000 cells per well. Cell culture and growth conditions were as described earlier (section 2.2.2.2.1). After 44 hours of cell incubation, used media was gently removed from the insert and cells were treated with media containing 50 ng/ml of vascular endothelial growth factor (VEGF-A$_{165}$) for an additional 4 hours. Post-VEGF treatment was followed by electrical measurements of the cell monolayer using two electrodes also known as chopstick electrode pair (Figure 2.2). To measure the electrical resistance across the GMVEC monolayer, one electrode was placed in the upper compartment (insert) and the other electrode was placed in the lower compartment (receiver plate) of the transwell plates and readings in volts were recorded. Blank measurements (R-Blank) were recorded using media only without cells. Data was analysed by subtracting blank measurements (R-Blank) from cell measurements (R-Total) to get the actual GMVEC monolayer resistance. The recorded data in volts was translated to ohms.

Thus, the equation used in calculating the monolayer resistance was:

$R_{GMVEC} = R_{Total} - R_{Blank}$
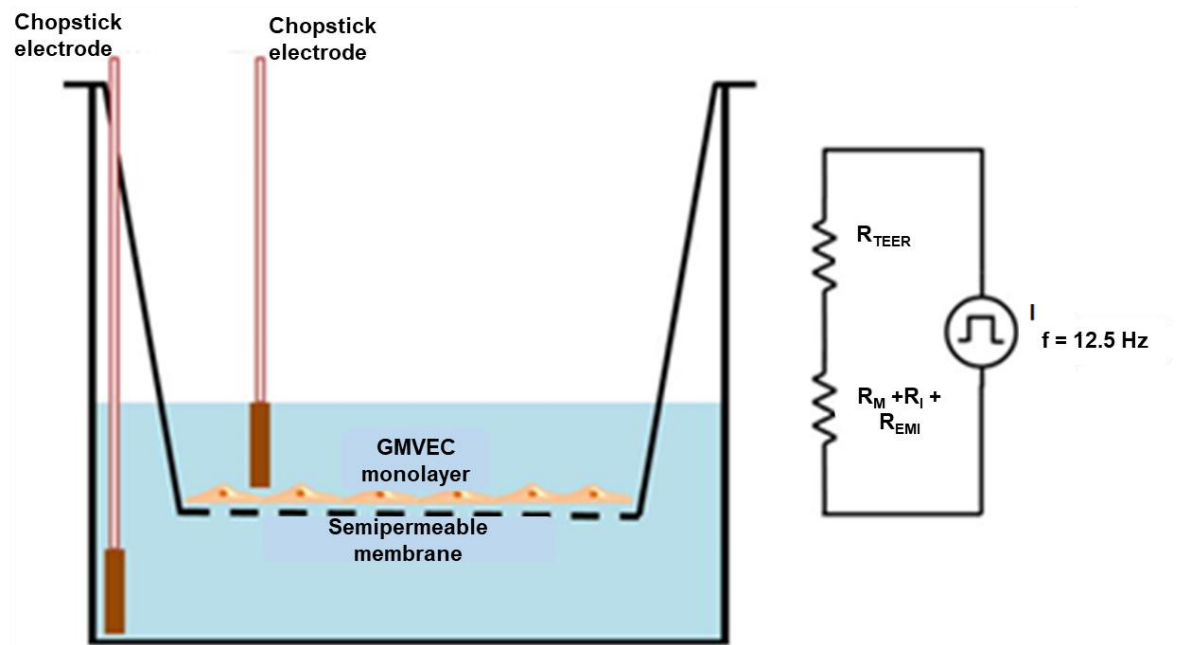
Figure 2. 2: **Diagrammatic representation of TEER assay**

The GMVEC barrier integrity was assessed using EVOM meter with chopstick electrodes and an alternating current of 12.5 Hz. The total electrical resistance includes the ohmic resistance of the cell layer R_{TEER}, the cell culture medium R_M, the semipermeable membrane inserts RI and the electrode medium interface R_{EMI}. (Adopted from Srinivasan, et al., 2015).

2.2.2.6 Investigating the effect of artificial sweeteners on GMVEC monolayer

Based on the results from the preliminary studies on the permeability-inducing effect of VEGF-A$_{165}$, the 20 KDa FITC-dextran molecular weight was selected as the tracer for further permeability studies (see Figure 3.6). The individual effect of artificial sweeteners, aspartame, saccharin, sucralose and neotame on GMVEC monolayer permeability was then determined by plating 20,000 cell suspension of GMVEC on the insert of transwell plate (400 µl/well) and cultured in a humidified incubator at 37°C and 5% CO$_2$ for 24 hours. The cells were then treated with varying doses; 0.1, 1, 10, 100 and 1000 µM of aspartame, saccharin, sucralose and neotame respectively in the absence of VEGF and incubated for additional 24 hours. The GMVEC monolayer formed was then tested for leak using the FITC-dextran (20 KDa) for 180 seconds as described earlier (section 2.2.2.5.1). The plates were measured at excitation and emission wavelengths of 485 and 535 nm respectively. This assay was carried out in 6 independent repeats. Data was analysed as previously outlined in section 2.2.2.5.1.

To study the protective effect of artificial sweeteners on VEGF-A$_{165}$ induced barrier leak, GMVEC were seeded and cultured as previously described for permeability studies (section 2.2.2.5.1) but for 20 hours to correspond with the total time of the assay for previous permeability assay. Following GMVEC culture, the cells were treated with varying doses of artificial sweeteners; 0.1, 1, 10, 100 and 1000 µM of aspartame, saccharin, sucralose and neotame followed by incubation for additional 24 hours. The cell monolayer on the insert was then exposed to 50 ng/ml of VEGF-A$_{165}$ (400 µl/well) while the receiver plate was replaced with 1500 µl/well of fresh media and kept in the incubator for additional 4 hours. The timings for the artificial sweeteners and VEGF incubation were optimised in preliminary studies prior assay. Then, 80 µl of used media was aspirated off and replaced with 80 µl of FD20, pipetted up and down to mix gently, allowing the fluorescent molecules to pass through the endothelial cell monolayer. Thereafter, at 180 seconds, 100 µl of the solution mix from both the base and insert were aliquoted into a 96-well plate. The plates were measured using the fluorescent plate reader at an excitation and emission wavelengths of 485 nm to 535 nm respectively.

The controls for this assay were untreated cells representing negative controls, while cells treated with VEGF-A$_{165}$ only were used as positive control. The resulting data were analysed as in section 2.2.2.5.1 with an n-number of 6.

2.2.2.7 Immunofluorescence (IF) imaging studies
2.2.2.7.1 Determining the internalisation of FITC-dextran

Imaging of GMVEC was carried out to determine whether any of the FITC-Dextran dye was internalised in the cell. To assess that, GMVEC were plated and cultured as in permeability studies (section 2.2.2.5.1). When the GMVEC monolayers had formed, used media was replaced with FITC-Dextran (4, 20, 40 and 70) in media at 5 µg/µl and left for 180 seconds. The media was then removed, and cells rinsed with DPBS, followed by addition of PFA (4%) for 10 minutes at room temperature to fix the cells. The GMVEC were washed and then 2 drops of DAPI (4', 6-diamidino-2-phenylindole) was added to the wells after which the GMVEC images were captured by Zoe fluorescent cell imager using scale bar of 100 µM.

2.2.2.7.2 Effect of VEGF on surface expression of VE-cadherin

This assay was performed to determine how VEGF-A$_{165}$ affects expression of the cell-cell adhesion protein, VE-cadherin, in GMVEC adopting a protocol by Chichger, et al. (2015). The assay was carried out by seeding 50,000 cultured GMVEC into a 12-well plate which was first pre-rinsed with attachment factor (100 µl/well). The cells were incubated in a humidified incubator at 37°C and 5% CO_2 for 44 hours followed by VEGF-A$_{165}$ treatment (50 ng/ml) for additional 4 hours. The cells were fixed in 1% PFA for 10 minutes at room temperature followed by the addition of 0.5% Triton-X 100 in DPBS for 15 minutes at room temperature to permeabilised the cells. To block the cells from any interference, 2% bovine serum albumin (BSA) was prepared by dissolving 2 g of BSA in 100 ml of PBS. The cells were then blocked in 2% BSA-PBS (200 µl/well) and incubated at 37°C for 1 hour. Then, cells were incubated with 1:50 anti-VE-cadherin primary antibody for an

additional 2 hours at 37ºC. Following the primary antibody binding, GMVEC were rinsed with DPBS and then the fluorescent linked-secondary antibody (Donkey anti-goat) was added, followed by an additional incubation for an hour. Following binding of the secondary antibody, used solution in the well was removed and cells were rinsed with PBS. Finally, the cells were counterstained with 3 drops of 4′,6-DAPI and mounted in prolong-gold antifade reagent for 10 minutes at room temperature and viewed using the Zoe imager using scale bar of 100 µM.

2.2.2.8 Immunochemistry assays
2.2.2.8.1 Cell surface expression T1R3 using indirect ELISA

The expression of sweet taste receptor, T1R3, was examined in glomerular endothelial cells using indirect whole cell ELISA (Chichger, et al., 2015). Cultured GMVEC were seeded unto a 96-well plate (5000 cells per well) and incubated in a humidified incubator at 37°C and 5% CO_2 for 24 hours. Next, the GMVEC were treated with 3 µM of lactisole for 10 minutes prior to treatment with artificial sweeteners. The cells were then treated with aspartame (10 µM), saccharin (0.1 µM), sucralose (0.1 µM) and neotame (0.1 µM) and incubated for an additional 24 hours. Following exposure of the cells to the artificial sweeteners at the designated time, used media was removed, and 50 µl/well of 1% PFA was added gently to the wells to fix the cells. The fixed cells were left to sit at room temperature for 10 minutes followed by aspirating of the PFA solution and rinsing of the cells twice with DPBS. The cells were blocked from any interference with 2% bovine serum albumin (section 2.2.2.8.2). After blocking the cells, GMVEC were rinsed three times with dH_2O followed by incubation with anti-T1R3 primary antibody (Table 2.4). The anti-T1R3 primary antibody mix was prepared in a ratio of 1:50 in 1% BSA-PBS. 50 µl of the primary antibody mix was added to each well except for the controls where 1% BSA-PBS was used instead and then incubated for 2 hours at 37ºC. The cells were rinsed 3x with dH_2O after incubation with primary antibody. The secondary antibody (Table 2.4) was prepared with 1% BSA-PBS in the dark. The ratio of secondary antibody used was 1:300 from which 50 µl/well was added to each well, then incubated at 37ºC for 1 hour.

The control plates were filled with 1% BSA-PBS. After incubation with the secondary antibody, the cells were rinsed with dH_2O and the plate was wrapped in foil and chemiluminescence was measured at 20 seconds exposure time using fluorescent plate reader.

2.2.2.9 Inhibition of sweet taste receptor by lactisole effect on VEGF-induced permeability

The activation of the sweet taste receptor by the artificial sweeteners in the glomerular endothelial cell was validated using the sweet taste inhibitor, lactisole. The GMVEC (20000 cells/well) were plated into insert of the transwell (plate pre-rinsed with attachment factor). The seeded cells were then incubated in a humidified incubator at 37°C and 5% CO_2 for 20 hours. The used media was pipetted off the well and cells were treated with 3 µM of lactisole (200 µl /well) and incubated at 37°C for 10 minutes. After exposure of cells to lactisole, 200 µl of each artificial sweetener (10 µM aspartame; 0.1 µM saccharin; 0.1 µM sucralose and 0.1 µM neotame) was pipetted into each well and incubated at 37°C for additional 24 hours. Following the treatment of GMVEC, permeability assay was carried out as detailed in section 2.2.2.7.

2.2.2.10 Investigating the effect of artificial sweeteners on intracellular cyclic AMP

Cyclic adenosine monophosphate (cAMP) levels in GMVEC were measured, utilising forskolin (FSK), a broad activator of cAMP, as a positive control. Cultured GMVEC were seeded (5000 cells per well) in a pre-treated 96-well plate and incubated in a humidified incubator at 37°C and 5% CO_2 for 20 hours. Thereafter, cells were treated with 100 µl of artificial sweeteners; aspartame, saccharin, sucralose and neotame at 1 and 1000 µM concentrations for 24 hours followed by VEGF (50 ng/ml) exposure for additional 4 hours followed by cAMP assay. Regarding the assay with sweeteners only, after 24 hours incubation at 37°C, cells were treated with 1 µM and 1000 µM of aspartame, saccharin, sucralose and neotame for another 24 hours, followed by the cAMP assay using the

cAMP-Screen direct system (Applied Biosystems #4412186) assay kit. As a positive control, forskolin was used to pre-treat the control prior to assay protocol. Although, the assay protocol was carried out according to manufacturer's guidelines, the concentration of the sweeteners used was based on pre-trial assays and time of incubation was selected to reflect those in permeability assay in order to maintain consistency in the exposure time of sweeteners to GMVEC (section 2.2.2.6).

The cAMP assay protocol was performed as outlined in the manufacturer's guidelines. That is, used media from the cells were first replaced with 60 µl per well of the assay lysis buffer and incubated at 37°C until cells lysed (10 minutes). Following cell lysis, the cAMP-AP conjugate was diluted at 1:1000 ratio with conjugate dilution buffer (40 µl cAMP-AP conjugate + 3.96 ml conjugate dilution buffer) and 30 µl of the diluted cAMP-AP conjugate was added into each well (including standards), mixed repeatedly by pipetting up and down gently. Then, 60 µl of cAMP antibody was added to each well, mixed gently, and incubated on a mini-plate shaker at room temperature for 1 hour. Following the incubation, the solutions from the wells were aspirated off and the wells were washed six times using the wash buffer. Thereafter, 100 µl of CSPD/Sapphire II RTU solution was added into each well and incubated for an additional 30 minutes at room temperature on the mini-plate shaker. To measure cAMP level, the plate backing sheet was carefully applied to the bottom of each microplate prior to luminescence measurement for 1 second on the Victor plate reader set up at 535/590 nm.

Additionally, alongside each experiment, there was a standard curve preparation which was done by adding 60 µl of the prepared standards to empty wells on the plate. The kit used was the cAMP-Screen direct system with pre-coated assay plate which are tissue-culture treated and pre-coated with capture antibody. The assay was carried out in four independent repeats performed with duplicates. Additionally, the cAMP standard supplied with the kit was 1 mM cAMP (1,000 pmol/µl) which was prepared in a 7-serial 1:10 dilution with the assay/lysis buffer resulting in a range of concentrations between 0.006 to 6,000 pmol cAMP per 60 µl for the standard curve.

2.2.2.11 Determining the effect of artificial sweeteners on reactive oxygen species

A known probe for the measurement of intracellular ROS, 2,7-dichlorodihydrofluorescein diacetate (DCFDA), is oxidised to dichlorofluorescein (DCF) through the action of intracellular esterases upon permeation into cell (Tampo, et al., 2003; Hempel, et al., 1999). This probe was utilised in this research due to the simple technique and sensitivity of the probe. To investigate the effect of artificial sweeteners on reactive oxygen species (ROS) levels in glomerular endothelial cells, cultured GMVEC (20,000 cells/well) were plated into black-walled, clear-bottomed, 96-well plates pre-rinsed with an attachment factor and cells were incubated in a humidified incubator at 37°C and 5% CO_2 for 24 hours. The used media was aspirated off and cells were washed with 100 µl/well 1x buffer (diluted from 10X buffer supplied by manufacturer) by swirling gently. The cells were then stained with 10 µM of DCFDA (100 µl/well) and incubated in the dark for 30 minutes at 37 °C. The DCFDA solution was then removed and cells were washed with 1X buffer, followed by exposed to 0.1, 1, 10, 100 and 1000 µM concentrations of aspartame, saccharin, sucralose and neotame respectively and further incubated for 2 hours. Additionally, 10 µM of hydrogen peroxide (H_2O_2) was used as a positive control for the assay while 10 µM of the antioxidant, N-acetyl-L-cysteine (NAC), was used as an inhibitor of ROS. The GMVEC fluorescence measurements were taken on a fluorescent plate reader (PerkinElmer 2030 Manager) for 1 second at an excitation/emission of 485/535nm. This assay had an n-number of 6.

2.2.2.12 Effect of artificial sweeteners on H_2O_2-induced ROS

To determine the protective effect of artificial sweeteners on the glomerular microvasculature against H_2O_2-induced ROS, GMVEC were seeded as above, (section 2.2.2.11). The cells were incubated for 24 hours in a humidified incubator at 37°C and 5% CO_2. Following on from the cell culture, the GMVEC were treated with 100 µl of 10 µM NAC, 0.1, 1, 10, 100 and 1000 µM doses of aspartame, saccharin, sucralose and neotame respectively for additional 2 hours. The DCFDA staining, experimental controls and fluorescence measurements were performed as in section 2.2.2.11. The difference between both methods is the time of DCFDA staining. In section 2.2.2.11, the cells were stained with the DCFDA probe first, followed by exposure to artificial sweeteners and other controls. In section 2.2.2.12, the cells were first pre-treated with artificial sweeteners and NAC for 2 hours prior staining with DCFDA followed by exposure to 10 µM of H_2O_2. In addition to the controls set during the experimental procedure of the ROS assay, a control with vehicle only (media only) was set up to determine maximal signal in the absence of compound while another set of control were non-stained cells (cells without DCFDA) in order to determine background fluorescence. To determine the absolute ROS generated, the blank readings were subtracted from all measurements (control and treated) and change in ROS level between control and treated cells was then determined.

2.3 Analytical chemistry materials and methods

2.3.1 Materials
2.3.1.1 Chemicals and reagents

The chemicals and reagents utilised for the analysis of sucralose using GC-MS are mentioned on Table 2.4. The reagents and chemicals utilised in the application of analytical techniques to cell studies has previously been outlined in Table 2.1.

Table 2. 4:**Derivatising reagents and solvents used for the research**

Product	Supplier
N-Methyl-*N*-(trimethylsilyl) trifluoroacetamide (MSTFA)	Sigma Aldrich (UK)
Trimethylphenyl ammonium Hydroxide (TMAH-33358-U)	Supelco Sigma Aldrich (UK)
N, O-Bis(trimethylsilyl)trifluoroacetamide (BSTFA)	Supelco Sigma Aldrich (UK)
Sucralose, 98% HPLC analytical grades	Sigma Aldrich (UK)
Sucralose-d6 (SC-220145)	Santa Cruz Biotechnology (USA).
Methanol and Ethyl acetate	Sigma Aldrich (UK)

2.3.1.2 Equipment
2.3.1.2.1 Gas Chromatography-Mass spectrometry

Instrumental analysis was done on a Perkin Elmer Clarus 500 GC-MS. The column used for most of the experimental work was Rtx ®-5 (30 m x 0.25µm x 0.25 mm i.d). Another column ZB-5 (30 m x 0.25µm x 0.25 mm i.d) was used during the preliminary studies to determine robustness of the method. The stationary phase on the Rtx ®-5 column was 5% diphenyl/95% dimethyl polysiloxane. The carrier gas was helium (BOC, 99.95 %) at a flow rate of 0.6 mL/min. The mass analyser was a single quadrupole, with an electron impact ionization of 70 eV operated in full scan mode from 40-600 m/z. All data collection, analysis and processing were carried out using TurboMass™ 5.4 GC-MS software. The Instrumental parameters and method set-up were adopted from Qiu, et al. (2007) as outlined on Table 2.5. The GC-MS was subjected to regular tuning (calibration) and maintenance (change of filament, liner, septum, column and needle as well as baking the column and cleaning the source) as necessary. Additional consumables and equipment used include GC-MS autosampler vials (Fisher Scientific, UK), Mivac (for drying samples) and heating blocks.

Table 2. 5: **Instrumental parameters for GC-MS**

Parameter	Setting
Flow Gas/Rate	He 0.6mL/min
Column	Rtx ®-5: 30m length, 0.25mm ID, 0.25µm thickness
	ZB-5: 30m length, 0.25mm ID, 0.25µm thickness
Injection	Auto
Injection Volume	1.0µL
Injection Mode	Split (9:1)
Injection port temperature	270 ^{O}C
Solvent delay Time	3.5 minutes
Oven temperature Parameters	180°C for 2 minutes, 6°C / minutes until 250 ^{O}C, hold for 20 minutes
Transfer Line Temperature	280 ^{O}C
Mode	EI, 70 eV and Scan 40-600m/z
Experiment time	34 minutes

2.3.2 Methods

2.3.2.1 Stock Solution preparation of sucralose and internal standards

Sucralose (5 mg) powder was dissolved in 10 ml of methanol to make 0.5 mg/ml (500 µg/ml) stock solution. The stock solution of sucralose was then diluted down with methanol to get desired concentrations of 0.04, 0.4, 5, 10, 25 and 100 µg/ml amongst which 100 µg/ml was used for the preliminary detection of sucralose prior to the selection of internal standards. The concentrations of sucralose used for the calibration curve were 2.5, 5, 7.5, 10, 12.5, 15, 20, 25, 30, 40, 50, 100, 200 and 400 µg/ml. To prepare the first internal standard, myo- inositol, (2 mg) was dissolved in 10 ml of methanol to make 200 µg/ml stock solution. Different myo-inositol working concentrations of 25, 50 and 100 µg/ml were prepared to determine the best concentration suitable for use as an internal standard. To prepare the second internal standard, deuterated-sucralose (sucralose-d6), supplied as 1 mg/ml solution of sucralose-d6, was diluted down with methanol to different concentrations of 25, 50 and 100 µg/ml.

2.3.2.2 Selection of derivatisation reagent

2.3.2.2.1 Derivatisation of sucralose with BSTFA and MSTFA

Two different derivatisation reagents; *N*-Methyl-*N*-(trimethylsilyl) trifluoroacetamide (MSTFA) and *N, O*-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) were studied for their suitability at derivatising sucralose. To derivatise sucralose with BSTFA, 100 µl of 100 µg/ml sucralose was transferred into a glass vial and solvent was evaporated using Mivac. This was followed by the addition of 100 µl of BSTFA and the mixture was allowed to derivatise (react) at 70 °C for 30 minutes. Following derivatisation, the analyte mix was transferred into GC vials, capped and analysed on the GC-MS using the set-up protocol for sucralose on Table 2.5. The same protocol was also utilised in derivatising sucralose with MSTFA.

2.3.2.2.2 Optimisation of sucralose derivatisation reaction

Based on the results from the derivatisation reaction with BSTFA and MSTFA (Figure 5.4), the selected reagent carried forward for derivatising sucralose was MSTFA. Hence, the derivatisation of sucralose with MSTFA needed to be optimised to determine the optimum reaction temperature and time. 100 µg/ml of sucralose was made up with methanol from stock solution and 100 µl was aliquoted into 3 separate glass vials and evaporated using Mivac. Once the analytes were dry, 100 µl of MSTFA was added and derivatised at 70 ^{O}C for 30, 45 and 60 minutes respectively and in triplicate making a total of 9 vials for each experiment. The derivatised analyte were then transferred onto GC-vials and analysed by GC-MS. To determine if 70 ^{O}C was the optimum reaction temperature, same protocol for derivatisation reaction time was followed without heating but instead the analyte was derivatised at room temperature for 30, 45 and 60 minutes. For each temperature (70 ^{O}C and room temperature), all time points were studied in triplicates with at least three independent repeats (n=3).

2.3.2.3 Selection of internal standard
2.3.2.3.1 Internal standard myo-inositol and sucralose-d6

The two internal standards tested for suitability during this research were myo-inositol and deuterated sucralose (sucralose-d6). To assess their suitability as an internal standard, 100 µl of 25, 50 and 100 µg/ml of both myo-inositol and sucralose-d6 were aliquoted into glass vials, dried, derivatised with MSTFA at 70 ^{O}C for 30 minutes and analysed by GC-MS protocol outlined in Table 2.5.

2.3.2.3.2 Myo-inositol stability test

A stability test for myo-inositol was performed by analysing the selected concentration (25 µg/ml) of myo-inositol over a period of 24 hours. 25 µg/ml of myo-inositol was prepared and 100 µl was aliquoted into 3 separate glass vials, dried and derivatised with MSTFA at 70 ^{O}C for 30 minutes, followed by GC-MS analysis.

2.3.2.3.3 Stability test of deuterium-sucralose

To determine the stability of the second internal standard,100 µl of 50 µg/ml sucralose-d6 was aliquoted into glass vials and dried using Mivac. After the analytes was dried by evaporation, 100 µl of MSTFA was added to derivatise the sample, followed by an analysis of the mixture as described in section 2.3.2.3.1. The stability test was carried out for a duration of 24 hours (intra-day) and 3-days (inter-day). The instrument auto sampler stability test for sucralose-d6 was further studied by analysing the result output of the selected concentration (50 µg/ml) of the internal standard over a period of 72 hours. This experiment has a technical repeat of at least 36 with generated data collected every 6 hours within the 3 days run time. To clarify, these stability tests were performed with only the internal standards. As a deuterated analogue, sucralose-d6 is like sucralose with six added atoms and hence, expected to behave in a similar manner.

2.3.2.4 Optimisation of sucralose with internal standard

To further optimise the derivatising reaction of sucralose with the selected internal standard, 100 µg/ml of sucralose was aliquoted into a glass vial, followed by addition of 50 µg/ml of internal standard, mixed and dried (both analyte and internal standard had equal volumes of 100 µl). When the mixed standard was evaporated (dried), 100 µl of MSTFA was added to the standard mix, heated at 70 ^{O}C for 30 minutes to derivatise, followed by injections into GC-MS for analysis.

2.3.2.5 Calibration standards

Linearity and linear range were determined by preparing different concentrations of sucralose standards, ranging from 2.5 to 400 µg/ml. Each sucralose standard (100 µl) was mixed with equal volume of 50 µg/ml internal standard (sucralose-d6) and prepared for GC-MS analysis as previously discussed (2.3.2.4). To avoid the analyte being carried over in the GC column, solvent blanks (methanol) were injected between each run. A quality control (made up of C18, 20 and 22; 50 µg/ml in ethyl acetate) was also injected at the

start, middle and end of each batch run. Peak area ratio (PAR) was calculated by dividing the peak area of analyte (sucralose) with the peak area of internal standard (sucralose-d6). A calibration graph of PAR of sucralose and internal standard was plotted against concentration of sucralose. Standard deviation and relative standard deviation (RSD) were also calculated. Linearity was established using the calibration curve slope equation to calculate the correlation coefficient value, (R^2) while the relative standard deviation (RSD) was used to determine repeatability and precision of the method. Each sucralose concentration was analysed in triplicate in 3 independent experimental repeats.

2.3.2.6 Determining limits of detection and quantification

The linear calibration was further used to evaluate the limit of detection and limit of quantification. Concentrations within the linear range (5-400 µg/ml) standards of sucralose were studied as explained in section 2.3.2.5. The linear regression method assumes that instrument response, y, is linearly related to the standard concentration with detection and quantification limits calculated based on this relationship (Sanagi, et al., 2009). The equation used for calculating limit of detection (LOD) was:

LOD = 3.3 σ/S; whilst limit of quantification (LOQ) = 10 σ/ S,

where σ = the standard deviation of the response and S = the slope of the calibration curve (Sanagi, et al., 2009; SWGTOX, 2013).

2.3.2.7 Matrix matched calibration of sucralose in cell and media

The optimised GC-MS method was then applied in the detection of sucralose in cell media. The glomerular endothelial cells require media for their growth. Thus, as a prerequisite, the media containing 4, 40,100 and 400 µg/ml sucralose concentrations, were analysed using the GC-MS developed method to assess the matrix effect of the cell media. To imitate the condition of the cell growth, these growth media containing sucralose without cells were incubated for 24 hours prior to sample preparation and analysis by GC-MS (section 2.3.2.4). These sucralose concentrations for the matrix match effect were

selected to correspond with the concentrations utilised in the cell biology section of this research.

2.3.2.8 Detection of sucralose in cell media with glomerular endothelial cells
2.3.2.9 Preparation of glomerular endothelial cells for GC-MS Analysis

Glomerular microvascular endothelial cells (GMVEC) were cultured as described earlier in section 2.2.2.2.1. Viable GMVEC were plated onto 6-well plates at 20,000 cells/well and incubated for 24 hours. The cells were treated with 500 µl of media containing varying concentrations of sucralose (4, 40,100, 200 and 400 µg/ml) and incubated for an additional 24 hours. The negative control was media only, and cells containing media only, while the positive control was media containing sucralose only with no cells.

The analysis of sucralose in GMVEC was carried out in two stages. First, it was determined whether sucralose remained in the cell media used in culturing GMVEC. Secondly, the lysate of GMVEC was extracted and analysed. To investigate whether sucralose was in the cell media, GMVEC were cultured and exposed to sucralose. After 24 hours of post sucralose treatment, the media from each well was removed and pipetted into glass vials. 100 µl of the used media was mixed with 100 µl (50 µg/ml) of internal standard. The sample mixture was evaporated using Mivac. The dried sample mix was then derivatised as described in section 2.3.2.4**,** cooled at room temperature, transferred into GC vials, capped and analysed.

To determine whether sucralose crossed the glomerular endothelial cell barrier, GMVEC were cultured and exposed to sucralose concentrations. GMVEC extraction was adapted from Zhong, et al. (2017) with modifications in sample preparations for GC-MS analysis. The cells pre-incubated with sucralose for 24 hours were washed 6 times with phosphate buffered saline (PBS) after aspirating off the used media. The GMVEC were then soaked in 400 µl methanol for 5 minutes followed by scraping using a cell scraper. The cell suspension was transferred into glass tubes. The cells were then sonicated twice for 10 minutes, which was done at 5 minutes interval. Cell lysate was vortexed (5 minutes) and

centrifuged at 212 g force (RCF) for 10 minutes. The cell lysate supernatant was then transferred into glass vials, mixed with 100 µl of the internal standard and dried using Mivac. The dried sample was subsequently derivatised with 100 µl MSTFA at 70 °C for 30 minutes and analysed by GC-MS protocol for sucralose as outlined in Table 2.5.

2.4 Statistical analysis

All data sets were statistically analysed using GraphPad Prism7/8 software (USA). Key tests such as one-way and two-way ANOVA with post hoc tests; Kruskal-Wallis test, Turkey comparison test and Dunn's multiple comparison test were carried out as appropriate. N-value (n-number) denotes the number of independent experimental repeats and is stated in the figure legend for each graph in the results section with each experiment having an n-number of at least 3. The data was presented as mean ± standard error mean (S.E.M.) except where stated otherwise. Statistically significant points represented by a p-value < 0.05 are denoted with an (*) and in some cases, the exact p-value was given. The number of (*) depends on how far away the data was significant with (****) meaning *p-value* was < 0.0001 and extremely significant; (***) denoting *p-value* < 0.001 and highly significant; (**) and (*) meaning *p-value* was significant and = 0.001-0.01 and 0.01-0.05 respectively.

CHAPTER 3

ARTIFICIAL SWEETENERS ATTENUATE VEGF-A INDUCED VASCULAR PERMEABILITY IN A CELL MODEL OF GLOMERULAR MICROVASCULATURE

3.1 Introduction

Microvascular permeability is the hallmark of vascular complications seen in metabolic diseases such as DKD (Deissler, et al., 2013; Weil, et al., 2012). The endothelium plays essential role in maintaining the vasculature through regulation of plasma proteins and solute transport. However, in a pathological state such as DKD, integrity of the endothelial barrier is compromised and dysfunctional resulting in vascular permeability. As discussed in the introduction (section 1.8.1), both *in-vivo* and *in-vitro* studies have linked increased levels of serum VEGF-A and proteinuria to progression of DKD, thereby indicating an increased intracellular levels of VEGF-A as a predictor and marker of DKD (Hovind, et al., 2000; Santilli, et al., 2001; Rodriguez, et al., 1998; Jelkmann, 2001; Wasada, et.al, 1998; Lenz, et.al, 2003; Veron, et al., 2010). These studies suggest that inhibition of VEGF-A is a possible treatment option for DKD. However, an experimental study by Eremina, et al. (2003), showed that inhibition of VEGF-A leads to significant renal pathology, exacerbating the condition and possibly the reason no such agent has been approved for the treatment of DKD. Therefore, the need for a novel and effective therapeutic agent targeted at attenuating VEGF-A induced permeability. The novel agents investigated in this research for their potential therapeutic effects on DKD were artificial sweeteners, aspartame, saccharin, sucralose and neotame. The increase in usage of sugar substitutes are based on the suggestions that artificial sweeteners aid in maintaining a "close to normal" level of blood glucose in individuals living with diabetes (section 1.9.1).

Since the impact of these artificial sweeteners when in circulation and on glomerular microvasculature following ingestion is still lacking, the rise in the number of individuals using artificial sweeteners has prompted investigations into their effects as sweet taste agonist and other possible physiological roles.

Whilst the hypothesis for this research are outlined in section 1.19, this chapter focused on addressing aim 1; establishing the protective effect of artificial sweeteners, aspartame, saccharin, sucralose and neotame on VEGF-A induced permeability in glomerular microvasculature. The chapter aim was achieved by addressing the following objectives;

- Culturing of glomerular microvascular endothelial cells and determination of their viability in the presence of varying physiological concentrations of artificial sweeteners

- Assessing and validating the *in vitro* permeability assay for glomerular microvascular endothelial cells in the presence and absence of VEGF-A as a model for mimicking permeability observed in DKD

- Using the validated permeability assay to determine whether artificial sweeteners affect the barrier integrity of the glomerular endothelium

- Application of the permeability assay protocol to determine the protective effects of artificial sweeteners against VEGF-A induced leak in the glomerular endothelial microvasculature.

3.2 RESULTS

3.2.1 Establishing endothelial cell numbers for viability assay

Initial experiments assessed the effect of the artificial sweeteners (aspartame, saccharin, sucralose and neotame) on glomerular endothelial cell viability. A preliminary study was performed to establish appropriate conditions for the viability assay. Proliferation assay was carried out as detailed in section 2.2.2.3. The results of the cell proliferation assay showed the growth pattern of the GMVEC under an incubation period of 24 hours (Figure 3.1a-3.1d), 48 hours (Figure 3.2a-3.2d) and 72 hours (Figure 3.3a-3.3d).

The selected parameter for viability studies was the culture time of 48 hours and 2 hours of CCK-8 incubation (Figure 3.2b) with a cell number of 5000 GMVEC as highlighted in Figure 3.2b. These parameters were chosen because at that time point, GMVEC has complete attachment to the culture plate and cells were at their optimum growth curve, giving ample time for other experiments that may go beyond the 2-hour time period. The 24-hour cell culture time period was not selected because as shown in Figure 3.1, GMVEC were just starting to grow following attachment, as seen in the irregular growth pattern of the cells at the various CCK-8 incubation times.

A peculiar feature of primary endothelial cells is their early onset of senescence (Pan et al., 2009). Thus, a prolonged culture time of 72 hours would not be an ideal experimental parameter as observed in Figure 3.3a-d because at this culture time frame, the GMVEC might enter lag phase prior viability assay. Therefore, these results do not only demonstrate the suitable conditions for viability assay, but also highlighted the growth pattern of GMVEC.
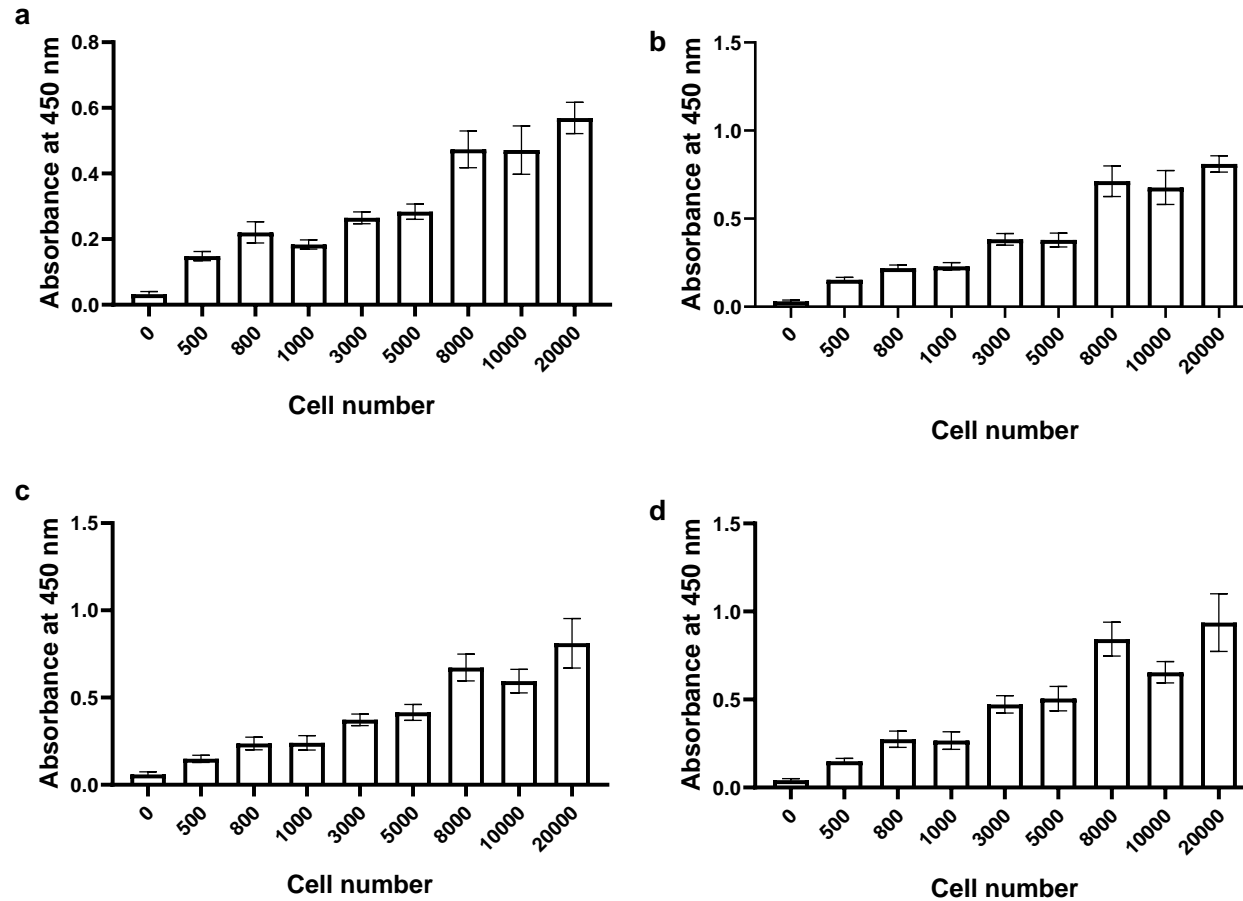
Figure 3. 1: **Glomerular microvascular endothelial cell proliferation after 24-hour incubation using CCK-8**

Varying cell numbers of GMVEC ranging from 500-20,000 cells were plated and incubated for 24 hours followed by CCK-8 assay. After treating the cells with CCK-8 dye, they were incubated for an additional 1 hour (a), 2 hours (b), 3 hours (c) and 4 hours (d) followed by measuring absorbance at 450 nm to determine the proportion of viable cells. Data is expressed as mean ± S.E.M and n=9.
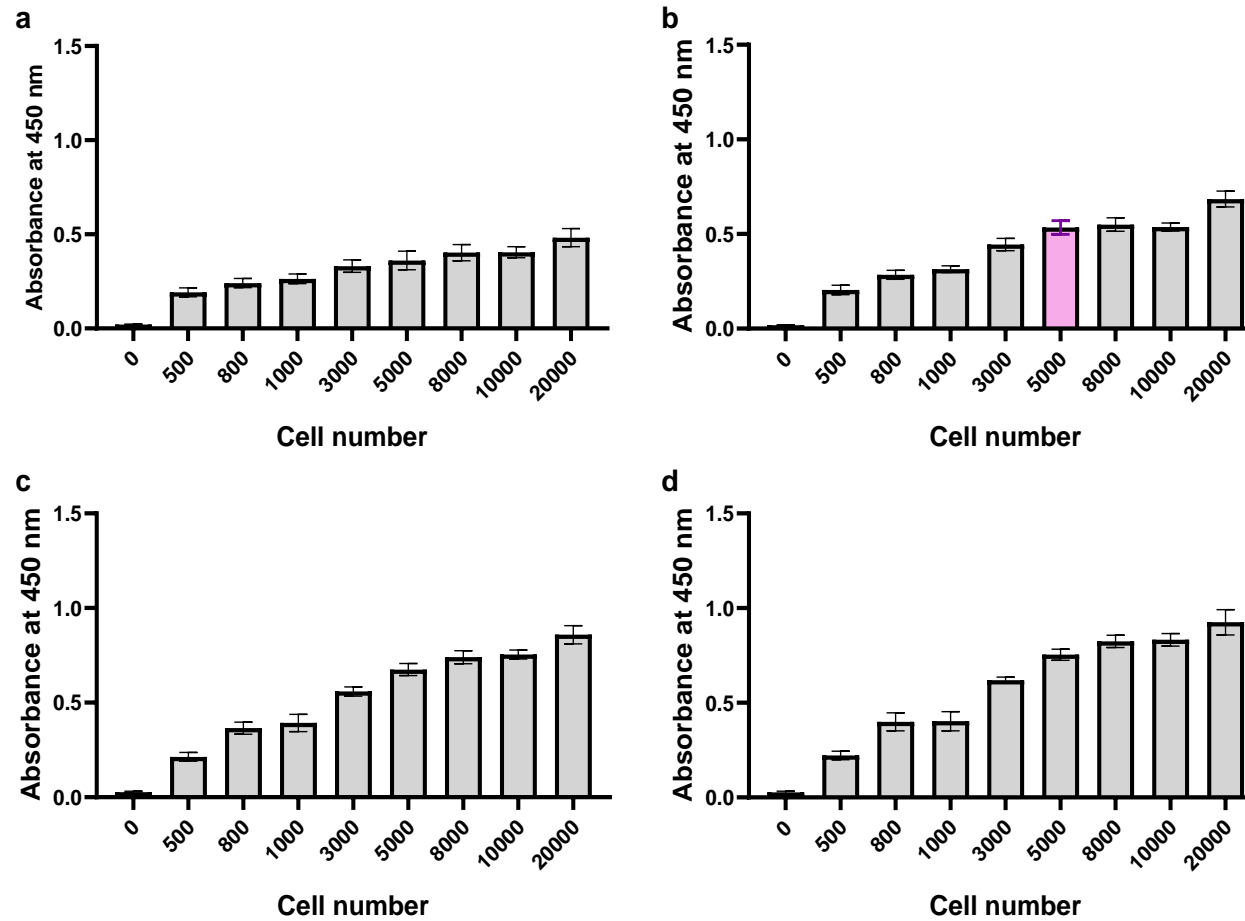
Figure 3. 2: **48-hour Glomerular microvascular endothelial cell proliferation and cell number selection**

The assay was performed as in Figure 3.1 but incubated for 48 hours prior to CCK-8 assay. Data was expressed as mean ± S.E.M and n=9. The data point

highlighted in purple represents the chosen cell number and incubation time for viability studies.
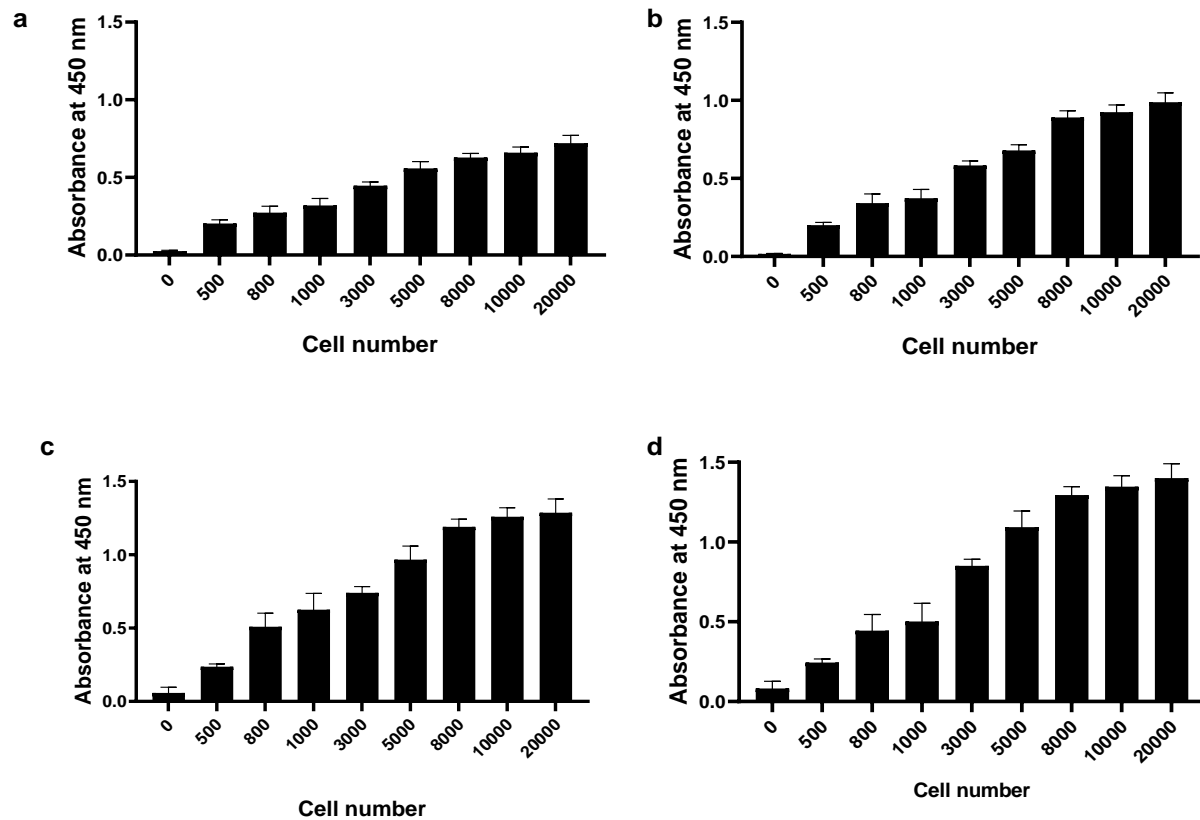
Figure 3. 3**: Glomerular microvascular endothelial cell proliferation following a 72-hour incubation using CCK-8**

Varying cell number of GMVEC ranging from 500-20,000 cells were plated and incubated for 72 hours. Next was the CCK-8 assay for additional

1 hour (a), 2 hours (b), 3 hours (c) and 4 hours (d) followed by measuring absorbance at 450nm to determine viable cells. Data was expressed

as mean ± S.E.M and n=9.

3.2.2 Cytotoxicity effect of aspartame, saccharin, sucralose and neotame

The cytotoxic effects of the artificial sweeteners, aspartame, saccharin, sucralose and neotame, were studied as outlined in section 2.2.2.4 of chapter 2. The parameters used for this study were based on the results of the cell proliferation assay (Figure 3.2b). The results presented in Figures 3.4a-d showed percentage of viable GMVEC treated with aspartame (Figure 3.4a), saccharin (Figure 3.4b), sucralose (Figure 3.4c) and neotame (Figure 3.4d) relative to untreated cells. The percentage of viable cells represents the absolute viability effect of these sweeteners while accounting for any interference from the CCK-8 dye. The findings from the cytotoxicity studies indicated that none of the artificial sweeteners, aspartame, saccharin, sucralose and neotame affected the viability of GMVEC at the studied concentrations (Figure 3.4). The solvent (culture media) for the sweeteners has no effect on cell viability as the control (0 µM) indicates. As noted in Figure 3.4, there were no statistical difference in cell viability when comparing the effects of different concentrations of sweeteners on glomerular endothelial cells. Therefore, neither aspartame, saccharin, sucralose nor neotame affected the growth rate or viability of glomerular endothelial cells at concentrations up to 1000 µM.
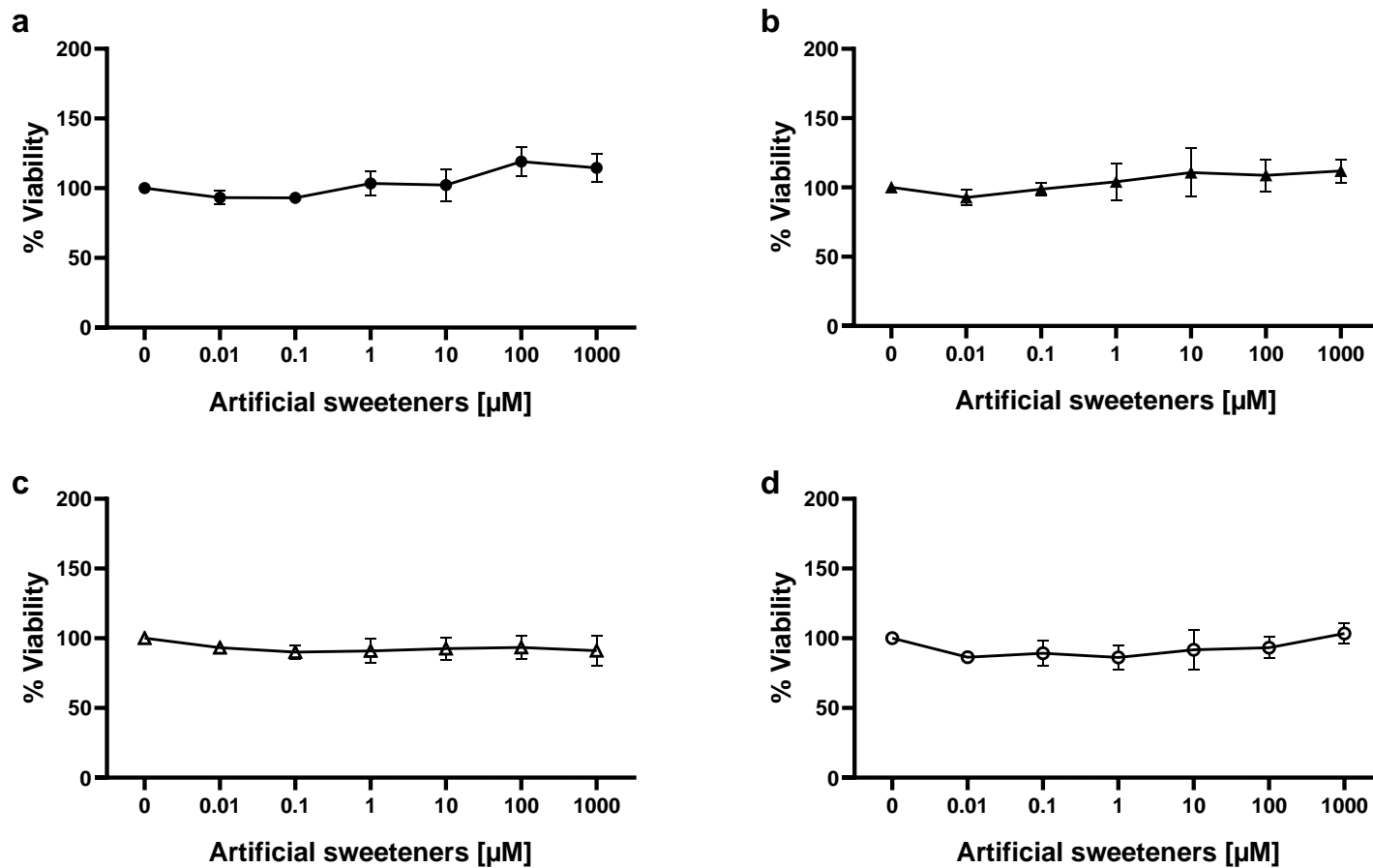
Figure 3. 4: **Artificial sweeteners have no effect on glomerular microvascular endothelial cell proliferation viability**

Viability of GMVEC was measured using CCK-8 assay following GMVEC exposure to artificial sweeteners; aspartame (a), saccharin (b), sucralose

(c) and neotame (d) at physiologically relevant concentrations ranging from 0.01 to 1000 µM. Absorbance was denoted as percentage viability. There

were no statistically significant differences when compared with the control (vehicle). n=9 and data, presented as mean with S.E.M.

3.2.3. Assessing the effect of lactisole on glomerular endothelial cell viability

Lactisole, an antagonist of the sweet taste receptor inhibits the sweet taste sensing of cells (Jiang, et al., 2005; Nakagita, et al., 2019) and since it will be utilised in conjunction with the sweeteners, it was vital to examine its effect on the viability of GMVEC. The assay was determined as previously explained (section 2.2.2.4). The results represented in Figure 3.5 demonstrated that lactisole, significantly reduced cell viability at 100 and 1000 μM concentrations with a *p-value* < 0.05 when compared with control (0 μM). This finding suggests using low dose (below 10 μM) of lactisole when treating primary glomerular microvascular endothelial cells. Moreover, given that less than 10 μM of lactisole has been shown to inhibit the T1R2/T1R3 sweet taste receptors (Nakagita, et al., 2019), and 1-10 μM concentrations of lactisole has no effect on GMVEC viability, the chosen concentration of lactisole used for further experimentation of the research was 3 μM. In addition, this concentration of lactisole (3 μM) has been shown to inhibit the activation the sweet-sensing receptor, T1R3 by its agonist, an artificial sweetener sucralose (Hamano, et al., 2015). Therefore, following optimisation assay, this concentration was utilised for further studies in determining the physiological functions of artificial sweeteners in relation to glomerular microvascular permeability.
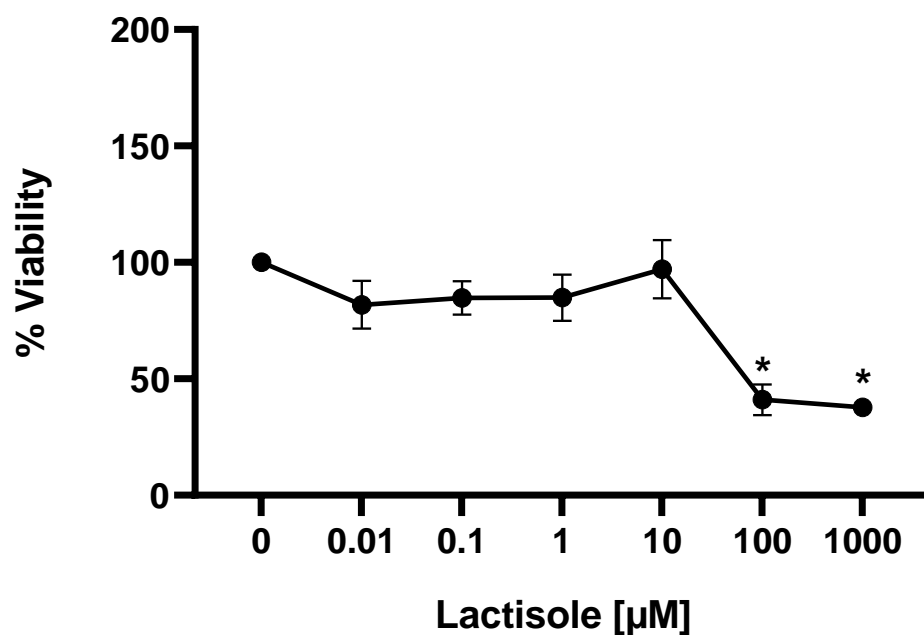
Figure 3. 5*: **Lactisole reduced viability of glomerular endothelial cell at high concentration**

Viability of GMVEC in the presence of sweet taste inhibitor, lactisole, was measured using CCK-8 assay following exposure to lactisole at physiologically relevant concentrations ranging from 0.01μM to 1000 μM. Absorbance was denoted as percentage viability. Data was presented as mean with S.E.M. *p<0.05 when compared with the control (vehicle) and n=9.

3.2.4 Vascular endothelial growth factor, VEGF-A, disrupts glomerular monolayer

Permeability of proteins is the hallmark of DKD with VEGF-A implicated in this leak as supported by some studies (Veron, et al., 2010). Since glomerular endothelial permeability is caused by overexpression of VEGF-A, responses to this growth factor, in the glomerular microvascular endothelium was determined to validate the experimental parameters before studying the effects of artificial sweeteners on this VEGF-A induced leak. To achieve that aim, permeability assays with and without VEGF-A was carried out in a cell model of glomerular endothelium using FITC-dextran as previously explained in methods (section 2.2.2.5.1). The results shown in Figure 3.6b indicates that VEGF-A, induced leak in the glomerular microvasculature. Another finding from this study was the difference in the level of VEGF-A induced permeability using different molecular weights of the (FITC-dextran) tracer. While 4 KDa (Figure 3.6a) of the FITC-dextran didn't show any measurable leak, 20 KDa (Figure 3.6b), 40 KDa (Figure 3.6c) and 70 KDa (3.6d) showed increased leak at different concentrations of VEGF-A. Furthermore, another parameter measured in this assay was the time of maximum leak using the molecular tracers. The result showed that the amount of VEGF-A induced leak measured at various time points differs. The time point,180 seconds showed the highest VEGF-A induced leak at 50 ng/ml using FD 20 (Figure 3.6b) with a *p-value* < 0.0001. Whilst FD40 showed significant leak at all studied VEGF-A concentrations but only at 60 seconds time point with *p-value* < 0.05; FD70 (Figure 3.6d) showed a measurable VEGF-A induced leak at 60 and 180 seconds with a *p-value* < 0.01. Based on these statistically significant findings, it was evident that VEGF-A induced leak in glomerular microvascular endothelium at the studied concentrations. Hence, subsequent experimentation of permeability assays was done using 50 ng/ml of VEGF-A, FD20 as tracer with leak measured at 180 seconds time point (Figure 3.6b). These results, therefore, demonstrated that the growth factor, VEGF-A, induced permeability in the glomerular microvascular endothelium *in vitro,* thereby providing a model for mimicking the VEGF-A induced permeability observed in DKD.
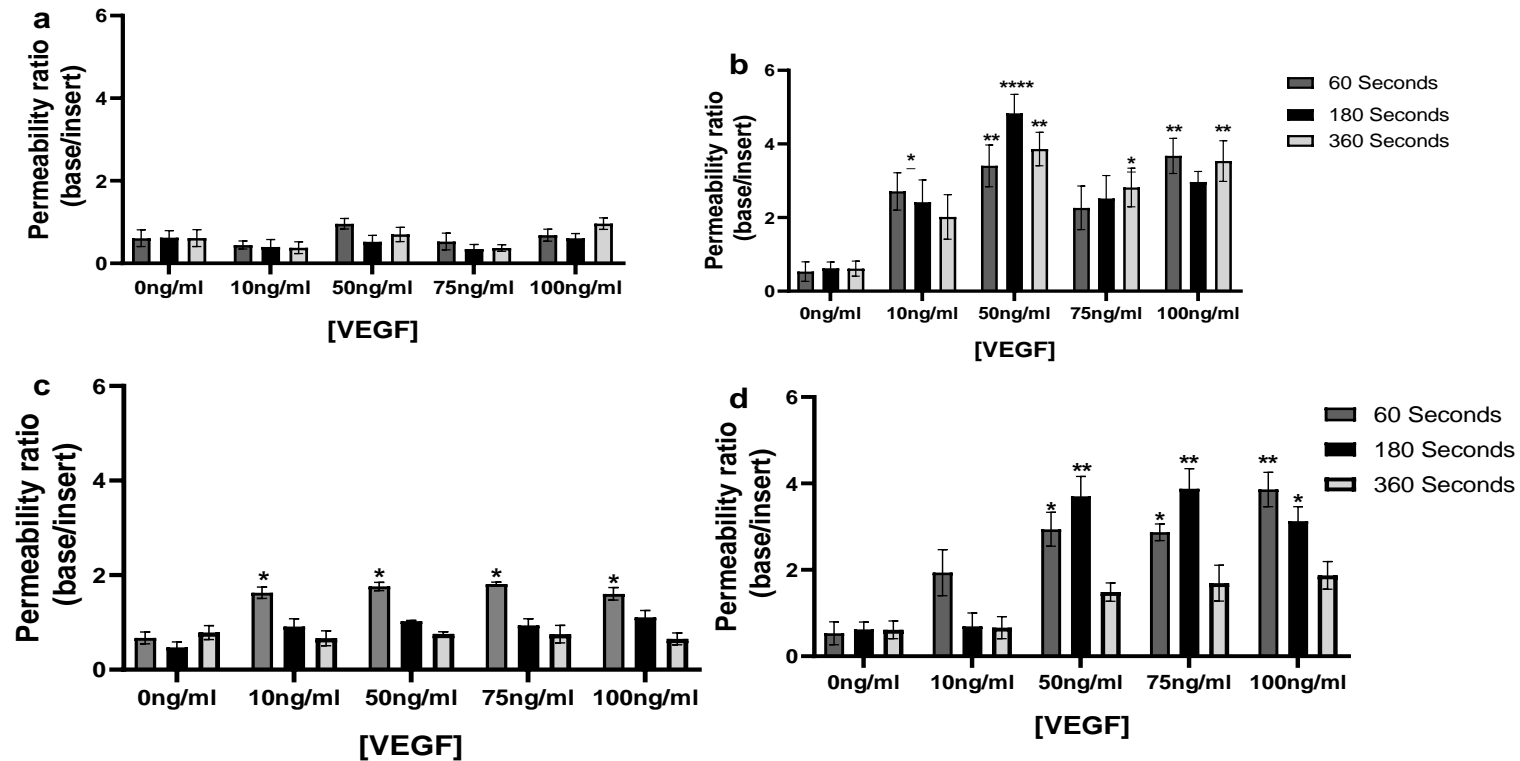
Figure 3. 6: **Vascular endothelial growth factor (VEGF-A) induces leak in an *in vitro* model of glomerular endothelium**
*Exposure of GMVEC to VEGF-A increased permeability significantly as demonstrated using FITC-dextran permeability assay. GMVEC were exposed to 10, 50, 75 and 100 ng/ml concentration of VEGF-A, incubated for 4 hours, followed by addition of 5 mg/ml of varying molecular weight of FITC-dextran (FD), 4kDa (a), 20kDa (b), 40kDa (c) and 70 kDa (d) to the insert. Aliquots were taken from the base and insert at 60, 180 and 360 seconds. FD4 data shows no significant difference in permeability with studied VEGF-A concentrations while FD20, FD40 and FD70 showed permeability effects on GMVEC. n=4 for each FD, Data is normalised to 0, from ratio of base to insert and presented as mean with S.E.M. p = * < 0.05, **< 0.01, ****<0.0001 vs 0 ng/ml.*

3.2.5 Determination of fluorescein isothiocyanate (FITC)-dextran internalisation

Fluorescein labelled dextrans are invaluable tracer tools in quantifying vascular permeability as it allows evaluation of how much the permeability of the barrier system is altered or increased (Bischoff, et al., 2016). The result in Figure 3.6a suggests that the lowest molecular weight (4KDa) of the FITC-dextran used might have given a false negative result of no VEGF-induced permeability in GMVEC. This is so because low molecular weight FITC-dextrans are expected to extravasate more in comparison to higher molecular weight dextrans (Desideri, et al., 2018). However, as seen in Figure 3.6a, the FD4 dextran was not detected to have crossed the glomerular endothelial cell membrane in the presence of VEGF. The reason for this might be because smaller dextrans could be readily washed away or dissolved in cell media (Hoffmann, et al., 2010). This then led to an investigation to determine whether these fluorescently tagged dextrans are internalised in the glomerular microvascular endothelial cell. The assay protocol was as described in section 2.2.2.7.1. As Figure 3.7 shows, upon staining the cell nuclei with DAPI, only blue colours were visible on all result panels (Figures 3.7a, 3.7b, 3.7c and 3.7d) which implied that the FITC dye did not penetrate the cell membrane to be internalised in the glomerular endothelium. This result, therefore, suggests that the 4-kDa FITC–dextran may have been dissolved or washed out in the presence of the sweeteners and cell media (Hoffmann, et al., 2010).
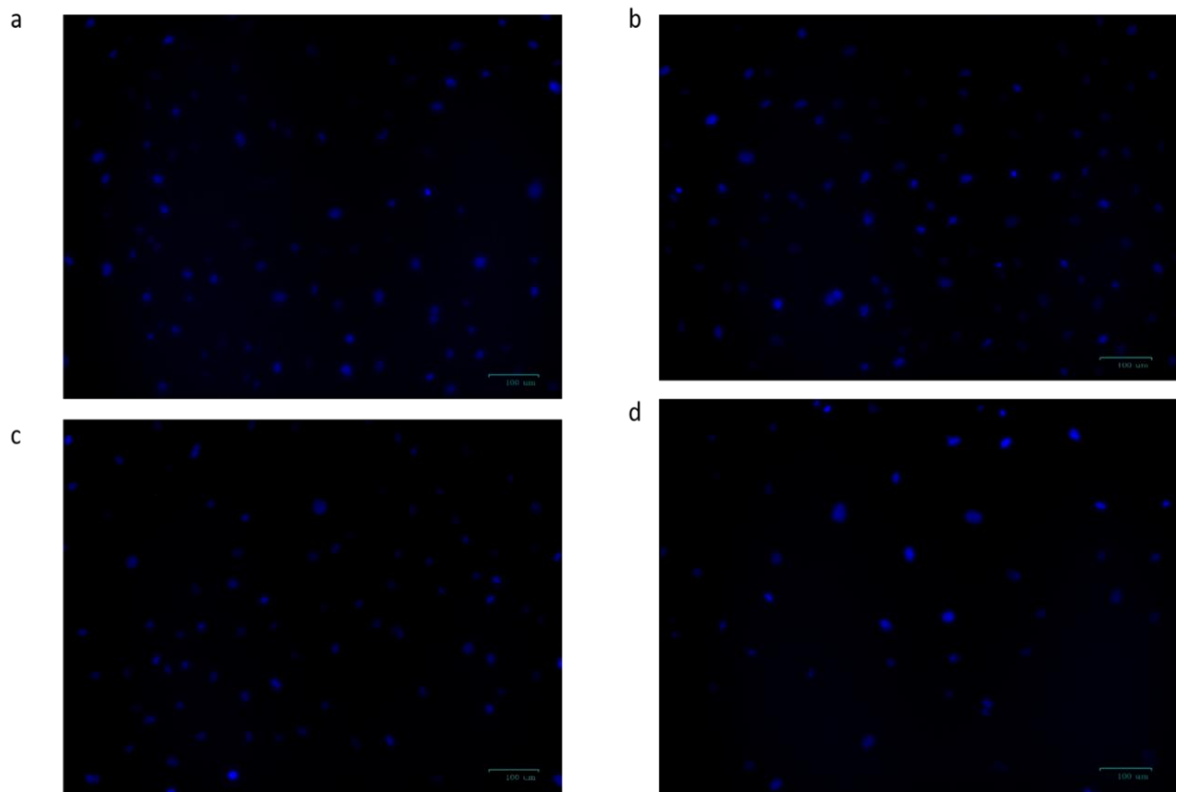
Figure 3. 7: **FITC-Dextran is not internalised in glomerular microvascular endothelial cell**

Representative images of GMVEC prefixed with FITC-dextran FD4 (a), FD20 (b), FD40 (c)

and FD70 (d) and stained for nuclei with DAPI (blue). Images captured at 40x magnification

and merged with images captured at 488nm wavelength using the Zoe Imager. Scale bar;

100 µm.

3.2.6 Validation of VEGF-A induced permeability using trans-endothelial electrical resistance technique

The two experimental techniques considered as gold standard for the last decades in the measurement of endothelial barrier permeability include the permeation of macromolecule using FITC-dextran and the measurement of the ion flux by trans-endothelial electrical resistance (Bischoff et al., 2016). Fluorescence-labelled dextran is the commonly used technique in permeability studies and the results in Figure 3.6b demonstrated its effectiveness in determining VEGF-A induced permeability across glomerular endothelial monolayer. However, it was important to validate the selected permeability parameters such as concentration of VEGF-A and duration prior measuring leak by using another technique; the trans-endothelial electrical resistance, (TEER). The experimentation was as previously described in section 2.2.2.5.2. The result in Figure 3.8 indicated that VEGF-A decreased GMVEC monolayer resistance thereby significantly increasing permeability. When comparing the endothelial electrical resistance of GMVEC at baseline (control) with those cells exposed to 50 ng/ml of VEGF-A, GMVEC resistance was decreased by almost 50% with a statistical significance, *p-value* of < 0.01. This validation result, therefore minimised the generation of any false negative or positive results and demonstrated that the chosen method (FITC-dextran) and parameters to determine paracellular permeability in the glomerular microvascular endothelial monolayer was appropriate for this research.
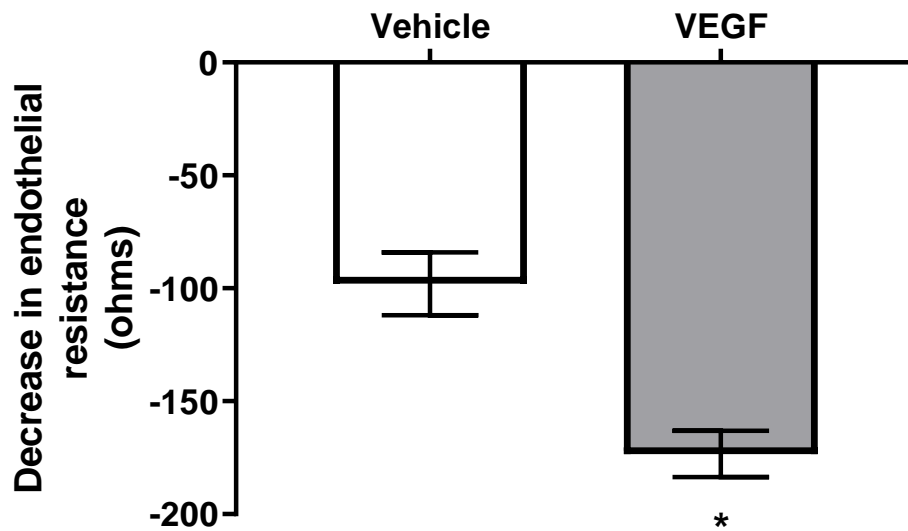
Figure 3. 8: **Validation of FITC-dextran permeability assay using trans-endothelial electrical resistance**

*Changes in glomerular microvascular endothelial monolayer barrier resistance was measured by exposing the cells to 50 ng/ml of VEGF-A prior to permeability assessment of the electrical resistance across the glomerular monolayer. The resistance of the GMVEC was measured in units of ohms (Ω). The assay had an n = 6 and data is presented as mean with S.E.M. with * p < 0.05 vs vehicle using Mann-Whitney test.*

3.2.7 Saccharin and sucralose do not affect the tight monolayer barrier of glomerular endothelium

The findings from the viability studies (section 3.2.2) indicated that neither saccharin nor sucralose affected the viability of the glomerular microvascular endothelial cells thereby making them suitable for further experimentation. This research was based on the rationale that these sweeteners could attenuate the permeability effect of VEGF-A in GMVEC. However, prior to determining the protective effect of these sweeteners against VEGF-A induced leak, their effect on GMVEC tight monolayer barrier was first established. The data presented in Figure 3.9 demonstrated that neither saccharin (Figure 3.9a) nor sucralose (Figure 3.9b) disrupted the GMVEC monolayer at all studied concentrations. When comparing the effect of saccharin and sucralose to the control (0 μM), there was no statistical difference between the treated and untreated glomerular endothelial cells. Based on these findings, it was evident that, the artificial sweeteners, saccharin and sucralose have no permeability-inducing effect on the glomerular endothelial monolayer.
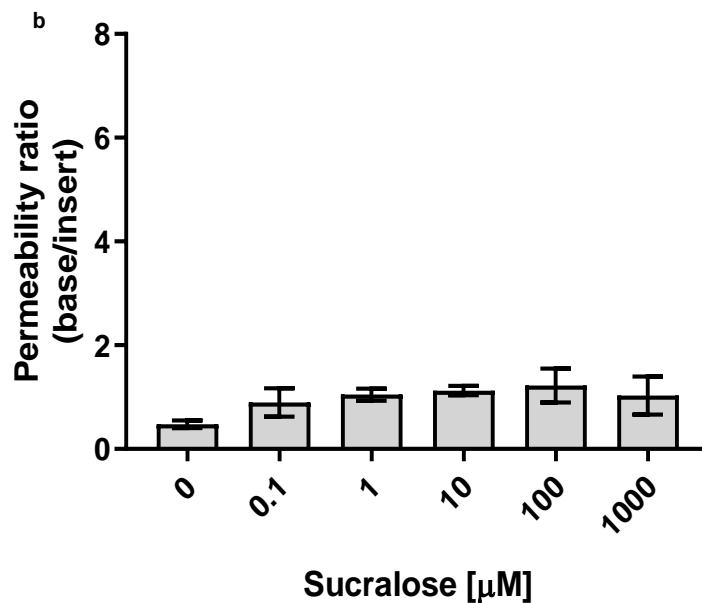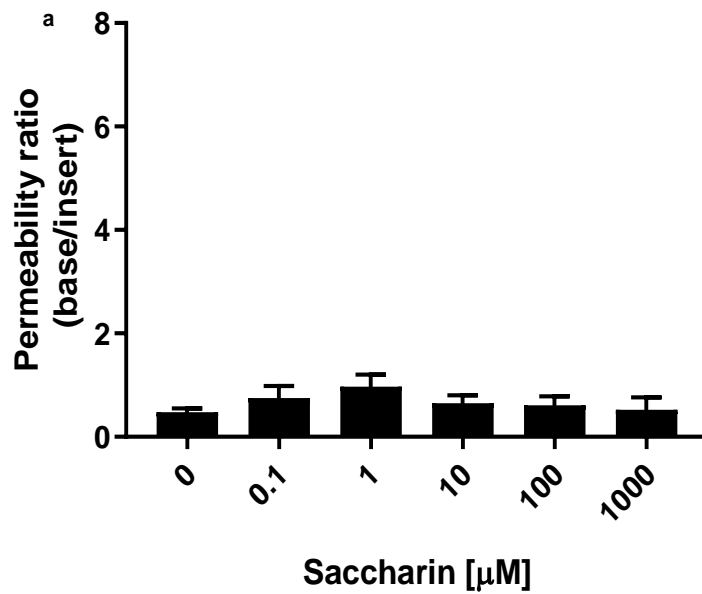
Figure 3. 9: **Saccharin and sucralose do not disrupt the tight barrier of glomerular microvascular endothelial cell**

Changes in glomerular microvascular endothelial cell (GMVEC) monolayer barrier resistance were measured by exposing the cells to a range of concentration (0.1,1, 10,100 and1000 µM) of saccharin (a) and sucralose (b). Permeability was assessed as the movement of FITC-dextran (FD20) across the glomerular endothelial monolayer. n= 6 and data is presented as mean with S.E.M. There was no statistical difference between the sweeteners and vehicle (0 µM).

3.2.8 Low concentration of aspartame and neotame do not disrupt glomerular microvascular endothelial monolayer barrier *in vitro*

Based on the data presented in Figure 3.10, aspartame (Figure 3.10a) and neotame (Figure 3.10b) did not disrupt the GMVEC monolayer at 0.1 - 100µM concentrations. However, at 1000 µM, aspartame and neotame had a contrary effect on the GMVEC monolayer, as permeability was significantly increased (p = 0.002). This result was surprising because, it was contrary to the viability studies, where neither aspartame (Figure 3.4a) nor neotame (Figure 3.4d) affected GMVEC viability even at a concentration of 1000 µM. Furthermore, although, neotame and aspartame does not cause any significant increase in permeability when exposed to GMVEC at concentrations below 1000 µM (Figure 3.10a and Figure 3.10b), when compared to those of saccharin and sucralose, their effect was above the base line (control) group.

As evident from the result in Figures 3.10a and 3.10b, the permeability induced by aspartame at 1000 µM seems higher than the leak induced by neotame at same concentration. However, a statistical analysis of both sweeteners at 1000 µM, using Mann Whitney test showed there was no significance between the induced leak with an exact p-value of 0.4.These findings, therefore, indicated that aspartame and neotame exhibit a dose-selective permeability effect on the glomerular endothelial cell monolayer.
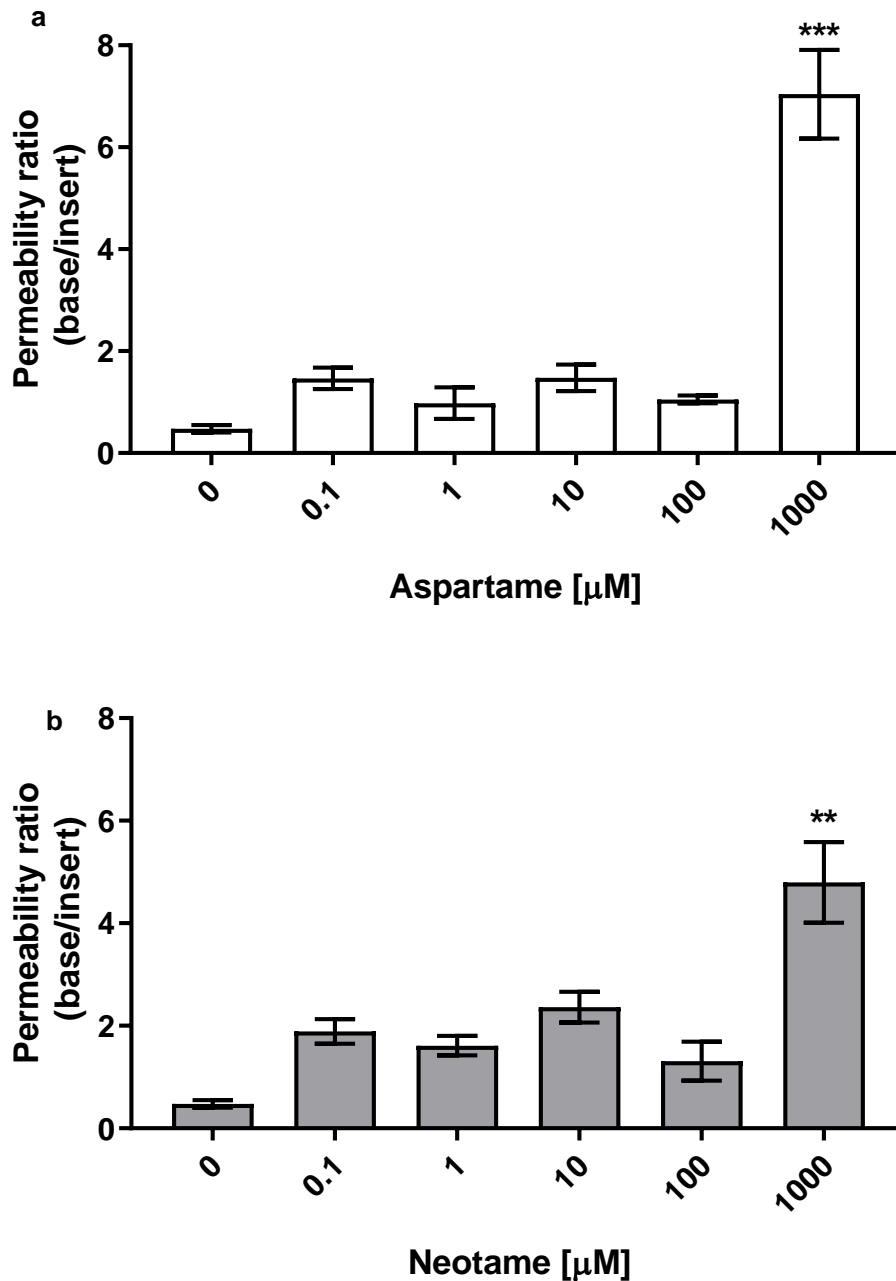
Figure 3. 10: **Aspartame and neotame have dose-dependent effect on the permeability of glomerular endothelial monolayer**

Changes in glomerular endothelial monolayer barrier resistance were measured by exposing the cells to a range of concentration (0.1 ,1, 10,100 and 1000 µM) of aspartame (a) and neotame (b). Permeability was assessed as the movement of FITC-dextran (FD20) across the glomerular endothelial monolayer. n=6 and data presented as mean with S.E.M. **p =0.002, ***p <0.0001 vs vehicle (0µM).

3.2.9 Artificial sweeteners saccharin and sucralose attenuates VEGF-A induced leak in a cell model of glomerular microvascular endothelium

The vascular endothelial growth factor (VEGF) has been shown to be a causative agent in GMVEC permeability (section 3.2.4). Hence, it was hypothesised that artificial sweeteners saccharin and sucralose could counteract the permeability-inducing effect of VEGF-A by protecting the glomerular endothelial microvasculature. That hypothesis was addressed through experimental studies as detailed in section 2.2.2.6. The results presented in Figure 3.11a and Figure 3.11b showed the attenuating effect of saccharin and sucralose on permeability in the presence of VEGF-A.

As shown in Figure 3.11, when GMVEC were exposed to VEGF-A in the absence of saccharin and sucralose (0 µM), a significant increase ($P<0.0001$) in GMVEC monolayer permeability was recorded. However, treatment of GMVEC with saccharin and sucralose for 24 hours prior to exposing the cells to VEGF-A abolished the leakiness caused by VEGF-A. Both saccharin (Figure 3.11a) and sucralose (Figure 3.11b) protected the glomerular endothelial cells from the permeability-inducing effect of VEGF-A, as there was a significant difference between treated and untreated cells (0 µM). This novel finding demonstrated that pre-treatment of GMVEC with artificial sweeteners, saccharin and sucralose protected the glomerular endothelium at all studied concentrations.
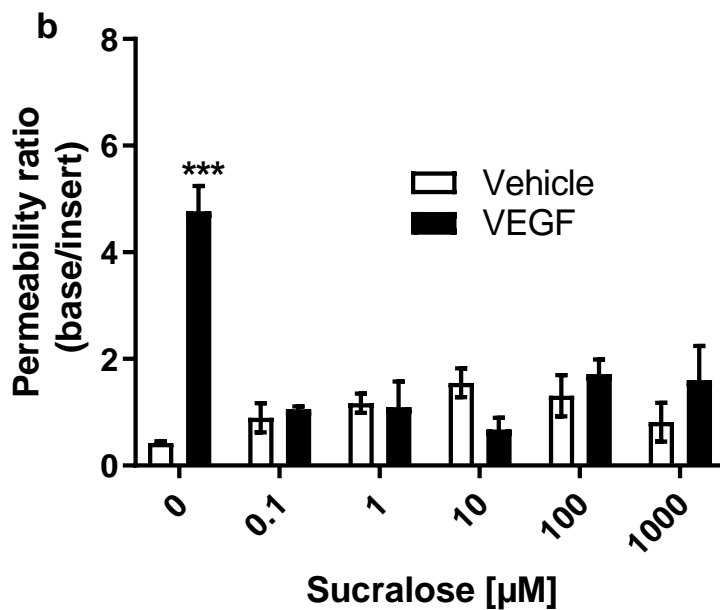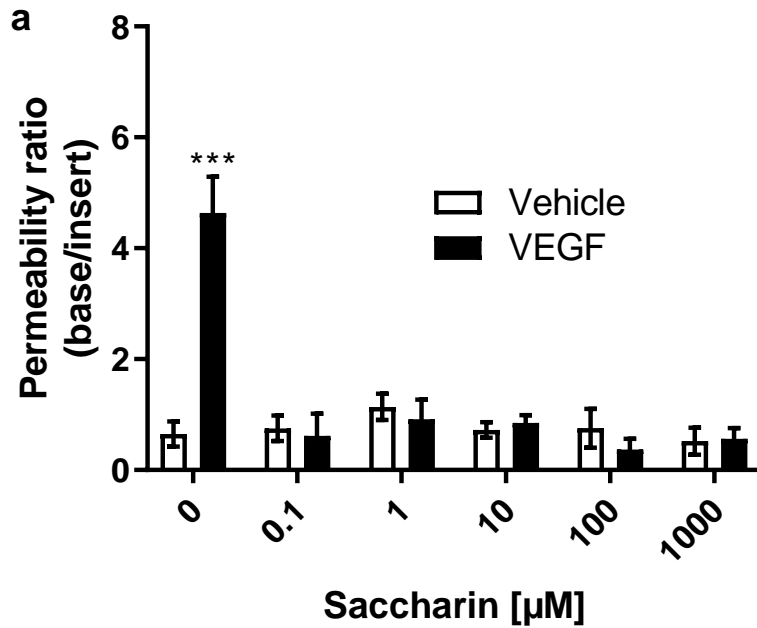
Figure 3. 11: **Saccharin and sucralose attenuates VEGF-A induced permeability in glomerular microvascular endothelium**

*Saccharin and sucralose protected against VEGF-A induced leak in an in-vitro model of glomerular microvascular endothelium. GMVEC monolayers were exposed to artificial sweeteners saccharin (a) and sucralose (b) for 24 hours followed by treatment with 50 ng/ml of VEGF-A$_{165}$ for 4 hours. Permeability was assessed as the movement of FITC-dextran across the endothelial cell monolayer. n=6. Data is normalised from ratio of base to insert and presented as mean with S.E.M. ***p<0.0001 vs 0 μM.*

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3.2.10 Differing protective effect of aspartame and neotame on VEGF-A induced permeability

In a similar manner to saccharin and sucralose, the effect of aspartame and neotame on VEGF-A induced leak in the glomerular microvascular endothelial cell monolayer was assessed and the results (Figure 3.12a and Figure 3.12b) showed a dose-selective effect of the artificial sweeteners, aspartame and neotame against VEGF-A induced leak. As evident in Figure 3.12a, only 10 µM and 100 µM of aspartame were able to protect the GMVEC monolayer from VEGF-A induced permeability while other concentrations (0.1, 1 and 1000 µM) tend to exacerbate the effect of VEGF-A. Furthermore, when GMVEC were exposed to VEGF-A following treatment with neotame concentration, only 0.1 µM and 100 µM concentration of neotame seems to attenuate VEGF-A induced permeability. Hence, this result shows that while aspartame and neotame alone did not disrupt the monolayer of the glomerular endothelium at concentrations below 1000 µM, the introduction of VEGF-A to GMVEC pre-treated with same concentrations of aspartame and neotame showed a different outcome. It is unclear why aspartame and neotame showed disparity on VEGF-A induced permeability compared to sucralose and saccharin (Figure 3.11). Hence, this result suggests that aspartame and neotame has a dose-selective attenuating effect against VEGF-A induced permeability and this was taken into consideration when using these sweeteners for further experiments.
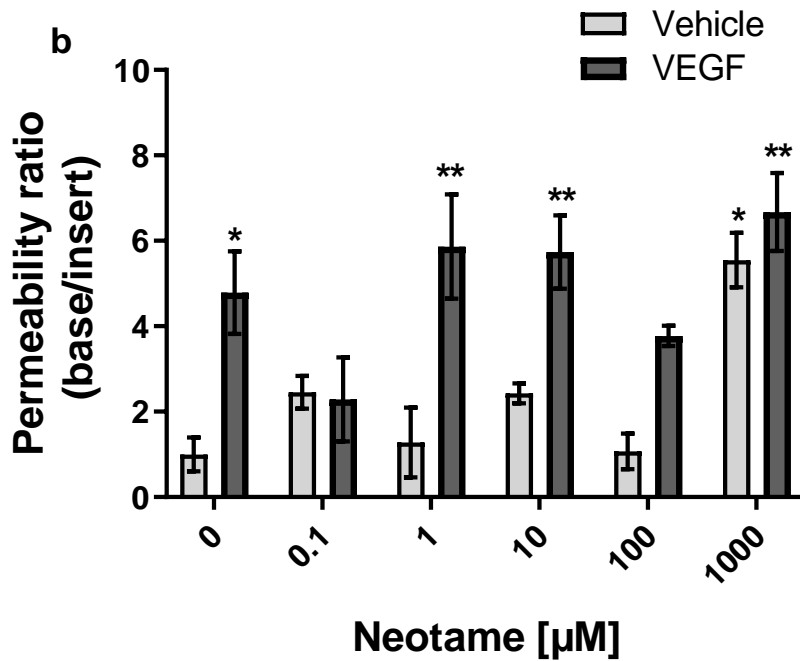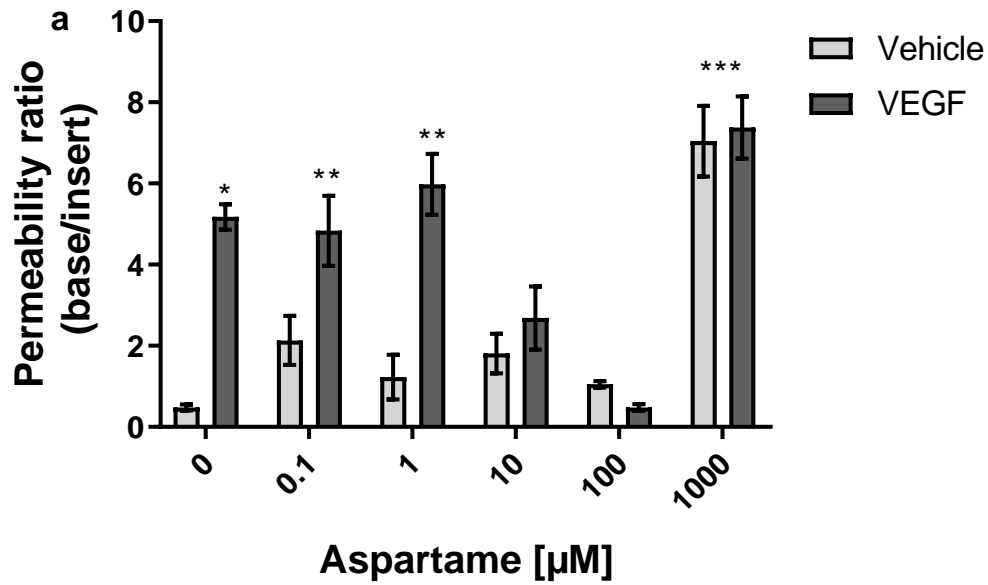
Figure 3. 12: **Dose-dependant effect of aspartame and neotame on VEGF-A induced permeability**

Changes in glomerular endothelial monolayer barrier resistance were measured following exposure of the cells to a concentration range of aspartame (a) and neotame (b) or vehicle (media) followed by 4 hours of VEGF-A (50 ng/ml) treatment. Permeability was assessed as the movement of FITC-dextran across the glomerular monolayer. n= 6 and data is presented as mean with S.E.M, *p<0.05; **p= 0.001; ***p=0.0001 vs 0 µM Vehicle.

Therefore, considering the findings from these experiments, further studies with the artificial sweeteners, aspartame, saccharin, sucralose and neotame were performed with the lowest concentrations shown to be protective against VEGF-A induced permeability. For aspartame, that dose was 10 µM (Figure 3.12a), saccharin; 0.1 µM (Figure 3.11a), sucralose 0.1 µM (Figure 3.11b) and neotame; 0.1 µM (Figure 3.12b). Taken together, these results from the permeability studies provide new insights and understanding into other physiological role of the artificial sweeteners; saccharin, sucralose, aspartame and neotame in a cell model of glomerular endothelium.

3.3 Discussion

The substitution of normal table sugar (sucrose) to artificial sweeteners either for pleasure or for individuals living with certain health conditions such as diabetes, a dietary assessment tool, have gained increasing attention in the last decade (Liauchonak, et al., 2019). Artificial sweeteners provide sweet taste sensation without the extra calories associated with the use of normal table sugar (Wee, Tan and Forde, 2018; Sharma, et al., 2016). The research question, therefore, was based on the hypothesis that artificial sweeteners may have physiological role on vascular permeability in individuals living with DKD other than the pleasure and satisfaction in the perception of sweet taste. The vascular endothelial barrier permeability observed in DKD patients has been linked with oedema and kidney failure (Tanabe, et al., 2017). Thus, having established the link between VEGF-A and permeability observed in DKD, this research was aimed at determining whether artificial sweeteners; aspartame, saccharin, sucralose and neotame could protect against VEGF-A induced endothelial barrier permeability in a cell model of the glomerular microvasculature.

There are four key findings from the studies in this chapter which contributes new knowledge to the physiological role of the studied artificial sweeteners, aspartame, saccharin, sucralose and neotame. First, these sweeteners at 0.01 to 1000 µM concentrations did not affect cell growth and viability of glomerular microvascular endothelial cells but the sweet taste inhibitor, lactisole reduced cell viability at concentration above 10 µM.

Secondly, it was established that VEGF-A induced leak in glomerular endothelial monolayer at concentrations >10 ng/ml. Third, artificial sweeteners saccharin and sucralose did not disrupt the glomerular microvascular endothelial cells monolayer at all concentrations up to 1000 µM while aspartame and neotame has same effect at concentration <1000 µM. Fourth, pre-treatment of GMVEC with saccharin and sucralose, ameliorated the permeability induced by VEGF-A in glomerular endothelium whereas aspartame and neotame showed differing effects over the range of concentrations studied.

3.3.1 Artificial sweeteners do not affect the viability of glomerular microvascular endothelial cell

Since the glomerular endothelial cells utilised in this research were different from immortalised cells (Pan et al., 2009), the preliminary assay on GMVEC proliferation was essential as it provided crucial findings on GMVEC features, culture and growth pattern required for planning and conducting subsequent experiments. Cell-based cytotoxicity assays are amongst the important biological evaluation techniques used in screening of compounds or drugs which might influence proliferation and viability, leading to cell death *in vitro* (Riss, et al., 2016).

Therefore, it was vital to use a reliable and reproducible viability assay such as the cell counting kit-8 (CCK-8) to determine the effect of aspartame, saccharin, sucralose and neotame concentrations on GMVEC. While it can be argued that cytotoxicity test using CCK-8 assay does not differentiate between the cause of cell death, this assay was the best because the detection sensitivity of CCK-8 is suggested to be higher than other tetrazolium salts such as MTT (Jo, 2015; Jiao, et al., 2015). Thus, the cell survival graph in Figures 3.4a-d indicated that artificial sweeteners, aspartame, saccharin, sucralose and neotame had no cytotoxic effect on GMVEC at the studied concentrations. In addition, since 1-10 µM concentrations of lactisole has no effect on GMVEC viability, the selection of 3 µM concentration of lactisole, utilised for the rest of the research was justifiable given that less than 10 µM of lactisole has been shown to inhibit T1R2/T1R3 receptors (Nakagita, et al., 2019).

The viability assay, therefore demonstrated that GMVEC were unaffected by the sweeteners at concentrations between 0 µM to 1000 µM. The glomerular microvascular endothelial cells utilized in this study were primary endothelial cells with specific features (Satchell, et al., 2006), and in order to mimic the normal physiological state, the selected dose of aspartame, saccharin, sucralose and neotame were aimed to be within the physiologically relevant range as outlined on Table 1.4 (chapter 1).

The selection of the working concentrations of artificial sweeteners used in this research was based on the expected doses seen by the glomerular endothelial cells as the expected daily intake ranges from 150 to 500 µM in soft drinks (Franz, 2010; Gardner, et al., 2012). Furthermore, the expected amount of these sweeteners to reach the glomerular endothelium upon consumption, using sucralose as an example was demonstrated in an experimental study by Slyvesky, (2017). When test subjects were given 68 mg of sucralose contained in 355 ml of diet cola, about 0.0004 mg was identified in the plasma which is equivalent to 1µM of sucralose. Hence, about 1% of the ingested sweetener is presumed to be seen by microvascular endothelial cells.

This indicates that the concentrations of sucralose used for this research was in line with the expected level or dose of sweeteners reaching the glomerular endothelium. Additionally, the results from the permeability studies in section 3.2.9, demonstrated that low concentrations (<1 µM) of artificial sweeteners, saccharin and sucralose had an effect on glomerular microvascular endothelial cells because of their sweetness intensity compared to normal table sugar (Li, et al., 2002; Fujiwara, et al., 2012).

The novel finding on the non-cytotoxicity effect of these artificial sweeteners; aspartame, saccharin, sucralose and neotame on glomerular microvascular endothelium was supported by other studies in different cells. The non-cytotoxic effect of artificial sweetener, sucralose on GMVEC corresponds with our previous study in the retinal microvasculature (Lizunkova, Enuwosa and Chichger, 2019), which demonstrates that the artificial sweetener, sucralose (1nM-1mM) has no cytotoxic effect on the retinal endothelium. Additionally, the work of Alleva, et al. (2011) and Schiano, et al. (2020), showed that aspartame has no cytotoxic effect *in vitro*. Therefore, under normal conditions, artificial sweeteners, aspartame, saccharin, sucralose and neotame are not likely to have any impact on glomerular microvascular endothelial cell viability at the studied concentrations.

3.3.2 VEGF-A$_{165}$ induced permeability in the cell model of the glomerular microvascular endothelial monolayer

The progression of DKD has been linked with overexpression of VEGF-A which in turn increases vascular permeability (Tanabe, et al., 2017; Kanesaki, et al., 2005; Hovind, et al., 2000; Satchell, et al., 2004 and Nakagawa, 2009; Wasada, et.al, 1998; Lenz, et.al, 2003). Therefore, knowledge of the association between VEGF-A and DKD is not lacking. However, strategies targeted towards the management of this VEGF-A induced leak in GMVEC is not yet known. Hence, this research, was focused on developing a novel therapeutic mechanism targeting the attenuation of permeability induced by VEGF-A. Thus, it is important to establish first, whether VEGF-A causes leak in glomerular microvascular endothelial monolayer prior introduction of artificial sweeteners.

The two experimental techniques utilised in this research are considered as gold standard for the measurement of endothelial barrier permeability; the permeation of macromolecules using FITC-dextran, and the measurement of the ion flux by the trans-endothelial electrical resistance (Bischoff et al., 2016). To mimic the endothelial permeability associated with DKD *in vitro*, FITC-dextran was utilised. The results of the study as presented in Figure 3.6b and its validation by the TEER results (Figure 3.8) are consistent with other studies that demonstrates the permeability-inducing effect of VEGF-A on vascular endothelial cells (Garvard and Gutkind, 2006; Deissler, et al., 2013; Lizunkova, Enuwosa and Chichger, 2019).Therefore, since VEGF-A induced barrier leak has been established in an *in vitro* model of GMVEC, it sets the platform for the next aspect of the research; the role of artificial sweeteners on this VEGF-A induced leak.

3.3.3 Artificial sweeteners saccharin and sucralose maintains the tight monolayer barrier of the glomerular microvascular endothelial cell

Since maintaining a tight glomerular endothelial monolayer under DKD is of importance in halting the progression of the disease, the individual effects of the artificial sweeteners; aspartame, saccharin, sucralose and neotame alone on GMVEC monolayer was assessed prior to the introduction of VEGF-A. The goal was to ensure their interactions alone with the glomerular microvascular endothelial cells does not induce leak as suggested by few studies in different cells (Santos, et al., 2018; Suez, et al., 2014). The results in Figures 3.9a, 3.9b, 3.10a, and 3.10b showed that each of the studied sweeteners; saccharin, sucralose, aspartame and neotame has differing effect on glomerular endothelial cell monolayer. Whilst there are very limited studies on the effect of these sweeteners on endothelial monolayer, the available literatures are on different cell types. The work by Santos, et al. (2018) showed that saccharin increased paracellular permeability through a non-cytotoxic mechanism in Caco-2 cells. Although the finding from this research on the effect of saccharin on the GMVEC monolayer contradicts those by Santos, et al. (2018) , it is of importance to note that the study by Santos, et al. (2018) , was carried out on a different cell type; Caco-2 cells which are neither endothelial nor microvascular cells but immortalized epithelial cells with morphological and functional properties characteristic of the small bowel enterocytes. The results in Figure 3.9b demonstrates that the artificial sweetener, sucralose maintains the integrity of the glomerular microvascular barrier, which is supported by the findings of Harrington, et al. (2018).

In contrast to saccharin and sucralose, the other studied artificial sweeteners, aspartame and neotame only does not disrupt the GMVEC monolayer at concentrations below 1000 μM. Although these sweeteners (aspartame and neotame) are both dipeptide sweeteners, their composition is different (Magnuson, et al., 2007; Liauchonak, et al., 2019) and thus, the similarity in their concentration-based (1000 μM) effect on the GMVEC monolayer was surprising. Therefore, while it has been demonstrated that the artificial sweeteners,

saccharin and sucralose has no effect on the permeability of glomerular endothelial cell monolayer, the effect of aspartame and neotame differs at high concentration of 1000 µM.

3.3.4 Artificial sweeteners (saccharin and sucralose) attenuated the permeability effect of VEGF-A$_{165}$ in an *in vitro* model of the glomerular microvasculature

The importance of VEGF-A in the local control of vascular permeability in the context of DKD has been previously discussed in detail (introductory section) and either VEGF-A knockout (Eremina, et al., 2013), or overexpression (Veron, et al., 2010), worsen DKD thereby leaving a gap for a novel and alternate therapeutic strategy for managing this leak. Hence, the goal of this research was to assess the protective role of artificial sweeteners, aspartame, saccharin, sucralose and neotame against VEGF-A induced permeability on the glomerular microvasculature.

As demonstrated in the results section, the data presented in Figure 3.11a and 3.11b, indicates that artificial sweeteners, saccharin and sucralose attenuated permeability in GMVEC at all studied concentration while aspartame and neotame (Figure 3.12 a and 3.12 b) has a dose-selective effect on VEGF-A induced leak in the glomerular endothelium. The differing protective role on the studied sweeteners; aspartame, saccharin, sucralose and neotame demonstrated against VEGF-A induced permeability on the glomerular endothelium could be linked to differences in their structural composition and metabolism.

Comparing this finding with existing studies, available few studies that focused on the use of artificial sweeteners as a therapeutic agent in diseases characterised by leak were those of Harrington, et al. (2018) and Lizunkova, Enuwosa and Chichger (2019). The findings from this current research mirrors those by Harrington, et al. (2018), which demonstrated that at 100 µM, sucralose attenuated LPS-induced permeability in pulmonary endothelium.

Additionally, the attenuating effect of the artificial sweeteners, saccharin and sucralose against VEGF-A induced permeability in the glomerular endothelium was corroborated by the work of Lizunkova, Enuwosa and Chichger (2019). The authors demonstrated the blockage of VEGF-A induced permeability and vasculogenic processes by sucralose in the retinal endothelium and showed that in the healthy endothelium, in the absence of VEGF-A, sucralose has no effect on permeability or vasculogenesis. Therefore, notwithstanding the controversy that exist over the safety of artificial sweeteners, the findings from this present research has demonstrated that these sweeteners, saccharin and sucralose has a physiological role other than being sweet and strengthens the argument that artificial sweeteners might have positive physiological impact on glomerular endothelial microvasculature. Hence, based on the availability and cost effectiveness of both saccharin and sucralose, in addition to its protective effect against VEGF-A induced permeability on GMVEC, these sweeteners could be valuable agents when seeking novel therapeutic approaches for individuals living with DKD.

In contrast to the complete attenuating effect of saccharin and sucralose, aspartame and neotame demonstrated a dose-selective protection of the glomerular endothelium against VEGF-A induced leak. Based on the results from this current study, aspartame seems to exacerbate VEGF-A induced permeability at 0.1, 1 and 1000 µM concentrations. Although studies (Palmnas, et al., 2018; Saleh, et al., 2015; Alleva, et al., 2011) exists on the damaging effects and permeability-inducing effect of aspartame, some of these studies were on the gut microbiota (Suez, et al., 2014) with none on the glomerular endothelium. It is uncertain why aspartame increases leak. However, a possible explanation for the controversial effect of aspartame on VEGF-A induced permeability could be attributed to its metabolic fate in the body as it is broken down to aspartic acid (40%), phenylalanine (50%) and methanol (10%) (Butchko, et al., 2002; Magnuson, et al., 2016).

Therefore, aspartame might not be identified as an intact molecule by the glomerular endothelial cells as shown by Magnuson, et al. (2007). According to the author, irrespective of the high concentration of aspartame (>200 mg/kg) ingested, there was no intact

aspartame found in the blood due to the rapid breakdown of aspartame into its primary metabolites; aspartic acid, phenylalanine and methanol which are readily absorbed and does not reach the large bowel. Therefore, it could be said that these metabolites of aspartame especially methanol is the causative agent of the controversial behavioural pattern of aspartame in the presence of VEGF-A and future studies should address this proposition (Ashok, Sheeladevi and Wankhar,2014).

The other sweetener with similar effect to aspartame was neotame as shown in Figure 3.12b. The glomerular endothelial cells treated with neotame prior to exposure to VEGF-A showed that neotame has protective effect against VEGF-A induced permeability only at 0.1 and 100 µM. Surprisingly, at other studied concentrations, neotame tends to exacerbate the permeability effect of VEGF-A. While neotame could also be metabolised to methanol, although at a significantly lower level compared to aspartame (Chi, et al., 2018), it could be the possible explanation to its dose-selective protection against VEGF-A induced permeability. Neotame, although, yet to be commercially available like other sweeteners (aspartame, saccharin and sucralose) studied, this new finding on neotame adds to the scientific knowledge on its effect on glomerular microvascular endothelium. Hence, the observed differences in the physiological role of the artificial sweeteners (aspartame, saccharin, sucralose and neotame) on VEGF-A induced permeability in the glomerular endothelial monolayer could be due to differences in their absorption and metabolism. As stated in section 1.11.1, each of the artificial sweeteners utilised in this research has its own structure which influences its metabolism and other physiological effects. Whilst sucralose and saccharin are not metabolised and thus, retains their sweetness in the glomerular endothelial cells (Gardener, et al., 2012). Therefore, it could be speculated that the similarity in effect seen between aspartame and neotame in their inability to protect the glomerular endothelium against VEGF-induced leak might be due to similarities in their chemical structure. Whilst aspartame is a dipeptide consisting of two amino acids, aspartic acid and phenylalanine, neotame is formed from aspartame and 3, 3-dimethylbutyraldehyde. Although, neotame yields minute amount of phenylalanine due to

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the presence of dimethylbutyraldehyde in its chemical structure, both sweeteners (aspartame and neotame) might lose their sweetness as they are degraded to other metabolite such as methanol and might not remain as intact sweet molecules once inside the cell (Whitehouse, et al., 2008; Magnuson, et al., 2016). Hence, further studies to better understand the effects of these artificial sweeteners based on their chemical composition in the glomerular microvasculature may have vital implications for their use.

These novel findings on the three commercially available artificial sweeteners; aspartame, saccharin, sucralose and a very high sweetening-potency sweetener, neotame on GMVEC was the first of its kind. These findings from this research would no doubt add knowledge on the physiological impact of these sweeteners when in circulation and on the glomerular microvasculature. Taken together, these research findings indicate that saccharin and sucralose based on their availability, affordability and their protective role against VEGF-A induced permeability on the glomerular endothelium, could be valuable agents when seeking novel therapeutic approaches for managing leak associated with progression of DKD. Having addressed the principle research question of this study, it is therefore necessary to determine the mechanism through which artificial sweeteners protects the glomerular endothelium from VEGF-A induced leak.

3.4 Conclusion

Although artificial sweeteners are considered safe by food standards authorities (EFSA and FDA) and well tolerated, their effects on glucose intolerance, weight gain, obesity, the activation of sweet taste receptors, permeability, endothelial vascular health and alterations to the composition of the intestinal microbiota are controversial and conflicting (Jacquillet, et al., 2018; Gardener, et al., 2016; Suez, et al., 2014; Uebanso, et al., 2017; Magnuson, et al., 2016; Lizunkova, Enuwosa and Chichger, 2019; Cabral, et al., 2018; Ruiz-Ojeda, et al., 2019; Liauchonak, et al., 2019; Santos, et al., 2018; Spencer, et al., 2016; Schiano, et al., 2020; Chi, et al., 2018). In this chapter, VEGF-A was used as an *in vitro* model to mimic the glomerular endothelial leak observed in DKD. Results have demonstrated the novel protective effects of saccharin, sucralose and the dose-*selective* effects of aspartame and neotame on VEGF-A induced permeability in glomerular microvasculature. Although this study was particularly focused on DKD, these novel findings about artificial sweeteners attenuating VEGF-A induced vascular permeability; could be applied to other disease models characterised by increased permeability and overexpression of VEGF-A, such as cancer metastasis.

CHAPTER 4

ARTIFICIAL SWEETENERS ATTENUATES VEGF- INDUCED PERMEABILITY ON GLOMERULAR ENDOTHELIUM THROUGH THE SWEET TASTE RECEPTOR.

4.1 Introduction

Natural (glucose, fructose, sucrose) and artificial sweeteners elicit a distinctive perceptual quality-termed sweetness in humans through binding to the sweet taste receptor (T1R 2/3) (Fujiwara, et al., 2012). In humans, the sweet taste receptor, belongs to the T1R class C of G protein-coupled receptors (GPCRs). The sweet taste receptor acts through the traditional GPCR-signalling pathway to activate the $\beta\gamma$ G-protein subunits. Upon the binding of sweet taste activators, heterotrimeric gustducin dissociates into its α and $\beta\gamma$ subunits with the $\beta\gamma$-subunit activating the phospholipase Cβ_2 (PLC β_2).The activation of phospholipase C-β_2 leads to the formation of 1,4,5-triphosphate (IP$_3$) through the hydrolysis of phosphatidylinositol 4,5-bisphosphate (Masubuchi, et al., 2013; Lindemann, 2001; Nakagawa, et al., 2009; Gilbertson, et al., 2000). A detailed review of the transduction of sweet taste can be found in section 1.10 of chapter 1.

The heterodimeric T1R2 and T1R3 sweet taste receptors binds to the large extracellular venus fly-trap domain (VFTD), linked by a short cysteine-rich domain (CRD) to an α-helical transmembrane domain (TMD) of the GPCR (Pin, Galvez and Prezeau, 2003; Kim, et al., 2017; Mahalapbutr, et al., 2019). Activators of the sweet taste receptors such as sucrose and some artificial sweeteners bind to receptors at sites considered to be an orthosteric domain which lies within the extracellular venus flytrap domain of T1R2 (T1R2-VFTD) (Cui, et al., 2006). The specific interaction site of the artificial sweeteners utilised in this research, aspartame, saccharin, sucralose and neotame on the sweet taste receptors, are mentioned earlier in section 1.11.1 of the introductory chapter. The other binding sites lies in the transmembrane domain of the T1R3 (T1R3-TMD), which also enables interaction with not only the sweeteners but also enables binding of the sweet taste inhibitors such as lactisole (Nakagita, et al., 2019).

The human sweet taste inhibitor, lactisole is an arakyl carboxylic acid possessing a 2-phenopropionic acid skeleton and docks at the binding pocket within the T1R3-TMD region of the sweet taste receptors through a selective competitive reaction, thereby suppressing the perception of sweet taste (Jiang, et al., 2005; Nakagita, et al., 2019; Nakagawa, et al., 2015). The binding of sweeteners to the sweet taste receptor, induces major conformational changes which includes the transformation of the transmembrane domain (TMD) from TMD 56 interface to the TMD 6 interface (Kim et al., 2017). This conformational change is necessary for perception of sweet taste and the ability of lactisole to block this conformational change, by restricting the movement of the 3, 5 and 6 helices of the T1R3 transmembrane domain, locks the receptor from being stimulated by sweet molecules (Jiang, et al., 2005). Lactisole has been shown to inhibit sweet taste receptor response to a range of chemically diverse sweet molecules including artificial sweeteners (Schiffman, et al., 1999). Therefore, the selection of this pharmacological inhibitor of sweet taste perception, lactisole, to assess the possibility of the artificial sweeteners, aspartame, saccharin, sucralose and neotame protecting the glomerular endothelium through activation of sweet taste receptors.

Chapter 3 of this research demonstrated the protective role of artificial sweeteners on VEGF-A induced vascular permeability in a cell model of the glomerular endothelium. However, the mechanisms through which this protection occurs is yet to be elucidated. This chapter, therefore, focused on the possible factors and their mechanism of actions associated with maintaining tight endothelial barrier and the role of artificial sweeteners in these settings. While several sweet taste molecules can trigger the sweet taste receptors, T1R2/T1R3, a certain concentration of sweet taste molecules is required for activation of the sweet taste receptors. As previously detailed in section 1.10.2, the binding of natural sugars to the sweet taste receptors requires very high concentrations >300mM which are detrimental to the viability of the target cells whereas, lower concentration (<1mM) of artificial sweeteners is required to activate the sweet taste receptors (Li, et al., 2002; Fujiwara, et al., 2012). Moreover, an experimental study by Schiano, et al. (2020)

demonstrated that a high concentration of D-glucose (>15mM) decreased the number of viable endothelial cells, thus making them unsuitable for this research. Hence, the hypothesis behind the second research aim; artificial sweeteners activates the sweet taste receptors in the glomerular endothelium, triggering other events in relation to the endothelial barrier.

The knowledge in the transduction of sweet taste through the sweet taste receptors upon its activation by sweet taste molecules is not lacking, with several studies agreeing to the notion of GPCR (Class C) as the main protein involved in sweet taste mechanism (Kim, et al., 2017; Zhao, et al., 2003; Li, et al., 2002). However, the impact of sweet taste receptors following activation by artificial sweeteners on downstream signalling pathways and glomerular microvascular endothelial cell membrane stabilisation is not yet known. A novel study by Harrington, et al. (2018) showed that blocking of the G-alpha gustducin, a G-protein subunit led to loss of protection by artificial sweetener, sucralose against VEGF-induced permeability. Thus, prompting the rationale that artificial sweeteners may protect the glomerular microvascular endothelium against VEGF-induced leak through the Gα pathway. This Gα-subunit triggers an increase of intracellular cAMP level, which is a known stabiliser of the endothelial cell barrier (Beese, et al., 2010). In addition, activation of the sweet taste receptors by sweet molecules have been shown to affect intracellular cyclic AMP level in other cell types but the glomerular microvascular endothelial cells (Kojima, et al., 2014).

Cyclic AMP plays important role in the modulation of endothelial barrier function through reorganisation of the actin molecules and adherens junctional proteins associated with cell-cell adhesion (Ramos, et al., 2018; Gunduz, et al., 2019). This cell-cell adhesion protein, VE-cadherin (detailed in section 1.8.2) plays a key role in maintaining the integrity of the microvascular endothelial barrier. In the presence of an external stimuli such as VEGF-A, internalisation of VE-cadherin ensues, followed by endothelial barrier disruption and consequently increase in vascular endothelial barrier permeability (Deissler, et al., 2013; Gavard, 2013; Garrett, et al., 2016; Wetering, et al., 2002). In addition to VEGF-A, another

stressor of the VE-cadherin protein is increased level of intracellular ROS with studies linking elevated ROS generation to tyrosine-phosphorylation of the VE-cadherin. This phosphorylation consequently leads to dissociation of the VE-cadherin catenin complex and loss of cell- cell adhesion, resulting in vascular leak (Monaghan-Benson and Burridge, 2009; Rhee, et al., 2018). Therefore, while it has been demonstrated that artificial sweeteners sucralose and saccharin attenuated VEGF-induced vascular permeability in GMVEC (chapter 3), it is not yet known if the sweet taste receptor, T1R3 is localised in the glomerular endothelium and activated by the sweeteners to effect other signalling events associated with maintaining endothelial barrier; cAMP, VE-cadherin and ROS .

As previously outlined in section 1.19, this chapter focused on addressing aims II: To determine the molecular mechanisms, linked to sweet taste receptor, T1R2/T1R3, cAMP signalling and oxidative stress, through which artificial sweeteners impact the glomerular endothelium. To achieve these aims, the first experiment was focused on the expression of the sweet taste receptor, T1R3 on the glomerular endothelium. Next, was to investigate whether artificial sweeteners attenuated VEGF-A induced leak through the activation of the sweet taste receptors. Furthermore, other downstream mechanisms associated with barrier permeability were studied such as the level of VE-cadherin expression on the extracellular surface of the glomerular endothelium and the generation of cyclic adenosine monophosphate (cAMP). The chapter then concludes with an investigation into effects of artificial sweeteners on intracellular level of reactive oxygen species (ROS) and their effects on exogenous H_2O_2-induced ROS in glomerular microvasculature.

4.2 Results

4.2.1 expression of sweet taste receptor, T1R3 in glomerular microvascular endothelium

To determine whether artificial sweeteners used in this study (aspartame, saccharin, sucralose and neotame) attenuated VEGF-A induced permeability in GMVEC through the sweet taste receptor, T1R3, it was necessary to first assess its expression on glomerular microvascular endothelial cell surface. Expression of the sweet taste receptor was assessed alongside a chemical inhibitor of T1R3, lactisole. The result in Figure 4.1 showed the expression of the sweet taste receptor, T1R3 in the glomerular microvascular endothelial cell surface. Whilst the sweet taste inhibitor, lactisole has been shown to inhibit the sweet taste sensing of the T1R3 receptor to sweet taste agonist (Hamano, 2015), this experiment was carried out to determine the impact of lactisole on T1R3 expression in the glomerular endothelial cell surface. The data in Figure 4.1 showed that lactisole does have an impact on the sweet taste receptor as it reduces the T1R3 expression. These findings, therefore, indicate for the first time, expression of the T1R3 sweet taste receptor on the cell surface of glomerular microvascular endothelium.
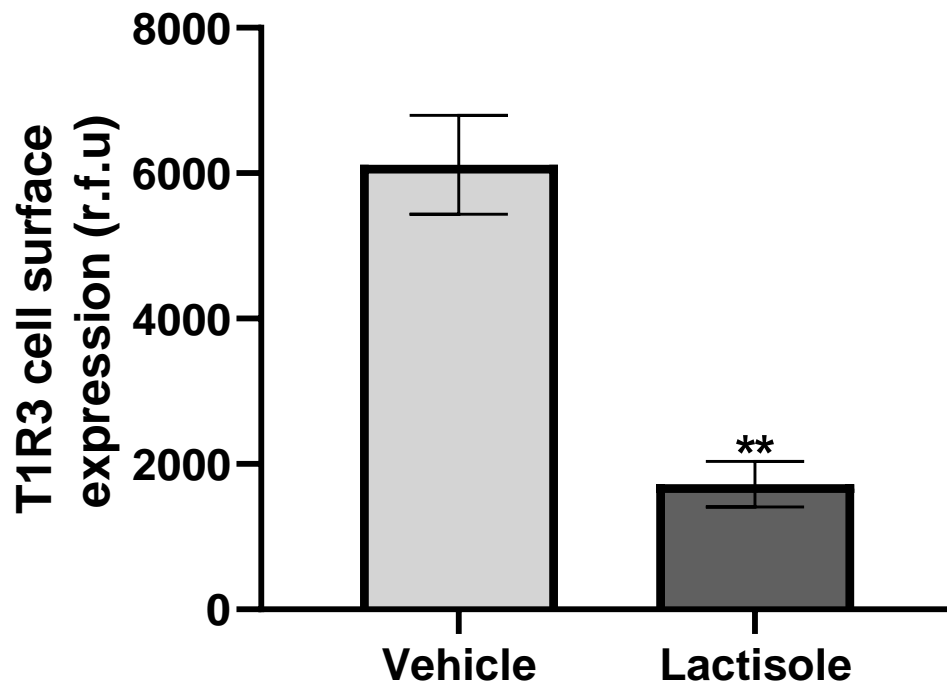
Figure 4. 1: **Expression of sweet taste receptor, T1R3 in glomerular microvascular endothelial cell**

Surface protein expression of the sweet taste receptor in untreated GMVEC was measured using T1R3-specific extracellular antibodies and indirect whole cell ELISA (chemiluminescence). The exposure of the cells to sweet taste inhibitor, lactisole (3 µM) for 10 minutes reduced expression of the T1R3 protein at the GMVEC cell surface. n=3 and data are presented as mean with S.E.M. **, P=0.0095 vehicle vs lactisole.

4.2.2 Saccharin and sucralose but not aspartame and neotame binds to the sweet taste receptor T1R3 on glomerular microvascular cell surface

To determine the impact of artificial sweeteners; aspartame, saccharin, sucralose and neotame, on expression of the sweet taste receptor, T1R3, protein assay was carried out as described in section 2.2.2.8.1. The results in Figure 4.2b and Figure 4.2c shows that both saccharin and sucralose bind to the sweet taste receptor and tend to enhance the basal expression of T1R3 in GMVEC. When comparing the result of GMVEC treated with saccharin and sucralose in the presence and absence of the sweet taste inhibitor lactisole, it was evident that lactisole reduces T1R3 expression by these sweeteners. In contrast to saccharin and sucralose, aspartame and neotame have a different effect. As Figure 4.2a and Figure 4.2d shows, aspartame and neotame do not stimulate an increase in expression of T1R3 sweet taste receptor. These results therefore demonstrate that artificial sweeteners, saccharin and sucralose are recognized by the glomerular microvascular endothelial cells as sweet molecules. On the contrary, aspartame and neotame, although sweet taste agonist, have no effect on the sweet taste receptor, T1R3 expression in an *in-vitro* model of glomerular microvascular endothelium.
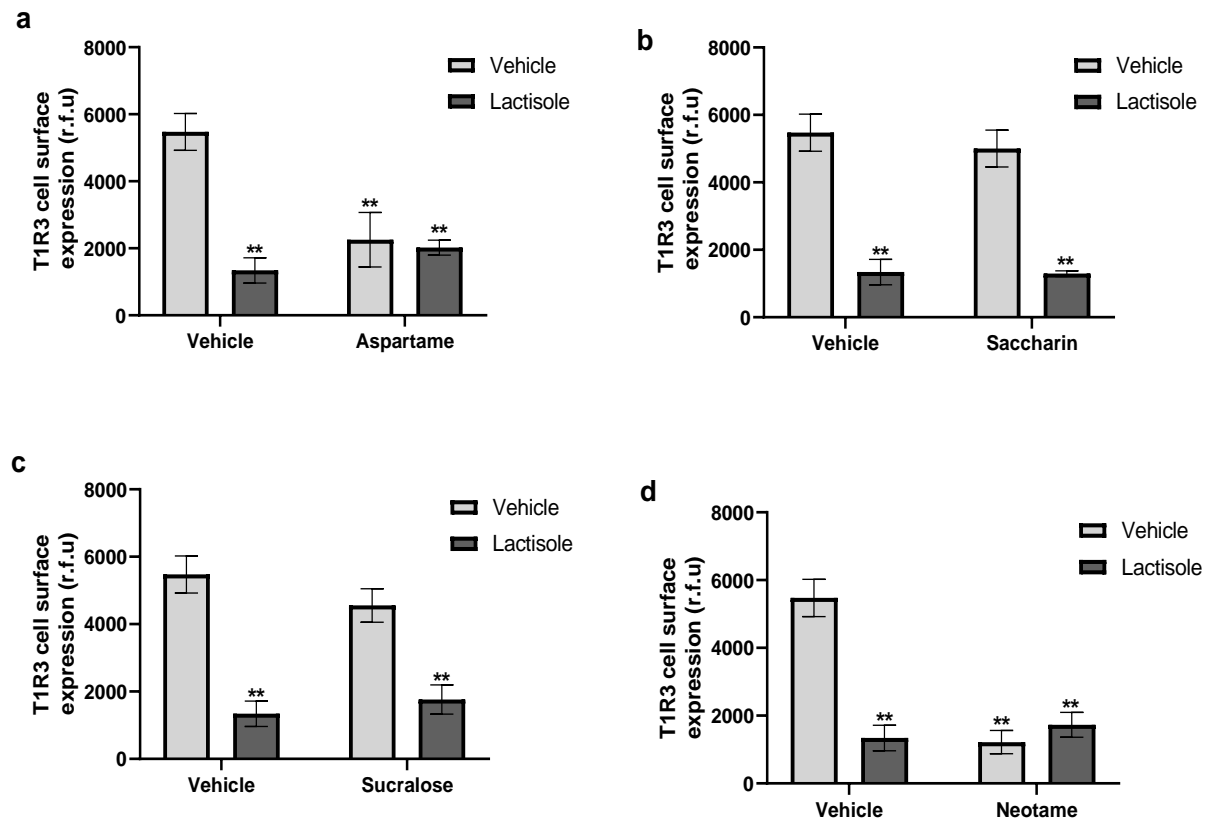
Figure 4. 2*:* **Saccharin and sucralose modulate the expression of sweet taste receptor, T1R3 in glomerular microvascular endothelial cell surface**

Surface protein expression of the sweet taste receptor in GMVEC was determined by exposing GMVEC to artificial sweeteners; 10 µM aspartame(a), 0.1 µM saccharin (b), 0.1 µM sucralose (c) and 0.1 µM neotame (d) for 24 hours followed by indirect whole cell ELISA (chemiluminescence) using T1R3-specific extracellular antibodies. The assay was further assessed using the sweet taste inhibitor, lactisole in the presence and absence of the artificial sweeteners. The data is presented as mean with S. E.M. and n=3 **p <0.005 vs vehicle.

4.2.3 Saccharin and sucralose binds to the sweet taste receptor T1R3 to attenuate VEGF-induced permeability

To determine whether saccharin and sucralose protects the glomerular endothelial monolayer against VEGF-A induced leak through T1R3 binding, a chemical inhibitor of T1R3 receptor, lactisole, was used to inhibit the sweet taste receptors on GMVEC followed by permeability studies as detailed in section 2.2.2.9. The results in Figure 4.3 showed that artificial sweeteners saccharin and sucralose attenuate VEGF-A induced permeability across the glomerular endothelial barrier through binding to the sweet taste receptor, T1R3. As evident from the results, the protective effect of saccharin observed in Figure 4.3a and of sucralose in Figure 4.3c were significantly inhibited by lactisole (Figure 4.3b and 4.3d). Although the protective effect of saccharin and sucralose were blocked by lactisole upon exposure to VEGF-A, the pre-treatment of GMVEC with saccharin (Figure 4.3a) and sucralose (4.3c) prior exposure to VEGF-A did not reduce permeability to base line value when compared with cells exposed to vehicle alone. Another observation evident in Figure 4.3b and 4.3d, was the increase in permeability on GMVEC in the presence of VEGF-A, upon inhibition of the sweet taste receptor with lactisole, compared with cells exposed to vehicle only. A visual observation of the graph in Figure 4.3b showed that permeability seems to be increased when cells were pre-treated with lactisole prior exposure to VEGF when compared to Figure 4.3a. However, a statistical analysis showed no significant difference between the permeability of those two groups with an exact *p-value* of 0.100. Comparing permeability in the presence of VEGF-A without lactisole, to those cells exposed to lactisole and saccharin, there was an increase in permeability with a *p-value* of 0.001. Thereby suggesting, a possible reaction between lactisole and saccharin that drives up permeability in the presence of VEGF-A.

 Contrary to these observation, Figure 4.3c and 4.3d indicates no statistical difference in VEGF-induced permeability, with or without lactisole. These results therefore indicate that artificial sweeteners, saccharin, and sucralose exerts protective effects against VEGF-A induced leak in the glomerular endothelium through potential activation of the T1R3 sweet taste receptor.
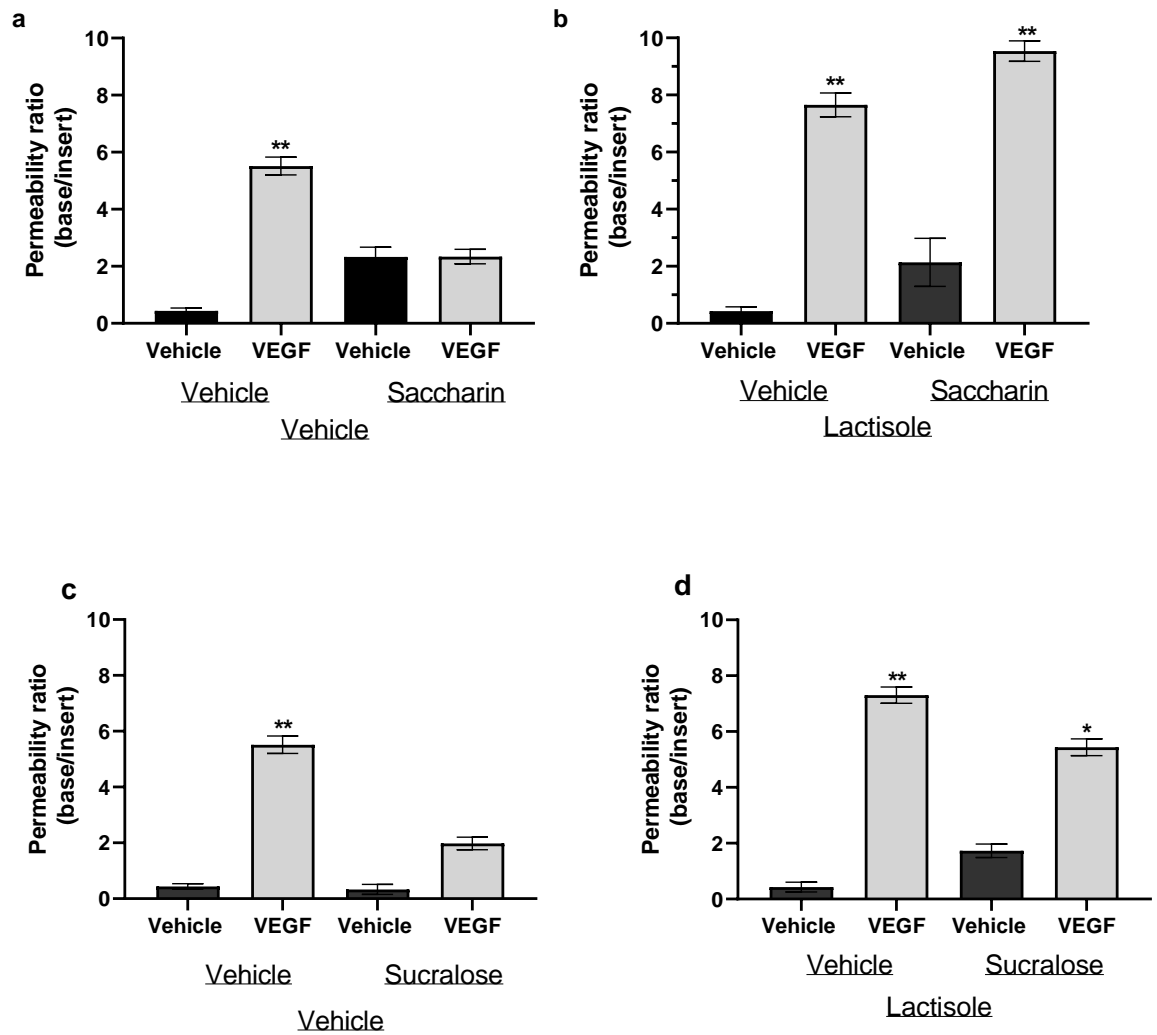
Figure 4. 3: **Inhibition of sweet taste receptors by lactisole blocks the protective effect of saccharin and sucralose on VEGF- A induced permeability**

Panel a: GMVEC were exposed to 0.1 µM saccharin in the presence and absence of VEGF-A (50 ng/ml). Panel b: GMVEC were pre-treated with lactisole (3 µM) for 10 minutes followed by 0.1µM saccharin in the presence and absence of 50 ng/ml of VEGF-A and permeability was assessed using FITC-dextran. Panel c: treatment of GMVEC with 0.1 µM sucralose and 50 ng/ml VEGF-A; panel d: pre-treatment of GMVEC with lactisole (3 µM) followed by 0.1µM sucralose in the presence and absence of 50 ng/ml of VEGF-A and permeability was assessed using FITC-Dextran. n = 6. Data was normalised and expressed as mean with S.E.M, *p < 0.03, **p < 0.002 vs vehicle.

4.2.4 Inhibition of sweet taste receptor T1R3 has differing effect on VEGF- A induced permeability in the presence of aspartame and neotame

The artificial sweeteners, aspartame and neotame were not able to protect the glomerular endothelium from VEGF-A induced leak at studied concentrations as shown in the data presented in Figure 4.4. This result was surprising because as previously discussed in chapter 3, the selected concentration (10 µM) of aspartame and neotame (0.1 µM) attenuated VEGF-A induced leak in GMVEC. Contrary to the findings in chapter 3, aspartame alone (Figure 4.4a) does induce leak in the endothelium. Therefore, upon GMVEC exposed to VEGF-A in the presence of aspartame, the sweetener was not able to protect GMVEC from permeability inducing effect of VEGF-A. Since aspartame was unable to protect GMVEC from VEGF-A induced leak, inhibiting the sweet taste receptor with lactisole has no identifiable positive effect on GMVEC permeability as Figure 4.4b demonstrates. Furthermore, Figure 4.4c and Figure 4.4d indicates that neotame not only increases leak but seems to exacerbate the permeability effect of VEGF-A as the pre-treatment of GMVEC with lactisole blocked this effect. Hence, as previously brought out in section 4.2.2, it could be that aspartame and neotame does not activate the sweet taste receptor, T1R3 in the glomerular microvasculature, thus the inability of these sweeteners to protect GMVEC against VEGF-A induced permeability. Consequently, these results showed that artificial sweeteners aspartame and neotame have no positive effect towards the attenuation of VEGF-A induced permeability in a cell model of glomerular microvascular endothelium even at low concentrations initially seen to be protective.
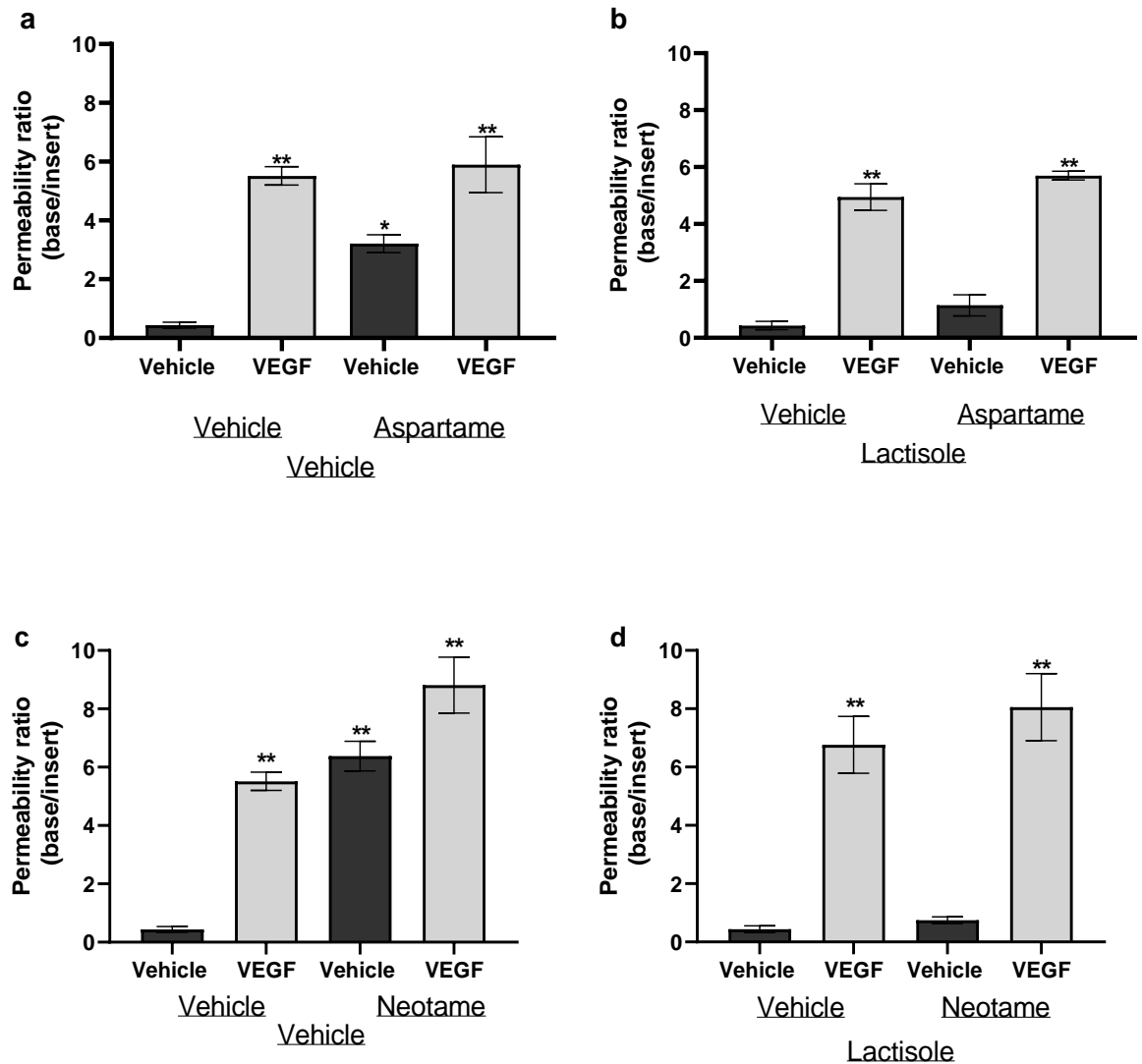
Figure 4. 4: **Aspartame and neotame effects on VEGF-A induced permeability in the presence of lactisole**

Panel a and c: GMVEC were exposed to 10 µM aspartame and 0.1 µM neotame in the presence and absence of VEGF-A (50 ng/ml) followed by FITC-Dextran permeability assessment. Panel b and d: GMVEC were pre-treated with lactisole (3 µM) followed by 0.1 µM aspartame and neotame respectively in the presence and absence of 50 ng/ml of VEGF-A and permeability was assessed using FITC-Dextran. $n = 6$. Data was normalised and expressed as mean with S.E.M, *P <0.05, **P 0.002 vs vehicle.

Taken together, these results demonstrated for the first time the expression of the sweet taste receptor, T1R3, in a cell model of the glomerular microvascular endothelium. Whilst artificial sweeteners, saccharin and sucralose protected the glomerular endothelium from VEGF-A induced permeability through activation of the sweet taste receptor, T1R3, aspartame and neotame showed a contrary effect.

4.2.5 Vascular endothelial growth factor facilitates the internalisation of VE-cadherin in a cell model of the glomerular microvascular endothelium

The impact of VEGF as a stressor of the microvascular endothelium through destabilisation of the endothelial cell-cell tight junctions (TJ) and adherens junctions (AJ) proteins has been well documented in the literature (Deissler, et al., 2008; Wang, Dentler and Borchardt, 2001; Gavard and Gutkind, 2006) as detailed in section 1.8.4 of chapter 1. Whilst the claudins, occludins and junctional adhesion molecules (JAMs) make up the TJ transmembrane proteins, the AJ is made up of the vascular endothelial (VE)-cadherin and its intracellular components; p120-catenin, beta-catenin and alpha-catenin (Chiba, et al., 2008; Ho, et al., 2018; Rincon-Choles, et al., 2006; Kiuchi-Saishin, et al., 2002). Although the tight junction proteins are vital for maintaining the endothelial barrier, their functionality is dependent on the maturation of adherens junctions. This was demonstrated in a study where blocking of the adherens inhibits correct organisation of the tight junction proteins (Azzi, et al., 2013; Ohsugi, et al., 1997). Therefore, the impact of VEGF on the adherens junction protein, VE-cadherin was assessed in the current study.

To determine other possible mechanisms through which artificial sweeteners protects GMVEC, an investigation into their effects on the surface expression of VE-cadherin was carried out. However, prior to determining the effect of artificial sweeteners, aspartame, saccharin, sucralose and neotame on the surface expression of the VE-cadherin in GMVEC, it was vital to also determine how the permeability inducing factor, VEGF-A affects VE-cadherin expression. The results in Figure 4.5 shows the internalisation of VE-cadherin by VEGF-A using an immunofluorescent imager. Figure 4.5a depicts an expression of VE-

cadherin on glomerular cell surface. However, upon exposure to VEGF-A, it was observed that the level of VE-cadherin expression was reduced (Figure 4.5b). Since this was a qualitative assay, it is vital to investigate how artificial sweeteners affect VE-cadherin expression in the glomerular endothelium using quantitative measures.
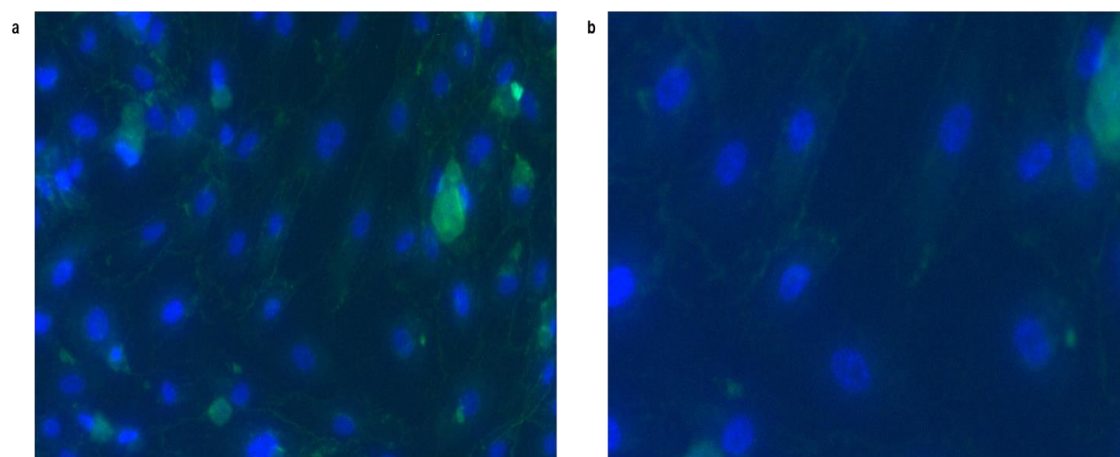
Figure 4. 5: **VEGF-A induces internalisation of VE-cadherin**

An immunofluorescent (IF) assay was used to determine the effect of VEGF-A on GMVEC cell surface expression of VE-cadherin following exposure to vehicle (media) (a) and 50 ng/ml VEGF-A (b). Cell Images was captured at 40x magnification and merged with images captured at 488nm wavelength using the Zoe Imager. Scale bar: 100 µm.

4.2.5.1 Artificial sweeteners saccharin and sucralose increases the expression of membrane adhesion protein; VE-cadherin

Following on from the results in Figure 4.5, the effect of artificial sweeteners on glomerular endothelial cell surface VE-cadherin expression in the presence and absence of VEGF-A was investigated. GMVEC exposed to saccharin and sucralose increased the level of endothelial VE-cadherin surface expression (Figure 4.6a and Figure 4.6b). Interestingly, the VEGF-A induced loss of VE-cadherin surface expression was significantly attenuated following exposure of GMVECs to saccharin and sucralose (Figure 4.6a and 4.6b). As evident in Figure 4.6b, sucralose demonstrated more protective effect against VEGF-A internalisation of VE-cadherin. Blocking of the sweet taste receptor, T1R3 by its inhibitor, lactisole has no effect on GMVEC VE-cadherin surface expression nor was it able to suppress the internalisation effect of VEGF-A as shown in Figure 4.6b. The results from these studies demonstrated that, artificial sweeteners, saccharin and sucralose not only increased the surface level expression of VE-cadherin, they also attenuated VEGF-A induced loss of VE-cadherin in glomerular microvasculature.
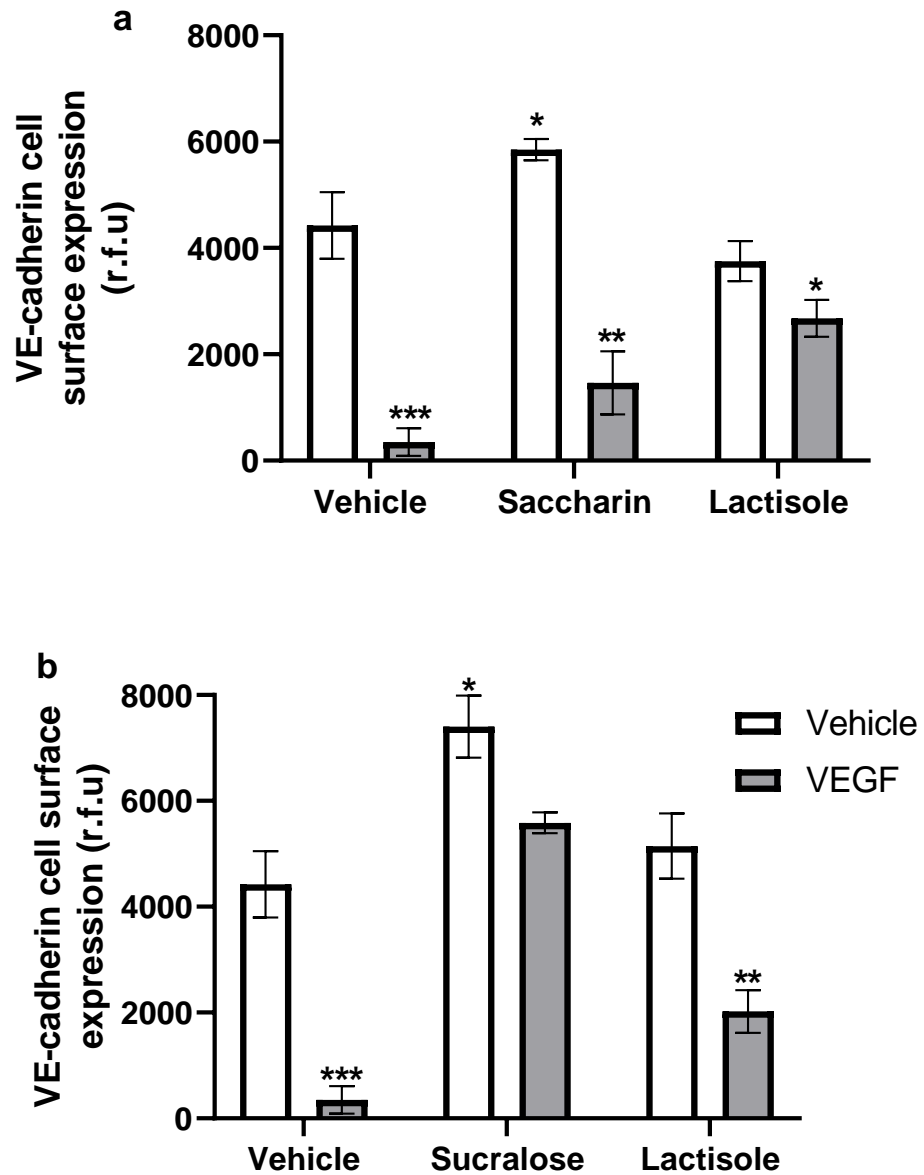
Figure 4. 6: **Saccharin and sucralose attenuates VE-cadherin internalisation in vitro through the stimulation of the sweet taste receptor**

*Whole cell indirect ELISA was used to determine GMVEC cell surface expression of VE-cadherin following exposure to 0.1 µM of saccharin (panel a), sucralose (panel b) and vehicle (media) in the presence and absence of 50 ng/ml VEGF-A and 3 µM of sweet taste inhibitor, lactisole. n=4. Data is expressed as mean with S.E.M *p 0.03, **p =0.002, ***p = 0.001 vs vehicle.*

4.2.5.1 Aspartame and neotame reduces the cell surface expression of VE-cadherin in a cell model of the glomerular microvascular endothelium

The findings presented in Figure 4.7a shows that GMVEC treated with aspartame significantly reduced the expression of VE-cadherin while cells exposed to neotame alone (Figure 4.7b) reduced the expression of VE-cadherin but seems to attenuate VEGF effect. Meanwhile the sweet taste inhibitor, lactisole has no effect on GMVEC VE-cadherin surface expression in the presence of aspartame. Blocking of the sweet taste by lactisole prior to exposure of GMVEC to neotame leads to increased level of VE-cadherin. This result is surprising as it suggests, there might be a possible reaction between lactisole and neotame, favouring the expression of VE-cadherin in the glomerular endothelial cell surface. Therefore, exposure of GMVEC to aspartame and neotame reduced the surface level expression of VE-cadherin, whilst the other sweeteners, saccharin and sucralose increases its expression.
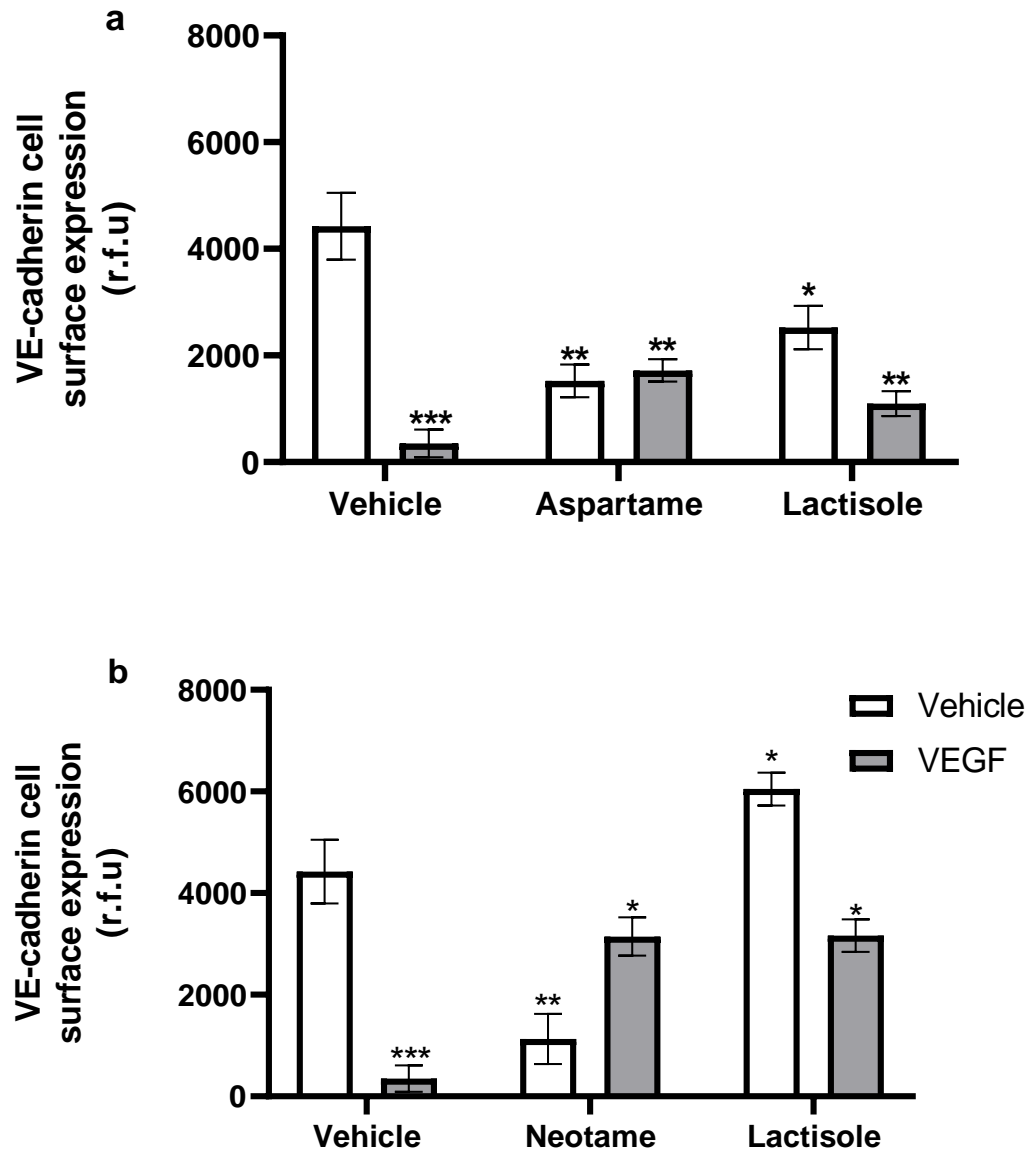
Figure 4. 7*: **Aspartame and neotame effects on the VE-cadherin surface level expression**

*Whole cell indirect ELISA (chemiluminescence) was used to measure cell surface expression of VE-cadherin following exposure to aspartame (10 μM) (panel a), Neotame (0.1 μM) (panel b) and vehicle (media) in the presence and absence of 50 ng/ml of VEGF-A and 3 μM of sweet taste inhibitor, lactisole. n=4. Data is expressed as mean with S.E.M ***p 0.002, ***p <0.001 vs vehicle.*

4.2.6 Artificial sweeteners follow a non-traditional signalling cascade upon activation of sweet taste receptor.

In normal physiological conditions, the activation of the sweet taste receptors, T1R2/3 by natural sugars such as sucrose triggers the dissociation of the heterotrimeric G-protein with the Gα subunit stimulating all adenyl cyclase (ACs), giving rise to increased level of cyclic adenosine monophosphate (cAMP) (Trubey, et al., 2006). This downstream signalling second messenger have been associated with improved endothelial barrier function with studies linking elevated level of intracellular cAMP to reduced permeability of endothelial cells *in vitro* and *in vivo* (Fu, et al., 2015).

Since cyclic adenosine monophosphate (cAMP) is a known modulator of endothelial barrier integrity, this aspect of the research sought to determine whether artificial sweeteners, aspartame, saccharin, sucralose and neotame affects the intracellular level of cAMP in glomerular microvascular endothelium. To address that hypothesis, the assay protocol was carried out as detailed in section 2.2.2.10 in the methods chapter. Contrary to the research hypothesis, results showed that artificial sweeteners, aspartame (Figure 4.8a), saccharin (Figure 4.8b), sucralose (Figure 4.8c) and neotame (Figure 4.8d) does not increase intracellular cAMP level in glomerular microvascular cells. Although the cells exposed to sweeteners showed a statistically significant difference from the control, it should be noted that according to the assay design, the higher values of relative fluorescence unit (rfu) indicate lower generation of intracellular cAMP (detailed in appendix). Additionally, VEGF-A and the sweet taste inhibitor, lactisole has no effect on cAMP level as shown in Figure 4.9 and Figure 4.10. The two concentrations, one lower range (1 μM) and the other higher (1000 μM) of each sweeteners (aspartame, saccharin, sucralose and neotame) selected for the cAMP assay was based on the notion that if cAMP was not generated at low dosage, then, an effect might be seen at a higher concentration. An inducer of cAMP, forskolin was utilised as a positive control and results shown in Figures 4.8a - Figure 4.8d, indicates that, forskolin significantly increased intracellular cAMP level. The goal of using forskolin was to eliminate the generation of false negative result and to validate the experimental technique.

Standards of cAMP was utilised as a guide in assessing the generation of cAMP by the sweeteners. The higher the amount of cAMP, the lower the relative fluorescence unit (R.F.U). Hence, the reason forskolin's RFU was statistically significant with a *p-value* < 0.0001 indicating the production of intracellular cAMP. These results, therefore, indicate that artificial sweeteners, aspartame, saccharin, sucralose and neotame follows a different signalling pathway to the traditional route of triggering an increase of cAMP upon activation of the sweet taste receptors.
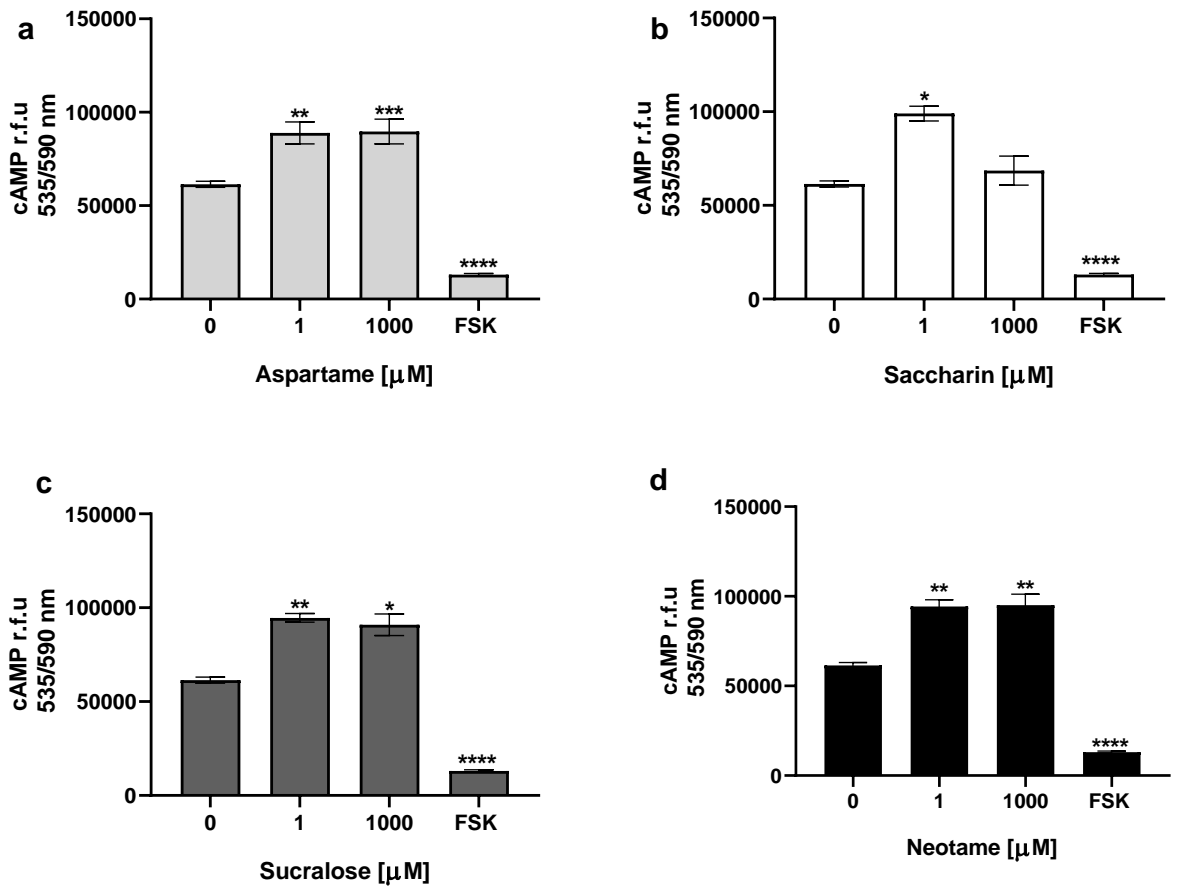
Figure 4. 8: **Artificial sweeteners do not stimulate the generation of cyclic AMP**

Glomerular microvascular endothelial cells were measured for the generation of cAMP upon exposure to artificial sweeteners; aspartame (1 and 1000 µM), saccharin (1 and 1000 µM), sucralose (1 and 1000 µM) and neotame (1 and 1000 µM) for 24 hours using the cAMP-Screen Direct chemiluminescent ELISA assay. The amount of cAMP produced was measured using the Victor plate reader (luminometry) set up at 535/590 nm for 1 second. Forskolin (FSK), 10 µM was used to validate the assay. n=4. Data is expressed as mean with S.E.M **p 0.002, ***p <0.001, ****p <0.0001 vs vehicle (0 µM).
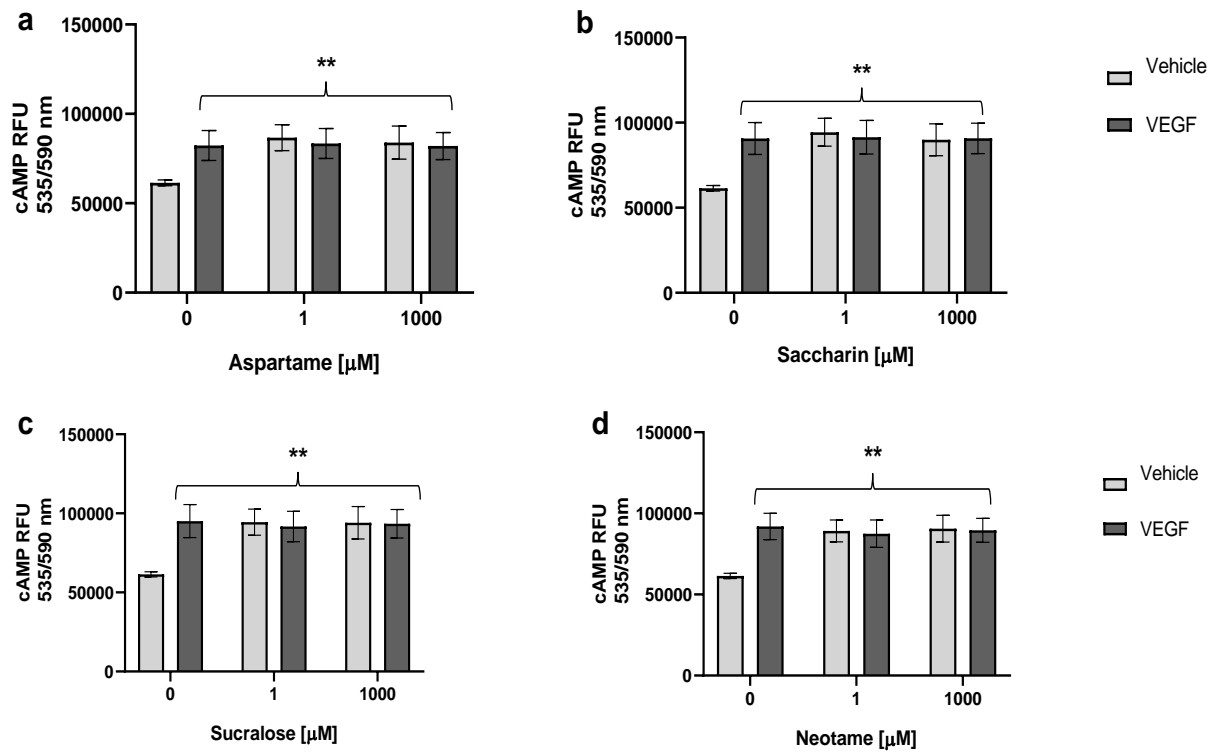
Figure 4. 9: **VEGF-A has no effect on cyclic AMP**

Glomerular microvascular endothelial cells were measured for the generation of cAMP upon exposure to artificial sweeteners; aspartame (1 and 1000 µM), saccharin (1 and 1000 µM), sucralose (1 and 1000 µM) and neotame (1 µM and 1000 µM) in the presence and absence of 50 ng/ml VEGF-A using the cAMP-Screen direct ELISA chemiluminescent assay. The amount of cAMP produced was measured using the Victor plate reader (luminometry) set up at 535/590 nm for 1second. n=4 and data are presented as mean with S.E.M. **p= 0.003 vs vehicle (0 µM).
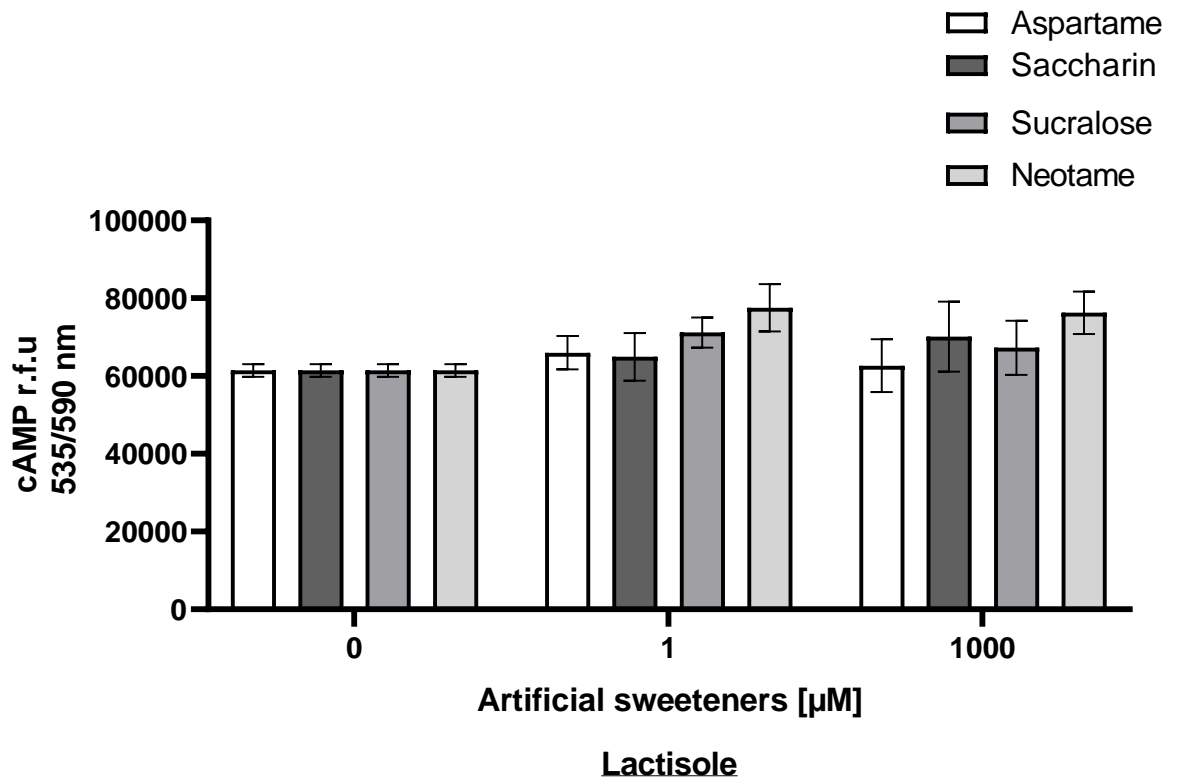
Figure 4. 10: **Effect of lactisole on intracellular cyclic AMP level**

Glomerular microvascular endothelial cells were measured for the generation of cAMP upon exposure to artificial sweeteners; aspartame (1 μM and 1000 μM), saccharin (1 μM and 1000 μM), sucralose (1 μM and 1000 μM) and neotame (1 μM and 1000 μM) in the presence and absence of lactisole for 24 hours. The cAMP-Screen direct ELISA chemiluminescent assay was then carried out. The amount of cAMP produced was measure using the Victor plate reader (luminometry) set up at 535/590 nm for 1second. n=4 and data presented as mean with S.E.M.

4.2.7 Effects of artificial sweeteners on intracellular ROS production in glomerular microvasculature

To address the hypothesis regarding effects of artificial sweeteners on GMVEC intracellular ROS level, DCFDA ROS assay was performed as detailed in section 2.2.2.11. The results presented in Figure 4.12a- Figure 4.12d, indicated that none of the tested artificial sweeteners increased cellular ROS level in the glomerular endothelium. The findings in Figure 4.11 indicates that H_2O_2 increases cellular ROS level in the glomerular endothelium. The assay was further validated using a known inhibitor of ROS, N-acetylcysteine (NAC) with results demonstrating that H_2O_2-induced ROS was abrogated by the addition of 10 µM N-acetylcysteine (NAC), an antioxidant and a precursor in glutathione formation (Park, et al., 2015; Halasi, et al., 2013). The findings from this research has demonstrated, for the first time, the effect of the artificial sweeteners on cellular ROS generation in a cell model of glomerular microvascular endothelium.
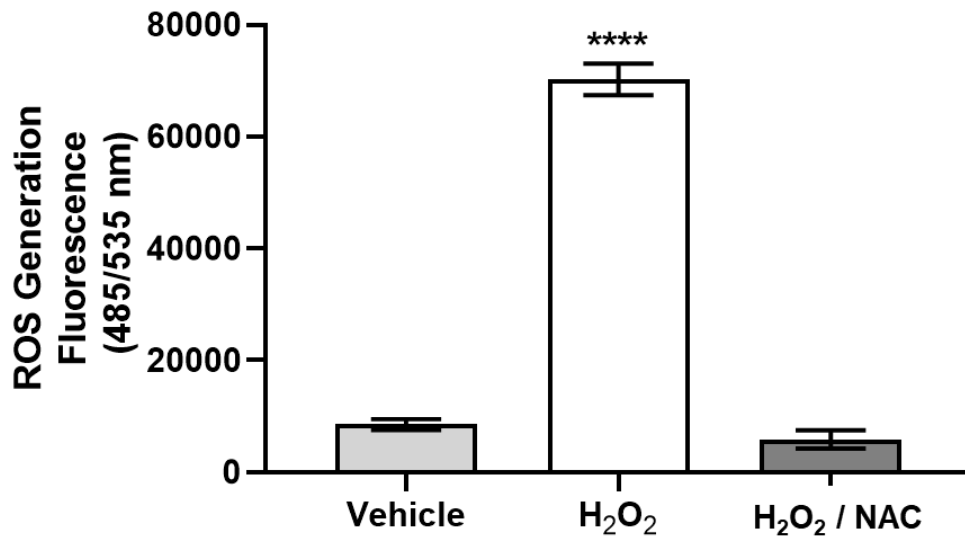
Figure 4. 11: **N-Acetyl cysteine inhibits intracellular ROS production in glomerular endothelial microvasculature**

*Glomerular endothelial cells were pre-incubated with N-Acetyl cysteine (NAC) for 2 hours, stained with DCFDA and followed by treatment with 10 μM H_2O_2. ROS production was assessed by measuring the fluorescence level of DCF following staining with DCFDA. Absolute ROS value was derived by subtraction of unstained values from stained values. n=6 and data is presented as mean with S.E.M, ****p <0.0001 vs vehicle (media). DCFDA = 2', 7' –dichlorofluorescin diacetate and DCF (2', 7'–dichlorofluorescein).*
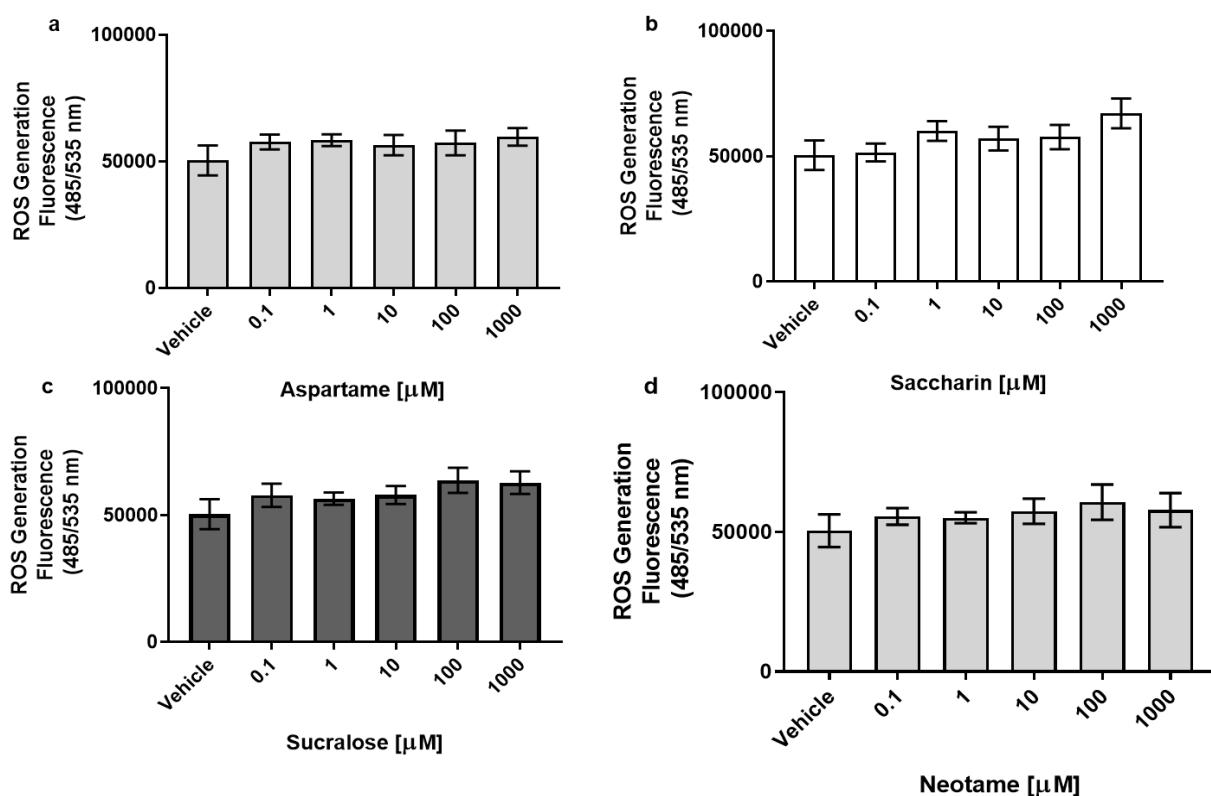
Figure 4. 12: **Artificial sweeteners has no effect on ROS generation**

The generation of ROS in GMVEC was studied in the presence of artificial sweeteners, aspartame(a), saccharin (b), sucralose (c) and neotame (d). Following the culture of the GMVEC, the cells were stained with the DCFDA probe followed by exposure to artificial sweeteners at a range of doses between 0.1 μM to 1000 μM and incubated for 2 hours. ROS production was assessed by measuring the fluorescence level of DCF. n=6 and Data is presented as mean with S.E.M. DCFDA = 2', 7'– dichlorofluorescin diacetate and DCF (2', 7'–dichlorofluorescein). Difference between ROS generated fluorescence value for the vehicle observed in Figure 4.11 and Figure 4.12a-d could be attributed to the methodological differences.

4.2.8 Saccharin, sucralose and neotame abrogated H_2O_2-induced ROS production in glomerular endothelial microvasculature

Following N-Acetyl cysteine (NAC) scavenging effect on H_2O_2-induced ROS in Figure 4.11, the effects of artificial sweeteners (aspartame, saccharin, sucralose and neotame) on H_2O_2-induced ROS was studied. The results indicated that saccharin (Figure 4.13a), sucralose (Figure 4.13b) and neotame (Figure 4.14b) attenuated the H_2O_2-induced ROS in glomerular microvascular endothelium *in vitro*. Interestingly, when the effect of these sweeteners (saccharin, sucralose and neotame) at the studied concentration was compared to the 10 µM effect of NAC, there was no significant difference, thereby suggesting that these sweeteners could be a competitive agent in ameliorating the cellular oxidative effect of ROS in the glomerular vasculature. Aspartame on the other hand, showed a *dose-selective* effect as only 0.1 µM attenuated the oxidative effect of H_2O_2-induced ROS (Figure 4.14a). These findings demonstrated the possible attenuation of VEGF-A induced leak in the glomerular endothelium by artificial sweeteners through the scavenging of cellular ROS.
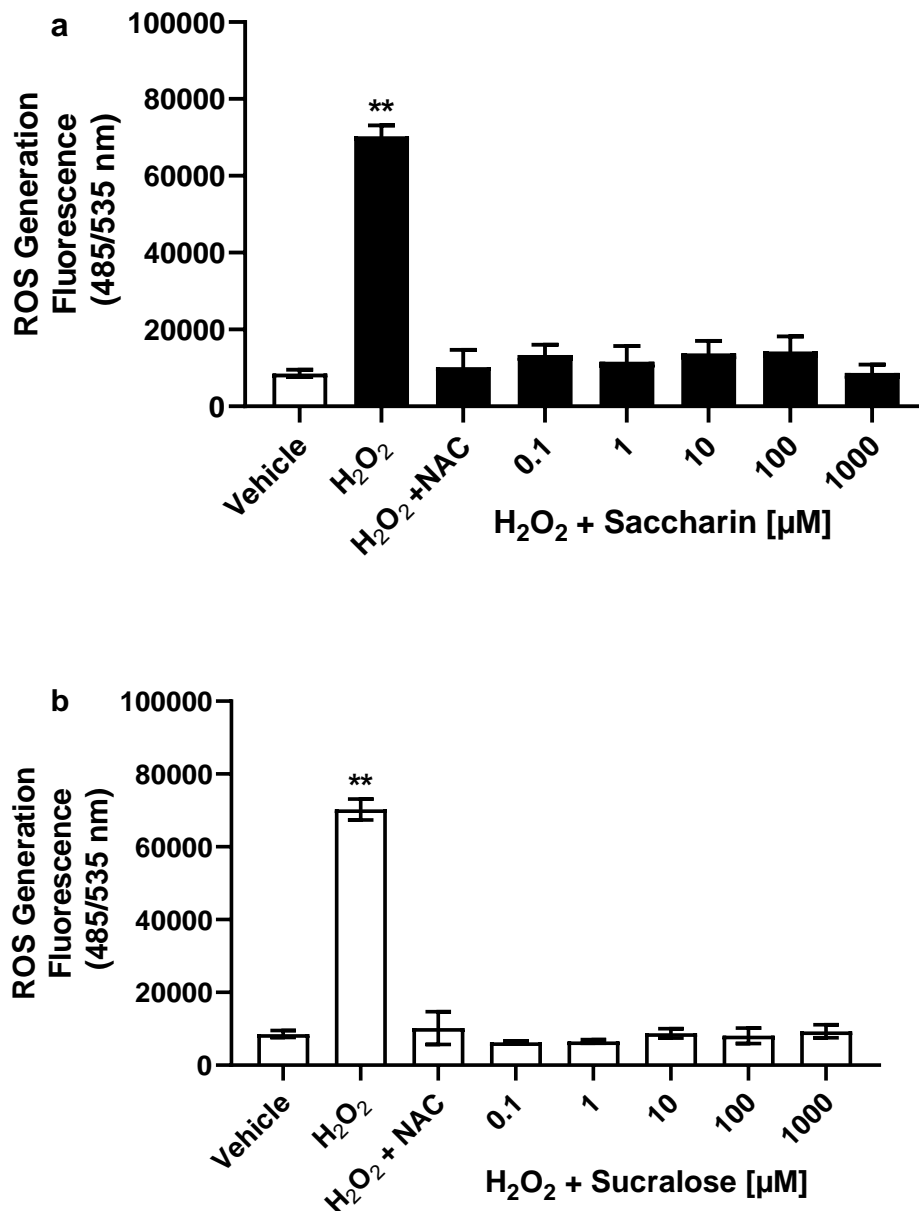
Figure 4. 13: **Saccharin and sucralose inhibit H_2O_2-induced ROS production in glomerular endothelium**

Glomerular endothelial cells were pre-incubated with 0.1, 1, 10, 100 and 1000 μM doses of saccharin (panel a) and sucralose (panel b) respectively for 2 hours followed by treatment with 10 μM H_2O_2. An inhibitor of ROS, NAC (10 μM) was used as control to validate results. Intracellular GMVEC ROS production was assessed by measuring the fluorescence level of DCF after staining with DCFDA. Absolute ROS value was calculated by subtraction of unstained values from stained values. n=6 and data is presented as mean with S.E.M, **p <0.05 vs 0μM. DCFDA = 2', 7'–dichlorofluorescein diacetate and DCF (2', 7' – dichlorofluorescein).
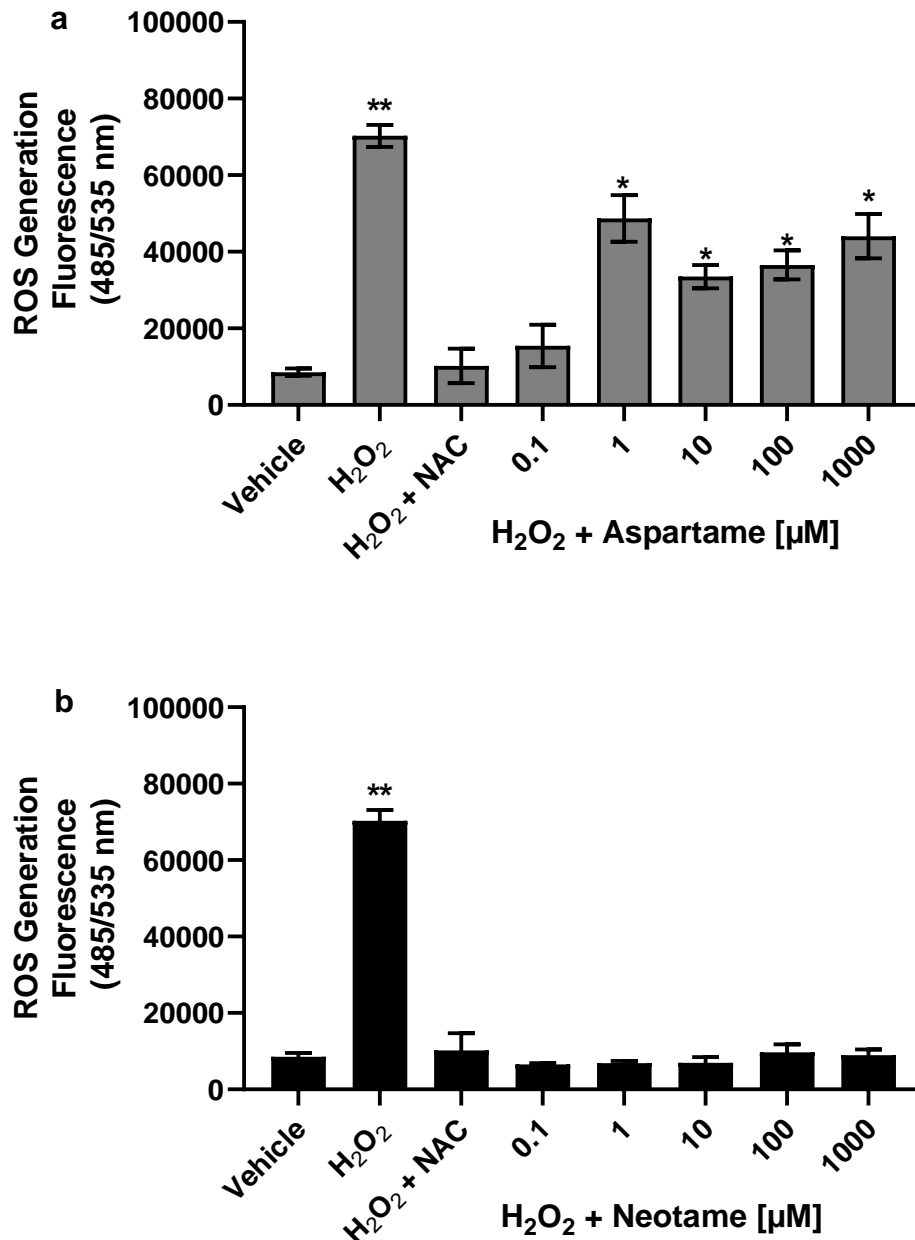
Figure 4. 14: **Neotame but not aspartame attenuates H_2O_2-induced cellular ROS production in glomerular endothelial microvasculature**

Glomerular endothelial cells were pre-incubated with 0.1, 1, 10, 100 and 1000 µM doses of

aspartame (panel a) and neotame (panel b) respectively for 2 hours followed by treatment

with 10 µM of H_2O_2. 10 µM NAC was used as control to validate results. ROS production

was assessed by measuring the fluorescence level of DCF following staining with DCFDA.

Absolute ROS value was derived by subtraction of unstained values from stained values.

n=6 and data is presented as mean with S.E.M, **p <0.05 vs vehicle (media). DCFDA = 2',

7' –dichlorofluorescin diacetate and DCF (2', 7' –dichlorofluorescein).

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4.3 Discussion

Sweet taste receptors, was initially thought to be located primarily in the oral cavity where its activation leads to a cascade of transductions and signal transmission to the brain, generating sweet taste sensation (Lasconi, et al., 2019) as previously detailed in section 1.10.1. However, studies have shown that these sweet taste receptors are expressed in other parts of the body, mirroring the results from this research (Young, et al., 2009; Dyer, et al., 2005; Elliot, Kapoor and Tincello, 2011; Freund and Lee, 2018; Kojima, et al., 2014; Nakagawa, et al., 2009; Meyer, et al., 2012; Lizunkova, Enuwosa and Chichger, 2019). Although the localization of the sweet taste receptors in other organs outside oral cells have been demonstrated, their full physiological roles and endogenous ligand activation is not yet known. Hence, the results discussed in this section of the research do not only demonstrate a new understanding on the expression of the sweet taste receptor T1R3, in the glomerular endothelium, but also indicate possible activation of the sweet taste receptor by artificial sweeteners as potential therapeutic target for endothelial permeability associated with diabetic kidney disease.

4.3.1 Expression of sweet taste receptors in the glomerular microvascular endothelium

The human sweet taste receptor (hT1R2–hT1R3) is a heteromeric complex composed of two subunits, T1R2 and T1R3 (Kim, et al., 2017; Masuda, et al., 2012) and natural sugars (e.g., sucrose and glucose) and artificial sugars (saccharin and sucralose) binds to specific domains of both T1R2 and T1R3 receptors (Nie, et al., 2005; Xu, et al., 2004). These sweet taste receptors have also been linked to roles in regulating fuel metabolism and nutrition in the body (Behrens and Meyerhof, 2014; Kojima and Nakagawa, 2011). However, no published research has studied the expression of the sweet taste receptors in glomerular endothelium nor the activation of the sweet taste receptors by artificial sweeteners, aspartame, saccharin, sucralose and neotame in one combined study. While only two of

the studied sweeteners, saccharin and sucralose seems to stimulate expression by binding to the sweet taste receptor, T1R3, the effect of aspartame and neotame also adds novel knowledge. Additionally, the inability of the artificial sweeteners, aspartame and neotame to activate the T1R3 sweet taste receptor suggests their binding to the T1R2 cleft domains of the T1R2/T1R3 heterodimer as shown in some studies (Masuda, et al., 2012; Xu, et al., 2004; Maillet, et al., 2015). The results from these studies therefore supports the notion that the localisation of sweet taste receptor, T1R3 is not limited to oral cells but exist in other cells including glomerular microvascular endothelium (Bezencon, LeCoutre and Damak, 2007; Masubuchi, et al., 2013; Harrington, et al., 2018).

4.3.2 Saccharin and sucralose attenuate VEGF-A induced permeability through potential activation of the sweet taste receptor T1R3

The role of the sweet taste receptor in regulating VEGF-A induced permeability in glomerular endothelium was investigated for the first time. The surface level expression of the sweet taste receptor in the glomerular microvasculature was a novel finding which was further explored to determine whether artificial sweeteners abrogated VEGF-A induced permeability through its potential activation. Hence, the binding of the sweet taste receptors by artificial sweeteners, saccharin and sucralose was demonstrated. This binding of artificial sweeteners, saccharin and sucralose to the T1R3 sweet taste receptor in glomerular microvasculature was further validated using lactisole, a chemical inhibitor. Lactisole is an aralkyl-carboyxlic acid, a competitive inhibitor specific to the human sweet taste receptors, hT1R2/ hT1R3 (Xu, et al., 2004). Thus, pharmacological inhibition of the sweet taste receptor, was carried out using lactisole, a proven inhibitor of the sweet taste receptors (Jiang, et al., 2005; Nakagita, et al., 2019).The exposure of GMVEC to lactisole, significantly blocked the protective effect of the artificial sweeteners, saccharin and sucralose against VEGF-A induced leak suggesting that the protective effect of saccharin and sucralose were mediated through their binding to and potential activation of the sweet taste receptor, T1R3.

The ability of the artificial sweeteners; saccharin and sucralose to attenuate VEGF-A induced permeability in the glomerular microvasculature through their binding to the sweet taste receptor, T1R3 as shown in this research mirrors studies by Harrington, et al. (2018) and Lizunkova, Enuwosa and Chichger, (2019). In line with current research findings, the study by Lizunkova, Enuwosa and Chichger, (2019) demonstrated that targeting the sweet taste receptor T1R3 using an artificial sweetener, sucralose blocks permeability induced by the growth factor, VEGF-A in an *in vitro* model of retinal microvasculature. Although these studies were conducted in different target organs and utilised only sucralose, their research was also performed on microvascular endothelial cells targeted by diabetic injury and could therefore be compared to current findings on the glomerular microvasculature. Another interesting finding was on aspartame and neotame. The inability of aspartame and neotame to activate the sweet taste receptor, T1R3, corresponds with other research finding on their binding to the T1R2 of the T1R2/T1R3 heterodimer (Nelson, et al., 2001). This phenomenon could be due to ability of each subunit of the T1R2/T1R3 sweet taste receptors to function independently (Nuemket, et al., 2017), despite existing as a heterodimer (Welcome, et al., 2015).

Additionally, Lizunkova, Enuwosa and Chichger, (2019), further indicates that sweeteners can bind to any of the heterodimer as their study demonstrates the inability of sucralose to activate the sweet taste receptor, T1R2, but rather stimulates the T1R3 dimer in retinal microvasculature. Thus, a likely explanation to the lack of protection against VEGF-A induced leak by aspartame and neotame could be because they bind only to the T1R2 of the sweet taste receptor. Although the results of the artificial sweeteners, aspartame and neotame were contrary to those of saccharin and sucralose, the knowledge on their inability to protect the glomerular microvasculature against VEGF-A induced permeability would aid in making informed decisions about these sweeteners. Researchers and other scientist with an interest in these sweeteners would also benefit from this new knowledge when proposing further studies.

So far, in this study, the localization of the sweet taste receptor T1R3 and its potential activation through binding of saccharin and sucralose to protect the glomerular endothelial cells against VEGF-A induced permeability has been evident. However, other possible mechanisms associated with the protective effect of artificial sweeteners, saccharin and sucralose on glomerular endothelial cell barrier against VEGF-A induced leak such as the cyclic AMP and VE-cadherin levels were also investigated.

4.3.4 Artificial sweeteners maintain the expression of membrane adhesion protein, VE-cadherin

The microvascular endothelium comprises of various pools of proteins that forms membrane domains, creating the cohesive structure that accounts for the barrier properties of the vessel wall (Rahimi, 2017; Rodrigues and Granger, 2015). The function of the endothelial junctions is not limited to cell-cell adhesion but also includes the collection, modification and conveyance of signals (Satchell, et al., 2006). The most important of the barrier-forming adhesive structures are the adherens junction proteins which consist mainly of the VE-cadherin and an alteration to this membrane component of the endothelium underlies increased vascular permeability (Rodrigues and Granger, 2015). As demonstrated in an *in vivo* study by Wessel, et al. (2014), VE-cadherin selectively regulate the induction of vascular permeability through phosphorylation/dephosphorylation of specific tyrosine residues. It was, therefore, crucial to determine whether this regulation of VEGF-A induced permeability by the artificial sweeteners occurs through maintenance of the surface adhesion protein, VE-cadherin. The findings from this current study showed that artificial sweeteners, saccharin and sucralose maintains the VE-cadherin surface level expression in glomerular endothelium and attenuates VEGF-A internalisation of the protein. The internalisation of VE-cadherin by 50 ng/ml of VEGF-A in GMVEC justifies the selected modulators by which the endothelial barrier is destabilised. The internalisation of VE-cadherin by VEGF-A in the glomerular endothelium as demonstrated in this research was supported by other studies (Gavard and Gutkind, 2006; Li, et al., 2002). In addition, it has

been established that artificial sweetener, sucralose attenuation of VEGF-A induced permeability could be based on the prevention of Src/PAK/ -mediated cell-contact disassembly and Src/MLC2/HSP27-mediated actin remodeling of the VE-cadherin (Harrington, et al., 2018). Therefore, it could be said that the studied sweetener, sucralose protects the glomerular endothelium against VEGF-A induced permeability through the same mechanism of action, preventing the phosphorylation of VE-cadherin by VEGF-A. In contrast to the effects of saccharin and sucralose, the plausible explanation to the controversial effect of aspartame and neotame on VE-cadherin expression could be their (aspartame and neotame) inability to activate the sweet taste receptor, T1R3, expressed in the glomerular microvascular endothelium. Thus, this finding indicates that the protection of GMVEC by these sweeteners against VEGF-A induced leak could be based on their potential to protect VE-cadherin against VEGF-A internalisation, thereby maintaining the glomerular endothelial barrier integrity.

Altogether, it has been demonstrated by the findings in this chapter that low concentrations of artificial sweet molecules, saccharin and sucralose activates the T1R3 sweet taste receptor. The studies further demonstrated the relationship between endothelial permeability and adherens junction formation as increased and maintained VE-cadherin surface levels was observed with same concentration of sucralose and saccharin shown to be barrier protective in the glomerular microvasculature.

4.3.5 Artificial sweeteners abrogated H$_2$O$_2$-induced ROS effect in an in vitro model of glomerular microvascular endothelium.

Redox imbalance caused by excess glucose (hyperglycaemia) in both systemic and intra-renal inflammation, plays a critical role in the pathogenesis of DKD (Basile, Anderson and Sutton, 2012; Amorim, et al., 2019). The hyperglycaemic condition of the microvascular endothelium under diabetic condition and the resulting vascular dysfunction has been linked to elevated ROS (El-Daly, et al., 2018). As extensively discussed in the introductory section of this research (1.8.4.3), there are several inducers of oxidative stress with the prevalent type in the cytoplasm of the cell being hydrogen peroxide (H$_2$O$_2$). In addition, studies have shown that hydrogen peroxide (H$_2$O$_2$) is the most interesting agent to use when determining cellular ROS level (Forman and Bernardo, 2016; Sanchez-Gomez, et al., 2015). Hence, H$_2$O$_2$ was utilised as a ROS inducer in the presence and absence of artificial sweeteners. The results in Figure 4.12 indicates that none of the artificial sweeteners at the studied concentration were able to induce ROS production in the glomerular endothelial cell.

Also, the artificial sweeteners, saccharin, sucralose and neotame exhibited a complete clearance of H$_2$O$_2$-induced ROS, mimicking the scavenging effect of a well-known ROS inhibitor and a proven scavenger of H$_2$O$_2$-induced ROS, N -acetylcysteine (NAC) (Odetti, et al., 2013; Halasi, et al., 2013). This study, therefore, demonstrated the potential antioxidant effect of the artificial sweeteners, saccharin, sucralose and neotame on the glomerular microvasculature.

Furthermore, the findings on the inability of the artificial sweetener, aspartame to protect the glomerular endothelial cells from H$_2$O$_2$-induced ROS could be linked with production of methanol as a by-product. This link between methanol produced as a metabolite from aspartame ingestion and oxidative stress was demonstrated by Ashok, Sheeladevi and Wankhar, (2014) .That study showed an observable induction of oxidative stress following acute exposure of aspartame *in vivo* due to detectable level of methanol in circulation after 24 hours of aspartame exposure.

The findings in Figure 4.12, indicates that artificial sweeteners do not stimulate intracellular ROS production, which is contrary to available studies on the effect of artificial sweeteners on cellular generation of ROS with aspartame being the most studied of the sweeteners (Pandurangan, Enkhtaivan and Kim, 2016; Alleva, et al., 2011). The contradictory effect of these artificial sweeteners in the induction of ROS generation could be attributed to the cell type. Alleva, et al. (2011) demonstrated that incubation of aspartame (20 µM −100 µM) induces intracellular ROS formation in HUVEC cells but not in fibroblast (IMR-90) cells, which are different from the cell type used in this research. Therefore, studies on ROS-inducing effect of aspartame is controversial and depends on the cell type, study parameters and the length of aspartame exposure to target cells (Mourad and Noor, 2011).

Furthermore, the effect of ROS-related vascular dysfunction has been linked to its effect on the adherens junctional proteins as demonstrated by Gong, et al. (2014). The authors demonstrated that H_2O_2 activates the src-cascade in endothelial cells, promoting the dissociation of p120-catenin from VE-cadherin, disassembly of the catenin, internalization of VE-cadherin and consequently, barrier leak.

Therefore, since the work of Gong, et al. (2014) linked intracellular ROS to endothelial dysfunction and leak, the ability of the named sweeteners to abrogate H_2O_2-induced ROS in the glomerular microvasculature could be a potential target in maintaining endothelial barrier integrity. This novel research finding therefore, answers part of the questions raised in aim II of this research (section 1.19); the cellular effects of artificial sweeteners on intracellular ROS. Since the increase in cellular ROS was abrogated by saccharin and sucralose, these sweeteners might aid in the management of other diseases characterised by increased production of ROS and not just in the management and treatment of DKD.

4.4 Conclusion

This chapter has demonstrated some key findings into the mechanism through which artificial sweeteners attenuates VEGF-A induced permeability in a cell model of the glomerular endothelial microvasculature. While the sweeteners do not follow the traditional signalling route of increasing cAMP in order to protect the glomerular endothelial barrier, they however, abrogated VEGF-A induced permeability through stimulation of sweet taste receptor, T1R3. This research also indicated that saccharin and sucralose maintained the surface level expression of the adhesion protein, VE-cadherin and attenuated its internalisation by VEGF-A. Saccharin, sucralose and neotame do not induce ROS generation, another modulator of endothelial barrier integrity, but instead, acts as a scavenger of ROS in GMVEC since they attenuated exogenous H_2O_2-induced ROS effect. The perception of sweet taste through potential activation of the sweet taste receptors by artificial sweeteners and the possibility of these sweeteners having other physiological effect at a molecular level were the research questions addressed in this chapter. The results discussed so far, demonstrated that, potential activation of the sweet taste receptor, T1R3 in an *in vitro* model of the glomerular microvasculature could be a target for new treatment strategy in managing leak and delaying the progression of DKD. These findings could, therefore, lead to practical interventions in relieving the social, economic and personal burden of individuals living with DKD especially in developing countries where the cost of healthcare is high (Bommer, et al., 2018; Beissenger, et al., 2013; Umanath and Lewis, 2018).

CHAPTER 5

DEVELOPMENT AND APPLICATION OF GAS CHROMATOGRAPHY-MASS SPECTROMETRY METHOD FOR DETECTION OF SUCRALOSE IN GLOMERULAR ENDOTHELIAL CELLS

5.1 Introduction

There are several analytical methods for the detection of artificial sweeteners as previously mentioned in section 1.13.4 and includes ion chromatography (Zhu, et al., 2005), capillary isotachophoresis (Herrmannová, et al., 2006), high performance liquid chromatography with evaporative light scattering detection (Wasik, et al., 2007; Buchgraber and Wasik, 2009; Yang and Chen, 2010) and gas chromatography-mass spectrometry (Qiu, et al., 2007). However, none of these analytical techniques have been employed in the detection of an artificial sweetener, sucralose in cultured glomerular cell samples as most studies focused on detecting sucralose in drinks (Queiroz Pane, et al.,2015), water (Ferrer, et al., 2013), table top sweetener; Splenda (Qiu, et al., 2007) and other foods (Zygler, et al., 2009). Hence, the need to develop an analytical method that is sensitive and specific in detecting sucralose in cultured samples of glomerular endothelial cells. As outlined in section 1.19, this chapter will address aim III; method development and validation using gas chromatography-mass spectrometry (GC-MS) for the detection and identification of sucralose, and aim IV; application of the validated GC-MS method to assess whether sucralose acts independent of the sweet state receptor to protect glomerular microvascular endothelial cell.

In this chapter, results from experiments carried out to achieve the above stated aims are discussed. The developed method for the detection of sucralose was adapted from Qiu, et al. (2007). The method was then optimised and validated using analytical parameters such as linearity, limit of detection, limit of quantification, repeatability, reproducibility, stability, selectivity and precision. In addition, selection of an internal standard was carried out to minimise errors which might arise during sample preparation or analysis. The chapter then concludes with application of the optimised GC-MS method to investigate whether selected concentrations of sucralose crosses the glomerular endothelial cell membrane.

5.2 Results

5.2.1 Identification of sucralose

Polar compounds, like sucralose requires sample processing to ensure detection by the GC-MS. As reviewed in section 1.16 of chapter 1, derivatisation is an essential reaction for polar compounds with low volatility prior analysis with GC-MS (Ruiz-Matute, et al., 2010). Derivatisation improves detectability of the derivatised product. As depicted in Figure 5.1a, upon derivatising a sample, a modified analyte is generated, known as the derivative and in this case, sucralose was converted to its trimethyl silyl ether (TMS) when derivatised with MSTFA. The hydroxyl molecules present in sucralose were replaced with a silyl group, changing sucralose's relative molecular mass (RMM) which increased from 397 to 757 (Figure 5.1a). Upon injection of derivatised sucralose into the GC column, a peak at 22.2 minutes retention time was detected (Figure 5.1b). This identification of the chromatogram as sucralose-TMS was further confirmed through the examination of its mass spectra as seen in section 5.2.1.1.
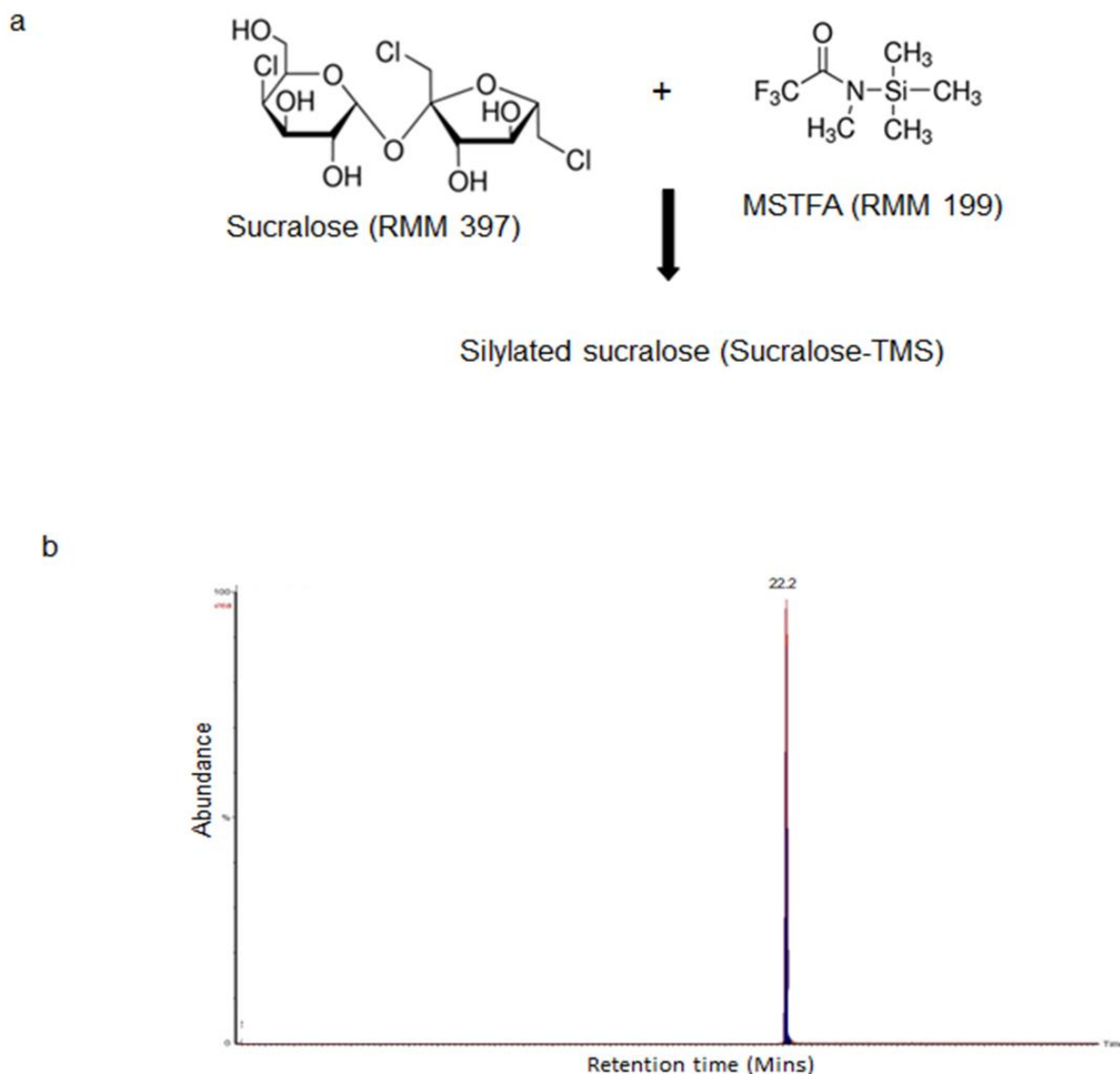
Figure 5. 1: **Gas chromatogram of derivatised sucralose with MSTFA**

Sucralose in an underivatized state with its hydroxyl group reacts with a derivatising reagent, MSTFA (Figure 5.1a) to yield sucralose-TMS. The generated chromatogram of derivatised sucralose (sucralose-TMS) (Figure 5.1b) when 100 µg/ml of sucralose was derivatised with MSTFA at 70°C for 30 minutes and the total ion chromatogram was recorded with Perkin Elmer Clarus 500 GC-MS. The elution time was at 22.2 minutes, with stated GC conditions (column Rtx ®-5 (30 m x 0.25 µm x 0.25 mm i.d.); 270 °C injection port temperature; column temperature: 180°C for 2 minutes, 6°C/ minutes until 250°C, hold for 20 minutes with 3.5 minutes solvent delay time. X-axis of the gas chromatogram represents retention time (the time it takes for the analyte to elute) and Y-axis represents the intensity or abundance of the signal.

5.2.1.1 Identification of sucralose based on mass spectra and diagnostic ions

To identify the structure of sucralose, mass spectra and diagnostic ions were used. Sucralose was eluted at 22.2 minutes and m/z s (m/z) of 207, 308 and 343 (Figure 5.2a) were detected. The tallest peak with a m/z of 73 is a trimethyl silyl group and thus not used for analyte (sucralose) identification. The mass spectrum and extracted ions of sucralose as shown in Figure 5.2a was compared to that of Qiu, et al. (2007) in Figure 5.1b which demonstrates that the analyte from this present study (Figure 5.2a) was sucralose-TMS. The mass spectra from both studies have similar m/z s. Furthermore, simply detecting the same ions does not confirm that the ions are specific to sucralose. Thus, fragmentation pattern of sucralose needs to be examined and compared with a standard. Results in Figure 5.3a and Figure 5.3b highlights the proposed fragmentation pattern of sucralose-TMS which corresponds with those of the detected analyte (Figure 5.2a). Additionally, as shown in Figure 5.3a, sucralose was converted to its TMS ether following derivatisation with MSTFA. As previously explained, in section 5.2.1, the relative molecular mass of the new derivative of sucralose (sucralose-TMS) increased to 757 but there was no m/z of 757 present in the mass spectrum (Figure 5.2a). Therefore, the plausible explanation is that the m/z of 757 might have undergone fragmentation as Figure 5.3b depicts. Since the intact molecular ion of sucralose-TMS (m/z 757) was not observed in the mass spectrum (Figure 5.2a), the other possible main m/z s resulting from sucralose-TMS fragmentation were m/z 412, 397 and 343. Hence, these m/z s and their fragmentation pattern, the chromatogram and the retention time confirms the detected analyte to be sucralose-TMS.
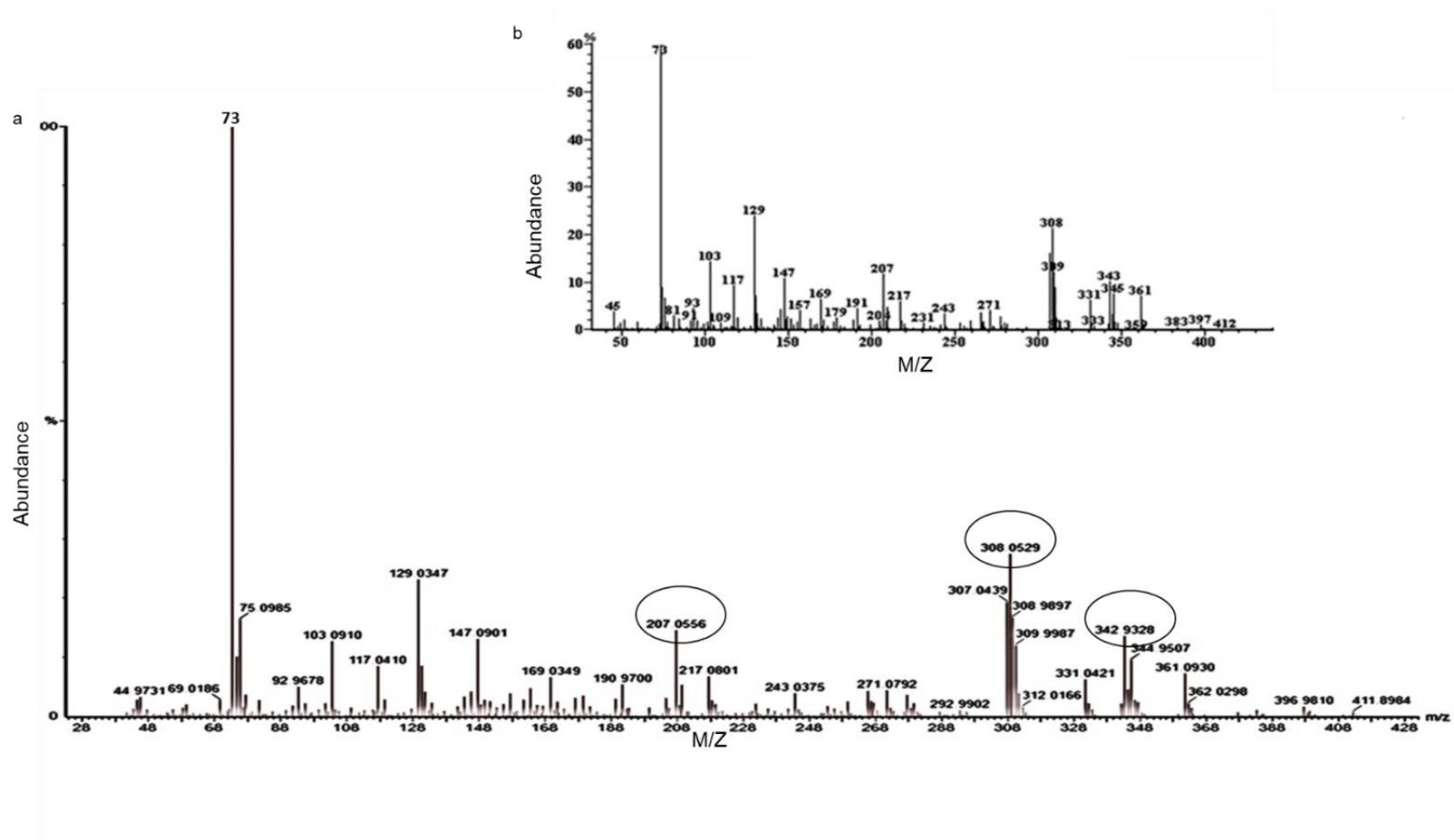
 Figure 5. 2: **The Electron ionisation mass spectra of sucralose–TMS**

The mass spectrum of MSTFA-derivatised sucralose (panel a) recorded with the TurboMass™ 5.4 GC-MS software with an electron ionization impact of 70 eV from 70-600 m/z. The spectrum shows the m/z s; 207, 308 and 343 m/z (circled) at a retention time of 22.2 minutes. Panel b is a comparative mass spectrum of sucralose-TMS (Qiu, et al., 2007) derivatised using a silylation reagent, HMDS.
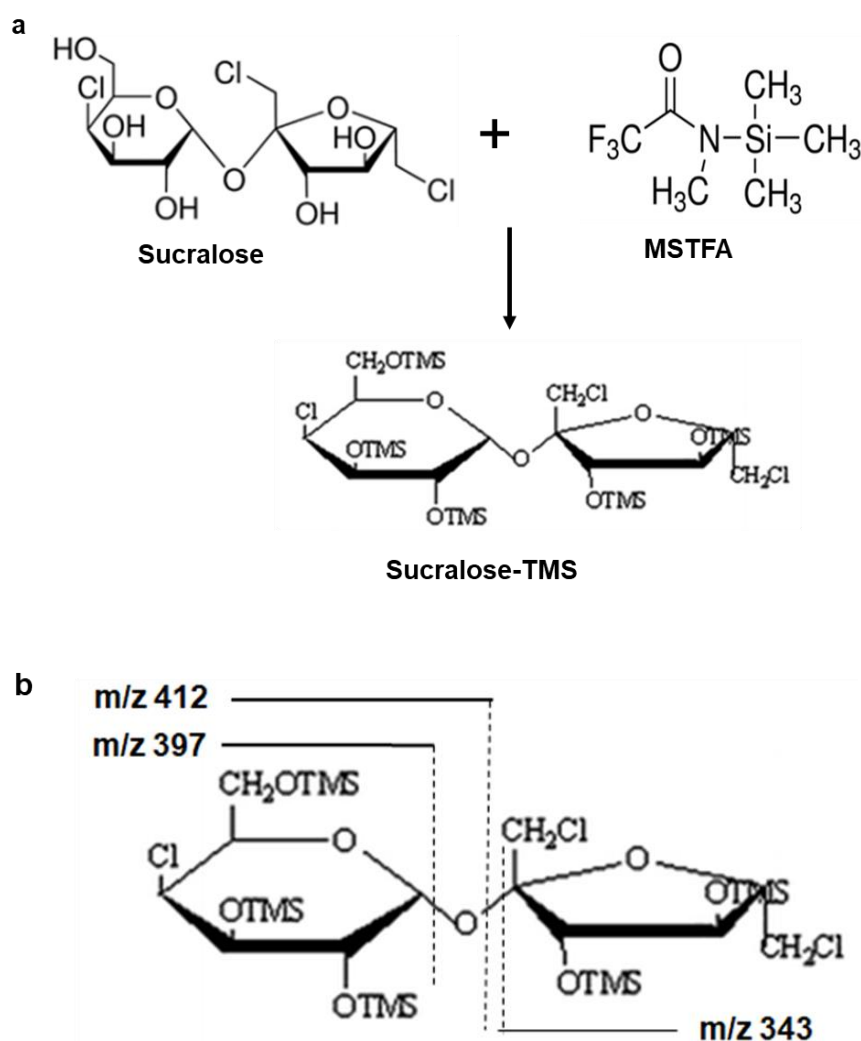
Figure 5. 3: **Proposed structures of derivatised sucralose fragmentation**

The Figure represents proposed structures (a) of sucralose following MSTFA derivatisation and fragmentation paths (b) of the principal fragment ions in the EI mass spectrum of sucralose–TMS ether. The molecular ion of sucralose following derivatisation with MSTFA was m/z 757 which could be fragmented to smaller m/z s of m/z 412, 397 and 343 (Qiu, et al., 2007).

5.2.2 Derivatisation of sucralose by silylation reaction to sucralose-TMS

Silylation improves the detectability and efficiency of the derivatives because the resultant analyte becomes more volatile, stable with narrow and symmetrical peaks (Orata, 2005), as evident in Figure 5.4b. It is noteworthy that when sucralose was analysed without derivatisation, it was undetectable with the GC-MS (no data generated).

5.2.2.1 Comparison of sucralose derivatisation with BSTFA and MSTFA

Two different silylation reagents, BSTFA and MSTFA were utilised in derivatising sucralose. As depicted in Figure 5.4a, the use of BSTFA yielded an incomplete derivatisation generating several silylation adducts. Based on the result (Figure 5.4a), it can be said that although BSTFA is a highly reactive derivatisation reagent, it was unable to completely derivatise sucralose and therefore the generation of silylation by-products or adducts with no peak detected for sucralose-TMS. In contrast to the chromatogram of sucralose derivatised by BSTFA, the chromatogram presented in Figure 5.4b demonstrates the complete derivatisation of sucralose by MSTFA at a retention time of 23 minutes with a clean single peak, devoid of any silylation by-products. This result therefore demonstrates that the derivatisation of sucralose by silylation reaction, using MSTFA achieved complete and optimal quality of detectable peak therefore making it (MSTFA) the selected derivatisation reagent for further studies. This choice of derivatisation reagent was further supported by results in section 5.2.2.2.
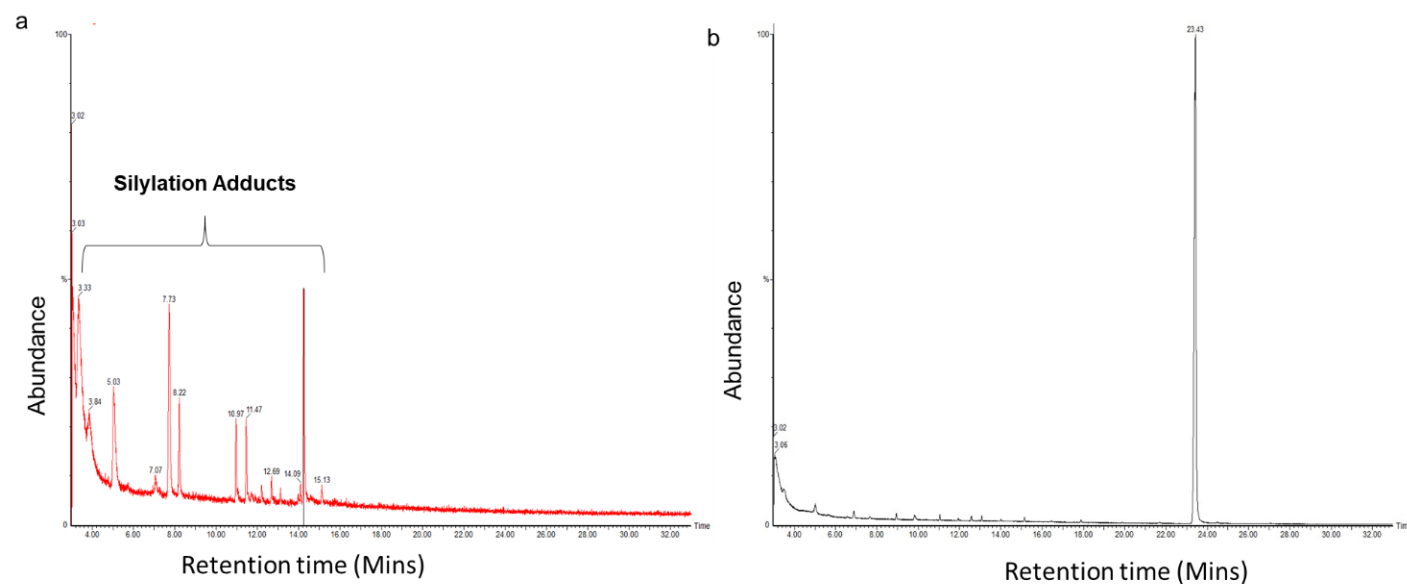
Figure 5. 4 **Selection of derivatisation reagent for sucralose**

Total ion chromatogram of sucralose derivatised with BSTFA (a) and MSTFA (b). Two different derivatisation reagents, BSTFA and MSTFA were used to derivatise 100 µg/ml of sucralose. BSTFA (panel a) was not able to derivatise sucralose as only silylation adducts present in the analyte mix were detected indicating likely an incomplete derivatisation while MSTFA (panel b) completely derivatised sucralose at 70^oC for 30 minutes. The retention time of sucralose was 23.43 minutes because the column used was ZB-5 (30 m length, 0.25 mm ID, 0.25 µm thickness). Subsequent studies used a Rtx ®-5 (30 m length, 0.25 mm ID, 0.25 µm thickness) column which elutes sucralose at 22.2 minutes.

5.2.2.2 Time dependent derivatisation of sucralose at room temperature and at 70°C

To select the optimum temperature and time for the derivatisation of sucralose with MSTFA, the temperature and time of derivatisation reaction were varied. Sucralose was derivatised at room temperature and under heat at 70 °C for time period of 30, 45 and 60 minutes. The result in Figure 5.5a showed that at room temperature, the peak area seems to increase as derivatisation time increases. Also, as evident from Figure 5.5a, at 45 minutes, there was an increase in peak area compared to the 30 minutes derivatisation time while at 60 minutes, the peak area value has tripled in comparison to the 30 minutes derivatisation time. Thus, if sucralose was to be derivatised at room temperature, it would require longer time, maybe 90 or 120 minutes to be comparable with the peak area value of those derivatised at 70 °C for 30 minutes (Figure 5.5b). On the other hand, derivatising sucralose at 70 °C for 45 and 60 minutes showed a decline in response as peak area values decreased with an increase in derivatisation time, therefore indicating possible degradation. Additionally, these parameters are essential because if sucralose was derivatised for longer period, as it is the case for derivatising at room temperature, the compound could degrade and if not given enough time, the derivatisation might be incomplete. Therefore, based on these results, the optimal parameter for derivatisation of sucralose was 70 °C for 30 minutes since it has better response and higher peak area value.
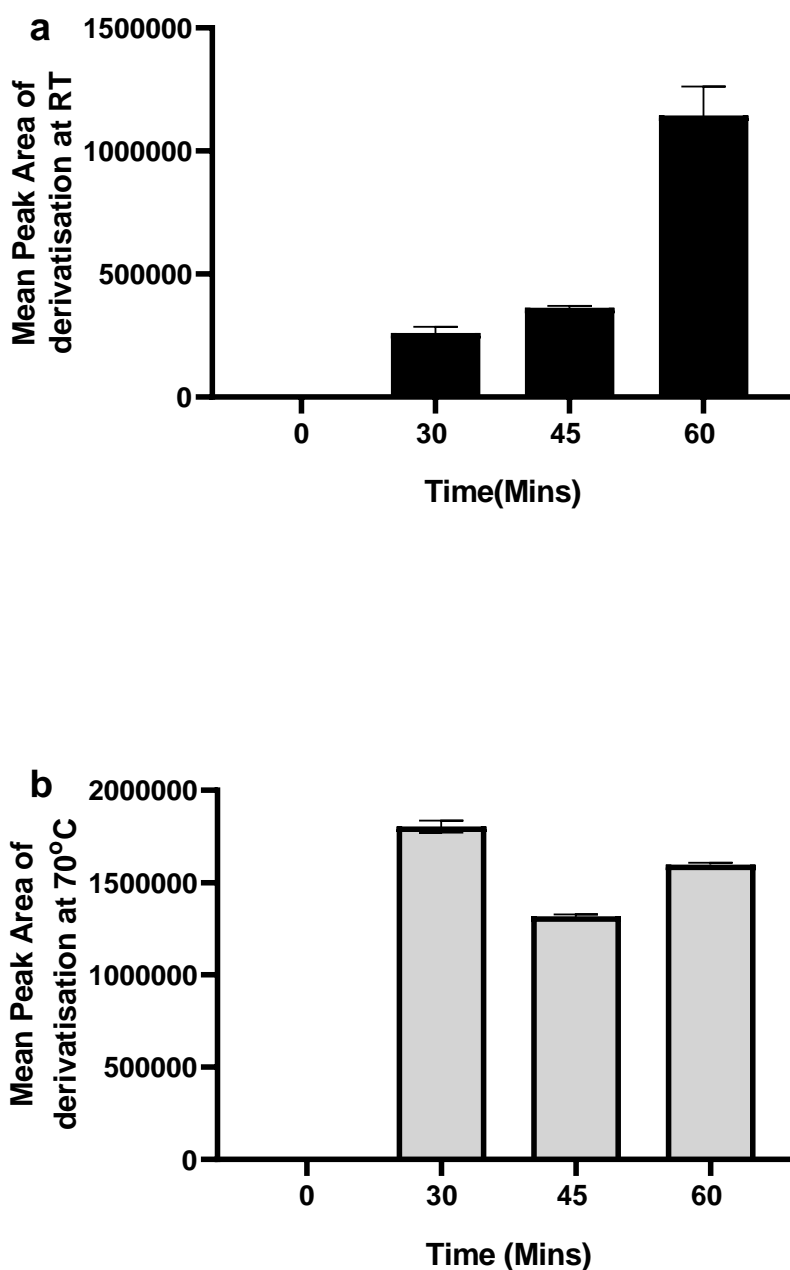
Figure 5. 5:**Optimisation of sucralose derivatisation with MSTFA**

Optimisation of sucralose derivatisation with MSTFA was done by derivatising 100 µg/ml of sucralose at room temperature (without heating) for 30, 45 and 60 minutes (panel a) and at 70 °C for 30, 45 and 60 minutes (panel b). Derivatisation at 70 °C for 30 minutes was selected as the most suitable for the method. n=3 (3 independent experimental repeats) with each time point studied in triplicates and data are presented as mean with error bars representing standard deviation. Since only sucralose was used for this experiment, mean peak area was utilised in Y-axis instead of mean peak area ratio.

Following the selected time and temperature for sucralose derivatisation with MSTFA, different concentrations of sucralose were derivatised using the selected derivatisation parameters to further optimise method. This experiment was vital as it will be a valuable guide on the detectable concentration of sucralose by the GC-MS method. The results in Figure 5.6 depicts the observable mean peak area of different concentrations of sucralose. Amongst the concentrations of sucralose tested; 0.04, 0.4, 5, 10, 25 and 100 µg/ml, there was no observable peak area value for 0.4 and 0.04 µg/ml. The lack of peak detection for 0.04 and 0.4 µg/ml concentrations of sucralose could be due to the split ratio (9:1) as only 1 in 9 parts of sucralose-TMS from the injected volume (1 µl) reached the GC for separation. Thus, 5 µg/ml of sucralose was the lowest observable concentration of sucralose detected amongst the analysed sucralose concentrations. Whilst 5 µg/ml was the lowest, observable concentration of sucralose detected at this time, it should be noted that this is not the limit of detection. At this stage, no internal standard was used, and the reason Y-axis data was not presented as peak area ratio.
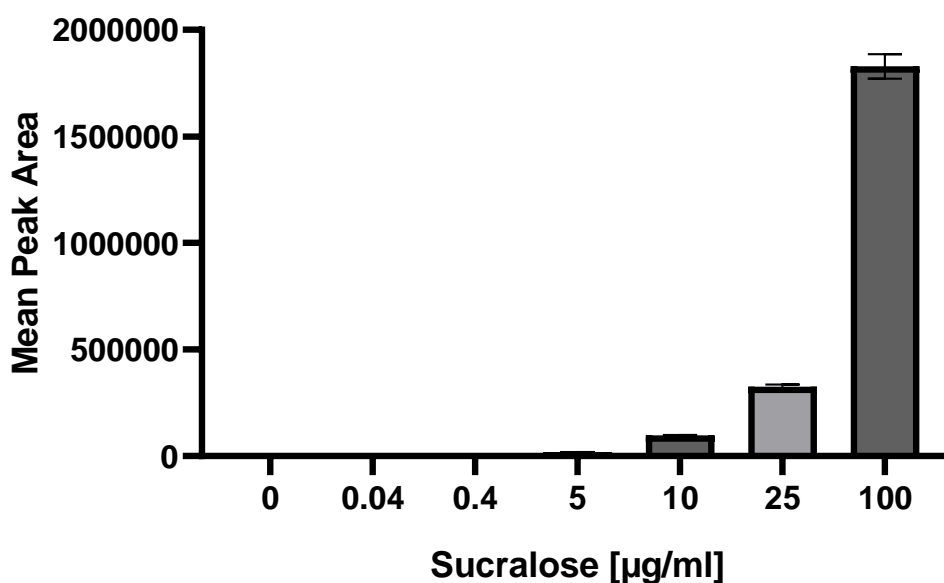
Figure 5. 6: **The representation of sucralose-TMS only at differing concentrations**
The mean peak area of sucralose-TMS plotted against 0 to 100 µg/ml sucralose concentrations. The above-mentioned concentrations of sucralose were made up from 1 mg/ml stock solution of sucralose, dried and derivatised with 100 µl MSTFA at 70ºC for 30 minutes and ran on the GC-MS using set up parameters as outlined in Figure 5.2. n=3 with error bars representing standard deviation.

Taken together, these results have demonstrated that the artificial sweetener, sucralose was completely derivatised with MSTFA at 70ºC for 30 minutes. Additionally, it was also evident that low concentrations of sucralose up to 5 µg/ml was detected at the experimental condition. This low concentration of sucralose can be converted to its mass on column to generate the lowest possible detectable sucralose concentration by applying the split ratio (9:1) and injection volume of the analyte (1 µl) as detailed in section 5.2.5.2.

5.2.3 Selection of internal standard

Internal standard compensates for any irregularities resulting from sample preparation. Thus, rather than calculate the analyte response alone, the ratio of the analyte to the internal standard is considered (Bressolle, Bromet-petit and Audran, 1996). Two internal standards tested for their suitability for use in this research were myo-inositol and deuterium-labelled sucralose (sucralose-d6).

5.2.3.1 Identification of internal standard (sucralose-d6)

The chromatogram of sucralose-d6 presented in Figure 5.7 shows a retention time of 22.1 minutes which is a second difference with the analyte of interest, sucralose-TMS. The close retention time of the internal standard with the main analyte, sucralose-TMS (22.2 minutes) was as expected since the internal standard is deuterated sucralose. Further identification of the internal standard was carried out using m/z s as Figure 5.8 illustrates. The internal standard extracted m/z s of interest were m/z 211, 312 and 347. Following identification of the internal standard, the next question addressed was the concentration to use since it has to be constant throughout the study. The resulting response of the internal standard should neither be too high nor too low as either way would affect the peak area ratio (PAR) and result quality. Therefore, preliminary tests were performed on the internal standards to determine appropriate concentration for use with the analyte of interest (sucralose-TMS) and this was 50 µg/ml for sucralose-d6 (see appendix).
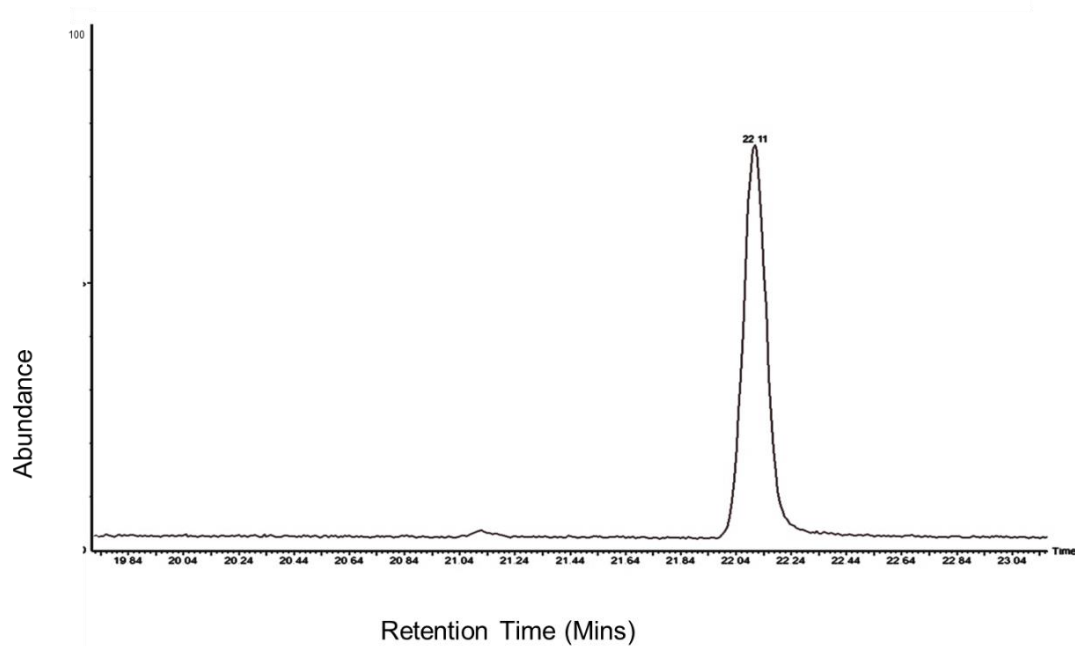
Figure 5. 7: **GC-MS chromatogram of internal standard (sucralose-d6)**

The total ion chromatogram of internal standard was recorded with Perkin Elmer Clarus 500

GC-MS (column Rtx ®-5 (30 m x 0.25µm x 0.25 mm i.d.); 270 °C injection port temperature,

column temperature: 180°C for 2 minutes, 6°C/minutes until 250°C, hold for 20 minutes with

3.5 minutes solvent delay time. The retention time of internal standard was 22.1 minutes.
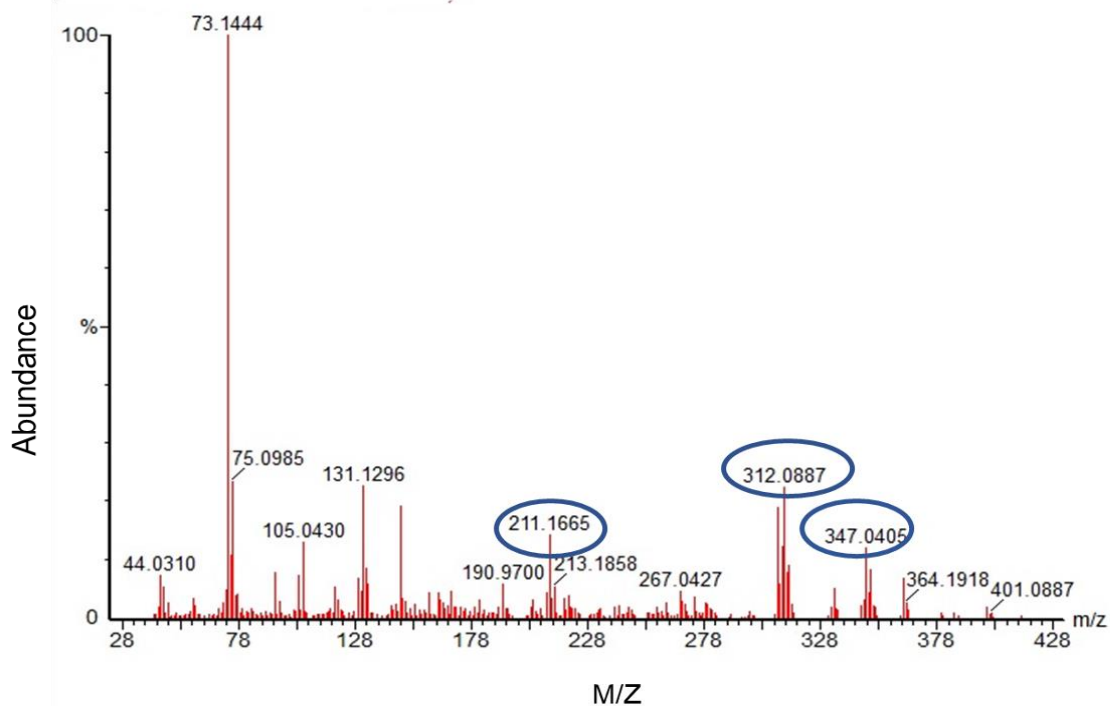
Figure 5. 8*: **The mass spectrum of internal standard (sucralose-d6)**

The mass spectra of internal standard recorded with the Turbo-Mass™ 5.4 GC-MS software

with an electron ionization impact of 70 eV operated in full scan mode from 40-600 m/z. The

m/z s (circled in blue) m/z 211, 312 and 347 were used to identify the internal standard at a

retention time of 22.1 minutes.

5.2.3.2 Stability of internal standards

Whilst it is important to use an internal standard to minimise technical and human error, its stability over a period of analysis in the instrument is likewise vital. As the method was adapted from Qiu, et al. (2007), initially, myo-inositol was used as an internal standard. The stability test of myo-inositol was carried out using 25 µg/ml of myo-inositol for 24 hours as described in section 2.3.2.3.2 while that of sucralose-d6 was performed by analysing 50 µg/ml concentration of sucralose-d6 for 24 hours (section 2.3.2.3.3). The generated peak area of each internal standard was plotted against selected concentration to determine its stability.

The results presented in Figures 5.9 and 5.10 and table 5.1 highlights the stability of the internal standards using statistically generated *p-value*. Statistically, the *p-value* denotes the degree of difference between the peak area of selected concentrations against zero or control. The result in Figure 5.9 showed that myo-inositol was unstable because the generated peak area values following different injections over a period of 24 hours were statistically different from control with a *p-value* of <0.0001. In contrast to myo-inositol, the result in Figure 5.10 showed the stability of sucralose-d6 as an internal standard. This result therefore indicates that sucralose-d6 was a better choice of internal standard.
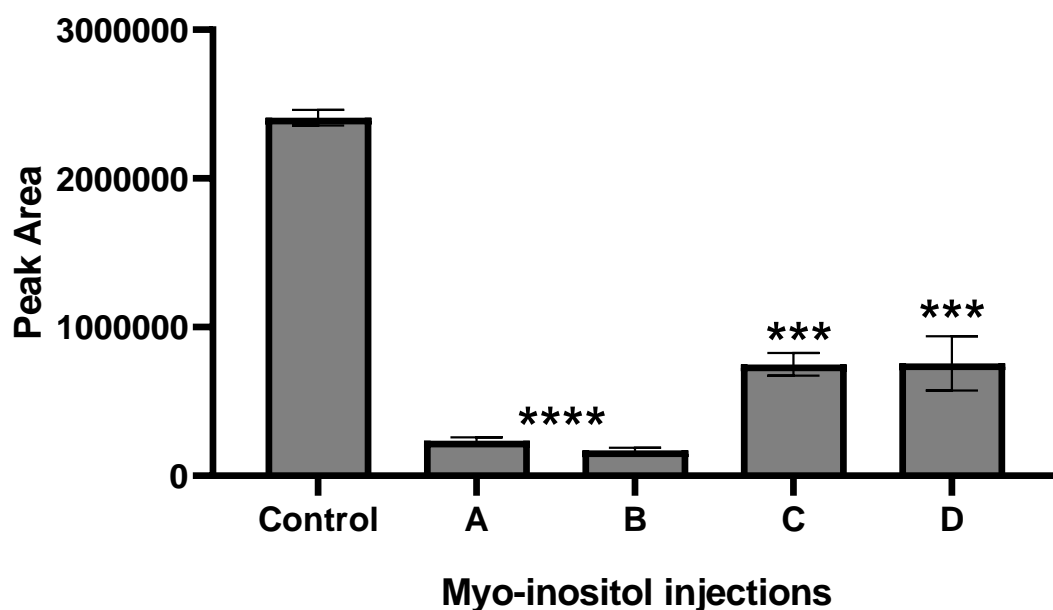
Figure 5. 9**: Myo-inositol is unstable in short term stability test**

The stability test for myo-inositol as an internal standard was done by repeated measure of same concentration of myo-inositol (25 µg/ml) from 4 separate vials over a period of 24 hours (A = 6, B= 12, C=18, D= 24 hours). n=3 with error bars representing standard deviation. The control data was collected based on myo-inositol selection of appropriate concentration. **** p-value < 0.0001 means statistically significant in comparison with control.
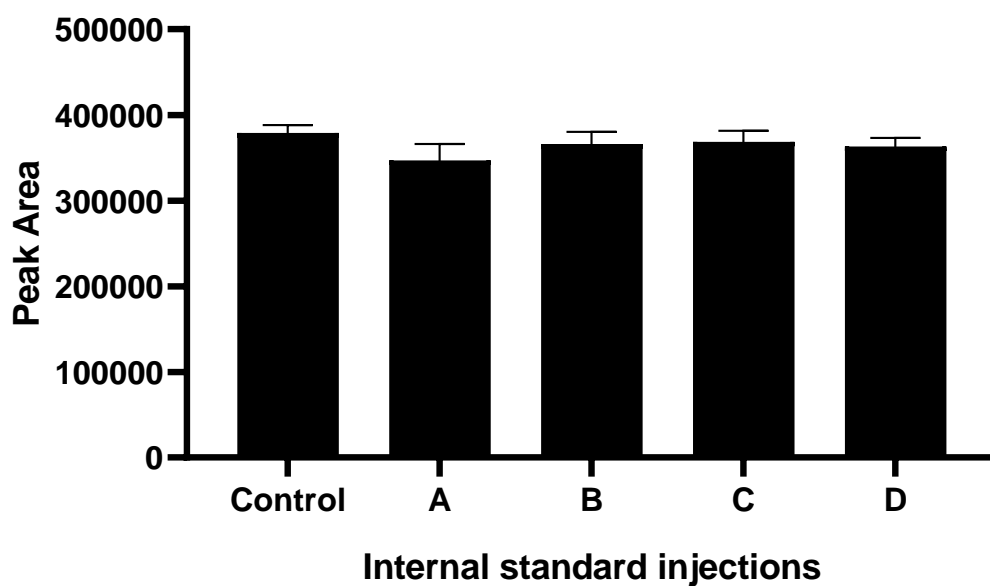
Figure 5. 10: **Stability of internal standard; deuterium-labelled sucralose(sucralose-d6)**

The stability test for sucralose-d6 as an internal standard was done by repeated measure of same concentration of sucralose-d6 (50 µg/ml) from 4 separate vials (A, B, C, D). n=3 with error bars representing standard deviation. The control data was collected based on sucralose-d6 selection of appropriate concentration (50 µg/ml). There was no statistically significant difference between the injections and control.

Table 5. 1: **Internal standards stability test**

Analyte	Concentration (μg/ml)	*P-value*	Analyte Stable?
Myo-inositol	25	0.0001	NO
Sucralose-d6	50	0.8893	YES

Note: To be stable, p-value need to be > 0.05.

Additionally, as part of the method optimisation with the internal standards, an instrument autosampler stability for the internal standard (sucralose-d6) was carried out over a period of 72 hours as shown in Figure 5.11. The analysed result which was collected within 6-hourly injections showed no statistical significance between the injections over the studied time period. The determination of instrument response using sucralose-d6 is suitable as this internal standard (sucralose-d6) would behave in similar manner as the analyte of interest (sucralose-TMS). Therefore, based on these findings, sucralose-d6 was selected as the internal standard utilised throughout this research and subsequent mention of internal standard will refer to sucralose-d6.
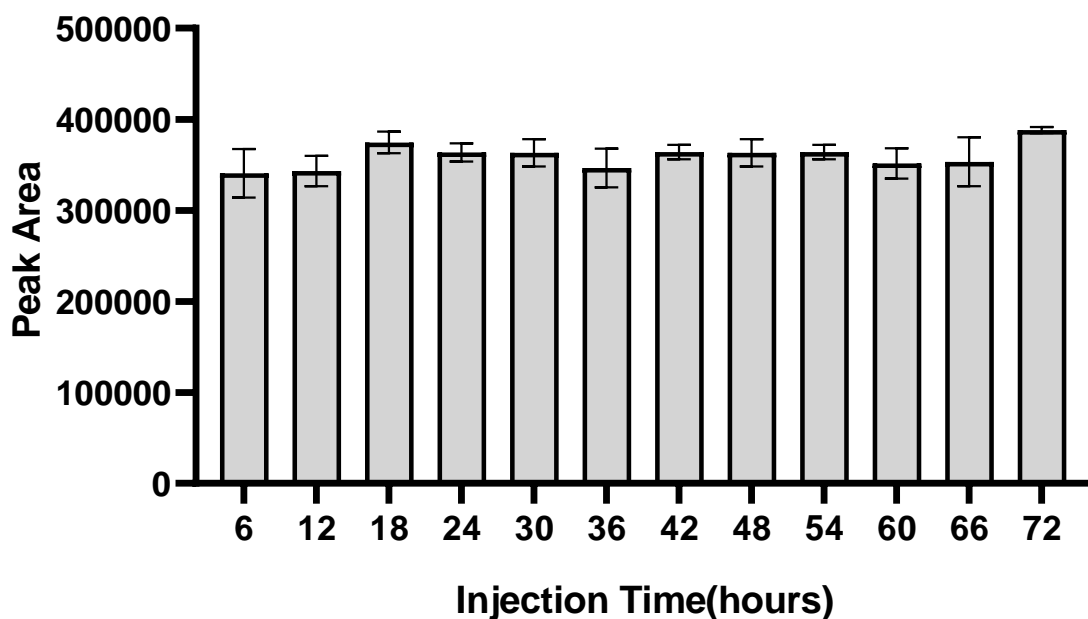
Figure 5. 11**: Instrument auto-sampler stability using the internal standard**

The bar graph of peak area of internal standard against its injection time at (50 µg/ml) to determine the auto sampler storage stability of the instrument (GC-MS) analysis. There was no statistically significant difference between the injections using two-way ANOVA. The experiment was conducted in triplicates for each injection time making a technical repeat of 36 with error bars representing standard deviation.

5.2.4 Identification of sucralose and internal standard (sucralose-d6) mix

Sucralose mixed with internal standard were analysed with the set method as described in section 2.3.2.4. Internal standard eluted first at 22.1 minutes followed by sucralose-TMS at 22.2 minutes as the chromatogram in Figure 5.12 shows. Examining the result, it was observed that the analyte (sucralose-TMS) and internal standard almost co- eluted with a time difference of 1 second. This occurrence is expected and normal behavioural pattern for deuterated internal standards. In such an occurrence, extracted ion chromatogram (in this case m/z ion of both sucralose and internal standard) were utilised for quantification. Therefore, the mass spectra of both compounds were analysed separately with Figures 5.13a and 5.13b showing the mass spectra of internal standard and sucralose-TMS. Hence, the extracted ion (the main ions of interest); 207, 308 and 343 (Figure 5.13b) of the analyte was utilised to differentiate it from the internal standard whose main ions were m/z 211, 312 and 347 as highlighted in table 5.2. Whilst the base ion (that is the tallest m/z ion in the mass spectrum) used for quantification of the analyte standard was m/z 308, that of internal standard was m/z 312. Therefore, sucralose-d6 was a good choice as an internal standard for sucralose detection as it does not interfere with detection of sucralose-TMS.
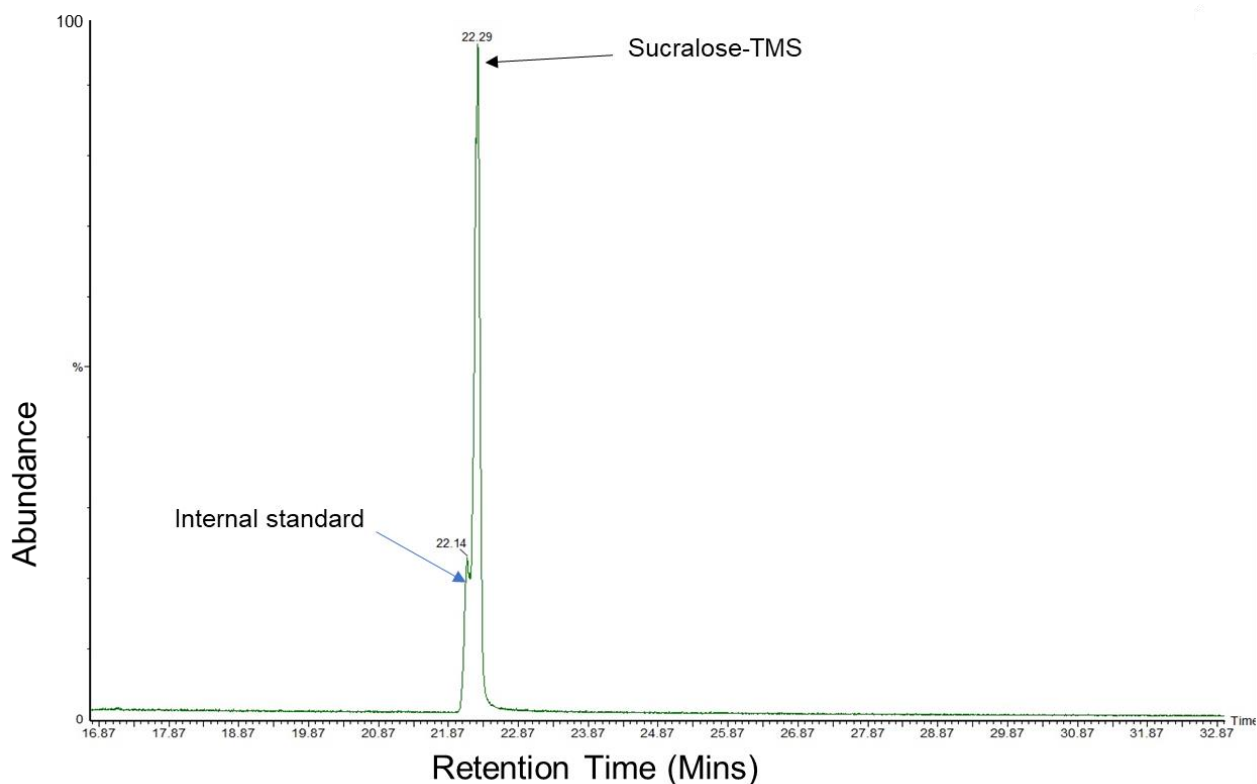
Figure 5.12: **Chromatogram of sucralose-TMS and its internal standard**

The chromatogram was recorded with Perkin Elmer Clarus 500 GC-MS. GC–MS conditions: column Rtx ®-5 (30 m x 0.25µm x 0.25 mm i.d.); 270 °C injection port temperature; column temperature: 180°C for 2 minutes, 6°C/ minutes until 250°C, hold for 20 minutes with 3.5 minutes solvent delay. Arrows (black) shows peak detection of sucralose-TMS which elutes at retention time of 22.2 minutes while internal standard (blue arrow) was eluted at retention time of 22.1 minutes.
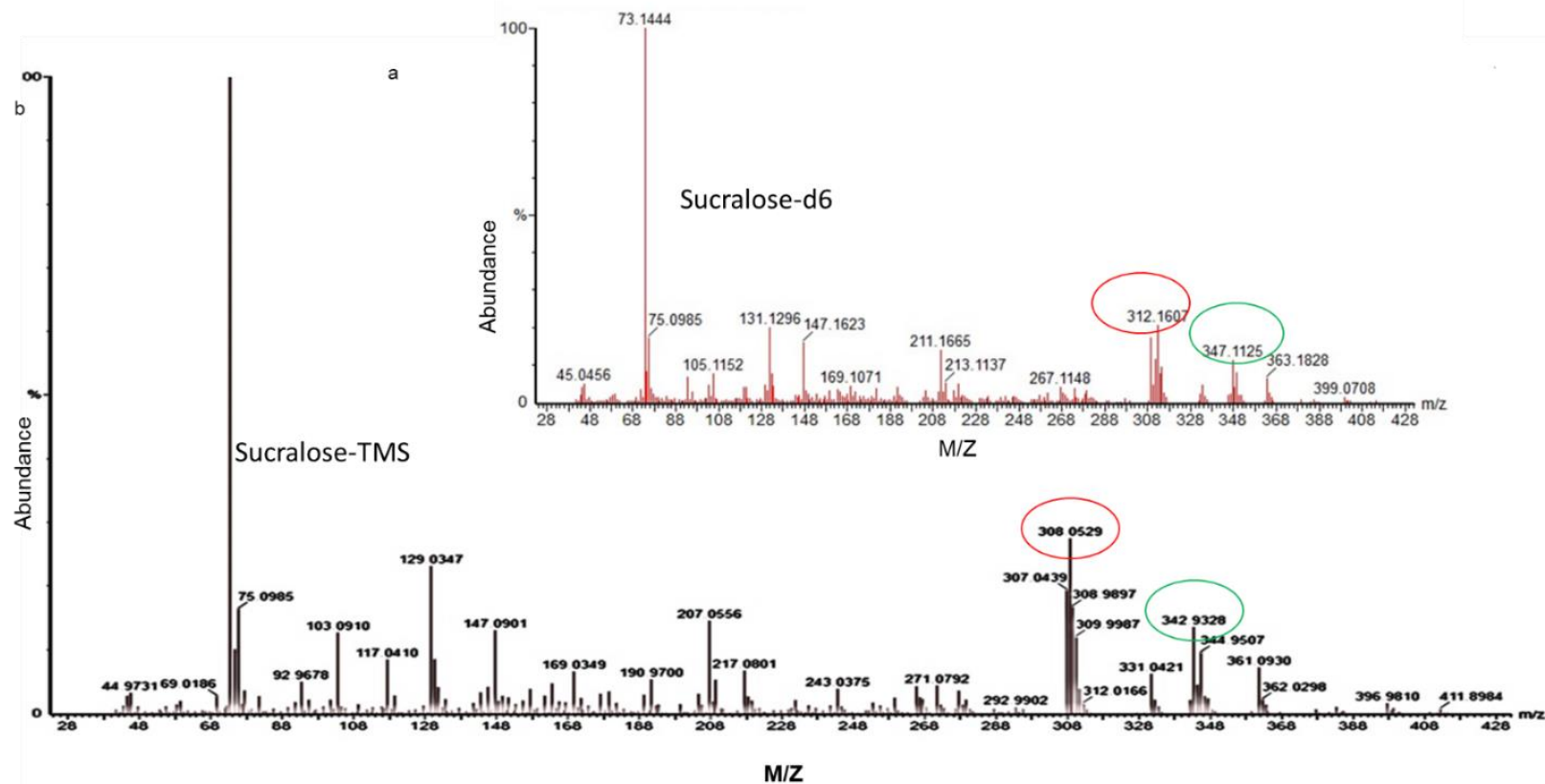
Figure 5. 13: **The electron ionisation mass spectrum for the TMS ether of internal standard, sucralose d-6 and sucralose**

The mass spectrum for the internal standard, sucralose d-6 (panel a) and sucralose-TMS (panel b). The base ions of both mass spectra are m/z 312 (circle red) for internal standard and m/z 308 for sucralose-TMS. The qualifier ions (that is selected ions that are specific to analyte, generally taller and heavier) are m/z 347 (circled green) for internal standard and m/z 343 for sucralose-TMS following GC-MS analysis.

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Table 5. 2: **Identification parameters of sucralose-TMS with internal standard**

Analyte	Retention Time (RT) in minutes	Relative Retention Factor (RRF)	Major ions (m/z)
Sucralose-TMS	22.2	1.005	207, <mark>308</mark> and 343
Internal standard	22.1	1.005	211, <mark>312</mark> and 347

Note: RRF =RT of sucralose/RT of internal standard. M/z s highlighted in blue are base ions used for quantification.

5.2.5 Instrument linearity and other method development criteria

So far, it has been demonstrated that the artificial sweetener, sucralose was detected using the optimised method and the lack of multiple peaks indicates the appropriateness of the selected derivatisation method. It is therefore vital to consider results from other key evaluation criteria for analytical method validation such as linearity and linear range; limit of detection; limit of quantification; selectivity; specificity and precision (Belouafa, et al., 2016; Mannocchi, et al., 2015). The linearity relationship of the analyte was determined using the regression plot presented in Figure 5.14. Concentrations of standards for linear regression, within the range of 2.5 - 400 µg/ml was chosen to closely match the concentration range of previous studies in chapter 3 and 4 of this research. The controls for calibration curve include a blank sample (methanol without internal standard), a zero sample (methanol with internal standard).

The calibration graph was plotted as mean peak area ratio against standard concentrations (2.5 - 400 µg/ml) with peak area ratio calculated by dividing the peak area of analyte by the peak area of the internal standard. The internal standard concentration, 50 µg/ml was constant for the entire study. Linearity was then assessed using statistical analysis (ANOVA) which yields a regression correlation coefficient, (R^2) of 0.9844 using the formula, $Y = 0.02151X + 0.1030$ as Figure 5.14 depicts. Although, the regression correlation coefficient, (R^2) coupled with a visual evaluation of the standard calibration plot (Figure 5.14) indicates that the plot was linear. However, it was evident that not all standard points were on the line, making it vital to perform further test on the standard concentrations to determine the final instrumental linearity range.
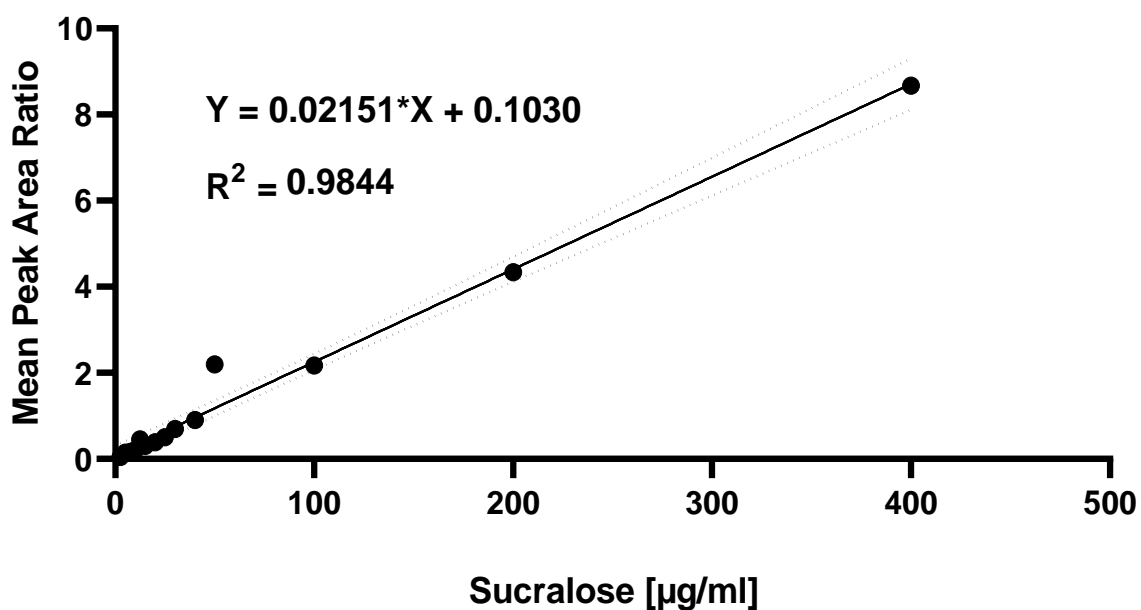
Figure 5. 14: **The initial standard curve of sucralose-TMS**

Calibration graph of peak area ratio against standard concentrations (2.5 - 400 µg/ml) of analyte. The standards were prepared as outlined in section 2.3.2.5, ran over a period of 4 days using GC-MS method settings in Table 2.6 and the correlation coefficient, was calculated to be $R^2 = 0.9844$. A triplicate injection was performed for all studied concentrations with error bars representing standard deviation.

5.2.5.1 Test of instrumental linearity

The linear range shown in Figure 5.14 was further tested using log-response plot (ANOVA) to determine the final instrumental linearity and linear range. Runs rest was also performed on the final linear concentrations using linear regression analysis (Belouafa, et al., 2016). The Runs test indicates no statistically significant deviation from linearity with a *p-value* of 0.9286. A final linear regression graph was plotted following the removal of standard points that were not within range as shown in Figure 5.15. Comparing the initial linear plot with final plot, it was evident that correlation coefficient, R^2 improved. The R^2 of 0.9844 (Figure 5.14) which was below ICH (1995) acceptance value, was improved to R^2 of 1.000 (Figure 5.15) thereby validating the linearity of GC-MS method used in this research. The reproducibility of this method was also evident in the very low percentage of standard deviation as presented in Table 5.3.

To further optimise the method, relative standard deviation of all final standard concentrations shown in Table 5.3 were used to assess the precision of the instrument. Relative standard deviation (RSD) were below 10%, indicating that using this current method, an RSD of ≤20% mark criteria set for low concentration ranges and ≤15% criteria for high concentrations were achieved (Aparicio-Ruiz, et al., 2018; Sanchez, 2018; ICH 1995). These results therefore demonstrated the linearity, range, repeatability and precision of the research method.
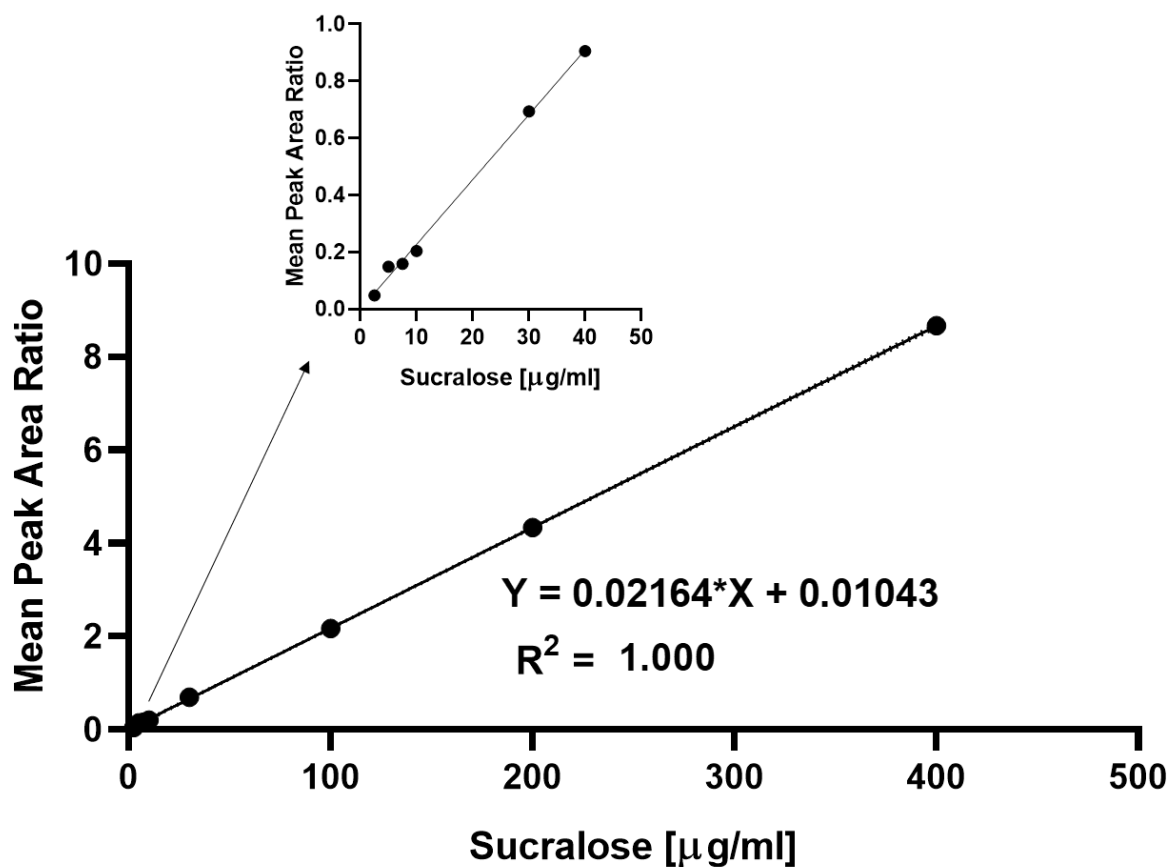
Figure 5. 15: **The final standard linear curve of sucralose-TMS**

The final linear range was achieved by plotting a calibration graph of mean peak area ratio (MPAR) against standard concentrations of analyte. The correlation coefficient was calculated to be $R^2 = 1.000$. A triplicate injection was performed for all studied concentrations with error bars representing standard deviation and the lower concentrations were further plotted (top centre) to also confirm linear range.

5.2.5.2 Calculating limit of detection and quantification

Detection and quantification limits were determined using the standard deviation of the response and the slope evaluation from the 3 separate calibration curves with the formula previously outlined in section 2.3.2.6. The slope and standard deviation were evaluated using the resulting formulas from three separate standard curves (A, B and C) as shown in Figure 5.16 and calculations are shown in Table 5.3.
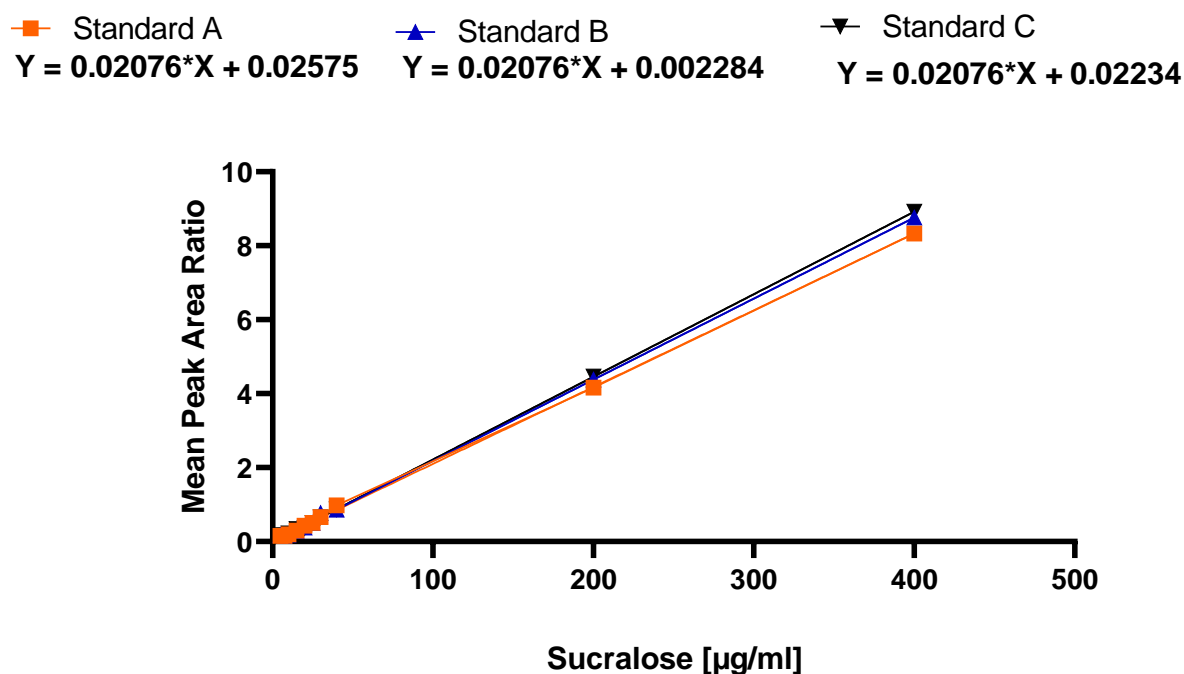
Figure 5. 16: **Regression plot for limit of detection (LOD) and quantification (LOQ)**
Three different regression plots (A, B, C) of sucralose were used to calculate the limit of detection and limit of quantification respectively.

Table 5. 3: **Detection and Quantification limit calculation**

PAR	Gradient/Slope	Intercept/Blank
Standard A	0.021	0.025
Standard B	0.022	0.002
Standard C	0.022	0.022
Average	0.022	0.016
Standard Deviation	0.001	0.013

The limit of detection (LOD) was calculated using the equation; 3.3*STD.DEV of blank/slope

=3.3 * 0.013/0.022 = 1.95 μg/ml. However, the analytical parameters used for the detection such as the injection volume and split ratio were considered in calculating the actual limit of detection of sucralose.

Therefore, if 1 ml contains 1.95 μg of sucralose, then the amount of sucralose in 1 μl (the injected volume) will equal   1.95 /1000 = 0.00195 μg. However, this was not a split-less injection as the method split ratio outlined in Table 2.6 was 9:1. Considering the split ratio used, the limit of detection would be 0.00195/9 =0.00022 μg. Therefore, the actual mass of sucralose detected on column was 0.0002 μg or 0.2 ng.

Quantification Limit (LOQ) was calculated using the equation; 10*STD.DEV of blank/slope =10*0.013/0.022 = 5.91 µg/ml. Following same principle used to calculate the actual LOD, the true quantification limit of sucralose on column was 0.00065 µg or 0.6 ng.

Therefore, these results demonstrated that using this method, the minimum detectable amount of sucralose, 0.2 ng shows the sensitivity of the GC-MS method.

5.2.5.3. Determination of method precision and accuracy

The developed method was further tested for precision and accuracy using the regression plot equation; **Y = 0.02164 *X + 0.01043** (from final linearity plot in Figure 5.15). Where Y represents the mean PAR. Three standard concentrations; low (5 µg/ml), medium (40 µg/ml) and high (400µg/ml) assessed for precision using acceptable recommended guideline for RSD, which is less than 20% for the lowest standard in determining accuracy and precision (Boulanger, et al., 2003). As the result on Table 5.4 shows, RSD of low, medium and high standard concentrations of sucralose were below 10% thereby, confirming the accuracy and precision of the method. To further validate the precision of the method, an intra-day and inter-day instrument precision was performed. The results in Figure 5.17 a and 5.17b indicates minimal RSD. Whilst the intraday (Figure 5.17a) was assessed within a 24-hour period, the inter-day (Figure 5.17b) precision was investigated over a period of 3 days with continuous run. The calculated RSD of both analyses indicate minimal standard deviations with the inter-day precision having lower RSD of 1.2% in comparison to the 4.6% RSD of inter-day runs. This result therefore shows the repeatability and accuracy of the GC-MS method optimised for sucralose detection. The method validation parameters are summarised in Table 5.5.

Table 5. 4: **Method accuracy and precision calculation using regression equation of sucralose**

Concentration (µg/ml)	Mean PAR	Std dev	RSD (%)
5 (Low)	0.158	0.009	5.357
40 (Medium)	0.981	0.066	7.293
400 (High)	8.678	0.305	3.518

Mean	Std	RSD
355452.4	16308.2	4.6

a

Mean	STD	RSD
359576.1	4374.688	1.2

b
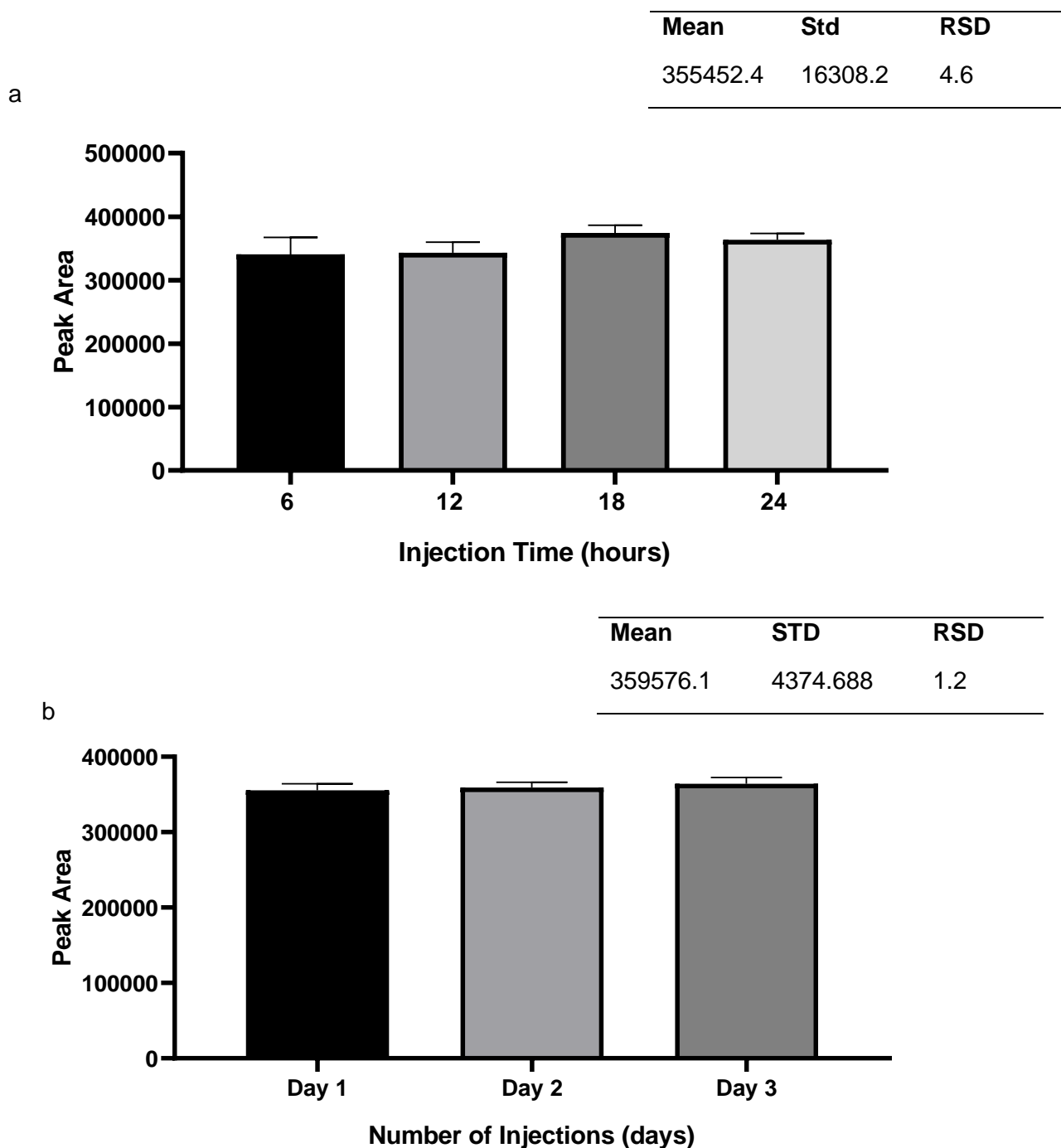
Figure 5. 17: **Inter and Intra-day precision of sucralose-d6**

The instrument precision was determined by running both intra and inter-day samples of sucralose-d6. The intra-day (panel a) covers data generated within a short time of 24 hours while inter-day (panel b) covers an analysis time of 3 days. The results were analysed using two-way ANOVA and error bars presented as standard deviation with 36 technical repeats. The calculated RSD is shown on the top right-hand corner depicting minimal deviations between repeat runs. The GC-MS method used were as stated in Figure 5.12.

Table 5. 5:**Summary of method validation parameters for this research**

Compound	Retention time(mins)	Quantifier ion (m/z)	Linearity (µg/ml)	LOD (µg)	LOQ (µg)	Intra-day %RSD	Inter-day %RSD
Sucralose	22.2	308	5 - 400	0.0002	0.0006	4	1.2

Additionally, selectivity and sensitivity of the method was assessed using blanks (methanol) and sample matrix as controls. The blanks showed absence of peaks at the same retention time as sucralose with no m/z s detected. A run of the matrix for the cell studies (media) also displayed no peaks nor m/z s at the retention time of sucralose. This indicates no interference from the matrix thereby making the developed method specific and sensitive.

Taken together, it has been demonstrated that the method optimised and validated for the detection and identification of sucralose, achieved most of the required and acceptable validation criteria, thus, its application to cell studies.

5.2.6 Application of the optimised GC-MS method for detection of sucralose in glomerular microvascular endothelial cells

To investigate if sucralose acts independent of the sweet taste receptors, the optimised and validated GC-MS method was utilised to determine if sucralose crosses the glomerular endothelial cell. The glomerular endothelial cell sample preparations were carried out as detailed in the methods chapter (section 2.3.2.9). To further assess the effect of the sample matrix, in the absence of GMVEC, the cell growth matrix (cell media) was spiked with same concentrations of sucralose (section 2.3.2.7). To ensure no interference from sample matrix, mean peak area ratio of media only was subtracted from the mean peak area of GMVEC treated with sucralose.

5.2.6.1 Detection of sucralose in culture media of glomerular cells

The result shown in Figure 5.18, indicated that sucralose used to treat GMVEC remained in the media. Upon statistical analysis of the resulting mean peak area ratio, p-value was < 0.0001 when compared to 0 µM of sucralose (Figure 5.18). This result therefore suggests that the artificial sweetener, sucralose does not cross the glomerular microvascular endothelial cell membrane following a 24-hour co-incubation of cells with sucralose.
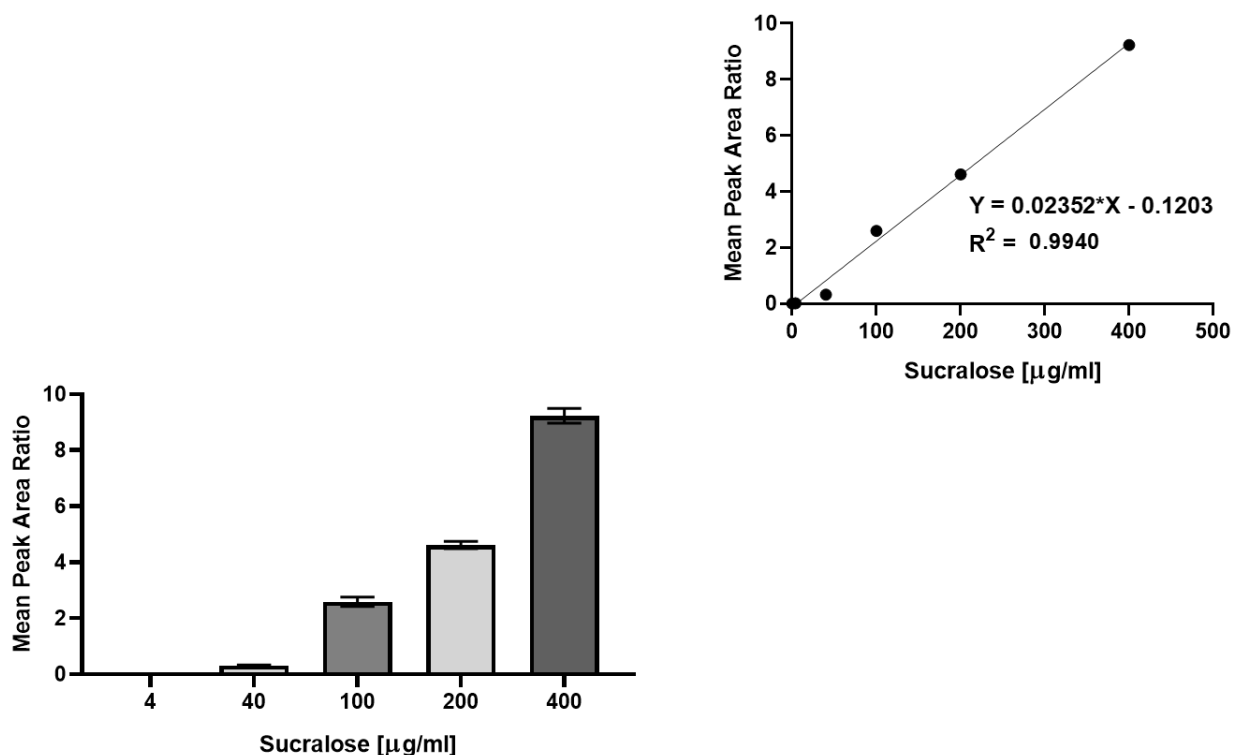
Figure 5. 18: **Detection of sucralose in cell cultured media**

Glomerular microvascular endothelial cells (GMVEC) were plated and incubated at 37 ^{o}C for 24 hours, followed by exposure to (500 µl) sterile-filtered sucralose concentrations ranging from 4, 40, 100, 200 to 400 µg/ml and incubated for additional 24 hours. The used media was aliquoted into glass vials, mixed with internal standard, evaporated to dryness, derivatised with MSTFA at 70 ^{o}C for 30 minutes and analysed. The instrument (GC) conditions were as outlined on Table 2.6. The mean peak area ratio of the mixed analyte was plotted against sucralose concentrations. n=3 (for 3 independent repeats with each sample concentration analysed in triplicates). The results were presented in different formats (line and bar graphs) to show the very low standard deviations since error bars were not visible on the linear graph.

5.2.6.2 Sucralose does not cross the cell membrane of glomerular endothelium

To further validate the presence of sucralose in the cell media and not in the glomerular cells, the cell lysates was prepared for GC-MS analysis as detailed in section 2.3.2.9. The resulting data presented in Figure 5.19, showed that sucralose was not detected in the GMVEC lysate. Thereby indicating that sucralose does not cross the glomerular endothelial cell membrane but effects its protection against VEGF-A induced permeability by acting on the cell surface, possibly through binding to the sweet taste receptor, T1R3 as discussed in chapter 4 of this research.
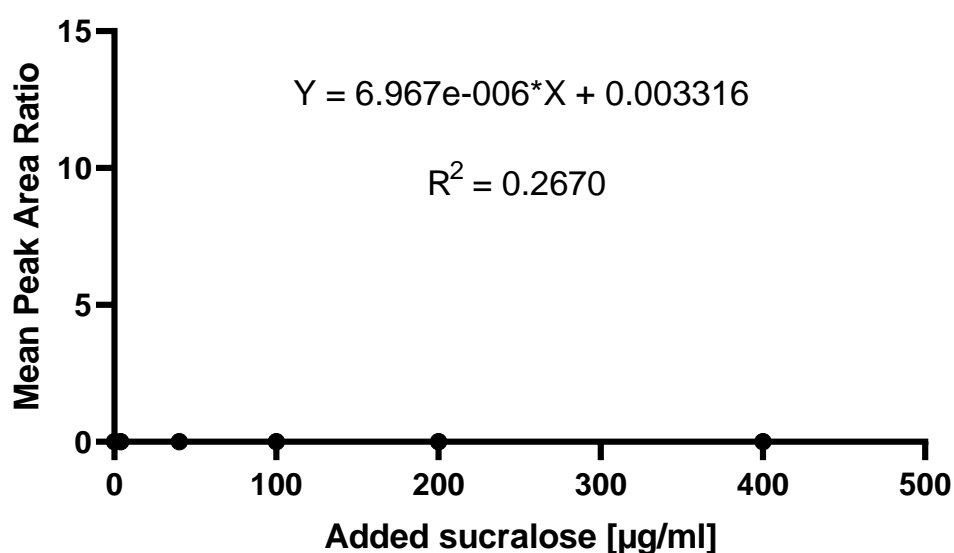
Figure 5. 19**: Sucralose does not cross the glomerular endothelial cell membrane**
Glomerular microvascular endothelial cells (GMVEC) were plated and incubated at 37 ^oC for 24 hours, followed by exposure to sterile-filtered sucralose (500 µl) at physiologically relevant concentrations ranging from 4, 40, 100, 200 to 400 µg/ml for additional 24 hours in the incubator. Following sucralose treatment, used media was aspirated off, cells were washed 6x with DPBS and extracted as described in the methods section (2.3.2.9). GC-MS analysis was as outlined on Table 2.6.

5.2.6.3 Matrix match effect of cell sample

In order to assess the effect of sample matrix on sucralose in the absence of GMVEC, cell media was spiked with same concentrations of sucralose used for treatment of GMVEC. The result presented in Figure 5.20 highlights the detection of sucralose in cell media in the absence of GMVEC. This finding shows that cell sample matrix, media has no effect on the GC-MS detection and quantification of sucralose from cell samples.
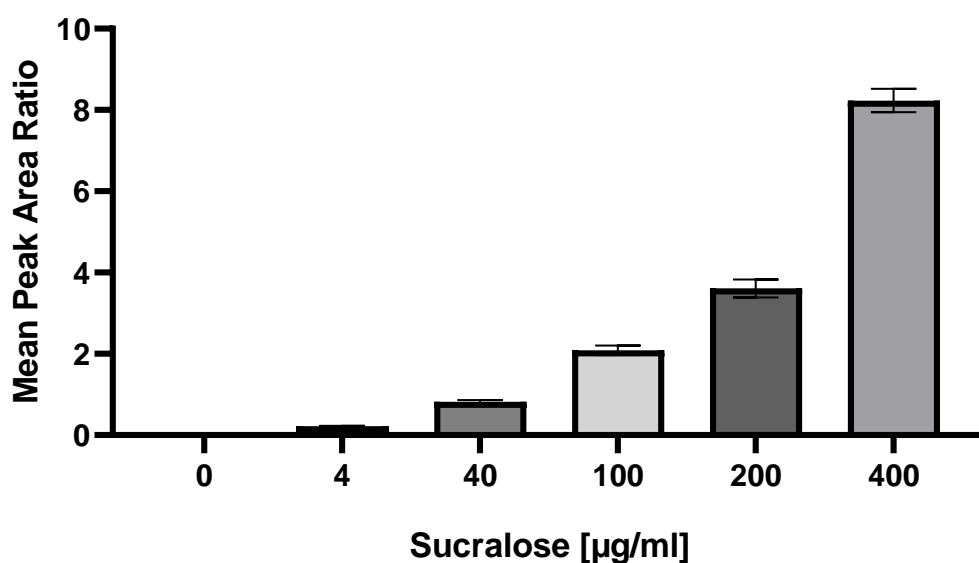
Figure 5. 20: **Peak area ratio of sucralose-TMS in media only after 24 hours incubation**

Sucralose was added to culture media without cells and incubated at 37 °C for 24 hours. Post incubation, 100 µl was aliquoted into glass vials, mixed with internal standard, evaporated, derivatised with MSTFA at 70°C for 30 minutes and prepared for GC-MS analysis as previously described. The mean peak area ratio of the mixed analyte was plotted against sucralose concentrations. Error bars represent standard deviation with an n-number of 3.

5.3 Discussion

The use of chemical analytical techniques in the detection of sugars might be for structural elucidation or understanding of metabolic processes (Becker, et al., 2013). In this research, the latter was the case as the study sought to determine whether artificial sweetener, sucralose crosses the glomerular microvascular endothelial cells to attenuate VEGF-A induced permeability. Whilst different analytical techniques have been employed in the detection of sucralose in various matrices such as foodstuffs (Chang, 2014; Hanko and Rohrer, 2004), water (Ferrer, et al.,2013), table top sweetener (Qiu, et al., 2007) and urine (Farhadi, et al., 2003), GC-MS has not been used for sucralose detection in cell lysate. The selection of gas chromatography (GC-MS) as a technique to address the question raised in this chapter was due to its specificity, sensitivity and practicality (Xia, et al., 2018). Sucralose was selected for this assay amongst the other artificial sweeteners (aspartame, saccharin and neotame) used in this research based on its stability under heat and ability to remain an intact molecule within cells (section 1.13). In contrast to sucralose, aspartame and neotame can be degraded to their primary metabolite. Hence, if the cells were treated with either aspartame or neotame, the extracted molecule from the cell sample might not reflect either of the sweeteners but rather their metabolites such as aspartic acid or methanol (Magnuson et al., 2016). In this study, a GC-MS based analytical method using silylation derivatization was optimized and validated. The method used a deuterium-labeled internal standard (sucralose-d6) which helps in minimizing method imprecision due to reaction and sample processing. The limit of detection was 0.0002 µg based on a set of linear range and competes with other methods in published literatures.

5.3.1 Detection and identification of sucralose

To identify sucralose, retention time, relative retention factor (RRF), peak area and m/z s were used. The retention time or the time it takes for the analyte of interest to be eluted from the GC-MS can be used as reference for detection and quantification studies (Boulanger, et al., 2003). Additionally, since the relative retention factor (RRF) is more reliable than retention time, it was therefore essential to add an internal standard which was utilised in calculating the RRF of sucralose-TMS. Furthermore, the area under each peak was used for quantification measures assuming equal interaction with the detector since each peak is proportional to the quantity of a molecule. Moreover, as evident from the chromatogram in Figure 5.1b, sucralose-TMS yields a single peak which was detected at retention time of 22.2 minutes. The selectivity of the method was demonstrated by the quality of detected peak of sucralose-TMS with no interference peak at the retention time. The detection of sucralose was achieved following its derivatisation with MSTFA and the clean, single peak of sucralose-TMS indicates complete derivatisation as multiple peaks are usually an indication of an incomplete silylation reaction (Zarate, et al., 2016). The derivatisation reagent and protocol utilised in this research, although different from those in some published literatures for detection of sucralose, the resulting chromatogram and mass spectrum were similar (Ferrer, et al., 2003; Qiu, et al., 2007).

Other criteria used for identification of sucralose was the m/z s produced from the mass spectra. The conversion of sucralose to its TMS ether, as highlighted in Figure 5.1a indicates that sucralose molecular m/z increases from 397 to 757 because the silyl group (with TMS) replaced all hydroxyl (OH) group of the analyte transforming it to a more volatile and stable compound, sucralose-TMS which is more suitable for GC-MS analysis. However, the mass spectrum in Figure 5.2 has no m/z of 757 because of some possible reasons. First, the instrument capacity used in this research has a GC-MS mass scanning range from m/z of 40 to 600, thus, unable to detect a m/z of 757. The second plausible explanation could be the fragmentation of sucralose-TMS into smaller main ion fragments. The possible fragmentation path of sucralose-TMS structure was shown in Figure 5.3b.

If sucralose-TMS was fragmented from the glycosidic bond towards the glucose moiety, then two m/z of 412 and 343 were expected but if the fragmentation of sucralose-TMS occurs at the fructose unit of the molecule, then m/z of 397 and 343 would be expected (Qiu, et al., 2007). Comparing these to the mass spectrum of sucralose-TMS in Figure 5.2b, the presence of the fragmentation m/z (412, 343 and 397) were observed but with very low intensities suggesting further fragmentation of these m/z as proposed in Figure 5.3.

To determine these lower m/z of the fragmented sucralose-TMS, a full scan of ions was studied and ions identifiable with sucralose, were m/z   207, 308 and 343 (342.9) (Figure 5.2a) with m/z 308 , the quantifier ion, being   the tallest ion besides m/z 73. These fragmentation ions of sucralose-TMS from this research corresponds with those observed by Baird, et al., (2000) and Qiu, et al., (2007). Although it could be argued that the m/z of 73 should be the quantifier as it has the tallest peak (Figure 5.2a). However, in line with previous studies (Halket and Zaikin, 2003; Qiu, et al., 2007) the base ion, m/z 73 (tallest ion) cannot be used as an identification ion because the mass spectra of all TMS esters or ethers show rather abundant (sometimes base peak) ions with m/z 73.These studied parameters therefore aids in the identification and confirmation of the analyte of interest as sucralose-TMS.

5.3.2 Selection of derivatisation reagent

The ability of the GC-MS to detect polar compounds such as simple sugars and artificial sweeteners depends on conversion of such compounds to a more volatile and less polar form (Becker, et al., 2013). The detection of polar compounds by GC-MS depends on appropriate derivatisation reagent and method to achieve separation, detection and identification of the analyte of interest. In order to select the derivatisation reagent for sucralose, two commonly used reagent known to derivatise sugars (BSTFA and MSTFA) were tested for their ability to derivatise sucralose (Ruiz-Matute, et al., 2010; Lin, et al., 2008).The good volatility and stability characteristics of trimethylsilyl (TMS) ethers derivatives formed makes them the popular option applied to GC analysis of saccharides and polyalcohols (Orata, 2012). The resulting chromatogram in Figure 5.4a (BSTFA) and

Figure 5.4b (MSTFA) showed that MSTFA completely derivatised sucralose to sucralose-TMS as there was only one single peak and no silylation adducts. In contrast, BSTFA, also a silylation reagent and referred to as a universal derivatising reagent for organic compounds (Zaikin and Halket, 2003), was unable to achieve complete derivatisation of sucralose. The use of MSTFA as a derivatisation reagent was further optimised to determine the optimal temperature and time required for sucralose derivatisation. Thus, sucralose was derivatised at room temperature (Figure 5.5a) and at 70°C (5.5b) for time period of 0, 30, 45 and 60 minutes. The result demonstrates that derivatisation at 70°C for 30 minutes (Figure 5.5b) was optimum. On the other hand, derivatising at room temperature with MSTFA, shows an increase in peak value with increase in derivatisation time (Figure 5.5a). Hence, to achieve a complete derivatisation at room temperature, it would require longer time between 90 to 120 minutes in order to achieve peak area value that is similar to derivatising at 70°C for 30 minutes. Therefore, the selection of MSTFA for the derivatisation of sucralose at 70°C for 30 minutes. In comparison to the work of Qiu, et al. (2007) where the silylation reagent used was HMDS-TMS-Cl mixture after dissolving sucralose in pyridine following a 5-steps reaction. This current method used MSTFA in a direct reaction after desired concentration of sucralose is dried by evaporation. The use of MSTFA as a derivatisation reagent was better due to less steps in sample preparation, minimal usage of chemicals such as pyridine and less time consuming without compromising on the quality of peak generated. Thus, the method utilised in this research reduces the time and cost of the experimental work which are the main drawbacks to using GC-MS for the detection of polar compounds.

5.3.3 Selection of appropriate internal standard

An internal standard is an essential part of analytical separation. Therefore, selecting the right internal standard can facilitate the accuracy of analysis and minimise any adverse influence due to sample preparation since analysis of the internal standard and the analyte of interest are processed simultaneously (Wang and Han, 2017). Internal standards which maybe chemically or structurally similar to the analyte of interest is added in constant amount to correct analyte losses. Based on the study by Qiu et al., (2007), myo-inositol was tested for its suitability as an internal standard alongside a labelled analogue of sucralose, (sucralose -d6). Whilst myo-inositol (Figure 5.9) was not stable with a p- value < 0.0001, sucralose-d6 was stable when tested and as depicted in Figure 5.10, there was no statistical difference in the peak area value over several injections within 24 hours. These findings are interesting because it does not only show the best internal standard but also questions the suitability of myo-inositol as an internal standard for GC-MS detection of sucralose. The first internal standard, myo-inositol was selected based on literature review as it was indicated to be a good choice for the detection of sucralose in a study by Qiu, et al. (2007). However, contrary to the results in this research, myo-inositol was not stable, demonstrating the importance of carrying out preliminary tests on suggested reagents and protocols in some published literatures before engaging in actual studies to avoid wasting time and resources. Hence, the need to treat some published literatures with caution as some experimental procedures might not be reproducible like the case of using myo-inositol. The other internal standard used for the study was sucralose-d6 which was also completely derivatised to its TMS by the derivatisation reagent, MSTFA and remained stable. The similarities between sucralose-d6 and the analyte of interest (sucralose) was evident in the close retention time of both compounds as they were separated by 1 second (Figure 5.12). Therefore, it was evident from these studies that the most suitable internal standard for GC-MS detection of sucralose-TMS was its labelled analogue, sucralose -d6.

5.3.4 Precision and repeatability

The method used in this research again fulfilled the acceptable standard for an RSD of 20% at lowest and highest concentrations (ICH, 1995) because the RSD at lowest (5 μg/ml) and highest (400 μg/ml) standard concentrations were 5% and 4 % (Table 5.4). In addition to the determined RSDs, instrument precision of the optimised method was further assessed by intra- and inter-day testing (Figure 5.17). Both intra-day and inter-day results indicate good repeatability and precision under same instrumental conditions for a period of 24-hours (intra-day) and 3 days (inter-day) with an RSD of 4.6 % and 1.2 %, achieving the acceptable level of precision (SWGTOX, 2013). This value (< 5%) of RSD achieved with the research method was below the mark criteria of RSD for method development (Sanchez, 2018; ICH 2005). Therefore, the method utilised in this research for the detection and identification of sucralose-TMS was repeatable and accurate.

5.3.5 Linearity and detection limits

According to ICH, (2005), other parameters in method development and optimization are linearity, limit of detection and quantification. The instrumental linearity was determined by plotting an initial linear regression graph of 14 different standard concentrations. However, due to the correlation coefficient, R^2 value of 0.9844 being below the acceptable value of 0.990 (ICH, 2005), the standard concentrations that were not within the linear range where deleted using a plot of relative response against log concentration. The final standard concentrations were within the linear range of 5-400 and demonstrates linearity with an R^2 value of 1.000, thereby achieving the acceptance criteria for linearity in method validation (SWGTOX,2013; ICH, 2005). Although, R^2 value is not a reliable measure of linearity, the R^2 value of 1.000 achieved with this research method was better compared to the work by Qiu, et al., (2007). Moreover, the correlation value (R^2) of 1.000 indicate minimal deviations and ability of method to derive a wide range of concentrations consistently (UNODC, 2009). Since R^2 value is not a reliable measure of linearity, another post-hoc test of linearity, Runs test was performed, further confirming linearity of standards.

To determine the detection and quantification limits, the regression plot shown in Figure 5.16 and calculations in Table 5.3 were utilized to determine the values. According to the ICH guideline and a study by Sanagi, et al., (2009), the acceptable methods for LOD and LOQ are signal-to-noise ratio, visual observation, blank methods and use of linear regression. In this research, the latter (linear regression) was applicable generating an on column LOD of 0.0002 µg and LOQ of 0.0006 µg. The detection limit of this research method was lower compared to that of Qiu, et al., (2007) which was 0.00025 µg and competes with other methods irrespective of the type of scan used in spectrum analysis. Whilst a full spectrum analysis scans all chromatogram peaks within a spectrum, selective ion monitoring (SIM) monitors selected peaks associated with a specific substance as the name implies and, in this research, full scan was used. Since main objective of this aspect of the research was detecting sucralose in glomerular endothelial cells, full scan was used for this study because of its usefulness in determining unknown compounds in a sample and provides better information during the confirmation and resolving of analyte of interest than SIM (Himabindu, et al., 2013). Furthermore, the limit of detection (0.0002 µg) calculated in this study is competitive and sensitive in comparison to other methods and techniques used for the detection of sucralose as shown in Table 5.6.

Table 5. 6: **Comparison of sucralose detection using various analytical techniques**

Author	Analytical technique	Derivatisation reagent	Linear range (µg/ml)	Mass on column detection limit (µg)
Current Research	GC-MS	Silylation using MSTFA	5-400	0.0002
Chang and Yeh (2014)	Liquid chromatography/ tandem mass chromatography (LC–MS/MS)	Not applicable	0.01–0.5	0.5
Qiu et al., (2007)	GC–MS and GC–FID	HMDS	5–60	0.00025
Hanko and Rohrer, 2004	High performance anion-exchange (HPAE)	Not applicable	16 -25	0.01
Farhadi et al., (2003)	Capillary column gas chromatography (CCGC)	Silylation with of *N*-trimethylsilyl-imidazole	0.2-40	0.0002

GC-MS = Gas chromatography with mass spectrometry; GC-FID = Gas chromatography with flame ionisation; HPLC-RI=High performance liquid chromatography with refractive index detectors; HMDS = Hexamethyldisilane; MSTFA = N-methyl-N-(trimethylsilyl)trifluoroacetamide.

The results from this research was interesting because not only does it demonstrates that GC-MS is sensitive and suitable analytical technique for the detection of sucralose, it

reduces the time required for sample preparation when compared to those of Farhadi, et al. (2003) and Qiu, et al., (2007). Thus, this optimised and validated GC-MS method with low LOD utilised in this research justifies its application in detection of sucralose in GMVEC. Therefore, since very low concentration of artificial sweetener, sucralose was expected to be seen by glomerular endothelial cells, a technique with high sensitivity to signal response such as GC-MS, was the best option to achieving desired result.

5.3.6 GC-MS detection of sucralose in glomerular microvascular endothelial cells

So far, method development and optimisation parameters for GC-MS detection of sucralose have been determined, from detection, identification, stability, linearity and quantification limits to precision and reproducibility. Thus, creating a platform for the identification of sucralose in glomerular endothelium.

The second phase of this chapter was the application of the optimised GC-MS method to determine if sucralose crosses the glomerular microvasculature endothelial cell membrane. The presented results in Figures 5.18 and 5.19, indicated that sucralose does not cross the glomerular endothelial membrane as sucralose used in treating cells were retained in the analysed cell media (Figure 5.18). To further determine matrix effect on sucralose, the result in Figure 5.20 shows the detection of sucralose introduced into the media without GMVEC, thereby validating that sucralose was not affected by other molecules present in the cell sample matrix. This finding is novel, and relevant in addressing an aspect of the research question as to artificial sweeteners reducing permeability by other means but crossing the glomerular endothelial membrane.

The inability of sucralose to cross the endothelium was supported by some studies in human and other species (Roberts, et al., 2000; Baird, et al., 2000; Grice and Goldsmith, 2000; Sims, et al., 2000; Wood, et al., 2000). These studies demonstrated that sucralose and its glucuronide conjugates are eliminated from the body without measurable bioaccumulation. Although there is limited GC-MS based detection of sucralose on other matrix, there are no study demonstrating the application of this technique in detecting or identifying sucralose

from glomerular endothelial cell samples *in vitro* thereby making this study the first. This research, therefore, adds to the knowledge that GC-MS technique can be used for the detection of artificial sweetener; sucralose from tissue cultured cell samples.

Taken together, these findings have shown that GC-MS is sensitive in detecting sucralose and its application to other compounds and matrixes including cell sample lysates. These research findings further demonstrated that the artificial sweetener, sucralose does not cross the glomerular endothelial cell membrane but rather as chapter 4 of this research demonstrates, binds to the sweet taste receptors to attenuate the VEGF-A induced permeability in the glomerular microvascular endothelial cells.

5.4 Conclusion

To summarise, the shift towards the use of artificial sweeteners either for satiation or health reasons warrants a robust and sensitive analytical technique to determine its effects in the cellular compartments and in this case, the glomerular microvascular endothelium. GC–MS technique was used for the detection and identification of sucralose following the derivatisation of sucralose with MSTFA to sucralose-TMS. The final range of linearity for sucralose determination was between 5-400 µg/ml with correlation coefficient of ($R^2 =$ 1.000). The calculated on column limit of detection and quantification were 0.0002 and 0.0006 µg. While precision, defined as the degree of scatter between measurements (ICH, 2005) and determined as relative standard deviation (RSD), set to be below 20 %, in this study, it was below 10%, indicating the quality of results obtained. These findings therefore demonstrated that GC-MS, despite its drawback (derivatisation), is a sensitive method to use in detection of MSTFA-derivatised sucralose and its application to glomerular endothelial cell lysate shows it can be used for several analytical studies in various fields other than chemistry.

CHAPTER 6

GENERAL DISCUSSION

6.1 Discussion

The increasing economic and personal cost of DKD globally, highlights the importance of developing alternative management and treatment strategies targeted at the molecular level of the disease (Beissenger, et al., 2013). Current DKD treatment strategies are focused on controlling blood sugar to delay progression and prevent further renal damage. The first approved treatment for delaying the progression of DKD was an SGLT2 inhibitor, Canagliflozin in 2019 (Perkovic, et al., 2019). However, this drug is associated with adverse effects such as hyperkalaemia, bone fracture and amputation (Perkovic, et al., 2019). Thus, the importance of investigating the impact of novel agents that could potentially attenuate leak in DKD, as it could not only save millions of lives but also reduce the economic burden of managing the disease and this, was the focus of current research.

The main biomarker for progressive DKD is proteinuria (Heerspink, et al., 2019), resulting from leaky endothelial membrane. Endothelial barrier integrity is crucial for vascular homeostasis but in a pathological state, increased secretion of a growth factor, VEGF-A destabilises the endothelial barrier (Garrett, et al., 2017; Onions, et al., 2019). The primary aim of this research was to assess the protective effect of artificial sweeteners; aspartame, saccharin, sucralose and neotame on VEGF-A induced barrier disruption and permeability in a cell model of glomerular microvascular endothelium. While certain responses are most appropriately examined *in vivo,* the application of cell studies in scientific research such as those carried out in this current work allows for careful analysis of the behaviour of primary glomerular endothelial cells and their interactions with mediators (Satchel, et al., 2006).

This research targeted the activation of GPCR by artificial sweeteners as a signalling pathway in protecting the glomerular endothelium from barrier disrupting cytokine prevalent in DKD, VEGF-A. Amongst the artificial sweeteners assessed in this research for their effect on mitigating endothelial barrier leak in the glomerular microvasculature, only

saccharin and sucralose showed potential benefits in halting permeability *in vitro* as shown in Figure 6.1.
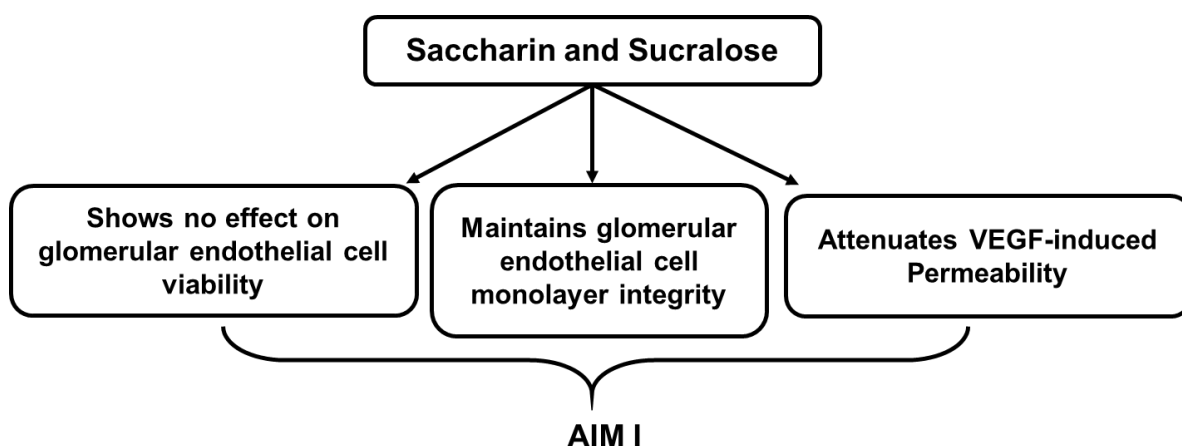
Figure 6. 1: **Artificial sweeteners, saccharin and sucralose as potential therapeutic agents for ameliorating leak in DKD**
A summary of some key findings from this research on the potential therapeutic effect of artificial sweeteners, saccharin and sucralose as they protect the glomerular endothelial microvasculature from VEGF-A induced leak.

As previously stated in section 1.12, a pioneer study linking artificial sweeteners to attenuation of leak caused by the endothelial barrier-disruptive agent, LPS, was by Harrington, et al. (2018) which demonstrated that artificial sweetener, sucralose protects the pulmonary microvasculature *in vitro* and *in vivo* against LPS, through a sweet taste receptor-dependent pathway. However, at the time of writing this thesis, there was no study targeting the impact of artificial sweeteners on leak in the microvascular endothelium linked with DKD, making current research novel. Findings from this research has demonstrated that artificial sweeteners, saccharin and sucralose have no effect on normal glomerular endothelial monolayer, supporting previous studies by Lizunkova, Enuwosa and Chichger (2019).

In contrast to normal table sugar, which is a major culprit in the development of diabetes and its complications, and also linked with increased vascular permeability (Hoshi, et al.,2002; Doronzo, et al., 2012; Schiano, et al., 2020; Montonen, et al., 2007), artificial sweeteners, saccharin and sucralose has no effect on glomerular endothelial cell barrier. Whilst the use of artificial sweeteners as substitutes for table sugar, is not without controversies, the negative health effects of natural sugar, sucrose is far more than some of the concerns about these sweeteners as cases of diabetes continues to rise.

The discovery of the sweet taste receptors, T1R2/T1R3, in extraoral cells has led to re-examination of the metabolic consequences of artificial sweeteners with studies in *both in vivo* and *in vitro* showing contradictory and inconclusive results. This variability has been observed in studies regarding the effects of artificial sweeteners on short term glucose absorption in the small intestine, glucose intolerance in the long term via alterations in the gut microbiota (Mace, et al., 2007; Pepino, 2015 ; Renwick and Molinary, 2010; Suez, et al., 2014), toxicity (Rahiman and Pool, 2014; Chattopadhyay, et al., 2011; Weihrauch and Diehl, 2004), adiposity changes (Laverty, et al., 2015) and permeability. Although the primary role of these artificial sweeteners is to generate the sense of sweet taste through the activation of its receptors, this research has demonstrated that these sweeteners do cause other physiological events besides sweet taste even at low concentrations.

However, the differing effects of aspartame, saccharin, sucralose and neotame on the parameters studied was made clear. Saccharin and sucralose attenuated permeability induced by VEGF-A in the glomerular microvascular endothelium through potential activation of the sweet taste receptor, T1R3 and increased surface level expression of the adhesion protein, VE-cadherin. Aspartame and neotame has contradictory effects to saccharin and sucralose on some of the studies carried out in this research. Although results from this research showed that aspartame and neotame have no effect on GMVEC viability which was supported by the work of Rahiman and Pool, (2014), their effects on other physiological parameters studied in this research was inconsistent. Studies carried out in this research were repeated multiple times to minimise technical and human error,

but the disparity exhibited by artificial sweeteners, aspartame and neotame was a cause for concern.

Thus, it could be speculated that aspartame and neotame lack of effect on VEGF-induced permeability might be linked to similarities in their chemical structure and metabolism. Hence, it is not plausible to make any conclusion on their protective effects against VEGF-A induced leak in the glomerular microvascular endothelium. Therefore, in line with other studies on differing effects of aspartame, on its safety (Choudhary, et al., 2017), appetite and glucose intolerance (Romo-Romo, et al., 2016) cytotoxicity (Alleva, 2011; Magnuson, et al., 2007) and now permeability, further studies should be carried out to decipher the cause of this differences.

Next, chapter 4 assessed the mechanisms by which artificial sweeteners protect against VEGF-A induced permeability on glomerular endothelial barrier. The novel finding on the localisation of the sweet taste receptor, T1R3, at the glomerular microvascular endothelial cell surface supports the notion that sweet taste receptors are not limited to the extra-oral cells (Malki, et al., 2015; Harrington, et al.,2018; Margolskee, et al.,2007; Lizunkova, et al.,2019; Masubuchi, et al., 2013; Rozengurt, et al., 2006; Kiuchi, et al., 2006).

The artificial sweeteners studied have different structural and physical compositions which might account for the observable differences in their effects at the different experimental studies carried out in chapter 4 of this work. First, it was demonstrated as a novel finding that the artificial sweeteners, saccharin and sucralose binds to the T1R3 sweet taste receptors in primary glomerular endothelial cells while aspartame and neotame does not.

The study went further to determine how these sweeteners affects other modulators involved in maintaining the glomerular endothelial barrier such as ROS, VE-cadherin, and cAMP. Cyclic adenosine monophosphate (cAMP) is amongst the signalling molecules that improve endothelial barrier function as elevated level of intracellular cAMP effectively reduced the permeability of endothelial cells *in vitro* and *in vivo* (Chava, et al., 2012; Fu, et al., 2015). Hence, the rationale to assess the effects of artificial sweeteners on intracellular cAMP level, was based on the knowledge that this downstream modulator of the endothelial

barrier, cAMP is increased upon activation of the sweet taste receptors (Trubey, et al., 2006). However, contrary to the anticipated result, findings from this research demonstrated that artificial sweeteners have no effect on intracellular cAMP in glomerular microvasculature. This difference on the level of intracellular cAMP in the presence of normal sugar and artificial sweeteners could be attributed to the differential recognition pathways of these sweeteners as agonist of sweet taste (Frank, et al., 2007). Therefore, the protective effect of saccharin and sucralose against VEGF-A induced permeability occurs through signalling mechanism other than cAMP. It was further demonstrated that sucralose and saccharin increased the surface level expression of VE-cadherin, another modulator of endothelial barrier (Yang, et al., 2015), and attenuated its internalisation by the growth factor, VEGF-A. In addition, the other modulator of microvascular permeability studied was ROS and result demonstrated that none of the studied sweeteners (sucralose, saccharin, aspartame and neotame), increased intracellular ROS in glomerular endothelium. However, under H_2O_2-induced ROS state, the artificial sweeteners, saccharin, sucralose and neotame were able to act as ROS scavengers in a similar manner to an antioxidant, N-acetylcysteine (Voghel, et al., 2008), while aspartame displayed a *dose-selective* effect. Hence, this research has not only demonstrated the role of these artificial sweeteners beyond the extra oral cavity but also adds new knowledge to the differing behavioural patterns. These differential physiological effects of the studied sweeteners might be attributed to differences in their chemical compositions and metabolism (Magnuson, et al., 2016; Kuhn, 2004; Gardener, et al., 2012; Grotz, et al., 2017; Whitehouse, et al., 2008).

Furthermore, it was shown that artificial sweeteners, saccharin and sucralose binds to and potentially activates the sweet taste receptor, T1R3, to affect their protection on glomerular endothelium against VEGF-A induced permeability. This protection was lost upon blocking of sweet taste receptor with a chemical inhibitor, lactisole but the sweeteners were unable to reduce VEGF-A induced permeability to baseline value in the absence of lactisole. This observation led to questions as to the artificial sweetener's exclusive binding to the sweet taste receptor. To determine whether these sweeteners act independent of the sweet taste

receptors, chapter 5 of this research focused on optimising and validating an analytical tool for the detection of artificial sweetener, sucralose and application of the method to cell studies.

The application of analytical chemistry technique in the field of biomedical science is not new with GC-MS amongst the most widely used technique because of its sensitivity and accuracy (Xia, et al., 2018). Chapter 5 addressed aims III and IV of the research questions raised in section 1.19, through optimisation and validation of GC-MS method for sucralose detection, identification and application of that technique to answer a cell-based question. The GC-MS method optimisation and validation were focused on first, finding a less time-consuming, yet effective derivatisation reagent for the analyte of interest, sucralose and its internal standard. This was followed by various method validation studies as set out by analytical science regulatory bodies (ICH, 1995; 2005; SWGTOX, 2013). These method optimisation and validation parameters, such as linearity, relative standard deviation, detection and quantification limits, precision and repeatability were carried out for sucralose detection and identification (Aparicio-Ruiz, et al., 2018; Mannocchi, et al., 2015). The results showed that the developed method for sucralose detection utilised in this research corresponds to the acceptable standards set by analytical regulatory bodies.

Sucralose was identified based on its retention time and fragmentation of molecular ions. These m/z from the mass spectrum of sucralose were utilised for its quantification and the application of an internal standard minimised any irregularities from sample preparation and analysis. The regression plot from the final linearity range of standard concentrations has an R^2 value of 1.000 corresponding to the acceptable value of 0.99 (SWGTOX, 2013). Comparing the GCMS sample preparation and validated methods utilised in this research to those of other analytical techniques, this current research was competitive. The resulting equation from the mean calibration plots was used to evaluate the limit of detection (LOD) and quantification (LOQ), with instrumental sucralose mass on column LOD of 0.0002 µg and LOQ, 0.0006 µg. These LOD and LOQ values are competitive when compared to other GC-studies (Farhadi, et al., 2003) and lower when compared to other chromatographic techniques (Hanko and Rohrer, 2004) for sucralose detection. The current research method

also demonstrated good precision with RSD values below 10%, indicating accuracy and reproducibility.

Since the overall outcome of chapter 5 was to determine whether artificial sweetener, sucralose crosses the endothelial cell membrane, the use of GC-MS was justified because of its sensitivity since low concentration of sucralose was estimated to be seen by endothelial cells and other techniques might not provide such sensitivity. Although a study by Musto, Lim and Suslick, (2009), on the detection of artificial sweeteners using a colorimetric sensor array, showed that very high concentrations (about 25 mM) is required to detect the sweeteners, such high concentration is not physiologically feasible to be seen in circulation by primary endothelial cells. The use of low concentration of any agent is recommended as it minimises potential side effects (Daughton and Ruhoy, 2013). Hence, low concentrations of sucralose (< 0.01 mM) was shown to activate the sweet taste receptor in this research, which is supported by a pioneer study (Harrington, et al., 2018), thereby making it vital to continue subsequent experiments with this concentration of sucralose. As evident from the findings in chapter 3 of this research, this low concentration of sucralose ameliorated VEGF-A induced permeability in the glomerular endothelium. Comparing this protective concentration of sucralose, to its LOD, 0.2 ng (0.0002 µg) using GC-MS, it was evident that GC-MS could detect very low concentrations of sucralose. With limited publications on this subject area, this finding was compared to the available study on the expected concentration of sucralose (400 ng) in circulation upon ingestion of 355 ml of diet cola containing 68 mg of sucralose (Slyvetsky, et al., 2017).

The limit of detection of sucralose (0.2 ng) from this research, was well below the author's level of circulating sucralose in plasma. Thus, the sensitivity and selectivity of the validated method was appropriate for detection and identification of sucralose in glomerular endothelial cell lysate. The result indicated that sucralose does not cross the glomerular endothelial cell membrane. Therefore, as summarised in Figure 6.2, the protective effect of sucralose against VEGF-A induced permeability was through binding to the sweet taste receptor, T1R3 and potentially activating it as supported by the work of others (Harrington, et al., 2018; Masuda, et al., 2012).
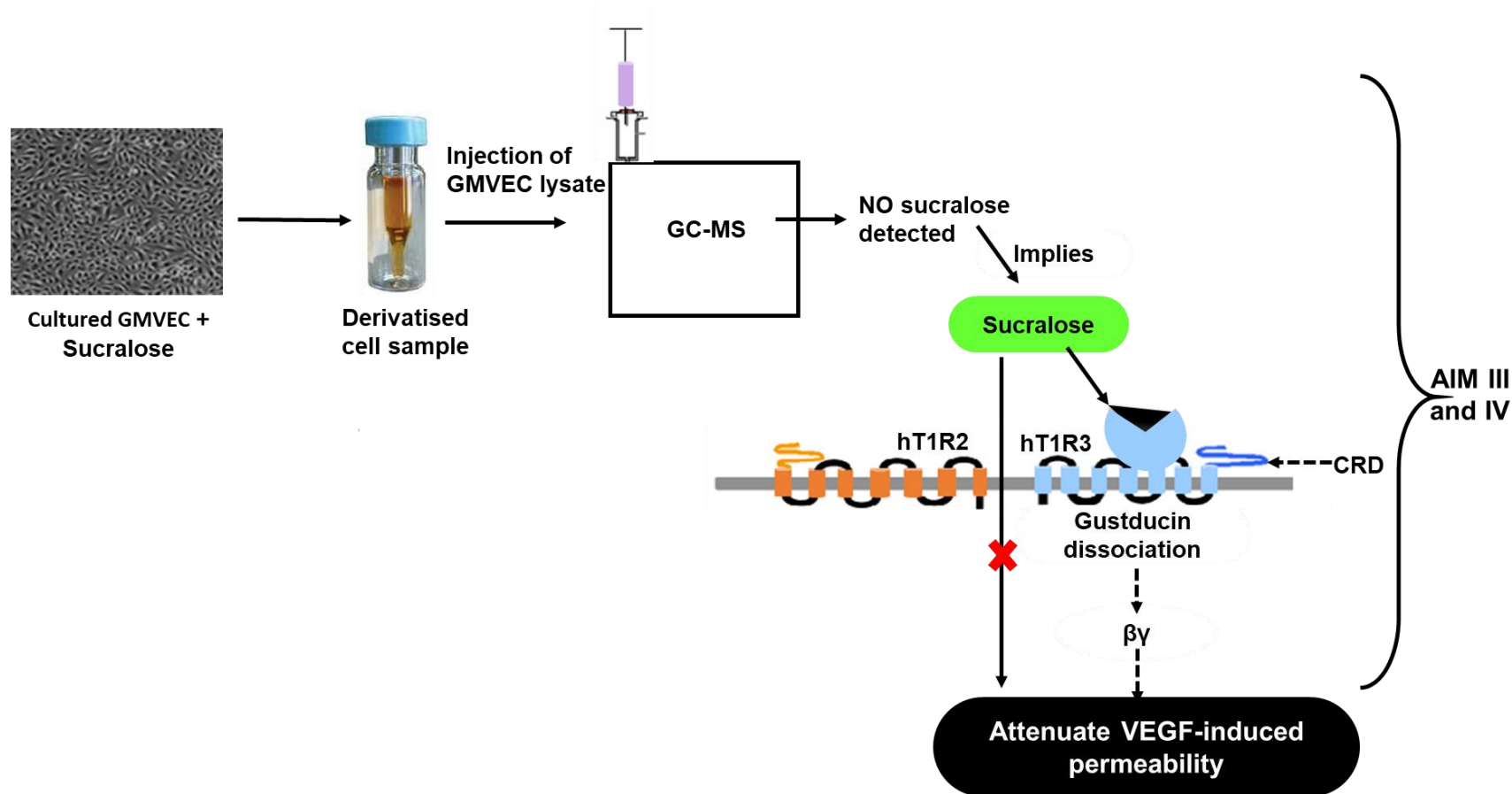
Figure 6. 2: **Sucralose, does not protect GMVEC independent of the the sweet taste receptor, T1R3**

The GC-MS method developed to detect and identify sucralose was applied to assess if the sweetener crosses the glomerular endothelial cell membrane. As demonstrated by the results, sucralose remained in the cell media and does not cross the endothelium, thereby indicating its action is through binding to the sweet taste receptor, T1R3 at the glomerular microvascular endothelial cell membrane.

Contrary to the assumptions in the last decade, on the consumption of artificial sweeteners by a particular set of individuals (Pena, 2010; Humphries, Pretorius and Naude, 2008; Piernas, Ng and Popkin, 2013; Schiano, et al., 2020), this is no longer the case. Since artificial sweeteners are now found in almost any products claimed to be "reduced" or "zero sugar", from chewing gums, cereals, ice creams to some drugs (Malek, et al., 2018; Kumari, et al., 2016), even unsuspecting individuals might consume some products containing these sweeteners (Sylvetsky, et al., 2017). Therefore, the findings from this research, that sucralose, an artificial sweetener does not get into glomerular endothelial cell even at high concentration of about 1mM was interesting and might have positive implications for individuals worried about sucralose effects when consumed. Admittedly, this research was an *in vitro* study and cannot be translated to same possible outcomes *in vivo*, it however gives an insight into the impact of sucralose on glomerular microvasculature when in circulation.

Thus, as diagrammatically summarised in Figure 6.3, this research has been able to provide insights into other physiological roles of artificial sweeteners, aspartame, saccharin, sucralose and neotame besides their influence on appetite, glycaemic control, BMI and obesity on which most studies with artificial sweeteners are centred on (Rosales-Gomez, et al., 2018; Tellez, et al., 2013; Fowler, et al., 2008).
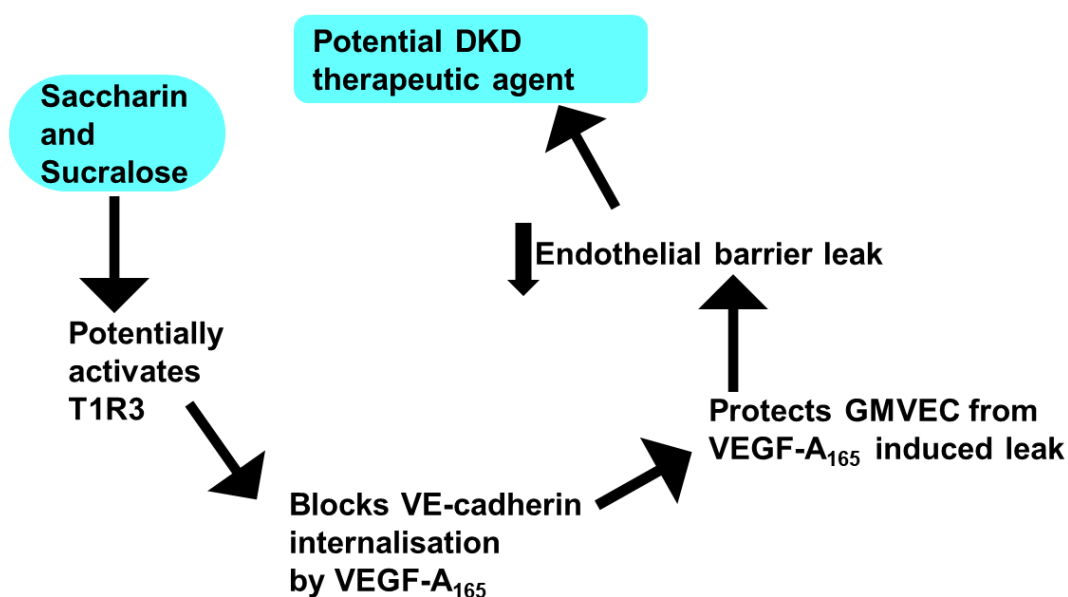
Figure 6. 3: **Summary of key research findings**
The diagram highlights the findings from the various aims of the research hypothesis raised during the work. The findings demonstrated that artificial sweeteners, sucralose and saccharin attenuates VEGF-induced permeability through the possible activation of the sweet taste receptor, T1R3 and blocking internalisation of VE-cadherin, therefore an alternative potential treatment strategy for DKD.

Although some experimental outcomes from this research were not as expected, just as it is with some scientific research, there are novel findings which can be considered as preliminary steps towards the development of novel therapeutic strategy in the management of permeability associated with DKD.

6.2 Further work

The main Limitation of this research, it was an *in vitro* study. However, findings from this research are novel with plausible prospect for *in vivo* studies to establish its potential in halting the progression of DKD. At present, the only approved pharmacological agent to lower the progression of DKD is canagliflozin (Perkovic, et al., 2019), which is an adjunct treatment to diet and exercise. Hence, further studies on the effects of saccharin and sucralose in the presence of other agents such as canagliflozin is required, since target sites for these treatments are different and such combined study might have success as therapeutic agent of DKD with less side effects.

The use of primary endothelial cells for *in vitro* model studies has gained interest for many pathological and physiological processes such as angiogenesis as they can be easily related to *in vivo* .However, the drawback with primary cells is their inability to be used for long term studies due to early senescence (Bouis, et al., 2001). Another limitation of this research was the use of one cell type which is primary glomerular endothelial cells. Therefore, the effects of these sweeteners on other cells affected by DKD should be considered. As detailed in the introductory section of this work, the glomerular cell comprises of three layers which are in crosstalk with each other, working collectively in maintaining glomerular barrier integrity. Whilst the permeability inducing effect of VEGF is predominantly seen in the glomerular endothelium which expresses the VEGF receptor, the podocyte secretes VEGF (Satchell, Anderson and Mathieson, 2004). Thus, the podocyte does not only repel against albumin, but also establishes the crosstalk between the other layers, GBM and endothelial cells. Moreover, some modulators of vascular permeability associated with DKD such as ROS has been linked with impairment of glomerular selective permeability through damage, detachment or apoptosis of the glomerular podocyte (Liu, et al., 2013; Susztak, et al., 2006). It would, therefore, be of interest to investigate the effect of saccharin and sucralose on the glomerular podocyte in the presence and absence of exogenous VEGF and ROS. In addition, since VEGF is predominantly expressed in the

podocyte, further works should assess the impact of saccharin and sucralose on VEGF expression in the glomerular podocyte.

Another limitation of this research was the concentration of sweeteners used which was selected to reflect the estimated doses expected to be seen in circulation (Robert, et al., 2000; Sylvetsky, et al., 2017). Also, the study was limited by lack of a combination of the artificial sweeteners as seen in the range of products on the market today. For example, saccharin is often combined with cyclamate or acesulfame-K, to mask off the after taste of both sweeteners (Behrens, Blank and Meyerhof, 2017; Taylor, Richards and Wiegand, 1968). Therefore, further *in vivo* studies should consider studying the level of sweeteners present in circulation following the consumption of products containing these sweeteners, either alone or as a combined mixture.

Considering the multifactorial hyperglycaemic mechanisms involved in the progression of DKD, such as the formation of advanced glycation end products, which has also been associated with other diabetic complications (Fukami, et al., 2013; Yamamoto, et al., 2001; Yamagishi, et al., 1997; El-Mesallamy, et al., 2011), further investigations on the effect of saccharin and sucralose on these mediators should be considered. This research has demonstrated that saccharin and sucralose ameliorated VEGF-induced permeability significantly. Therefore, clinical studies should be considered in order to assess the efficacy of saccharin and sucralose in ameliorating glomerular microvascular permeability in individuals living with DKD. Such study will not only provide proof of concept but also identify any side effects of these sweeteners in long term consumption and determine any withdrawal effects.

Furthermore, findings from this research, supported by other studies (Harrington, et al., 2018; Lizunkova, Enuwosa and Chichger, 2019), have demonstrated the potential therapeutic effects of artificial sweetener, sucralose in attenuating microvascular leak through binding to the sweet taste receptor, T1R3. Hence, further studies focused on the effects of natural sweeteners that activates the sweet taste receptors with no caloric content, such as steviol glycosides should be investigated.

Permeability is the hallmark of other diseases besides DKD, such as Ischemic diseases (Lee, et al., 2007) and cancer metastasis (Numnum et al., 2006). As an example, in cancer metastasis, it was found that secretion of VEGF by tumour cells disrupts endothelial junction proteins exposing the underlying basement membrane and increasing permeability and tumour cell adhesion in the micro vessel (Fu, et al., 2015). Therefore, additional studies on the effects of these artificial sweeteners (saccharin and sucralose) on permeability associated with these diseases would be vital and could be achieved through a coculture of cells involved in some of those diseases such as cancer cells.

The downstream signalling mechanism mediated through binding and activation of the sweet taste receptor, T1R3 may depend on the cell type. Findings from this research has shown that artificial sweeteners, aspartame, saccharin, sucralose and neotame does not elicit an increase in the level of intracellular cyclic AMP in the glomerular microvasculature. Whilst in other vascular beds, such as the pulmonary endothelium, the potential activation of kinases such as the inhibitory Src kinase leads to attenuation of sucralose-mediated protection from LPS, possibly through PLCβ2 recruitment, following the release of Gα gustducin. In other cells, activation of the sweet taste receptor, T1R3 by sweeteners led to stimulation of adipogenesis through Akt phosphorylation in the adipocyte and increase in insulin release through elevation of intracellular calcium level in the pancreatic β-cell (Nakagawa, et al., 2009; Simon, et al., 2013). However, there is still gap in knowledge on the exact signalling pathway through which the artificial sweeteners maintain the microvascular endothelium following activation of the sweet taste receptor, T1R3. Therefore, further studies are required to establish the signalling mechanism involved downstream of the cell membrane as it not yet clear if other cross talks exist following binding of the artificial sweeteners to the sweet taste receptors in the glomerular endothelial cell membrane. A potential signalling pathway for future studies might be the DAG-PKC pathways since activation of the T1R3 stimulates intracellular DAG, through hydrolyses of the inositol, 4,5 bisphosphate (IP2) (Kim, et al., 2017; Masubuchi, et al., 2013; Lindemann, 2001; Nakagawa, et al., 2009). The rise in intracellular DAG in turn affects PKC, which is amongst the regulators of VEGF (Xia, et al.,1994). Studies have demonstrated that DAG-

PKC pathway affects the level of inflammatory cytokines, amongst which is VEGF, a mitogenic growth factor and an inducer of permeability in diabetic kidney disease (Ziyadeh, et al., 1995; Koya, et al., 1997; Geraldes and King, 2010). Further studies should, therefore, examine the effects of artificial sweeteners, saccharin and sucralose on PKC and any potential cross talk between the sweet taste receptors, PKC and VEGF on the glomerular endothelial cell membrane.

The GC-MS method developed in this work was for sucralose, but with very limited studies on GC-MS analysis of other commercially available artificial sweeteners, there is need for similar studies using other artificial sweeteners such as saccharin. Since the main drawback of using GC-MS centres on derivatisation of polar compounds, the developed method mitigated that effect through reduction of sample preparation time. Further studies could therefore adopt this method to detect these sweeteners, saccharin and sucralose in other biological samples such as urine and blood.

Collectively, the suggested further studies will aid in understanding the mechanisms involved in managing leak in DKD and to develop novel and effective treatment strategies for the disease.

6.3 CONCLUSION

With the increasing number of diabetic patients suffering from DKD, there is a significant need to identify novel therapies to target key pathologies in the disease. Studies presented in this thesis demonstrated the potential of artificial sweeteners, saccharin and sucralose, to reduce vascular leak seen in DKD. These findings, coupled with the mechanistic studies to understand how this is regulated and the development of a tool to measure sweetener levels, provide a basis for future studies on targeted interventions aimed at developing a new treatment for the vascular leak seen in this debilitating disease.

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Appendices

Appendix I: Effect of Artificial sweeteners on GMVEC viability at high physiological dose

Viability of GMVEC was measured using CCK-8 assay following GMVEC exposure to artificial sweeteners; aspartame, saccharin, sucralose and neotame at high concentration of 10,000 μM. Absorbance is denoted as percentage viability, n=9 and data is presented as mean with S.E.M.
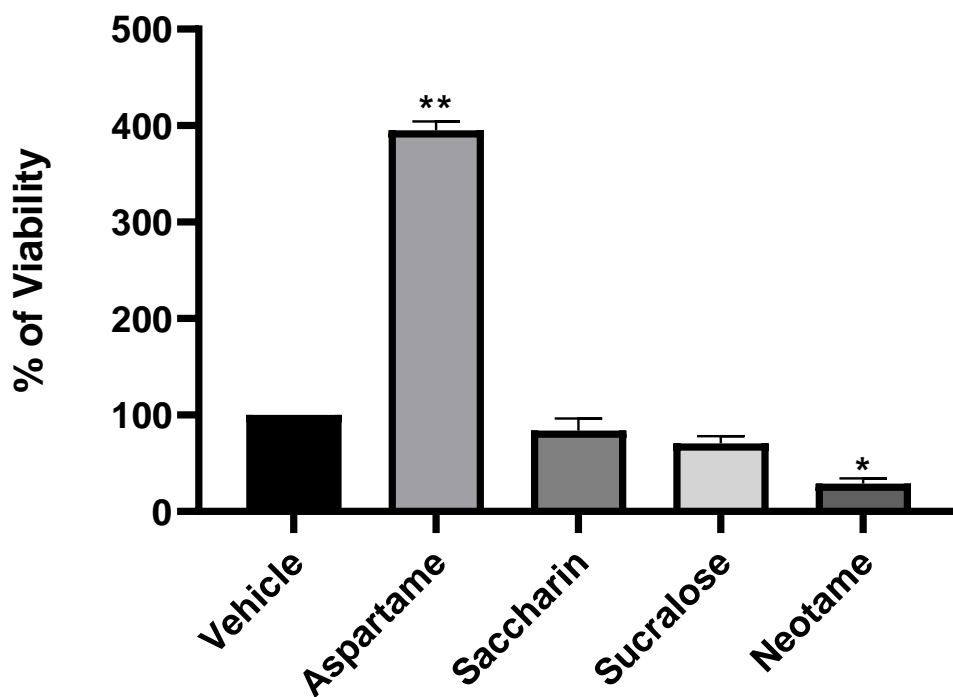
Appendix II

Calculations for working concentrations of reagents

Reagents/Molar Mass (g/mol)	Mass used (g)	Solvent volume(ml)	Stock concentration (µM unless otherwise stated)
Aspartame (294)	0.0294	Media / 10 ml	10,000
Saccharin (183.18)	0.0183	Media / 10 ml	10,000
Sucralose (397.64)	0.0398	Media / 10 ml	10,000
Neotame (378.46)	0.0378	Media / 10 ml	10,000
Lactisole (218.188)	0.02182	Ethanol / 10ml	10,000
VEGF		1 ml of dH_2O	10 µg /ml
NAC (163.19)	0.0163	Media / 10 ml	10, 000

In order to calculate the required working concentration for each of the reagent, the formula $C_1V_1 = C_2V_2$ was used where C_1 = Known Concentration; $V1$ = Unknown volume; C_2 = Desired concentration and V_2 = Desired volume.

For example; the desired working concentration (C_2) for lactisole was 3 µM, the stock concentration (C_1) was 10,000 µM, desired volume (V_2) was 20,000 µl and unknown volume (V_1) = X?

Inputting the values into the equation; $C_1V_1 = C_2V_2$

10,000 * X = 3 * 20,000

10,000 * X = 60,000

Therefore, X = 60,000 / 10,000 = 6

So, the unknown volume is 6 µl. Therefore, 6 µl of the lactisole stock solution (10,000 µM) was made up with media to give a final volume of 20,000 µl and a final working concentration of 3 µM. Similar calculations were carried out for other reagents prior to use in studies.

However, regarding other reagents used in this study such as tert-butyl hydrogen peroxide (TBHP) and 2′,7′-dichlorodihydrofluorescein diacetate (DCFDA), the calculations for the working solution are different from those in table 2.4. For DCFDA, 5 µl of 20 mM DCFDA solution (from supplier) was made up to 10,000 µl in 1x buffer to give a 10 µM working concentration while 1.82 µl of 55mM TBHP stock solution was made up to 10,000 µl using complete media.

**Appendix III: Cyclic AMP standard dilution and standard curve**

The cAMP Standard was 1 mM cAMP (1,000 pmol/µl). Which was prepared in a 7-serial 1:10 dilutions in the assay/lysis buffer to give a range of concentrations between 0.006 to 6,000 pmol cAMP per 60 µl (table) for the standard curve. The resulting data was used to plot the standard curve. As evident from the graph, the higher the amount of cAMP, the lower the relative fluorescence unit (r.f.u) at 535/590 nm and vice versa. Thus, in determining the production of cAMP, this standard was used as a guide to determine the level of cAMP produced by its agonist (sweeteners). Standards were diluted just before use. n=4.Data is expressed as mean with S.E.M *$*p \, 0.002, ***p <0.001, ****p <0.0001$* vs vehicle (0 µM).

Dilution	cAMP pmol/60µl	cAMP-standard	Assay/Lysis Buffer
1	6000	30µl of cAMP standard	270 µl
2	600	30 µl of dilution 1	270 µl
3	60	30 µl of dilution 2	270 µl
4	6	30 µl of dilution 3	270 µl
5	0.6	30 µl of dilution 4	270 µl
6	0.06	30 µl of dilution 5	270 µl
7	0.006	30 µl of dilution 6	270 µl
Blank	0	0 µl	300 µl
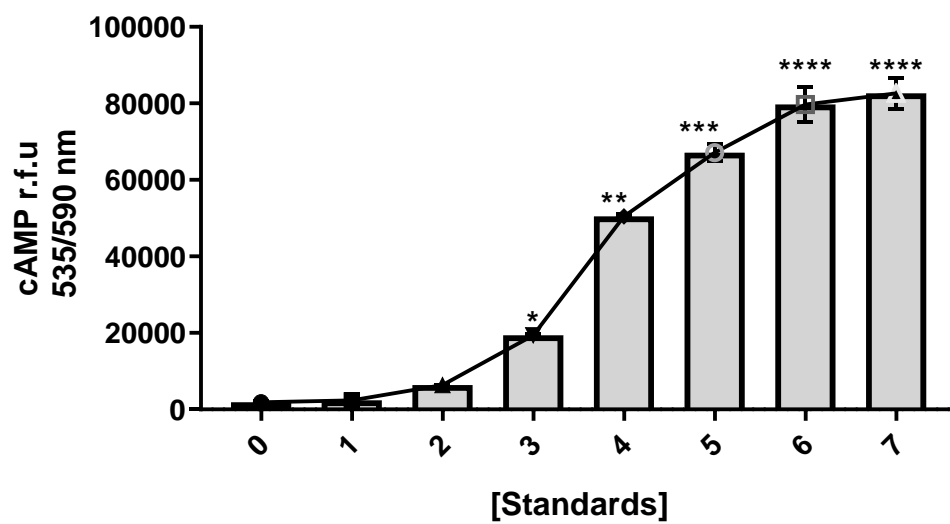
Appendix IV: Selection internal standard; deuterium-labelled sucralose

Based on the instability of myo-inositol, a deuterium-labelled sucralose was tested as an internal standard to account for imprecision during analyte processing. 25, 50 and 100 µg/ml of the internal standard was diluted down from 1 mg/ml stock solution, dried using vacuum extractor, derivatised with MSTFA at 70 $^{\circ}$C for 30 minutes and analysed in GC-MS using full scan mode. n=3 with error bars representing standard deviation.
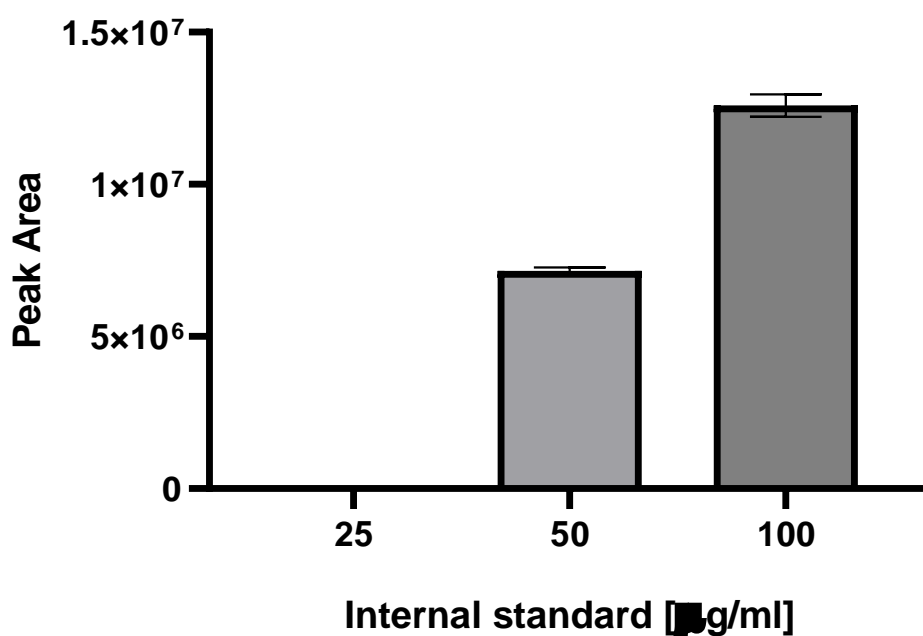
Appendix V: Chromatogram of myo-inositol with sucralose-TMS

The chromatogram shows myo-inositol was detected as an internal standard of sucralose-TMS. Hence, eliminating lack of detection or incomplete derivatisation as possible reason behind its unstable nature over continuous run. Myo-inositol was derivatised in like manner to sucralose, with 100 μl MSTFA at 70°C for 30 minutes.